Gas–Liquid Chromatography: the Separation and Identification of the Methyl Esters of Saturated and Unsaturated Acids from Formic Acid to *n*-Octadecanoic Acid

By A. T. JAMES and A. J. P. MARTIN
National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

(*Received* 21 *July* 1955)

The initial limitation of the gas–liquid chromatographic technique for the separation of fatty acids was due to the use of titration in an aqueous medium for detection and estimation of the acids (James & Martin, 1952). The elevated column temperatures necessary for a reasonable rate of movement of the long-chain acids preclude the use of water as the titration medium, and we did not discover a suitable high-boiling solvent to replace it.

The development of the sensitive gas-density meter for the detection of vapours in gas streams (Martin & James, 1956) provided the means for the extension of the technique to the higher-boiling fatty acids. Nevertheless, we have preferred to use the methyl esters rather than the free acids, as difficulty was encountered in finding a stationary phase for the column that would prevent dimerization of the free acids, with its consequent impairment of the separations (see James & Martin, 1952).

Other methods of separating the long-chain fatty acids, e.g. reversed-phase liquid–liquid chromatography (Howard & Martin, 1950; Zbinovsky, 1955) and countercurrent distribution (Ahrens & Craig, 1952), can resolve only acids differing by two carbon atoms in chain length, and overlapping of the zones of the common unsaturated and saturated acids always occurred. In an earlier attempt at separating the long-chain acids by gas–liquid chromatography (Cropper & Heywood, 1954), the efficiency of the columns used was so low that the separations achieved showed little or no improvement over the techniques already referred to. However, by the use of our 4 ft. × 4 mm. straight columns of the type already described (James & Martin, 1952) we have been able to separate not only acids differing in chain length by one carbon atom, as well as *iso*- or *anteiso*-acids, from their straight-chain isomers, but also unsaturated acids from saturated acids of the same chain length.

This paper is offered, not as providing complete analyses of any natural fat, but as indicating a method which is probably the only one at present capable of so doing. The evidence here presented shows that the problem is vastly more complicated than has previously been appreciated.

EXPERIMENTAL

The apparatus used is described elsewhere (Martin & James, 1956; but see James, 1955). Separation of the methyl esters of fatty acids from formic to *n*-caproic (*n*-hexanoic) acid can be carried out on columns with the following stationary phases: (*a*) at 78·7°, liquid paraffin (B.P. pond) or dioctyl phthalate [di-(2-ethylhexyl) phthalate], or (*b*) at 100°, paraffin wax, congealing point 49°, benzyldiphenyl (see James & Martin, 1956) or dioctyl phthalate. The Celite 545 support for the stationary phase was size-graded (see James & Martin, 1952), and pretreated with dilute ethanolic alkali as described by James, Martin & Smith (1952).

Separation of the methyl esters of fatty acids from *n*-pentanoic to *n*-octadecanoic acid was carried out at 197° or 205° on two types of column. The first had as stationary phase Apiezon M vacuum stopcock grease (Shell Chemicals Ltd.) and the second an extract of an aromatic character from a heavy lubricating oil (kindly supplied by Dr S. M. Birch of British Petroleum Ltd.). In both cases weighed amounts of the stationary phase (8–20 g. of Celite 545) were heated in the oven and stirred continuously until the product had cooled to room temperature. Packing of the columns was done as previously described (James & Martin, 1952).

The mixed esters were applied to the column with a micropipette (James & Martin, 1956), solid esters being melted and taken up in a previously heated pipette. Column temperatures were maintained by the appropriate solvent in the boiler (ethanol for 78·6°, water for 100°, ethylene glycol for 197° and tetralin for 205°). Silicone-rubber gaskets must be used on the columns at 197° instead of natural rubber.

The methyl esters of the acids present in olive oil were obtained by inter-esterification in ethyl ether with methanol in the presence of KOH as described by Kurz (1937).

The fatty acids from *Pseudomonas aeruginosa* were obtained by ether extraction of an acetone-soluble fraction of the culture medium. The ether-extracted material (acid value 181) was distilled under reduced pressure and esterified with diazomethane. This material was presented by Dr D. S. Bhate of this Institute.

