Separation & Purification Reviews
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597294

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To cite this Article Kuksis, Arnis(1977) 'Gas-Liquid Chromatography of Free Fatty Acids', Separation & Purification Reviews, 6: 2, 353 — 395
To link to this Article: DOI: 10.1080/15422117708544706
URL: http://dx.doi.org/10.1080/15422117708544706

SEPARATION AND PURIFICATION METHODS, 6(2), 353-395 (1977)

GAS-LIQUID CHROMATOGRAPHY OF FREE FATTY ACIDS

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I. INTRODUCTION

Fatty acids may be separated by gas-liquid chromatography (GLC) as the free acids if appropriate packings are used. This was first recognized by James and Martin[1], who studied the separation of C_1-C_{12} acids and found it necessary to add organic or inorganic acids to the liquid phase to reduce tailing of the peaks. Subsequent work has confirmed the correctness of these measures and has led to the preparation of improved columns for an effective separation of both saturated and unsaturated fatty acids of short and long chain length in the free form. The latter developments have been fostered by the requirement for a rapid and direct determination of individual free fatty acid levels in extracts of clinical, biochemical and industrial sources. This need cannot be readily met by a conventional preparation and GLC analysis of the methyl esters of the fatty acids, which is laborious and frequently leads to a contamination of the sample due to methylation of any glyceryl esters that may be present in the sample or on the equipment.

The present review surveys the more recent developments in both methodology and application of GLC to analysis of free fatty acids in the underivatized form. In view of the extraordinary importance of the relative polarity of the acids in GLC the discussion is opened with a brief consideration of the pertinent physico-

chemical properties of free fatty acids. An excellent recent review on GLC of the free short-chain acids has been written by Cochrane[2].

II. PHYSICO-CHEMICAL PROPERTIES OF FREE FATTY ACIDS

Both theoretical considerations and practical experience have taught that fatty acids are best resolved by GLC in the form of their simple esters[3]. Nevertheless, certain experimental needs can be met only by analyzing the acids in the free form, and it is therefore instructive to examine the physico-chemical properties of the free acids that are known to cause difficulties in their separation and quantitation in a GLC system.

Boiling Point

Of the various physico-chemical properties of the free fatty acids none is more important for GLC than the vapour pressure and the related properties, boiling point and heat of vaporization. Selection of the operating temperatures and the choice of the liquid phase is directly dependent upon it. Systematic determinations of the boiling points at various pressures have shown that the free fatty acids are much less volatile than the corresponding methyl esters[4]. Table I shows that the difference in the boiling points is greatest for the shorter chain acids (50-60°C)[4,5] but that significant differences (20°C) persist among the medium and long chain acids and esters[4]. Furthermore, the order of GLC elution of the saturated acids is consistent with the order of the boiling points[1]. Other things being equal, it is obvious that the elution of the free fatty acids from a GLC column will require considerably higher temperature than the recovery of the methyl esters. Comparisons of boiling points at a given pressure have shown that those of the free long chain acids differ by 10-20°C compared to 15-20°C for the corresponding methyl esters, with lower differences being characteristic of the higher molecular weight acids[4]. It would therefore be expected that the chain length resolution of the free acids would be slightly inferior to that of the methyl esters. In the range 1-20 mm pressure, methyl linoleate boils about 3°C lower

TABLE I

BOILING POINTS OF FREE FATTY ACIDS AND THEIR METHYL ESTERS[4,5]

Components	Boiling Points, °C *	
	Acids[5]	Esters[4]
Acetic	118	56
Propionic	141	79
Isobutyric	154	91.5
Butyric	162	103
Trimethylacetic	164	
3-Methylbutyric	177	115.5
(±) 2-Methylbutyric	177	
Valeric	186	126.5
2,2-Dimethylbutyric	186	
3,3-Dimethylbutyric	190	
(±) 2,3-Dimethylbutyric	190	
2-Ethylbutyric	190	
(±) 2-Methylpentanoic	193[a]	
(±) 3-Methylpentanoic	198	
4-Methylpentanoic	199[b]	
Caproic	205	148.5
Heptanoic		171
Caprylic		192
Capric		228
Lauric		261.5

*748-774 torr.

than methyl oleate, which in turn boils about 3°C lower than methyl stearate[4]. It has not been established whether or not a similar relationship exists among the corresponding free acids, but the unsaturated free acids are eluted from a non-polar GLC column slightly ahead of the corresponding saturated acids[6].

The lower molecular weight fatty acids possess sufficient vapour pressure to undergo a significant lowering of the boiling point in the presence of steam and are carried over by steam distillation.

They are collected in amounts that are proportional to their vapour pressure. The higher molecular weight acids of much lower vapour pressure come over in relatively small amounts compared with the amount of steam used[7]. In this connection of interest would be the boiling points of true azeotropic mixtures of free short chain fatty acids, which might form separate peaks during GLC of aqueous solutions of these acids. Wills[8] has suggested that formation of an acetic acid-water azeotropic mixture could explain the irregularities encountered in the GLC analysis of aqueous solutions of this acid. For the present purposes, steam distillation also provides an effective method for the isolation of specific groups of fatty acids which can then be subjected to GLC analysis under conditions more rigidly defined than those applicable to total free acid mixtures.

Ionization

Unlike the esters, free fatty acids undergo dissociation of the molecule into ions in a manner analogous to inorganic acids. The degree of dissociation, however, is much smaller than that of inorganic acids. The degree of dissociation is often affected by temperature, but in the case of the fatty acids variation of the value of the dissociation constant with temperature is small[4]. Table II gives the dissociation constants and equivalent conductivities for normal fatty acids in aqueous solution at 25°C[9]. Formic acid undergoes dissociation to an extent about 10 times greater than acetic acid. Acetic acid has a dissociation constant of 1.8×10^{-5} and is therefore a weak and sparingly dissociated acid. Propionic acid is even more feebly ionized and as the series is ascended the dissociation constants decrease but slightly. All of the acids are stronger than carbonic acid ($K_1 = 3.4 \times 10^{-7}$). The relative dissociation constants of the fatty acids indicate that they will be liberated from the alkali salts by hydrochloric acid, which is completely dissociated, and that they in turn will liberate carbonic acid from sodium bicarbonate.

TABLE II

DISSOCIATION CONSTANTS AND EQUIVALENT CONDUCTANCES FOR NORMAL CHAIN FATTY ACIDS IN AQUEOUS SOLUTIONS AT 25°C[9].

Acid	Dissociation Constant, k x 10^5	Equivalent Conductance, Δo
Formic	21.0	404
Acetic	1.813	300.8
Propionic	1.32	386
Butyric	1.50	383
Valeric	1.56	381
Caproic	1.40	379
Heptanoic	1.30	378
Caprylic	1.41	377
Nonanoic	1.1	377

merization or Self-association

The normal aliphatic acids or at least the lower molecular ight members, which have been extensively investigated, are rongly associated, and at temperatures just below their boiling ints have twice the molecular weight corresponding to the empiri-l formulae[4]. Because of the tendency of the fatty acids to exist dimers they are capable of forming salts of the formula $_nH_{2\,n+1}COONa \cdot C_nH_{2\,n+1}COOH$. Introduction of various substituents, pecially halogen and hydroxyl groups into the fatty chain rkedly affects the dissociation and the effect is greatest the oser the substituent is to the carboxyl group.

Szabo and Dijkstra have shown, as cited by Beerthuis et al[10], at the self association varies with dilution of the acid and that fferent dilutions are differently affected by temperature. In ese studies the association of the fatty acids was determined by frared absorption in an inert solvent (paraffin) from 25°-220°C. e infrared absorption of the C=O stretching frequency displayed, r the C=O dimer, an absorption at 1717 cm^{-1};for the C=O monomer, absorption at 1767 cm^{-1}. It was concluded that even in dilute

solution, complete association is asymptotically approached. At room temperature 90% of the molecules of a 0.007 M stearic acid solution are associated. In undiluted solution only very slight dissociation occurs with increasing temperature. At 220°C at least 80% of the stearic acid is still associated. In dilute solution, however, stronger dissociation occurs and approaches complete dissociation asymptotically. At 200°C only 0.5% of the stearic acid in 0.007 M solution is associated. The association was constant for all chain lengths and the degree of unsaturation of the chain had no influence on the degree of dissociation. These data indicate that separation of free acids by GLC could be improved by increasing the temperature and maintaining low concentrations of acid in the liquid phase. The self-association of the free fatty acids is believed to be responsible for the tailing and ghosting of peaks during GLC. Self-association does not occur with the corresponding fatty acid esters.

