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GAS–LIQUID CHROMATOGRAPHY OF LIPIDS

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I. INTRODUCTION

Lipids and their component aliphatic alcohols, aldehydes, and acids form one of the most complex groups of naturally occurring compounds; for this reason, over the past few decades workers in many countries have used various types of chromatography such as adsorption, displacement, partition, and reversed-phase chromatography to study these compounds. Our ideas on the structure and metabolism of lipids in the living organism have been greatly extended by the use of these methods. However, there are many disadvantages associated with their use in lipid chemistry: much time is required to get a satisfactory separation, the analysis is not sufficiently reproducible, it is laborious, the distribution parameters for unsaturated and saturated lipids overlap, etc.

In the search for methods of effectively separating complex mixtures of lipids from the animal body, that sometimes contain as many as 60 components, the English biochemists James and Martin discovered in 1952 an entirely new type of chromatography, gas–liquid chromatography[1]. In this method separation of the mixture is based on the different distribution of the components between two phases, a stationary liquid phase supported on a solid carrier forming

the chromatographic column and a gas passing through the column. At pressures around atmospheric, gases have much lower densities and viscosities than liquids, and the resistance of the column to the gas stream is relatively low. This permits very rapid analysis with a highly effective separation, which is ensured by the rapid mass exchange on the surface of the stationary phase. The concentration of organic vapour issuing from the column may be recorded continuously by an automatic device measuring a particular physical property. The advantages of gas–liquid chromatography have given it a leading position compared with other types of chromatography. This review does not contain a detailed presentation of the principles of gas–liquid chromatography or a description of chromatographic apparatus; for these the reader is referred to a number of books [2-9].

Gas–liquid chromatography is used to separate methyl esters of fatty acids, the free acids, alcohols, aldehydes, ketones, amines, nitriles, hydrocarbons, and to some extent triglycerides, derivatives of mono- and di-glycerides, etc. It is the best method of separating and quantitatively determining the compounds mentioned. The method has also been used for the study of long-chain aliphatic radicals (residues of acids, alcohols, aldehydes, etc.), which enter into the composition of non-volatile lipids — waxes, phospholipids, glycolipids, sulpholipids, lipoproteins, etc. During the past ten years a large number of experimental studies in lipid gas–liquid chromatography have been made and a number of reviews have appeared [10-17].

Our aim in the present review is to describe the present state of this very topical problem. Numerous papers in which gas–liquid chromatography was used only for lipid analysis, with no original work on the theory or technique of the method, have not been covered. Papers dealing with studies of short-chain (to C_8) aliphatic compounds, sterols, essential oils, and other isoprene derivatives are hardly touched upon. The review covers the most important papers on gas–liquid chromatography of the methyl esters of fatty acids, the free acids, glycerides, and some other lipids, published up to 1st July 1963.

II. GAS–LIQUID CHROMATOGRAPHY OF METHYL ESTERS OF FATTY ACIDS

1. Conditions for Separating Methyl Esters

In the first gas-chromatographic studies to determine the acid composition of lipids, chromatography of the free fatty acids was used [1]. However, in 1953 it was found that use of the less polar methyl esters has a number of advantages: a more effective separation is achieved at a lower temperature in a shorter time, and more precise results are obtained in identification and quantitative determination [18,19]. At present, gas–liquid chromatography of the methyl esters of higher fatty acids is the standard method for investigating the composition and structure of natural and synthetic lipids [20]. The method covers the most diverse fatty acids in the C_1–C_{30} range and higher, including saturated, branched, and unsaturated acids (*cis*- and *trans*-isomers), hydroxy-acids, epoxyketo-acids, etc. By modern methods all these acids can be converted rapidly and quantitatively into methyl esters. The esters obtained are separated on columns filled with adsorbent, or capillary columns, heated to 150–300°; the column is packed with a solid carrier (usually kieselguhr) on which is a high-boiling non-polar or polar liquid phase. Nitrogen, helium, or argon is used as the carrier gas, which passes through the column at 30–100 ml min^{-1}. The methyl esters are detected and quantitatively determined by means of a katharometer or an ionisation detector. Table 1 gives some examples of the separation of methyl esters.

TABLE 1. Gas–liquid chromatography of methyl esters of higher fatty acids.

Solid carrier	Liquid phase and its content	Temp., °C	Carrier gas	Gas flow, ml min^{-1}	Detector	Composition of sample and duration of analysis, min	Ref.
Sterchamol 52–85 mesh	Silicone DC	210	N_2	5.5	katharometer	C_8–C_{34}	21
Celite 545 50–100 μm	Apiezon L, 10–20%	197–231	N_2	20–30	»	C_{12}–C_{28} 80–180	22
Celite 545 100–210 mesh	Apiezon L, 20%	197	Ar	60	ionisation	C_{12}–C_{28} 72	23
Ditto	poly(ethylene glycol adipate), 24%	180	Ar	40	»	C_{12}–C_{18} 70	23
Celite 545 140–170 mesh	poly(ethylene glycol succinate), 20%	203	He	200	katharometer	C_{10}–C_{26} 38	24
Capillary column 30–60 m × × 0.25 mm	Apiezon L	200	Ar	0.5–1	ionisation	*cis-trans*-isomers of methyl linoleate and methyl linolenate, 60 ditto, 40	25
Ditto	poly(diethylene glycol succinate)	168–188	Ar	0.5–1	»		25

The synthesis of the methyl esters, the chromatographic separation conditions, and also the methods of their identification, quantitative analysis, and determination of radioactivity are considered in detail below.

2. Synthesis of Methyl Esters for Chromatographic Separation

At present there are two methods of synthesising methyl esters of fatty acids for chromatographic purposes: (*a*) catalytic transesterification of lipids in methanol solution; and (*b*) saponification of the lipids and esterification of the free fatty acids.

(*a*) Transesterification of fatty acids. The lipids are dissolved in absolute methanol; if they are not completely soluble, benzene or toluene is added [26]. To ensure a quantitative yield of esters, all the reagents must be carefully purified [27]. Reaction conditions used by different workers are given in Table 2. This shows that methanolysis is completed much more rapidly in a basic medium than in an acidic one; HCl and H_2SO_4 have about the same catalytic activity, but K is much more active than

TABLE 2. Conditions for transesterification of triglycerides in methanol.

Catalyst	Duration, h	Temp., °C and gaseous medium		Yield of esters, %	Ref.
5% HCl	2	64–65	air	—	28
0.8 *N* HCl	2	64–65	air	—	23
5% HCl	2	80–100	air	95	26
5% H_2SO_4	18	64–65	air	—	29 *
0.005% Na	0.5	—	—	96	30
0.1 *N* Na or K	1.5–2	64–65	N_2	98	31 *
1% Na	2 min	60	N_2	99	32

* Methanolysis of phospholipoids and sterol esters.

Na.[31] In a 1% solution of Na in primary alcohols, alcoholysis of triglycerides is completed in 2^m min, where m = = 1–6 is the number of carbon atoms in the alcohol molecule[32]. Methanolysis of phospholipoids and sterol esters requires more time and a larger excess of methanol and catalyst than does the methanolysis of triglycerides. Final purification of the methyl esters before chromatography may be achieved by fractional distillation[33], vacuum sublimation[26,34], adsorption chromatography on a silic acid column[31], or thin-layer chromatography[32]. The methyl esters obtained must be fully protected from oxidation; they can be kept for long periods in benzene solution below 0° in the dark under an inert gas[35].

