Preparative fractionation of triglyceride mixtures according to acyl carbon number, using hydroxyalkoxypropyl Sephadex

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Abstract The present paper describes the use of hydroxyalkoxypropyl Sephadex in a liquid chromatography system. When the column is held at 40°C, and when elution is made with a linear gradient of two solvents, an excellent separation of saturated triglycerides in the region C_9–C_{56} is obtained in 24 hr, even with sample loads as high as 0.5 g/cm^2 of column.

Triglycerides containing unsaturated fatty acids are eluted more rapidly than their saturated homologs, one C-C double bond being equivalent to −1.42 fatty acid carbon atoms.

Supplementary key words gradient elution · unsaturated triglycerides · reversed phase · hydrophobic gel

For investigation of, for example, the crystallographical and rheological properties of the triglycerides of natural fats, it is necessary to have a triglyceride separation method with high resolution power and the ability to handle large amounts of starting material. The same applies to the investigation of the composition of minor components of natural fats.

Although gas–liquid chromatography provides the necessary resolution, it is impractical to use because of the small sample loads permitted. With liquid column chromatography, however, it is possible to separate natural fats with a satisfactory resolution, using much larger amounts of sample than with GLC. Nickel and Privett (1) reviewed the current situation when they presented their triglyceride separation method in 1967. They used a column of silanized Celite, 125 × 2.5 cm, and were able to separate 15 mg of a triglyceride mixture. By means of a simple elution procedure they obtained a separation of triglycerides depending on the chain length of the fatty acids. The separation was very efficient. Shortly after, Ellingboe, Nyström, and Sjövall (2, 3) described a method of separation in which they used two types of hydroxyalkoxypropyl Sephadex as column material. A recalculation of their data shows that about 250 mg of triglyceride mixture could be separated on a column of the same size as that used by Nickel and Privett (1).

This paper describes an improved version of the method of Ellingboe et al. (2, 3). By the use of this method it is easy to get enough material, in a short time, for a closer physical investigation of the triglycerides of a natural fat. The method permits 2.5 g or more of a sample to be separated into groups of triglycerides according to their total carbon chain lengths. A column 100 × 2.5 cm is used for the separation.

The separation is effective for triglycerides within the range tributyrin to tristearin and can be carried out with smaller volumes of eluting solvents and in a much shorter time than has previously been possible.

METHODS

Instruments

The equipment for preparative separation is shown in Fig. 1. As effective performance of these instruments is vital to the success of the method, a specification for each is given.

Gradient mixer. For tests with nonlinear gradients, an LKB 11300 Ultrograd was used together with one or two LKB 11310 valves. In routine work with linear gradients, two cylinders of stainless steel were used, each of internal diameter 73 mm and height 550 mm. The vessels were connected at the bottom by a short length of Teflon tubing of internal diameter 6 mm. One vessel is equipped with a motor-driven stirrer with a long shaft, so that the impeller works very close to the bottom of the vessel. This vessel also has a connection for an outlet tube to the pump. The connection of the other vessel to the first can be broken by

Abbreviations: PTFE, polytetrafluoroethylene; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

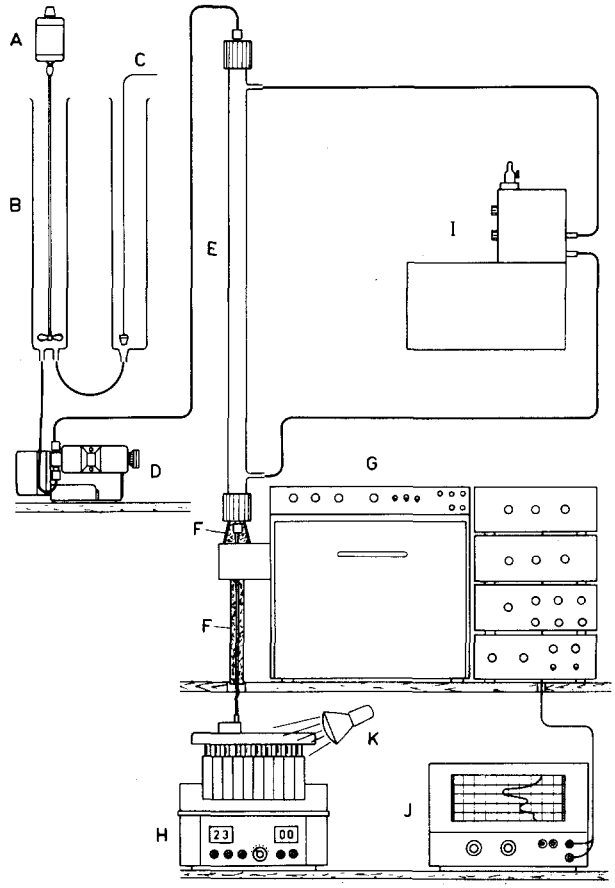

Fig. 1. Schematic diagram of the instrumental setup. *A*, stirrer; *B*, gradient vessels; *C*, stopper for connecting tube; *D*, pump; *E*, column; *F*, heat-insulated effluent tubes; *G*, Pye liquid chromatograph system 2 (moving wire detector); *H*, fraction collector; *I*, thermocirculator; *J*, recorder; *K*, heating lamp.

inserting into the outlet a PTFE plug at the end of a shaft 75 cm long. After filling the connecting tube with eluent 1, this plug is used to enable both vessels to be filled with their respective eluents. The plug is removed at the start of a run.