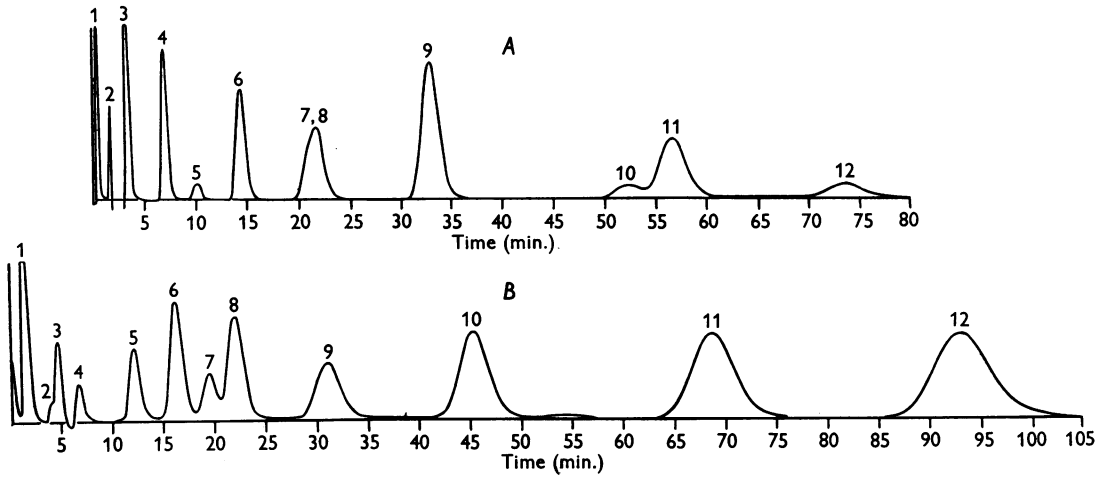

Fig. 1. Curve *A*. Separation of methyl esters of acids from formic to *n*-caproic acid at 78·6° on a 4 ft. column with dioctyl phthalate as stationary phase. N_2 pressure 44 cm. Hg, N_2 flow rate 40 ml./min. Peaks in order of appearance: (1) air, introduced on loading the column, (2) formate, (3) acetate, (4) propionate, (5) *iso*butyrate, (6) *n*-butyrate, (7) α-methylbutyrate, (8) *iso*valerate, (9) *n*-valerate, (10) 3-methylvalerate, (11) *iso*caproate, (12) *n*-caproate. Curve *B*. Separation of methyl esters of acids from formic to *n*-caproic acid at 100° in a 4 ft. column with dioctyl phthalate as stationary phase. N_2 pressure 14·5 cm. Hg, N_2 flow rate 10·1 ml./min. Peaks in order of appearance: (1) air, (2) formate, (3) methanol, (4) acetate, (5) propionate, (6) *iso*butyrate, (7) trimethylacetate, (8) *n*-butyrate, (9) *iso*-valerate, (10) *n*-valerate, (11) *iso*caproate, (12) *n*-caproate.

Bromination of the mixed esters from goat-milk fat, olive oil and the *Ps. aeruginosa* culture medium was carried out by dissolving 1 g. of the mixture in 10 ml. of ether at −10° and adding bromine drop by drop until a permanent coloration was produced. The solution was then evaporated below 30° with a rotary evaporator, and the residual oil used for the chromatographic experiments.

RESULTS AND DISCUSSION

It is not possible to cover the whole range of acids from C_1 to C_{18} by operating the column at any one temperature. We have preferred to separate the methyl esters of the acids from C_1 to C_6 at either 78·6° (Fig. 1, curve *A*) or 100° (Fig. 1, curve *B*) and to separate the methyl esters of the acids from C_5 to C_{18} at 197° or 205°. The esters can be separated into two such overlapping fractions by azeotropic distillation in toluene. A mixture of the complete range can be run at the lower temperature to separate the more volatile esters. The column will then need to be cleaned up by running until the blank has settled down to an acceptable level. Another run at the higher temperature on a different column will separate the longer-chain esters, and the more volatile esters will not interfere. It is of course possible to arrange a short length of cooled glass capillary (below −80° for very volatile esters) at the exit of the apparatus and so collect the fast-running volatile esters; they alone can then be distilled on a column at the lower temperature and separated.

Table 1. *Effect of temperature on the separation factor for an increase in chain length of one* $-CH_2-$ *group (the time of emergence of a substance relative to its next lowest homologue) in paraffin-hydrocarbon-type stationary phases*

Temp. (°)	$-CH_2-$ separation factor
23	3·40
58·6	2·76
65	2·46
78·6	2·38
100	2·10
137	1·90
197	1·56

It is not possible with our apparatus to separate the whole range of acids by continuously raising the column temperature, since the gas-density meter will show a zero changing with the temperature. Furthermore, as the factor of separation per $-CH_2-$ group decreases with temperature (see Table 1) it becomes less easy to identify an unknown acid by time of emergence alone when the column temperature is rising continuously. Nevertheless, this method would have great advantages in routine work. A great saving of time would result, since all the zones would be equally sharp. It should not be technically difficult provided that the detector was maintained throughout at the highest temperature reached by the column.

Saturated acids

The relative times of emergence of a number of compounds depend only on the temperature and nature of the stationary phase, and are thus highly reproducible. The retention volumes (the volume of nitrogen emerging from the column before the centre of the peak emerges) vary also with the amount of stationary phase and the nitrogen flow rate [which can, however, be corrected for the nitrogen pressure applied to the column by the formula given by James & Martin (1952)]. In Table 2 are given the retention volumes of the methyl esters of a variety of acids from formic to *n*-caproic acid relative to methyl *n*-butyrate at two temperatures and in a variety of stationary phases. In the paraffin stationary phases the separations are due primarily to differences in the Van der Waals free energies of solvation, whereas in the aromatic stationary phases other more specific forces are also involved (see James & Martin, 1956). It will be seen from the table that the separation factors for esters of normal and *iso*-acids are larger in the paraffinic stationary phases, but the esters of more highly branched acids such as αα-dimethylpropionic acid and ethylmethylacetic acid are better separated from esters of similar boiling point such as *n*-butyric and *iso*valeric esters in the aromatic stationary phases. In general the less viscous dioctyl phthalate results in columns of higher efficiency than the more viscous paraffinic phases, so that what is lost by fall in separation factor is gained in greater zone sharpness.

The separation of the isomeric branched-chain esters such as those of ethylmethylacetic acid and *iso*valeric acid is not as good as the separation of the free acids described by James & Martin (1952). This would suggest that an additional factor apart from the difference in Van der Waals interactions of the two branched chains was operating to assist the separation. It is probable that the association of the volatile acids with the stearic acid in the stationary phase is influenced by the side chain in such a way as to improve the separation.

Quantitative estimation of the esters is carried out by measuring the area under the peak in question, and this is more laborious than the simple method of step-height measurement used when titration is the detecting technique (James & Martin, 1952). For this reason we would recommend the latter technique where quantitative estimations of the more volatile acids are needed. Furthermore, if methanol rather than diazomethane is used to esterify the acids, it is necessary to remove any excess of methanol before running the esters, as it will interfere with the estimation of methyl formate as is shown in Fig. 1, curve *B*.

