We wish to thank Dr R. Pitt-Rivers and Dr J. Elks for gifts of tri-iodothyronine and di-iodothyronine, respectively.

REFERENCES

Acland, J. D. (1952). *Nature, Lond.*, **170**, 32.
Gross, J. & Pitt-Rivers, R. (1952). *Brit. med. Bull.* **8**, 136.
Taurog, A., Chaikoff, I. L. & Tong, W. (1950). *J. biol. Chem.* **184**, 99.
Tong, W., Taurog, A. & Chaikoff, I. L. (1951). *J. biol. Chem.* **191**, 665.
Tong, W., Taurog, A. & Chaikoff, I. L. (1952). *J. biol. Chem.* **195**, 407.

The Resolution of Mixtures of C_{16}–C_{24} Normal-chain Fatty Acids by Reversed-phase Partition Chromatography

By M. H. SILK* and H. H. HAHN†
National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa

(*Received* 28 *July* 1953)

Stand oils (fish drying oils) made from the body oil of the pilchard (*Sardina ocellata* Jenyns) are of commercial importance in South Africa and, as part of a general study of these and other polymerized oils (cf. Joubert & Sutton, 1952), the need arose for an analytical method suitable for the separation of mixtures of saturated and unsaturated acids having from 16 to 24 carbon atoms in the chain. For convenience, it was desirable that the method should be applicable on a semi-micro scale.

While the displacement chromatographic method of Holman & Hagdahl (1950) offered a possible approach, it was felt that the elaborate apparatus and technique involved would impair its ready applicability to our problem. For a similar reason an extension of the method of Vandenheuvel & Hayes (1952) seemed unlikely to be suitable, while that of Ramsey & Patterson (1948) was also rejected on the grounds that the higher fatty acids would have partition coefficients too greatly in favour of the less polar phase in the solvent system used.

The method of reversed-phase partition chromatography on benzene-rubber columns using methanol:acetone (3:1, v/v) containing varying amounts of water as mobile phase, has been applied by Boldingh (1950) to the quantitative semi-micro determination of saturated normal fatty acids from C_6 to C_{18}. Chromatography using rubber columns has been claimed by Boekenoogen (1952, not published in detail) to be satisfactory for resolution of mixtures containing saturated acids up to C_{24}, as well as for mixtures of various C_{18} unsaturated acids. Since 'Mealorub' rubber powder was unfortunately not available at the beginning of our work, the choice remained between elution chromatographic methods (Riemenschneider, Herb & Nichols, 1949; White & Brown, 1948; Herb, Witnauer & Riemenschneider, 1951), and the reversed-phase partition chromatographic method of Howard & Martin (1950). Recourse was had to the latter, which has been successfully extended to cover the resolution of mixtures of higher saturated fatty acids from 16 to 24 carbon atoms in chain length. Only acids with an even number of carbon atoms have been used for the investigation.

Preliminary experiments have indicated that the method should also be satisfactory for the resolution of unsaturated acids of differing chain length, as well as for acids of the same chain length but differing degrees of unsaturation (cf. Howard & Martin, 1950).

METHODS

The aqueous acetone-medicinal paraffin system of Howard & Martin (1950) was used throughout the investigation. Aqueous acetone concentrations are expressed on a v/v basis (e.g. 70% (v/v) acetone in water).

Preparation of materials

Non-wetting kieselguhr. 'Hyflo Super-cel' (Johns Manville, U.S.A.) was submitted to a process of flotation in water to remove the finer particles. About 5 lb. of kieselguhr were thoroughly mixed with 3 gal. of water and allowed to stand 1 hr., after which the suspended particles were decanted. The process was repeated on the sediment once, and the final product so obtained was dried at 110°. When cool, the material was exposed to the vapour of dimethyldichlorosilane in a partially evacuated desiccator. After 2 hr., the kieselguhr was washed free of acids with absolute methanol and dried at 110°. The product was completely non-wetting when shaken with water.

Medicinal paraffin. Colourless liquid paraffin (sp.gr. 0·880–0·895) was dissolved in pure pentane and percolated through a column of Brockmann grade I alkaline alumina to ensure removal of any acidic material. Evaporation of the pentane gave pure paraffin, used as stationary phase.

