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HIGH-SPEED LIQUID CHROMATOGRAPHY OF NON-POLAR LIPIDS

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SUMMARY

The rapid separation of non-polar lipid classes on columns of polystyrene gel with aqueous acetone as mobile phase is described. The influence of mobile phase composition, column temperature and gel pore size on performance is investigated and the advantages of operating the column system at elevated temperatures discussed. The quantitation of the separated lipid classes based on cholesterol as an internal standard and the modified moving wire/flame ionization detector is discussed. Examples of the quantitation of standard mixtures show both the precision and accuracy of the technique to be within 5% relative. The advantages and disadvantages of the system are considered.

INTRODUCTION

The separation of lipids into functional classes using liquid column chromatography is a well established technique[1–3]. Most methods described have been based on liquid–solid chromatography using adsorbents such as alumina and silica. A variety of eluents has been used dependent on the activities of the adsorbents and the polarities of the lipids being investigated. The composition of the eluent is commonly varied during elution to optimise the separations and the analysis speed. Separation methods based on liquid–liquid partition techniques have also been described[4,5]. Of these, the most promising in the light of advances in column, column packing and detector technology is the Factice chromatography of Hirsch. Factice is a polymerized vegetable oil which is broken up into relatively small particles for packing. As it is strongly hydrophobic, it takes up acetone, diethyl ketone, dioxane, ethanol, diethyl ether and similar organic solvents in preference to water. Thus two liquid phases can be obtained in a packed column; the mobile phase containing water and an organic solvent and the stationary phase being principally the organic solvent retained in the swollen gel matrix. Reversed-phase partition chromatography can thus be performed with such a gel system. Hirsch[4] described several binary solvents giving lipid class separation, finding acetone–water to be most effective. The principal disadvantage in such a gel system is that it is dimensionally unstable to changes in pressure, mobile phase composition and temperature. However, with advances in column packing materials, it is now possible to obtain rigid and semirigid gels of small, uniform particle size. The separation and quantiation of lipid classes using hydrophobic gels

of this type allied to increased column inlet pressures and a sensitive quantitative detection system is described below.

EXPERIMENTAL

Apparatus

A standard liquid chromatographic system consisting of a solvent reservoir, pump, thermostatted column and continuous detector was used.

The pump, which was developed in our laboratory, was continuous, pulse-free and capable of operating at up to 68 atm. The column (1 m × 4 mm I.D.) was glass, fitted with a Pye GLC injection head (Pye Unicam, Cambridge). The column inlet was a 6.35-mm Kovar-to-glass seal, the injection head being slightly modified to take a compression fitting to the Kovar. The column outlet was a standard Pye GLC fitting connected directly to the detector. A water jacket, maintained at a constant temperature in the range 30 to 65° by circulation from a water bath, enclosed the column. A modified Pye moving wire detector[6] provided a direct trace of the column eluent composition on a potentiometric recorder.

Materials

The pure lipids used as standards were obtained from Sigma, St. Louis, Mo. The glyceride samples contained a mixture of C_{16} and C_{18} fatty acids.

The polystyrene gel was Poragel 200A or 60A obtained from Waters Associates Ltd., Stockport. The packing procedure was either to allow a slurry of the gel in the initial developing solvent to settle under gravity, aliquots of the slurry being added as necessary to fill the column or to dry pack the column by tapping, rotating and bumping successive small portions of the gel until no further settling could be observed.

Operating conditions

The lipid mixture (mono-, 1,3-di- and tripalmitin, cholesterol and palmitic acid) was eluted at a number of mobile phase compositions (2.5–10% aq. acetone) and temperatures (30–65°). Sample injection was from a 10-μl syringe, with the col-

TABLE I

EFFECT OF MOBILE PHASE COMPOSITION AND COLUMN TEMPERATURE ON THE RETENTION VOLUMES OF NON-POLAR LIPIDS

Column, 1 m × 4 mm I.D.; packing, Poragel 200A.

