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THE ANALYSIS OF FATTY ACID MIXTURES BY GAS-LIQUID CHROMATOGRAPHY:

Construction and Operation of An Ionization Chamber Instrument*

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I. Introduction

The purpose of this publication is to describe the application of gas-liquid chromatography (GLC) to the analysis of complex mixtures of C_7 - C_{24} fatty acids and, in addition, to provide details on the construction and operation of an exceedingly sensitive apparatus for GLC. This instrument has proved to be dependable in laboratory operation for more than a year. The entire apparatus including an ionization chamber detector can be constructed from readily available components, utilizing the mechanical and electronic skills generally available to research establishments.

It is a pleasure to acknowledge with gratitude the exceedingly helpful cooperation of three scientists whose work at the National Institute for Medical Research, London, England, pioneered this new approach: the inventors of the basic technic, Drs. A. J. P. Martin and A. T. James who built and installed our first apparatus, and Dr. J. E. Lovelock who designed the improved detector described herein. In addition, we appreciate the advantage of sharing our experience in this new and rapidly evolving technology with Drs. S. R. Lipsky (Yale), J. E. Callen (Procter and Gamble Company) and G. Popják (Medical Research Council, London), who have contributed useful ideas which have unquestionably influenced our own application of this method.

Developments in gas-liquid chromatography (GLC) have been rapid and the basic reports are dispersed in numerous publications. In part, it is the purpose of this report to consolidate the most important practical points from these many sources, but it does not pretend to go into historical detail nor into the theoretical issues which have been presented by others. A selected bibliography is given in Appendix A, which any worker embarking in GLC should regard as essential and minimal.

In this report all temperatures are reported on the centigrade scale, and measurements are given in the metric system whenever it is appropriate.

II. General Principles; Detection Devices

Gas-liquid chromatography is an outgrowth of the development of liquid-liquid chromatography

and is closely analogous in principle. Compounds which are volatile at the temperature of the column are distributed between a moving gas phase and a stationary liquid phase. The latter is supported in the column on a porous bed of non-reactive material. Differences in partition coefficients cause the volatile compounds to move through the column at different rates. By means of a suitable detection system the concentration of eluate in the effluent gas is plotted against time. Extremely effective separations of highly complex mixtures can be made by utilizing various stationary phases and column temperatures in successive chromatographic runs.

Rapid progress has been made in designing detection devices of increasing sensitivity and dependability. The ingenious gas-density balance of Martin and James (15) was one of the first in this sequence. The ionization chamber of Lovelock (7) is a recent development, and with its enormous sensitivity it is now possible to take full advantage of high column efficiencies in resolution of structurally similar compounds. The relative simplicity of construction and operation, the ruggedness and low cost of the ionization chamber instrument recommend it for laboratories which aim at high precision in analysis on a microgram scale. An additional virtue of this detection device is that less than one part in 10^8 of the applied sample is destroyed during the analysis (7). Accordingly this instrument can be utilized when the investigator desires to collect samples for further chemical analysis.

In principle, the ionization chamber detector detects changes in ionization caused by the entrance of ionizable molecules foreign to the argon carrier gas stream. During the operation of GLC, argon flows through the chamber, radiation from a sealed radioactive source partly ionizes this gas and produces a steady and stable ionization current when a high voltage is applied across the chamber. In addition to the ionized argon molecules, metastable (excited but non-ionized) argon atoms are formed whose concentration depends on the strength of the source and on the amount of voltage applied to the chamber. These metastable argon atoms act in a sense as

a reservoir of energy and are responsible for the unique sensitivity of this detector. Any molecules entering the chamber which have a lower ionization potential than the excitation potential of argon will, on collision, accept energy from metastable argon atoms to form ion pairs, thus producing an increased ionization current which is amplified and recorded. Collisions are relatively very infrequent, therefore the bulk of the sample is not destroyed during passage through the detector. Fortunately, most volatile organic compounds have ionization potentials below 11.6 electron-volts (the excitation potential of argon) and accordingly will be detected. In general, the detector responds in a linear fashion to increasing concentrations of many volatile organic substances with molecular weights over 150 (7), such as alcohols, ketones, esters, etc. However, some low molecular weight compounds with high ionization potentials (e.g. nitrogen, carbon dioxide and methane) will not be detected.

The concentration of metastable argon atoms can be increased by increasing the voltage applied to the detector; this leads to a greater probability for collision of foreign gas particles with metastable argon atoms. Consequently the sensitivity of the detector can be controlled by varying the applied voltage. Similarly, the amount of radiation given off by the ionizing source also affects the concentration of metastable argon atoms. In the detector to be described the radiation source is a constant (10 millicuries of Sr^{90}); the voltage across the cell is variable. The steady ionization current due to the presence in the detector of pure argon is balanced electrically to give a zero base-line reading on the recorder.

III. Construction of Apparatus

The main components of the apparatus are as follows: 1) argon, the moving carrier gas, 2) the column which contains the stationary liquid and solid support into which the unknown material is introduced, 3) the heating system to maintain constant temperatures uniformly over both column and detector, 4) the detector to signal and to measure the concentration of components emerging in the effluent gas stream, 5) the high voltage supply to produce a high field strength within the detector cell and 6) the amplifier-recorder unit to amplify the weak electrical signals of the detector and to record the electrical events on moving graph paper.

The components to be described were selected

to provide reliable operation over long periods of time, as well as a high degree of versatility to meet everchanging requirements. Other equipment of similar technical specifications should also be adequate.

A. Gas Supply

Commercial argon gas is used as the moving gas phase. Helium has slight theoretical advantages over argon, as pointed out by Lovelock (7); unfortunately, the trace impurities (including argon) in commercial helium prevent its use unless it is purified. Argon is carried from a high pressure cylinder with an ordinary reducing valve (Fig. 1) through $\frac{1}{2}$ inch rubber tubing to a low-pressure gauge (Fig. 9) and then to the head of the column. Special gas-flow regulating devices are not required.

Although Keulemans (10, p. 57) has recommended that commercial gas be passed through silica towers to ensure dryness, we have not found this step necessary. A soap bubble flow-meter (Fig. 2) is essential for measurement of flow rates. Continuous recording of gas flows has not been necessary: once a column has been "conditioned", the gas flow rate does not change over a period of hours if the column temperature and pressure of gas inflow are held constant.

The argon is purchased in large high pressure cylinders¹. With a single chromatographic apparatus in constant use, one cylinder lasts about three weeks; it is convenient to have four or five such tanks on hand.

B. Chromatographic Column

Columns are cut from 1.83 meter lengths of straight pyrex tubing. One end is constricted and the column is cut to a final length of 1.42 meters, a length determined by the size of the heating system. Diameters may be varied, depending upon application. Columns used for analytical work are 4 mm. internal and 6 mm. external, but larger internal diameters are used when increased column capacity is required. A 5 mm. length of 2.7 mm. internal diameter silicone rubber tubing² is positioned over the constricted end of the column (Fig. 3) and acts as a gasket to

¹ Obtained as "Laboratory Grade Argon 99.998% pure", in cylinder size 1A which contains 224 cubic feet of gas.

² Obtained as "Silatube" Grade 50 HT from Reiss Manufacturing Corporation, 111 Fourth Avenue, New York, N. Y.

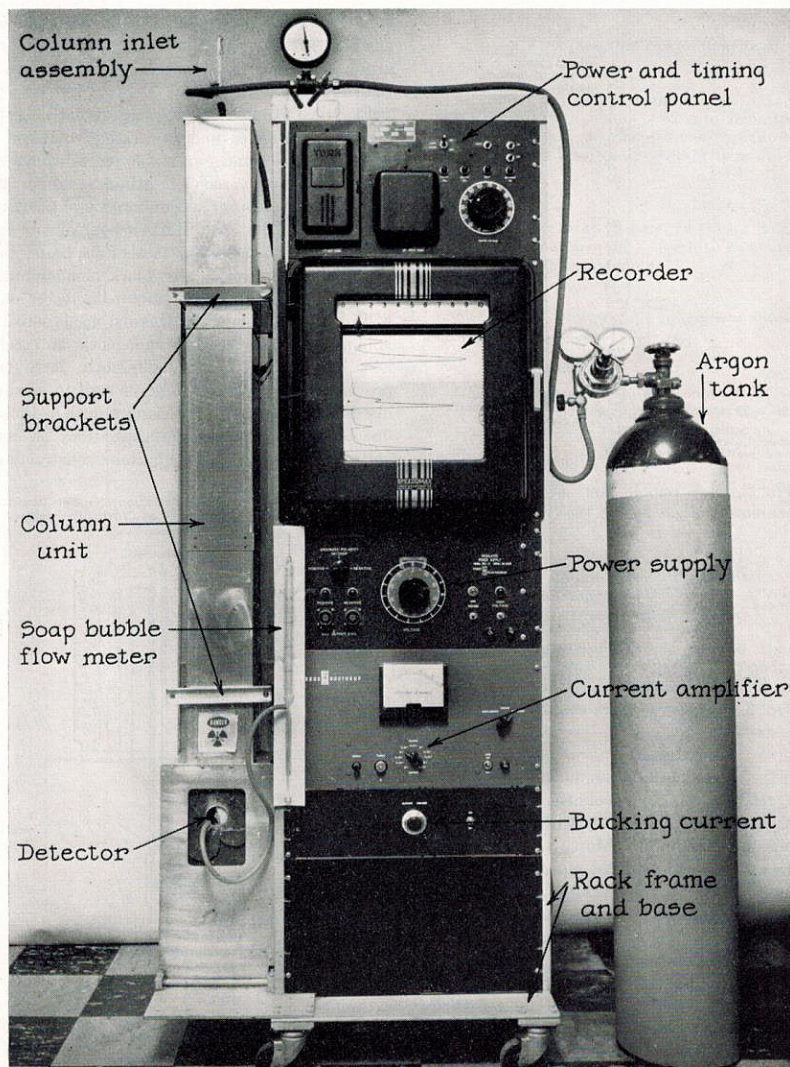


FIG. 1. Gas-liquid chromatography instrument, with a vapor-jacketed column and strontium-90 ionization chamber detector.

prevent loss of effluent gas at the point of insertion of the column into the detector.

A variety of stationary phases and packing methods will be described in a later section.

C. Heating System

The temperature of column and detector is held constant throughout any given chromatographic run. The temperature must be uniform

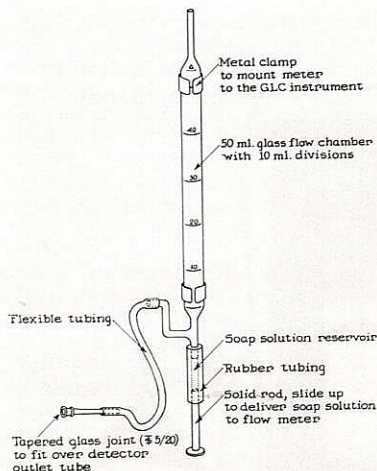


FIG. 2. Soap bubble flow meter for periodic measurement of gas flow at the effluent outlet.

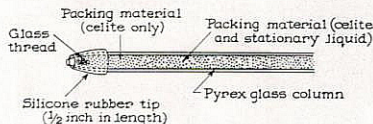


FIG. 3. Diagram of glass column tip with silicone gasket.

over the length of the column, since the absolute and relative retention volumes of fatty acid methyl esters vary markedly with a few degrees' change in temperature. Moreover, the detector temperature must be held up to that of the columns in order to minimize the condensation of substances from the effluent gas stream. The vapor jacket of James and Martin (15) is an efficient method of attaining stability and even distribution of high temperatures. However, it is made somewhat inflexible by the limited choice of thermally stable liquids of widely varied boiling points (but see Appendix E), and it requires periodic cleaning and refilling. Figure 1 shows a vapor-jacketed heating system in which the vapors are condensed on a coil connected to the laboratory compressed air line. Figure 4 shows the construction details. With this assembly, thermocouple measurements at 10 cm. intervals

throughout the length of the chromatographic channel revealed $\pm 0.25^\circ$ variation. The temperature within the detector was only 3 degrees lower than that of the column.