Solubility

The solubility difference of the fatty acids and their esters in organic solvents also is of considerable interest to GLC of the free acids. Two striking phenomena have been noted in the results of the work of Ralston et al.[11] on the organic solvent - fatty acid system: a tendency toward pairing of the solubility curves and a formation of eutectics between some of the solvents and fatty acids. Eutectic formation with benzene, cyclohexane, and acetic acid is observed in the case of all the acids investigated. Without exception, the solubility curve of the even numbered carbon acid lies above the curve of the next higher odd numbered carbon acid throughout all, or a considerable part, of the temperature range. Thus, myristic acid is less soluble than pentadecanoic, and palmitic acid is less soluble than heptadecanoic acid in the same solvent at any given temperature.

In the solvents, which are more polar than trichloromethane, the adjacent homologs are also paired but the solubility curves intersect at moderate dilution[11]. Thus, the next higher odd-numbered homolog is the less soluble of each pair at temperatures below the

intersection, and the solubility of the acids decreases without alteration as the series is ascended. In the less polar solvents, this intersection occurs above lauric acid at ordinary temperatures, while in the more polar solvents it occurs also in the lower acids. Except in alcohols, the normal saturated fatty acids show a marked correlation between their solubilities and the polarities of the solvents. In non-polar solvents, the solubilities of the acids are almost linearly dependent upon temperature, but as the polarity of the solvent increases, the relation between concentration and temperature deviates considerably from linearity.

Kolb and Brown[12] determined the solubilities of a number of structurally different fatty acids. The results led them to conclude that the solubility of the acids increased with decreasing chain length and increasing degree of unsaturation. They also found that the solubility increased with distance of the point of unsaturation from the carboxyl group. Acids with cis-configuration were more soluble than the corresponding trans-isomers.

The mutual solubility effect of one acid on another is of very common occurrence and of extreme practical importance, but has received little experimental attention[4,11]. This phenomenon is responsible for the difficulty and sometimes impossibility of obtaining a pure component by crystallization of a mixture of several fatty acids. It is also responsible for the separation during crystalization of palmitic-stearic acid mixtures having the properties of margaric acid. This phenomenon is the cause of the inability to prepare absolutely pure unsaturated acids from mixtures of unsaturated acids by low temperature crystallization. It may also be responsible for some of the tailing and ghosting of free fatty acids during GLC.

III. COLUMN REQUIREMENTS

The columns for the GLC separation of mixtures of free fatty acids require a high degree of deactivation and a stationary phase which permits complete resolution of all components as well as a rapid elution of the sample. These requirements have been met to

a large extent by using all glass systems and supports and liquid phases with highly acidic additives.

Supports

The diatomaceous earths which are commonly employed as chromatographic supports cause extensive adsorption and tailing of free fatty acids. The main reason for tailing of acid peaks is believed to be adsorption of the acids at those sites where hydroxyl groups are attached. Horning et al[13] have advocated the masking of such groups by silanization, which has led to a wide-spread use of silanized supports in GLC. Withers[14], however, has shown that the use of silanized supports for free acid analysis leads to selective losses of acids. These findings have been confirmed by Ottenstein and Bartley[15], who have suggested that the poor wetting properties of silanized supports may prevent complete deactivation of active sites of adsorption by acid additives. Some silanized supports have been shown to be compatible with deactivation by formic[2] and phosphoric[16] acid.

James and Martin[1] succeeded in minimizing the adsorption by washing the support with dilute aqueous phosphoric acid (1%, v/v) before final drying. This helpful effect of H_3PO_4 was subsequently confirmed by Metcalfe[17], who extended its use to combinations with polyester type of liquid phases. Likewise, Nikelly[18] has reported that a treatment with phosphoric acid also helps to remove natural alkalinity from glass bead supports, resulting in reduced peak tailing.

The difficulties associated with adsorption have also been overcome by inclusion of a strong volatile acid vapour in the carrier gas. Ackman and Burgher[19] passed helium over the surface of strong formic acid before entering the column for analysis of C_2-C_6 acids dissolved in water or 30% acetone. Much improved recoveries and reduced peak tailing was obtained with columns containing 25% Tween- 25% NPGA and 25% DC-50 silicone oil supplemented with 5% stearic acid. It was observed, however, that occasionally two peaks were obtained for acetic acid with all the columns. This double peak formation appeared to depend on the amount of water

resent and may have been related to the formation of a water-cetic acid aezotrope[8]. The findings of Ackman and Burgher[19] have een confirmed by Cochrane[2,20], who has employed a formic acid quilibrated carrier gas to elute C_2-C_{12} acids from 25% NPGA oated Chromosorb W (AW DMCS, 80/100 mesh) support.

There is ample evidence, however, that the addition of strong cids to the liquid phase may also reduce peak tailing and asymmetry by minimizing dimerization of the free acids. James and artin[1] considered the selfassociation or dimerization of the free atty acids in the vapour phase as the main obstacle to their atisfactory separation by GLC. They minimized this effect by incorporation of an organic acid of low vapour pressure into the liquid phase. The beneficial effect was attributed to the reduction of the proportion of free acid molecules present in the monomer form, but potential inactivation of the active sites on the support also appeared to make a significant contribution to the improved resolution. The idea of selfassociation of the acid in the liquid phase was supported by the improvement in peak symmetry realized when dimer acid (a condensation product of two C_{18} unsaturated acids) was added to the silicone oil liquid phase deposited on a very inert fluorocarbon surface[21].

Subsequent work by Kirkland[22] with the fluorocarbon, Teflon 6, support coated with Carbowax 400, without a special acid additive, showed that relatively high concentrations of C_1-C_3 acids in aqueous solution could be successfully separated without any apparent tailing. Confirmation that an acid additive is not required when Teflon is used as the support material was claimed by Kabot nd Ettre[23]. Examination of the manufacturer's literature (see)ttenstein[24]), however, has shown that Carbowax 400 can vary from)H 4 to 7. It is possible therefore that some acid was present in he Carbowax used in these studies. Likewise, the presence of acid ust be suspected in the Polypropylene Glycol 600 and PEG-1000 liquid phases employed in combination with Chromosorb W (regular) oth of which have also been claimed not to require the addition

of acid for the separation of free short chain acids[25]. Ottenstein and Bartley[15] have subsequently claimed to have confirmed Kirkland's findings that with a glass column and Teflon support the acid addition may not be necessary to eliminate peak tailing due to any dimerization of the acids. However, Gas Pak, which is a Teflon impregnated diatomite support, gave severe tailing when it was coated with Carbowax 400 and used for GLC of C_2-C_5 acids, yet it was found to be non-absorptive when coated with a keto acid polymer[26].

Another alternative to an inert support is the use of porous polymers as shown by Hollis[27], but some problems remain. Ackman[28] has evaluated three types of porous polymer beads, Chromosorb 101 (Johns-Manville), Porapak-QS and Porapak N (Waters Associates) for GLC of the volatile free fatty acids under loads of about 10^{-8} moles, which are often encountered in flame ionization detection of analytical samples. The non-polar surfaces of these styrene divinylbenzene polymer beads serve as the stationary phase and do not require the addition of a special liquid phase. It was found that the addition of formic acid vapour to the carrier gas suppressed peak tailing and increased peak recovery without modifying the retention times or shortening the column life. Without the benefit of formic acid vapour the acetic acid was barely detectable with the Chromosorb 101 packing and there was tailing of all acids. After formic acid, the tailing of the volatile fatty acids was much reduced and a reasonable peak appeared for acetic acid. Furthermore, there was a distinct butyric acid peak apparent, which was made up of the impurity contributions from the propionic, isobutyric and valeric acids. Comparable observations were made with the other porous bead polymers. The general separation of acids favoured the use of Chromosorb 101, but this packing required a conditioning period of up to 24 hours at 160°C before baseline drift diminished enough to allow use at low attenuations. Recently, Tyler and Dibdin[29] have successfully quantitated the C_2-C_5 acids using a column containing Phase-

ak Q-2% H_3PO_4 in the range 10-40 nanograms without the use of ormic acid. Ghosting and peak tailing were not detected. The cids, however, were dissolved in 1 M HCl, which may have provided ufficient acid vapour to have a beneficial effect on the nalysis[2].

According to Van Eenaeme et al.[30,31] performing quantitative nalysis of the free volatile fatty acids by GLC without the ddition of formic acid either to the sample or to the carrier gas eems impossible. The requirement is most serious for the short hain acids which show the greatest tendency to dimerize, while he medium and long chain acids can be satisfactorily resolved ith suuports inactivated by phosphoric acid or terephthalic acid dded to the liquid phase.

iquid Phases

The requirements of the liquid phases differ for the short nd long chain free fatty acids, although both types of phases how the need for acid additives. It is assumed that the more olar the liquid phase is, the less chance there is for dimeriza- ion of the acids to occur, as well as for an increased tendency or removal of adsorption sites on the support material.

