recording type; column—eight feet of ¼-in. copper tubing packed with polyester succinate on −40 + 65 Tyler mesh red Chromosorbe; carrier gas—helium.

The results indicate practically no difference in the fatty acid compositions of the various glyceride fractions. The slight down-trend of linoleic acid with a compensating up-trend of palmitic acid would have no significance if it were not repeated in both series. It is interesting also to note that the percentage of linoleic acid in Sample 4 in Table I is 48.8 as compared with a weighed cumulative value of Fractions 1 to 5, inclusive, of 49.2. These two results represent values from two similar samples from different flakes and extractions. It is apparent that there are no significant variations in the total fatty acid composition of the first 95% of the oil extracted under the conditions studied. This agrees with the general conclusion from the work of Chang (2) on soybeans.

REFERENCES

1. Bull, W.C., and Hopper, T.H., Oil and Soap, *18*, 219–222 (1941).
2. Chang, H.Y., "Relationship of Soybean Oil Quality with Completeness of Extraction," Ph.D. dissertation, Iowa State University of Science and Technology, Ames, Ia., 1960.
3. Eaves, P.H., Spadaro, J.J., D'Aquin, E.L., Crovetto, A.J., Cirino, V.O., Stansbury, M.F., and Hoffpauir, C.L., J. Am. Oil Chemists' Soc., *33*, 639–645 (1956).
4. Eaves, P.H., Molaison, L.J., Pollard, E.F., and Spadaro, J.J., J. Am. Oil Chemists' Soc. *34*, 156–169 (1957).
5. Herb, S.F., Magidman, Paul, Riemenschneider, R.W., J. Am. Oil Chemists' Soc., *37*, 127–129 (1960).
6. Karnofsky, George, J. Am. Oil Chemists' Soc., *26*, 564–569 (1949).
7. Rao, R.K., and Arnold, L.K., J. Am. Oil Chemists' Soc., *35*, 277–281 (1958).

[Received August 3, 1960]

Partial Fractionation of Fatty Acid Triglycerides on a Silicic Acid Column

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A silicic acid column chromatographic method for the fractionation of glyceride types, employing an all-glass apparatus which permits gradient changes in solvent mixtures under pressure is described. Data on seven natural fats are discussed on the basis of the iodine values of individual fractions. The general trend in elution with the increasing concentration of ethyl ether in n-hexane is governed by a) chain length and b) unsaturation of the constituent fatty acids. Glycerides containing short-chain acids are more strongly adsorbed than those containing long-chain acids and glycerides containing unsaturated fatty acids are more strongly adsorbed in direct relation to unsaturation than those containing saturated acids of the same chain-length.

IN RECENT YEARS a number of techniques have been applied to the separation of natural triglycerides, prominent among which are the low-temperature crystallization (3,5,15) and countercurrent distribution (6,7). Separation of glycerides has also been attempted on adsorption columns of alumina with some success (17,23).

Silicic acid has been used for lipid separations by a number of workers (2,8,9,14,21,22,24). One of the more recent publications on the subject is that of Hirsch and Ahrens (12), who demonstrated the separation of complex lipid mixtures into chemical classes by elution from a single column.

The present work was undertaken in an attempt to standardize a method for the fractionation of the triglyceride types on a silicic acid column and to study the fatty acid distribution in natural fats. This report is a description of the method and its application to some representative fats and oils.

Experimental

Apparatus. The all-glass apparatus used in the study is shown diagramatically in Figure 1. It is essentially a modification of the one used by Sahasrabudhe and Tuckey (18) in their studies on organic acids. The apparatus is made up of 3 parts: a reservoir (A), a mixing flask (B), and the column (C). The different parts are provided with ball and socket joints and are held together with mechanical clamps.

The reservoir is a 3-liter flask with a standard ball joint on the upper end and a socket at the lower end. The lower end of the flask is sealed into a 1-mm. capillary (E) which extends through the socket into the mixing flask (B). An 8-mm. tube (F) opening at one end in the upper part of the reservoir, extends through the joint at the base into the mixing flask. The length of the tube extending into the mixing flask is such that the lower tip is about 5 mm. below the 500-ml. level of solvent in the mixing flask.

The mixing flask is a three-necked, 1-liter flask with ball joints on the necks and a socket joint at the lower end. It is also provided with a capillary tubing (E) and an 8-mm. tube (F) extending into the column through the adapter (D).