Mobile phase (%)	*Retention volume (ml)*											
	Monoglyceride			*Diglyceride*			*Triglyceride*			*Monopalmitin*		
	30°	*47°*	*62°*	*30°*	*47°*	*62°*	*30°*	*47°*	*62°*	*30°*	*47°*	*62°*
10		8.46			24.92			96.68			8.31	
7.5		8.32	8.16		17.08			42.34	30.93		8.02	7.79
6		7.91	7.68		13.79	11.61		28.78	20.91		7.89	7.41
5	7.98	7.58	7.49		11.98	10.66	31.18	22.07	17.55	8.11	7.59	7.31
4		7.62	7.09		10.86	9.24		17.34	13.18		7.47	7.34
2.5		7.08	7.03		8.88	8.42		12.10	10.96		6.95	6.86

umn flow stopped, into a 5-cm layer of fine glass beads above the column packing to give an even application to the gel. Column inlet pressure varied from 7 to 27 atm.

RESULTS

The principal factor influencing separation of lipid classes in the gel system is the mobile phase composition. Its variation may also alter the stationary phase composition due to acetone/water solubility effects even within the gel matrix. In addition, the effect of column temperature was investigated, it being particularly important in this case as the lipids investigated are relatively insoluble in aqueous acetone at ambient temperatures. Tables I and II illustrate the effects of mobile phase composition (2.5–10% aq. acetone) and column temperature (30–65°) on the retention volumes and relative retention volumes (to cholesterol) of a number of lipids.

Fig. 1 shows the separation of a number of pure lipids (monopalmitin, palmitic acid, 1,3-dipalmitin, cholesterol and tripalmitin) in 30 min. Fig. 2 compares the separation of these lipids at a number of mobile phase compositions. The chromatograms in Figs. 1 and 2 are not exactly comparable as they were obtained on different columns using in one case (Fig. 1) a dry packing technique and in the other (Fig. 2) a slurry packing technique. The similarity of the chromatograms demonstrates the ease with which columns on which a satisfactory separation is obtained can be packed and the simplicity of the system for obtaining adequate separations.

Table III compares the retention volumes of the lipids on columns of Poragel 60A (exclusion limits 2,400 for polystyrene in THF) and Poragel 200A (exclusion limits 8,000 for polystyrene in THF). Although the values obtained are similar, there is a general tendency to smaller retention volumes and separation factors for the 60A.

A series of experiments was carried out to determine the linearity of the detector response to samples of the different lipid classes. Fig. 3 illustrates two typical responses for monopalmitin and cholesterol. The straight lines shown are those for a plot of peak area (by triangulation) against the volume of lipid solution injected. Table IV lists the gradients, calculated by regression analysis, of the best straight lines for each of the lipids. In addition, Table IV gives the gradient related to the carbon content of each lipid, *i.e.* the rate of change of peak area with mass of carbon. These

(Retention volume (ml))

1,3-Dipalmitin			*Tripalmitin*			*Palmitic acid*			*Cholesterol*		
30°	*47°*	*62°*	*30°*	*47°*	*62°*	*30°*	*47°*	*62°*	*30°*	*47°*	*62°*
	19.99			83.56			9.98			19.67	
	14.24	12.40		37.00	27.41		9.50	8.88		17.21	15.59
	12.06	10.31		25.61	18.80		9.01	8.51		15.65	13.80
13.12	11.22	9.75	29.86	21.60	15.95	9.28	8.73	8.11	16.52	14.34	13.10
	9.89	8.97		15.89	13.04		8.31	8.32		13.31	12.76
	8.35	8.11		11.63	10.38		8.03	7.91		12.16	11.16

TABLE II

EFFECT OF MOBILE PHASE COMPOSITION AND COLUMN TEMPERATURE ON THE RETENTION VOLUMES OF NON-POLAR LIPIDS RELATIVE TO CHOLESTEROL

Mobile phase (%)	*Retention volume (ml)*													
	Mono-glyceride		*Di-glyceride*		*Tri-glyceride*		*Mono-palmitin*		*1,3-Di-palmitin*		*Tri-palmitin*		*Palmitic acid*	
	47°	*62°*	*47°*	*62°*	*47°*	*62°*	*47°*	*62°*	*47°*	*62°*	*47°*	*62°*	*47°*	*62°*
10	0.430		1.267		5.017		0.422		1.016		4.248		0.507	
7.5	0.484	0.524	0.978		2.424	1.987	0.473	0.502	0.841	0.799	2.183	1.767	0.553	0.572
6	0.519	0.553	0.905	0.835	1.889	1.505	0.504	0.539	0.771	0.750	1.636	1.368	0.575	0.619
5	0.534	0.564	0.844	0.802	1.555	1.322	0.524	0.562	0.792	0.750	1.490	1.227	0.602	0.624
4	0.562	0.555	0.801	0.724	1.278	1.032	0.562	0.575	0.743	0.703	1.195	1.021	0.625	0.652
2.5	0.582	0.630	0.730	0.754	0.995	0.982	0.572	0.615	0.687	0.721	0.956	0.930	0.660	0.709

TABLE III

EFFECT OF GEL FORM ON RETENTION VOLUME OF NON-POLAR LIPIDS AT VARYING MOBILE PHASE COMPOSITION

Column, 1 m × 4 mm I.D.; temperature 47°. 60 = Poragel 60A; 200 = Poragel 200A.