Acids. Palmitic, stearic and lignoceric acids were prepared from natural sources by distillation. Arachidic acid

* Marine Oils Research Fellow.
† Assistant in the Fellowship.

was synthesized from ethyl hydrogen adipate and palmitic acid, and behenic acid from ethyl hydrogen adipate and stearic acid using the electrolytic method of Greaves, Linstead, Shepheard, Thomas & Weedon (1950). All acids were purified by crystallization before use.

Preparation of columns

The mull used for packing the columns was prepared in batches. Non-wetting kieselguhr (93 g.) was suspended in a solution of paraffin (75 ml.) and anhydrous ether (500 ml.). The ether was slowly evaporated with constant agitation of the slurry, until a coarse homogeneous powder was obtained, which was then dried for 2 hr. at 60° in a vacuum oven.

In earlier experiments, a column 1·3 cm. in diameter and 30–35 cm. in height was found satisfactory for separation of component acids in mixtures, but it was later shown that resolution could be considerably improved by use of a column 85 cm. in height and 0·8 cm. in diameter.

Owing to difficulty in preparing air-free columns, special precautions were taken during packing. The mull (19·5 g.) was placed in a top-drive macerator, together with 83% aqueous acetone (200 ml.) previously equilibrated with paraffin. After thorough agitation, the slurry was transferred to a 500 ml. separating funnel constructed with a wide-bore stopcock and a ground glass neck into which a tap was fitted after introduction of the material. To expel all air, the slurry was boiled by holding the separating funnel in a horizontal position on a water bath. Next the column was filled completely with boiled, 83% aqueous acetone to give a positive meniscus at the top. The stem of the separating funnel was then closed with the tip of the forefinger and, by inclining the vessel, was filled with aqueous acetone. The funnel was attached to the column by judiciously sliding the stem over the liquid meniscus and withdrawing the finger at the same time. With the column and separating funnel stem completely full of liquid, the mull was allowed to fall into the column by setting it to flow. After some settling had taken place, the contents were compressed with air at an excess pressure of 50 mm. During subsequent settling, the column was vigorously tapped and shaken to ensure homogeneity of packing. A neatly fitting disk of filter paper was then placed on top of the packed mull. Satisfactory air-free columns were obtained by this method. All columns were jacketed and maintained at a constant temperature of 35°.

Loading of columns

The procedure employed by Howard & Martin (1950), of loading the acids dissolved in the developing solvent, was found unsuitable for our purpose owing to the low solubility of the longer-chain acids in aqueous acetone. Accordingly, the mixture to be chromatographed (containing 6–8 mg. of each component, accurately weighed) was dissolved in paraffin with gentle warming (0·2 ml. paraffin to each 7 mg. of acids). When cool, the solution was taken up in anhydrous ether (15 ml.). Non-wetting kieselguhr was then added (0·26 g. for each 0·2 ml. paraffin), and the ether was evaporated with continuous stirring. The powdery mull was then dried overnight under vacuum at 60°.

For loading, the mull was thoroughly slurried with 50% aqueous acetone (10–15 ml.) previously equilibrated with paraffin. The mixture was boiled to expel air and poured on to the column. After some settling, the solid mull was gently bedded by the slow downward motion of a plunger fitted with a perforated, stainless steel disk. After a filter-paper disk had been placed on top of the acid-containing band, the supernatant 50% aqueous acetone solution was run into the column. To prevent disturbance of the band and loss of acids during changes of developing solvents, a small protecting band of acid-free mull was placed on top of the column. For this purpose, a small quantity (about 1 g.) of mull was boiled with 50% aqueous acetone and packed with the plunger, as above, to a height of approx. 2 cm.

Developing solvents

Ordinary commercial acetone of low acidity was used to prepare developing solvents. Before use, all solvents were equilibrated with paraffin, and clarified by removing suspended oil droplets by percolation under pressure through a 3 cm. bed of kieselguhr-paraffin mull. The clear solutions were placed in separating funnels fitted with stopcocks and connected to a manifold of capillary tubing. To facilitate the changing of the developing solvents and also to provide a means of obtaining samples for blank titre determination, the column top was fitted as in Fig. 1.