(*b*) Esterification of free fatty acids. Transesterification conditions are quite suitable for the esterification of fatty acids obtained by saponification of lipids[36]. However, there are more rapid methods of esterification that require only a few minutes[15,16]. For a number of years methyl esters have been prepared by esterifying fatty acids with diazomethane[37]. Previously the danger of handling diazomethane and the possibility of the formation of pyrazolines by addition of CH_2N_2 to the double bonds of fatty acids[26] were considered disadvantages of this method. It has recently been found that when a freshly prepared solution of CH_2N_2 in a mixture of CH_3OH and ether (1 : 9) is used, the methyl esters are obtained in quantitative yield in 10 min, free from decomposition and polymerisation products; the methanol in this reaction appears to act as a catalyst[38]. It has also been recommended[39] that the esters should be synthesised by keeping the fatty acids in a weak solution of diazomethane at -34° for $2\frac{1}{2}$ days. Esterification of fatty acids in a solution of boron trifluoride in methanol is complete in only 2 min, but the yield of methyl esters does not exceed 80–85%; epoxy-acids and acids with conjugated double bonds cannot be esterified by this method[40]. In the formation of esters of higher fatty acids, CH_2N_2, HCl, and BF_3 show very similar activities[35,41]. To ensure a quantitative yield of the more volatile esters of the lower acids (C_1–C_{10}) diazomethane[41] should be used with docosane as solvent[42], or the ethyl esters may be prepared instead of the methyl[43].

The reaction with 2,2-dimethoxypropane in acidified methanol has been successfully used for the synthesis of methyl esters of 2-hydroxy-acids from cerebrosides and the esters of the acetates of these acids[44,45]. Aliphatic epoxy-acids are destroyed on saponification, being converted into the corresponding dihydroxy-acids; if epoxy--acids are present in lipids, methanolysis in sodium solution should be used[46].

There are other methods of synthesising esters. Hydrogenolysis of triglycerides with lithium tetrahydroaluminate gives free glycerol and LiAl alkoxides of the higher alcohols corresponding to the individual fatty acids. The acetylation products of these compounds — triacetin and acetates of fatty alcohols — have been determined by gas–liquid chromatography[47]. The reaction of silver salts with methyl iodide in an inert gas for 8 h has been proposed for the preparation of esters of volatile and non-volatile organic acids; the yield of methyl esters was 96–100% with this method[48].

3. Introduction of Sample and Temperature of Separation

Volatile components in the liquid phase and water vapour are removed from the column by maintaining it at 5–10 deg above the working temperature for 48–96 h and streaming carrier gas through it[24]. Sometimes the column with the solid carrier containing 10–50% of liquid phase is stabilised by being heated to 300° at a pressure of 10 mmHg for 5 h.[6]

The sample is introduced into the column as rapidly as possible, preferably without interrupting the carrier-gas stream. If only a small amount of material is available for analysis, the sample is dissolved in volatile organic compounds of the methyl tridecanoate type[23]. A device has been described for introducing small samples of methyl esters into a chromatographic column with a closed injection system[49].

Before the chromatographic separation commences, the methyl esters introduced into the column that are liquid or solid at ordinary temperature must be vaporised[15,16,46,50]. For this purpose, many chromatograph models are equipped with vaporisation chambers maintained at a temperature 50–100 deg above that of the column[24]. With capillary columns this temperature difference may reach 150 deg[25,51]. If the vaporisation of the sample at the entrance to the column is not instantaneous, the effectiveness of the separation is reduced, especially with components having a large retention volume V_R.[49] Esters of saturated and unsaturated acids are not usually affected by vaporisation[52]. However, unsaturated acids with conjugated double bonds and hydroxy--groups may undergo *cis–trans* isomerisation or dehydrogenation[53] in the vaporisation chamber. Esters of tung-oil acids, containing over 70% of elaeostearic acid, polymerise in the chromatographic column at high temperatures[15].

Correct selection of the working temperature and its control throughout the experiment determine the effectiveness of the separation and the accuracy of the quantitative determination of fatty acids. Sometimes a high column temperature is achieved by means of a vapour bath using high-boiling liquids such as polyethylene glycol (b.p. 197°)[22], but more often an air thermostat is used.

The temperature conditions for gas-chromatographic separation depend on the composition of the methyl esters in the sample and on the chemical nature of the liquid phase in the column. The best separations of aliphatic esters up to and including C_{30} on non-polar liquid phases (Apiezon M and L, Silicone) are achieved with a column temperature of 250–300°.[16,21,54] At still higher temperatures the rate of analysis increases, but the separation is less effective[23,25]. With polyester phases, the threshold temperature seldom exceeds 200–210°;[16,39,43] further heating may result in removal of liquid phase from the column, which would spoil the detector[55]. On capillary columns the same mixture can be separated at a temperature 40–50 deg lower than on columns packed with sorbent[12,25,56,57].

The temperature of the column has a considerable effect on the volume of the separate components retained. Thus methyl oleate and methyl stearate are not separated on a polyester column at 125°, but at 158–168° a good separation is achieved[12]. The "separation factor" (ratio of retention volume V_R of a component to that of the preceding component[58]) decreases with increasing working temperature for esters of saturated and branched-chain fatty acids; for unsaturated esters it increases (relative to the preceding saturated esters)[57]; this behaviour may be used for the chromatographic identification of fatty acids. When mixtures of methyl esters were being separated on a polar column without preliminary calibration of the katharometer an increase in temperature led to high results for readily volatile components and low results for esters with a high

retention time; as the temperature was lowered the divergence was largely eliminated[24,51] [see Section II, 8(*a*)].

If a mixture of methyl esters contains components differing markedly in volatility, it cannot be analysed at a single column temperature. In such cases the more volatile C_2–C_{10} esters are determined at 100–120°, and the esters of the higher fatty acids are separated at 180–200°; the esters of the lower acids emerge as one peak at the beginning of the chromatogram[15,58–60]. Recently chromatography of derivatives of lower and higher acids has been carried out with automatic programming of the working temperature of the column[15,61].

The katharometer should be carefully thermostatted to obtain reliable quantitative results[51]. The temperature of the katharometer is usually 25–75 deg above that of the column[24]. In some cases such a temperature gradient is set up for ionisation detectors[25,50].

The working life of a column is greatly increased if the temperature is reduced to 100° when it is not in use[11,25].

4. The Solid Carrier

In gas–liquid chromatography of lipids various kinds of kieselguhr (diatomaceous earth), such as Celite-545[22,30,62] or Chromosorb W[33,55,63–65] have been used most frequently; other materials used include Sterchamol[12], ground firebrick[46], glass beads[66], Embacel[34], and sodium chloride[19]. Carriers of particle size 50–150 mesh have been used in different investigations; the range of particle size in any column should not exceed 20 mesh. Before deposition of the liquid phase, the solid carrier is washed through successively with concentrated hydrochloric acid, KOH solution, NH_4OH solution, and water; it is then thoroughly dried[51,67]. Sometimes it is recommended that the column should be treated with dichlorodimethylsilane vapour[66,68,69], but this procedure may promote the retention of methyl esters on polyester columns[70].

Chromatographic columns can be packed with carrier containing the liquid phase by means of a vibrator and under pressure of the gas that is to be used in the separation[24,51]. In other cases packing is achieved by creating a vacuum inside the column[22].

