richer in longer chain acids than does the urea method.

Application of the urea procedure to total pilchard fatty alcohols affords a satisfactory stepwise fractionation, resulting in a concentrate rich in C_{16} and C_{20} unsaturated alcohols with a relatively small proportion of the C_{18} and C_{22} chain lengths.

Each of the four procedures investigated would appear to have its respective merits depending upon the nature of the unsaturated concentrate required. Material relatively rich in any desired chain length can be prepared for subsequent isolation of individual acids or alcohols by choice of the appropriate procedure. For convenience in analysis, fractionations involving the acids rather than the alcohols are to be preferred.

Urea fractionation of acid esters was not undertaken, although this has been claimed by Abu Nasr & Holman (1951-2) to result in the removal of material more highly unsaturated than is the case when the free acids are fractionated. The reduced tendency of the esters to associate is offered as an explanation of this behaviour.

SUMMARY

1. The urea complex fractionation and lithium soap-acetone techniques have been applied to the segregation of a concentrate of highly unsaturated fatty acids from the body oil of the South African pilchard. For comparison, the alcohols resulting from lithium aluminium hydride reduction of pilchard glycerides have also been fractionated by the urea complex method.

2. A comparison has been made between the unsaturated concentrates prepared by each procedure. The urea complex method affords an excellent stepwise fractionation resulting in a concentrate of high average unsaturation rich in the shorter C_{16} and C_{18} chain lengths. The lithium soap-

acetone procedure affords a concentrate of lower average unsaturation than does the urea method, but the segregate is relatively richer in the longer C_{20} and C_{22} chain lengths. Urea fractionation of total pilchard fatty alcohols affords a concentrate of high average unsaturation rich in the C_{16} and C_{20} chain lengths.

The authors wish to thank Dr D. A. Sutton and Dr W. S. Rapson, Director of the National Chemical Research Laboratory, for their interest and advice throughout the undertaking of this work. Part of 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 with the permission of the South African Council for Scientific and Industrial Research.

REFERENCES

- Abu Nasr, A. M. & Holman, R. T. (1951-2). *Rep. Hormel Inst. Univ. Minn.* p. 38.
Farnsteiner, K. (1903). *Z. Untersuch. Nahr. -u. Genussm.* **6**, 161.
Hilditch, T. P. & Riley, J. P. (1945). *J. Soc. chem. Ind., Lond.*, **64**, 204.
Kaufmann, H. P. (1939). *Fette u. Seif.* **46**, 268.
Kaufmann, H. P. (1940). *Angew. Chem.* **53**, 98.
Ligthelm, S. P., von Rudloff, E. & Sutton, D. A. (1950). *J. chem. Soc.* p. 3187.
Lovern, J. A. (1942). *Spec. Rep. Fd Invest. Bd, Lond.*, no. 51. London: H.M. Stationery Office.
Pollard, A., Chibnall, A. C. & Piper, S. H. (1931). *Biochem. J.* **25**, 2111.
Schlenk, H. & Holman, R. T. (1950). *J. Amer. chem. Soc.* **72**, 5001.
Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **56**, 406.
Swern, D. & Parker, W. E. (1952). *J. Amer. Oil Chem. Soc.* **29**, 431.
Tsujimoto, M. (1920). *J. chem. Ind., Tokyo*, **23**, 1007.
Tsujimoto, M. & Kimura, K. (1923). *J. chem. Ind., Tokyo*, **26**, 891.
Twitchell, E. (1921). *J. industr. Engng Chem.* **13**, 806.
Voorhies, S. T. & Bauer, S. T. (1943). *Oil & Soap*, **20**, 175.

South African Pilchard Oil

3. THE FATTY ACID COMPOSITION OF SOUTH AFRICAN PILCHARD OIL

By M. H. SILK* AND H. H. HAHN†

National Chemical Research Laboratory, South African Council for Scientific and Industrial Research,
Pretoria, South Africa

(Received 2 February 1954)

The complete and unambiguous analysis of the fatty acid composition of marine oils is a matter of extreme complexity owing to the diverse nature of the component acids. Not only are acids of differing chain length and unsaturation encountered, but

also acids which have the same chain length and a varying degree of unsaturation. Acids of from 12 to 28 carbon atoms in chain length have been reported; the higher members containing as many as six or seven double bonds per molecule (Lovern, 1942).

