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Gel filtration liquid chromatography of fatty acids and other fat samples using a Pye liquid chromatography detector

The analytical and experimental aspects of molecular-sieve chromatography, including the use of lipophilic gel filtration media such as Sephadex* derivatives and Bio-Beads** for molecular size separation problems, have often been reviewed[1,2].

In most reports of separations achieved with Sephadex LH-20 the gel permeation effect was superseded to some extent by adsorption or liquid–liquid partition phenomena[2–5], and in many cases Sephadex LH-20 has been used deliberately for the adsorption or partition chromatographic separation of samples of different polarity such as prostaglandins[6] or mono-, di-, and triglycerides[2,7]. Only a few papers describe a more genuine gel permeation chromatography (GPC) of fatty acid polymers on Sephadex[8,9].

CHANG[10] had used Bio-Beads SX-2 for the separation of tall oil fatty acid dimers because strong adsorption was found with LH-20 in tetrahydrofuran, and MULDER AND BUYTENHUYS[11] separated triglycerides on columns of Bio-Beads SX-1 + SX-2. Separations of polymeric fatty acids by GPC using the Waters chromatograph*** have also been described[12].

In used frying fats and other heated fats, thermal dimers and polymers of fatty acids are present and may be analysed *e.g.* by PC[13] or TLC[14] of urea-non-adduct methyl esters. Most of the fatty acid dimers in the fat appear to be present originally in the form of dimeric triglycerides[15]. In frying fats heated with access to air, some of them are oxidized, *i.e.* contain polar functional groups which may give rise to adsorption or partition effects if a polar gel such as Sephadex LH-20 is used with a less polar solvent or solvent mixture[4–8].

In this investigation, the potential of Sephadex LH-20 and of Bio-Beads SX-1 for the preparative isolation and analytical characterization of dimeric fatty acid methyl esters and triglycerides was further explored using model compounds.

Materials and methods

Reagent grade ethanol and benzene (E. Merck, G.F.R.) were used for swelling the Sephadex LH-20 and Bio-Beads SX-1, respectively.

Analytical columns were made from 3.5 mm I.D. glass tubing. The equipment used has been described[16] in connection with the liquid chromatography of frying fats. It consisted of a solvent reservoir under gas pressure (N_2, usually 0.5 atm), a column with a Pye injection head and a Pye System II liquid chromatography (LC) detector with flame ionization detector[17] for recording the analytical chromatograms. All connections were made with PTFE tubing.

For preparative purposes Sephadex SR-25/100 columns were used in conjunction with an LKB fraction collector operated with a 5-ml siphon. The preparative columns were usually run overnight and off-line monitoring was carried out in the

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following way: An aliquot (usually 100 μl) of each collector fraction was injected with an Eppendorf pipette into a constant flow of ethanol rinsing the walls of a narrow glass tube which has an opening to accept the pipette tip. The ethanol-flow serves to transport the sample through the LC coating block and to rinse the tubing and coating block between subsequent injections. At a flow rate of 30–60 drops/min, 2–4 injections could be made per minute.

Methyl oleate and triolein (Fluka, Switzerland) were used as test samples. Dimers and trimers were prepared with di-*tert.*-butylperoxide according to the method of PASCHKE AND WHEELER[18] and separated by distillation and silica gel chromatography. Polymeric trioleins were separated by silica gel column chromatography and purified by preparative GPC on Bio-Beads SX-1 in benzene.