The stirring device in the mixing flask is a modification of the one used by Hirsch and Ahrens (11). The glass rod holding the Teflon collar and the stirring bar is fused into a socket to form a cap. Rapid mixing is achieved with the rotating magnetic bar held close to the externally-placed magnetic field.

The column is 60 mm. in diameter and 210 mm. long. At one end it is connected with a stopper and a Beketel adapter. On the top is a ball joint 65/40. The column is enclosed in a water jacket.

The adapter (D), with a 35/20 ball joint on top and 65/40 socket joint at the bottom, connects the column to the mixing flask.

The length of the capillary (E) and the solvent-levelling tube (F) are so adjusted that the capillary reaches to within 1 cm. of the solid packing, and the tube (F) gives the required solvent head of the column. The three air pockets in the apparatus are interconnected and thus help maintain constant levels in the mixing flask and the column head.

Adsorbent. Silicic acid [1] (100 mesh powder) is used (16). A 5-lb. lot of silicic acid is suspended in dis-

[1] Mallinckrodt Chemical Company, Montreal, Canada, labelled as "suitable for chromatography by the method of Ramsay and Patterson."

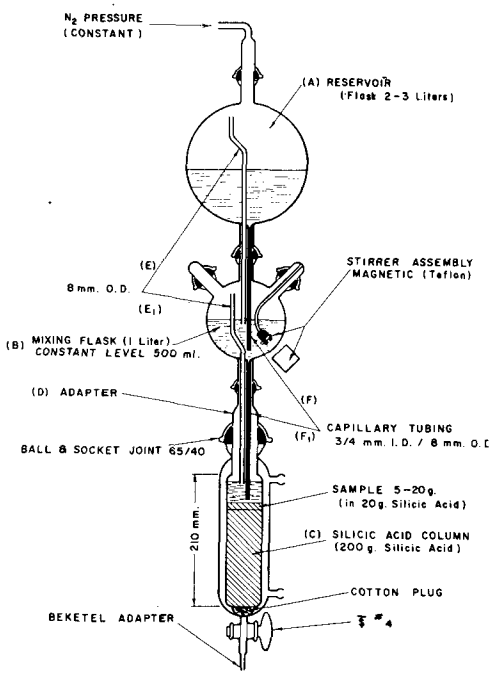

FIG. 1. Apparatus. Mechanical clamps used are not shown.

tilled water and agitated. After 20–30 min. of settling all the suspended silicic acid is decanted, and the process is repeated three times. Two liters of methanol are then added with agitation, and the slurry is allowed to settle for 30 min. Supernatant methanol is then decanted, and the silicic acid is dried in three stages: first on a water bath, then at 100°C. for 8–12 hrs., followed with activation at 200°C. for 8–12 hrs. Silicic acid treated in this manner is stored in bottles and mixed on a rotor before use. Acidity of the silicic acid was investigated in the manner described by Kay and Trueblood (13). No free acid was present.

Solvents. The two solvents used are n-hexane [2] and ethyl ether.[3] The solvents are freshly distilled before use; ethyl ether is distilled over potassium hydroxide.

Column Preparation. Two hundred grams of silicic acid are weighed in a beaker, and 25 ml. of distilled water are carefully mixed with a stainless steel spatula. Properly-activated silicic acid warms up when water is added. Two hundred and fifty ml. of n-hexane are then added, and the mixture is worked up with a stainless steel spatula. Two hundred grams of silicic acid adsorb about 250 ml. of n-hexane, resulting in a solvent-saturated mixture that can be easily transferred.

Two hundred and fifty ml. of n-hexane are placed in the column with a cotton-wool plug at the bottom. The plug is held in place with a long glass rod, and about 20 g. of prepared silicic acid are added with the help of a powder funnel. Gentle suction applied from below gives a solid packing of silicic acid at the bottom. The stopcock is then closed, and the remaining silicic acid mixture is added and the slurry agitated with the glass rod. Gentle tapping removes all trapped air.

The column is then connected to the reservoir through the adapter. Two hundred and fifty ml. of n-hexane are placed in the reservoir and developed with nitrogen pressure from the top (7–8 cm./Hg). The silicic acid shrinks to a column height of 13½ to 14 cm. When the solvent level on the top of the column comes to within 1 cm. above the silicic acid, the stopcock is closed and the reservoir removed.