A large number of the more highly unsaturated acids of marine oils have been isolated and character-

* Marine Oils Research Fellow.

† Assistant in the Fellowship.

Table 1. *Molecular distillation of the unsaturated acid concentrate*

Fraction	Distillation temperature (°)	Weight (g.)	Equiv. wt.	Refractive index (n_D^{20})	No. of double* bonds/mol.
Unsaturated acids	—	29.5	311	—	4.62
I	96	3.40	271	1.4806	3.55
Intermediate	105	0.72	274	1.4832	3.62
II	105	2.84	276	1.4848	3.65
Intermediate	120	0.59	287	1.4879	4.08
III	120	2.51	287	1.4886	4.18
Intermediate	130	0.47	306	1.4913	4.65
IV	130	4.83	307	1.4933	4.86
V	135	3.57	315	1.4950	5.20
VI	145	2.27	327	1.4972	5.42

Residue: 7.0 g. Hold-up and loss: 1.3 g.

* H_2 uptake over Pd-BaSO₄.

ized, chiefly by the Japanese workers (e.g. Toyama & Tsuchiya, 1935*a, b*; Tsujimoto, 1906; Toyama & Yamamoto, 1953; Ueno & Iwai, 1934; Ueno & Yonese, 1936). While such work has been of very great value, the results are nevertheless open to some criticism on the grounds that the more labile components could easily have been modified during the vacuum distillation used for their isolation. The prolonged heating of acids with more than three double bonds per molecule is known to result in isomerization and polymerization reactions (cf. Norris, Rusoff, Miller & Burr, 1941, 1943).

For analysis of fatty acid mixtures without isolation of individual components, the well established method of Hilditch has been almost universally employed. With all its merits, this method does nevertheless suffer from a disadvantage in regard to thermal modification of the components, and is moreover only empiric in regard to the assignment of unsaturation between the different chain lengths in the case of marine oil acids.

The present communication represents a new approach to the analysis of marine oils without isolation of individual component acids. The techniques of lithium soap-acetone segregation, molecular distillation, reversed-phase partition chromatography and urea complex fractionation have been employed in successive subdivision of pilchard acid fractions. By suitable application of these techniques it has proved possible to obtain a fraction whose composition could be calculated unambiguously without isolation of an individual component. The procedures employed can reasonably be expected to have caused little or no modification of the original acids.

EXPERIMENTAL

General methods. The preparation of the total pilchard fatty acids and the concentration of the unsaturated fatty acids were as described in the preceding paper (Silk, Sephton & Hahn, 1954).

The concentrate of the more highly unsaturated acids prepared using the lithium soap-acetone technique repre-

Table 2. *Chain length distribution of fractions I-VI*

Fraction	Molar percentages			
	C ₁₆	C ₁₈	C ₂₀	C ₂₂
I	62.8	13.2	17.0	7.0
II	41.3	18.2	32.1	8.4
III	19.7	20.8	49.2	10.3
IV	5.1	10.3	64.2	20.4
V	1.9	4.4	64.5	29.2
VI	2.0	2.6	44.9	50.5

sented 29.5% of the total acids; it had an average equiv. wt. of 311, and an average number of double bonds/molecule, 4.62 (H_2 uptake over Pd-BaSO₄).

Molecular distillation of the unsaturated acid concentrate

A three-stage falling-film molecular still, especially designed for the purpose (Sutton, 1953), was used to separate the unsaturated acid concentrate into six fractions (I-VI) of increasing boiling point and equivalent weight. The distilland was recycled twice with all distilling stages at the same temperature before removal of each fraction, and the temperature of the stages was increased by use of a higher boiling solvent after each successive fraction had been removed.

Results are shown in Table 1.