The nature of the solid carrier considerably affects the separation of methyl esters; the esters of linolenic and arachidic acids are not separated with poly(ethylene glycol succinate) on Celite, but when the same phase is used on Chromosorb W, the arachidic ester appears on the chromatogram between the linoleate and linolenate peaks[71]. In contrast, the use of Chromosorb R and Celite-545 saturated with poly(vinyl acetate) gave similar results in the separation of artificial mixtures of methyl esters of fatty acids[66]. Considerable destruction of methyl *cis*-9,10-epoxyoctadecanoate on a column with silicone oil at 235° can be avoided by using Celite instead of firebrick C-22 as solid carrier[46].

Methyl esters of saturated and unsaturated acids may be adsorbed during separation on a column of Sterchamol or kieselguhr impregnated with a non-polar or polar liquid phase. Later the carrier gas desorbs the esters and sweeps them out of the column. Thus with Sterchamol 8% of the radioactivity of methyl [1-^{14}C]palmitate is eluted after the main peak. To reduce its adsorptive tendency Sterchamol was treated with hexamethyldisilazane or a 2% solution of sodium laurate, and kieselguhr was washed with hydrochloric acid and methanolic KOH. After such treatment only 4% of methyl [1-^{14}C]palmitate was adsorbed[72].

5. Chromatography on Non-Polar Liquid Phases

In work on the gas chromatography of methyl esters of higher fatty acids, high-boiling non-polar vacuum greases began to be used in preference to other liquids; these were organosilicon or paraffin in nature, *e.g.* Silicone DC 550, Apiezon M, Apiezon L, and polybutene[1,18,22,54,58,73].

The values of the retention volume of methyl esters are determined by differences in the partial pressure of the vapours of these compounds when they are dissolved in the liquid phase on the column; the partial pressure in turn depends on the nature and magnitude of forces of interaction between the molecules of the ester and those of the liquid phase.

At a vapour concentration of the organic compound in the gas stream of 3–5 $\mu g\ ml^{-1}$ or less, London dispersion forces are the only forces acting between the ester molecules and those of non-polar phases, and these forces weaken as the molecular weight is reduced and with branching of the aliphatic chain[23]. These phases ensure complete separation of esters of normal saturated acids differing in chain length by one carbon atom (separation factor 1.56) in the C_1–C_{34} range[58,74]. The retention time is comparatively large, and for methyl stearate it often exceeds 60 min. Esters of branched-chain acids emerge from the column before unbranched esters containing the same number of carbon atoms[14,23]; iso-forms of branched acids move more rapidly than the corresponding anteiso-forms[11,75,76]†.

Bromination is used before chromatography to separate esters of unsaturated acids: the brominated esters (except methyl oleate) are non-volatile at the separation temperature[58]. The chain length of an unsaturated acid can be determined by the preparative isolation of the ester with subsequent catalytic hydrogenation; the saturated ester obtained is identified by its retention volume[77]. Transesterification during hydrogenation with an Adams catalyst may be avoided by carrying out the reaction in methanol[78]. Other methods of identification are considered in Section II, 7(*b*) below.

In separations on non-polar fatty phases, the esters of unsaturated fatty acids are found on the chromatogram in front of the esters of the corresponding saturated acids. Saturated and monounsaturated esters are completely separated; di- and tri-unsaturated esters form a mixed peak, which is partly separated from the monounsaturated ester peak[79]. Separation of methyl linoleate and methyl linolenate was not achieved even on an Apiezon L capillary column of 200 000 theoretical plates[56]. When the distance between the carboxyl and the double bond is reduced, the retention volume V_R of monounsaturated esters increases somewhat[23,58]. The isomers of methyl linoleate — the 8,11-, 9,12-, 10,13-, and 11,14-octadecadienoic esters — have been partly separated on a polybutene capillary column[73]. Polyunsaturated esters with conjugated double bonds emerge from the column more slowly than do esters with isolated bonds[6]. The isomers of methyl linoleate

† In the iso-form of branched acids the aliphatic chain terminates in an isopropyl group, but in the anteiso-form it terminates in an isobutyl group. [An s-butyl group is probably meant here (Ed. of Translation).]

and methyl linolenate with conjugated bonds separate well on a column of Apiezon L; under the same conditions the above two esters themselves cannot be separated[34].

Non-polar liquid phases are used successfully to separate geometric isomers of unsaturated acids, formed by hydrogenation under industrial conditions; V_R for the *cis*-isomers of mono-, di-, and tri-unsaturated esters with isolated or conjugated double bonds is less than that of the corresponding *trans*-isomers[12,22,23,58,63]. The geometric isomers of methyl linoleate have been separated, on a capillary column of Apiezon L with an ionisation detector, into *cis-cis*, *cis-trans*, and *trans-cis* + *trans-trans* forms; methyl *cis-cis-cis*-linolenate moves in front of the *trans-trans-trans*-isomer. Geometric isomers of octadecadienoic acid with conjugated double bonds have also been separated[25].

Methyl *cis*-9,10-epoxyoctadecanoate from uredospores of a rust fungus has been separated from its acetylation product, the acetoxyhydroxyoctadecanoate, on a silicone column[46]. The separation of methyl esters of 2-hydroxy--acids has also been described[45].

At present non-polar liquid phases are mainly used for the preliminary fractionation of methyl esters according to the number of carbon atoms, for the determination of *cis*- and *trans*-isomers, and for comparative identification. Gas–liquid chromatography on polar liquid phases is now more often used to study the composition of complex fatty acid mixtures from natural sources.

6. Chromatography on Polar Liquid Phases

(*a*) Preparation of columns. Polar liquid phases were proposed for the separation of esters of saturated and unsaturated acids in 1958[80,81]. The first polar phases, which consisted of derivatives of adipic acid — poly(ethylene glycol adipate) (LAC-1-296) and poly(oxyalkylene adipate) (Reoplex-400) — are still widely used. Polyesters of this kind $[-O-CO-(CH_2)_n-CO-O-]_m$‡ are synthesised by the polycondensation of succinic[30], adipic[23,82], and other dibasic acids with ethylene glycol or other polyhydric alcohol at 150–180°, usually in the presence of a catalyst (toluene-*p*-sulphonic acid, zinc chloride, etc.) in an inert atmosphere; the product is dissolved in alcohol or acetone, and purified from catalyst impurity and free acid by ion-exchange chromatography on Amberlite IR-120 or Duolite A-4.[30,65,67,70,73] The more thermostable high--molecular polyester fractions may be separated by reprecipitation from acetone solution with hexane[30]. The vapour pressure of the liquid phase at the working temperature should not exceed 0.5 mmHg.[83]

Poly(vinyl acetate) (Vinylite AYAC) with a molecular weight of 1500–23 000 is also used as a polar liquid phase. An advantage of this polymer is that on being heated it liberates only a small amount of acetic acid, which can be easily removed from the fractions collected in preparative work; under these conditions the noise level of the katharometer does not exceed 0.2 mV.[55] Decomposition of dimethyl malonate has occurred on poly(vinyl acetate) columns; when silicone oil was used no decomposition was observed[84]. In recent years acylated β-cyclohextrins have been used in the chromatography of methyl esters; these are characterised by low oxygen content, absence of terminal polar groups, uniform molecular weight, and thermal stability up to 236°.[85,86]

The polar liquid phase (5–25%) is added to the solid carrier, and the column material is conditioned as described above (Section II, 3). In this case the column must be stabilised with particular care, since the polyesters are unstable at high temperatures. If conditioning is not satisfactory or the temperature is too high, the liquid phase enters the detector and puts it out of action. Volatilisation of the stationary phase from the column leads to a reduction in the efficiency of separation and it also produces drift and "noise" in the zero line of the detector. The carrier gas entering the column should be carefully dried to avoid hydrolysis of the polyesters[30,67]. If the column is well stabilised and correctly operated, it should last for several months[23].