The connection between gradient mixer and pump is made with Teflon tubing of 1.6 mm internal diameter. Tubing with larger diameter may, however, be preferable. The gradient mixer should be placed so that the bottom is level with the bottom end of the column.

Pump. The pump feeding eluent from the gradient mixer to the column represents the weakest link in the chain of components. After testing several types of pumps the following setup proved satisfactory. We used a Dosapro Milton Roy Minipump type Simplex 19631 with a special plunger packing substituted for the ordinary Viton A packing (408A). The special plunger packing consists of an expandible packing of Rulon (a glass-fiber-filled PTFE packing with a stainless steel spring inserted).

To prevent gas locks on the suction side of the pump, the pump should be placed about 1 m below the gradient vessels. Even under such conditions the pump will keep the flow rate constant to ±0.5% only when pumping near its maximum rate (150 ml/hr).

Column. A precision, moderate pressure column (LKB 4220) with internal diameter 2.5 cm and working length of 100 cm was used. Each column is tested at 10 kg/cm^2 before delivery. The actual working pressure is normally below this, but can, under unfortunate circumstances, much exceed it with consequent rupture. Ordinarily the damage is limited to the column itself.

The Viton sealing rings (LKB 4264) on the top and bottom plugs will swell under the influence of the eluent. The rings can, in spite of this, be used if a method of working is used that eliminates the need to remove and reinsert the plugs between the runs. When, however, a plug is removed, a new Viton ring must be inserted.

During the actual analysis the column is maintained at 40°C by circulating water through the jacket by means of a thermocirculator (Heto 08C 623K). The column should be mounted on a frame so that during repacking or regeneration it may be swung away from the working position over the detector inlet into a more accessible position. The connection to the detector is very critical. The column outlet should be placed as close to the detector inlet as possible. We have used as a connection between the detector and the column a 2-cm length of stainless steel tubing (internal diameter 0.8 mm) with a 0.5-cm length of Teflon sleeve slid over the stainless steel tube. With this short connection and good heat insulation, the heat from the detector will prevent crystallization of higher triglycerides in the tube.

Due to the risk of bubble formation in the column, when the column is not in use, it cannot be plugged at the outlet in the ordinary way. It should be connected to a vertical expansion tube of the same size as the inlet tubing, reaching about 0.5 m above the top of the column. The same applies to the column inlet, which cannot be left attached to the pump when the column is not used but should be connected to an expansion tube similar to that just described.

It is also necessary to stop the water circulation in the jacket and to let the column cool down completely before the pumping pressure is released. Otherwise, rupture of the column material occurs due to boiling and release of gas.

Detector. A Pye liquid chromatograph system 2 combined with a gas control system, Pye PV 4100, and a Texas Servowriter II recorder have been used as a detector system. A slight modification was made to the electronics of the "system 2." The "event marker" function was changed to give negative pulses from the working level of the recorder pen and to give smaller event marks. A further change in this function is to let the event mark signal from the fraction collector (LKB 7000 UltroRac)

trigger the event marker of "system 2" instead of using manual triggering.

The working parameters of the detector system have been set as follows: temperature of evaporator, 140°C; temperature of pyrolyzer, 650°C; temperature of flame ionization detector, 200°C; wire velocity, 6 cm/sec. Attenuation depends on the sample size but is usually 1×10^2 for 2.5 g of a natural triglyceride mixture and for individual reference substances and 5×10^1 for 0.5 g of a natural triglyceride mixture. Paper velocity was 1 inch/hr.

Fraction collector. The part of the eluate not used by the detector is led by gravity to a fraction collector (LKB 7000 UltroRac) positioned as near as possible below the detector. The eluate flows from the "coating block" of the detector through a heat-insulated Teflon tube of 1.8 mm internal diameter to the collector. For a normal run of about 20 hr and using 3 l of eluent, the collector is loaded with 12 racks, each containing 10 rimless test tubes 18 mm in external diameter and 18 cm long. With a pumping rate of 150 ml/hr and with automatic tube change every 10 min, each tube will contain 25 ml of eluate. To prevent crystallization of higher triglycerides at the tip of the Teflon tubing, a small heating lamp is focused on the tip.

