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THE RESOLUTION OF A MIXED ALKYL ETHER AND MONOGLYCERIDE SYSTEM BY GAS–LIQUID CHROMATOGRAPHY

THE IDENTIFICATION OF MONOGLYCERIDE CONTAMINATION IN ALKYL ETHER PREPARATIONS

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SUMMARY

The resolution of a mixed system of alkyl ethers and monoglycerides, as their trimethylsilyl ether derivatives, has been studied by gas–liquid chromatography on several different types of liquid phase.

Individual monoglyceride and alkyl ether derivatives up to C18:0 were resolved on non-polar columns in under 25 minutes and best separations were achieved on JXR methyl silicone liquid phase. Semi-polar and polar columns did not separate saturated and mono-unsaturated TMS derivatives of either alkyl ethers or monoglycerides.

Contamination detected in alkyl ether samples prepared by acid hydrolysis of the glycerogalactolipid fraction of sheep brain has been identified as monoglyceride.

Recently, a detailed study on the gas–liquid chromatography (GLC) of the trimethylsilyl (TMS) and trifluoroacetate (TFA) derivatives of isomeric alkyl ethers has been reported[1] and limitations involved in the preparation and analysis of the isopropylidine, dimethoxy and acetylated forms described in earlier reports on the gas chromatography of lipids of this class[2–4] have been overcome. The TMS and TFA derivatives are formed in reactions which go to completion rapidly at room temperature and require no further purification steps prior to analysis. Also the 1- and 2-isomers of alkyl ethers can be successfully resolved as their TFA derivatives on semi-polar liquid phases[1].

Monoglycerides have also been analysed by GLC as their TMS ethers[5,6] but little work has been reported on the resolution of a mixture of these two closely related groups of lipids by gas chromatography. In connection with a problem of contamination encountered in alkyl ether preparations isolated from the glycerogalactolipid fraction of brain tissue[7], the behaviour of a mixed system of alkyl ethers, characteristic of nerve tissue, and some common monoglycerides has been studied by gas chromatography on several types of liquid phase. The results of this study and also the identification of the contamination observed in alkyl ether preparations as monoglyceride are discussed in this report.

EXPERIMENTAL

*Standards**

Chimyl alcohol (C16:0), batyl alcohol (C18:0) and selachyl alcohol (C18:1) have been identified as the main alkyl ether components of nerve (personal observations) and standards of these lipids were obtained in about 75 % purity (Calbiochem), the impurities in each case being alkyl ethers of longer and/or shorter chain lengths. In some experiments the retention times and values of the C14:0 and C15:0 alkyl ether components of the chimyl alcohol standard have been recorded. Monomyristin (Applied Science Labs.), monopalmitin, monostearin and monoolein (Hormel Institute) were all about 99 % pure. Standards were predominately in the 1-isomer form and resolution of the 1- and 2-isomers of monoglycerides and alkyl ethers has not been studied, having been described by other workers[1,5].

Silylation

Lipids were chromatographed as their trimethylsilyl ether derivatives and the silylation reagent was prepared freshly when required by mixing anhydrous pyridine (distilled over BaO and stored over KOH), hexamethyldisilazane and trimethylchlorosilane in a ratio of 10:2:1, v/v/v[8]. This mixture was well shaken and allowed to stand for 20 min when the precipitate which formed on mixing was removed by centrifugation. The clear supernatant was then used to prepare TMS ethers by the addition of 5 ml of the silylating reagent to 5 mg of each individual lipid standard weighed out in 15 ml centrifuge tubes. Tubes were stoppered, shaken well and allowed to stand at room temperature for 15 min.

TMS ethers were then extracted into hexane as described by WOOD *et al.*[5] and hexane solutions were made up to a final concentration of 2 μg lipid/μl. An aliquot of each standard was then pooled to produce a mixed system which contained all the alkyl ethers and monoglycerides studied as their TMS derivatives.

Gas chromatography

An F and M Model 402 gas chromatograph fitted with a hydrogen flame ionisation detector was used and TMS derivatives of the standards described above were resolved on the four types of liquid phase indicated in Table I. Liquid phases were prepared by the flash evaporation technique and were packed in glass columns, 6 ft. $\times$ 1/4 in. O.D. and conditioned prior to use by the usual methods. Carrier gas (helium) flow rate for all columns was 50 ml/min and the ratio of hydrogen: helium: air was 1:2:7. Attenuation and range settings were 2 and 10^2 respectively and the flash heater and detector were set about 30–40° above the operating temperature of the column in use.