In Fig. 2 is shown the separation of a few mg. of the methyl esters of acids from C_5 to C_{13}. When a column 4 ft. long at 197° is used with the lubricating-oil extract as stationary phase, the separation is completed in only 70 min. A similar result can be obtained with Apiezon M vacuum stopcock grease as stationary phase. In Fig. 3 saturated acid esters of chain lengths from C_7 to C_{18} are separated with a higher rate of flow of nitrogen through the same column. Good separations of branched- and straight-chain isomers are obtained on these columns. In Table 3 is given a list of retention volumes of methyl esters of branched- and straight-chain acids from C_5 to C_{18} relative to methyl myristate on two types of column, the predominantly paraffinic stationary phase Apiezon M vacuum grease and the extract of aromatic character from lubricating oil. The results in the two types of column are very similar, as might be expected, but the aromatic-type

Table 2. *Retention volumes of methyl esters of saturated fatty acids from formic to* n-*caproic acid relative to methyl* n-*butyrate in a variety of stationary phases at two temperatures*

	Temp. 78·6° Stationary phase		Temp. 100° Stationary phase		
Methyl ester	Liquid paraffin	Dioctyl phthalate	Paraffin wax	Benzyldiphenyl	Dioctyl phthalate
Formate	0·071	0·098	0·09	0·117	0·124
Acetate	0·177	0·216	0·228	0·261	0·256
Propionate	0·445	0·485	0·495	0·53	0·51
*iso*Butyrate	0·724	0·71	0·75	0·67	0·706
n-Butyrate	1·0	1·0	1·0	1·0	1·0
αα-Dimethylpropionate	1·04	0·87	1·0	—	0·875
α-Methylbutyrate	1·69	1·51	1·52	—	1·45
*iso*Valerate	1·63	1·56	1·53	1·34	1·45
n-Valerate	2·42	2·31	2·15	2·13	2·12
3-Methylvalerate	4·25	3·66	3·53	—	—
*iso*Caproate	4·5	3·96	3·60	3·03	3·24
n-Caproate	5·86	5·16	4·70	4·37	4·36

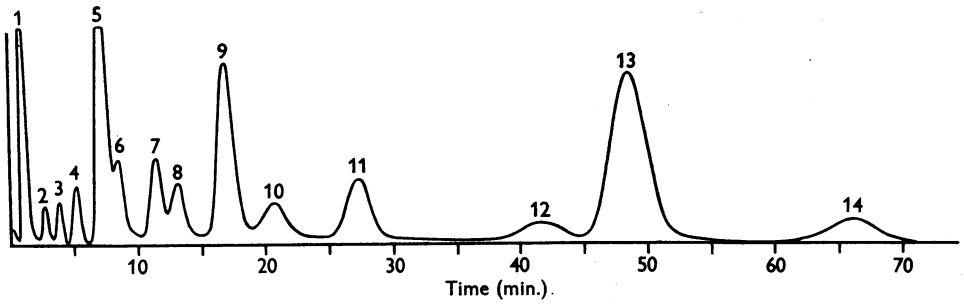

Fig. 2. Separation of the methyl esters of branched- and straight-chain saturated acids from C_5 to C_{13} on a 4 ft. column at 197° with a high-boiling-point lubricating-oil extract as stationary phase. N_2 pressure 12 cm. Hg, N_2 flow rate 14·1 ml./min. Peaks in order of appearance: (1) air, (2) *n*-pentanoate, (3) *n*-hexanoate, (4) 4-methylhexanoate, (5) 6-methylheptanoate, (6) *n*-octanoate, (7) 6-methyloctanoate, (8) *n*-nonanoate, (9) 8-methylnonanoate, (10) *n*-decanoate, (11) 8-methyldecanoate, (12) 10-methylundecanoate, (13) *n*-dodecanoate, (14) 10-methyldodecanoate.

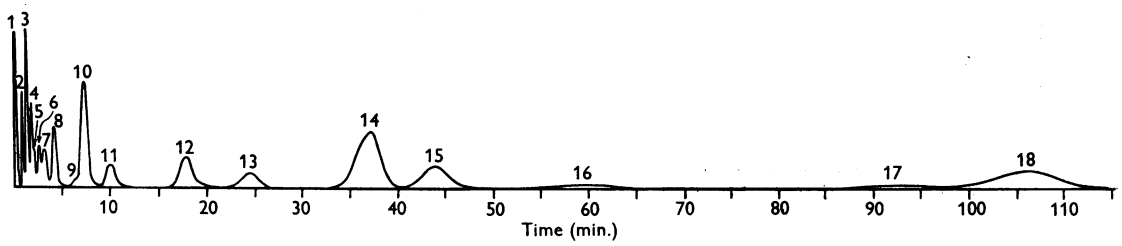

Fig. 3. Separation of methyl esters of branched- and straight-chain saturated acids from C_7 to C_{18} on a 4 ft. column at 197° with a high-boiling-point lubricating-oil extract as stationary phase. N_2 pressure 76 cm. Hg, N_2 flow rate 133 ml./min. Peaks in order of appearance: (1) air, (2) *n*-heptanoate, (3) *n*-octanoate, (4) 6-methyloctanoate, (5) *n*-nonanoate, (6) *n*-decanoate, (7) impurity probably a C_{11} acid, (8) 8-methyldecanoate, (9) 10-methyldodecanoate, (12) *n*-tetradecanoate, (13) 12-methyltetradecanoate, (14) 14-methylpentadecanoate, (15) *n*-hexadecanoate, (16) 14-methylhexadecanoate, (17) 17-methylheptadecanoate, (18) *n*-octadecanoate.

Table 3. *Retention volumes of methyl esters of longer-chain saturated fatty acids relative to methyl myristate in two stationary phases at* 197°

Methyl ester	Stationary phase: Apiezon M vacuum grease	Stationary phase: Heavy-lubricating-oil extract
n-Pentanoate (valerate)	0·0194	0·0148
n-Hexanoate (caproate)	0·0311	0·240
4-Methylhexanoate	0·0407	0·0354
6-Methylheptanoate	0·0567	0·052
n-Octanoate (caprylate)	0·0715	0·064
6-Methyloctanoate	0·0965	0·0861
n-Nonanoate (pelargonate)	0·112	0·100
8-Methylnonanoate	0·144	0·133
n-Decanoate (caprate)	0·173	0·169
8-Methyldecanoate	0·235	0·229
10-Methylundecanoate	0·354	0·344
n-Dodecanoate (laurate)	0·426	0·396
10-Methyldodecanoate	0·567	0·55
n-Tetradecanoate (myristate)	1·0	1·0
12-Methyltetradecanoate	1·35	1·37
14-Methylpentadecanoate	2·04	2·09
n-Hexadecanoate (palmitate)	2·41	2·45
14-Methylhexadecanoate	3·24	3·32
n-Octadecanoate (stearate)	5·60	5·92

stationary phase has a lower vicosity than the vacuum grease and gives a chromatogram of slightly higher efficiency.