Mobile phase (%)	*Retention volume (ml)*															
	Monoglyceride		*Diglyceride*		*Triglyceride*		*Monopalmitin*		*1,3-Dipalmitin*		*Tripalmitin*		*Palmitic acid*		*Cholesterol*	
	60	*200*	*60*	*200*	*60*	*200*	*60*	*200*	*60*	*200*	*60*	*200*	*60*	*200*	*60*	*200*
7.5	7.29	8.32	14.97	17.08	35.91	42.34	7.03	8.02	12.03	14.24	30.65	37.00	7.77	9.50	14.97	17.21
5	7.25	7.58	10.32	11.98	17.86	22.07	7.12	7.59	9.30	11.22	16.30	21.60	7.31	8.73	13.10	14.34
2.5	6.24	7.08	7.52	8.88	9.94	12.10	6.08	6.95	7.28	8.35	9.57	11.63	6.78	8.03	10.74	12.16

responses are essentially constant as would be anticipated from the operating principle of the detection system.

The accuracy and precision of quantitation of various lipid mixtures was assessed. In each case, cholesterol was used as an internal standard. Examples illustrating the accuracy and precision of the method are given in Table V.

DISCUSSION

The tables and figures illustrate the effectiveness of this method of lipid class separation and confirm that the mechanism is principally reversed-phase partition and not gel permeation. It is apparent from Table I and Fig. 2 that increasing water content in the mobile phase leads to increasing retention volumes, most notably in the case of the triglycerides. The increasing retention volumes are caused by in-

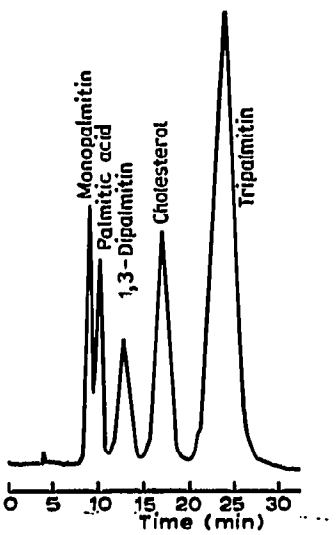

Fig. 1. Separation of non-polar lipids. Column, 1 m × 4 mm I.D.; packing, Poragel 200A; flow-rate, 0.66 ml min^{-1} at 14 atm; mobile phase, 5% aq. acetone; column temperature, 45°.

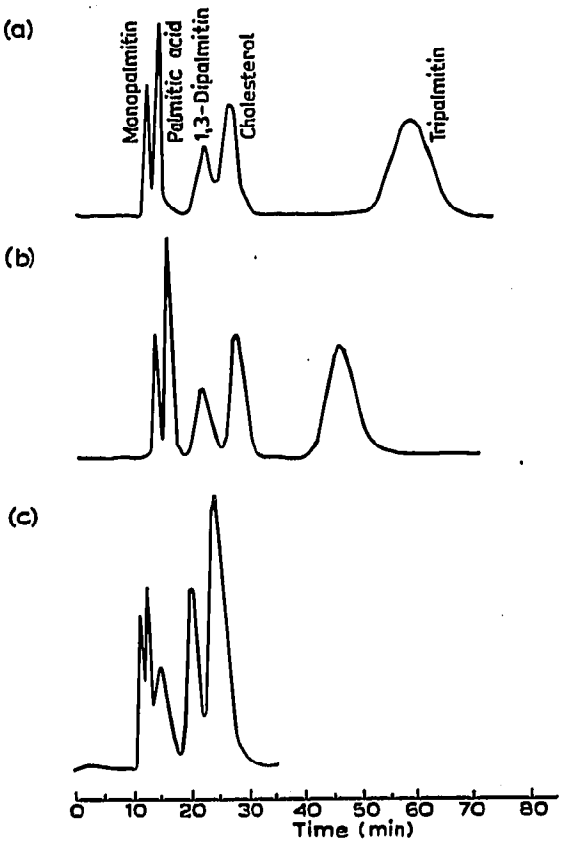

Fig. 2. Comparison of the separation of non-polar lipids at different mobile phase compositions. Column, 1 m × 4 mm I.D.; packing, Poragel 200A; flow-rate, 0.60 ml min^{-1} at 7 atm; column temperature, 45°. Mobile phase: (a) 7.5% aq. acetone; (b) 6% aq. acetone; (c) 4% aq. acetone.