Elution. Twenty grams of the adsorbent are separately weighed in a beaker and mixed with 3 ml. of water. Twenty grams of the fat or oil to be eluted are then added to the beaker and mixed with a spatula. The mixture is quantitatively transferred to the top of the column. A circular filter paper disc (Whatman No. 1, 5.5 cm.) is placed on top without pressure. One hundred and twenty ml. of n-hexane are then placed on the column head, and the column is connected to the assembly. A constant pressure of 7–8 cm./Hg is maintained on the top of the column. The flow rate is regulated to give about 100 to 125 ml. per hour.

Two elution schemes may be used:

a) Stepwise elution. The following eluant mixtures are used.
   1. 250 ml. of n-hexane
   2. 250 ml. of 2.5% ethyl ether in n-hexane
   3. 250 ml. of 5.0% ethyl ether in n-hexane
   4. 250 ml. of 10.0% ethyl ether in n-hexane
   5. 500 ml. of 15.0% ethyl ether in n-hexane
   6. 250 ml. of 25.0% ethyl ether in n-hexane

b) Gradient elution
   1. upper reservoir: 1,000 ml. of 15% ethyl ether in n-hexane
      mixing flask: 500 ml. of n-hexane
   2. upper reservoir: 250 ml. of 28% ethyl ether in n-hexane

Twenty-five-ml. fractions are collected on an automatic fraction collector. Each fraction is individually evaporated on a steam bath, and the residue is weighed in tared dishes. Care is taken not to heat the sample over 50°C. A stream of nitrogen is used whenever possible.

Iodine values are determined on individual fractions according to the American Oil Chemists' Society Official Method Cd-1-25 (1). In some cases where the sample quantity was not sufficient, the volumes of the reagents were proportionately scaled down to allow proper excess of Wijs reagent. In others two to four consecutive fractions were pooled.

Results and Discussion

The fats and oils studied are listed in Table I along with the iodine values. Table II gives the theoretical iodine values for C_{18} glyceride types, based on four acids normally present in natural fats.

TABLE I

Figure number	Fat and oil	Iodine value
3	Simple glycerides	
	Tristearin	0
	Triolein	83.6
	Trilinolein	173.3
	Trilinolenin	263.0
	Tripalmitin	0
	Tricaproin	0
4 and 5	Corn oil	122.1
6	(A) Butter fat	40.8
	(B) Hydrogenated corn oil	60.4
	(C) Olive oil	86.7
	(D) Seal oil	140.1
	(E) Horse fat	86.4
	(F) Linseed oil	184.0

[2] British Drug Houses, Toronto, Canada, reagent grade.
[3] Anachemia Chemicals Ltd., Montreal, Canada.

TABLE II

Theoretical C_{18}-Glyceride Types

Glyceride type	Number of double bonds	Iodine values
SSS	0	0
SSO	1	28.54
SOO } SSL }	2	57.21
SSLe } OOO } SOL }	3	86.01
SOLe } OOL } SLL }	4	114.95
OLL } OOLe } SLLe }	5	144.01
SLeLe } OLLe } LLL }	6	173.21
OLeLe } LLLe }	7	202.54
LLeLe	8	232.00
LeLeLe	9	261.60

S–stearic, O–oleic, L–linoleic, and Le–linolenic.

In the initial stages of the study three different batches of silicic acid were compared. When treated in the manner described above, all three batches gave comparable peak effluent volumes for the fat studied. The present study is carried out with one 100-lb. batch of silicic acid obtained from the Mallinckrodt Chemical Company. Water (12.5%) is incorporated into the silicic acid in order to obtain a partition between degrees of unsaturation. With less water the conditions would be more favorable for adsorption on the solid surface. The separations can be best explained in terms of a combination of adsorption and partition mechanisms (13).

Choice of Solvents. In order to determine whether a mixture of ethyl ether and n-hexane or ethyl ether and petroleum ether would be more satisfactory for the fractionation of the triglycerides, comparative adsorption properties of the silicic acid in the presence of these two solvent systems were studied, using a modification of the method described by Hirsch and Ahrens (12). Two hundred milligram aliquots each of tristearin, trilinolein, and tricaproin were individually mixed in a series of 4-g. aliquots of silicic acid (containing 12.5% water, prepared as described under column preparation). The silicic acid-glyceride mixture is then transferred to a stoppered glass tube and extracted with 25 ml. of test solvent mixtures. The filtered extract is then evaporated, and the percentage of adsorbed glyceride is computed from the residual weight.