Our earlier publications on the gas–liquid chromatogram (James & Martin, 1952; James, 1952) showed that plotting log retention volume (or retention volume relative to a standard substance) against the number of carbon atoms in the molecule for members of a homologous series produces a straight line. (In this way it is possible to identify an acid on the basis of time of emergence alone.) This shows that the free energy of solution of an additional $-CH_2-$ group is independent of its position in the chain. In earlier studies this was shown to be true for the free fatty acids from C_3 to C_{12}. In Fig. 4 is shown the result obtained for methyl esters of saturated acids from C_5 to C_{18} and branched-chain *iso*- and *anteiso*-acids from C_8 to C_{17}. Slight differences in rate of movement are shown between the *iso*- and *anteiso*-acid esters, but these are insufficient to permit separation on a 4 ft. column at this temperature. For separations such as this a longer column is necessary.

Unsaturated acids

In Fig. 5, curve *A* is shown the separation of the methyl esters of a variety of saturated acids from C_7 to C_{18} and also of *cis*-palmitoleic and oleic acids.

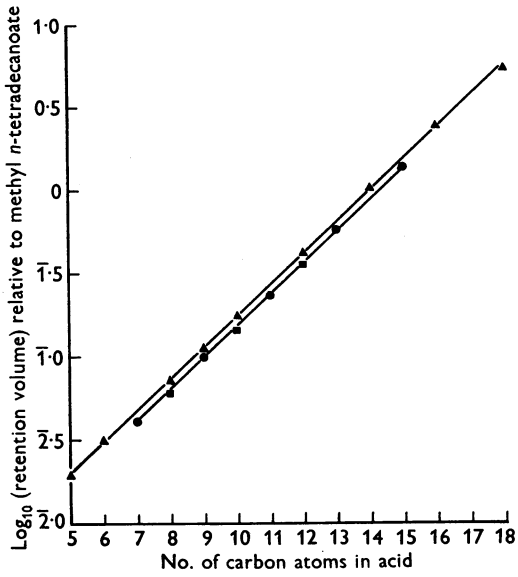

Fig. 4. Relationship between log (retention volume) relative to methyl *n*-tetradecanoate and number of carbon atoms in the molecule for the methyl esters of straight- and branched-chain acids from C_5 to C_{18}. ▲, Straight-chain acids; ●, *iso*-acids; ■, *anteiso*-acids.

The *cis*-palmitoleic ester and palmitic esters are almost completely resolved. This separation with a 4 ft. column is good enough to allow estimation of the relative amounts of the two acids by measurement of the peak heights.

In Fig. 5, curve *B* is shown a separation of even-numbered saturated acids from C_8 to C_{18}, and also linoleate and elaidate, demonstrating the clear differentiation between the di- and mono-unsaturated and the saturated C_{18} acids.

Table 4 shows a list of retention volumes of some unsaturated acids (as methyl esters) relative to methyl myristate in the paraffinic stationary phase Apiezon M grease. The unsaturated acid esters with the *cis*-configuration run faster than those with a *trans*-configuration; unsaturated acid

Table 4. *Retention volumes of methyl esters of some unsaturated acids relative to methyl* n-*tetradecanoate in two stationary phases at* 197°

Methyl ester	Stationary phase: Apiezon M vacuum grease	Stationary phase: Heavy-lubricating-oil extract
cis-Palmitoleate	2·09	2·21
trans-Palmitoleate	2·16	2·26
Linoleate	4·55	5·05
Oleate	4·75	5·22
Petroselinate	4·87	5·15
Elaidate	4·95	5·40
cis-Δ^4-Octadecenoate	4·95	5·42
trans-Δ^4-Octadecenoate	5·15	5·52