TABLE IV

RESPONSE OF THE MODIFIED MOVING WIRE DETECTOR TO VARIOUS LIPIDS

Lipid	*Gradient (area/unit mass)*	*Response (area/unit mass C)*
Palmitic acid	24.6	32.8
Cholesterol	25.6	30.5
Monopalmitin	22.7	32.9
Dipalmitin	23.9	32.3
Tripalmitin	23.8	31.4

TABLE V

ACCURACY AND PRECISION OF QUANTITATION OF STANDARD MIXTURES

Compound	*Mixture 1*				*Mixture 2*				*Mixture 3*				*Mixture 4*			
	Actual wt. (mg)	*Wt. found (mg)*	*Error (%)*	*S.D. (%)*	*Actual wt. (mg)*	*Wt. found (mg)*	*Error (%)*	*S.D. (%)*	*Actual wt. (mg)*	*Wt. found (mg)*	*Error (%)*	*S.D. (%)*	*Actual wt. (mg)*	*Wt. found (mg)*	*Error (%)*	*S.D. (%)*
Glycerol Monopalmitin	10.5	10.4	1.0	1.4	12.9	12.3	4.7	4.6	5.7	5.8	1.7	3.6	5.4	5.6	3.7	3.7
Monostearin									9.8	9.8	0	2.0	7.3	7.1	2.7	2.2
1,3-Di-palmitin	15.1	15.8	4.6	1.8	9.7	10.1	4.1	3.0								
Distearin									15.0	15.3	2.0	2.4	11.9	12.5	5.0	3.6
Tripalmitin	15.0	15.4	2.7	3.0	67.0	65.1	2.8	2.6					8.5	8.5	0	1.0
Tristearin									15.0	15.3	2.0	3.1				

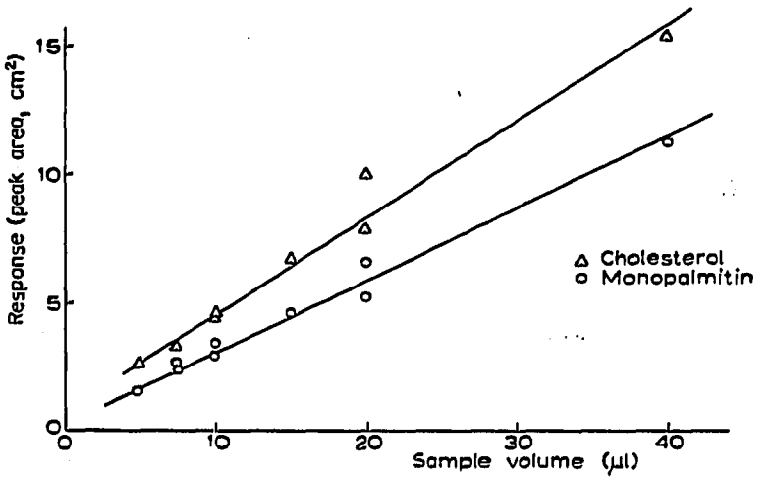

Fig. 3. Response of moving wire detector to cholesterol and monopalmitin. Operating conditions as for Fig. 1. Concentration of components ~ 10 mg ml^{-1}.

creasing distribution coefficients between the stationary and mobile phases. At any mobile phase composition, this effect may be reduced by increasing the column temperature which correspondingly reduces the distribution coefficient.