Eluents. The eluents we used are based on those described by Ellingboe et al. (3) but are modified to fulfill certain additional requirements. With the eluent used by Ellingboe et al. (3) for separation of triglycerides, the elution of tristearin was difficult. By increasing the working temperature to 40°C, good elution of tristearin was obtained but the elution conditions for lower triglycerides were adversely affected. The lower triglycerides could, however, be fairly well eluted at 40°C by use of the eluent used by Ellingboe et al. (3) for the separation of monoglycerides, but, conversely, the resolution of higher triglycerides was adversely affected. After certain modifications of the eluents, a gradient of the two eluents proved satisfactory for elution of both low and high molecular weight triglycerides. The eluent system used was: eluent 1, 1500 ml of isopropanol–chloroform–heptane–water 115:15:2:35 (v/v); and eluent 2, 1740 ml of heptane–acetone–water 4:15:1 (v/v). To both eluents 0.1% dimethyldisulfide was added as an antioxidant in order to protect the column material from decomposition. All reagents were of analytical reagent quality and were used without further purification.

The two volumes, 1500 ml and 1740 ml, are in hydrostatic equilibrium when filled into the gradient vessels. Vessel 1, i.e., the one with the impeller and outlet to the pump, should be filled with eluent 1, and the other vessel with eluent 2.

Column material. Hydroxy(C_{15}–C_{18})alkoxypropyl Sephadex[1] was synthesized as described by Ellingboe et al. (3) from Sephadex LH-20 (lot no. 4116, with a particle size of 25–100 μm) and Nedox 1518 (lot 050-M180-021). The degree of substitution was 55%, estimated from the increase in weight of the material.

Column packing. If dry, the packing material was allowed to swell overnight in an excess of eluent 1. The column was fitted with an extension tube (LKB 4290-57) and the column was filled to half its height with eluent 1. The outlet was closed by a vertical expansion tube as described earlier. The column material was transferred into the tube as an air-free suspension and was allowed to settle under the influence of the streaming eluent when the expansion tube was lowered to permit flow from the column. A total hydrostatic pressure of about 2.5 m of solvent was used. Further portions of suspension were filled into the extension tube to keep the level constant to within about 10 cm.

The column was filled to a height of only 87 cm. The packing was then compressed by circulating eluent 1 by means of the pump at a flow rate of 150 ml/hr for 24 hr at 40°C. The level of the gel was then adjusted to 85 cm by addition or subtraction of material. The remaining 15 cm of column length was reserved for the swelling of the gel under the influence of eluent 2.

The details of the packing procedure are given for the sake of convenience but are not particularly critical for the performance of the column. Even a poorly packed column will work satisfactorily after a few cycles of use and regeneration. Thus, a column packing that is ruptured by gas bubbles can be restored to effectiveness, without repacking, merely by cycling the eluents.

After a run, the column is regenerated by pumping eluent 1 through it until the eluate has the same refractive index as eluent 1. This measurement is carried out with a simple butterfat refractometer.

Analytical procedure

Sample treatment and application. The test substances were obtained from butter or margarine of different types after isolation of the pure fat by melting, centrifugation, and warm filtration. The isolated fat was used either directly or after hydrogenation. In addition to triglycerides, these fats contain minor quantities of other lipid components, e.g., mono- and diglycerides, branched-chain and other unusual fatty acids, and sterols. These do not usually give rise to well-defined peaks on the chromatograms but may possibly contribute to the "background." The reference substances used were of a quality corresponding to Sigma grade 1. These were tributyrin, tricaproin, tricaprin, trilaurin, trimyristin, tripalmitin, and tristearin. Triolein, trilinolein, and trilinolenin have also been used. Triglycerides containing different fatty acids were not tested.

[1] Now commercially available from Becker Delft N.V. Chemical Division, Duinkerkenstraat 32, Groningen, The Netherlands.

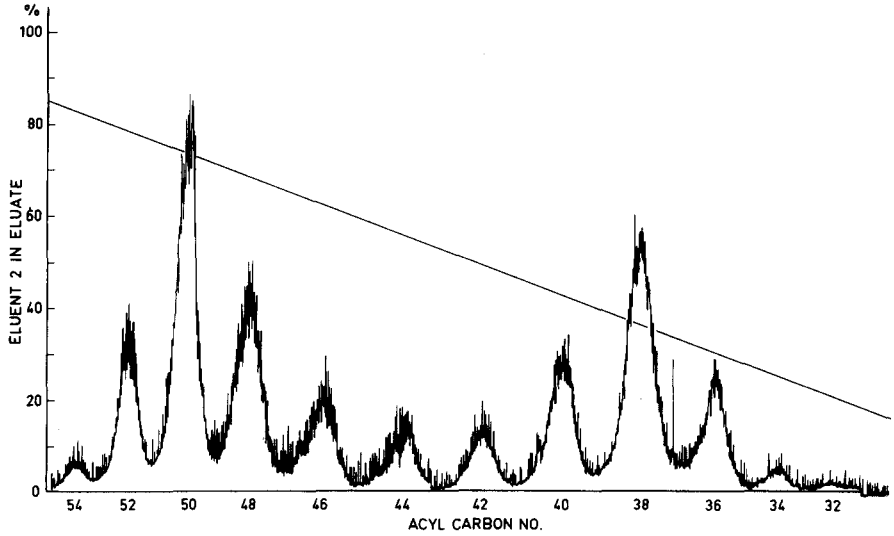

Fig. 2. Separation of triglycerides from 0.5 g of butterfat. Elution velocity, 75 ml/hr; detector attenuation, × 20; paper speed of recorder, 0.5 inch/hr. Other conditions are as given in the text. Ordinates in this figure and in Figs. 3 and 7 are detector responses in arbitrary units.