In experiments designed to study the relationship between detector response and lipid concentration TMS ethers of chimyl alcohol and monopalmitin were chromatographed directly in pyridine and peaks were quantitated by the method of CARROLL[9]. Other results are the means of data collected over a period of several days.

Acid hydrolysis of diglycerides

To study the hydrolysis products formed from diglycerides treated with acid

* The shorthand nomenclature system of WOOD AND SNYDER[1] is used in this work.

TABLE I

LIQUID PHASES, SUPPORTS AND OPERATING TEMPERATURES OF COLUMNS USED TO STUDY THE RESOLUTION OF ALKYL ETHERS[a] AND MONOGLYCERIDES[a] BY GLC

Liquid phase	*Percentage*	*Support*	*Operating temperature (°C)*
Apiezon L	5	Gas-Chrom Q[b]	240
JXR[c]	5	Gas-Chrom Q[b]	225
XE-60	3	Gas-Chrom Q[b]	185
DEGS	20	Diatoport W[d]	200

[a] As their TMS ether derivatives.
[b] 100–120 mesh (Applied Science Labs.).
[c] Routinely used for alkyl ether analysis.
[d] 80–100 mesh, acid washed.

under conditions similar to those used for glycerogalactolipid degradation, 5 mg samples of synthetic diglycerides, including oleoyl-1-palmitoyl glycerol (about 90 % 1,2-isomer) and oleoyl-1-steroyl glycerol (about 60 % 1,3-isomer) were dissolved in 0.5 ml methanol and hydrolysed with 2 ml 3 *N* H_2SO_4 for 2 h at 100° in sealed tubes. Samples of monopalmitin were treated similarly.

Lipid-soluble hydrolysis products were extracted into diethyl ether which was washed and dried over anhydrous Na_2SO_4. The solvent was then removed in a stream of nitrogen and residual material, dissolved in a small volume of 5 % diethyl ether in hexane, was applied to a 5 g column of silicic acid (Bio-rad, 200–325 mesh) and free fatty acid, diglyceride and monoglyceride fractions eluted according to the method of BARRON AND HANAHAN[10]. Fatty acids were methylated with BF_3–MeOH reagent[11] and methyl esters were extracted into hexane and the extract washed and dried as above.

Fractions were analysed by thin-layer chromatography (TLC) on Silica Gel G in solvent systems of (1), chloroform–methanol (185 : 15, v/v) to resolve monoglycerides and (2) hexane–diethyl ether (6 : 4, v/v) to resolve monoglycerides, diglycerides and methyl esters. Standards of monopalmitin, diglyceride and methyl palmitate were chromatographed at the same time. The monoglyceride fraction was further analysed by TLC on plates of Silica Gel G impregnated with 5 % boric acid and developed in chloroform–methanol (98 : 2, v/v)[12]. Standards of 1- and 2-monopalmitin were chromatographed simultaneously. In all cases lipids were located by sulphuric acid charring.

RESULTS AND DISCUSSION

In studies on the glyceryl ether-containing lipids of nerve tissue gas chromatography has proved to be the most satisfactory technique for the characterisation and quantitation of alkyl ethers. During preliminary work on the glycerogalactolipid fraction of sheep brain[7], alkyl ether preparations isolated from this source by acid hydrolysis were found to contain a component which could not be identified as an alkyl ether. Non-polar columns of JXR methyl silicone are usually used for alkyl ether resolution (Table I). With this system the unidentified component had a retention value of 2.55 (relative to chimyl alcohol), close to but not identical with that

expected for a C19:0 alkyl ether and was also found to account for as much as 11% of the total sample. Recent work has revealed that alkyl ether preparations from the same source which have been treated with alkali prior to silylation and gas chromatography lack the contamination observed in unsaponified samples and that the C19:0 alkyl ether component occurs in very low concentrations only.

It is unlikely that fatty acid contamination was present since alkyl ether samples are purified prior to analysis by methylation and thin-layer chromatography. Furthermore, under the conditions of gas chromatography used, fatty acid methyl esters are eluted more rapidly than alkyl ethers. Earlier work[7,13] has also revealed that the glycerogalactolipid fraction does not contain long chain fatty acids such as lignoceric acid (C24:0) or hydroxy fatty acids.