Electrical column heating has recently been designed and tested under operating conditions. Although a wide range of stable temperatures is permitted, the speed of attaining different temperatures is slower and uniformity of temperature throughout the heated components is difficult to achieve. This method is based on electrical heating of a large block of aluminum which encloses both the column and detector and is, in turn, surrounded by thermal insulation. It is important that the aluminum block, an excellent heat conductor, be sufficiently large to provide a thermal mass large enough to ensure insensitivity to short term changes, such as those due to ambient temperature variations or line voltage fluctuations. Figure 5 shows construction

Modification of Lovelock-James Ionization Chamber Detector and Vapor Heating Jacket for Gas Chromatography

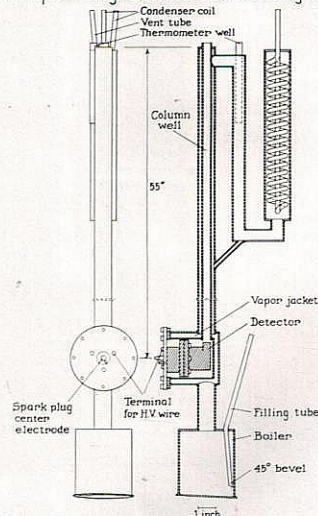


FIG. 4. Vapor-jacket heating system. Notes: a) Place $\frac{1}{4}$ " lead plates around detector housing. b) Surround entire unit with $\frac{1}{8}$ " aluminum sheeting and glass wool insulation. c) Vapor jacket sealed with silver solder. d) Install hot plate under the vapor jacket boiler (Catalogue #61725, Precision Scientific Company, Chicago, Illinois).

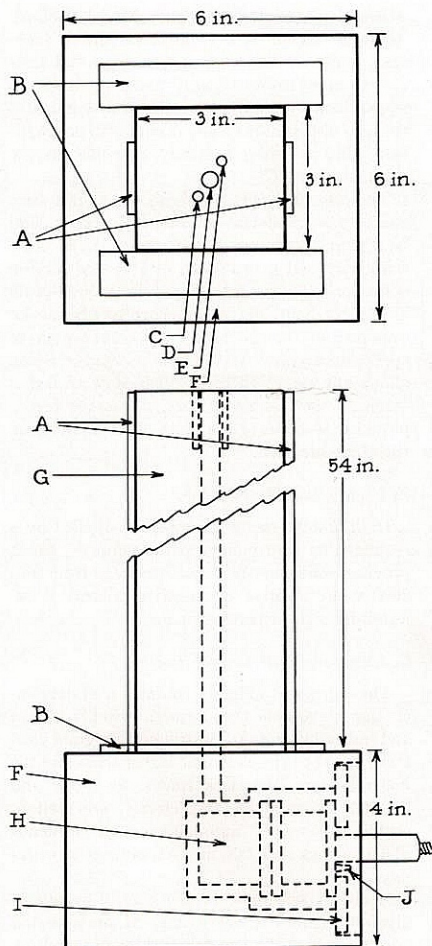


FIG. 5. Electrical heating system

LEGEND (See text for further details)

- A. Electrical strip heaters - 54 in. long by 1 1/2 in. wide. Attach to separate variable transformers and connect only one of these to the temperature controller.
- B. Electrical strip heaters - 4 in. long by 1 1/2 in. wide. Attach to a separate variable transformer.
- C. Drill hole 3 3/16 in. deep for a Hg thermometer.
- D. 1/2 in. axial hole for column insertion. Hole is milled along length of two 54 in. x 3 in. x 1 1/2 in. aluminum bars.
- E. Drill hole 3 3/16 in. deep for spring-loaded thermister probe.
- F. Aluminum detector block - 6 in. x 6 in. x 4 in. (Must have cavity of dimensions to fit detector).
- G. Aluminum housing for column. Bolt to detector block. Cover column and detector with a double layer of 1 1/2 in. thick "Marinite No. 23".
- H. Detector.
- I. Mycalex insulator.
- J. Stainless steel effluent gas tube.

details. Two long electrical strip heaters³ are bolted to the outside of the assembled aluminum block prior to the attachment of the insulating material. Two short 4 inch strip heaters, connected to a separate variable transformer, are attached to the top of the detector block (Fig.

³ 54 inches long x 1 1/2 inches wide, "chrome steel strip heaters", E. L. Wiegand Company, Pittsburgh, Pennsylvania.

5). Uniform temperature along the length of the column is attained by independent control of power through the three variable transformers⁴. In practice, it is only necessary to control a small portion of the total power required at any one temperature. Thus, a variable transformer is used to adjust power to one of the two long

⁴ Variac transformer—type V-10, General Radio Company, Cambridge, Massachusetts.

electrical heaters while the temperature controller⁵, attached to the second long heater through another variable transformer, controls the remaining required power. During operating conditions, the temperature remains constant at any setting⁶, and the variation of temperature down the column length and in the detector is $\pm 0.5^\circ$. Temperature control with this system is independent of variations in line voltage and of variations in ambient temperature.

D. The Detector System

Construction details of the detector, closely patterned after the ionization chamber described by Lovelock (12) are shown in Figure 6. The central anode, electrically insulated from the chamber body, is conveniently supplied by a commercially available demountable spark plug⁷. A thin disc is fixed to the conductor portion to allow high field strengths for moderately low applied chamber voltages. Before using a particular spark plug, it should be tested for electrical conductivity. The dimensions of the chamber and central electrode are designed to achieve proper "geometry" of electrical response and should not be modified unless one is prepared to cope with resulting alterations in detector performance.

The source of ionizing radiation (strontium-90—10 millicuries) was sealed in gold foil leaf of 0.001 inches thickness which was brazed into the inside of the brass cylinder.⁸

E. Amplifier-Recorder Unit

With a steady flow of argon through the detector a very small current (about 10^{-8} amperes) is produced when a high voltage (from 500 to 2000 v) is applied across the detector. Then, very small increments in current are produced by the increase in ion pairs which results when ionizable molecules appear in the argon gas stream. The electronic requirements for amplification of these currents are well served by a Leeds and Northrup

Micromicroammeter Amplifier, Model 9836-A⁹. This instrument is a chopper-stabilized, high-gain, low noise dc amplifier covering a full scale current range from 10^{-9} to 10^{-6} amperes in eleven steps. Chopper stabilization effectively eliminates the zero drift that normally occurs with dc amplifiers, thus ensuring a steady base-line on the chromatogram.

The amplified current is displayed on a strip chart potentiometric recorder, a Leeds and Northrup Speedomax G (10 millivolt full scale sensitivity and a pen speed of 1 second for full deflection). The recorder has a chart speed of 30 inches per hour, but other chart speeds can be obtained in the laboratory by substitution of appropriate gears. An automatic changer which allows any set speed to be doubled or tripled is available for this instrument. A marker pen is provided to indicate the start of an analysis on the chromatogram.

F. High Voltage Supply

High voltage to the chamber is supplied by a regulated dc high voltage power supply¹⁰, which provides continuously variable voltage from 0 to 2000 volts. Positive or negative polarity is selected by a front panel switch.

G. Electrical Power Control System

The top panel (Fig. 1) contains a system including a variable transformer, switches, relays and indicator lights to control and program electrical power to the electronic instruments and the boiler heater. Two clock timers, for 7-day and 24-hour programming respectively, are used to switch power OFF automatically on weekends and evenings and ON in the mornings of work-days.

A circuit schematic of the control system is given in Figure 7. Clock motors, M, are operated continuously with main power through switch S_1 . With the operation of the clock switches, the high voltage power supply, current amplifier and heater are energized. The recorder is energized upon manual operation of switch S_4 . This arrangement prevents the chart from running when the

⁵ Fenwall Model 560 temperature controller, Fenwall, Inc., Ashland, Massachusetts.

⁶ No change in temperature can be detected during weeks of operation at any desired temperature from 50° through 250° .

⁷ Champion 20, Champion Spark Plug Company, Toledo, Ohio.

⁸ Prepared, installed and tested for radiation safety by the U. S. Radium Corporation, Morristown, New Jersey.

⁹ Leeds and Northrup Company, Philadelphia, Pennsylvania.

¹⁰ A satisfactory power supply is Fluke Model 404M, from John Fluke Manufacturing Company, Seattle, Washington.

machine is automatically turned on in the morning. In the event of loss of liquid from the boiler, the resulting increase in boiler temperature opens thermal switch S_2 . This in turn, through relays K_1 and K_2 , causes power to the system to be interrupted. It is impossible to restore power without manually operating the reset switch S_3 , after filling the boiler.

IV. Assembly of Apparatus

A. Mechanical Assembly

To conserve bench space, the components are assembled vertically on a standard 19-inch channel type relay rack¹¹. Swivel type casters¹¹ with 3 inch diameter wheels are mounted on the bottom of the frame to make the rack mobile. Supports are constructed to attach the column and detector housing to the instrument rack. Hinges and brackets are installed to allow forward tilting of the housing in order to insure sufficient head room for column replacement. (A ceiling height of 10 feet is required if columns are to be inserted with the vapor jacket in the vertical position.) The top panel contains the power control system (III, G, above), while immediately below it the recorder is placed at eye-level. Below the recorder the high voltage power supply is shown, then the current amplifier, and last the bucking current control. At the back of the frame are mounted electrical power strips¹² to provide convenient outlets for 115 volts ac.

B. Electronic Assembly

The circuit diagram of the ionization chamber is shown in Figure 8. High voltage from the power supply (#2) is connected to the chamber (#1); the chamber wall is negative with respect to ground, and the central electrode positive. The cable¹³ from the power supply to the chamber is commercially available high voltage insulated wire of the type used in the high voltage section of television receivers, and is rated at 10,000 volts dc or higher. Teflon-insulated wire is used inside the vapor jacket to withstand the high temperatures, and connection to the high voltage cable

is made by means of a high voltage connector mounted on the aluminum sheet metal frame surrounding the vapor jacket. Connection to the detector is made by attaching the high voltage lead to one of the screws securing the Mycalex¹⁴ insulator to the brass chamber wall.

Current from the center electrode is measured and amplified by the current amplifier (#8) and is recorded on the strip chart recorder (#9). A resistor (#7) is inserted in series with the ionization chamber and the current amplifier in order to produce linearity of chamber response.

In order to provide zero adjustment of the base-line on the recorder, a bucking current circuit is included. It consists of a dry cell battery (#3), a ten-turn precision potentiometer (#4), a capacitor (#5) and a high meg-ohm resistor (#6). This network forms an adjustable current generator that produces a current of direction opposite to that from the ionization chamber. The bucking current is varied by means of the potentiometer and has a range that is slightly greater than the usual current (3×10^{-8} amperes). The current drain from the battery is negligible; therefore, the life of the battery is determined only by its shelf-life characteristics. The battery used is a 6 volt dry cell¹⁵ whose shelf-life is about one year. If preferred, a mercury battery¹⁶ could also be used, in which case a shelf-life of the order of two years would be obtained.

The series calibration resistor (#7) and 2×10^8 ohm resistor (#6) are mounted on insulated stand-off terminals inside a small standard aluminum chassis box¹⁷. The remainder of the bucking current circuit is mounted on a plate, providing front panel access to the potentiometer. In all wiring connections made in accordance with the circuit diagram, special thermal-free solder¹⁸ is used to minimize electrical disturbances due to changes in ambient temperature. Connection from the series resistor to the ionization chamber electrode is made with

¹⁴ Mycalex-400, $\frac{1}{4}$ -inch thick, Mycalex Corporation of America, New Jersey.