While in use, the supply funnels were fitted with soda-lime guard tubes. The column eluate was also protected from contact with atmospheric CO_2 by means of a rubber collar and guard tube placed between the column tip and the siphon cup.

Siphon

A siphon was constructed to deliver 1·0 ml. samples. To prevent precipitation of higher acids in the siphon during cooler weather, it was wound with a 15 Ω length of nichrome wire and heated with a current of 0·35 A.

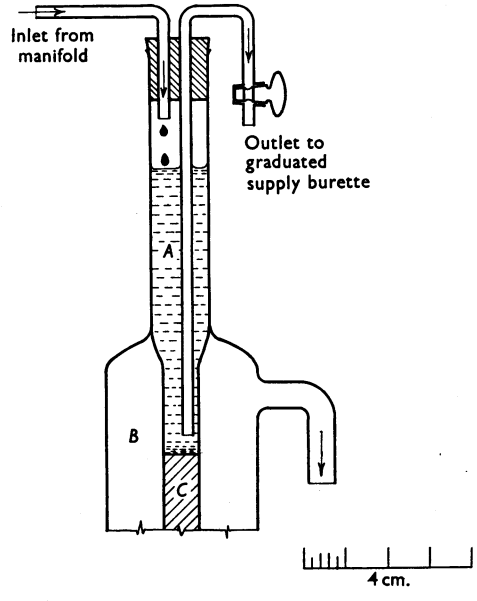

Fig. 1. Diagram of column head. *A*, developing solvent; *B*, column jacket; *C*, top of protecting band.

Titration of eluates

The titration vessel with N_2 circulation was used as described by Howard & Martin (1950). The $CHCl_3$ tube and light system were replaced by an ordinary 25 w globe built into a lamp-housing which surrounded the titration vessel, and was fitted with suitable filters to give a background of white light.

It was found necessary to saturate the N_2 stream with acetone vapour to counteract evaporation effects in the titration vessel. The N_2 stream was accordingly passed over Cu turnings at 700°, then through 40% aqueous KOH solution, and finally through a sintered-glass wash bottle containing neutral acetone, maintained at 30° by means of a small 1·5 w immersion heater.

Methanolic 0·01 N-KOH was protected at all points from contact with atmospheric CO_2. Bromothymol blue, as a 0·2% neutral solution in 70% aqueous acetone, was used as indicator.

Procedure

For chromatography of a mixture of C_{16}–C_{24} acids, development was begun with 70% aqueous acetone. This was continued until the C_{16} peak just began its downward slope, when a change was made to 75% aqueous acetone. The solvent was changed to 80% aqueous acetone on the downward slope of the C_{18} peak, to 83% aqueous acetone on the downward slope of the C_{20} peak, and finally to 90% aqueous acetone on the downward slope of the C_{22} peak. The hold-up was determined by running a narrow band of $KMnO_4$ solution down the column after the experiment. After each solvent change, the blank titre of the previous solvent was determined by titration of 10 ml. samples, under N_2, without boiling. Individual acids were chromatographed with solvent concentrations corresponding to those used for their elution from a mixture. A flow rate of 35 ml./hr. was used in all chromatograms.

RESULTS

A typical curve demonstrating resolution of a mixture of five acids is shown in Fig. 2. The areas underneath each peak were measured with a planimeter. The relative proportions of the areas gave the molar ratio of the component acids, while if each area was multiplied by the equivalent weight of the respective acid before proportionation, the weight ratio was obtained.

In an ideal case, where 100% recovery is achieved for all acids, the actual weight of each present can be determined if standardized alkali is used. In actual fact, recoveries were lower than the 95–100% reported by Howard & Martin (1950). In individual chromatograms, recoveries of C_{16}, C_{18}, C_{20} and C_{22} acids were, on the average, 25% low, while recovery of C_{24} acid was not greater than 60%. Efforts to improve recoveries by 'saturating' columns with higher homologues, before chromatography of individual acids, were unavailing. Some lost acid could be recovered and determined by stripping the column mull with ether, and titrating the acid:paraffin mixture in 50% benzene:neutral ethanol solution.

Attempts to eliminate the solvent blank titre determination, by using solvents neutralized either directly with alkali, or by percolation through beds of Amberlite I.R.A. 400, resulted in loss of chromatographic resolution.