Results

Fig. 1 shows the separation, on LH-20, of a test mixture consisting of monomeric and dimeric methyl oleate, and of a silica gel column chromatography fraction

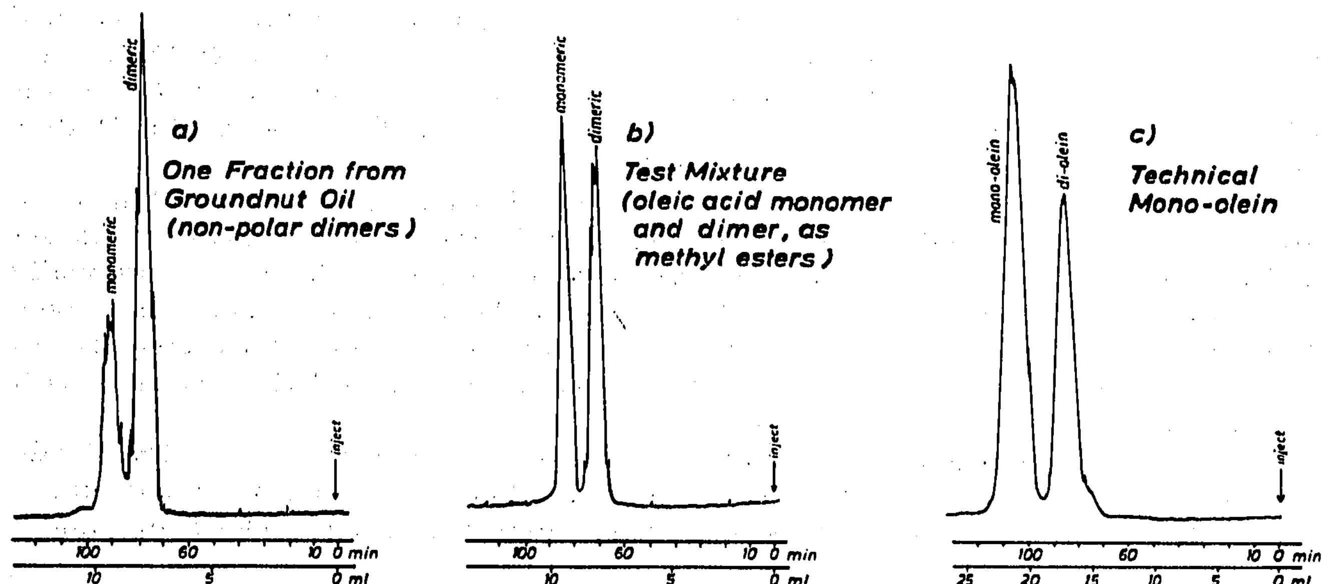

Fig. 1. Analytical gel permeation: Sephadex LH-20 in ethanol. Columns: (a) and (b) = 0.35 × 105 cm; N_2 pressure 0.5 atm; sample, 15 μl of a 10% ethanol solution; (c) = 0.6 cm × 112 cm; 0.5 atm N_2; sample, *ca.* 3–4 μl of undiluted mono-olein.

obtained from the fatty acid methyl esters of groundnut oil used for frying which contained all the non-polar dimers. It also shows an LH-20 gel permeation chromatogram of a technical mono-olein which contained *ca.* 40% diolein.

Fig. 2 demonstrates, by TLC, the separation obtained on a preparative LH-20 column with a test mixture consisting of dimeric fatty alcohols (prepared by $LiAlH_4$ reduction of dimeric methyl oleate) and methyl oleate.

Fig. 3 shows the off-line monitoring of preparative LH-20 GPC fractions from an overnight run. The sample, a silica gel fraction of more polar fatty acid methyl esters obtained from a frying fat, contained both non-polar dimers and polar monomers.

Fig. 4 shows the GPC analysis, using the equipment previously described[16], of three preparative fractions of polymerized triolein, and the chromatogram obtained