¹⁵ Burgess dry cell, Catalogue #F4BP, Burgess Battery Company, Freeport, Illinois.

¹⁶ Mercury battery, $7\frac{1}{2}$ volts, Catalogue #TR-145R, Mallory and Company, North Tarrytown, New York.

¹⁷ Minibox, Catalogue #CU-2103, Bud Radio, Inc., Cleveland, Ohio.

¹⁸ Solder, #107-1-0-1, Leeds and Northrup, Philadelphia, Pennsylvania.

¹¹ Bud Radio, Inc., Cleveland, Ohio. (Rack: Catalogue #RR-1364; Casters: Catalogue #RC-7756.)

¹² Wiremold Snapicoil 19C03, Wiremold Company, Hartford, Connecticut.

¹³ High voltage cable, Belden Manufacturing Company, Chicago, Illinois. Catalogue #8869.

Ion Chamber Detector - Wiring Diagram

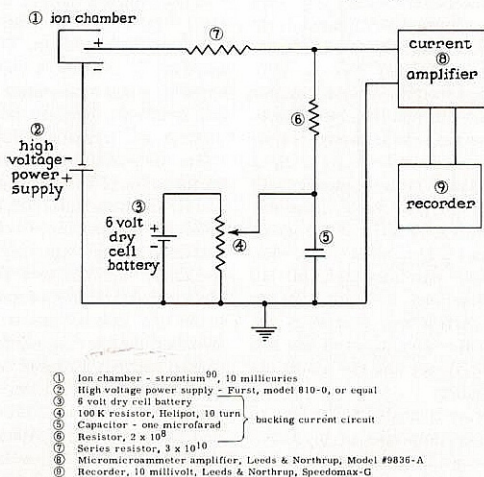


FIG. 8. Ionization chamber circuit diagram

shielded Teflon-insulated cable¹⁹, to withstand the high temperatures inside the vapor jacket. Connection from the series resistor to the current amplifier is made using the special twin lead-shielded cable provided with the instrument. Shielding is used on all signal cables to prevent the effects of stray electric fields.

V. Column Preparation

Since the ultimate success of gas-liquid chromatography analysis depends on column efficiency, the procedures of column packing will be described in detail. The three components of the packed column are: 1) the glass column, 2) the solid support and 3) the stationary liquid. The dimensions of the glass column have been described (III, B, above).

A. Preparation of Solid Support

The function of the solid support is to furnish a porous bed of small, evenly sized particles to which the stationary liquid adheres. Uniform particle size allows even distribution of the adsorbed stationary liquid and prevents formation of channels of flow due to irregular

spacing. The porosity of the solid support increases its effective surface area and allows the stationary liquid to distribute as a thin film.

The solid support in most general use is diatomaceous earth (Celite-545)²⁰ which is size-graded and processed in the following manner. A 3 liter beaker is filled to the half-way mark with raw Celite, 2 liters of water are added and the mixture is stirred vigorously (not swirled). After a 3 minute period for settling, the entire turbid supernatant water is decanted. This flotation process, which is repeated two additional times, removes about 50% of the unwanted small Celite particles (about 90% of the original Celite is smaller than the desired particle size). Then, the material is treated with acid to remove acid-soluble inorganic salts and metals. An excess of concentrated hydrochloric acid is added to the material for at least a 3 hour period. Acid is removed by repeated suspension in water and decantation, then by washing to neutrality on a Buchner funnel. The filter cake is dried with acetone. After oven-drying at 100°, the material is stored in clean glass bottles.

The flotation-graded acid-washed Celite is then

¹⁹ Size #22 AWG, Tensolite Insulated Wire Company, Tarrytown, New York.

²⁰ Johns Manville Corporation, 22 East 40th Street, New York 16, N. Y.

size-graded through standard "ASTM" 8 inch diameter sieves in a mechanical shaker. Although the selection of sizes is currently empirical, there is a theoretical basis for use of very small particles for maximum column efficiency (10, p. 146). However, there is also a theoretical advantage for use of larger particles (10, pp. 139, 146) due to the low inlet pressures that the increased permeability of larger particles allows. Our most satisfactory results have been obtained with either 120-140 or 140-170 mesh fractions. Keulemans recommends a 30-50 or 50-80 screen fraction of Sterchamol 1-22 fire brick (10, p. 145), while James and Webb²¹ currently prefer 80-110 and 100-120 mesh fractions of Celite. Clearly, the final choice of particle size represents the compromise between the advantages of the two extremes suggested by theory and the results obtained by the investigator.

The size-graded Celite is treated with alkali to decrease the adsorptive properties of the Celite by removal of silicic acid. Alkali treatment is performed by suspending 50 gm. of dry size-graded Celite in approximately 400 ml. of 0.5 *N* methanolic KOH. After the mixture settles, it is decanted and then washed to neutrality with methanol on a Buchner funnel. The Celite is then spread on a flat pan and dried in an oven at 100°. The product is screened for the second time, usually with some losses due to both larger and smaller grades (as much as 25% loss). Dried Celite of the desired mesh fraction may be stored over solid KOH in a desiccator, but also can be mixed immediately with stationary phase.

B. Preparation of Apiezon Stationary Phase for Non-Polar Columns

Apiezon-M or -L²² is weighed into a 400 ml. beaker, which is placed in an oven at 100°. Size-graded, alkali-treated Celite of the desired size range (either 120-140 or 140-170 mesh) is separately weighed and brought to 100° in a similar manner. When the stopcock grease is thoroughly melted, the Celite is added and stirred gently for at least 5 minutes to ensure complete mixing of the two substances. When cool the mixture can be used immediately for the packing of columns, or it can be stored in a

desiccator for future use. Alternatively, the weighed Apiezon may be dissolved in petroleum ether (30-60°) on a steam bath; the Celite is added to this solution. While evaporating the solvent, the mixture is vigorously agitated (not swirled). Final evaporation of solvent is carried out in a 100° oven. In both methods the final product is a dry, pale yellow powder.

The proportion of Apiezon to Celite depends on the needs of the investigator. Keulemans (10, p. 147) discusses theoretical and practical points involved in the choice of the proper ratios. Increased column efficiency results if the film of stationary liquid is very thin, since this allows more rapid transfer of methyl esters between liquid and gaseous phases. Since the ionization chamber detector is highly sensitive, columns packed with low ratios of Apiezon to Celite have adequate capacity: thus, for optimal column efficiency in analytical GLC, it is recommended that the proportion of Apiezon to Celite be in the range of 15/100. When large sample loads of methyl esters are applied in preparative GLC, a proportion of 25-40/100 may be used. The less sensitive gas-density balance detector demands columns of higher capacity, and proportions of Apiezon to Celite of 25-40/100 may be required for the larger sample loads needed to produce satisfactory detector response.

C. Preparation of Polyester Stationary Phases for Polar Columns

Polyester stationary phases are prepared in much the same manner as described for Apiezon. Reoplex-400 (polyoxyalkylene adipate polyester)²³ mixed with 120-140 or 140-170 mesh Celite (acid- and alkali-treated) has produced satisfactory results in proportions of 20-25/100. Ethylene glycol adipate polyester (EGA) (for preparation, see Appendix D) is dissolved in chloroform and filtered to remove insoluble matter. An appropriate amount of this solution, to give a final ratio of 20-25 parts EGA to 100 parts Celite, is mixed with the solid support and the chloroform is evaporated off on a rotary still or steam bath. The product is freed of chloroform in a 100° oven.

²¹ Personal communication.

²² Metropolitan-Vickers Electrical Company, Ltd. Product distributed in U. S. by James G. Biddle Company, Philadelphia, Pennsylvania.

²³ Geigy Pharmaceuticals, Ardsley, New York. (The exact chemical composition of this material has not been furnished by the Geigy Company.)

D. Packing of Columns

The stationary phase mixtures are packed into the glass columns as follows: 20 cm. of glass thread is inserted as a wad into the tip of the 1.42 meter glass column and tamped down, then 2 to 3 cm. of uncoated 140–170 mesh Celite is poured in the column and tamped down. The column is filled to half its length with coated Celite; this is packed down by vibration. Vibration is carried out by holding the glass firmly against a rapidly rotating metal rod with a flat along one side to enhance vibratory action. Vibration is started at the drawn tip and proceeds in one direction only, the column being drawn past the rod and simultaneously rotated. The total length of time for this vibratory pass is held at about 15 seconds. Usually the material settles approximately 10 cm. The column is then filled to the top and packed by vibration in a second 15-second pass, then refilled until the packing totals 1.34 meters. Finally, a second 20 cm. length of glass thread is wadded and placed on top of the packing with gentle tamping. Each column should be carefully labeled, and appropriate records kept of its history and performance.

E. Conditioning the Columns

More or less stationary phase "bleeds" from every column when it is heated to the high temperatures used in GLC. It is helpful to condition the column at the highest temperature at which it is to be used (at least 12 hours (Apiezon) to several days (polyesters)). This can be done in the vapor jacket itself, but possible instability of the base-line during this period may prevent completion of analytical runs until the column is thoroughly conditioned. For conditioning new columns it is helpful to use a 1.37 meter tubular heating jacket²⁴ in which the columns can be heated at any desired temperature while nitrogen or argon is passed through at a slow rate (15 ml./minute).

F. Inserting and Changing Columns

The column is wiped free of grease and fingerprints with petroleum ether, the silicone gasket is applied, and the assembled column is inserted into the heating jacket. When the tip reaches the detector it is seated firmly by pressing it in with

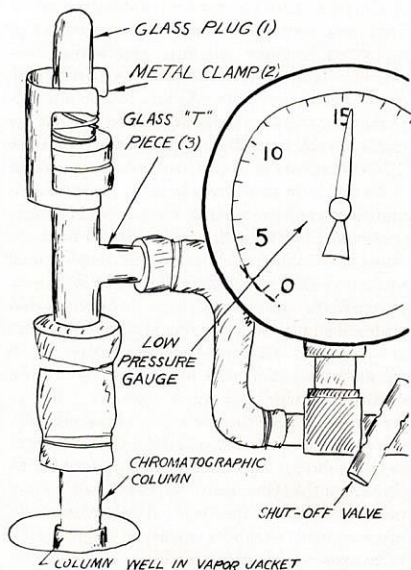


Fig. 9. Details of column inlet assembly

a twisting motion. Argon is led into the column through the assembly shown in Figure 9. The smooth-lapped glass plug²⁵ permits rapid opening of the column when samples are to be charged to the column.

Columns are removed while hot by disconnecting the gas flow, permitting the pressure to drop, and then lifting out the column using asbestos gloves or fingers. The column is unseated with a twisting motion.

When not in use, columns should be capped at both ends to prevent admission of water vapor and dust.

VI. Preparation of Samples of Fatty Acid Methyl Esters for Chromatography

The calibration of newly installed GLC apparatus and the interpretation of analytical results is greatly facilitated if certain precautions are observed in the preparation of samples of fatty acid methyl esters. After extraction of

²⁵ Components 1, 2, and 3 (Fig. 9) are available as "Gas Inlet Assembly", O. H. Post Scientific Instrument Company, 68-22 60th Road, Maspeth, Long Island, N. Y.

²⁴ 260 watts, 110 volts, serial # 27.5004, Glas-Col Apparatus Company, Inc., Terre Haute, Indiana.

lipids, it is convenient to separate the various fatty acid-containing fractions by chromatography on columns of silicic acid (16). Non-esterified fatty acids are isolated by partitioning out of organic solvents into an alkaline aqueous phase (17). It is then necessary to form the methyl esters of each fraction, and to free these of other lipid contaminants. It has been found convenient and reliable to form the esters by transmethylation in superdry methanol, and to free them from contaminants by sublimation (18). This procedure has been preferred to saponification followed by methylation with diazomethane, for it appears to avoid the structural changes in polyethenoid acids which may occur during alkaline hydrolysis and diazomethanolysis. The methyl esters washed off the sublimation cold finger are produced in quantitative yield and are colorless.