The results of chromatographing eight artificial mixtures of C_{16}–C_{24} saturated acids are given in Table 1.

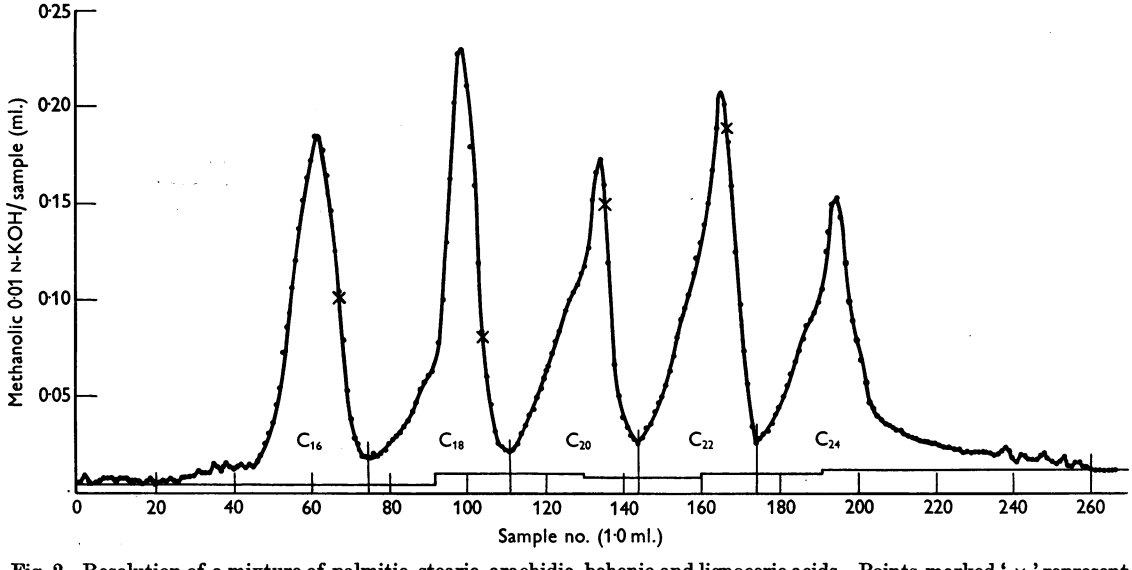

Fig. 2. Resolution of a mixture of palmitic, stearic, arachidic, behenic and lignoceric acids. Points marked ' × ' represent points of change to higher concentrations of aqueous-acetone developing solvent. The blank titre of the solvent in the eluate is represented by the lower line. Steps correspond to effective solvent-change points. Column 82 × 0·8 cm. diameter. Hold-up, 25 ml. (Note how effective solvent-change points correspond to steeper rises in the curve.)

Table 1. *Resolution of mixtures of* C_{16}–C_{24} *fatty acids*

(The weight percentage of each acid added in the mixture is shown in column *a*. The weight percentage experimentally determined is shown in column *b*. The percentage error is shown in column *c*.)

Expt. no.	Total wt. of acids (mg.)	Components														
		C_{16}			C_{18}			C_{20}			C_{22}			C_{24}		
		a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1	32·20	26·2	24·4	−6·9	26·6	22·8	−14·3	24·1	26·8	+11·2	23·1	26·0	+12·5	—	—	—
2	40·70	20·0	18·4	−8·0	21·0	19·5	−7·1	20·0	21·6	+8·0	19·8	19·0	−4·0	19·2	21·4	+11·5
3	39·92	17·6	16·6	−5·7	20·4	18·5	−9·3	19·5	20·0	+2·6	21·7	21·8	+0·5	20·8	23·1	+11·0
4	39·23	18·4	16·4	−10·9	20·2	19·4	−4·0	19·4	19·5	+0·5	20·4	20·7	−3·3	20·6	24·0	+16·5
5	39·07	18·7	17·6	−5·9	20·1	19·7	−2·0	19·2	20·2	+5·2	21·2	20·9	−1·4	20·8	21·6	+3·8
6	31·42	23·0	23·9	+3·9	26·4	28·0	+6·1	25·4	23·5	−7·5	25·2	24·6	−2·4	—	—	—
7	66·59	18·9	18·4	−2·6	20·8	18·3	−12·0	19·9	19·2	−3·5	21·0	24·7	+17·6	19·4	19·4	0·0
8	33·82	17·9	16·4	−8·4	20·1	17·8	−11·4	18·9	17·1	−9·5	20·7	23·3	+12·5	22·3	25·4	+13·9
Av. error (%)		—	—	−5·6	—	—	−6·8	—	—	+0·9	—	—	+4·0	—	—	+9·5