It is more probable, however, that alkyl ether preparations were contaminated with monoglyceride, formed by the action of acid on the diacyl glyceryl galactoside component of the glycerogalactolipid fraction. The glycosidic bond in such glycolipids is readily labile under the acid conditions used (3 N H_2SO_4, 100°, 2 h) but diglycerides, remaining after release of galactose would be more resistant to acid especially in aqueous medium[14] and quantitative conversion to fatty acids and glycerol is unlikely to be achieved.

Indeed, hydrolysis of synthetic diglycerides with acid revealed that monoglyceride is formed under these acid conditions and can be recovered in the ether-soluble phase following hydrolysis (Fig. 1A). Also of interest was the finding that, following hydrolysis, about 50% of the original diglyceride could be recovered intact in the ether-soluble extract indicating that conversion of diglyceride to glycerol and fatty acids is only partially achieved under the acid conditions used. Diglyceride recovered following acid hydrolysis of 1,2-diglyceride was mainly the 1,3-isomer (Fig. 1A) indicating that acyl migration occurs readily under these hydrolysis conditions. TLC on borate-impregnated Silica Gel G plates (Fig. 1B) revealed that monoglyceride formed by the action of acid on diglyceride was exclusively in the 1-isomer form. Monoglyceride was also recovered in the ether-soluble phase following hydrolysis of monopalmitin.

The results indicate that monoglyceride is formed as a partial hydrolysis product by the action of acid on diglycerides under the conditions described above. It is likely, therefore, that monoglycerides are also produced during acid hydrolysis of the glycerogalactolipid fraction. Monoglyceride thus formed would be extracted into the ether-soluble extract following hydrolysis along with alkyl ethers and other lipid-soluble hydrolysis products and, having similar chromatographic properties, would be resolved with alkyl ethers during subsequent purification procedures. Alkyl ether samples would therefore contain monoglyceride contamination on silylation and gas chromatography. The presence of monoglyceride could account for the lability of the unidentified contaminant peak to alkali.

It was therefore necessary to study the chromatographic behaviour of monoglycerides under the conditions usually used for alkyl ether analysis. At the same time this work was extended to study the resolution of a mixed alkyl ether and monoglyceride system by GLC on several different types of liquid phase including Apiezon L and JXR (non-polar), XE-60 (semi-polar) and DEGS (polar) under the operating conditions shown in Table I. Retention values, relative to chimyl alcohol, of individual monoglyceride and alkyl ether TMS derivatives are reported in Table II and typical