Reversed-phase partition chromatography of fractions from the molecular distillation process

The six fractions (I-VI) from the molecular distillation process were chromatographed (23 mg. each) on a column 31.5 cm. in height $\times$ 1.4 cm. diameter packed with non-wetting kieselguhr supporting liquid paraffin as the stationary phase (cf. Silk & Hahn, 1954*a*). Aqueous acetone (60%, v/v) was used to develop the chromatograms at a flow rate of 35 ml./hr. and a temperature of 10°. The curves obtained are shown in Fig. 1.

Chain length analysis of the six unsaturated acid fractions (I-VI) from the molecular distillation process

Each of the fractions was completely hydrogenated over Pd-BaSO₄ catalyst, and the resulting mixture of saturated acids analysed for chain length distribution by reversed-phase partition chromatography (Silk & Hahn, 1954*a*). Results are given in Table 2.

Table 3. Urea complex fractionation of fraction I

Fraction	Weight (g.)	Equiv. wt.	No. of double bonds/mol.	Molar percentages			
				C ₁₆	C ₁₈	C ₂₀	C ₂₂
I (total)	2.02	271	3.55	62.8	13.2	17.0	7.0
I α	0.14	281	1.66	69.0	19.0	10.5	1.5
I β	0.18	273	2.55	59.5	13.8	22.6	4.1
I γ	0.08	276	3.14	—	—	—	—
I δ	0.04	275	3.34	—	—	—	—
Residue	1.5	263	3.68	79.3	11.0	9.7	0

Fig. 1. Reversed-phase partition chromatography of unsaturated acid fractions I-VI from molecular distillation. Solvent system: aqueous acetone (60%, v/v)-medicinal paraffin supported on non-wetting kieselguhr. Column: 1.4 x 30.0 cm. Temp. 10°. Flow rate: 35 ml./hr. Samples 1.4 ml. Load: 23 mg. each fraction.

*Urea complex fractionation of fraction I
from the molecular distillation*

Fraction I was further fractionated by removal of the components of lower unsaturation as their complexes with urea. Thus, urea (3 g.) was dissolved in absolute ethanol

(80 ml.) and the solution cooled to 15°. Fraction I (2.02 g.) was added to the solution and the whole left overnight at 4°. The crystalline precipitate was filtered off and washed with cold ethanol. The filtrate and washings were concentrated to a volume of 65 ml. and left to deposit a second crop of complexes which were removed by filtration. Urea (0.5 g.)

Fig. 2. Reversed-phase partition chromatography of unsaturated acids segregated from fraction I by urea complex fractionation. Stationary phase: medicinal paraffin supported on non-wetting kieselguhr. Moving phase: aqueous acetone. Fraction I, 60%, v/v; fraction I α , 70%, v/v; fraction I β , 67.5%, v/v; fractions I γ and I δ , 65%, v/v; residue, 55%, v/v. Column: 1.3 $\times$ 35 cm. Temp. 10°. Flow rate: 35 ml./hr. Samples: fraction I, 1.4 ml.; fractions I α –I δ and residue, 1.0 ml. (Residue curve: molar percentage peak X, 52.7%; peak Y, 26.6%; peak Z, 20.6%.)

was added to the filtrate and washings, which were allowed to deposit a third crop of complexes on standing. A fourth crop was obtained by evaporation of the filtrate from the previous crop to a volume of 30 ml. and again allowing to stand overnight at 4°. The acids which did not form urea complexes were recovered from the final filtrate by evaporating it to dryness, adding water and extracting with *n*-pentane. The complexes precipitated at each stage in the fractionation were decomposed with water, and the acids recovered by extraction with *n*-pentane.

The subdivision of fraction I is illustrated in Table 3. Chain length analyses of fractions removed as their urea complexes are shown.

Reversed-phase partition chromatography of subfractions I α -I δ and residue

The subfractions I α -I δ and the residue obtained by urea complex fractionation of fraction I were subjected to reversed-phase partition chromatography.

The results are illustrated in Fig. 2.