The principle of like dissolves like has been of value in hoosing suitable stationary phases for the short chain fatty cids. Thus, free fatty acids up to C_5 were well separated by Jackson[32] using behenic acid or sebacic acid phases in packed olumns, while Averill[33] used a phase based on a trimer acid (a C_{54} tribasic acid derived from condensation of 3 unsaturated C_{18} cids) for capillary work. Slight tendency for tailing, however, emains suggesting insufficient acidity because addition of a hi- hly polar dinonylnaphthalenedisulfonic acid results in efficient ail reduction with the trimer acid phase[33]. Others[34] have found hat addition of phosphoric acid to EGA or terephthalic acid to arbowax also produce less tailing and higher recoveries of short hain fatty acids than do the same phases without the addition of he acids. A comprehensive evaluation of different acid additives

was carried out by Nikelly[18] who found that of several inorganic and organic acids tested, the best packings for the resolution of C_2-C_{18} acids were those composed of 0.25% Carbowax 20M and 0.5% isophthalic acid. Carbowax 20M with added or crosslinked terephthalic acid has been extensively employed[35-37] on a variety of supports for the analysis of free fatty acids. Particularly effective has been the product from the combination of Carbowax 20M and 2-nitroterephthalic acid [37]. The latter material is available commercially under the name of "Free Fatty Acid Phase" (Varian Associates).

Clarke and Fredericks[38] have pointed out that the presumably inert silicone phases may cause peak tailing and component loss because they deposit silica inside flame detectors, where the free acids become adsorbed.

A comprehensive evaluation of 13 different types of packing in stainless steel columns was made by Doelle[39] for determining C_2-C_7 acids from bacterial fermentations. Best results were obtained from 3.1% PEGA column, which surprisingly gave much less peak tailing than a similar one containing H_3PO_4. Hrivnak and Palo[40] have evaluated six polyester type phases in glass columns with H_3PO_4 for the analysis of free fatty acids from dairy products. All the columns were found to be suitable for the lower acids, although the separation of C_3 and iso-C_4 acids was not very good. An NPGA column was the best in this respect.

The development of the porous polymers of styrene divinylbenzene provided a mjor advancement in analyses of small polar molecules[27]. The separation results from a surface area of 400-800 m^2/g, but it also makes it necessary to use temperatures of 150-200°C for analyses of compounds which can be performed at room temperature with conventional coated packings[28]. By changing the chemical composition of the polymer, materials such as Porapak Q, Chromosorb Century Series, Porapak S and otherpackings have been prepared.

Table III compares the separation factors obtained for the more difficulty resolved pairs of free short chain fatty acids on the

TABLE III

COMPARISON OF SEPARATION FACTORS FOR SHORT CHAIN ACIDS AND MCREYNOLDS NUMBER[41,42]

Liquid Phase	Column Temperature, °C	Separation Factors			McReynolds Number, 2-Me-2Pentanol
		iso-C_4/C_3	n-C_4/iso-C_4	3-Me-C_4/2-Me-C_4	
EGS*	110	1.08	1.38		633
CHDMS	110	1.08	1.39		351
DEGA	110	1.12	1.37		479
BDS	110	1.13	1.38		457
EGA	110	1.14	1.37		462
R-400	110	1.14	1.37		482
FFAP	110	1.14	1.37		423
PEG-20M	110	1.18	1.36		387
TW-80	110	1.18	1.37		310
NPGS	110	1.21	1.38		371
SP-1200	110	1.37	1.26		145
DOS	160	1.38	1.26		132
POR-Q	220	1.79	1.16		
FT-G+0.15% H_3PO_4	110	2.13	1.48	1.23	
+0.4% PEG-20M	160	1.97	1.33	1.18	
Graphon+0.5% H_3PO_4	110	2.98	1.58	1.29	
+3% PEG-20M	160	2.28	1.41	1.19	
FT-G+0.3% FFAP	185	1.97	1.33	1.15	

* EGS, polyetheleneglycol succinate; CHDMS, polycyclohexanedimethanol succinate; DEGA, polydiethyleneglycol adipate; BDS, polybutanediol succinate; EGA, polyethyleneglycol adipate; R-400, Reoplex 400, PEG-20M, polyethyleneglycol 20M; TW-80, Tween 80; NPGS, polyneopentylglycol succinate; DOS, dioctyl sebacate; POR-Q, Porapak Q; FFAP, free fatty acid phase (Varian); FT-G and Graphon, Preparations of of graphatized carbon black. SP-1200, Synthesized by Supelco, Inc.

more frequently employed liquid phases[41,42]. A critical factor is the use of small sample size. The separation factors for iso-C_4/C_3 increased with decreasing polarity of the phase as measured by the McReynolds constant for 2-methyl-2-pentanol. Table III also includes the separation factors for the porous organic polymer Porapak Q which may also be classified with the chromatographic supports.

The ability of the liquid phase to withstand the attack of water may also be a valid criterion in the selection of the phase[41]. The samples of short chain free fatty acids are frequently admitted to the column in aqueous solutions. Several packings, which have given reasonable separations of free short chain acids, such as dilauroyl phthalate and Castorwax, have been excluded from the above listing because of their susceptibility to hydrolysis.

In dealing with free fatty acids C_{14} and higher, the major problem becomes the separation according to degree of unsaturation. The liquid phases of moderate polarity, such as Carbowax 20M, employed in the separation of the short chain fatty acids possess very low separation factors for the resolution of the saturated and monounsaturated acids of the same carbon number, although the separation of the saturated and polyunsaturated fatty acids is adequate. Incorporating the free acid tail-reducers into the liquid phase allows the long chain acids to be eluted without tailing but does not change the saturate-monoene separation ratio.

Metcalfe[17,34] extended the use of phosphoric acid to combinations with polyester liquid phases to obtain adequate separations of saturated and monounsaturated long chain fatty acids in the underivatized form. Metcalfe[34] has suggested to precondition this type of packing before filling the column. After solvent is evaporated, the packing is placed in a tray and heated in air in an oven at 200-250°C for several hours. Although the packing turns dark, it shows less bleed and gives better results than a packing that has not been treated this way. Metcalfe[17,34] has shown that the separations obtained are in the order of the degree of unsatu-

:ation as is the case of the fatty acid methyl esters. DEGA was :ound to be the most stable polyester of a series tested. The DEGS ınd NGS packings were less stable and could be used up to 180°C for :he separation of acids up to C_{18}. Metcalfe[34] suggested that phos-)horic acid might form a phosphate ester with the terminal groups)f the polyester thus stabilizing it. According to Ottenstein ınd Supina[16] EGS with H_3PO_4 is less stable than EGS without H_3PO_4.)ttenstein and Supina[16] have described the properties of two com-ıercially available preparations of EGS-H_3PO_4(SP-216-PS) and)EGS-H_3PO_4(DEGS-PS). All were coated on 80-100 mesh Supelcoport, ın acid washed and DMCS-treated diatomaceous earth support (Supelco, Inc.).

Table IV compares the separation factors for 18:1/18:0 and :0:0/18:2 on several polyester liquid phases fortified with phos-)horic acid along with the appropriate McReynolds factors[16]. It is ıeen that the stearic/oleic acid separation increases in direct)roportion to the polarity of the liquid phase. The most rapid ınd complete separation was obtained with SP-216-PS, but this phase ;ave no separation for the 20:0/18:2 pair. In case of the DEGA-PS, TAP and 20M-TPA, the analysis took considerably more time and the ıeparation was quite poor for the 18:0/18:1 pair, but the 20:0 acid ras well resolved from both 18:2 and 18:3 by being eluted last.)ttenstein and Supina[16] have shown that the 18:1/18:0 separation :actors are decreased as the loading of the liquid phase is reduced :rom 10% to 5% .

At the temperatures used (200°C) only DEGS-PS has the proper)olarity to avoid overlapping of 18:0 and 18:1, and 20:0 and 18:2. Vith the free acids, separation factors for 18:1/18:0 of 1.20 and ..12 are found for the DEGS and DEGA compared to 1.18 and 1.10 for :he methyl esters, respectively. The DEGS-PS and the SP-216-PS .olumns tended to show overloading more readily than the lower po-.arity columns.