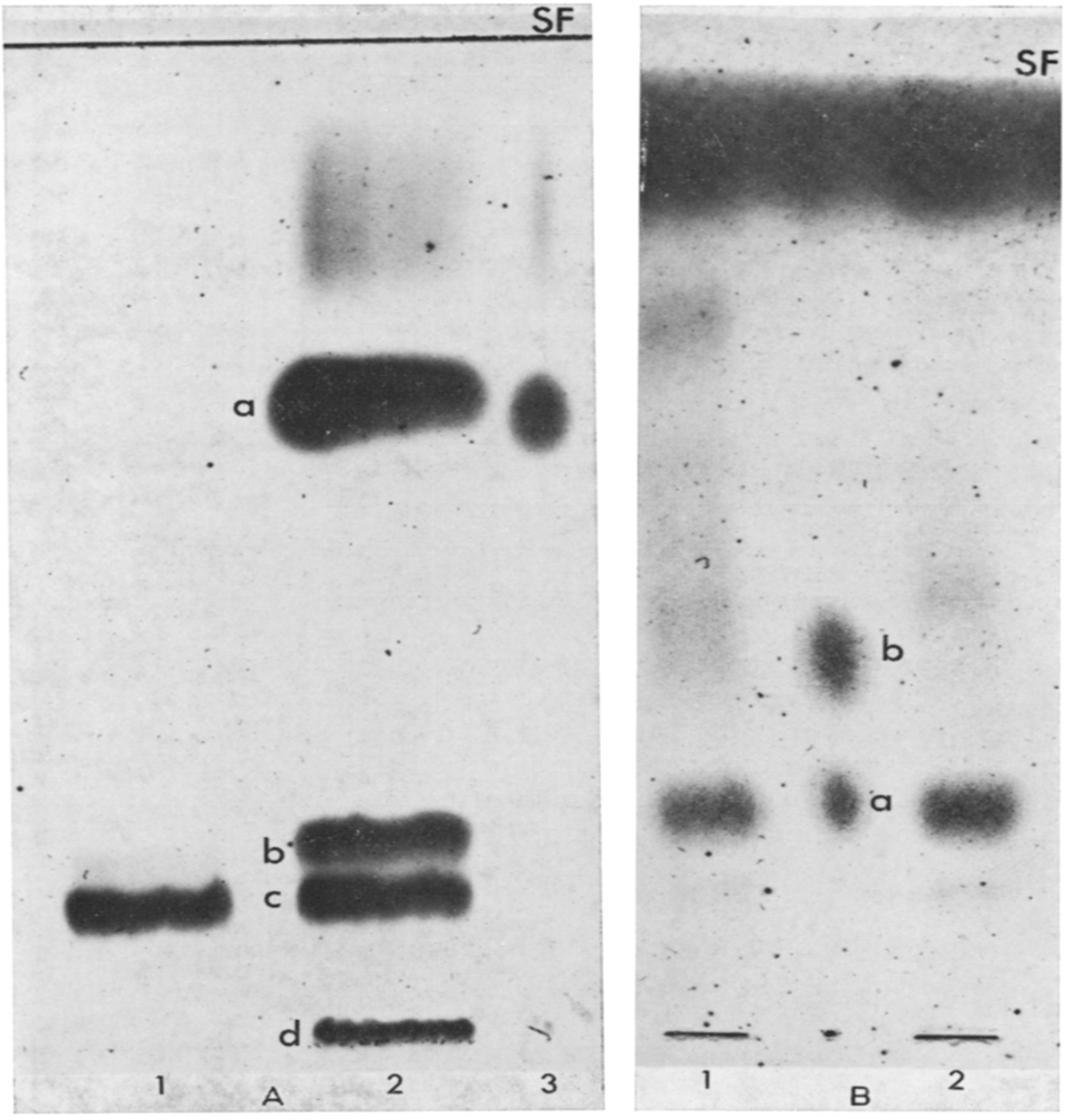

Fig. 1. (A) TLC of 1,2-diglyceride before hydrolysis (1), ether-soluble hydrolysis products (2) and methyl ester standard (3). Hydrolysis products identified as fatty acid methyl esters (a), 1,3-diglyceride (b), 1,2-diglyceride (c), and monoglyceride (d). Developing solvent: hexane–diethyl ether (6:4, v/v). SF = Solvent front. (B) TLC on borate-impregnated silicic acid of monoglyceride fractions (1 and 2) isolated following acid hydrolysis of 1,3- and 1,2-diglycerides respectively and standards of 1-monopalmitin (a) and 2-monopalmitin (b). Developing solvent: chloroform–methanol (98:2, v/v). SF = Solvent front.

separations of the mixed standard on the different liquid phases are shown in Figs. 2a, 2b, 3a and 3b.

In the present study TMS derivatives of alkyl ethers and monoglycerides were prepared with a clear silylation reagent with the precipitate of ammonium chloride, which forms on addition of trimethylchlorosilane to pyridine and hexamethyldisilazane, removed by centrifugation. CARTER AND GAVER[15] have reported that peak areas of TMS ethers of sphingolipid bases formed with the uncentrifuged silylation reagent decreased as time after addition increased while, with the clear centrifuged

TABLE II

RETENTION VALUES OF ALKYL ETHERS[a] AND MONOGLYCERIDES[a] RELATIVE TO C16:0 ALKYL ETHER[b]

Standard	*Apiezon L*	*JXR*	*XE-60*	*DEGS*
C14:0 monoglyceride	0.65	0.73	1.23	1.00
C14:0 alkyl ether	0.50	0.53	0.50	0.50
C16:0 monoglyceride	1.36	1.35	2.43	2.07
C16:0 alkyl ether	1.00	1.00	1.00	1.00
C16:1 alkyl ether	0.92	0.94	[c]	[c]
C18:0 monoglyceride	2.64	2.57	4.70	4.13
C18:0 alkyl ether	2.02	1.88	1.95	2.05
C18:1 monoglyceride	2.31	2.34	4.70	4.38
C18:1 alkyl ether	1.80	1.70	1.94	2.10

[a] As their TMS ether derivatives.
[b] C16:0 alkyl ether derivative resolved in the following average times: Apiezon L, 7.75 min; JXR, 9.5 min; XE-60, 4.25 min; DEGS, 6.5 min.
[c] C16:1 alkyl ether not resolved on XE-60 and DEGS liquid phases.

reagent, reproducible and constant height ratios were obtained up to 2.5 h after derivative formation. The clear silylation reagent was usable for about 6 days after preparation.