(*b*) Properties of polar liquid phases. Intermolecular attraction arises between the polarisable double bonds of unsaturated fatty acids and the ester groups of the polar liquid phases, and this increases with the number of double bonds in the aliphatic chain. This attraction specifically retards unsaturated methyl esters on the chromatogram, and they are found after the corresponding saturated esters, *i.e.* in the reverse order to that observed in chromatography on non-polar liquid phases[23].

Because of the weakening of London dispersion forces on polyester columns, the retention volume of the methyl esters of saturated acids, for the same temperature and weight content of liquid phase, is lower by a factor of 2–3 compared with columns of Apiezon L, and the chromatographic separation is correspondingly accelerated[15]. The free energy of solvation per CH_2 group is also reduced, and the separation factor of esters differing by a CH_2 group becomes smaller than on a non-polar column (1.40 instead of 1.56)[23]. Esters of branched-chain saturated acids, the distribution of which is governed by London forces, are found in front of the corresponding straight-chain saturated esters, as with non-polar phases[33,87]. The arrangement of double bonds in the chain and their geometric configuration have much less influence on V_R than when non-polar liquid phases are used[23].

The solubility of methyl esters of unsaturated fatty acids increases in *all* polar liquids as the number of double bonds in the chain increases. For this reason, mono-, di-, and tri-unsaturated esters emerge from the column (in that order) immediately after the saturated esters containing the same number of carbon atoms. At the same time, differences in the polarity of polyesters of differing composition considerably influences the effectiveness of the separation.

If the number of carbon atoms in the dibasic acid residue linked to the polyethylene glycol is reduced, or the monobasic acid residue in acylated β-cyclodextrins is shortened, the polarity of the polyester and the separating power of the column are increased; the thermal stability of the liquid phase and the absolute retention volume of methyl esters are reduced[14,86]. Methyl oleate can be completely separated from methyl stearate on columns with poly(ethylene glycol succinate), and they can be partly separated on columns with poly(ethylene glycol adipate)[12,24,30,51]. On strongly polar phases the relative retention of unsaturated esters increases to such a degree that the peak of the triunsaturated methyl ester overlaps the peak of the saturated ester containing two more carbon atoms. Thus on columns containing poly(ethylene glycol succinate) or poly(ethylene

‡ A second polymethylene chain should presumably have been included at one end of this unit (Ed. of Translation).

glycol pentaerythritol adipate) (LAC-2-R446) the ratio of the retention volume of methyl linolenate to that of methyl arachidate is unity; this ratio becomes greater than unity when the polarity of the column is increased, with an increased amount of liquid phase and a higher working temperature. Pairs of methyl esters of this kind are usually separated on less polar polyester phases such as poly(ethylene glycol adipate), poly(butanediol succinate), and poly(diethylene glycol sebacate)[24,30,51,88].

When a polyester column is kept at a high temperature for several weeks, the polarity of the phase is reduced[15]; this suggests the possibility of using columns that have been heated in this way to separate pairs of methyl esters that cannot be separated on "fresh" columns of the same polyester[33].

(*c*) Separation on polar liquid phases. Polyester phases have given the best results in the analysis of methyl esters of animal and vegetable fatty acids differing in chain length from C_1 to C_{30} and having 1–6 double bonds[14].

Nine branched-chain methyl, dimethyl, and trimethyl isomers of methyl heptadecanoate have been separated on columns of β-cyclodextrin valerate, butyrate, propionate, and acetate[86].

Unsaturated esters with the same number of carbon atoms and double bonds but differing in the positions of the bonds in the chain were difficult to separate on a packed column, though they could be separated on capillary columns. The 8,11-, 9,12-, 10,13-, and 11,14-isomers of methyl linoleate were completely separated on a capillary column of poly(ethylene glycol glutarate) from one another and from the methyl esters of stearic, oleic, and linolenic acids; on an ordinary column of poly(ethylene glycol adipate) only the 11,14-isomer and the 8,11- + 9,12-isomers were separated V_R slightly increased as the distance from the double bond to the carboxyl increased, but the methyl esters of oleic and petroselinic acids could not be separated on either column[73]. The methyl esters of linolenic and linoleic acids with conjugated double bonds were well separated from the corresponding esters with isolated bonds[34].

The *cis*- and *trans*-isomers of unsaturated acids are not separated[12,63] on a packed column. Methyl oleate has been separated from methyl elaidate, which had a lower V_R, on a capillary column of poly(ethylene glycol succinate). The geometric isomers of methyl linoleate have been separated into *trans*-*trans*, *cis*-*cis* + *cis*-*trans*, and *trans*-*cis* forms, and the isomers of methyl linolenate into *trans*-*trans*-*trans*, mono-*trans*, di-*trans*, *cis*-*cis*-*cis* + + di-*trans*, and mono-*trans* + mono-*cis* forms. The separation of geometric isomers of methyl linoleate with conjugated double bonds was as effective as on a non-polar column[25].

The chromatography on a polyester column of methyl *cis*-9,10-epoxyoctadecanoate and its derivatives has been described[46]. Methyl *erythro*- and *threo*-dibromooctadecanoates have been separated on a succinate polyester column; the *threo*-form with the larger dipole moment was found to have the higher retention volume. V_R for the *threo*-form was 20 times the value of V_R for methyl stearate[64].

7. Identification of Fatty Acids

Methods of identifying fatty acids in complex natural mixtures may be divided into two main groups — (*a*) identification from chromatographic results obtained under different conditions, varying with respect to polarity of the liquid phase, composition of the solid carrier, type of detector, separation temperature, speed of gas, etc.; and (*b*) identification by a combination of chromatographic and non-chromatographic methods (oxidative cleavage, halogenation or catalytic hydrogenation of double bonds, ultraviolet and infrared spectrophotometry, mass spectrometry, etc.).

(*a*) Gas-chromatographic identification. The retention volumes V_R of the methyl esters of fatty acids vary considerably with the parameters of the experiment; for identification purposes the separate components of a mixture are usually characterised by the relative retention volume V_R^{rel}, which is equal to the ratio of V_R for the given ester to V_R for a known compound (methyl ester of myristic, palmitic, or stearic acid[51]). V_R is measured from the centre of the air peak[28]. The simplest method of chromatographic identification is that based on the value of V_R^{rel}; however, because two or more components may be contained in one chromatographic band, this method of identification is not always reliable in the analysis of complex mixtures of fatty acids[14]. Identification of branched-chain and unsaturated acids from the separation factor has been described in Section II, 3.

Like other processes of partition chromatography, the gas-chromatographic separation of lipids follows an additive rule[58,60-86]. According to this rule, the addition of a new functional grouping changes the partition coefficient by an amount depending only on the nature of this grouping and its distribution between the phases of the chromatographic system, but independent of the composition of the remainder of the molecule. On non-polar and polar phases the values of $\log V_R^{rel}$ when plotted against the number of carbon atoms in a homogeneous series m give a family of parallel lines. This holds for the methyl esters of normal saturated fatty acids[51], branched-chain saturated acids, iso and (+)-anteiso branched-chain acids[87], monounsaturated acids[51,87], di-, tri-, and poly-unsaturated acids[23], brominated fatty acids[64], mono- and di-basic short-chain acids[77], etc.; it holds also for hydrocarbons[90], aliphatic aldehydes[91], triglycerides[92], and other lipoids. For methyl esters of the same chain length a linear relation between $\log V_R^{rel}$ and the number of double bonds has been established[51]. The functional dependence for saturated acids is described by an equation of the type $\log V_R^{rel} = a + bm$, where a and b are constants depending on the experimental parameters[19,21]. As the separation temperature rises, the slopes of the parallel lines decrease[93]. Fatty acids can be identified from the linear dependence of $\log V_R^{rel}$ on m, if the homologous series to which they belong is known.