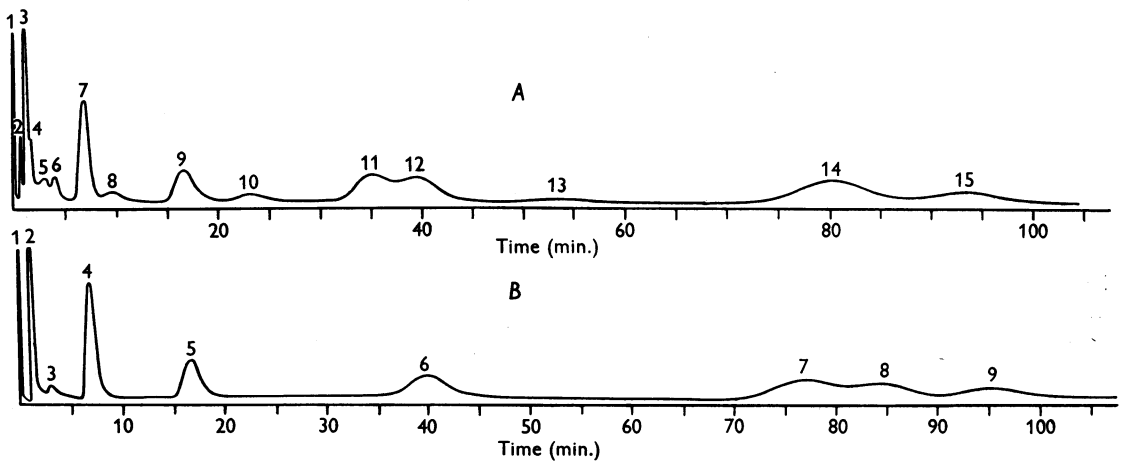

Fig. 5. Curve *A*. Separation of methyl esters of some branched- and straight-chain saturated acids from C_8 to C_{18}, and also palmitoleic and oleic acids on a 4 ft. column with Apeizon M vacuum grease as stationary phase at 197°. N_2 pressure 76·5 cm. Hg, N_2 flow rate 98 ml./min. Peaks in order of appearance: (1) air, (2) *n*-heptanoate, (3) *n*-octanoate, (4) *n*-nonanoate, (5) *n*-decanoate, (6) 8-methyldecanoate, (7) *n*-dodecanoate, (8) 10-methyldodecanoate, (9) *n*-tetradecanoate, (10) 10-methyltetradecanoate, (11) *cis*-palmitoleate, (12) *n*-hexadecanoate, (13) 14-methylhexadecanoate, (14) oleate, (15) *n*-octadecanoate. Curve *B*. The separation of methyl esters of the even-numbered saturated acids from C_8 to C_{18} and methyl linoleate and elaidate, under the same conditions as with curve *A*. Peaks in order of appearance: (1) air, (2) *n*-octanoate, (3) *n*-decanoate, (4) *n*-dodecanoate, (5) *n*-tetradecanoate, (6) *n*-hexadecanoate, (7) linoleate, (8) elaidate, (9) *n*-octadecanoate.

esters emerge before the corresponding saturated acid ester, and the higher the degree of unsaturation the more rapid the movement. The nearer the double bond is to the carboxyl group the slower the movement. These generalizations are in agreement with the chromatographic behaviour of unsaturated and saturated hydrocarbons (James & Martin, 1956).

In column 2, Table 4, the list of retention volumes of methyl esters of the unsaturated acids relative to methyl myristate shows that the unsaturated esters are perceptibly retarded relative to the nearest saturated acid in the aromatic-type stationary phase. The effect is not so large as that described for unsaturated hydrocarbons in a purely aromatic stationary phase (James & Martin, 1956), presumably because of the low aromatic content of the lubricating-oil extract. A stationary phase with greater aromatic content should produce a larger effect, so that unsaturated acids could be clearly identified on this basis.

It is not possible to separate esters of *iso*- or *anteiso*-saturated acids from an unsaturated normal acid ester with the same number of carbon atoms on either type of column. More positive identification of such esters can be obtained by chemical modification of the unsaturated acid, e.g. reduction to the corresponding saturated acid, oxidation to a hydroxy acid of lower volatility, or further oxidation to the corresponding mono- and di-carboxylic acids, or by bromination to give the much-less-volatile dibromo acids. Esters of the resulting products will in all cases behave differently from the esters of the original acids on a gas chromatogram. The change in chromatographic behaviour produced by bromination on the reversed-phase columns of Howard & Martin (1950) was insufficient to be of much value.

Some applications of the technique

In Fig. 6, curve *A* is shown the result obtained with 8 mg. of a mixture of methyl esters of fatty acids isolated from goat milk by Popják, Glascock & Folley (1952) run on a 4 ft. column with the

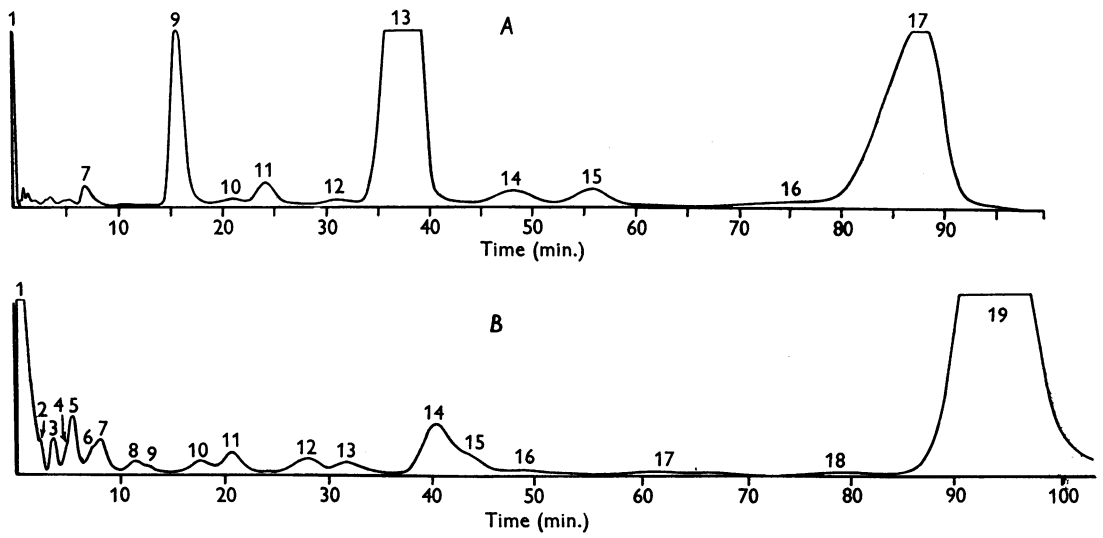

Fig. 6. Curve *A*. Analysis of 8 mg. of the methyl esters of acids present in goat-milk fat (Popják *et al.* 1952) on a 4 ft. column with an aromatic extract of a high-boiling lubricating oil as stationary phase at 205°. N_2 pressure 74 cm. Hg, N_2 flow rate 96 ml./min. Peaks in order of appearance: (1) air, (2)–(6) are shown further resolved in curve *B*, (7) *n*-dodecanoate, (8) probably *n*-tridecanoate, (9) *n*-tetradecanoate, (10) probably the methyl ester of a branched-chain C_{15} acid, (11) probably *n*-pentadecanoate, (12) probably the methyl ester of a branched-chain C_{16} acid, (13) *n*-hexadecanoate, (14) probably the methyl ester of a branched C_{17} acid, (15) probably *n*-heptadecanoate, (16) probably the methyl ester of a branched C_{18} acid, (17) *n*-octadecanoate. Curve *B*. Chromatogram of similar material to that shown in curve *A* but with a lower flow rate of N_2 (11·7 ml./min., N_2 pressure 12 cm. Hg). The peaks identified are as follows: (1) air, (2) *n*-pentanoate, (3) *n*-hexanoate, (4) *n*-heptanoate, (5) probably the methyl ester of a branched C_8 acid, (6) *n*-octanoate, (7) probably the methyl ester of a branched C_9 acid, (8) *n*-nonanoate, (9) probably the methyl ester of a highly branched C_{10} acid, (10) *n*-decanoate, (11) probably the methyl ester of a highly branched C_{11} acid, (12) probably *n*-undecanoate, (13) probably the methyl ester of a highly branched C_{12} acid, (14) *n*-dodecanoate, (15) probably the methyl ester of a highly branched C_{13} acid, (16) probably the methyl ester of a branched C_{13} acid, (17) *n*-tridecanoate, (18) probably the methyl ester of a branched C_{14} acid and (19) *n*-tetradecanoate.