Samples ranging from 0.1 to 2.5 g were dissolved in 0.5–2.0 ml of chloroform. The solutions were applied to the column by sucking them up with a Luer syringe fitted with a 25-cm-long needle of 0.9 mm external diameter. The needle was inserted through the coupling cone of the top plug of the column and lowered to within 5 mm of the surface of the gel, previously regenerated and maintained at 40°C. The space over the gel up to the top plug was completely filled with eluent 1. The solution containing the sample was applied very slowly to the surface of the gel, where, due to its higher density, it formed a sharp layer. The sample will, to a certain extent, penetrate the top layer of the gel causing it to swell additionally by about 2 cm.

TABLE 1. Peak positions for saturated triglycerides with various numbers of carbon atoms in the acyl groups

Acyl Carbon Number	Mean Eluate Volume	Standard Deviation
	ml	*ml*
18	354	
30	700	17.0
32	819	38.8
34	978	18.6
36	1147	26.0
38	1329	12.1
40	1534	10.7
42	1750	17.4
44	1967	22.4
46	2168	20.8
48	2353	14.6
50	2506	11.7
52	2653	16.4
54	2796	18.5
56	2930	15.7
		Mean 18.6

Immediately after the syringe is emptied, the connection between pump and column is restored. Then, in sequence, the recorder (the detector should be switched on about 1 hr before), the fraction collector, and the pump are started. The functioning of the detector is tested by touching the running wire with the finger as outlined in the Pye manual.

Fat hydrogenation. The hydrogenation was carried out exactly as described by Hadorn and Zürcher (4).

Treatment of the eluate. Guided by the peak positions and the tube change marks of the recorder output, the tubes with the eluate were pooled to form fractions corresponding to the peaks. The fractions were stored directly at −18°C or were evaporated in a flash evaporator. The dry material was then stored in an argon-filled freezer.

RESULTS

Resolution

The first objective of the investigation was to find a method with high resolution for triglycerides differing only by two CH_2 groups. For this, fat isolated from butter was used as a test substance. The ultimate resolution reached is shown in Fig. 2. In this case the amount of sample applied to the column was only 500 mg, i.e., 100 mg/cm^2, and a low rate of pumping (75 ml/hr) was used. For most practical purposes the resolution attained is more than adequate.

Usually, the resolution illustrated in Fig. 3 is sufficient for quantitative and qualitative analysis. This curve was obtained from an experiment using 2.5 g of isolated butterfat and a pumping rate of 150 ml/hr.

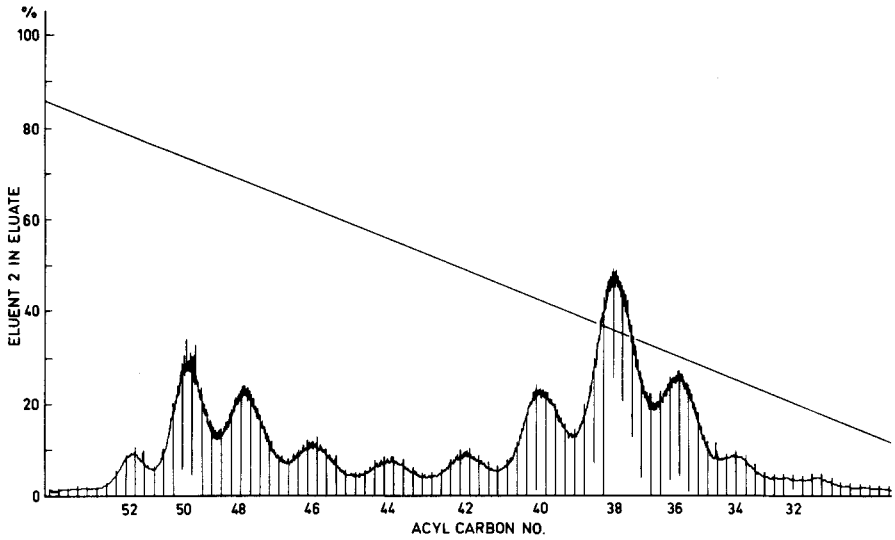

Fig. 3. Separation of 2.5 g of butterfat. Working conditions as given in the text.

Reproducibility of the separations

It has been found that the reproducibility of the peak positions is influenced by the state of the packing material, the column temperature, the functioning of the gradient mixer and the pump, and by the skill of the operator when applying the sample to the column.

The reproducibility of the peak positions is also dependent on the working conditions. To obtain reliable results the rules given previously must be followed, and attention is especially drawn to the importance of checking the pumping rate before each run. This is done by measuring the volume leaving the column during 10 min and is best done at the end of a regeneration cycle.