Storage of polyethenoid methyl esters in solution in petroleum ether (30–60°) at 4° or lower in the dark appears to be satisfactory for at least three months. One-ounce screw-top bottles are used with plastic caps lined with aluminum foil. Stoppers made of rubber or cork are emphatically to be avoided. Samples stored in petroleum ether are conveniently freed of solvent in a stream of nitrogen in a hot alcohol bath, with last traces removed under reduced pressure at lower than 25°, or at room temperature under a stream of nitrogen.

All preparative steps are carried out under a blanket of nitrogen. Evidence has been presented that structural changes do not occur in polyethenoid methyl esters during GLC at 197° on columns of Apiezon (19).

VII. Sample Loading

Gas flow to the column is interrupted by closing the valve of the low-pressure gauge (Fig. 9). A period of 15–20 seconds is required to lower the head of pressure of argon to about 4 lbs./sq. in., at which point the column is opened. By opening the shut-off valve slightly a very slow flow of argon is run continuously to the column head. The loading pipette is inserted to rest upon the wadded glass thread at the head of the column, and the sample is milked out by gentle pressure on a short piece of soft lightweight rubber tubing²⁶ attached to the pipette. The con-

tinuous flow of argon prevents admission of excessive amounts of air into the system which occurs when the loading pipette is withdrawn from the column. The column head is closed and gas flow re-established.

Speed in loading is advantageous, especially in the case of polyester columns. The longer the interruption of gas flow, the more stationary phase bleeds off when gas flow is begun again, hence the more distortion of base-line at the beginning of the run. Only when the pressure has decreased to approximately 4 lb./sq. inch should the column be opened: if too little time is allowed, the continuity of the column will be disrupted by sudden release of pressure; if too much time elapses, unnecessary base-line distortion occurs. With practice it becomes simple to keep the total loading time to 40 seconds or less.

VIII. Calibration of Instrument

In order that the instrument be effective for quantitative analysis, it is essential to test whether the ionization current recorded on the moving chart is directly proportional to the amount of fatty acid methyl ester applied to and eluted from the column. This "linearity of response" must be assured for a fairly wide range of voltages and sample sizes. Since one desires the greater sensitivity present at higher voltages (7), one need not test for linearity at the lower range of voltage. Tests for linearity of response of the detector can be carried out only when the columns and the detector are not overloaded. Records which are typical of column overload and detector overload are shown in Figure 10.

Tests of linearity of response are most satisfactorily performed on Apiezon columns by chromatographing a mixture of methyl esters, the exact composition of which is known. This "standard mixture" is best composed of three or four components, each present in a different concentration, such as: myristate 7.5, palmitate 50 and stearate 42.5, or myristate 25, palmitate 25 and stearate 50, all as percentages by weight. *These proportions are purposely chosen in order that the peak heights vary widely.* Clearly, it is not a proper test of linearity when the peak heights of successive components are all the same. The composition of the standard should be determined by 1) accurately weighing in known amounts of pure reference compounds, or 2) determination of

²⁶ Obtained as "Red-Stitched Rubber Tubing" #896 $\frac{1}{16}$ " \times $\frac{1}{32}$ ", Hospital Accessories Company, Woodside, N. Y.

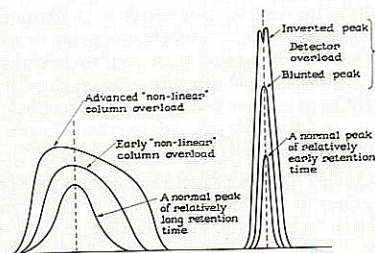


FIG. 10. Example of patterns typical of various degrees of column and detector overload.

the composition of an approximate mixture of these acids (not necessarily pure) by GLC with gas-density balance detection. In the latter case, any minor components appearing as contaminants are neglected in calculating the percentage composition of the major components.

In the absence of column and detector overload, the ideal or linear response is demonstrated when the percentage composition of the standard mixture is the same as that known to be present. Linearity of response is achieved by finding the proper balance between applied voltage and magnitude of series resistor²⁷. With the geometry of the detector chamber shown in Figure 6, the response was linear with voltages of 700–1800 and with series resistors of 1×10^9 to 3×10^{10} ohms. This linearity was present through a 50-fold range of sample loads applied (0.01–0.5 μ l. of standard mixture). With series resistors of 1×10^{10} ohms or less, the response was non-linear when more than 0.2 μ l. of standard mixture was applied; the component showing the highest peak height was over-estimated. With series resistors of more than 3×10^{10} ohms the same component was under-estimated.

If construction details given above are followed, the response of the instrument should be linear if tested under similar circumstances. However, if changes in chamber design or electronic circuitry are made, one must be prepared to find the range of voltages and resistances which

ensures linearity. *In any circumstances, linearity of response must be evaluated with each ionization chamber detector.*

IX. Sensitivity of Instrument

The small sample sizes (0.1 to 0.05 μ l. of mixtures containing about 40 components) which are applied to the instrument attest to the remarkable sensitivity of this argon ionization detector. When these small samples are chromatographed, individual components of approximately 0.05 percent of the total by weight are measured with accuracy. To define this sensitivity in other terms: one can readily detect 1×10^{-3} moles of methyl laurate, or one part of methyl laurate per 1×10^5 parts of argon, when 1200 volts is applied across the detector. No attempt has been made to define maximal sensitivity, particularly since this necessarily includes measurement of base-line stability and background "noise", two terms that lack adequate definition. If one desires to increase the sensitivity, this can readily be achieved by an increase in the strength of the strontium source or by an increase in operating voltage. Further increases in sensitivity, necessary if capillary columns are used, can be attained by a modification of the chamber design (32).

X. Operation of Instrument

A. Selection of the Column

For preparative runs, columns of high capacity must be used (wide bore columns with large ratios of stationary phase to solid support). The sensitivity of the circuitry must be reduced so as to record all the peaks within the width of the chart. It can be advantageous to make the run at reduced temperatures; this will increase the separation and resolution of components on both non-polar (20) and polar columns (see Appendix C).

For analytical runs the primary requirement is a column of great efficiency, with at least 3500 theoretical plates for Apiezon and 2000 plates for the polyester packings (measured at 18:0, see below). Capacity is not required since the strontium-90 detector is capable of sensing extremely small quantities of effluent esters. Thus, it is possible to use low ratios of stationary phase to solid support; these give low capacity but high efficiency. If the recommended packing methods and column lengths are used, the chromatograms are rapidly developed. Under

²⁷ Series resistors obtained as "Glass-sealed, Hi-Meg resistors, Type RX-1", from the Viotreen Instrument Company, Cleveland, Ohio. The following resistors are needed to perform a test for linearity: 5×10^8 and 5×10^9 ohms, one of each; 1×10^9 and 1×10^{10} ohms, three of each; and 1×10^{11} , one.

such conditions it is helpful in measuring areas precisely to use chart speeds of 24-60 inches per hour.

The efficiency of one column can be compared with another by determining the theoretical plates of each (see Appendix B for the method of calculation). This calculation is commonly made for the methyl stearate band by biochemists working with most mixtures of long-chain fatty acids of biological origin, but any component can be selected. A triangle is fitted by eye to the curve of the ester chosen for this calculation.

The resolution of two adjoining peaks on a column is calculated (see Appendix B) when one wishes to determine the ability of a particular column to resolve components from each other.

B. Selection of Sample Size

For preparative runs on columns of 4 mm. internal diameter, samples of 5-10 μ l. can be loaded; even larger samples can be accommodated on wider columns packed with larger concentrations of stationary phase, particularly if the components to be separated do not require highly efficient columns.

For analytical runs, the choice of sample size depends on the number of components in the sample and their emergence times. Thus, esters of high volatility appear early in the run in a high narrow peak, whereas the same amount of a higher molecular weight ester will appear later in a flatter broader peak. In the former case the detector is presented with a higher concentration of effluent ester at the time of its emergence than in the latter case, and thus the detector and column are more easily overloaded by esters appearing earliest in the run. These considerations require that the operator apply small loads when components are few, or when the components emerge early in the run; larger loads are tolerated when the sample contains many esters and when these components emerge late in the run. With samples of unknown composition it may be necessary to do a pilot run to learn the approximate make-up of the mixture, in preparation for a second run under optimal conditions. In this laboratory it has been expedient to use loads of 0.1-0.2 μ l. when the mixture contains up to 40 components, but as little as 0.025 μ l. may be required when the components are few in number and rapid in emergence. It is a good general rule to apply the smallest sample which permits accurate calculation of percentage areas, thus, under-

loading the column and detector as much as possible. In practice, detector overload is not encountered if samples are kept so low that optimum column efficiency is approached.

Micropipettes are available down to 0.025 μ l.²⁸

C. Selection of Gas Flow

The gas flow which gives optimum column efficiency is determined empirically for each column. The resistance to gas flow of most 4 mm. columns is such that from 10-15 lb./sq. inch of argon pressure is needed for optimum flow rates (30-50 ml. of argon/minute). Increase of flow above optimum leads to less of a decline in column efficiency than is the case for reductions of flow rates (10, p. 142). One may wish to operate at higher than optimum flow rates, if the time thus saved warrants the reduction in column efficiency that occurs. However, it is rarely wise to lower the flow below the optimal rate, since the efficiency declines rapidly under conditions which permit longitudinal diffusion of vapors to occur in the column.

D. Selection of Column Temperature

Because the earlier heating systems were vapor-jacketed, many investigators have had experience with GLC at 197°, the boiling point of ethylene glycol, used originally by James and Martin (20). Runs made at 197° have given exceedingly useful information, and tables of retention volumes have been published for a wide variety of fatty acid esters run on Apiezon and on Reoplex-400 (19, 20, 29). After proper conditioning, Apiezon-M columns "bleed" very little at 197°, Apiezon-L even less, but many of the polyesters bleed excessively at this temperature, limiting their usefulness for preparative purposes. Lower temperatures are now being used for polyester columns. This has the advantage of reducing the amount of bleeding and lengthening the life-expectancy of the column. An additional advantage results from improved resolution between components; this is partly offset by the slower rate of emergence of all the acids at lower temperatures (for example, note the differences in specific retention volumes at three different temperatures—Appendix C, Table C-1). To suit

²⁸ Scientific Glass Company, 100 Lakewood Terrace, Bloomfield, New Jersey, or W. G. Pye and Company, Cambridge—distributed by Jarrell-Ashe Company, Newtonville 60, Massachusetts.

the needs of his laboratory the investigator may choose to improve resolution at the expense of increasing the time of analysis, or vice versa.

It is to be expected that tables of relative retention times and specific retention volumes will be available in the near future for a wide variety of working conditions (stationary phases and column temperatures) (but see Appendix C). This work will be greatly facilitated by the general availability of a wide assortment of pure acids of known chemical structure.

E. Selection of Voltage and Sensitivity

Clear recommendations cannot be made for selection of voltage and sensitivity, for it is not known how closely the performance of one apparatus designed as described above will duplicate that of another. In this laboratory it has been found most useful to work at one voltage (1400 V) and to obtain variations in sensitivity over a 1000-fold range by use of the 11-step range selector of the amplifier. This is much to be preferred to the use of several voltages in a single run, where the response at different voltages must be determined experimentally in order to obtain a table of factors to equate voltage steps with gain in sensitivity.

F. Selection of Chart Speed

When using a single chart speed, a compromise must be made between drawing overly narrow peaks at early retention times and overly long flat peaks late in the run. Thirty inches per hour has proven generally useful in this laboratory, where major attention has been given to esters emerging later than methyl laurate. It is more desirable to run the chart at 30 inches/hour through the C_{18} acids, then at 15 or 10 inches/hour through the C_{22} acids.