aromatic-type stationary phase at 205°. Unsaturated fatty acids had previously been removed by the conventional lead-salt technique. The column has been deliberately overloaded to reveal components present in only trace amounts. An unexpected range of acids has been revealed, at least twenty peaks being visible. Treatment of the mixture in ether solution with bromine at $-10°$, followed by rechromatographing the mixture after removal of excess of bromine and ether *in vacuo*, gave an identical picture; indeed, the two results could be superimposed. This result confirms the absence of significant amounts of unsaturated acids from the mixture.

From knowledge of the time of emergence of the methyl esters of some pure even-numbered saturated acids and the $-CH_2-$ separation factor (see Table 1) for this column and temperature (1·52), the times of emergence of the methyl esters of odd-numbered acids were calculated. In this way it was shown that peaks 7, 9, 13 and 17 represented methyl dodecanoate, methyl tetradecanoate, methyl hexadecanoate and methyl octadecanoate; peaks 11 and 15 fell in the places expected for methyl esters of *n*-pentanoic and *n*-heptanoic acids; peaks 10, 12, 14 and 16 correspond to the positions expected for methyl esters of simple branched-chain (*iso*- or *anteiso*-) acids C_{15}, C_{16}, C_{17} and C_{18}.

The complex of peaks shown at the beginning of the chromatogram is shown further resolved in Fig. 6, curve *B*, by running the column more slowly and with a still higher load (14 mg.). Nineteen peaks can be seen when the analysis is carried as far as methyl myristate. From the positions of the peaks the tentative identifications indicated in the figure legend have been made.

Three homologous series can be fitted to the results: (*a*) the straight-chain ester peaks 2, 3, 4, 6, 8, 10, 14, 17 and 19; (*b*) branched-chain esters (possibly with the *iso*-configuration), peaks 5, 7, 16 and 18; (*c*) branched-chain esters of a different configuration (probably more highly branched), 9, 11, 13 and 15. Peak 12 does not apparently belong to any of these series. These results can be regarded as only tentative until the individual acids are isolated, chemically degraded and the fragments identified. There is no reason why all this cannot be carried out on a micro-scale by using the gas chromatogram both as a means of preparing each substance and as a tool to analyse the products of chemical degradation.

Another example of the application of this technique to the study of the fatty acid composition of a natural fat is shown in Fig. 7, curve *A*. The fat is olive oil, the methyl esters being prepared by the inter-esterification technique (Kurz, 1937). The unmodified esters (8 mg.) consist of a mixture of at least fourteen components. The main component, peak 13, is methyl oleate, and the next largest component is methyl palmitate, peak 12. Peak 11 could be either methyl palmitoleate or a saturated isomer of methyl palmitate. Peak 14 is too slow to be methyl stearate and must therefore represent

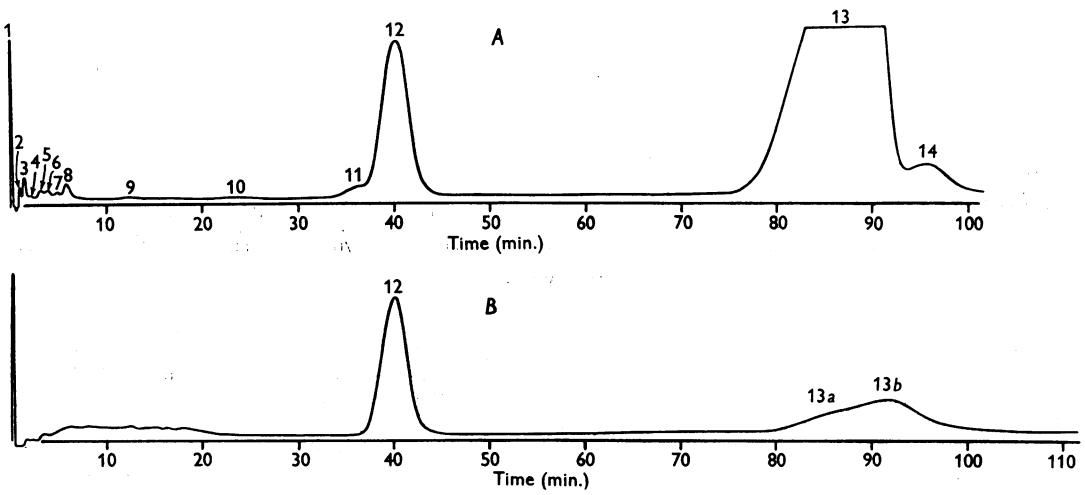

Fig. 7. Curve *A*. Analysis of 8 mg. of the methyl esters of acids present in olive oil. Conditions as for Fig. 6, curve *A*. Peaks in order of appearance: (1) air, (2)–(10) methyl esters of saturated and unsaturated acids from C_6 to C_{15}, (11) methyl palmitoleate or the methyl ester of a saturated branched-chain C_{16} acid, (12) methyl palmitate, (13) methyl oleate, (14) methyl ester of a highly unsaturated or branched saturated C_{19} acid. Curve *B*. Analysis of 8 mg. of the methyl esters of acids present in olive oil after bromination. Conditions as for Fig. 6, curve *A*. Peaks in order of appearance: (12) palmitate, (13*a*) probably the ester of a branched-chain C_{18} acid, (13*b*) stearate.