Even if the rules are carefully followed, some variation in the positions of the peaks is observed. In Table 1 the magnitude of these variations is presented. The values given are corrected for observed deviations in pumping rate and for differences in the starting conditions. It should be noted that the standard deviation of the position of a triglyceride is roughly independent of the nature of the triglyceride. On the average, the standard deviation is 3.15 mm on a curve that is 500 mm long, corresponding to 18.6 ml for a total eluate volume of about 2950 ml.

Triglycerides differing in chain length by two CH_2 groups differ in average 29 mm or 171 ml. One standard deviation thus represents 0.22 CH_2 group.

Peak positions

The peak positions given in Table 1 when plotted against the total number of carbon atoms in the fatty acids of the triglyceride do not form a straight but an S-shaped curve. In Fig. 4 the values taken from Table 1 are plotted, together with the positions of some saturated triglycerides that are not present in butter. One possible reason for the S form of the curve is discussed later.

Unsaturated triglycerides have been investigated to a limited extent only. Fig. 5 shows the results obtained during separation of C_{18} triglycerides with varying degrees of unsaturation. The peak positions of triolein, trilinolein, and trilinolenin give an approximately straight line when plotted against the number of double bonds. Interpolation of the curve gives a peak deflection of -1.42 CH_2 groups per double bond. It has not been established whether this applies only to this example or is valid for triglycerides with other distributions of chain length and double bonds.

Assuming that the ratio of -1.42 CH_2 groups per double bond is valid within the triglyceride region in question, it is possible to predict graphically the peak position for some unsaturated triglycerides of butter, which have been described in the literature (5). This is done in Fig. 6.

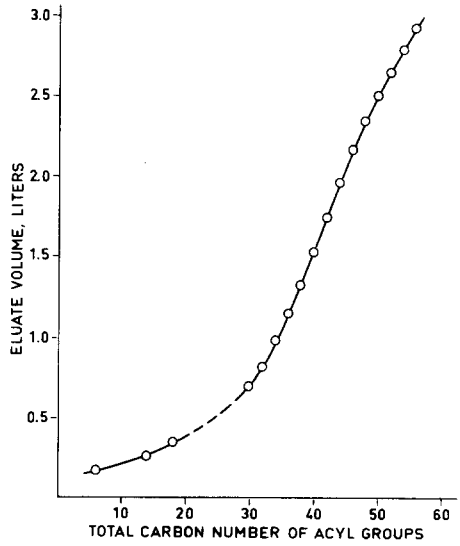

Fig. 4. Eluate volume for triglycerides with increasing numbers of carbon atoms of the acyl groups of the fatty acids.

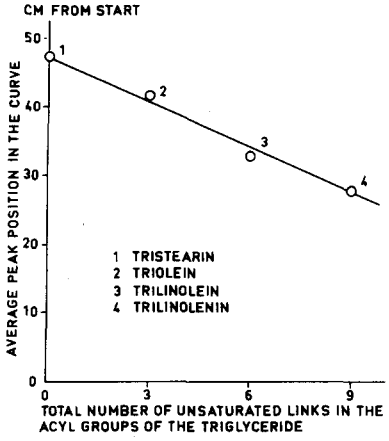

Fig. 5. Relative positions of tristearin, triolein, trilinolein, and trilinolenin.

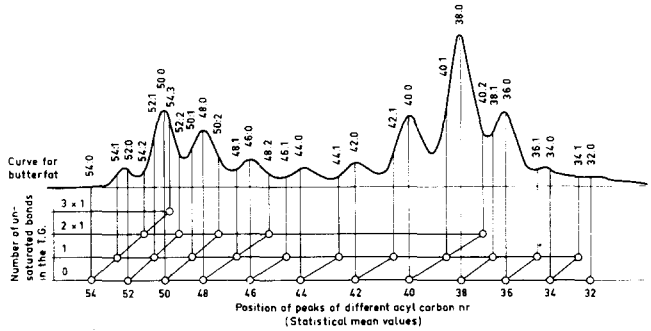

Fig. 6. Calculated peak positions for various saturated triglycerides and their corresponding unsaturated homologs compared with the experimental curve. The triglycerides selected are those that mathematically can be shown to be present in milk fat in a concentration higher than 3% (5). These are formed by the following acids: $C_{4:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$.

It is obvious that the resolution of the method is insufficient for use in analysis of such a complicated triglyceride mixture.

The validity of this conclusion was verified when attempts were made to analyze vegetable oils and special types of margarine that have a high content of polyunsaturated fatty acids. The recorder curves obtained were short and lacked significant peaks, with much overlap of peaks due to individual components.

It is also easy to see from the record of a typical analysis of butter, e.g., that of Fig. 3, that the peaks stand against a "background" of what can be assumed to be an unresolved mixture of unsaturated triglycerides. Analysis of the same sample after hydrogenation shows a record with reduced background (Fig. 7). The residual background that can be distinguished is probably due to overlapping parts of the "saturated peaks" as well as to traces of the minor components present. In Fig. 7 it can also be seen that the peaks corresponding to triglycerides C_{56}, C_{54}, C_{52}, C_{40}, and C_{42} are increased in height. The peaks corresponding to the triglycerides C_{50} and C_{48} and also C_{38} and C_{36} have decreased correspondingly. These changes are obviously due to hydrogenation of unsaturated triglycerides in the original mixture. Rechromatography of fractions isolated from the butterfat, both before and after hydrogenation, confirmed the presence of both unsaturated and saturated triglycerides.