Although the silylation reaction for monoglycerides is very rapid[5] a 15 min reaction period was allowed in the present study, after thorough mixing, to ensure solubility of the lipids and complete formation of TMS derivatives. Direct chromato-

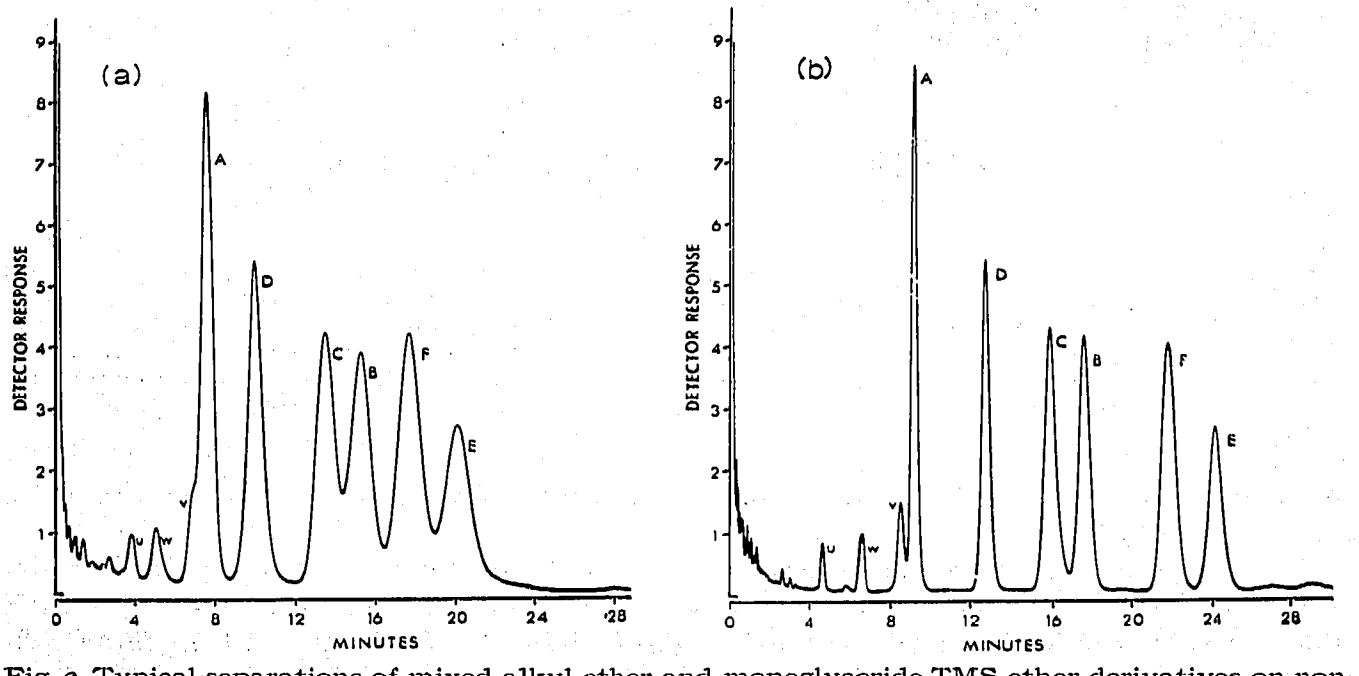

Fig. 2. Typical separations of mixed alkyl ether and monoglyceride TMS ether derivatives on non-polar liquid phases of (a) Apiezon L 240° and (b) JXR methyl silicone 225°. Peaks identified as alkyl ethers: u = C14:0; A = C16:0; v = C16:1; B = C18:0; C = C18:1 and monoglycerides: w = C14:0; D = C16:0; E = C18:0; F = C18:1.

graphy of TMS ethers in pyridine has been widely described but better results in terms of a smaller solvent peak, etc., are obtained from hexane. TMS ethers were therefore chromatographed in hexane and it has been reported[5] that such derivatives stored in hexane at low temperatures are stable for at least four months.