The stationary phases studied by Ottenstein and Supina[16] were ıighly susceptible to deterioration by moisture in the carrier gas. . 2 ft x 1 in. diameter metal tube filled with activated molecular

TABLE IV

COMPARISON OF SEPARATION FACTORS FOR LONG CHAIN FATTY ACIDS AND MCREYNOLDS NUMBER[16]

Liquid Phase *	Separation Factors			McReynolds Factor X
	16:1/16:0	18:1/18:0	20:0/18:2	
SP-216-PS	1.25	1.23	1.00	632
DEGS-PS	1.20	1.20	1.15	496
DEGA-PS	1.15	1.12	1.38	378
FFAP	1.12	1.09	1.53	340
20M-TPA	0.12	1.07	1.54	321

*SP-216-PS, polyethyleneglycol adipate; DEGS-PS, polydiethyleneglycol succinate; and DEGA-PS, polydiethyleneglycol adipate, all fortified with orthophosphoric acid; other liquid phases as in Table III. All were coated on 80-100 mesh Suplecoport, an acid washed and DMCS-treated diatomaceous earth support (Supelco, Inc.).

sieve 4A or 5A was found effective in removing the moisture. Without the drier, the SP-216-PS deteriorated in a week while the DEGS-PS deteriorated in about three weeks. Obviously aqueous solutions could not be properly analyzed with these liquid phases. There was no need for adding formic acid vapour to the carrier gas for the analysis of the long chain saturated and unsaturated acids and there may be doubt about the compatibility of formic acid vapour in the carrier and the long term performance of these liquid phases.

Column Material

In an extensive study of problems associated with the use of different column materials, Ottenstein and Bartley[15] showed that free short chain fatty acids can be rapidly analyzed using either a porous polymer in a glass column, or a low polarity stationary phase with H_3PO_4 in either a glass or stainless steel column. From various experimental observations it was concluded that acids

ı the range of 0.1% concentration are adsorbed by the metal column ıbing, metal in the inlet side of the column, and by the glass ›ol plugs with siliconized surface. Ottenstein and Bartley[15] :aimed that the role of the acid additive is to deactivate the :tal tube as well as the support. When stainless steel or alumin- ı was substituted for glass, the free acids of short chain length :re no longer eluted from a porous polymer column. Silanized sur- ıces appeared to be incompatible with the efficient separation of :ee fatty acids. Presumably silylation masks active sites other ıan those masked by phosphoric acid and prevents phosphoric acid :om reaching them if applied prior to it[14,15]. When treating the ıpport with a tail reducer (phosphoric acid), it may be necessary › treat the tubing and the glass wool with this type of material.

Metal at the front of the column appears to be more detrimental ıan metal at the exit. In practice metal inlets may be avoided by ıserting a glass liner in the injection port when working with a :tal column. Also a direct injection of the sample onto the pack- ıg may help to reduce adsorption in the metal injector or the ›per part of the column. Comparisons of various grades of stain- :ss steel have shown that the material supplied by some manufac- ıres was satisfactory for free acid analyses while that supplied r others was not[43].

In several instances satisfactory separations of free fatty :ids have been obtained with aluminum[44] and copper[25] columns, but ›mparative studies have demonstrated that stainless steel or glass › superior[38,15]. The most obvious disadvantage of copper and alumin- ı is that both form oxides which act as adsorbents or catalysts. compromise between metal and glass columns has been offered by ›mmercial sources in the form of glass-lined metal column tubing ılltech Associates, Inc., Arlington Heights, Ill.) but no applica- :ons of such columns appear to have been specifically reported in ıe separation of free fatty acids. It is generally conceded that lass columns give slightly higher efficiency compared to steel, ıt the reason for this is not clear.

Both glass[45] and stainless steel[5,46] tubing have been successfully used for the separation of the free short chain fatty acids by capillary GLC. Routine analyses of free fatty acids with more than six carbon atoms in natural materials have not as yet been achieved[47].

Ghosting

The selection of the packing, column material and the nature of the injector or preheater ports of the gas chromatograph are important in regards to ghosting. Ghosting is a phenomenon characterized by the appearance of small solute peaks upon injection of water or formic acid subsequent to the injection of a volatile fatty acid mixture[48]. Smith and Gosnell[49] have discussed the problem of ghosting of acids and have attributed it to the glass wool or organic debris in the injector port. Treating the wool with phosphoric acid appeared to solve the problem. Ackman and Burgher[19] recommended the addition of formic acid vapour to the carrier gas in order to promote the self-elution of the volatile fatty acids. This suggestion has been reiterated by Geddes and Gilmour[50], who have called attention to the insidious nature of ghosting, which is not easily detected in a sequential series of samples. Cochrane[2,20] has reinvestigated the use of formic acid vapour in the carrier gas for the elution of C_2-C_{12} fatty acids and has confirmed the beneficial effects claimed by Ackman and Burgher[19]. Cochrane has pointed out that the material and cleanliness of the injector port and the nature of the support for the liquid phase may also contribute to ghosting as they do to peak tailing. Van Eenaeme et al.[30,31] have conducted a systematic study on the influence of the potentially critical factors on the occurrence of ghosting, as well as on the precision and accuracy of calculated data. It was concluded that ghosting related to polarity or acid strength of the acids, the most polar exhibiting the largest ghosting. Peak shape and retention times of the ghosts indicated that the injection area which includes the injector, the column plugs and the column top, might be the principle cause of ghosting.

Ghosting depends on the nature and the strength of the ghost eluter, formic acid being stronger than water in the elution of the ghost peaks. Ghosting influences both precision and accuracy of the analysis. Under circumstances where ghosting is important, repeatability of sample injection is poor and precision suffers. Ghosting affects accuracy by holding back a fraction of the sample in the analytical system, and by negatively influencing linearity of calibration, resulting in a quantitative error.

According to Van Eenamae et al.[31] and Cochrane[2] when formic acid is not incorporated into the carrier gas, the problem of ghosting and tailing increases with decreasing chain length and with decreasing concentration of the free fatty acids. Cochrane[2] has estimated that satisfactory results may be obtained for free fatty acids at concentrations of 1,000 ppm, but unsatisfactory ones are often obtained from the same acids at lower concentrations. While the use of a tail and ghost reducer such as formic acid may be necessary in special circumstances, this practice should be avoided whenever possible by the use of more appropriate stationary phases and supports.

IV. GLC OF STANDARD FREE FATTY ACIDS

In addition to establishing optimum conditions for elution of separated peaks and quantitative recovery of the solutes, GLC of standard acids allows the selection of liquid phases and the determination of conditions for optimum separation of mixtures of free fatty acids. Markedly different recovery conditions are usually required for the short and long chain, and saturated and unsaturated fatty acids in the free form. The choice of the conditions of separation is influenced by the level of sensitivity at which the analyses are to be made and by the quality of the results required.

Short Chain Acids

The short chain species were the first free acids to be successfully recovered and separated by GLC columns[1]. Subsequent studies have shown that the common homologous series of acids of C_2 to C_6 can be readily resolved and recovered as symmetrical peaks from

most GLC columns provided the chromatographic system is free of active sites of adsorption[2] . The prevention of selfassociation of the acids by inclusion of an excess acid in the liquid phase or carrier gas is less important when working with relatively large sample sizes. At low levels of total sample, however, there is a marked improvement in the recovery and in the shape of elution of all peaks when non-volatile acids are included in the liquid phase, or formic acid is present in the carrier gas. There is also evidence that the phenomenon of ghosting is completely eliminated by the presence of formic acid vapour in the carrier gas[19,20].

Figure I shows the separation obtained for C_1-C_7 fatty acids on 10% SP-1000/1% H_3PO_4 coated on 100/120 mesh Chromosorb W AW and contained in a 6 ft x 4 mm I. D. glass column[51]. The analysis was complete in 24 minutes at 155°C. Except for formic acid, the acids are eluted in the order of their boiling points, or molecular weights. The branched chain acids are eluted ahead of the corresponding normal chain acids, as already established for the methyl esters of these acids[52]. Comparative elution patterns for these acids have been obtained on other columns packed with conventional packings fortified with acid additives[15,26,42,51,53,54], or with conventional packings[20] and porous polymers[28] but developed with carrier gases containing formic acid vapour.

The most complex mixtures of short chain fatty acids have been resolved by capillary GLC. TAble V gives the relative retention times of 16 short chain fatty acids on three different liquid phases suitable for capillary GLC[5]. With few exceptions, the order of elution of the free acids from all phases follows closely the order of increasing boiling points.

Table VI gives the results of a quantitative analysis of standard solutions of C_2 to C_6 acids obtained with columns developed without and with formic acid in the carrier gas[2]. The addition of formic acid to the carrier gas resulted in a significant improvement in the accuracy and precision of the analysis compared to the other methods.