If the homologous series is not known, the methyl ester is identified from the value of V_R^{rel} on columns with non-polar and polar liquid phases by means of a relationship observed by James for straight-chain saturated and unsaturated acids with isolated double bonds. He showed that there is a linear dependence between values of $\log V_R^{rel}$ for the methyl esters of a given homologous series on columns with Apiezon L and poly(ethylene glycol adipate); saturated and mono-, di-, tri-, and poly-unsaturated fatty acids form a series of parallel lines. The point of intersection of the value of $\log V_R^{rel}$ with one of these lines enables an unknown acid to be characterised with respect to the number of carbon atoms and the number of double bonds. The points of intersection corresponding to esters of the same chain length but differing in unsaturation lie along the perpen-

dicular drawn to the straight line for esters of saturated acids at the point corresponding to the methyl ester of the saturated acid with the given number of carbon atoms [23]. Similar relationships have been obtained for the separation of mixtures of fatty acids on two polyester columns differing in polarity [86].

The value of V_R^{rel} may be expressed for identification purposes by the number of carbon atoms in the saturated fatty acid; this quantity has been termed the "carbon number" [75] or the "equivalent chain length" (ECL) [94]. To calculate the ECL of an unidentified chromatogram peak, a reference line is drawn for the dependence of $\log V_R^{rel}$ of methyl esters of normal saturated fatty acids on the number of carbon atoms m for the given separation conditions, and from this line the value of m corresponding to the value of $\log V_R^{rel}$ for the component to be identified is read; the value of m obtained is the ECL of the methyl ester. In the absence of a reference line the ECL can be calculated from the equation

$$\mathrm{ECL} = (s_2 - s_1)\frac{\log(t_x : t_{s_1})}{\log(t_{s_2} : t_{s_1})} + s_1,$$

where s_1 and s_2 are the ECL's of the standard methyl esters and t_x, t_{s_1}, and t_{s_2} are the V_R^{rel} values for the ester under investigation and the standards [93]. Obviously, for saturated esters the values of ECL on polar and non-polar phases coincide and are equal to the number of carbon atoms in the aliphatic chain. The inclusion of a double bond or a certain functional group in the molecule gives a known increment to the ECL compared with that of the saturated ester having the same chain. Table 3 shows the increments in the ECL for several substituents.

When the ECL of an unknown component has been determined on columns with polar and non-polar liquid phases, the component and its functional groups may be identified. ECL values have been used to identify nine isomers of branched C_{17} fatty acids on polar phases consisting of acylated β-cyclodextrins [95].

Recently methods have been developed that permit not only the number but also the positions of isolated double bonds in a fatty acid chain to be determined from V_R^{rel} values obtained with highly polar liquid phases [96-98]. When the double bonds are displaced from the central position towards the carboxyl or methyl end of the aliphatic chain, V_R^{rel} on polyester phases increases in such a way that, when values of $\log V_R^{rel}$ for the methyl esters of the unsaturated acids are plotted against the number of carbon atoms, parallel lines between the points corresponding to fatty acids having the same number of unconjugated double bonds can be drawn only if these acids contain the same number of carbon atoms between the centre of the double bond most remote from the carboxyl and the terminal methyl group inclusive, *i.e.* when the increase in methylene groups in the homologous series occurs only at the carboxyl end of the chain [96]. Similar series of parallel straight lines have been obtained by grouping unsaturated fatty acids with the same number of carbon atoms between the carboxyl and the centre of the nearest double bond, and also acids with the same degree of unsaturation and an equal number of carbon atoms, in which the double bonds are regularly displaced from the carboxyl end of the aliphatic chain to the methyl [97]. The structure of the polyunsaturated acids of whale oil has been determined by using these graphs to compare experimental values of $\log V_R^{rel}$ with values for esters having a known arrangement of double bonds [98].

TABLE 3. Increments of equivalent chain length for various functional groups in the gas–liquid chromatography of methyl esters of fatty acids on non-polar and polar liquid phases.

Substituent	Apiezon	Poly(ethylene glycol adipate)	Ref.
cis-9,10-Double bond	−0.3	+0.4	94
cis-9,10-12,13- Double bonds	−0.4	+1.0	93
trans-9,10-11,12-Conjugated double bonds	+0.7	+2.6	94
Cyclopropene ring	−0.4	+1.0	93
$COOCH_3$ group	+2.6	+7.3	94
9-CH_3 group	−0.7	−0.8	93
4-CO group	+1.3	+1.8	93
9-CO group	+1.4	+5.7	93
12-OH group	+1.7	+6.3	94
9,10-$(OH)_2$ group	+3.3	+11.8	94
cis-9,10-Epoxy-group	+2.0 *	+4.0 **	46

* Chromatography on a silicone column.

** Chromatography on a column of poly(butane-1,4-diol succinate).

(*b*) Combination of chromatography with non-chromatographic methods. Esters of fatty acids with the same number of carbon atoms and the same number of double bonds in different positions on the chain cannot be separated on non-polar liquid phases; the separation of such esters is also sometimes impossible on polyester phases (methyl oleate and methyl petroselinate). To identify unsaturated acids in such cases, they are oxidised at the double bonds and the products are determined by gas chromatography.

Oleic, linoleic, and other fatty acids were oxidised with $KMnO_4$ in acetic acid at -70°, the excess of permanganate was destroyed with metabisulphite, and methyl esters of the di- and mono-basic acids obtained were separated on a column with Apiezon M. Azelaic and pelargonic acids were found among the oxidation products of oleic acid. The presence of numerous by-products of the oxidation shows that in this case the unsaturated acid chain was broken not only at the double bonds but also at other positions [77]. This method has been used also for the identification of unsaturated acids from fish oils [69]. Oxidation with a $KMnO_4$–KIO_4 mixture also gave many secondary degradation products [99]. No oxidation by-products were produced when unsaturated acids were oxidised with performic acid, the unsaturated dihydroxy-acids obtained were hydrogenated over a palladium catalyst, and the dihydroxy-grouping was ruptured with $KMnO_4$–KIO_4 mixture to give all possible mono- and di-basic acids. Fatty acids containing triple bonds underwent hardly any degradation in this series of reactions [100].

The chains of unsaturated acids may be ruptured by either oxidative or reductive ozonolysis. In the first case the fatty acids are ozonised in chloroform solution at -60°, and the ozonide is decomposed by water with simultaneous oxidation of the terminal carbon atoms to carboxyl under the action of Ag_2O. The acids obtained are converted into methyl esters, and separated by gas–liquid chromatography using programmed temperature conditions [101,102]. Oxidative ozonolysis of the decomposition products of polyunsaturated fatty acids containing non-conjugated double bonds has made possible the isolation of malonic acid, which is formed on elimination of a $-CHCH_2CH-$ group: periodate oxidation destroys malonic acid [103].