TABLE 2. Fatty acid composition of the spots obtained after argentation TLC analysis of peak C_{50} of butterfat

Spot Number	Fatty Acid						
	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
				%			
1	7.3	15.4	54.9		22.4		
2	2.3	16	35.6		6.8	39	
3	2.7	17.3	24.9	1.6	4.5	49	
4	0.7	12	20.3	2.8	4.8	59.5	
5		9.6	23.4	2.4	5.1	56.7	2.5

Only major peaks are considered. In addition to the acids given in the table, several other acids were observed in minor quantities in the gas chromatograms. C_{20} may be present in quantities (around 10%) but it was difficult to determine this acid quantitatively due to its position and due to the small quantities of sample.

Thin-layer and gas–liquid chromatography show[2] that the peaks in Fig. 6 are heterogeneous. Fig. 8 is an example of a TLC plate obtained after analysis of peaks C_{46}, C_{48}, and C_{50} by argentation TLC (6). For C_{50}, three strong spots together with three weaker ones are visible. Gas–liquid chromatographic analysis of the spots of C_{50} yielded the fatty acid compositions given in Table 2. For comparison, some theoretically possible compositions are given in Table 3.

According to Fig. 6, peak C_{50} should contain the triglycerides $C_{54:2}$, $C_{52:1}$, $C_{50:0}$, $C_{54:3}$, and $C_{52:2}$. In addition, some overlapping of $C_{50:1}$ from the C_{48} peak may be expected. In argentation TLC these triglycerides should combine into at least four spots, containing zero, one, two, and three or more double bonds in their triglycerides. Extra spots may occur due to the resolution of a group of similar triglycerides into species containing the unsaturated fatty acids in various relative positions in the molecules.

Spot 1 of Fig. 8 belongs, judging from its position in the chromatogram and from the GLC analysis, to the saturated triglycerides. The GLC values indicate that combination 2 of case 1 of Table 3 is the dominant among its triglycerides.

Spot 2 of Fig. 8 probably contains triglycerides with one double bond per molecule. Combination 1 of case 2 of Table 3 seems dominant, but there is reason to believe that also combination 3 should be represented together with some overlapping of $C_{50:1}$ from peak C_{48}.

Spot 3 of Fig. 8 should consequently contain triglycerides with two double bonds, either of the type SUU or

[2] Lindqvist, B. Unpublished results.

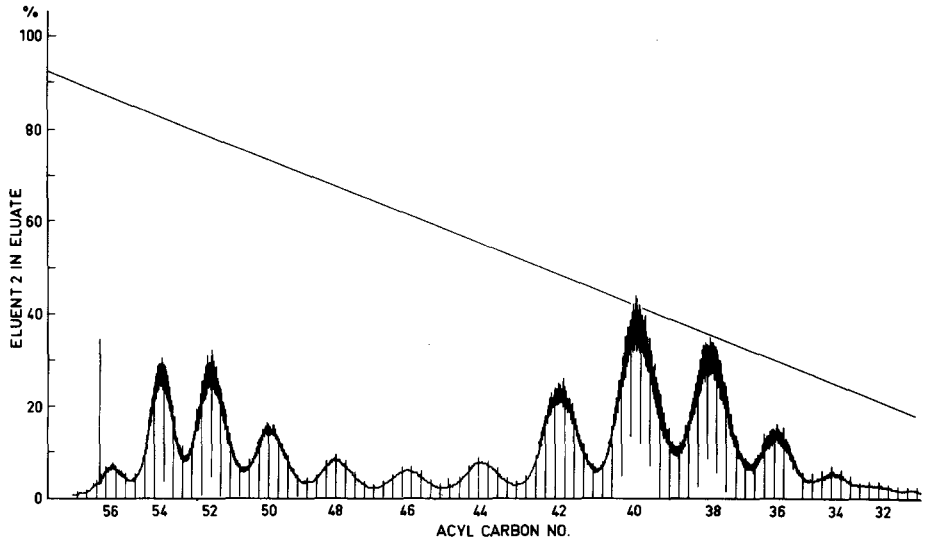

Fig. 7. Separation of the same sample as in Fig. 2, but after hydrogenation. Conditions of separation are as in Fig. 2.

USU,[3] judging from the GLC analysis, which shows the absence of linoleic acid. Then, only combinations 2 and 3 of case 3 of Table 3 are possible. Combination 3 seems to be dominant.

Spots 4, 5, and 6 are very weak, and the corresponding GLC values are not reliable. They may represent various forms of triglycerides containing from two to three double bonds, or more. Of spots 4 and 5, only the latter contains linoleic acid together with oleic and palmitoleic acids. Due to the very small amount of substance present in spot 6, GLC was unsuccessful.