Elevated temperatures play an important part in the separation system described. Their importance lies in increased solubility of all lipid classes in the mobile phase. For this reason, the column temperature was routinely maintained at 45°, no breakdown of individual lipids being observed. A further aspect of the problem of solubility is that of application of sample to the column. The low solubilities of glycerides and fatty acids in aq. acetone make it difficult to apply the small volume of concentrated sample to the column necessary to reduce band broadening due to injection effects. However, the column and detection system used are almost unaffected by other solvents so that it is possible to apply concentrated samples in small volumes of a different solvent. For this purpose, chloroform–methanol (2:1) was used in all the experiments described. The chloroform and methanol do not noticeably affect the column performance and are lost in the evaporation stage of the modified moving wire detector. This illustrates one advantage of the moving wire detector over differential refractive index detectors for this separation system. The solvent peaks would be detected by the differential refractometer and cause considerable problems in identification and quantitation of the early eluted peaks.

Table II illustrates the retention of the different lipids relative to cholesterol. Whereas the lipids are, in general, affected to similar degrees by both mobile phase composition and temperature changes, cholesterol is not affected to the same extent. The overall result is that the retention volumes relative to cholesterol are markedly influenced by such changes. This is particularly observable for the di- and triglycerides. Triglycerides are totally resolved from cholesterol at 7.5% aq. acetone whereas they begin to overlap at 2.5%. The changes for diglycerides relative to cholesterol are even more marked. At 2.5% aq. acetone there is complete resolution, with the diglyceride being eluted before the cholesterol. However, at 7.5% there is considerable

overlap and by 10% cholesterol is eluted before the diglyceride (distearin). The reason for this almost certainly lies in the different general molecular structures of glycerides and steroids resulting in the distribution coefficients of the steroids changing more slowly with mobile phase composition than those of the glycerides. The importance of these variations lies in the possibility of choosing experimental conditions for cholesterol or a cholesterol ester as internal standard and the knowledge that other sterols and vitamins D and E are eluted at similar retention volumes to cholesterol.

The effect of gel pore size on separation of the lipid mixture is indicated in Table III. Both gels are nominally $<37\ \mu m$ particle diameter, the differences between them lying in their exclusion limit which is somewhat lower for the Poragel 60A. This is almost certainly controlled by the pore dimensions, the wider the pore, the greater the exclusion limit. In this case, none of the lipid molecules have dimensions approaching those of the pores. Poragel 200A has a marginally larger pore volume than Poragel 60A, thus taking up a larger volume of acetone and influencing retention by virtue of the partition term, KV_s/V_m (where K is the partition coefficient and V_s and V_m are the volumes of stationary and mobile phases, respectively). An optimum in gel pore size may exist for this separation but no detailed investigation has been carried out to determine this.

The speed and efficiency of this separation technique are demonstrated in Fig. 1. Monopalmitin, palmitic acid, 1,3-dipalmitin, cholesterol and tripalmitin are adequately resolved for quantitative work in less than 30 min. The effect of increasing chain length of the substituent fatty acids is to increase retention volume so that in the case of a mixture of glycerides (*e.g.* dipalmitin and distearin), a single broadened elution peak of Gaussian shape is obtained with a retention volume intermediate vetween those of the component glycerides. It is in practice possible to separate indibidual glycerides at appropriate mobile phase compositions *e.g.* dipalmitin and distearin at 10% aq. acetone.

The quantitation of components separated from a mixture, whether these components are single compounds or mixtures of similar compounds, is simplified with the modified moving wire detector. Previous work using the wire detector to monitor carbohydrates has been described[7,8]. In this work and subsequent routine use it has been possible to achieve precisions of 2.5% relative standard deviation despite wide variations in the proportions of individual components present (<1%–>80%). Similar precisions should be achievable from lipid class analysis. Table IV illustrates that the response per unit mass of carbon of different lipids is identical

TABLE VI

TRIGLYCERIDE MIXTURES—MEAN CARBON FRACTION

Sample	*Acid (%)*				*Carbon content*	*Error (%) from* C_{18}
	C_{14}	C_{16}	C_{18}	C_{20}		
1			100		0.769	—
2		100			0.759	1.30
3		30	70		0.766	0.39
4	5	45	45	5	0.764	0.65
5	5	65	25	5	0.762	0.91
6	5	30	30	5	0.765	0.52

and so the problem of mixtures of glycerides of the same class but different carbon contents does not lead to significant error in quantitation. Table VI shows the mean carbon fraction of a number of different triglyceride types having C_{14} to C_{20} fatty acid substituents. The assumption that a mixture contains only C_{18} saturated fatty acids when it contains only C_{16} acids would lead to errors of only 1.3% in the analysis. However, such a case is unlikely to occur without the differences in retention volumes for each species being observed and therefore allowance being made. What is probable in practical analysis of oils and fats is that a range of lipids in each lipid class will be present, giving broader peaks. The samples which have been examined by this technique have had fatty acid ranges from traces of C_{14} and C_{20} to major amounts of C_{16} and C_{18}. In such a mixture the error in assuming a triglyceride peak to be only C_{18} rather than mainly C_{18} with C_{16}, C_{20} and mixed glycerides will normally be less than 1% (see Table VI). In practice the worst case in Table VI would probably be sample 4 (0.65% error), those cases in which there is a preponderance of C_{16} being noticeable from the chromatograms and retention volumes.