The most reliable information on the structure of unsaturated acids is obtained by reductive ozonolysis. The ozonides formed at low temperatures are decomposed with H_2 in the presence of a palladium catalyst or by reduction with triphenylphosphine; aldehyde groups are formed at the positions where double bonds have been broken. Gas chromatography of the aldehydes and dialdehydes is carried on an apparatus with a flame-ionisation detector, since malonaldehyde is difficult to observe with an ionisation detector [61,68,104].

Infrared spectroscopy (detection of *trans*-isomers) [33], mass spectrometry [105], and also a combination of gas–liquid chromatography with other types of chromatography [33,76]. have been used to identify acids after separation.

8. Quantitative Determination of Fatty Acids

Gas–liquid chromatography enables a quantitative determination of saturated and unsaturated fatty acids to be carried out with much greater speed and precision than any other method in lipid chemistry. For a quantitative analysis the signal of the detector must be directly proportional to the weight concentration of the given methyl ester, and also of methyl esters of fatty acids with different structures [10]. The relative area of the corresponding peak in the differential chromatogram usually serves as a measure of the quantity of a component in gas chromatography. There are a number of methods for measuring the areas of peaks — (1) weighing the paper, (2) planimetry, (3) calculation of the product of the height of the peak and its width at half its height, (4) calculation of the product of peak height and retention volume, and (5) continuous determination during chromatography by means of an integrator. Although method (3) is the most widely used [59] because of its simplicity and reliability, for speed and accuracy integration is superior to all other methods [106].

In their first paper on gas–liquid chromatography, James and Martin determined free fatty acids by using a titration cell with alkali solution containing an acid–base indicator; change in colour of the indicator when it absorbed volatile fatty acids from the column was measured by a colorimeter connected to a potentiometer that recorded an integral curve. The height of each step corresponded to the molar content of the given fatty acid [1].

Later the same workers used as detector a Martin balance to measure the density of the gas, which enabled the sample size to be reduced [58]. The balance gives figures directly proportional to the mass of the organic compound being separated, and therefore does not require calibration for different methyl esters; until recently, however, Martin balances have not been much used because of their complex construction [22,28,54].

Satisfactory results have been obtained by using a flame-ionisation detector, where the signal depends linearly on the concentration of fatty acids in the mixture [107]. Nevertheless, the katharometer and the Lovelock ionisation detector are most widely used; we shall consider their operation in detail.

(*a*) Katharometer. A katharometer, measuring the difference between the thermal conductivity of an inorganic carrier gas and that of an organic compound, was first used for the gas-chromatographic analysis of methyl esters of higher fatty acids in 1953 [18]. The sensitivity of the katharometer is $\sim 10^{-6}$ g. At higher separation temperatures the detector becomes less sensitive, so that the volume of the sample must be increased [15]. To determine the absolute amount of fatty acids, an internal standard is used in the sample. Before the analysis an aliquot of methyl heptadecanoate is added to a known volume of sample — this compound does not usually occur in biological material — and the ratio of the areas of the peaks of methyl esters to that of the standard is calculated [35,36,51].

In the fatty acid series a linear relation has been observed between the logarithm of the peak area and the logarithm of the corresponding weight [37]. Comparative quantitative determinations have been made of the composition of fatty acids by chromatography with a katharometer, and also by standard non-chromatographic methods [108,109]. It was found that on polyester liquid phases the katharometer gave high results in measurements on the more volatile saturated components, but for esters with a high retention volume the results were low [30,110,111]. An explanation suggested for this phenomenon was that more intense transesterification occurs between the high-boiling components and the column polyesters at a high separation temperature: in fact, when the temperature of the column was reduced to 200°, the difference in the results obtained by different methods was greatly reduced [51]. However, the same effect was later observed in chromatography on a non-polar liquid phase (Apiezon), where the possibility of transesterification was excluded [112,113]. Other workers have explained the differences by imprecise measurement of the peak areas with large retention volumes [24].

Detailed investigation of standard mixtures of methyl esters of known composition has shown that in the fatty acid homologous series the specific thermal conductivity is not proportional to molecular weight. Thus if the area of the peak relating to 1 mg of compound is taken as 1.00 for methyl decanoate, the area for methyl stearate is only 0.84. [112] The following empirical equations have been proposed to correct the thermal conductivity: $\log A = m \log M + \log K$, where A is the peak area, M the weight of compound, m the number of carbon atoms, and K a constant [114]; $R = 24.68 + 5.79m + 0.075m^2$, where R is the relative molar signal of the detector based on the signal for methyl palmitate [115]; $\log y = 0.001007x - 0.161$, where y is a correction factor and x the relative retention volume [116]. Kaufmann has observed a linear dependence between the calibration factor (ratio of weight content of methyl ester to peak area) and the number of carbon atoms in a given homologous series of fatty acids [106].

All these investigations have shown that precise quantitative results can be obtained in work with a katharometer only after the detector has been carefully calibrated with standard mixtures of methyl esters for the given conditions of sample size, current in sensitive cell, rate of flow of carrier gas, and temperature and nature of column [35,48]; after the same column has been working for some time the calibration is repeated, or corrections corresponding to new values of V_R are determined from a graph [116]. A Martin balance may be used as standard in calibrating the detector [11].

(*b*) Ionisation detector. In 1958 Lovelock [117] proposed a new type of detector, in which the organic compounds are detected and quantitatively determined by ionisation with metastable argon atoms produced by the action

of ionising radiation. The following reactions occur in the detector:

$$Ar + \beta \text{ particles} \rightarrow Ar^+ + e^-, \quad (1)$$

$$Ar + e^- \xrightarrow{\text{high voltage}} Ar^* \ (11.6 \text{ eV}), \quad (2)$$

$$\text{Organic compound} + Ar^* \rightarrow \text{organic compound}^+ + e^- + Ar, \quad (3)$$

$$e^- \xrightarrow{\text{to anode}} \text{ionisation current}. \quad (4)$$

When argon passing through the detector is irradiated with β particles from ^{90}Sr, metastable argon atoms with an excitation potential (11.6 eV) which is above the ionisation potentials of most organic compounds are formed in addition to argon ions. Ionisation of organic compounds leads to an increase in the ionisation current, which is registered on a recording potentiometer. The high sensitivity of the ionisation detector (10^{-9} g) enables it to be used for the separation of small quantities of material, which greatly increases the effectiveness of the column. For esters with a molecular weight of 150 and higher, the peak area corresponds to the weight content of a given methyl ester in the mixture; therefore the detector does not require calibration[12,35,118].

The linear dependence of the detector signal on the concentration of a compound in the stream has been confirmed within certain limits in the analysis of standard mixtures of methyl esters of higher fatty acids[63,119,120]. The relative error in measuring the concentration of the maximum peak did not exceed 1%.[35] As a check on the linearity of the signal, free C_4–C_{10} fatty acids were separated on a non-polar column with Apiezon L: if the carboxyl occupied a large volume in the acid molecule, the signal on the ionisation detector was somewhat less than that corresponding to the weight content of the component[113]. The peak areas of the lower fatty acids are proportional to their mole percentages[118]. An appreciable deviation has repeatedly been observed in the analysis of samples containing methyl linolenate[60]. Thus, the linolenic acid content of linseed oil was found to be 46.4% by ultraviolet spectrophotometry and 54.2% by gas chromatography with an ionisation detector[50]. The reason for this behaviour of linolenic acid is still obscure.