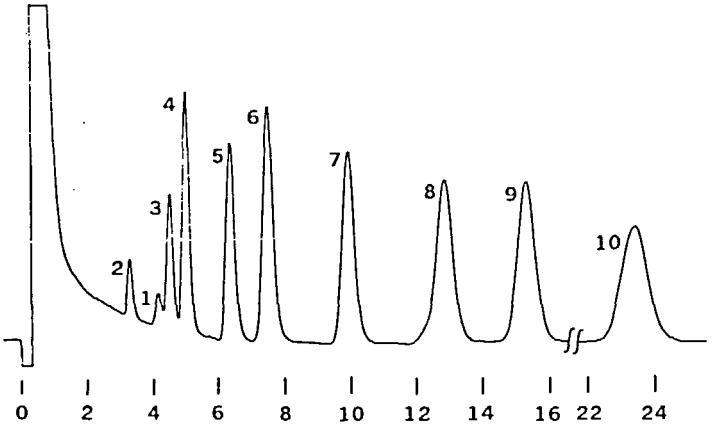

FIGURE 1

GLC Resolution of Underivatized C_1-C_7 Fatty Acids on SP-1000[51]. (Reproduced with permission from Supelco, Inc., Bellefonte, Pa.) GLC conditions: 10% SP-1000/1% H_3PO_4 on 100/120 mesh Chromosorb W AW, 6 ft. x 4 mm I. D. glass column. Temp.: 155°C, isothermal; Flow rate: 86 ml/min He. Sample size: 14 µl of a chloroform solution. Peak identification: 1, formic; 2, acetic; 3, propionic; 4, isobutyric; 5, n-butyric; 6, isovaleric; 7, n-valeric; 8, isocaproic; 9, n-caproic; 10, heptanoic.

The operating conditions were as follows: column, 5 ft x 4 mm I.D. glass tube, packed with 25% NPGA coated onto Chromosorb W (AW DMCS, mesh 80/100) was used and it incorporated a glass liner in order to prevent the inlet end of the column from becoming contaminated with inorganic and carbonaceous material. The column was conditioned at 250°C until satisfactory baseline was obtained. The carrier gas was admitted to the column after first passing over a formic acid solution in special reservoir. Temperature was programmed from 75°C at 6°C/min to 250°C. The acids were dissolved in a solution containing 20% of 0.5 N formic acid and 80% acetone prior to injection.

Quantitative analyses were carried out for all the methods by adding a known amount of internal standard (0.5 ml of isobutyric acid) to a weighed portion of the standard solution (4.5 ml) after

TABLE V

RELATIVE RETENTION TIMES OF FREE SATURATED C_2-C_6 FATTY ACIDS ON THREE STATIONARY PHASES AS OBTAINED BY CAPILLARY GLC[5].

Acid	Liquid Phases*		
	Trimer Acid (120°C)	Tricresyl Phosphate (110°C)	Ucon LB-550-X (125°C)
Acetic	0.138	0.114	0.178
Propionic	0.276	0.274	0.314
Isobutyric	0.405	0.374	0.428
Butyric	0.498	0.501	0.542
Trimethylacetic	0.519	0.441	0.521
3-Methylbutyric	0.697	0.694	0.742
(±)-2-Methylbutyric	0.759	0.717	0.776
Pentanoic	1.000	1.000	1.000
2,2-Dimethylbutyric	1.080	0.933	1.040
3,3-Dimethylbutyric	1.215	1.605	1.542
(±)-2,3-Dimethylbutyric	1.236	1.154	1.215
2-Ethylbutyric	1.286	1.267	1.308
(±)-2-Methylpentanoic	1.381	1.333	1.340
(±)-3-Methylpentanoic	1.431	1.449	1.427
4-Methylpentanoic	1.524	1.540	1.485
Hexanoic	1.855	1.933	1.816

*The liquid phase was applied by forcing a solution (0.4-0.6 ml) of the phase (90 mg) and orthophosphoric acid (10 mg, 85%) in acetone (1.0 ml) through the tubing (45 m x 0.25 mm I.D.) with the aid of nitrogen at a velocity of 0.8-1.2 cm/sec. The column was conditioned 6 hr at 20°C below the max limit of the phase.

response factors for each component relative to the internal standard had been determined. This method is also satisfactory for the analysis of the C_2 to C_{12} acids on a routine basis[20].

Reiss et al[55] have described 90-102% recoveries of radioactive octanoic acid from a GLC of free fatty acids on a 5 ft x 1/4 in.

TABLE VI

QUANTITATIVE ANALYSIS OF STANDARD SOLUTIONS OF C_2-C_6 ACIDS[2]

Statistic	Standard Acids				
	Acetic	Propionic	Butyric	Iso-Valeric	Caproic
			(ppm)		
Method 1*					
Standard Concentration	107	106	99	91	92
Found (Mean)	120	118	111	102	105
S.D.	5.35	4.83	5.05	12.31	6.02
Relative S.D. (%)	4.45	4.10	4.56	12.13	5.75
Method 2					
Standard Concentration	107	106	99	91	92
Found (Mean)	128	120	106	115	110
S.D.	7.97	4.93	12.34	16.46	7.08
Relative S.D. (%)	6.21	4.10	11.61	14.28	6.45
Method 3					
Standard Concentration	4.8	4.2	3.8	5.4	5.7
Found (Mean)	4.6	4.2	3.7	5.5	5.5
S.D.	0.20	0.11	0.15	0.19	0.20
Relative S.D. (%)	4.34	2.62	4.05	3.46	3.64

*Method 1, 0.3% SP-1000 + 0.3% H_3PO_4 on Carbopak A; Method 2, Chromosorb 101 without formic acid in carrier gas; Method 3, 25% NPGA on Chromosorb W AW DMCS with formic acid in the carrier gas.

Stainless steel column containing 15% DEGS and 3% phosphoric acid for this purpose the authors employed a specially designed collection system.

Long Chain Acids

When dealing with free fatty acids of long chain length it is necessary to separate them according to degree of unsaturation and to complete the separation rapidly enough to avoid decomposition of the acids and the liquid phase at the higher working temperatures. In order to obtain complete base line separation for the saturated and monounsaturated fatty acids an 18:1/18:0 separation factor of 1.18 is necessary. This can be obtained with the DEGS columns fortified with phosphoric acid without bringing about an undesirable overlapping of other peaks. Furthermore, the more polar DEGS column gives faster elution of the sample than the less polar EGA and DEGA columns at the same column temperature[16].

Figure 2 gives the separation of C_{14}-C_{20} fatty acids on a 10% DEGS-PS column[56]. A complete baseline separation of all components is obtained in 23 minutes of running time. An SP-216-PS column has given a complete baseline separation for the 18:1/18:0 pair in 12 minutes, but this liquid phase does not resolve the 20:0/18:2 pair of acids. Similar elution patterns have been obtained by other workers[57-60] for these acids on similar columns. Kuksis et al[60] have extended these separations to include the polyunsaturated fatty acids, 20:4, 20:5 and 22:6, which present no special difficulty in resolution on 5 or 10% DEGS-PS columns although the recoveries are rather low.

Table VII gives the relative retention data for the C_{14}-C_{20} saturated and unsaturated acids on several polyester columns containing added free acid[16]. Both DEGS-PS and SP-216-PS give adequate separation of the test mixture with 10% packing in 3 ft columns. By reducing the sationary phase loading to 5%, comparable separations are obtained in shorter time. In all instances the column temperature of 200°C was used.