The results of this study are presented in Figure 2. A comparison of adsorption properties of silicic acid in the presence of these two solvent systems shows that ethyl ether in n-hexane results in a relatively greater difference between the three simple glycerides studied than does ethyl ether in petroleum ether.

Standardization Experiment. In a preliminary study six simple glycerides were used for standardization of the column, individually as well as in a mixture. The glycerides were added in 0.5- to 2.0-g. quantities. These were mixed with 5 g. of silicic acid instead of 20 g. Fifteen grams of silicic acid were added above the sample to make up the column height, and the sample was eluted in the manner described below.

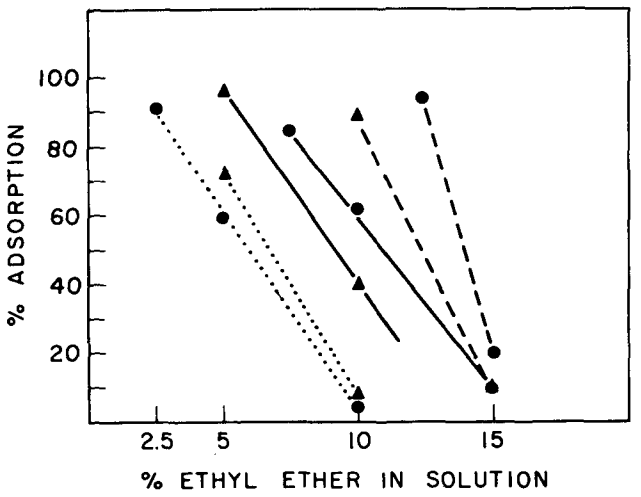

FIG. 2. Adsorption curves. Percentage of adsorbed glyceride on 4 g. of silicic acid. Tristearin (dotted line), trilinolein (solid line), and tricaproin (broken line), with ethyl ether in n-hexane (closed circles) and ethyl ether in petroleum ether (triangles).

In Figure 3 the weight-distribution patterns of individual simple glycerides are superimposed along with the pattern obtained with the mixture of the same glycerides. Iodine values are plotted against each fraction. Each simple glyceride was individually eluted, and the weight curves were plotted. The general trend of elution is based on a) chain length and b) unsaturation. Glycerides containing short-

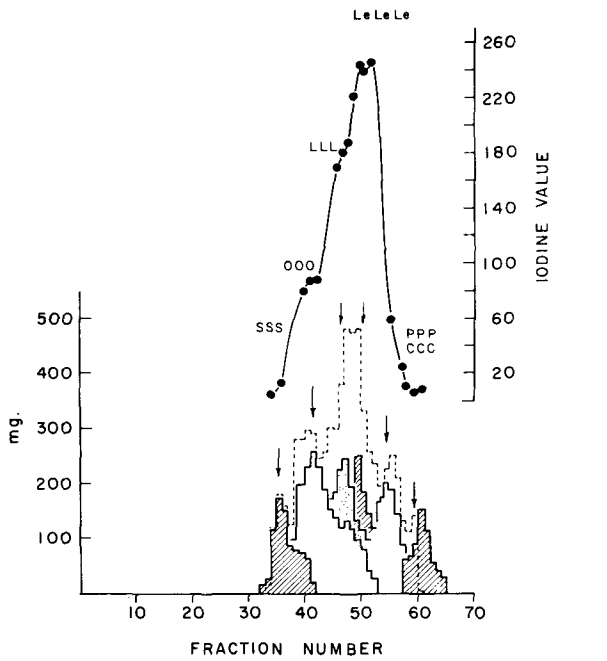

FIG. 3. Fraction weight and iodine value patterns obtained with a mixture of six simple glycerides: tristearin—SSS; triolein—OOO; trilinolein—LLL; trilinolenin—LeLeLe; tripalmitin—PPP; tricaproin—CCC. Pattern obtained with the mixture of simple glycerides is shown by dotted line. Iodine values were determined on fractions obtained from the mixture.

chain acids are adsorbed more strongly than those containing long-chain acids, and the glycerides constituting unsaturated acids are more strongly adsorbed in direct relation to unsaturation than those containing saturated acids of the same chain-length. Similar clear-cut separation of tristearin and tricaproin is demonstrated by Hirsch and Ahrens (12).