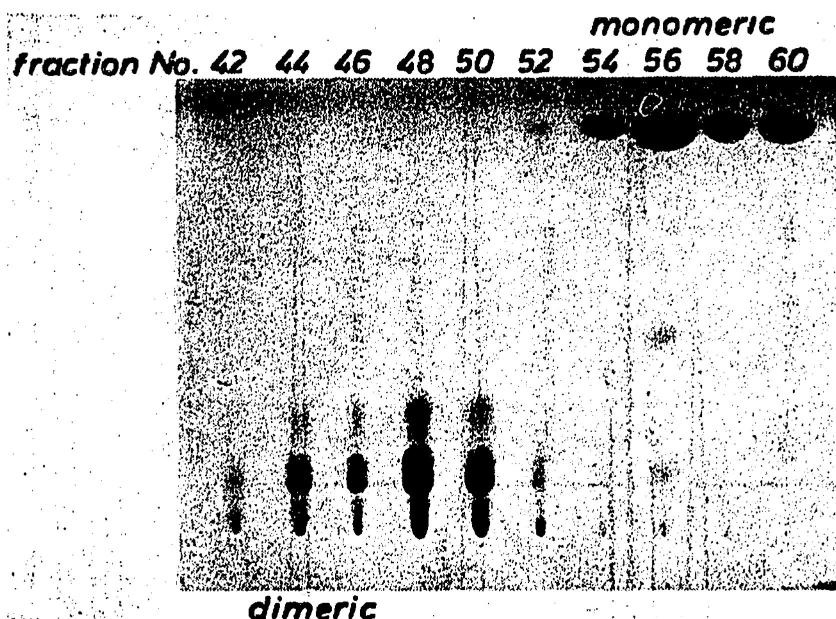

Fig. 2. TLC of fractions from a preparative SR-25/100 column with Sephadex LH-20: Methyl oleate and dimeric (C_{36}) fatty alcohols. Sorbent, Silica gel; solvent, benzene–chloroform–ethanol (60:30:2); visualization: 10% H_2SO_4, charred.

from a mixture of the three, on a column of Bio-Beads SX-1 in benzene. The triolein was dimerized in a similar manner to the method described by PASCHKE AND WHEELER[18] and the fractions analyzed here were obtained from an SR-25/100 column filled with Bio-Beads SX-1 in benzene.

Analytical chromatograms of used frying fats have been obtained in the same way. They normally show peaks in the molecular weight range of dimeric and trimeric triglycerides amounting to between 1 and 5% of the total peak area[15,19]. Some of these dimers may be linked by C–C bond or ring formation while others may contain C–O–C or peroxide linkages between the triglyceride units.

Discussion

The results described here show that Sephadex LH-20 is useful for the analytical and preparative separation of the dimeric from monomeric fatty acid methyl esters. The use of alcohols suppresses adsorption effects on the polar gel so that dimeric diols (Fig. 2) are eluted in the same fraction as non-polar dimeric methyl esters.

Initial experiments showed that in methanol a separation of dimeric from monomeric oleic acid methyl ester may be achieved even on Sephadex G-10. On SR-25/100 columns of LH-20 in methanol, a separation of trimer from dimer may be achieved, but in ethanol the trimers will often form only a shoulder on the dimer peak. Free fatty acids may also be separated, but slight tailing is observed. 12-Acetoxystearic acid methyl ester (mol. wt. 356, peak elution volume 259 ml) clearly ran ahead of oleic acid methyl ester (mol. wt. 296, peak elution volume 277 ml), but the peaks overlapped. Up to 1 g of sample could be applied on SR-25/100 columns of *ca.* 83-cm gel bed.

Lipophilic Sephadex types of a pore size larger than that of LH-20 are not available, and LH-20 itself does not resolve dimeric triglycerides (mol. wt. *ca.*