Two types of non-polar liquid phase were used and typical separations of the mixed standard on Apiezon L and JXR columns are shown in Figs. 2a and b. Retention values of the individual components are reported in Table II. The results indicate that non-polar columns are superior to semi-polar and polar liquid phases (Figs. 3a and b) for the separation of the mixed alkyl ethers and monoglycerides studied. Individual monoglyceride and alkyl ether derivatives up to C18:0 were completely resolved on either non-polar phase in about 25 min, peaks showing good symmetry especially on JXR columns. A better separation was achieved on the JXR methyl silicone liquid phase, routinely used for alkyl ether analysis, than on Apiezon L columns, TMS derivatives of monounsaturated alkyl ethers and monoglycerides being more completely resolved from their saturated analogues. As can be seen in Fig. 2b the detector response returned to zero during the separation of the monoolein (F) and monostearin (E) derivatives.

The complete resolution of 18:1 and 18:0 alkyl ethers achieved on JXR columns in this work is in contrast to the partial separation on SE-30 columns reported by WOOD AND SNYDER[1] and while the two types of liquid phase are little different, both being methyl silicone gums, the combination of a silanised support of finer particle size (100–120 mesh) and the slightly longer column may be responsible for the better separation. The same workers have also reported that TMS ethers of 18:2 and 18:1 alkyl ethers are not separated on SE-30 columns. WOOD *et al.*[5], have described the resolution of saturated monoglyceride TMS derivatives from C10:0 to C18:0 on a 2.6 % SE-30 column in under 30 min by temperature programming.

Peaks resolved on Apiezon columns (Fig. 2a) were broader and showed some

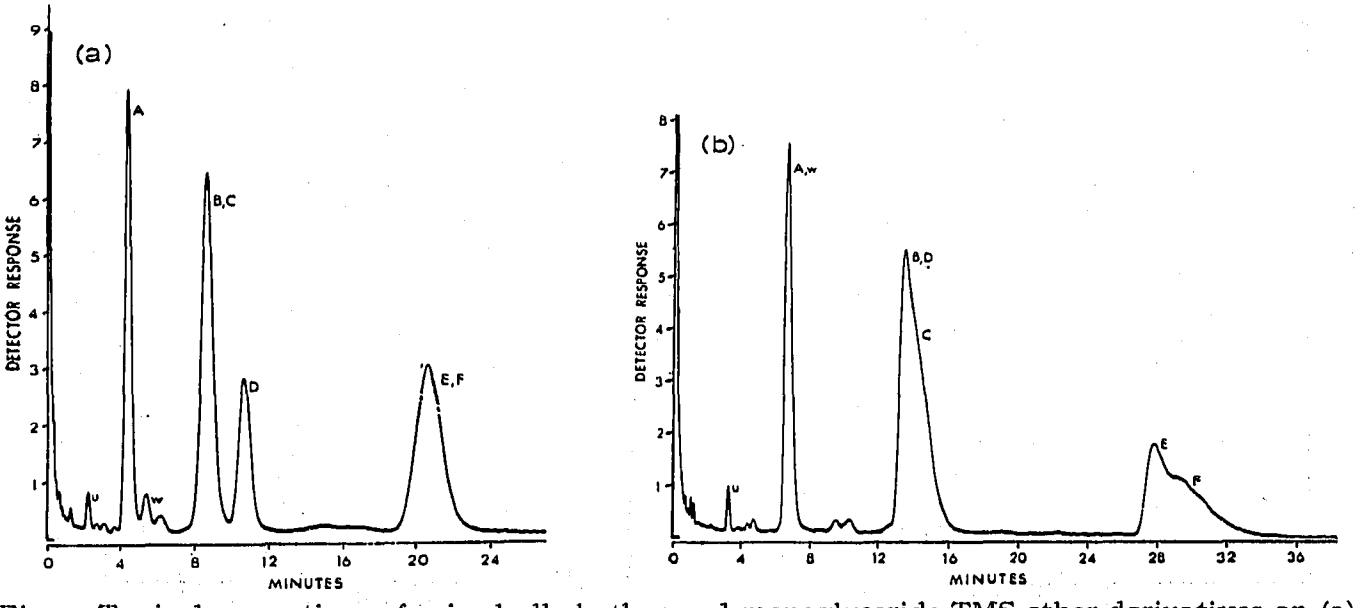

Fig. 3. Typical separations of mixed alkyl ether and monoglyceride TMS ether derivatives on (a) semi-polar XE-60 185° and (b) polar DEGS 200° liquid phases. Peaks identified as alkyl ethers: u = C14:0; A = C16:0; B = C18:0; C = C18:1 and monoglycerides: w = C14:0; D = C16:0; E = C18:0; F = C18:1.