G. On-off Arrangements

Although some laboratories maintain a steady flow of argon continuously, night and day, as well as continuous column heating, it has been the practice in this laboratory to switch the heater off each day when the instrument is not in use, primarily in order to preserve the life of the column. By means of the two clock-timers the heater can be switched ON 2-3 hours before the beginning of the workday, and OFF after completion of the last run each day. The "off" switch also interrupts the operation of the re-

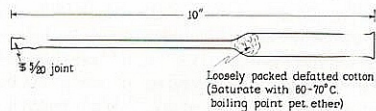


FIG. 11. Glass tube for collection of effluent methyl esters at outlet of ionization chamber.

corder, the high voltage supply and the current amplifier. As a minimum requirement, a timer is recommended to switch off the chart motor in the recorder; this permits a last run to be started at the end of the day and for the chart to be discontinued (to save paper) at a predetermined hour.

H. Collection of Effluent Esters in Preparative GLC

Methyl esters can be recovered almost quantitatively from the detector, individually or in groups, when it is desired to learn their chromatographic behavior on another stationary phase, or when further studies of structure are to be made by independent means. The esters emerge from the column at high temperature and condense rapidly from the vapor state on reaching a cooler environment. To obtain 85 to 90% recovery of an ester a glass collection device (Fig. 11) is inserted over the short, tapered stainless steel effluent tube. Trapping of vapor is incomplete at high flow rates unless a cotton plug is inserted in the wide end of the tube. This cotton should be saturated with petroleum ether (60-70°).

The design of the detector (Fig. 6) with its easily accessible gas outlet facilitates: 1) collection of effluent esters isolated during GLC, 2) periodic measurement of flow rates with a soap bubble flow meter, and 3) the attachment of other apparatus to the GLC instrument, such as the continuous scintillation counter of Popják *et al* (21) or the Bendix Time-of-Flight mass spectrometer.

XI. General Maintenance

A. Detection Chamber—Radiation Safety and Cleaning

The Atomic Energy Commission requires that the laboratory be licensed to possess the radioactive source and that certain safety precautions be observed. Among these requirements are that the personnel wear radiation film badges²⁹ and

²⁹ Supplied as "Film Badges" by Nucleonic Corporation of America, Brooklyn, N. Y.

that the radiation safety of the source be tested every six months.

These tests include: 1) a "wipe" test to determine if there is any leakage of strontium-90 through the foil in which it is enclosed (preferably performed by the supplier of the source) and, 2) measurements of external radiation of the gamma rays formed secondary to the impact of the beta particles with the brass chamber. (The primary radiation of the source consists of beta particles; these, of course, do not penetrate the chamber.)

The results of repeated wipe tests carried out by this laboratory and by U. S. Radium Corp.³⁰ indicate that investigators may have great confidence in the safety of this method of foil enclosure of strontium-90 at temperatures to at least 230°. The amounts of radioactivity detected in these wipe tests averaged <0.01% of the level considered permissible by the Atomic Energy Commission. In addition, when effluent gases from the instrument were passed through an aqueous solution of ethylene-diamine tetraacetic for periods up to one week, there was no evidence of radioactivity trapped in this solution.

A very low level of external radiation³¹ (maximum 1.4 milliroentgens/hour) is present at the surface of the $\frac{1}{4}$ inch lead shielding surrounding the chamber. One foot away this diminishes to 0.1 milliroentgens/hour, and at 30 inches from the detector the radiation is identical to the normal background level of approximately 0.02 milliroentgens/hour.

If a higher strength source is used, or if the detector is placed at eye or waist level, then it is advisable to increase the amount of lead shielding or to increase the thickness of the wall of the brass chamber. If very sensitive radioactive counting equipment, such as a scintillation counter, is used within 2 feet of the detector, increased shielding is advised.

During the eighteen months of constant use of this detector, no significant level of radiation has been detected by the monitoring badges worn by the personnel.

It is advised that the detector be cleaned every

³⁰ U. S. Radium Corporation, Morristown, New Jersey.

³¹ Measured with a Tracerlab SU-5A Survey Meter, Tracerlab Inc., Waltham, Massachusetts.

six months by immersion for 30 minutes in hot chlorobenzene, then rinsed in distilled water.

B. Vapor Jacket Heater

Boiler fluids are rarely completely thermostable, and must be topped up weekly and completely replaced each month with washing out of the boiler. The low vapor pressure, non-toxic fluids of different boiling points listed in Appendix E have proven acceptable as boiler fluids.

C. Silicone Gaskets for Columns

The silicone gasket fitted at the end of the column cracks after continuous exposure to high temperatures and should be renewed every two weeks. Each time a column is inserted, it is best to use a new gasket.

D. Care of Micropipettes

Pipettes for loading the column should be rinsed in acetone and thoroughly dried by suction before and after each use. Complete cleaning is facilitated by a rinse through hot sodium dichromate cleaning solution. It is advisable to clean the outside of the pipette with acetone before charging the column.

XII. Quantitation and Identification of Chromatograms

Using a recording system which draws a differential curve, it is necessary to measure areas under peaks and to calculate the percentage of the total represented by each peak. The identification of the various peaks must then be made. The problems of quantitation and identification will be considered in that order.

A. Quantitation

Areas under peaks can be measured by planimetry, by cutting out the curves and weighing the papers, by triangulation, or by automatic integration. In this laboratory the first three methods were critically compared: results were almost identical. Since triangulation is technically simpler and more rapid than the first two methods, this procedure (22) has been used routinely.

Straight lines are drawn by eye along the two slopes of a curve, so as to contact these slopes at their points of inflection. The base line of the

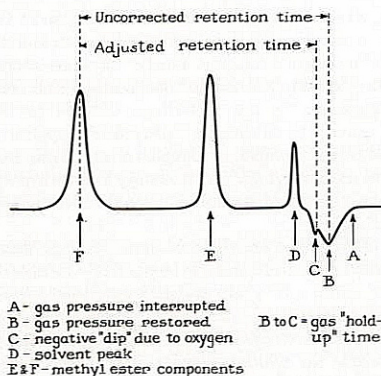


Fig. 12. Schematic diagram of chromatogram to show base-line changes at start of run. Corrected retention times are calculated from the air dip C.

entire chromatogram is drawn in; ideally, this is a straight line throughout the run, but sometimes there is a smooth downward-sloping curve to the true base-line shortly after the start of the run. The areas under all curves are calculated (height $\times \frac{1}{2}$ base) and recorded alongside the appropriate separation factors of each. The areas are totaled and the percentage of each component is calculated. A calculating machine greatly facilitates this laborious procedure.

Reliance can be placed on the quantitative nature of these chromatographic techniques because of the remarkable uniformity of replicate analyses. As an example of this close replication, the coefficients of variation³² of each of 5 components of a "standard" fatty acid methyl ester mixture were less than 1.5 percent. These data were calculated from 8 successive analyses on Apiezon-M, at the same temperature and applied load, and at a range of voltage from 700 to 1400. The mean and standard deviation of the individual components (calculated on each analysis as the percent of total esters) was: 14:0 = 12.8 ± 0.17 ; 16:0 = 22.9 ± 0.17 ; 18:1 = 32.9 ± 0.24 ; and 18:0 = 31.92 ± 0.27 .

B. Identification

A vertical line is drawn at the air "dip" from which all adjusted retention times (t_R') are calcu-

³² Coefficient of variation = (standard deviation \div mean) \times 100 per cent

lated (see Appendix B). The sequence of events at the start of a record and the appearance of the air dip are shown in Figure 12. The adjusted retention time of each peak (t_R') is calculated in terms of distance of each peak from the air "dip". (This "retention distance" is, of course, convertible into gas volume since one knows the gas flow rate, but this calculation is rarely necessary.) The t_R' of each peak is then related to any commonly observed component appearing midway in the run. In many analyses of fatty acids of biological origin methyl stearate is conveniently used as this internal standard. Thus, the separation factors (r) or relative retention factors of all peaks are calculated by dividing the t_R' of each component by that of the internal standard (see Appendix B for method of calculation). This method of identification of peaks corrects for variations in absolute retention volumes that occur with different columns of varied length and internal diameter, or with the same column at varied flow rates. Results also are independent of the proportion of stationary phase to solid support.

It is frequently useful to calculate the separation factor (r) of a $-\text{CH}_2-$ unit (20). The separation factor between successive straight chain saturated fatty acid esters on Apiezon columns at 197° is approximately $1.54 \left(\frac{t_R' 15:0}{t_R' 14:0} = 1.54 \right)$, whereas at this temperature on Reoplex and EGA columns r is about 1.33 (see Appendix C, Tables C-2 and C-3). Thus, the positions of all members of this series can be located after identifying any two members of the series.

Certain generalizations may be made on the retention behavior of fatty acid classes on the two types of columns. Figure F-1 (Appendix F) illustrates that on Apiezon the unsaturated esters precede their saturated straight-chain homologs, and never elute earlier than the saturated ester which is one CH_2 unit shorter. In addition, with increasing unsaturation retention times decrease progressively. However, there are some exceptions to this rule, such as the coincident elution times of the acids 18:2 and 18:3 and of 20:4 and 20:5 (20, Table C-2 and Fig. F-2). Isomers differing only in double-bond position generally have similar retention times on Apiezon (Table C-4).

In contrast, on the polar columns described herein the unsaturated fatty acids are eluted

after their saturated homologs, and without exception they are retained progressively more as unsaturation increases (26, 28, Tables C-3 and C-4 and Fig. F-2). Frequently isomers differing only in double-bond position will resolve partly or completely (19, 27 and Table C-4). Unfortunately, the unsaturated esters containing more than 2 double bonds will ordinarily be retained to such a degree that they elute close to the next higher chain length saturated component. Thus, troublesome overlaps should be anticipated between tri-hexaenes of one chain length and the less unsaturated acids of longer chain length. For example, 16:4 is imperfectly resolved from 18:0, and 18:4 appears between 20:0 and 20:1 on either Reoplex-400 or EGA. Improved resolution is obtained by a decrease in operating temperature, and by use of columns of highest possible efficiency.

On the basis of the studies thus far completed on simple *branched* saturated acids (iso-, ante-iso- and neo-acids, see Table C-1 and Figs. C-1, C-2, C-5 and C-6) it can be expected that branched acids will always have shorter retention times than their straight chain saturated homologs. Systematic study of more highly branched acids has not yet been made, but it can be expected that the greater the branching the shorter the retention time. Extremely complex situations can be anticipated with unsaturated branched acids.³³

Preliminary identification of peaks can be made by reference to tables of relative retention factors (Appendix C) *when column temperatures coincide*. Ideally, each laboratory should determine its own tables of *r*'s on esters of known purity, but published tables will be helpful in identifying unfamiliar peaks. Comparison with published tables of relative retention factors will be more meaningful if the components of an unknown mixture are analyzed on different stationary phases and at two or more temperatures. Figures C-1 to C-4 (Appendix C) illustrate variations in relative retentions at different temperatures. The fact that straight-line relationships between retention and temperature are shown for almost every acid examined indicates that one may safely predict retentions at temperatures between those listed.

Unknown components may be assumed to be unsaturated if they disappear when rechromato-

graphed following bromination (see Appendix G) or hydrogenation (see Appendix H) of an aliquot of the original mixture. Further steps are necessary to establish double-bond position and configuration. By use of techniques adapted to the relatively small samples collected in preparative GLC the number of double-bonds (alkali isomerization and UV spectroscopy) and their position (oxidative ozonolysis) can be determined (27, 27a).