There have been very few quantitative analyses published on the recovery of free fatty acid standards from GLC columns. Sampson and Hensley[57] have determined the calibration slopes and have estimated the minimal injected amounts detectable for C_{14}-C_{20}

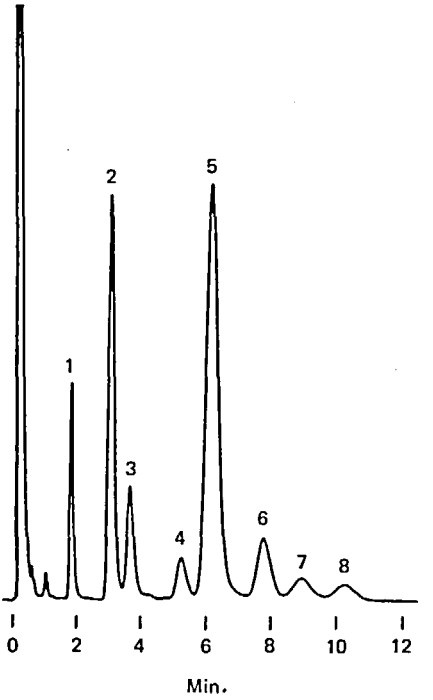

FIGURE 2

GLC Resolution of Underivatized C_{14}-C_{20} Fatty Acids on DEGS-PS[56]. (Reproduced with permission from Supelco, Inc., Bellefonte, Pa.). GLC conditions: 5% DEGS on 100/120 Supelcoport, 3 ft x 2 mm I.D. glass column. Temp.: 200°C, isothermal; Flow rate: 20 ml/min He. Sample size: 1.0 µl of a chloroform solution. Peak identification: 1, myristic; 2, palmitic; 3, palmitoleic; 4, stearic; 5, oleic; 6, linoleic; 7, arachidic; 8, linolenic.

turated and unsaturated fatty acids on 10% SP-1000 and 10% SP-6-PS columns using C_{15} and C_{17} acids as internal standards. The nimal injected amounts detectable varied from 0.3 nanomoles for e saturated acids to 1.0 nananomole for linolenic acid on the SP-00 column, all acids being detectable at about the same level of jection (0.1 nanomole) with the SP-216-PS column. The total ounts of injected acids varied from 0-28 nanomoles per peak. rth et al[58] have demonstrated an excellent correlation between

TABLE VII

RELATIVE RETENTION TIMES OF FREE SATURATED AND UNSATURATED C_{14}-C_{20} FATTY ACIDS ON FIVE STATIONARY PHASES AT 200°C[16]

Acids	Liquid Phases*				
	SP-216PS	DEGS-PS	DEGA-PS	FFAP	20M-TPA
14:0	0.41	0.34	0.29	0.27	0.26
16:0	0.64	0.59	0.54	0.52	0.51
16:1	0.80	0.71	0.62	0.58	0.57
18:0**	1.00	1.00	1.00	1.00	1.00
18:1	1.23	1.20	1.12	1.09	1.07
18:2	1.60	1.49	1.35	1.27	1.25
18:3	2.22	1.96	1.74	1.58	1.54
20:0	1.60	1.71	1.87	1.95	1.92

*Liquid phases identified as in Table IV.

**The absolute retention times of 18:0 were: SP-216-PS, 5.3; DEGS-PS, 11.5; DEGA-PS, 22.8; FFAP, 44.3; and 20M-TPA, 49.5 minutes.

the titration and GLC data for total free fatty acids over the range 250 to 1600 uEq/liter. The underivatized acids ranging from lauric to linoleic were analyzed on 10% DEGS-PS.

Kuksis et al.[60] have determined the relative recoveries of the underivatized polyunsaturated fatty acids from DEGS-PS columns at the 5% and 10% load levels. Only the 5% DEGS-PS columns allowed rapid elution and high recoveries of these acids. Table VIII compares the relative recoveries of 18:2, 18:3, 20:4, 20:5 and 22:6 acids from a 5% DEGS-PS column during isothermal and temperature programmed operation. Isothermal runs at 195°C required about 68 minutes for completion and resulted in about 50% loss of 22:6 acid. Temperature programming from 190-230°C over a period of 30 minutes resulted in a 40% loss of the 22:6 component. Since the 20:5 acid was eluted in about one half the time required for the elution of

TABLE VIII

RELATIVE RECOVERIES OF FREE POLYUNSATURATED FATTY ACIDS FROM 5% DEGS-PS COLUMNS[60]

Acids	Composition* (Weight, %)	Recoveries 195°C**	(Weight %) 190-230°C
18:2	20	27	26
18:3	20	25	23
20:4	20	21	20
20:5	20	17	19
20:6	20	10	12

*The original weight composition of the acid mixture was obtained from the manufacturer (NuCheck Prep, Elysian, Minn.). The free fatty acids were prepared from the methyl esters by saponification.

**195°C, isothermal runs; 190-230°C, temperature programmed runs over a 30 minute time interval. GLC columns were 3 ft x 2 mm I.D glass tubes packed with 5%DEGS on 80/00 mesh Supelcoport.

the 22:6 acid, the losses of the 20:5 acid were about one half those of the 22:6 acid. Analyses of the polyunsaturated fatty acids in the methyl ester form on these columns gave essentially correct weight proportions for all the acids except 22:6, which has about 10% low.

V. PRACTICAL APPLICATIONS

The various methods described for the separation of the standard mixtures of free fatty acids have been applied with various degrees of success to analyses of natural mixtures of free fatty acids. The greatest progress thus far has been realized in analyses of the free fatty acids of short chain length, although acids of medium and long chain length have also given satisfactory results. The free fatty acids of long chain length and a high degree

of unsaturation, however, have been recovered from polar GLC columns in low yields and have generally given unsatisfactory results when analyzed in the free form.

Sample Preparation

For a reliable qualitative and quantitative analysis it is essential to secure representative samples of free fatty acids. The best GLC system cannot recover the information lost during sample preparation. Due to the presence of the free carboxyl group, the free fatty acids are much more difficult to handle than the corresponding methyl esters. The volatility of the shorter chain lengths must be considered along with their increased water solubility and greater chemical reactivity. As yet there are no generally applicable methods of isolation and concentration of total free fatty acids. It is therefore essential that isolation methods are first tested with standard acids expected to be present in the sample before actual analyses are attempted.

A reliable method which is often used for extraction of free fatty acids from serum and plasma is the one developed by Dole[61] and by Dole and Meinertz[62], which were originally used for titrimetric techniques. Since then it has also been applied in GLC analyses[58,63,64]. Several investigators have found a recovery of more than 93%[61,65]. Detailed findings over interfering substances such as phospholipids, lactate, acetoacetate and β-hydroxybutyric acid have also been reported[61-63]. For the analyses of serum free fatty acids by direct GLC, Wirth et al.[58] recommend the addition of 5 ml of the extraction solvent (isopropan-2-ol/n-heptane/sulfuric acid, 40:10:1, v/v) to 1 ml of serum. The mixture is then shaken vigorously for at least 5 min. After standing for 10 min, 2 ml H_2O and 3 ml n-heptane are added and this mixture is shaken again for 3 min. After standing for 10 min the sample separates into 3 layers with upper layer containing the free fatty acids. The upper layer is separated and washed with 5 ml H_2SO_4 (0.05%). The heptane layer is separated by centrifugation and the n-heptane is removed by vacuum evaporation. The dry

residue is diluted with internal standard (a C_{15} or C_{17} acid) and an aliquot injected into the GLC column.

Sampson and Hensley[57] have employed chloroform/n-heptane/methanol 28:21:1 v/v as the solvent system for a rapid isolation of free fatty acids from plasma. A recovery of 75 to 80% was obtained for added standard fatty acids, which was thought to be satisfactory for rapid screening purposes. In this method 150 nmoles of C_{15} or C_{17} acid are added as internal standard to 1.0 ml plasma. The free fatty acids are extracted from the plasma into 20 ml of extraction solvent by shaking for 30 seconds on a Vortex mixer. The plasma and extraction solvent is left at room temperature for 15 minutes with occasional shaking, then mixed for 10 seconds on the Vortex mixer. After centrifuging the aqueous phase is discarded, the protein layer is pushed aside and the organic phase removed and evaporated to dryness under reduced pressure. The dry residue is dissolved in 100 µl n-heptane and 2 µl injected into the GLC column.

The above extraction methods are not suitable for the isolation of the volatile free fatty acids which are lost to variable extent during the evaporation of the extraction solvents. The latter acids may be recovered along with the medium and long chain acids by the solvent extraction procedure originally proposed by Drucker[66] and modified by Whitehead et al.[54] In this method an aliquot of plasma is made to 1 ml with water, 6.25 µg of isocaproic acid is added and the mixture extracted with 0.5 ml of diethyl ether. The ether layer is transferred to a small conical tube containing one drop of phenolphthalein and 5 µl of 4 M NaOH, which is sufficient to make the sample basic. The ether is evaporated at room temperature and the residue dried thoroughly at 60°C. After cooling the dried sample, the short chain fatty acids are extracted by adding 5 µl of 4 M H_2SO_4 and 50 µl of acidified ether. Before injection, ether samples are kept in capped tubes maintained at -25°C with alcohol-dry ice. Up to 2 µl of the ether extracts are injected into the GLC column with a 10 µl lock-pressure liquid

syringe. To avoid the appearance of ghost peaks, during subsequent injections, the syringe is washed thoroughly with acetone and ether between injections.