a C_{19} acid. In Fig. 7, curve *B* is shown the result obtained after bromination of the esters; peak 13 has also disappeared and has been replaced by two close peaks, 13*a* and 13*b*. Peak 13*b* can be identified as methyl stearate and peak 13*a* as a branched-chain C_{18} ester, but not methyl *iso*stearate, as it is too close to methyl stearate. Almost all the short-chain acids have disappeared on bromination, and by running a larger amount of material (9·2 mg.) at a slower rate there were found to be at least sixteen components up to peak 10 in Fig. 7, curve *A*. The results obtained suggest the presence of unsaturated C_6, C_8, C_{11} and C_{12} acids, C_6, C_8, C_9 and C_{10} straight-chain saturated acids, and traces of branched-chain C_8, C_9, C_{11}, C_{12} and C_{13} acids.

In Fig. 8, curve *A* is shown a separation of the methyl esters of the acids extracted from the *Ps. aeruginosa* culture medium. A total of at least twenty-four different acids can be distinguished. After bromination (Fig. 8, curve *B*) many of these components have disappeared and can be identified as unsaturated acids. From their chromatographic behaviour tentative identifications of the higher acids have been made. These are indicated in the figure legend.

From the results obtained with a higher load at a lower flow rate (shown in Fig. 9, curves *A* and *B*) the lower acids present would seem to be the following: peak no. (2) methyl ester of an unsaturated C_5 acid, (3) methyl ester of an unsaturated C_6 acid, (4) methyl *n*-hexanoate overlapping with the methyl ester of an unsaturated C_7 acid, (5) methyl *n*-heptanoate, (5*a*) methyl ester of a branched-chain saturated C_8 acid, (6) methyl ester of an unsaturated C_8 acid, (7) methyl ester of an unsaturated C_9 acid, (8) methyl ester of a branched-chain saturated C_{10} acid, (9) methyl ester of an unsaturated C_{10} acid, (9*a*) methyl *n*-decanoate, (10) methyl ester of an unsaturated C_{11} acid, (11) methyl ester of a branched-chain C_{12} acid, (12) methyl ester of an unsaturated C_{12} acid, (13) methyl *n*-dodecanoate, (14) methyl ester of a branched-chain saturated C_{13} acid, (15) methyl ester of an unsaturated C_{13} acid, (16) methyl ester of a branched-chain saturated C_{14} acid, (17) methyl ester of an unsaturated C_{14} acid, (18) methyl *n*-tetradecanoate. This gives a total of twenty-nine components in the original mixture.

These results obtained with natural fats from three different sources show clearly the presence not only of the expected even-numbered saturated

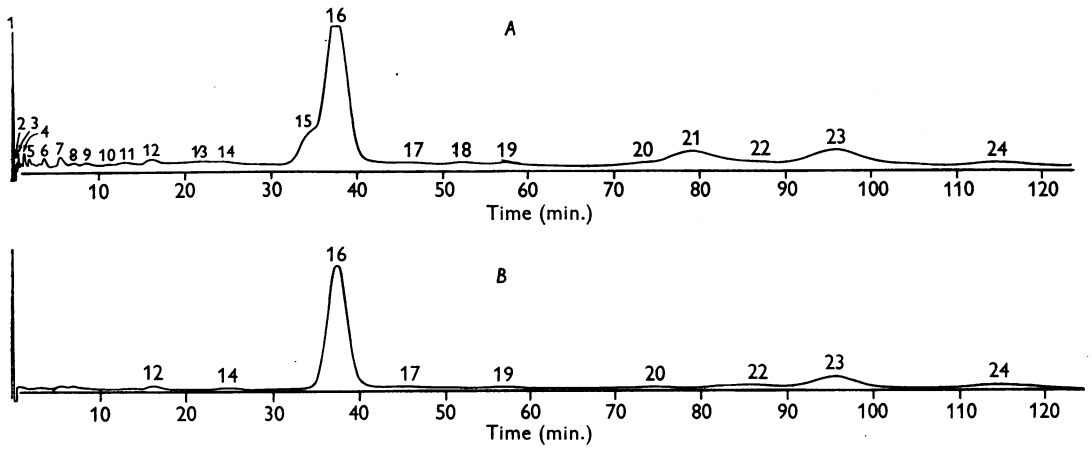

Fig. 8. Curve *A*. Separation of 2·3 mg. of the methyl esters of fatty acids extracted from a culture medium of *Ps. aeruginosa*. Column length 4 ft., temp. 205°, stationary phase lubricating-oil extract. N_2 pressure 75 cm. Hg, N_2 flow rate 96 ml./min. Peaks in order of appearance: (2)–(11) are shown further resolved in Fig. 9, curve *A*, (12) *n*-tetradecanoate, (13) methyl ester of a branched-chain saturated or an unsaturated C_{15} acid, (14) *n*-pentadecanoate, (15) palmitoleate or the methyl ester of a saturated branched-chain C_{16} acid, (16) *n*-hexadecanoate, (17) methyl ester of a branched-chain saturated or unsaturated C_{17} acid, (18) methyl ester of a branched-chain saturated or unsaturated C_{17} acid, (19) *n*-heptadecanoate, (20) methyl ester of a branched-chain saturated or a straight-chain diunsaturated C_{18} acid, (21) methyl ester of a branched-chain saturated or a straight-chain unsaturated C_{18} acid, (22) *n*-octadecanoate, (23) methyl ester of a highly branched saturated or highly unsaturated C_{19} acid, (24) methyl ester of a branched-chain saturated or unsaturated C_{19} acid. Curve *B*. Separation of 2·6 mg. of the methyl esters from curve *A* after bromination. Conditions as for curve *A*. A number of peaks have disappeared and the remaining saturated acids have been identified as the following: (12) *n*-tetradecanoate, (14) *n*-pentadecanoate, (16) *n*-hexadecanoate, (17) methyl ester of a branched-chain C_{17} acid, (19) *n*-heptadecanoate, (20) methyl ester of a highly branched saturated C_{18} acid, (22) *n*-octadecanoate, (23) methyl ester of a highly branched C_{19} acid, (24) methyl ester of a branched-chain saturated C_{19} acid.