This section ends on a word of warning. Many fatty acid esters remain to be positively identified; meanwhile, they are assigned structures which are suggested by their relative retention factors or specific retention volumes. These particularly include the odd-numbered and branched acids. Preliminary study of a few complex branched acids (other than those listed in Table C-1 and Figs. C-5 and C-6) indicates that multiple internal branching *markedly* decreases the relative retention time on Apiezon. Thus, 3,7,11-trimethyldodecanoic acid has a retention time (relative to 18:0) of 0.131 at 197° on Apiezon;³³ therefore, this C₁₅ acid emerges well ahead of 14:0. The hazards of assigning structure on the basis of a single relative retention time must be emphasized. However, by the use of retention volume data from analyses made on various stationary phases and at different temperatures, coupled with hydrogenation, bromination and degradation techniques, it is likely that GLC will play an increasingly important role in elucidating the structure of unknown substances.

C. Percentage Composition

It has proved expedient when resolving complex mixtures of C₈ to C₂₂ esters to calculate the percentage composition of the C₈ to C₁₈ esters from an Apiezon chromatogram, and the C₁₈ to C₂₂ esters from an analysis on EGA. A component which appears as a single peak on both runs is selected (*usually* methyl stearate) and the results of the two runs are intercalated by relating all areas to the area ratio of the common component $\left(\frac{\text{area of 18:0 on EGA}}{\text{area of 18:0 on Apiezon}} \right)$. Thus, if the area of 18:0 on the EGA run is one-half that on the Apiezon run, all single-component areas on the EGA analysis are doubled and added to the single-component areas of the Apiezon run. The proportion of each component in the total is then calculated (clearly, no component is counted

³³ G. Popják, unpublished data.

twice, and all single-component areas must be included). The accuracy of this calculation can be improved by using as common denominator the average area ratio of more than one common component (i.e., $18:0 + 18:1 + 18:2 + 18:3$).

The accuracy of these calculations can be checked in three ways. 1) The iodine value of the mixture determined from the calculated percentage composition data can be compared with its actual experimental value. 2) An aliquot of the original mixture can be hydrogenated completely (Appendix H), then this product chromatographed. The percentages calculated for each saturated ester in the hydrogenated sample should correspond to the total of all esters, saturated and unsaturated, assigned that chain length in the composition analysis of the original sample. 3) An aliquot of the original mixture can be brominated (Appendix G); the chromatogram of the product will show only the saturated esters originally present. The ratios of these to each other should correspond to the ratios of the saturated esters in the composition analysis of the original mixture.

XIII. Summary

The expanding technology of GLC now provides scientists with remarkably effective means for separation of organic compounds. Many applications of GLC to the separation, identification and quantitation of one class of organic substances (fatty acids) have been described in this report, and detailed instructions have been given on methods of construction and maintenance of a stable and sensitive GLC instrument.

The future will likely see extension of this form of chromatography to an increasing number of biological and chemical problems. Then as now, the very excellence of the techniques will undoubtedly reveal these problems to be of greater complexity than initially predicted, and continuing improvements in technique will be necessary to keep pace with the demands.

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Appendix A

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Appendix B

Glossary of GLC Nomenclature

Formulae Used in the Calculation of GLC Constants of Fatty Acid Methyl Esters

These formulae are in agreement with the recent recommendations made by a subcommittee of the International Union of Pure and Applied Chemistry (D. Ambrose, chairman).

1. Retention time (uncorrected) = t_R = time from sample injection to the midpoint of the symmetric elution curve of any component. When samples are loaded by the gas flow-interruption technique described herein, t_R is measured from the time of restoration of gas pressure. However the t_R is consequently made uncertain by the time taken to re-establish gas pressure at its equilibrium level.

2. Gas hold-up time = t_{gas} = time from injection of inert gas, such as air, to the beginning of the "air dip" (see Fig. 12).

3. Adjusted retention time = $t_R' = t_R - t_{\text{gas}}$. These parameters are conveniently measured in cm., rather than in min.

4. Number of theoretical plates = $n = 16 \left(\frac{t_R}{W} \right)^2$,

where W = the base width of the component (in same units as t_R). In this calculation t_R rather than t_R' is used.

5. Height equivalent to a theoretical plate = $HETP$ = column length (in cm.) \div n .

6. Relative retention or separation factor = $r = \frac{t_{R'} \text{ component A}}{t_{R'} \text{ component B}}$. To determine the relative retention factors of a series of components, B , the internal standard, should be chosen approximately midway in the series in elution time.

7. Retention volume (uncorrected) = $V_R = t_R F_c$, where F_c = flow of carrier gas at the temperature of the column, measured at the outlet pressure.

8. Retention volume (corrected for pressure

drop across column) = $V_R^0 = V_R f$, where

$$f = \frac{3/2 (P_i/P_o)^2 - 1}{(P_i/P_o)^3 - 1}, P_i = \text{inlet pressure and}$$

P_o = outlet pressure.

9. Adjusted retention volume = $V_R' = t_R' F_c$.

10. Net retention volume = $V_{R'}^0$ (or V_n) = $f V_R' = f t_R' F_c$.

$$11. \text{ Specific retention volume } (V_g) = \frac{F_m f z}{u} \cdot \frac{273}{T_m w}$$

where T_m = temperature of the flow meter in degrees absolute, F_m = flow in cc./min. at T_m , corrected for the vapor pressure of water (assume complete saturation in a soap bubble flow meter), u = recorder chart speed in cm./min., $z = t_R'$ in cm., w = total weight of stationary phase in grams; f = pressure gradient correction factor. V_g = volume of gas per gram of stationary phase to elute a given component. For every V_g the column temperature must be specified.

12. Performance index of Golay (11) = PI (at any component) = $\frac{(W)^4}{t_R^3} \cdot \frac{t_{\text{gas}} \Delta P}{t_R - \frac{1}{5} t_{\text{gas}}}$, where W = base width of component, and ΔP = the pressure drop across the column. The units of PI are in poise ($\text{cm}^{-1} \text{ sec}^{-1}$), a unit of viscosity. A low PI indicates a favorable column; speed of elution and column efficiency are both considered.

13. Component resolution = $\frac{2\Delta y}{y_a + y_b}$, where Δy = distance between any 2 peak maxima, and y_a and y_b = base widths of components a and b .

14. Partition coefficient K

$$= \frac{\text{wt. of solute/ml. stationary phase}}{\text{wt. of solute/ml. mobile phase}} = \frac{V_g T_c P_L}{273}$$

where T_c = temperature of column and P_L = density of stationary phase at T_c .

Appendix C

GLC Constants of Fatty Acid Methyl Esters

See references #34 and 35, Appendix A, and formulae in Appendix B, for suggested methods of presentation of gas-liquid chromatographic retention data.

A "shorthand designation" of structure of each fatty acid methyl ester is included in the Tables and Figures, i.e. 18:2 = a C_{18} acid with 2 double bonds, 18:0 = a saturated C_{18} acid, 17:0 br = a saturated branched chain C_{17} acid, 20:un = an unsaturated C_{20} acid of unknown structure, etc. When the precise chemical structure of an unsaturated acid is known, this is shown with asterisks; structures of these acids are given in Table C-4.

Column dimensions in all cases: length of packing = 137 cm., internal diameter = 4 mm.

In the following Tables all retention times are

calculated from the "air-dip" and are calculated relative to methyl stearate in Tables C-1, C-2 and C-3.

TABLE C-1—Retention times (relative to 18:0) of saturated normal and branched-chain fatty acids

| FATTY ACIDS | | STATIONARY PHASES | | | | | |
|---------------|-----------------------|-------------------|---------|---------|---------------|---------|---------|
| Familiar Name | Shorthand Designation | Apiezon m | | | EGA Polyester | | |
| | | 173.5°C | 182.5°C | 197.0°C | 173.5°C | 184.5°C | 197.6°C |
| LAURIC | 12:0 | 0.055 | 0.059 | 0.075 | 0.123 | 0.136 | 0.165 |
| | 13:0 | 0.088 | 0.097 | 0.112 | 0.178 | 0.197 | 0.222 |
| MYRISTIC | 14:0 | 0.144 | 0.154 | 0.174 | 0.252 | 0.272 | 0.302 |
| | 14:0 iso | 0.118 | 0.129 | 0.149 | 0.211 | 0.233 | 0.252 |
| | 15:0 | 0.232 | 0.245 | 0.272 | 0.354 | 0.372 | 0.401 |
| | 15:0 ante-iso | 0.198 | 0.213 | 0.233 | 0.319 | 0.342 | 0.367 |
| PALMITIC | 16:0 | 0.380 | 0.394 | 0.422 | 0.502 | 0.521 | 0.550 |
| | 16:0 iso | 0.312 | 0.329 | 0.370 | 0.429 | 0.449 | 0.472 |
| | 16:0 neo | 0.246 | 0.260 | 0.282 | 0.354 | 0.373 | 0.402 |
| | 17:0 | 0.615 | 0.630 | 0.650 | 0.706 | 0.715 | 0.733 |
| | 17:0 ante-iso | 0.523 | 0.547 | 0.565 | 0.645 | 0.656 | 0.676 |
| STEARIC | 18:0 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| | 18:0 iso | 0.824 | 0.836 | 0.845 | 0.860 | 0.864 | 0.858 |
| | 18:0 neo | 0.650 | 0.666 | 0.681 | 0.710 | 0.724 | 0.723 |
| | 19:0 | 1.62 | 1.59 | 1.56 | 1.41 | 1.38 | 1.35 |
| | 19:0 ante-iso | 1.38 | 1.38 | 1.35 | 1.28 | 1.26 | 1.24 |
| ARACHIDIC | 20:0 | 2.64 | 2.53 | 2.37 | 1.99 | 1.89 | 1.82 |
| | 20:0 iso | | | 2.02 | 1.69 | 1.66 | 1.59 |
| | 20:0 neo | | | 1.62 | 1.40 | 1.39 | 1.35 |
| | 21:0 | | | 3.65 | | 2.56 | 2.46 |
| | 21:0 ante-iso | | | 3.18 | | 2.40 | 2.29 |
| BEHENIC | 22:0 | | | 5.58 | | | 3.27 |
| | 22:0 iso | | | 4.71 | | | 2.91 |
| LIGNOCERIC | 24:0 | | | 13.10 | | | 6.14 |
| | 24:0 iso | | | 11.10 | | | 5.32 |
| | 24:0 neo | | | 9.00 | | | 4.52 |

Non-polar column: 0.88 grams of Apiezon-M stopcock grease to 5.5 grams of acid and alkali-washed, 140-170 mesh Celite-545. Specific retention volume of 18:0 (V_R 18:0) = 7.84 L/gram at 173.5°, 4.94 L/gram at 182.5°, and 2.43 L/gram at 197.0. Performance index = 27.9 poise at 173.5°, 31.6 poise at 182.5°, and 35.8 poise at 197.0° for 18:0.

Polar column: 1.2 grams of EGA to 5.5 grams of acid and alkali-washed, 100-120 mesh Celite-545. Specific retention volume of 18:0 (V_R 18:0) = 512 ml./gram at 173.5°, 298 ml./gram at 184.5°, and 175 ml./gram at 197.0°. Performance index = 243.6 poise at 173.5°, 207.2 poise at 184.5°, and 181.7 poise at 197.0° for 18:0.

Iso = terminal isopropyl group; ante-iso = terminal isobutyl group; neo = terminal neopentane group.