Notwithstanding the complications mentioned above, its high sensitivity, simplicity, and reliability make the ionisation detector superior to other types of detector. At present this detector is used more often than others in the quantitative determination of methyl esters of higher fatty acids.

9. Determination of Radioactivity of Fatty Acids with Gas-Chromatographic Separation

In biological investigations in which labelled atoms are used it is necessary to determine the separate compounds qualitatively and quantitatively and in addition their radioactivity must be measured. A number of methods have been proposed for solving this problem in gas–liquid chromatography. The methods may be divided into two groups: in the first group the separate fractions emerging from the column are preparatively trapped and their radioactivity is determined after their chromatography has been completed; in the second group the mass and the radioactivity of the fractions are measured simultaneously at the outlet of the column while the separation is in progress.

Traps for the preparative collection of fractions may consist of short glass tubes filled with anthracene crystals coated with silicone oil[70,121], tubes containing cotton wool moistered with toluene and kept at room temperature[132], collectors with a cooling gradient[123], spiral condensers with glass powder on the inner walls[69], or special microporous filters[124]. With an ionisation detector there is practically no degradation of the sample in the detector; samples of methyl esters emerging from the column can therefore be collected without loss[117]. As the fractions are collected the traps are changed by hand[121,122] or by means of an automatic fraction collector[70]. The methyl esters collected are eluted with a solution of scintillators (2,5-diphenyloxazole and 2-*p*-fluorophenyl-5-phenyloxazole) in toluene, and measurements are made to the required accuracy on a scintillation spectrophotometer. Usually ~90% of the radioactivity introduced into the column is trapped[122]; the remainder of the activity emerges from the column later because it is held up by transesterification of the methyl esters with the polyester liquid phase and their adsorption on the solid carrier[65,72]. This delay can be reduced if the carrier is treated before addition of the liquid phase with hydrochloric acid and alkali, the transesterification catalyst (toluene-*p*-sulphonic acid) is removed from the liquid phase, and the separation is carried out at the lowest possible temperature[65,70,72]. The method of collecting fractions is used in work with samples having low radioactivity.

If the activity of the separate methyl esters exceeds a count of 1000 min^{-1}, more rapid methods in which radioactivity is continuously recorded should be used[125]. The vapours emerging from the column condense in a cooled solution of scintillator circulating very rapidly under the action of a gas stream in a closed system of tubes between windows of one or two spectrometer photomultipliers. The radioactivity of the solution is recorded as an integral curve, the height of a separate step being proportional to the activity of a given methyl ester; this curve is combined in due course with a differential curve recording the mass, the peaks are identified, and the specific radioactivity of each ester is determined. The efficacy of counting on such an apparatus reaches 80% for ^{14}C and 28% for ^{3}H.[126,127] If the retention volumes of adjacent components on the differential chromatogram are close (methyl stearate and methyl oleate), the esters cannot be separately determined on an integral radioactivity curve[70].

In another method lipids at the outlet from the column are burned over CuO to CO_2 and water; the water is absorbed by activated $MgClO_4$ or reduced to hydrogen, and the activity of the $^{14}CO_2$ is determined on a proportional or scintillation counter, a differential radioactivity curve being recorded[128,129].

III. GAS–LIQUID CHROMATOGRAPHY OF FREE FATTY ACIDS AND THEIR DERIVATIVES (OTHER THAN METHYL ESTERS)

1. Separation of Free Fatty Acids

Gas–liquid partition chromatography was proposed in 1952 by James and Martin as a method for separating volatile C_1–C_{12} free normal aliphatic acids[1]. A column of kieselguhr impregnated with a mixture (9 : 1) of silicone oil and stearic acid was used; this acid was included in the liquid phase to suppress dimerisation of the aliphatic acids, the molecules of which are highly polar. The association of carboxyls and the formation of dimers, which lowers the

volatility of the acids and leads to the formation of unsymmetrical diffuse zones on the chromatogram, may also be reduced somewhat by changing the amount of test sample and the concentration of liquid phase on the column, as well as the working temperature[6]. At present separation in the free form is confined almost entirely to the lower aliphatic acids[14,130]. Only a few papers have been devoted to the gas chromatography of the free higher fatty acids. The conditions for separating these acids are given in Table 4.

As Table 4 shows, dimerisation of free acids has been suppressed by the use of polar liquid phases containing a non-volatile mineral acid[82,131]. Free fatty acids in low concentrations have been separated on a column with a non-polar phase, when an increase in working temperature reduced the asymmetry of the peaks[22]. On a polyester column the asymmetry of the peaks increased with rise in temperature and also as the concentration of the liquid phase was lowered[132]. Under all separation conditions considerable stretching of the rear of the peak ("tailing") was observed.

In comparison with the corresponding methyl esters, the free acids exhibit higher retention volumes V_R^{20}; the separation of saturated and monounsaturated fatty acids was less sharp, and quantitative determination with a katharometer was difficult, because of differences in thermal conductivity between the acids and the methyl esters[82,132].

2. Separation of Hydrocarbons, Alcohols, Aldehydes, and Amines

Higher hydrocarbons present in many vegetable and animal products are usually extracted by adsorption from the remaining lipoids as the least polar fraction, and are separated without further treatment on a column with a non-polar liquid phase at 270–300°;[133,134] no difference in V_R is observed between saturated and unsaturated hydrocarbons of the same chain length[135]. Hydrocarbons are much more volatile than alcohols: V_R for hydrocarbons with an even number of carbon atoms C_{2m} corresponds with V_R for the primary aliphatic alcohols having four fewer carbon atoms C_{2m-4}.[90] Usually organisms contain the normal C_{16}–C_{34} hydrocarbons with a preponderance of either even or odd components[133,136]. Sometimes alcohols or methyl esters of acids are reduced to hydrocarbons, and the latter are determined by gas–liquid chromatography[134,135].

Free aliphatic alcohols can be separated on a column with a liquid phase[136,137], but alcohols with different degrees of unsaturation are not separated from one another[135]. Free alcohols are poorly separated on columns with polar polyesters, because of transesterification with material of the liquid phase[138]: acetates of unsaturated and saturated C_5–C_{18} aliphatic alcohols have been separated well on polyester phases[139]. When the temperature of the column is raised to 180°, compounds with the same number of carbon atoms and one ester bond are separated, for example the methyl ester of a monobasic acid and the acetate of the primary alcohol, such as methyl laurate and undecyl acetate; this separation is impossible at 130°.[140]

Aliphatic aldehydes from brain plasmalogens form dimethylacetals on methanolysis in the presence of HCl, and these can be separated on columns with polar or non-polar liquid phases[91,138].

When chromatographed with non-polar liquid phases, aliphatic amines react with the material of the column; the reaction ceases when the solid carrier has been washed with methanolic KOH.[141]

3. Separation of Glycerides

Because of their high molecular weights, mono-, di-, and particularly tri-glycerides are only slightly volatile, and the gas–liquid chromatography of these compounds is therefore still at the stage of preliminary experiments. Conditions for the separation of triglycerides are given in Table 5. This shows that at the high temperatures required for the analysis of triglycerides only non-polar silicone liquid phases may be used. These phases separate triglycerides only according to the number of carbon atoms in the molecule; there is no separation based on unsaturation. Polyester phases cannot be used here because of their low thermal stability.