DISCUSSION

Inspection of the chromatographic curves for fractions I-VI shows that as peaks 1 and 2 on the left-hand side diminish in quantity, so peak 4 on the right-hand side increases, while peak 3 reaches a maximum and then again diminishes. This behaviour is paralleled by the variation in chain length distribution of fractions I-VI (Table 2), from which it is seen that as the molar percentage of lower boiling C₁₈ acids shows a steady decrease, so the percentage of higher boiling C₂₂ acids shows an increase, while the percentage of C₁₈+C₂₀ acids reaches a maximum and then decreases. A broad assignment of chain length to the peaks shown in the chromatographic curves is thus possible. Peaks 1 and 2 may well represent C₁₆ acids, peak 4 C₂₂ acids, peak 3 a mixture of C₁₈+C₂₀ acids, and peak 5 a complex mixture of variable composition probably consisting largely of C₂₂ acid in fraction VI.

Such correlation of chain length with peaks in a chromatographic curve is of no value unless the effect of unsaturation upon the resolution is taken into account. In our experience, the clear separation shown on chromatography of a mixture of oleic and linoleic acids has suggested that acids of the same chain length but different degree of unsaturation should be easily resolvable. The failure to resolve a mixture of palmitic and oleic acids has suggested that where one component has a double bond, and two methylene groups more than the other, resolution is not possible. These findings have recently been confirmed by Savary & Desnuelle (1953), and in their studies on the countercurrent distribution of fatty acids Ahrens & Craig (1952) have arrived at similar conclusions.

Further subdivision of one of the crude molecular distillates (fraction I) has been achieved by means of urea complex fractionation. Chromatography of

the urea segregates (fractions I α -I δ) shows curves (Fig. 2) in which a well defined variation of the peaks is apparent. Peak A increases throughout the series, while B and C diminish. These fractions are nevertheless still too complex to permit unambiguous calculation of their composition.

The non-complex forming residue obtained by urea fractionation of fraction I is, on the other hand, a relatively simple mixture of acids whose composition can be calculated without isolation of individual components. The chromatographic curve of this unsaturated material shows three peaks (Fig. 2) whose molar percentages are 52.7, 26.6 and 20.6 % respectively. The chain length analysis of this fraction (Table 3) shows that no C₂₂ acid is present. The molar percentage of C₁₆ acid (79.3 %) suggests that peaks X and Y in the unsaturated curve (Fig. 2) are both C₁₆ acid, since the sum of their molar percentages (52.7 + 26.6) is in agreement with the C₁₆ value determined. Peak Z must therefore represent a mixture of C₁₈ and C₂₀ acids, in the molar proportions of 11.0 and 9.7 respectively (cf. Table 3).

In the assignment of unsaturation to the peaks of the residue curve (Fig. 2), it is reasonable to assume that peak X is a C₁₆ tetraene acid and peak Y a C₁₆ triene, since resolution might be expected between such a pair of acids with the more highly unsaturated member being eluted first. The isolation and characterization of a C₁₆ tetraene acid are reported in the succeeding paper (Silk & Hahn, 1954b). Peak Z of the residue curve (Fig. 2) is likely to be a mixture of C₁₈ triene and C₂₀ tetraene acids, since these would be expected to give an overlapping peak and yet both be resolvable from C₁₆ tetraene and triene acids.

Assuming therefore that the residue curve (Fig. 2) represents a mixture of 52.7 % C₁₆ tetraene, 26.6 % C₁₆ triene, 11 % C₁₈ triene and 9.7 % C₂₀ tetraene, then the average equivalent weight calculated for such a mixture would be 259 and the average number of double bonds per molecule = 3.62. These values are in excellent agreement with the values of 263 and 3.68 experimentally determined (Table 3), and indeed it is not possible to postulate any other composition for the residue curve such that the theoretically calculated values for equivalent weight and average unsaturation agree with those experimentally observed.

Analysis of the residue curve (Fig. 2) indicates that by successive subdivision of pilchard acid fractions it is possible to obtain a mixture of a sufficiently simple nature to permit calculation of its composition without isolation of individual components. Other fractions obtained were still too complex in nature to allow of more than qualitative analysis, but it may be hoped that further subdivision of these mixtures by the techniques

suggested will result in less-complex fractions amenable to quantitative analysis. The mild procedures employed in fractionation of the acids might be expected not to cause modification of structure during processing. Consideration of the results obtained in this investigation illustrates that the composition of marine oils is of a far greater complexity than hitherto envisaged.