TABLE C-2—Retention times (relative to 18:0) of some saturated and unsaturated fatty acid methyl esters of menhaden body oil (Apiezon-M Stationary Phase)

| FATTY ACIDS | | STATIONARY PHASE | | |
|---------------|-----------------------|------------------|---------|---------|
| Familiar Name | Shorthand Designation | Apiezon - M | | |
| | | 173.5°C | 188.0°C | 197.0 C |
| LAURIC | 12:0 | 0.055 | 0.065 | 0.075 |
| | 13:0 | -- | 0.103 | 0.112 |
| MYRISTIC | 14:0 | 0.144 | 0.162 | 0.174 |
| | 14:0 br | 0.120 | 0.139 | 0.155 |
| | 14:1 | 0.125 | 0.146 | 0.159 |
| | 15:0 | 0.232 | 0.257 | 0.272 |
| | 15:0 br | 0.198 | 0.223 | 0.238 |
| | 15:0 br | 0.192 | 0.216 | 0.230 |
| | 15:0 br | 0.171 | 0.192 | 0.218 |
| PALMITIC | 16:0 | 0.380 | 0.405 | 0.422 |
| PALMITOLEIC | 16:1* | 0.322 | 0.350 | 0.366 |
| | 16:2* | -- | -- | 0.360 |
| | 16:3* | 0.278 | 0.308 | 0.324 |
| | 16:4* | 0.266 | 0.294 | 0.314 |
| | 17:0 | 0.615 | 0.638 | 0.650 |
| | 17:0 br | 0.455 | 0.481 | 0.500 |
| | 17:0 br | 0.511 | 0.538 | 0.554 |
| STEARIC | 18:0 | 1.00 | 1.00 | 1.00 |
| OLEIC | 18:1* | 0.815 | 0.844 | 0.860 |
| LINOLEIC | 18:2* | 0.754 | 0.780 | 0.792 |
| LINOLENIC | 18:3* | 0.754 | 0.780 | 0.792 |
| | 18:4* | 0.661 | 0.694 | 0.720 |
| ARACHIDIC | 20:0 | 2.64 | 2.48 | 2.37 |
| | 20:1* | -- | -- | 1.99 |
| | 20:2* | -- | -- | 1.76 |
| | 20:2* | -- | -- | 1.85 |
| | 20:3* | -- | 1.64 | 1.64 |
| ARACHIDONIC | 20:4* | -- | 1.46 | 1.46 |
| | 20:5* | -- | 1.46 | 1.46 |

Specific retention volumes (V_g of 18:0) closely approximate those stated for the non-polar column of Table C-1 for temperatures of 173.5° and 197.0°. At 188.0°, V_g 18:0 = 3.63 L/gram and the performance index at 18:0 = 52.6 poise.

TABLE C-3—Retention times (relative to 18:0) of some saturated and unsaturated fatty acid methyl esters of menhaden body oil (2 polyester stationary phases)

| FATTY ACIDS | | STATIONARY PHASES | | | |
|---------------|-----------------------|-------------------|---------|---------|------------------------|
| Familiar Name | Shorthand Designation | EGA Polyester | | 197.0°C | Rooplex-400 197.0°C |
| | | 173.5°C | 184.5°C | | |
| LAURIC | 12:0 | 0.123 | 0.136 | 0.165 | 0.180 |
| | | | | | |
| MYRISTIC | 14:0 | 0.252 | 0.272 | 0.302 | 0.320 |
| | 14:1 | 0.301 | 0.316 | 0.343 | 0.360 |
| | 15:0 | 0.354 | 0.372 | 0.401 | 0.430 |
| | Unknown | 0.428 | 0.445 | 0.471 | 0.501 |
| | | | | | |
| PALMITIC | 16:0 | 0.502 | 0.521 | 0.550 | 0.570 |
| PALMITOLEIC | 16:1 | 0.576 | 0.590 | 0.634 | 0.640 |
| | Unknown | 0.629 | 0.646 | - | - |
| | 16:2's* | 0.735 | 0.754 | 0.785 | 0.788 |
| | 16:3* | 0.860 | 0.894 | 0.904 | 0.900 |
| | 17:0 | 0.706 | 0.715 | 0.733 | 0.740 |
| | Unknown | 0.796 | 0.805 | 0.825 | - |
| | | | | | |
| STEARIC | 18:0 | 1.00 | 1.00 | 1.00 | 1.00 |
| OLEIC | 18:1* | 1.12 | 1.12 | 1.12 | 1.11 |
| | Unknown | 1.31 | 1.30 | - | - |
| LINOLEIC | 18:2* | 1.35 | 1.35 | 1.34 | 1.32 |
| | 18:2* | 1.45 | 1.43 | 1.43 | 1.40 |
| LINOLENIC | 18:3* | 1.76 | 1.73 | 1.72 | 1.66 |
| | 18:4* | 2.04 | 1.99 | 1.97 | 1.91 |
| | 19:0 | 1.41 | 1.38 | 1.35 | 1.32 |
| | Unknown | 1.56 | 1.54 | 1.54 | 1.50 |
| | 19:un | 1.68 | 1.66 | 1.64 | 1.59 |
| | | | | | |
| ARACHIDIC | 20:0 | 1.99 | 1.89 | 1.82 | 1.78 |
| | 20:1* | 2.16 | 2.09 | 2.02 | 1.96 |
| | 20:un ? | 2.34 | 2.25 | - | - |
| | 20:2* | 2.48 | 2.40 | 2.32 | 2.24 |
| | 20:2 ? | 2.66 | 2.52 | 2.45 | 2.36 |
| | 20:3* | 3.02 | 2.88 | 2.76 | 2.68 |
| ARACHIDONIC | 20:4* | 3.32 | 3.17 | 3.04 | 2.90 |
| | 20:un | 3.91 | 3.70 | 3.51 | 3.33 |
| | 20:5* | 4.33 | 4.08 | 3.85 | 3.67 |
| | 20:un | 4.75 | 4.42 | 4.11 | 4.00 |
| | Unknown | 5.15 | 4.78 | 4.38 | - |
| | 22:4 | 6.17 | 5.75 | 5.30 | 5.01 |
| | Unknown | 6.40 | 5.99 | - | - |
| | 22:5 | 7.43 | 6.75 | 6.09 | 5.80 |
| | 22:5 | 8.15 | 7.46 | 6.60 | 6.30 |
| | 22:5* | 8.40 | 7.69 | 7.00 | 6.67 |
| | 22:6* | 9.55 | 8.59 | 7.75 | 7.40 |

Column conditions for EGA identical to those in Table C-1. For Rooplex-400 the column conditions were not determined. * = chemical structure known, see Table C-4.

TABLE C-4—Chemical structure and retention times (relative to 18:0) of the unsaturated fatty acids and their percentage in menhaden body oil (29)

| No. | Fatty Acids | | Retention Times, $r \left(\frac{x}{18:0} \right)$ | | Per cent of total fatty acids |
|-----|-----------------------|--------------------------|--|---------------|-------------------------------|
| | Shorthand Designation | Position of Double Bonds | Apiezon 197.0° C. | EGA 197.0° C. | |
| 1. | 16:1 ⁶ to | 9 | 0.366] | 0.634] | 9.8 |
| 2. | 16:1 1 | 8 | 0.366] | 0.634] | |
| 3. | 16:2 4 | 9,12 | 0.360 | 0.785] | 2.0 |
| 4. | 16:2 1 | 6,9 | 0.330 | 0.785] | trace |
| 5. | 16:3 5 | 6,9,12 | 0.324] | 0.904] | 1.3 |
| 6. | 16:3 1 | 7,10,13 | 0.324] | 0.904] | |
| 7. | 16:4 20 | 6,9,12,15 | 0.314] | † | 2.0 |
| 8. | 16:4 1 | 4,7,10,13 | 0.314] | † | |
| 9. | 18:1 | 9 | 0.860 | 1.11 | 14.5 |
| 10. | 18:2] | 9,12 | 0.792 | 1.34 | 2.0 |
| 11. | 18:2] | 6,9 | 0.760 | 1.43 | 0.7 |
| 12. | 18:3] | 9,12,15 | 0.792] | 1.72 | 1.3 |
| 13. | 18:3] | 6,9,12 | 0.792] | † | |
| 14. | 18:4 | 6,9,12,15 | 0.720 | 1.97 | 3.2 |
| 15. | 20:1 | 11 | 1.99 | 2.02 | 2.1 |
| 16. | 20:2] | 8,11 | 1.76 | 2.32 | 0.6 |
| 17. | 20:2] | 11,14 | 1.85 | 2.45 | 0.6 |
| 18. | 20:3] | 8,11,14 | 1.64] | 2.76] | 2.0 |
| 19. | 20:3] | 5,8,11 | 1.64] | † | |
| 20. | 20:4] | 5,8,11,14 | 1.46] | 3.04] | 0.6 |
| 21. | 20:4] | 8,11,14,17 | 1.46] | † | |
| 22. | 20:5 | 5,8,11,14,17 | 1.46 | 3.85 | 12.5 |
| 23. | 22:5 | 7,10,13,16,19 | 3.30 | 7.00 | 2.0 |
| 24. | 22:6 | 4,7,10,13,16,19 | 3.02 | 7.75 | 8.9 |

When known, the molar ratio of isomers is given (i.e. 6 to 1, 4 to 1, etc). The symbol + indicates acids whose r (18:0) has not been clearly defined at the stated column conditions. The 16:4 acids 7 and 8, do not resolve from 18:0 on EGA at 197.0°. The double bond structure of acids 1 through 12, 14, and 20 through 24 were published previously (references 19 and 27). Double-bond positions are numbered from the carboxyl carbon at all times. Acids marked with an asterisk in Tables C-2, C-3 and of Appendix C refer to the acids in this Table.

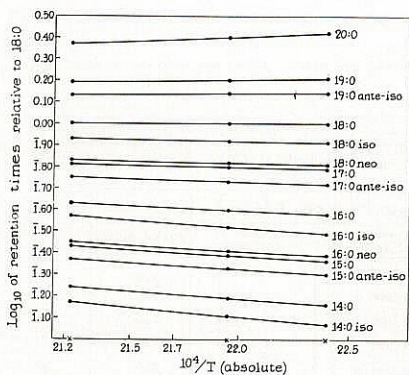


FIG. C-1. Effect of temperature variation on the \log_{10} of the retention times (relative to 18:0) of some saturated normal and branched-chain fatty acid methyl esters. Stationary phase—Apiezon-M. Numerical data and column conditions of Table C-1.

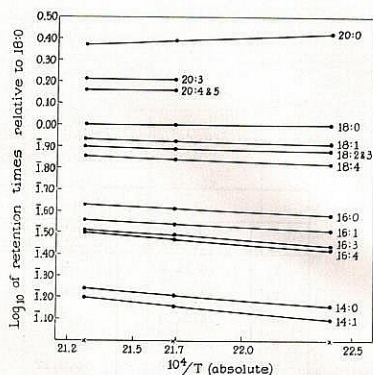


FIG. C-3. Effect of temperature variation on the \log_{10} of the retention times (relative to 18:0) of some fatty acid methyl esters of menhaden oil. Stationary phase—Apiezon-M. Numerical data from Table C-2, and column conditions of Table C-1.

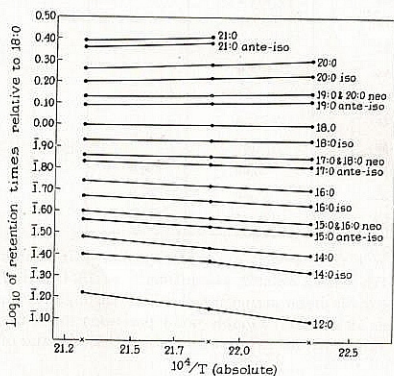


FIG. C-2. Effect of temperature variation on the \log_{10} of the retention times (relative to 18:0) of some saturated normal and branched-chain fatty acid methyl esters. Stationary phase—EGA. Numerical data and column conditions of Table C-1.

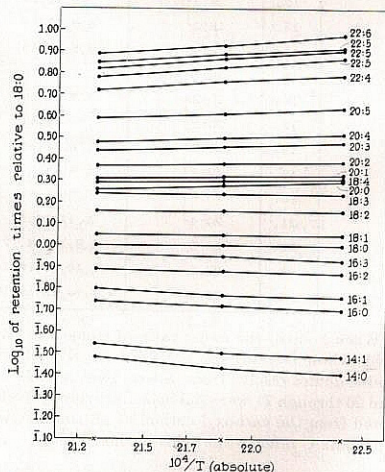


FIG. C-4. Effect of temperature variation on the \log_{10} of the retention times (relative to 18:0) of some fatty acid methyl esters of menhaden oil. Stationary phase—EGA. Numerical data from Table C-3, and column conditions of Table C-1.