slight tailing compared with the JXR separations (Fig. 2b). Operation of the Apiezon phase at higher temperatures only slightly sharpened the peaks and did not improve the resolution achieved between unsaturated and saturated monoglyceride and alkyl ether derivatives. In agreement with other workers[1] 18:0 and 18:1 alkyl ethers were not well resolved on Apiezon columns.

The results in Table II indicate that TMS derivatives of monoglycerides and alkyl ethers were less successfully resolved on semi-polar and polar liquid phases. The mixed standard was resolved on XE-60 columns (Fig. 3a) in about 22 min but, in agreement with other reports[1], the saturated and unsaturated derivatives of standards were not separated. Isomeric saturated alkyl ethers and monoglycerides were resolved however.

WOOD *et al.*[5], have described the resolution of the TMS ethers of C18:0, C18:1 and C18:2 monoglycerides (1-isomers) on a DEGS liquid phase at 215°. In closer agreement with earlier findings[1], however, DEGS columns used in this study generally failed to separate saturated and unsaturated derivatives of either alkyl ethers or monoglycerides (Fig. 3b) although slightly different retention values were observed for these standards when chromatographed individually (Table II). On DEGS columns the mixed standard was resolved in about 32 min but C16:0 monoglyceride and C18:0 and C18:1 alkyl ethers were eluted in one peak as were the C14:0 monoglyceride and C16:0 alkyl ether components (Fig. 3b).

A plot of $\log_{10}$ retention against the carbon number of the ether or ester linked hydrocarbon side chain for individual alkyl ether and monoglyceride derivatives (Fig. 4a, 4b) shows that for each liquid phase linear results are obtained over the molecular weight range studied. Monoglyceride and alkyl ether derivatives are widely differentiated on XE-60 and DEGS columns (Fig. 4b) while plots for the same standards on non-polar columns (Fig. 4a) lie closer together. With either Apiezon L or

