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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF FATTY ACIDS AS PENTAFLUOROBENZYL ESTERS

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SUMMARY

Using ultraviolet detection (254 nm), pentafluorobenzyl esters have been shown to be suitable derivatives for the semi-preparative separation of fatty acids by number of double bonds on silica columns and by chain length on reversed-phase columns. The two chromatographic systems are entirely complementary in that a critical pair in one of the two systems can be completely separated in the other system, thus allowing the isolation of any given fatty acid from a complex mixture following two sequential injections. The complete separation of pentafluorobenzyl *cis*-9,10-methylene-hexadecanoate and pentafluorobenzyl heptadec-10-enoate in both systems has also been achieved.

INTRODUCTION

Methods for the analytical separation of fatty acids as ultraviolet (UV) absorbing acyl esters in reversed-phase systems have been given in the literature [1, 2] as have methods for the preparative separation of various unsaturated methyl esters on silver-saturated ion-exchange resins [3]. However, there seems to be a dearth of methods for the convenient isolation of fatty acids from complex mixtures for, for example, liquid scintillation counting or for further identification. The analytical methods cannot be conveniently scaled up since critical pairs such as arachidic and erucic [1] or palmitoleic and arachidonic [2], or even palmitic and oleic acids [2] on the preparative scale would not be sufficiently separated to give clean isolations. On the other hand, the preparative method cannot be readily scaled down since the resolution and sensitivity is relatively low. In attempting to find a suitable semi-preparative method we initially turned our attention to normal-phase separations on two types of columns containing Ag^+. The results, particularly for polyunsaturated fatty acids, were not encouraging so we decided to investigate normal-phase separa-

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tions on silica. According to Schwarzenbach [4] separation, by number of double bonds, of underivatised fatty acids can be made on silica provided the ionisation of the carboxyl group is suppressed. We surmised that a similar separation could be made if the fatty acids were to be esterified such that the ester had no additional functional groups to interact with the silica. Since we wished to use a fixed-wavelength detector set at 254 nm, if possible, and since we were also aware of the hydrophobic properties of polyfluorocarbon compounds we decided to investigate pentafluorobenzyl (PFB) esters initially. These proved to be eminently suitable so this paper details the separations of the PFB esters of the various types of fatty acids in both normal- and reversed-phase systems.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment was from Waters Assoc. (Milford, MA. U.S.A.): M45 or M6000A pump, U6K injector and M440 detector with a filter for 254 nm detection. Samples for normal-phase separations were dissolved in benzene and for reversed-phase separations were dissolved in isopropanol. Two HPLC columns containing silver were investigated for their ability to separate fatty acid benzyl esters on the basis of the number of double bonds that they contained. First, silver nitrate coated silica (10 g per 100 g) was prepared from LC Porasil A (Waters Assoc.) essentially as described by Battaglia and Fröhlich [5]. This was packed into a 600 mm × 9 mm column (particle size 37—75 μm) using a column packer of the Micromeritics type and the Waters M6000A pump. Secondly, silver aluminosilicate was prepared according to the procedure of Lam and Grushka [6] and packed into a 300 mm × 3.9 mm column as above.

Benzyl esters of stearic (18:0), oleic (18:1), linoleic (18:2) and α-linolenic (18:3) acids were prepared from O-benzyl-N,N′-diisopropyl isourea as described by Knapp and Krueger [7]. This reagent was prepared from diisopropylcarbodiimide [8].

PFB esters were synthesised from 2,3,4,5,6-pentafluorobenzyl bromide (Fluka, Buchs, Switzerland) by a procedure based on the method of Ehrsson [9]. Up to 1 mg of fatty acid sample was dissolved in 1 ml of dichloromethane and 1 ml of a solution containing 0.1 mmol tetrabutylammonium hydrogen sulphate (Fluka) and 0.2 mmol sodium hydroxide was added. Pentafluorobenzyl bromide (20 μl) was then added and the mixture shaken vigorously at room temperature for 30 min. The dichloromethane phase was then evaporated. Ehrsson [9] has shown that the esterification is quantitative for octanoic and longer acids under similar conditions. The residue was taken up in hexane and loaded onto a silica Sep-Pak (20 × 10 mm plastic columns, Waters Assoc.) and the PFB esters were eluted with dichloromethane—hexane (3:17).

Four sets of standard PFB esters were prepared: (a) PFB 18:0; (b) an approximately equimolar mixture of PFB 18:0, PFB 18:1, PFB 18:2, PFB 18:3; (c) an approximately equimolar mixture of the straight-chain fatty acid PFB esters: C_2, C_3, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16} and C_{18}; (d) a mixture containing two parts PFB *trans*-18:1 and one part PFB *cis*-18:1. Mixture d was prepared as described above while the other three standards required a scaling up of the quantities. The first three standards were therefore purified by

application to a Merck (Darmstadt, F.R.G.) Lobar Lichroprep® silica gel 60 (particle size 40—63 μm) column (size A, 240 mm × 10 mm) followed by elution with dichloromethane—hexane mixtures and detection at 254 nm with a Pye Unicam SP-6 spectrophotometer (Cambridge, U.K.), rather than on Sep-Pak as described above.

Separations of PFB esters were carried out by normal phase on a μPorasil semi-preparative (300 mm × 7.8 mm; particle size 10 μm) column (Waters Assoc.) using dry dichloromethane—hexane half saturated with water (3:17 or 1:9) and by reversed phase on a C_{18} μBondapak semi-preparative (300 mm × 7.8 mm; particle size 10 μm) column (Waters Assoc.) with methanol—water (19:1).