Sample load

No systematic efforts have been made to elucidate the influence of sample size and working conditions on the efficiency of the separation. Figs. 2 and 3 suggest, however, that samples may range from 10 to 5000 mg of the triglyceride mixture. The lower value applies to a mixture containing only a few components, the higher to a mixture of many saturated triglycerides. At the 10 mg level the detector must be used at high sensitivity. As a consequence the curves become "noisy."

DISCUSSION

The system described here for the separation of triglycerides is the result of an empirical improvement of a previously described method. The working parameters given represent a compromise. Where conditions are different, a different compromise may be adopted, e.g., if high precision pumps that perform well at low pumping rates are available.

Compared with previous methods, the range of triglycerides that may successfully be analyzed has been extended both upwards and downwards. This has been achieved by using an eluent with low elution activity as the first component of a gradient elution system and by working at a sufficiently high temperature to make the higher triglycerides soluble in the second eluent.

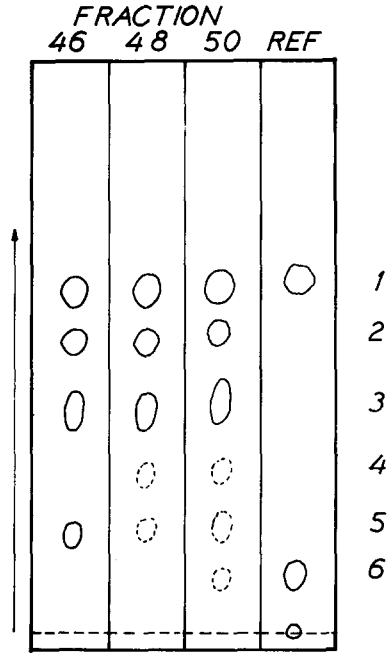

Fig. 8. TLC plate tracing from argentation TLC of the peaks C_{46}, C_{48}, and C_{50} from butterfat. Reference is a mixture of tristearin, triolein, and trilinolein. Trilinolein is retarded the most and remains at the origin.

[3] S denotes any saturated fatty acid and U any type of unsaturated fatty acid with one double bond.

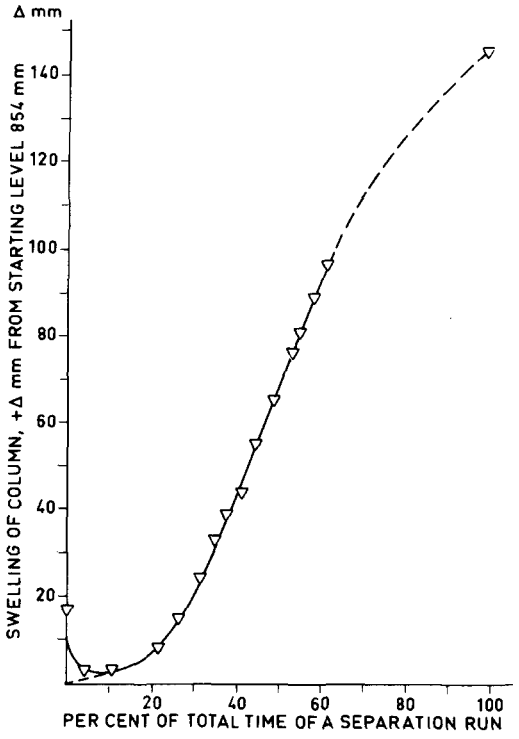

Fig. 9. Swelling of the modified Sephadex gel in the column during the analysis.

Triglycerides higher than C_{56} are still insufficiently soluble in the second eluent, so that work in the region C_{58}–C_{66} and above requires either the use of a third eluent or a much higher working temperature. Attempts to include such triglycerides within the separation range have been made at higher temperatures, but the problems associated with boiling eluents have not been successfully solved. New types of eluents are required for use at such temperatures, though the choice of components is limited. For instance, the use of chlorinated carbons must be restricted because they easily damage the heating wire of the pyrolysis oven.

The use of chloroform as a solvent for the sample also represents a problem. It is necessary to have a high density solvent to permit the sample application technique we have described. On the other hand, the solvent should preferably not exhibit greater elution power than eluent 1 and should not cause the gel to swell when the sample is applied. Chloroform suffers from all of these disadvantages. It is quite likely that the positions of the peaks below C_{34} in the diagrams would be different if another solvent were used.

The requirements of a good solvent for use with the sample are: a volatility comparable with that of isopropanol, density in the neighborhood of that of chloroform, and good solubility of higher triglycerides.

The approximately linear form of the curve in Fig. 3 within the range C_{30}–C_{56} is, from a practical point of view, more useful than the theoretically more interesting semilogarithmic form of the curve obtained with the system of Nickel and Privett (1). The deviation from a straight line at the lower end of the curve is partly due to elution by the solvent used for the sample. This results in elution of some of the components too quickly and causes swelling of the column at the start of the experiments. In other parts of the curve the deviation can be explained by nonlinear swelling of the column during the elution (Fig. 9). Still another reason for the deviation, especially in the upper part of the curve, may be the rather low solubility of higher triglycerides in the eluent used. There seem, however, to be no practical reasons for adjustment of the curve to obtain a straight line.