Satisfactory gas-chromatographic separation of triglycerides would provide the most direct and shortest path to a study of the composition and structure of these lipoids; for this reason, several attempts have been made in this direction. Initially, separation of the lipoids of butter-fat and coconut oil was achieved only as far as trimyristin C_{42}, and numerous decomposition products were obtained[142]; then the glycerides of palm-kernel oil and lard were separated as far as C_{52}.[143] Recently fractions of C_{26}–C_{60} triglycerides differing by two carbon atoms have been separated from various seed oils and butter-fat, as well as triglycerides of odd and branched fatty acids. There is no contamination with thermal decomposition products at the beginning of the chromatogram[92]. This method has been used on triglyceride fractions obtained by molecular distillation[144].

For gas chromatography mono- and di-glycerides must be converted into volatile derivatives, for example acetates. Monoglycerides up to monoarachin and diglycerides up to palmitomyristin have been separated as acetates; the acetates of 1-mono- and 1,3-di-glycerides are eluted

TABLE 4. Gas–liquid chromatography of free higher fatty acids.

Solid carrier	Liquid phase and its content	Temp., °C	Carrier gas	Gas flow, ml min^{-1}	Detector	Composition of sample and duration of analysis, min	Ref.
Celite 545, 50–100 mesh	Apiezon L, 20%	300	N_2	12	katharometer	C_{14}–C_{22}, 90	22
Celite 545 60–80 mesh	poly(ethylene glycol adipate), 25% + H_3PO_4, 2%	220–235	He	30–40	katharometer	C_8–C_{20}, 60–80	131
Kieselguhr	Reoplex-400, 5–20%	165	Ar	35–50	ionisation	C_4–C_{18}, 90	132
Glass beads 0.75–1.00 mm	poly(ethylene glycol succinate), 1% + + H_3PO_4, 0.4%	140–200	Ar	60	ionisation	C_6–C_{18}, 40	82

TABLE 5. Gas–liquid chromatography of triglycerides.

Solid carrier	Liquid phase and its content	Temp., °C	Carrier gas	Gas flow, ml × × min^{-1}	Detector	Composition of sample and duration of analysis, min	Ref.
Chromosorb W	silicone, 30%	347	He	200	katharometer	C_{20}–C_{42}, 90	142
Ditto	silicone rubber, 2%	programmed, 100–360	He	145	»	C_{18}–C_{52}, 25	143
»	methylsilicone rubber, 2.25%	ditto, 220–325	N_2	150	flame-ionisation	C_{26}–C_{60}, 30–50	92

more rapidly than the derivatives of 2-mono- and 1,2-di--glycerides respectively[145]. To determine 1- and 2-monoglycerides, a mixture of them is treated with methanesulphonyl chloride, and then with a solution of sodium iodide in acetone; the allyl esters of the fatty acids obtained are separated on a column with a non-polar liquid phase at 240° in 60 min. In NaI solution the acid residue of the 2-monoglyceride migrates to the extreme position; 1- and 2-monoglycerides therefore give products of the same composition. The fatty acids of 2-monoglycerides are determined after removal of the 1-monoglycerides by periodate oxidation[146]. This method might be useful in studying the specific hydrolysis of triglycerides by pancreatic lipase[6].

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In recent years there has been a continuous and ever increasing growth of gas-chromatographic methods in both theoretical and practical directions, and also in the wider application of this type of chromatography to the study of the most diverse problems in the chemistry and biochemistry of fatty acids and lipids.

Nevertheless, gas chromatography is still not a universal means of solving any problems that the development of science might present to the research worker. Like any other method, it has definite limitations, and it is only by skilfully combining gas chromatography with thin-layer, partition, and other types of chromatography, as well as with non-chromatographic methods — molecular and nuclear spectroscopy, mass spectrometry, X-ray analysis, specific enzyme hydrolysis, radioisotope methods, etc. — that we can hope to overcome successfully all the difficulties associated with the study of the chemical structure of plant, animal, and microorganism lipids and with the study of the part played by lipids in metabolism.

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OSCILLOGRAPHIC POLAROGRAPHY IN ORGANIC CHEMISTRY

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I. INTRODUCTION

The method of oscillographic polarography is based on the interpretation of curves obtained during the electrolysis of test solutions and recorded by a cathode-ray oscillograph. In ordinary or classical polarography the voltage applied to the polarographic electrolysis cell is varied according to a linear law, the rate of change of potential of the polarised electrode being deliberately selected at a low value of about 5 mV s^{-1} so that it has no influence on the shape of the polarogram. In oscillographic polarography the rates of change of potential vary from several millivolts to tens of volts per second. In some cases the rate of change of potential of the polarised electrode may reach several hundred volts per second. With a comparatively high rate of change of potential the current also changes rapidly, and a cathode-ray oscillograph is therefore used to measure the current; hence the name oscillographic polarography.

The methods of oscillographic polarography can be divided into two groups, depending on the form of the voltage applied to the polarographic cell. In the first group of methods, the voltage applied to the cell increases linearly. In the second group, it increases non-linearly, in particular sinusoidally.

The first papers on oscillographic polarography appeared about twenty years ago [1,2] and described chiefly the design and use of the instruments. Later, oscillographic polarography began to be employed as a highly sensitive analytical method. The most widely used methods have been those in which current–potential (i–E) curves are recorded with a voltage of triangular form applied to the cell, or in which curves for rate of change of potential as a function of potential, $dE/dt = f(E)$, are recorded with an applied voltage of sinusoidal form.

The general principles of oscillographic polarography and the theory of the method have been described in detail in a number of monographs [3-6] and reviews [7-12]. Oscillographic polarography is one of the newest instrumental methods of study, and is finding extensive application primarily in analytical chemistry. The value of the method is not restricted to analytical problems, however. Oscillographic polarography is widely used in organic chemistry, pharmacology [13-17], biochemistry [18,19], medicine [20,21], and oceanography [22]. In the present review an attempt is made to show how oscillographic polarography can be used to solve problems in theoretical organic chemistry. We shall deal chiefly with oscillographic polarography using alternating current, which gives $dE/dt = f(E)$ curves. This method is now the most fully developed with regard to both theory and apparatus. For comparison and comparative characterisation we shall give data from some work in which $i = f(E)$ curves were recorded.

II. PHYSICOCHEMICAL STUDIES BY OSCILLOGRAPHIC POLAROGRAPHY

For the sake of brevity we shall refer to the methods of oscillographic polarography in which the dependence of dE/dt on E and the dependence of i on E are recorded as the dE/dt method and the i–E method respectively.

The polarisable electrodes used in oscillographic polarography are the same as those used in classical polarography, *i.e.* the dropping mercury electrode, the hanging mercury drop [23-25], and solid electrodes [26]. In addition, jet electrodes are widely used in oscillographic polarography. Calomel electrodes, carbon electrodes, and the mercury macroelectrode are usually employed as reference electrodes.

To obtain oscillographic curves by the dE/dt method, an alternating current of about 0.5 mA with the dropping electrode and 5 mA with the jet electrode is passed through the polarographic cell. The mains are generally used as source of a.c. of frequency 50 c/s. [27]

In the absence of an electrochemically active substance, the $dE/dt = f(E)$ curve on an alkaline supporting electrolyte has an oval shape (Fig. 1). In the presence of a depolariser, the curve shows the appearance of incisions whose depth depends on the concentration of the depolariser and whose position along the potential axis depends on the nature of the depolariser.

The oscillogram for a solution containing a depolariser (Fig. 1) has two branches. The upper branch corresponds to the cathodic process and the lower branch to the anodic process. The extreme left-hand point of the curve corresponds to the dissolution potential of the mercury electrode, and the extreme right-hand point to the reduction potential of the cation of the base electrolyte.