A sharp rise in the iodine value from 4 to 258 units in the fractions obtained with the mixed sample indicates a good resolution. Arrowheads indicate the positions of individual glyceride peaks. Weights obtained are in good agreement with the calculated values of overlapping fractions. However the iodine values on fractions do not show signs of overlapping as much as is indicated in the superimposed curves.

Sample Size. The effect of the size of the sample on the elution pattern was studied with corn oil in 5-, 10-, 20-, and 40-g. quantities mixed with 20 g. of silicic acid. The result of this study is shown in Figures 4 and 5. While 5-g. and 10-g. samples gave a better

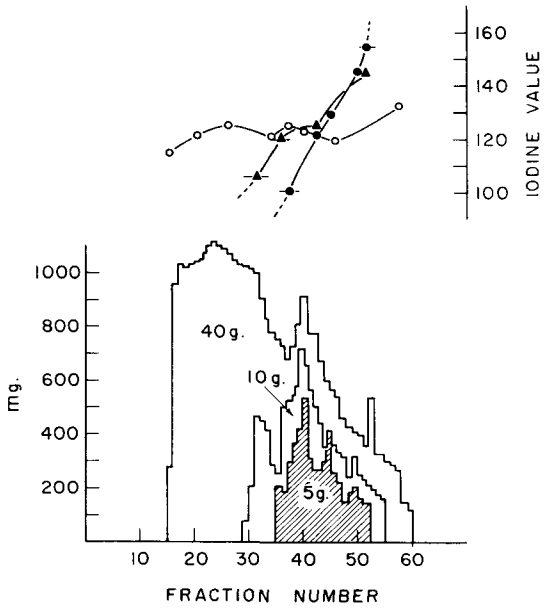

FIG. 4. Effect of sample size (5, 10, and 40 g.). Weight and iodine value patterns of fractions obtained with corn oil (I.V. 122.1).

resolution than the 20-g. sample, some of the fraction weights were not sufficient for satisfactory analysis. Judging from the iodine values of individual fractions, the 40-g. sample did not resolve into glyceride types. The 20-g. sample (Figure 5) not only gave good resolution but provided sufficient sample for convenient analysis.

Reproducibility. The reproducibility of the fractionation technique was studied by using corn oil. This oil was added to the column and eluted on three different occasions. The percentage of weights of the oil obtained in the three major fractions together with the total weight are shown in Table III. From the results it may be seen that there is good agreement between the amounts in each fraction. The percentage of recovery of the fats and oils studied is between 87 and 95.

In Figure 5 is shown a typical pattern obtained with corn oil. It indicates a clear-cut separation of

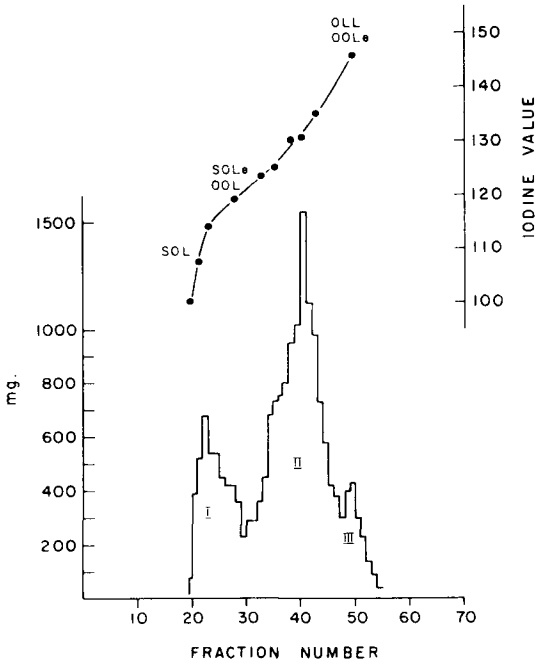

FIG. 5. Weight and iodine value patterns of fractions obtained from corn oil (I.V. 122.1) 20 g.

three glyceride types. The corn oil used is a commercial brand of salad oil. The three major fractions correspond to about 12%, 57%, and 20%, respectively. With low-temperature solvent fractionation Doershuk and Daubert obtained similar results (5). The iodine values are in general agreement with the above distribution and have a range in the iodine value of 50 units.