The isolation of acids by means of column or thin-layer adsorption chromatography usually constitutes a follow-up step to the solvent extraction, although in certain instances the entire original sample may be applied to the column or plate. Although more laborious, this method results in the isolation of the free fatty acids as a distinct lipid class. A rapid quantitative method for the separation of free fatty acids from other lipids by adorption column chromatography has been described by McCarthy and Duthie[67]. Silicic acid treated with iso-propanol-KOH and washed with ethyl ether serves as the column packing. The sample is introduced in ethyl ether, and neutral lipids are eluted with the same solvent. Free fatty acids are recovered from the column in 98% yield with 50 ml of 2% formic acid in ethyl ether followed by 75 to 100 ml of ethyl ether. This method has recently been tested by Wirth et al[58], who found incomplete recoveries for some acids.

Thin-layer adsorption chromatographic methods for the isolation of medium and long chain free fatty acids from plasma and tissue lipids have been extensively employed. Kuksis et al.[6] and Ko and Royer[63] have demonstrated that the GLC estimates of free fatty acids isolated by TLC give a close agreement with titration values obtained on free acids isolated by TLC. For the TLC separation Kuksis et al.[6] used hexane-diethyl ether 9:1 and Ko and Royer[63] hexane-diethyl ether-acetic acid 82:18:1, as the developing solvents. Rodrigues de Miranda and Eikelboom[68] have described a reversed phase TLC system for the isolation of individual free fatty acids or small groups thereof. A methanol-water-solvent system in proportions ranging from 10:90 for C_2-C_4 and up to 60:40 for C_7-C_9 free fatty acids gave excellent and reproducible resolution of the individual acids on silanized silica gel.

On a theoretical basis, anion exchange resins ought to offer the most promise for the isolation of free fatty acids from natural sources. In practice, however, difficulties have been experienced.

ornstein et al.[69] reported the isolation of free fatty acids from ipid mixtures by means of a weak anion resin (Amberlite IRA-400). he free fatty acids were recovered from the column by means of ethanolic-HCl, which converted them into methyl esters. Subse- uent studies by McCarthy and Duthie[67] and Duncombie and Rising[70] ave shown that only after the resin has been used several times nd regenerated is a quantitative gravimetric recovery of the ree fatty acids possible. GLC analyses have shown that fatty cids from previous runs exchange with the free fatty acids in the ample. Unsatisfactory experiences with the Amberlite-type of esins in the isolation of free fatty acids from plasma have been ecorded also by others[58].

The higher exchange capacity of the cross-linked dextran, EAE-Sephadex, has provided a superior ion exchange material for a ariety of applications. Zinkel and Rowe[71] have employed this ion xchanger in the free base form for the separation of acidic from eutral amterials in an organic solvent system. The free fatty cids and resin acids can be eluted readily with ether-methanol 0:10 saturated with CO_2. The acids are recovered in the free orm. Rouser et al.[72] have employed DEGA-cellulose in the acetate orm for the separation of free fatty acids from the bulk of lipid. he free fatty acids which bind quantitatively to the column are luted with chloroform-methanol-formic acid 200:100:3 v/v. oodridge[73] has employed the system for a successful isolation and uantitation of free fatty acids from hepatic tissues. Thompson id Markey[74] have compared various conventional methods of isola- ion and concentration of the volatile organic acids for subsequent C/MS and have found that the ion-exchange methods are superior to hers.

The short chain fatty acids C_1-C_7 can be selectively isolated steam distillation. Mahadevan and Zieve[75] have reviewed the rly methods of steam distillation of short chain fatty acids from ood and have recommended an improved method, which they have sted by determining the volatile fatty acids of normal human rum, plasma and red cells.

An initial chloroform-methanol extract is acidified with 25% acqueous metaphosphoric acid to pH 3.0, 5.0 g of $MgSO_4$ is added, and the mixture is steam distilled in a micro Kjeldahl distillation unit (Labconco, Kansas City, Mo.). At this pH, pyruvic, lactic, and β-hydroxy-butyric acids at concentrations normally encountered in blood are not distilled off and hence do not interfere with determination of short chain fatty acids. Values obtained were 97-102% of the expected for all acids studied (acetic, propionic, isobutyric, butyric, isovaleric and valeric).

Meijer and Hessing-Brand[76] have described a rountine method for the isolation of volatile fatty acids from biological materials using a simple direct distillation system. After addition of phosphoric acid and an internal standard, the sample is subjected to an isothermal distillation at 90°C in a Conway-Lips microdiffusion cell. The volatiles are absorbed by a small volume of an alkaline trapping agent, an aliquot of which is analyzed by GLC.

Specific Applications

GLC has become a useful aid for the identification of bacteria[77]. When many samples are to be analyzed speed is of prime importance and the analysis of the acids in the free form has become recognized as a time saving step. Especially well suited for this purpose are the short chain fatty acids.

Henkel[78] injected the supernatant fluid of aqueous cultures after centrifugation and filtration through a membrane filter, onto a Porapak N column without liquid phase. The fatty acids were eluted by temperature programming from 160° to 195°C. The author found his procedure useful for the identification and quantitation of the C_1-C_5 fatty acids. Bricknell and Feingold[79] have described a rapid method for the direct qualitative and quantitative assay by GLC of microbial metabolites produced in culture and biological specimens. For this purpose aqueous samples were injected directly onto a GLC column containing Resoflex, LAC-1-R-296 or 6% FFAP on Porapak Q and obtained effective separations for the shorter chain volatile fatty acids from acetic to caproic. The analysis was com-

plete in 16 minutes. The samples were prepared for assay by acidification of the culture broth with two drops of 50% H_2SO_4 per 4 ml of broth. Bricknell and Feingold[79] found that coating of Porapak Q with FFAP shortened the retention times of the acids.

Hauser and Zabransky[53] have described the use of an improved column packing (SP-1000, 1% H_2PO_4) for the separation of propionic and isobutyric acids as well as for demonstrating the presence of formic acid in the fermentation products of anaerobic bacteria. The column gave excellent separations of C_1-C_8 acids in a minimum of 12 minutes. The processing of about 600 extracts at 145°C over a period of three months left the packing stable and no variation in resolving power or elution time of the acids was observed. The findings of Hauser and Zabransky[53] have been corroborated in other laboratories[51], which have found that the SP-1220 column generally provides better separation and more sensitivity than Resoflex[79] for formic and propionic acids and faster elution for other acids. There are several instances where the bacteria produce amyl alcohols in significant quantities and these alcohols might be confused with the acids. This objection can be overcome by substituting the SP-1000 for the SP-1220 column. The SP-1000 column elutes the alcohols well in advance of the acids. The volatile free fatty acids for this purpose are extracted by adding 1 ml of diethyl ether to 2 ml of acidified culture medium, mixing and centrifuging to break emulsion, and freezing out the aqueous phase. The ether phase is decanted and dried before injection into the GLC column.

The determination of individual free fatty acid levels in plasma or serum is becoming increasingly relevant clinically, particularly when essential fatty acids are involved. A rapid assay is needed which is suitable both for individual cases and for the screening of relatively large numbers of samples. Sampson and Hensley[57] have developed a rapid method for quantitation of individual free fatty acids in plasma without derivatization or further purification. An internal standard (C_{15} or C_{17} acid) is added to the plasma before extraction to correct for day to day variation in extraction, injection and GLC conditions. Quantita-

tive estimates are obtained for 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 acids, which normally account for over 95% of plasma free fatty acids. The small amounts of shorter and longer chain fatty acids were not detected either because they were in the solvent front or were not present in sufficiently large concentration, or were not eluted from the 10% SP-1000 column. Among the latter ones must be included arachidonic acid (20:4), which occasionally occurs in plasma in the free form in significant concentration. The mean value obtained from 14 fasting laboratory members (295 μmoles/liter), was in the lower range of normal values reported in the literature (380 μmol/liter and 390 μmol/liter). The mean fasting value of patients with suspected lipid abnormalities (606 μmol/liter) was considerably higher.

Wirth et al.[58] have reported a rapid and precise method for the determination of total free fatty acids in the serum followed by the separation of individual acids by GLC. After extraction the acids are directly injected into the GLC column without esterification. The separations are performed on a 6 ft x 3 mm I.D. 10% DEGS-PS on 80-100 mesh Supelcoport at 200°C with N_2 as the carrier gas. The esterified fatty acids present in cholesterol esters, phospholipids and triacylglycerols do not interfere with the free fatty acid determination by the GLC method. This method is compared to other commonly used titrimetric and GLC procedures and the superiority of the present variation is demonstrated. A comparison of the total free fatty acid levels by means of titration and GLC showed that on the average the titration values were 1.1% higher than the GLC values with a correlation coefficient of 0.95. The method of isolation of the free fatty acids was modified from that described by Sampson and Hensley[57].