The distance, expressed as the number of carbon atoms, which corresponds to one double bond in the triglyceride is in our system −1.42 compared with −2.3 in the system of Nickel and Privett (1). The difference, and also the values themselves, are of no practical importance. In both systems some interference is to be expected between peaks due to saturated, and variously unsaturated, triglycerides. Other preparative measures have to be taken to avoid such interference.

Some efforts have been made at prior separation of the triglyceride mixtures according to the method of de Vries (7), but for reasons unknown the fractions obtained did not separate properly when subsequently analyzed in our chromatographic system. (Further work has been undertaken to elucidate this.) In spite of this failure the best solution to the preparative separation problem seems to lie in this direction. Analytical separation may well be obtained by gas–liquid chromatography.

TABLE 3. Some theoretically possible fatty acid compositions of the triglycerides in peak C_{50} of Fig. 6

Case	Triglyceride	Combination	Fatty Acids
1	$C_{50:0}$	1	$C_{18:0} + C_{18:0} + C_{14:0}$
		2	$C_{18:0} + C_{16:0} + C_{16:0}$
		3	$C_{20:0} + C_{18:0} + C_{12:0}$
		4	$C_{20:0} + C_{16:0} + C_{14:0}$
2	$C_{52:1}$	1	$C_{18:1} + C_{18:0} + C_{16:0}$
		2	$C_{18:0} + C_{18:0} + C_{16:1}$
		3	$C_{20:0} + C_{18:1} + C_{14:0}$
		4	$C_{20:0} + C_{16:1} + C_{16:0}$
3	$C_{52:2}$	1	$C_{18:2} + C_{18:0} + C_{16:0}$
		2	$C_{18:1} + C_{18:1} + C_{16:0}$
		3	$C_{18:0} + C_{18:1} + C_{16:1}$
		4	$C_{20:0} + C_{18:2} + C_{12:0}$
4	$C_{54:2}$	1	$C_{18:2} + C_{18:0} + C_{18:0}$
		2	$C_{18:1} + C_{18:1} + C_{18:0}$
		3	$C_{20:0} + C_{18:2} + C_{16:0}$
5	$C_{54:3}$	1	$C_{18:2} + C_{18:1} + C_{18:0}$
		2	$C_{18:1} + C_{18:1} + C_{18:1}$
		3	$C_{20:0} + C_{18:3} + C_{16:0}$
		4	$C_{20:0} + C_{18:2} + C_{16:1}$

Only the fatty acids that have been observed to be present in butterfat in concentrations at least occasionally greater than 2.5% are considered. Refer to the legend to Fig. 6.

For analytical purposes the use of columns with diameters smaller than 2.5 cm seems useful. Our experience indicates, however, two obstacles. One is the functioning of the pump and the second the operation of the gradient mixer. Assuming a 6-mm column the flow rate should be about 9 ml/hr. Under these conditions formation of gas bubbles in connecting tubes and other vital parts is quite a serious problem with the solvents that we have used. At such low flow rates, bubbles are not readily flushed out and easily clog parts of the system. It seems, however, not difficult to set up an analytical system using more advanced components, which will circumvent these obstacles and permit the use of smaller columns.

Hydroxy(C_{15}–C_{18})alkoxypropyl Sephadex has demonstrated its ability to resist mechanical and chemical degradation if the working procedure given is adhered to. If no antioxidants are present in the eluents, the gel will lose its separating power after about a month of continuous use. If an antioxidant is used the same column can be used for more than a year with no loss in resolution.

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REFERENCES

1. Nickel, E. C., and O. S. Privett. 1967. Fractionation of triglycerides by reversed-phase partition chromatography. *Separ. Sci.* **2:** 307–318.
2. Ellingboe, J., E. Nyström and J. Sjövall. 1968. A versatile lipophilic Sephadex derivative for "reversed-phase" chromatography. *Biochim. Biophys. Acta.* **152:** 803–805.
3. Ellingboe, J., E. Nyström and J. Sjövall. 1970. Liquid–gel chromatography on lipophilic–hydrophobic Sephadex derivatives. *J. Lipid Res.* **11:** 266–273.
4. Hadorn, H., and K. Zürcher. 1968. Die katalytische Hydrierung als Hilfsmittel zur Identifizierung und Bestimmung ungesattiger Fettsäuren. *Mitt. Geb. Lebensmittelunters. Hyg.* **59:** 78–107.
5. Nutter, L. J., and O. S. Privett. 1967. Structure of triglycerides of bovine milk serum. Short-chain triglycerides. *J. Dairy Sci.* **50:** 1194–1199.
6. Bandyopadhyay, C. 1968. Estimation of trisaturated glycerides in fats by argentation thin-layer chromatography. *J. Chromatogr.* **37:** 123–127.
7. de Vries, B. 1964. Separation of triglycerides by column chromatography on silica impregnated with silver nitrate. *J. Amer. Oil Chem. Soc.* **41:** 403–406.