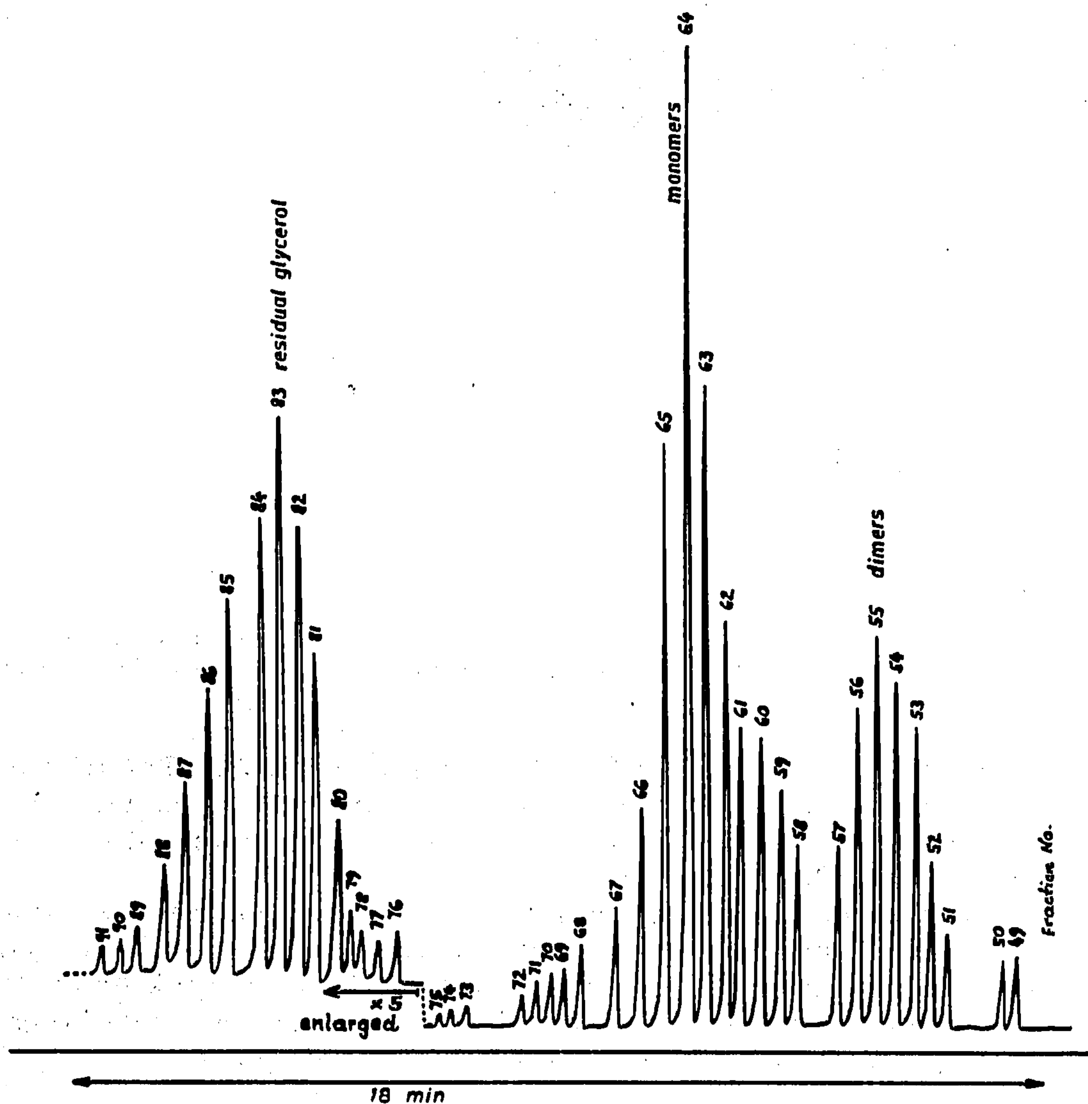

Fig. 3. Screening of collector fractions from a routine preparative run. LKB fraction collector (overnight at 5-ml fractions); Sephadex LH-20 in ethanol (2.5 × 80 cm column [SR-25/100]): Pye LC detector (100 µl each, injected into ethanol flow). Sample, a silica gel fraction of methyl esters from a frying fat, containing more polar monomers and residual phytosterols, dimers, and an unknown component of low molecular weight, possibly glycerol (this portion of the diagram is 5 times enlarged).