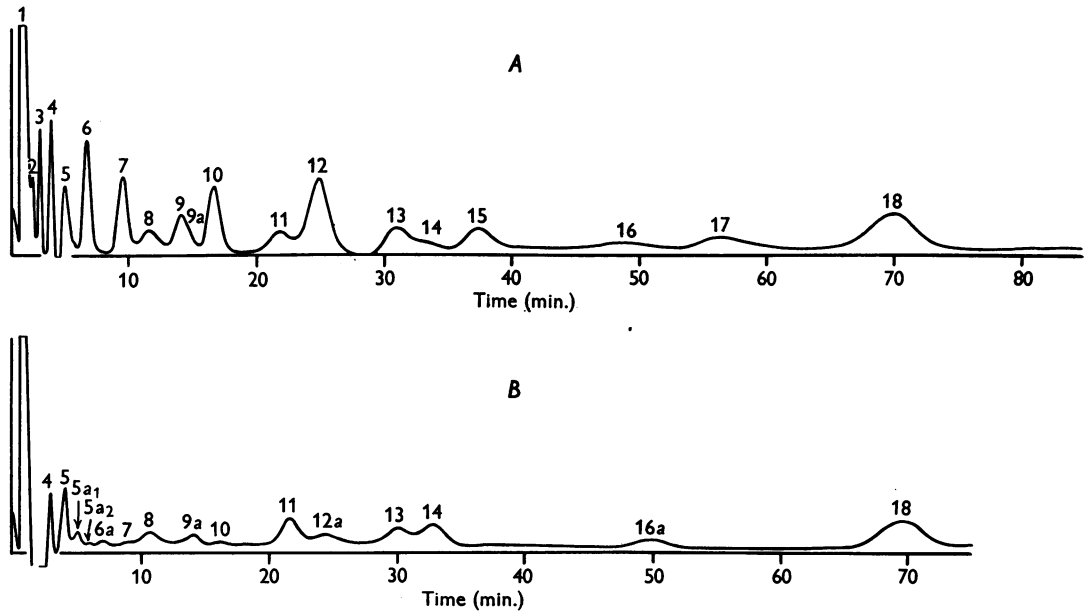

Fig. 9. Curve *A*. The peaks (2)–(12) from Fig. 8, curve *A*, are shown, further resolved by use of a lower N_2 flow rate. N_2 pressure 16·5 cm. Hg, N_2 flow rate 16·6 ml./min. Load, 6·9 mg. of the mixed esters; other conditions as for Fig. 8, curve *A*. Curve *B*. The result obtained under the same conditions as curve *A* after bromination of the esters (load 6·9 mg.). Peaks (2), (3), (6), (7), (9), (10), (12), (15) and (17) have either decreased or disappeared.

acids from C_6 to C_{18} but also the odd-numbered saturated acids from C_5 to C_{17}, two types of branched-chain saturated odd- and even-numbered acids, and odd- and even-numbered mono- and di-unsaturated acids of the same molecular-weight range. The presence of odd-numbered saturated acids in natural fats has recently been demonstrated by Hansen, Shorland & Cooke (1955). The biological significance of these newly described acids has yet to be determined.

A preliminary account of this work was presented at the International Conference on Lipids in Ghent 1955, and published in the Proceedings of the International Conference.

SUMMARY

1. Separation of micro amounts of methyl esters of saturated fatty acids from C_1 to C_6 at 78·7° or 100° and of esters of saturated and unsaturated acids from C_5 to C_{18} at 197° have been carried out by gas–liquid chromatography.

2. Good separation of normal and *iso*- or *anteiso*-saturated acids are obtained, and mono- and di-unsaturated acids can be resolved from the corresponding saturated acids on 4 ft. columns.

3. Methods are presented for distinguishing between saturated and unsaturated acids by chromatographic behaviour before and after chemical modification.

4. Application of the technique to some natural fats is described, showing the existence of odd-numbered saturated acids, two types of branched-chain saturated acids and odd- and even-numbered unsaturated acids from C_5 to C_{19}.

Thanks are due to Butterworths Ltd. for permission to reproduce diagrams already published. We would like to thank Dr J. Popják and Professor Folley for the acids from goat-milk fat, Dr E. V. Truter of The University, Sheffield, for the gifts of the branched-chain acids, Dr D. S. Bhate for the acids isolated from *Ps. aeruginosa*, and Mr H. Hadaway for the preparation of many of the esters used.

REFERENCES

Ahrens, E. H. & Craig, L. E. (1952). *J. biol. Chem.* **195**, 299.
Cropper, F. R. & Heywood, A. (1954). *Nature, Lond.*, **172**, 1101.
Hansen, R. P., Shorland, F. B. & Cooke, N. J. (1955). *Chem. & Ind.* p. 92.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
James, A. T. (1952). *Biochem. J.* **52**, 242.
James, A. T. (1955). *Research, Lond.*, **8**, 8.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
James, A. T. & Martin, A. J. P. (1956). *J. app. Chem.* **6**, 105.
James, A. T., Martin, A. J. P. & Smith, G. H. (1952). *Biochem. J.* **52**, 238.
Kurz, H. (1937). *Fette u. Seif.* **44**, 144.
Martin, A. J. P. & James, A. T. (1956). *Biochem. J.* **63**, 138.
Popják, G., Glascock, R. F. & Folley, S. J. (1952). *Biochem. J.* **52**, 472.
Zbinovsky, V. (1955). *Analyt. Chem.* **27**, 764.