TABLE III

Percentage of Three Fractions Obtained from Corn Oil

Elution	Fraction (%)			
	I	II	III	Total
1	12.2 + 7.5	57.0	19.8	96.5
2	13.2 + 6.5	61.6	14.5	95.8
3	16.3 + 7.0	59.2	15.5	98.0

Results obtained with six representative fats and oils (Table I) are compiled in Figure 6. The spread of fractions is indicated by horizontal bars; the distinctive peaks are marked by arrow-heads against fraction numbers.

Butter Fat. Peaks I. 1.5%; II. 23.5%; III. 32.5%; IV. 21.9%; V. 0.5%.) The fat shows a characteristic iodine value curve with a downward trend in iodine values because of the shorter-chain saturated acids.

Hydrogenated Corn Oil. (Peaks I. 11.2%; II. 25.4%; III. 51.3%.) The pattern of hydrogenated corn oil again is in agreement with the known glyceride types. The hydrogenated sample used was from the same source as corn oil. The restricted variation of iodine value units presumably results from the fact that oleic acid is the main unsaturated acid. The shift in the weight pattern and the disappearance of the high iodine value peaks of unsaturated glycerides are a clear indication of effective fractionation.

Olive Oil. (Peaks I. 62.8%; II. 25.0%.) With the high oleic acid content the olive oil (11) shows a sharp peak coinciding with the triolein. This is borne

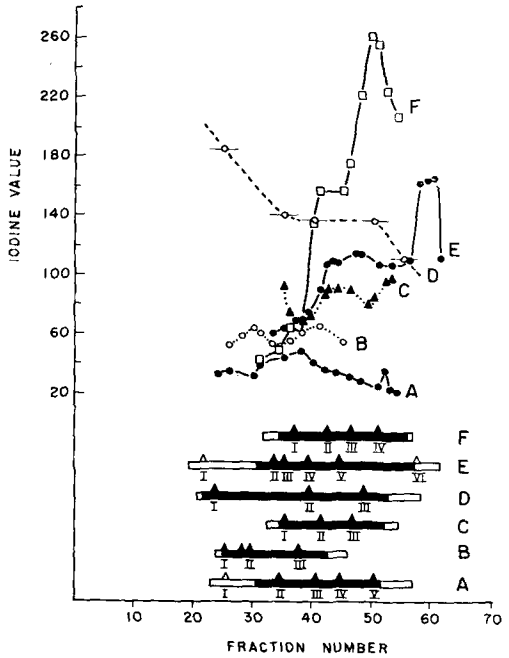

FIG. 6. Iodine value patterns and distribution of fractions obtained with six representative fats: (A) butter fat, (B) hydrogenated corn oil, (C) olive oil. (D) seal oil, (E) horse fat, and (F) linseed oil.

out by the iodine value of 83.5 in two adjacent fractions (Numbers 42 and 43). The initial high peak in the iodine value, which drops by 21 units within three fractions, needs an explanation. Olive oil is known to contain a relatively high percentage of palmitic acid (9.7 to 15.6%) (11). Since palmitic acid containing glycerides is adsorbed in preference to those containing stearic, it is likely that monostearodiunsaturated glyceride like SOL with a theoretical iodine value of 115 will be eluted before monopalmito diolein with a theoretical iodine value of 56.

Seal Oil. (Peaks I. 23.5%; II. 54.0%; III. 17.5%.) The presence of higher unsaturated acid is evidenced from the high iodine values for the earlier fractions with seal oil. A wide range of 17.6% to 40.8% for C_{20-22} unsaturated acid in seal oils is reported by Winter and Nunn (25). Iodine value determinations on seal oil were done on pooled fractions. The results may not give the value for individual fractions, but the trend is clearly demonstrated. The drop in the tail-end fractions is likely caused by the short-chain acids in fish oils (25).

Linseed Oil: (Peaks I. 35%; II. 28%; III. 12%; IV. 16%.) This is an example of a fat containing triunsaturated glycerides of the type, LLL, LLLe, LLeLe, and LeLeLe. Judging from the iodine values, Fraction IV constitutes trilinolenin while Fraction III seems to be made up of linoleo-dilinolenins.

Dutton and Cannon (7) for the first time demonstrated the separation of trilinolenin and linoleo-dilinolenins by countercurrent distribution. The present results indicate up to 11.2% (Fraction Numbers 49, 50, and 51) of trillinolenin in linseed oil. The linseed oil used is a sample of pure raw oil that has been in storage in our laboratory refrigerator more than four years.