1800) from monomeric triglycerides. Gels of larger pore size such as Bio-Beads SX-1, a cross-linked polystyrene, will serve the purpose.

In analogy to the separation of dimeric fatty acid methyl esters[14], non-polar dimeric and trimeric triglycerides may also be separated[20] from monomeric triglycerides by silica gel adsorption chromatography (TLC and column LC). In actual frying fats, however, they may be overlapped by more polar monomers. The same analytical equipment[16] may be used for GPC columns and adsorption LC columns. GPC will yield information on 'total dimeric compounds' (non-polar and polar) while adsorption LC will permit the estimation of 'total more polar' compounds. In the latter case this may or may not include the non-polar dimeric triglycerides

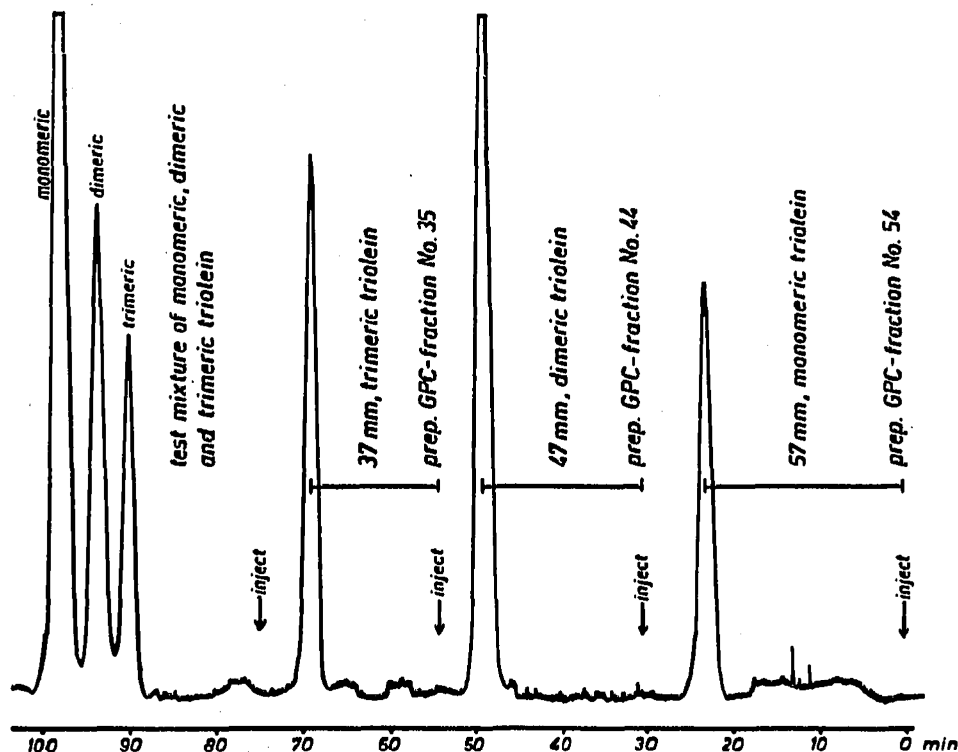

Fig. 4. Analytical GPC of polymeric trioleins. Column, Bio-Beads SX-1 in benzene; 0.35 cm × 112 cm. N_2 pressure 0.6 atm. Chart speed: 2.5 mm/min. From a triolein dimerization[18] experiment, a 1-g sample was fractionated using an SR-25/100 column of Bio-Beads SX-1 in benzene. Fractions of 5 ml each were taken by an LKB fraction collector. Shown here are analytical gel permeation chromatograms of three of these fractions (fraction No. 35, containing trimeric triolein; No. 44, containing dimeric triolein; and No. 54, containing triolein) and a chromatogram of a test mixture, containing all three.

depending on how the elution is programmed[20]. The Pye moving wire detector permits the recording of analytical chromatograms and is also of advantage for the off-line screening of preparative collector fractions.

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