A rapid and sensitive method for acetic acid determination in human plasma and in hemodialysis baths has been developed by Desch and Descomps[80]. The method is based on a GLC resolution of unesterified short chain acids on a 180 cm x 3.2 mm I.D. glass column containing 20% NPGS and 1.8% phosphoric acid at 200°C. Formic acid was injected in-between runs to inactivate those adsor-

tive sites which had been missed by the phosphoric acid impregnation of the liquid phase and support. Nevertheless, difficulties, ere occasionally encountered due to either a formation of acetic id-water azeotropes or adsorption of acetic acid at the top of ie column.

Whitehead et al.[54] have developed a simple method for quantitating C_2-C_6 acids in intestinal fluids, feces and blood. The method tilizes extraction with dietyl ether in combination with GLC on % FFAP in a 6 ft x 1/8 in I.D. column. The temperature of the olumn is programmed from 90° to 130°C at 2°C/min. Prizont et al[81] ave used this method to determine the luminal and plasma levels f short chain fatty acid products of bacterial fermentation in its with surgically produced, self-filling blind loops, located i the proximal small intestine. High levels of acetic, propionic, id butyric acids were detected in the blind loop segment and in ie distal small bowel regions, which in normal sham-operated rats ontain no short chain fatty acids. Isobutyric, isovaleric and aleric acids were also present. The free fatty acids were extrated and concentrated by a modification of the method of Drucker[66].

Porter[59] has obtained excellent GLC resolution in the free orm of the fatty acids of bovine milk fat. For this purpose a ft x 2 mm I.D. glass column containing 10% SP-216-PS was used ith temperature programming from 130-200°C, at 1.5°C/min. The ree fatty acids were prepared by saponification. After acidification with 2 N HCl the acids were extracted with petroleum ether id diethyl ether, and an aliquot of the extract admitted to the LC column. The author pointed out that the losses of the C_4 acid ere minimized.

An interesting application of GLC of free fatty acids has been escribed by Sniegoski[82] in the determination of precise rates of Cl-catalyzed esterification in methanol of various aliphatic cids. Specific comparisons were made of the rates of esterification of eight isomeric hexanoic acids in methanol by GLC utilizing artially esterified mixtures with normal heptanoic acid as interal standard. For this purpose a 6 ft x 1/8 in I.D. stainless

steel tube packed with Chromosorb 101 was employed at 200°C. The relative esterification rates and the rate constants were calculated for each isomeric hexanoic acid and compared to that of normal heptanoic acid using appropriate equations. It was observed that methyl branching on the α-carbon exerts more influence in retarding esterification than methyl branching on the β-carbon despite the generally accepted thesis that the reverse is true. Sniegoski[83] has subsequently extended these analyses to the alkyl substituted acetic acids using a 6 ft x 0.125 in I.D. stainless steel column packed with 20% DEGA and 3% phosphoric acid on 60/80 mesh Gas Chrom P. On the basis of the results obtained Sniegoski[83] has described a method whereby aliphatic acids are ranked according to expected relative esterification rates by a simple inspection of their structure. The demonstrated success of the scheme indicates that it is far superior to any presently available.

VI. FUTURE PROSPECTS

Assuming that there will remain a need for rapid analysis of free fatty acids in biological and industrial samples one may briefly reflect upon the areas where further advances should be made to facilitate their GLC assay, and upon the likelyhood of developing alternate methods.

Improved GLC Assays

Despite major advances in the development of liquid phases and chromatographic supports, the currently available materials fall far short of ideal performance. The acid-fortified polyether and polyester phases have given excellent resolution of the short chain fatty acids and the common saturated and unsaturated long chain fatty acids, respectively, but the separation processes are accompanied by relatively high losses of all components requiring larger samples and sample loads in comparison to the GLC of the corresponding methyl esters. The recovery of the polyunsaturated long chain fatty acids such as arachidonic and docosahexaenoic acids is unsatisfactory although the resolution is excellent. This is apparently due in part to the higher affinity of the free car-

yl group for the polar liquid phase and the higher losses from longed oxidation at the higher temperatures needed to complete elution in a reasonable time.

It is therefore necessary to develop new polar liquid phases ch possess adequate separation factors for saturated and unsatu-ed acids and allow faster elution at low temperatures, resulting higher recoveries of all solutes. Further developmental effort needed in the technology of open tubular columns. To date open ular columns have failed to yield any useful separations of the ium and long chain saturated and unsaturated acids in the un-ivatized form, although the lower molecular weight acids have n effectively resolved.

Presumably, at the higher operating temperatures required for id elution of the free fatty acids, the chemical activity of the port also becomes more important, and a higher degree of inacti-ion may be necessary than commonly employed in the analyses of ty acid methyl esters. The current methods of support inactiva-n are largely based on silanization, which may be incompatible h proper analysis of underivatized fatty acids. Furthermore, y methods of support inactivation lead to a drastic reduction the surface area of the particles. The substitution of organic ymer particles for the diatomaceous earths is also largely atisfactory because of the low surface area of such particles the tendency of the polymer to dissolve the solute and thus in-ase the total effective liquid phase proportion. Therefore, a elopment of chromatographic supports of high surface area and igh degree of inertness at elevated temperatures would also be eficial to improved resolution and recovery of free fatty acids m GLC columns.

The separation and recovery of the short chain free fatty ds from presently available liquid phases and chromatographic ports would appear to have been solved to the satisfaction of eral laboratories, all of which have recommended the inclusion formic acid vapor into the carrier gas as a tail reducer and

inhibitor of ghosting. It is unfortunate, however, that such a stop-gap measure is necessary to overcome a shortcoming essentially confined to the preparation of the support and the design of the column and injector ports. Hopefully the need for these extraordinary measures will disappear with the development of improved GLC packings and a more general utilization of open tubular columns for short chain fatty acid analysis which eliminates the need for conventional supports and injector ports.

Alternate Solutions

A good possibility is a rapid GLC analysis of free fatty acids in the form of some derivative, which can be prepared in the total lipid extract without transesterification of any glyceryl or steryl esters. At the present time, only the preparation of the trimethylsilyl esters is compatible[84] with such a requirement, because even diazomethylation[85] leads to some degree of transesterification. The TMS esters however are not sufficiently stable for use with polyester columns although separations based on molecular weight of the acids can be obtained with non-polar columns[84]. Hopefully more stable silyl esters will shortly become available for this purpose.

Another recent development bearing on the need for direct GLC of free fatty acids concerns the report of a successful analysis of mixtures of free fatty acids by chemical ionization mass spectrometry[26]. Although this method is not likely to become available in every laboratory that requires analyses of free fatty acids, it is nevertheless a potential solution to a difficult problem.

A further likely development is the high pressure liquid chromatographic resolution of free fatty acids along with other components of total lipid extracts or indeed acidified Millipore filtrates of aqueous extracts. These separations have already been demonstrated in principle[87], but problems of solute detection and rapid elution have prevented them from general acceptance.

VII. SUMMARY AND CONCLUSIONS

A review of the present methodology and results of GLC analysis of underivatized free fatty acids reveals that satisfactory routines exist for the identification and quantitation of the common normal and branched chain volatile fatty acids at moderate concentration. These routines include both conventional packed and open tubular capillary columns. Higher sensitivity analysis appears to require the use of formic acid in the carrier gas to prevent tailing and ghosting, and seem to be limited to operation of packed columns. Adequate methods are also available for the separation of medium and long chain length saturated and oligounsaturated fatty acids in the underivatized form. Polar liquid phases fortified with phosphoric acid have given the best results, but such columns are short-lived even when protected from moisture. The GLC analyses of free polyunsaturated acids, however, are considerably less sensitive than those of the corresponding fatty acid methyl esters, and at the present time are limited to conventional packed columns. Despite much effort, open tubular columns have given unsatisfactory results. Because of the low recovery of the long chain polyunsaturated fatty acids. Care must be used in applying GLC of free acids to the analysis of completely unknown samples.

A variety of effective routines has been described for the isolation of free fatty acids for GLC analysis, but none would appear to be generally applicable.

It is concluded that the claims of increased speed of analysis and simplified sample handling are true only if the quality of preparation and analysis of the sample are to be sacrificed. For many purposes this is acceptable. For other applications it is necessary to await the development of better suited liquid phases and chromatographic supports. A convenient preparation of volatile derivatives of free fatty acids in the presence of bound acids combined with effective GLC resolution of all components remains a desirable alternative.

VIII. ACKNOWLEDGMENT

The studies of the author and his collaborators referred to in the review were supported by funds from the Ontario Heart Foundation, Toronto, Ontario, and the Medical Research Council of Canada.

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