SUMMARY

1. A new approach has been suggested for the analysis of total marine oil acids without isolation of individual components.

2. The techniques of lithium soap-acetone segregation, molecular distillation, reversed-phase partition chromatography and urea complex fractionation have been applied in the successive subdivision of pilchard acid fractions. Relatively simple mixtures of acids have been obtained; the composition of one of which has been calculated without isolation of an individual component.

3. The techniques employed have been such as might be expected to have caused little or no modification of the component acids.

The authors wish to thank Dr D. A. Sutton for his interest and advice throughout the undertaking of this work which was made possible through the endowment of a Fellowship

by Messrs Marine Oil Refiners of Africa Ltd., Simonstown, Cape Province. This paper is published with the permission of the South African Council for Scientific and Industrial Research.

REFERENCES

- Ahrens, E. H. & Craig, L. C. (1952). *J. biol. Chem.* **195**, 299.
 Lovern, J. A. (1942). *Spec. Rep. Fd Invest. Bd, Lond.*, no. 51. London: H.M. Stationery Office.
 Norris, F. A., Rusoff, I. I., Miller, E. S. & Burr, G. O. (1941). *J. biol. Chem.* **139**, 199.
 Norris, F. A., Rusoff, I. I., Miller, E. S. & Burr, G. O. (1943). *J. biol. Chem.* **147**, 273.
 Savary, P. & Desnuelle, P. (1953). *Bull. Soc. chim. Fr.* **10**, 939.
 Silk, M. H. & Hahn, H. H. (1954a). *Biochem. J.* **56**, 406.
 Silk, M. H. & Hahn, H. H. (1954b). *Biochem. J.* **57**, 582.
 Silk, M. H., Sephton, H. H. & Hahn, H. H. (1954). *Biochem. J.* **57**, 574.
 Sutton, D. A. (1953). *Chem. & Ind.* p. 1383.
 Toyama, Y. & Tsuchiya, T. (1935a). *Bull. chem. Soc. Japan*, **10**, 411.
 Toyama, Y. & Tsuchiya, T. (1935b). *Bull. chem. Soc. Japan*, **10**, 539.
 Toyama, Y. & Yamamoto, T. (1953). *Mem. Fac. Eng., Nagoya*, **5**, no. 1.
 Tsujimoto, M. (1906). *J. Coll. Engng Tokio*, **4**, no. 1.
 Ueno, S. & Iwai (1934). *J. Soc. chem. Ind. Japan*, **37**, S. b. 251.
 Ueno, S. & Yonese (1936). *J. chem. Soc. Japan*, **57**, 180.

South African Pilchard Oil

4. THE ISOLATION AND STRUCTURE OF A HEXADECATETRAENOIC ACID FROM SOUTH AFRICAN PILCHARD OIL

By M. H. SILK* AND H. H. HAHN†

National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa

(Received 2 February 1954)

In the preceding communication of this series (Silk & Hahn, 1954), the presence of a C_{16} tetraene acid in the body oil of the South African pilchard (*Sardina ocellata* Jenyns) was posulated in order to account for certain observed analytical values. The isolation of such an acid is now reported, together with its characterization by physical and chemical means.

The techniques of lithium soap-acetone segregation, urea complex fractionation and molecular distillation have been applied in successive subdivision of total pilchard acids yielding a concentrate of highly unsaturated material. A C_{16} tetraene

acid has been isolated from this concentrate by reversed-phase partition chromatography on a preparative scale, and has been characterized as *hexadeca-6:9:12:15-tetraenoic acid*. The techniques employed for its isolation have been sufficiently mild in nature to render it unlikely that any modification of structure could have taken place during processing.

EXPERIMENTAL

General methods. See Silk, Sephton & Hahn (1954).

Ultraviolet spectra were determined on a Beckman model DU quartz spectrophotometer. Infrared spectra were determined on a Perkin-Elmer single-beam infrared spectrophotometer.

* Marine Oils Research Fellow.

† Assistant in the Fellowship.