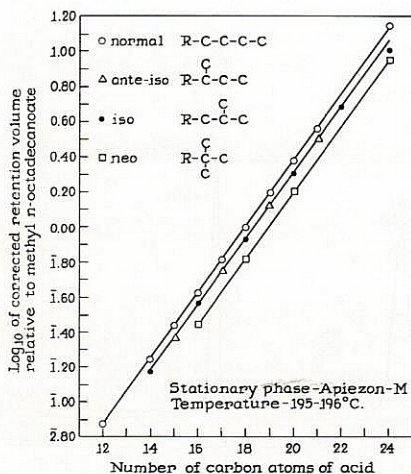


FIG. C-5. Relationship between chain-length and \log_{10} of the retention times (relative to 18:0) of some saturated and branched-chain fatty acid methyl esters at a single temperature (196.0°). Stationary phase—Apiezon-M. Numerical data and column conditions of Table C-1.

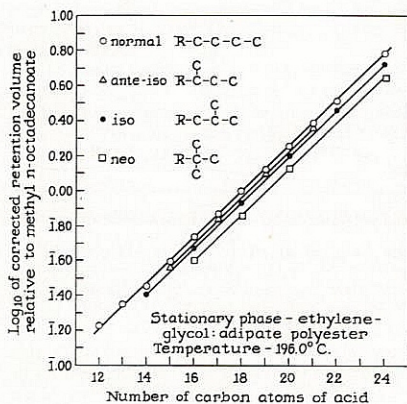


FIG. C-6. Relationship between chain-length and \log_{10} of the retention times (relative to 18:0) of some saturated and branched-chain fatty acid methyl esters at a single temperature (196.0°). Stationary phase—EGA. Numerical data and column conditions of Table C-1.

Appendix D

Preparation of Ethylene Glycol Adipate Polyester (EGA).³⁴

Ethylene glycol³⁵ (32.6 grams) and adipic acid³⁵ (73.1 grams) are mixed in a 250 ml. double inlet round bottom flask. These amounts represent a molar ratio of 1.05/1.00; the 5% excess of glycol forces the reaction to completion, theoretically leaving no free carboxyl groups of adipic acid.

The mixture is heated in a silicone bath to a temperature of 180° while passing a stream of nitrogen slowly over the surface of the reactants. When the materials have melted, 25 mg. of p-toluenesulfonic acid is added as a catalyst. Water evolved in the reaction is prevented by the nitrogen stream from condensing on the reaction vessel. The polymerization reaction is allowed to proceed for approximately 2 hours. For a third hour at 180°, the nitrogen stream is discontinued while the reaction vessel is subjected to about 15 mm. Hg pressure (water vacuum pump), in order to strip out water and excess glycol. The mixture is cooled and stored in a wide-mouthed glass-stoppered jar.

Appendix E

Stable Non-Toxic Liquids for Vapor Jacket

| Name | Boiling Point, °C |
|------------------------------|-------------------|
| 1. Ethanol* | 78.5 |
| 2. Water* | 100.0 |
| 3. sec-Butyl benzene* | 173.4 |
| 4. Cyclohexanol acetate | 175.0 |
| 5. 2-Ethyl hexanol* | 184.8 |
| 6. Propylene glycol* | 188.0 |
| 7. Ethylene glycol diacetate | 190.8 |
| 8. Ethylene glycol* | 197.0 |
| 9. Dipropylene glycol | 231.0 |

Appendix F

Chromatograms of Fatty Acid Methyl Esters of Menhaden Body Oil

Figures F-1 and F-2 are reproductions which illustrate the sensitivity of the detector and instrument, the efficiency and resolving power of two types of stationary phase, and steps in component identification and quantitation. The time axis is split in order that the complete chromatograms

³⁴ Method of Dr. A. T. James, National Institute for Medical Research, London (33).

³⁵ Eastman Organic Chemicals, Rochester, 3, N. Y.

³⁶ Mann Research Laboratories, Inc., New York 6, N. Y.

* Found to be satisfactory in this laboratory, others not yet evaluated.

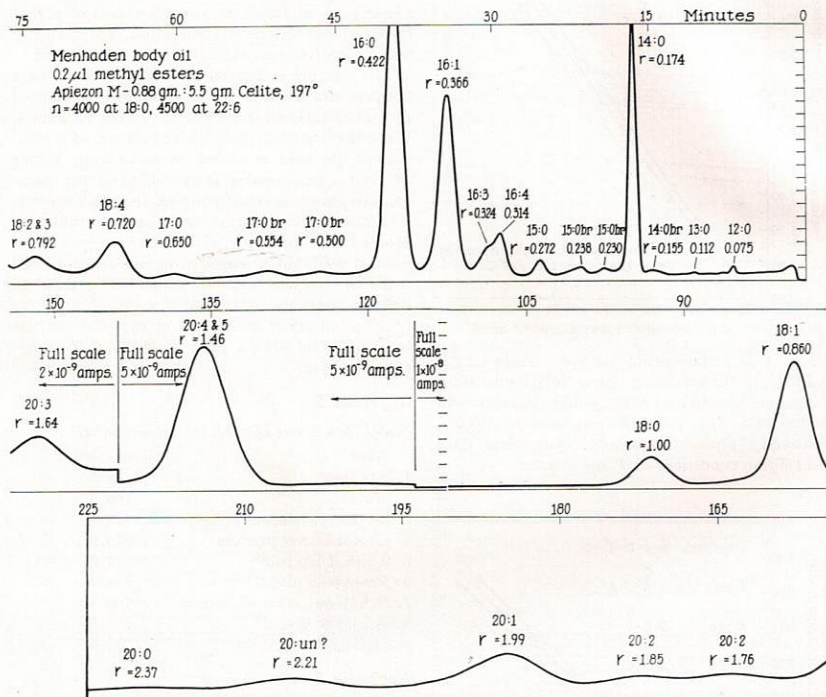


FIG. F-1. Chromatogram on non-polar stationary phase (Apiezon-M) of menhaden body oil methyl esters. $HETP$ (at 18:0) = 0.034 cm.; $HETP$ (at 22:6) = 0.030 cm. $r \left(\frac{18:1}{18:0} \right) = 0.860$; resolution $\left(\frac{18:1}{18:0} \right) = 2.12$.

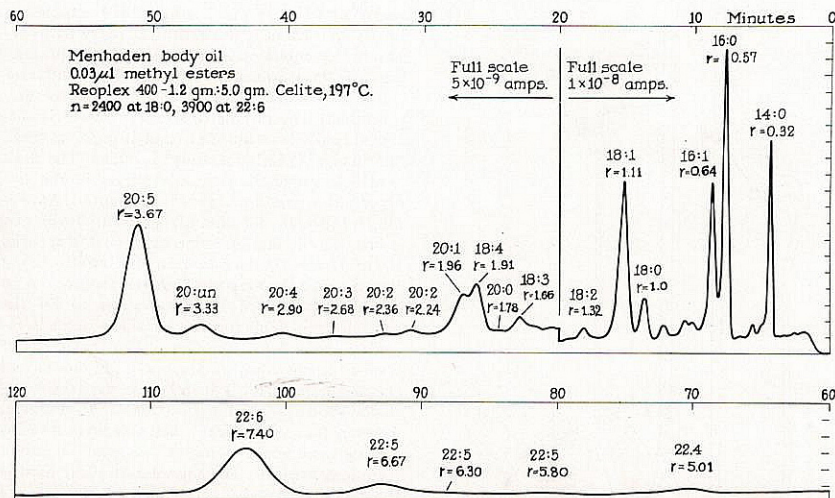


FIG. F-2. Chromatogram on polar stationary phase (Reoplex-400) of menhaden body oil methyl esters. $HETP$ (at 18:0) = 0.055 cm.; $HETP$ (at 22:6) = 0.035 cm. $r \left(\frac{18:1}{18:0} \right) = 1.11$; resolution $\left(\frac{18:1}{18:0} \right) = 1.08$

may be more conveniently displayed. Components elute, in time, from right to left.

The applied voltage was fixed at 1200 volts. Amplifier sensitivity was changed by adjustment of the current amplifier range to increase peak heights of components appearing later than 18:2 in order to facilitate their quantitation.

Appendix G

*Microbromination of Fatty Acid Methyl Esters**

Principle: Bromine bound at the double bonds of unsaturated fatty acid methyl esters increases their molecular weight and decreases their volatility. Thus, GLC of a brominated mixture of esters shows only the saturated components in the original mixture. By comparing the chromatogram of an unbrominated mixture of esters with that made after bromination it is possible to deduce which components of the original mixture are unsaturated and to measure accurately the saturated components.

Method: A small amount of methyl esters (0.5 to 5.0 μ l.) is dissolved in diethyl ether in a conical centrifuge tube. This tube is chilled to -10° or

lower in a beaker containing dry ice and 95% ethanol. A 22% solution of liquid bromine in diethyl ether is added dropwise to the methyl ester solution, the yellow color of the solution disappearing as double bonds are brominated. The reaction must be carried to completion, shown by persistence of yellow color. Excess bromine and solvent are then completely evaporated under a stream of air or nitrogen at a temperature less than 30° , the brominated fatty acid esters tending to crystallize and settle to the bottom of the tube. A sample of the supernatant saturated methyl esters is taken with a micropipette and applied to the column.

Appendix H

Microhydrogenation of Unsaturated Fatty Acid Methyl Esters

Principle: After complete hydrogenation, mixtures of saturated and unsaturated fatty acids or their methyl esters are converted to saturated compounds. By comparing chromatograms of such mixtures before and after hydrogenation it is possible to deduce which components of the original mixture were unsaturated. Moreover, the chain length of unsaturated acids can be shown conclu-

* Modification of the method of A. T. James, National Institute for Medical Research, London.

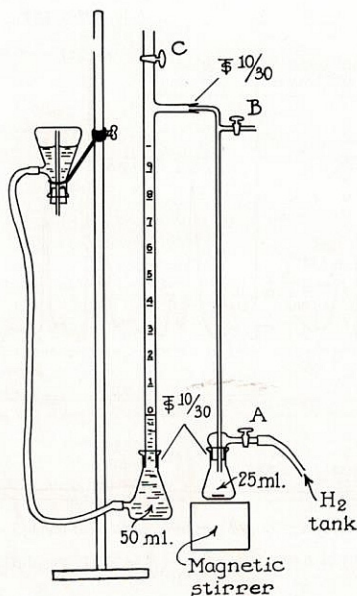


FIG. H-1. Microhydrogenation apparatus

sively by GLC of the hydrogenated sample. In both applications it is essential to carry hydrogenation to completion in order to avoid the production of *trans* isomers, for these have different retention volumes than the saturated homologs.

Method: The sample (0.5 mg. or more) is dissolved in 5 ml. absolute ethanol in a 25 ml. flask and 10 mg. of platinum oxide* is added. The flask is attached over the magnetic stirrer in the hydrogenation apparatus shown in Figure 1, Appendix H, in which all joints are greased.† Hydrogen is introduced through stopcock A with stopcocks B and C closed, the water in the burette being forced below the zero mark. After stopcock A is closed, B is opened to allow water to fill the burette again. This flushing of the system is repeated six to ten times. Finally, with the water level at about zero, all stopcocks are closed and the magnetic stirrer is switched on. Hydrogen uptake is shown by a rising water level in the burette. The reaction is complete when this level remains constant, at which point the catalyst begins to form long strands. The supernatant clear fluid is freed of catalyst by filtration and the ethanol evaporated at 15 mm. Hg and 40° or less.

* Amend Drug and Chemical Company, New York, New York

† Lubriseal, A. H. Thomas Company, Philadelphia, Pennsylvania.