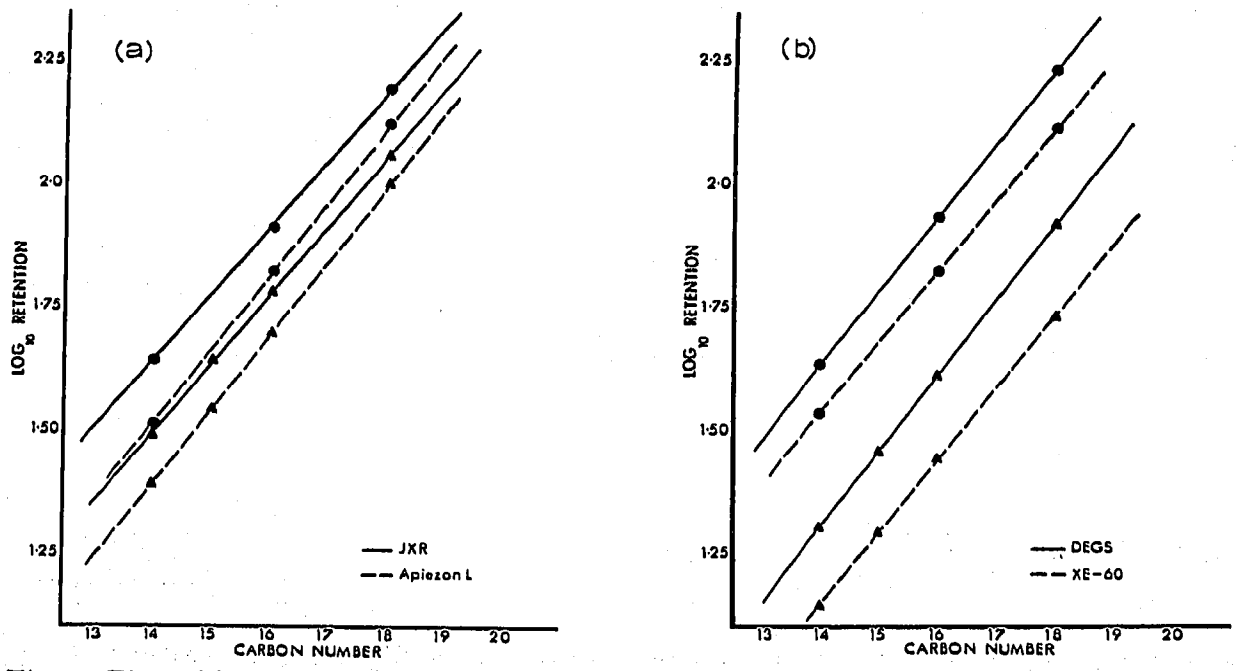

Fig. 4. Plot of $\log_{10}$ retention against carbon number for individual alkyl ethers (▲) and monoglyceride (●) TMS ether derivatives on (a) non-polar liquid phases and (b) semi-polar and polar liquid phases.

JXR liquid phases monoglycerides are resolved in approximately the same time as the respective alkyl ether containing an additional carbon unit in the side chain. Thus, on JXR columns (Figs. 2b, 4a), TMS derivatives of C16:0 monoglyceride and C17:0 alkyl ether, C18:0 monoglyceride and C19:0 alkyl ether, etc., have about the same retention.

These findings are of interest in relation to identification of the contamination detected in alkyl ether preparations described earlier. On JXR columns, the unidentified contaminant peak had a retention value of 2.55 relative to chimyl alcohol, a figure close to that expected for a C19:0 alkyl ether. However, it can be seen from Table II that this figure is in good agreement with that of 2.57 observed for C18:0 monoglyceride analysed under identical conditions while the retention value of a C19:0 alkyl ether resolved on the same system is 2.63 (Fig. 4a). These results indicate further that alkyl ether samples isolated from the glycerogalactolipid fraction of brain tissue contained monoglyceride contamination and, from the GLC studies above, the unidentified peak present in such samples has been resolved as monostearin.

Gas chromatography can be used for the quantitative assay of alkyl ether preparations and on JXR columns a linear response was obtained for the TMS ether derivatives of both monopalmitin and chimyl alcohol over a concentration range up to about 1 μg (Fig. 5). The plot tends to become non-linear at higher concentrations probably due to peak broadening. However, under the conditions of chromatography used injection of samples over 1 μg resulted in an off-scale detector response. In this experiment, as in routine alkyl ether analyses in which very low concentrations of sample are available, TMS ethers were injected in pyridine and the results achieved were similar to those described above, peak resolution and symmetry being as good as with hexane.

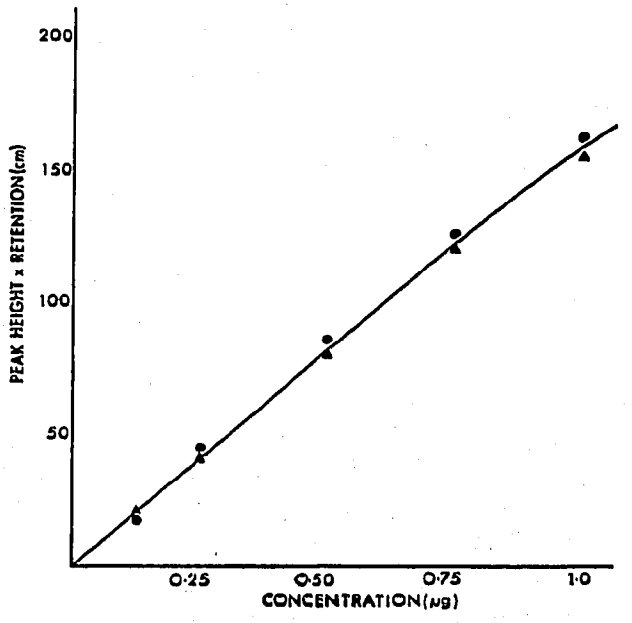

Fig. 5. Plot of concentration (μg) against peak height × retention (cm) for C16:0 alkyl ether (▲) and C16:0 monoglyceride (●) TMS ether derivatives. Lipids were chromatographed in pyridine.

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