Horse Fat. (Peaks I, 2.0%; II. 9.5%; III. 26.0%; IV. 27.6%; V. 30.1%; VI. 2.75%.) This is another example of fat with a high proportion of linoleic and linolenic acids (20) and follows a pattern similar in trend to linseed oil. The fat is extracted from fresh horse fatty tissue by the method described by Crowell (4). The separation is not as clear-cut as linseed oil, probably because of the even distribution of oleic and palmitic acids. However triunsaturated glycerides containing linolenic acid constitute about 2.5% (Fraction Numbers 56, 57, 58).

Conclusion

The results indicate a fractionation of glyceride types. By the method described above, an unknown fat could be fractionated into different types. In conjunction with a further fatty acid analysis on the fractions the method is believed to provide a good means for determining fatty acid distribution in natural fats.

Acknowledgment

The apparatus was fabricated at the National Research Council Laboratory, Ottawa, by G. Ensel. The authors gratefully acknowledge the technical assistance of John Frayne and R.G. Campbell. R.A. Chapman, assistant director, Food and Drug Laboratories, and R.P.A. Sims, Fats and Oils Laboratory, Canada Department of Agriculture, have been particularly helpful with their many suggestions during the course of the study.

REFERENCES

1. American Oil Chemists' Society, "Official and Tentative Methods," 2nd ed. (1958 Revision) Method Cd 1-25.
2. Borgstrom, B., Acta. Physiol. Scand., *25*, 101–110, 111–119 (1952).
3. Cama, J.S., Chakrabarty, M.M., Hilditch, T.P., and Meara, M.L., J. Sci. Food Agri., *4*, 321–328 (1953).
4. Crowell, G.K., J. Assoc. Offic. Agri. Chemists, *27*, 448–451 (1944).
5. Doerschuk, A.P., and Daubert, B.F., J. Am. Oil Chemists' Soc., *25*, 425–433 (1948).
6. Dutton, H.J., "Progress in the Chemistry of Fats and Other Lipids," Vol. 2, Ch. 7, R.T. Holman, W.O. Lundberg, and T. Malkin, editors, New York, Academic Press Inc., 1954.
7. Dutton, H.J., and Cannon, J.A., J. Am. Oil Chemists' Soc., *33*, 46–49 (1956).
8. Fillerup, D.L., and Mead, F.J., Proc. Soc. Exptl. Biol. Med., *83*, 574–577 (1953).
9. Frankel, E.N., Smith, L.M., and Jack, E.L., J. Dairy Sci., *41*, 483–491 (1958).
10. Hanahan, D.J., Dittmar, J.C., and Warashina, E., J. Biol. Chem., *228*, 685–700 (1957).
11. Hilditch, T.P., "The Chemical Constitution of National Fats," 2nd ed., New York, John Wiley and Sons Inc., 1947.
12. Hirsch, J., and Ahrens, E.H., Jr., J. Biol. Chem., *233*, 311–320 (1958).
13. Kay L.M., and Trueblood, K.N., Anal. Chem., *26*, 1566–1572 (1954).
14. Lea, C.H., and Rhodes, D.N., Biochem. J., *57*, XXIII (1954).
15. Magnusson, J.R., and Hammond, E.G., J. Am. Oil Chemists' Soc., *36*, 339–343 (1959).
16. Malmberg, E.W., Anal. Chem., *27*, 840–842 (1955).
17. Reinbold, C.L., and Dutton, H.J., J. Am. Oil Chemists' Soc., *25*, 117–120 (1948).
18. Sahasrabudhe, M.R., and Tuckey, S.L., J. Dairy Sci., *38*, 1392 (1955).
19. Schroeder, W.A., Ann. N.Y. Acad. Sci., *49*, 204–217 (1948).
20. Shorland, F.B., Bruce, L.W., and Jessop, A.S., Biochem. J., *52*, 400–407 (1952).
21. Trappe, W., Biochem. Z., *305*, 150–161 (1940).
22. Trappe, W., Biochem. Z., *306*, 316–336 (1940).
23. Walker, F.T., and Mills, M.R., J. Soc. Chem. Ind., *61*, 125–128 (1942).
24. Weiss, B., J. Biol. Chem., *223*, 523–534 (1956).
25. Winter, G., and Nunn, W., J. Sci. Food Agri., *4*, 442–448 (1953).
26. Wycoff, H.D., and Parsons, J., Science, *125*, 347–348 (1957).

[Received May 18, 1960]