For quantitative analysis of lipid classes, an internal standard (normally cholesterol) has been used in order that both relative and absolute amounts of material may be assessed. It is shown in Table IV that the responses of cholesterol and the glycerides are equivalent. If circumstances occurred in which it was inconvenient to use cholesterol, either because it was incompletely resolved from the di- or triglyceride peak or because it, or a compound of similar retention volume, was present in the mixture, the cholesteryl esters would provide a series of useful alternative standards. The accuracy and precision of the quantitation of standard mixtures are given in Table V. It is seen that the precisions are better than 4% relative independent of the amount of material present and the accuracies of the determination are better than 5%. These techniques have been applied to the quantitation of commercial samples with results in good agreement with independent methods of analysis.

In addition to the separation of the lipid classes listed, it is possible to carry out other useful separations with this system. Glycerol is eluted before the monoglyceride. Calciferols and tocopherols are eluted at a similar volume to cholesterol. Cholesteryl esters are eluted over a wide range of retention volumes, the acetate being eluted at a similar volume to tripalmitin (with 7.5% aq. acetone) whereas the palmitate is eluted much later.

One disadvantage of Hirsch's Factice was that it was dimensionally unstable to changes in solvent composition. This resulted in the need to prepare and pack a fresh column at each change in solvent composition. Poragel is extremely stable with only minimal changes in column packing volume between 2.5 and 10% aq. acetone mobile phase composition. A column has been in use in this laboratory with numerous mobile phase composition changes, temperatures varying from ambient to 65°, and at inlet pressures up to 34 atm with no significant decrease in column volume or performance.

This separation technique for non-polar lipid classes has several advantages over liquid–solid chromatography on silica gel. These are the simplicity of the column system, the ease of packing the column, the speed in preparing column packing material when compared with the sieving, clean-up and activation procedures necessary for silica gel, and the speed of the separation. In addition there is no necessity for gradient elution with subsequent column regeneration. There are also certain general

benefits of the technique. The detection system used simplifies both the application of small, concentrated volumes of sample directly to the column and the quantitation of the amounts of each lipid class present. The method also provides a rapid, simple means of obtaining known amounts of lipid classes for subsequent analysis, *e.g.* by thin-layer chromatography, gas–liquid chromatography and mass spectrometry. The main disadvantage lies, as with most liquid chromatographic separation methods, in the lack of separating power for individual glycerides, though it is thought that higher water contents in the mobile phase might enable such separations to be carried out.

CONCLUSIONS

Neutral lipid classes may be readily separated on columns of polystyrene gel with aqueous acetone as mobile phase. For solubility reasons, it is an advantage to operate at temperatures greater than ambient. The separated lipids may be collected for subsequent analysis. In addition, the lipid classes may be readily quantitated with good accuracy and a precision of better than 5 % relative without refined experimental or data handling techniques.

REFERENCES

1 J. J. Wren, *J. Chromatogr.*, 4 (1960) 173.
2 M. Lederer and E. Lederer (Editors), *Chromatography*, Elsevier, Amsterdam, p. 269.
3 L. J. Morris and B. W. Nichols, in E. Heftmann (Editor), *Chromatography*, Reinhold, New York, p. 446.
4 J. Hirsch, *J. Lipid Res.*, 4 (1963) 1.
5 J. Ellingboe, E. Nystrom and J. Sjovall, *J. Lipid Res.*, 11 (1970) 266.
6 R. P. W. Scott and J. G. Lawrence, *J. Chromatogr. Sci.*, 8 (1970) 65.
7 J. S. Hobbs and J. G. Lawrence, *J. Sci. Food Agr.*, 23 (1972) 45.
8 J. S. Hobbs and J. G. Lawrence, *J. Chromatogr.*, 72 (1972) 311.