DISCUSSION

Satisfactory qualitative resolution has been obtained for mixtures of acids from 16 to 24 carbon atoms in chain length. Where it is desired to determine only the weight ratio or molar ratio of the component acids in a mixture, there seems justification for assuming a quantitative accuracy of ± 10 %, as evidenced by the results in Table 1. Low recoveries of acids do not permit of absolute weight determinations being made.

Adsorption effects on the non-wetting kieselguhr may possibly account for losses of acid, but attempts to find other supports were unsuccessful. Among others, cellulose powder, glass powder, and silica powder were investigated. Attempts were made to render these materials non-polar by exposure to the vapour of either dimethyldichlorosilane or trimethylchlorosilane, and by use of 'Quilon' (cf. Kritchevsky & Calvin, 1950). In no case was the treated material able to retain the paraffin phase of the solvent system.

The use of bromothymol blue as indicator may to some extent also contribute towards the observed low recoveries of acids. When added during a pH titration of 0·002N lignoceric acid with methanolic 0·01N potassium hydroxide in 90 % aqueous acetone, the indicator showed a pK of 10·5, while the pH at the end point was found to be nearer 11·5. Thus bromothymol blue gives a low end point but all acids from C_{16} to C_{24} were found to titrate low by the same amount (10 %) when titrated in a variety of aqueous acetone concentrations. Thus, equivalent quantities of acids will give equivalent peak areas, if no losses are incurred on the column, but these areas will all be low by the same amount. Quantitatively, acid will appear to have been lost, but the apparent loss will be the same for all peaks. Since the purpose of our investigation was to determine relative proportions of acids, rather than actual amounts, bromothymol blue was considered a satisfactory indicator.

The method does not appear suitable for the separation of corresponding normal-chain and *iso* acids. For example, it was not possible to resolve a mixture of stearic and 16-methylheptadecanoic acids. Application of the method to crude mixtures of unsaturated acids obtained by molecular distillation of pilchard-oil acids, has shown excellent qualitative resolution, when a temperature of 10° and aqueous acetone concentrations of approximately 60 % are employed. Chromatography of such crude fractions in a saturated condition shows curves with fewer peaks, indicating that acids with the same chain length but differing degrees of unsaturation are resolvable. The application of the method to the total analysis of pilchard-oil acids, and its adaptation to a preparative scale, are being studied.

SUMMARY

1. The reversed-phase partition chromatographic method of Howard & Martin (1950) has been extended to cover the resolution of mixtures of even-numbered fatty acids from C_{16} to C_{24}, in fulfilment of a need for an analytical method capable of identifying acids above 18 carbon atoms in chain length.

2. The method has been shown to be of semi-quantitative as well as qualitative value, and is suitable for application to the analysis of natural mixtures of saturated and of unsaturated acids.

The authors wish to thank Dr D. A. Sutton of this laboratory for suggesting the work and for much encouragement and advice throughout its undertaking. The investigation was made possible through the endowment of a Fellowship by Messrs Marine Oil Refiners of Africa, Ltd., Simonstown, Cape Province, which is gratefully acknowledged. This paper is published by permission of the South African Council for Scientific and Industrial Research.

REFERENCES

Boekenoogen, H. A. (1952). *Angew. Chem.* **64**, 339.
Boldingh, J. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 247.
Greaves, W. S., Linstead, R. P., Shepheard, B. R., Thomas, S. L. S. & Weedon, B. C. L. (1950). *J. chem. Soc.* p. 3326.
Herb, S. F., Witnauer, L. P. & Riemenschneider, R. W. (1951). *J. Amer. Oil Chem. Soc.* **28**, 505.
Holman, R. T. & Hagdahl, L. (1950). *J. biol. Chem.* **182**, 421.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Joubert, F. J. & Sutton, D. A. (1952). *J. Amer. Oil Chem. Soc.* **29**, 287.
Kritchevsky, D. & Calvin, M. (1950). *J. Amer. chem. Soc.* **72**, 4330.
Ramsey, L. L. & Patterson, W. I. (1948). *J. Ass. off. agric. Chem. Wash.* **31**, 441.
Riemenschneider, R. W., Herb, S. F. & Nichols, P. L. (1949). *J. Amer. Oil Chem. Soc.* **26**, 371.
Vandenheuvel, F. A. & Hayes, E. R. (1952). *Analyt. Chem.* **24**, 960.
White, M. F. & Brown, J. B. (1948). *J. Amer. chem. Soc.* **70**, 4269.

The Metabolism of Short-Chain Fatty Acids in the Sheep

2. FURTHER STUDIES WITH RUMEN EPITHELIUM

BY R. J. PENNINGTON
Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 30 *July* 1953)

Short-chain fatty acids are produced in considerable quantities as a result of microbial activity in the fore-stomachs and large intestine of ruminants and are undoubtedly an important source of energy for these animals. In the first paper in this series (Pennington, 1952) it was shown that acetic, propionic and *n*-butyric acids can be utilized by many tissues of the sheep including the epithelia of the alimentary tract. Interest was focused principally upon the activity of the rumen epithelium, which proved to be a very useful tissue for *in vitro* metabolic studies. Some further experiments on the metabolism of short-chain fatty acids by this tissue are described in the present paper.

Particular attention has been given to propionic acid since little is known with certainty about the pathways of metabolism of this acid in animal tissues. It can be completely oxidized under suitable conditions by rat-liver preparations (Grafflin & Green, 1948; Huennekens, Mahler & Nordmann, 1951). The latter authors could demonstrate its oxidation by kidney preparations only when supplemented with a fraction from liver dispersions. Propionic acid is also known to be a precursor of carbohydrate in the dog (Ringer, 1912) and rat (Deuel, Butts, Hallman & Cutler, 1935–6). In this respect it may be of special importance to the ruminant, whose carbohydrate supply is limited owing to the nature of its diet and means of digestion. There is no generally accepted evidence that either acetic or butyric acid can give rise to a net increase of carbohydrate in animal tissues (Deuel, Johnston, Morehouse, Rollman & Winzler, 1945; Wood, 1946).

EXPERIMENTAL

The tissue was collected, prepared and incubated as described previously (Pennington, 1952) with the modification that the tissue sections, before incubation, were shaken vigorously for 5 min. in ice-cold Ringer solution in order to ensure a more complete removal of rumen micro-organisms possibly adhering to the tissue. In manometric experiments, where smaller amounts of tissue were needed, it was found convenient to use papillae, shaved from the rumen wall. These were pooled and usually 150 mg. (wet wt.) were introduced into each flask; since this quantity represents upwards of a dozen papillae, a uniform distribution of tissue between the flasks was ensured. Such preparations of papillae have a rate of respiration and fatty acid utilization somewhat higher than that of the tissue prepared in the usual manner. Chopping the tissue finely with scissors appreciably diminishes the rate of utilization of fatty acids.

Volatile fatty acids and ketone bodies were determined as before (Pennington, 1952). As previously mentioned, high blank values may be encountered in the determination of ketone bodies by the dinitrophenylhydrazine method of Greenberg & Lester (1944) unless the reagent is dissolved in a lower concentration of HCl (0·5 N, instead of 2 N recommended by these authors). Recrystallization of the reagent from butanol did not reduce the high blank values obtained when 2 N acid was used. Low blanks may also be obtained by dissolving the reagent in 2 N-HCl and extracting the solution twice with half its volume of CCl_4. The latter procedure is rather more convenient than the use of 0·5 N-HCl, owing to the difficulty in dissolving the reagent in acid of this concentration.

Glucose was determined by the method of Somogyi (1945) on $ZnSO_4/Ba(OH)_2$ filtrates. Glycogen was determined by the procedure of Good, Kramer & Somogyi (1933). Succinic acid was determined manometrically using a sheep-heart succinoxidase preparation (Umbreit, Burris & Stauffer, 1945).