PFB esters were collected from HPLC separations and identified by comparison with standards by gas chromatography (GC) (EGSS-X on Chromosorb W-HP, 100—120 mesh columns: 1.5 m × 4 mm at 180°C and a flow-rate of 20 ml/min; 5.5 m × 4 mm at 200°C and a flow-rate of 20 ml/min. Effluent gas was divided: 24 parts to a flame-ionization detector and 1 part to an electron-capture detector, Pye GCV). In some cases these identifications were confirmed by gas chromatography—mass spectrometry (GC—MS). A Model 3200 chemical-ionization (CI) system was interfaced to an Incos 2300 data system both from Finnigan-MAT (San Jose, CA, U.S.A.). GC carrier gas (flow-rate 20 ml/min) and CI reagent gas (source pressure 0.8 Torr) was methane. The ion source temperature was 130°C, the column was glass, 1.8 m × 2 mm, 3% OV-17, Gas Chrom Q, 100—120 mesh, programmed from 200°C at 10°C/min to 300°C.

Methyl *cis*-9,10-methylenehexadecanoate (Me 16:CH_2) and methyl *cis*-9,10-methyleneoctadecanoate (Me 18:CH_2) were purchased from Applied Science Labs. (State College, PA, U.S.A.). Methyl heptadec-10-enoate (Me 17:1) and methylnonadec-10-enoate (Me 19:1) were purchased from Nu Check Prep (Elysian, MN, U.S.A.). Each methyl ester was converted into the free acid by saponification with potassium hydroxide in methanol (200 g/l) for 40 min at 80°C and PFB esters synthesized as above.

RESULTS AND DISCUSSION

Initially the silver nitrate column separated benzyl (Φ) esters by number of double bonds, but this selectivity was lost, presumably by the leaching of silver nitrate from the column. Although this problem can be overcome [10] we felt we would be better served to take a new approach since silver nitrate columns are not particularly suitable for use with gradient elution [10] and the large proportion of saturates in our samples was likely to swamp the relatively small proportion of monounsaturates. The separation of the type of samples we are dealing with has been achieved on the preparative scale on silver-saturated ion-exchange resins [3] but this took much longer, some 26 h, and was on a larger scale then we required. Although separations of Φ 18:0, Φ 18:1, Φ 18:2 and Φ 18:3 were obtained on the silver aluminosilicate column, the peak shapes were poor and double peaks were sometimes seen. Presumably this behaviour was related, at least in part, to the destruction of the silica in the preparation of the silver aluminosilicate.

Using the reversed-phase column with methanol—water (93:7), on a preparative scale Φ 18:1 elutes just after Φ 16:0. The resolution (where resolution is

defined as the difference in elution volumes of the two peaks divided by the average peak width measured at their bases) is 0.37. With 10^{-2} *M* Ag^+ (as silver nitrate) [11] added to the mobile phase, Φ 18:1 elutes just before Φ 16:0 with a resolution of 0.38. Thus, in both systems Φ 16:0 and Φ 18:1 are too close to give a satisfactory isolation of either compound.

The PFB 18:0 standard was purified by HPLC on the μPorasil column which afforded a sample for a UV spectrum; the wavelength of maximum absorption (hexane) being 263 nm and with log ϵ = 2.78. PFB esters do not have a high molar absorptivity at 254 nm (log ϵ in hexane is 2.67). Thus 1 μmol with a retention of 15 min gives 0.113 a.u.f.s. However, this is quite adequate for semi-preparative work particularly since the calibration graph is linear. These derivatives have the additional advantage that aliquots can be taken directly from fractions collected from the chromatograph for analysis or identification by GC or by GC—MS.

Fig. 1A shows the separation of the PFB esters of 18:0, 18:1, 18:2 and 18:3 on the μPorasil column. Baseline separation of these compounds is obtained in less than 20 min. The peaks that elute prior to PFB 18:0 (Fig. 1A) appear to be butylated hydroxytoluene, excess pentafluorobenzyl bromide and perhaps a

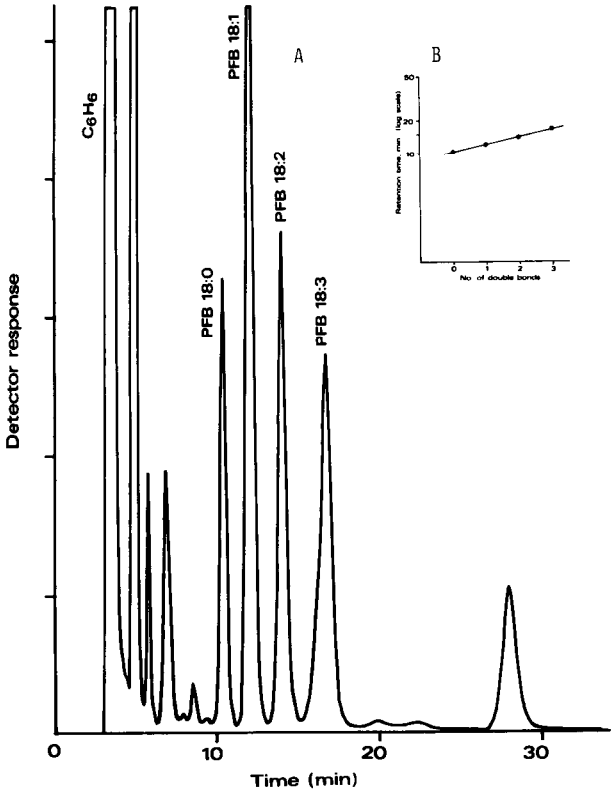


Fig. 1. (A) Normal-phase semi-preparative HPLC separation of PFB 18:0, PFB 18:1, PFB 18:2 and PFB 18:3, approx. 0.1 mg each. (B) Log plot of retention data. μPorasil semi-preparative column. Solvent: dry dichloromethane—hexane half saturated with water (3:17); flow-rate: 4 ml/min; UV detection at 254 nm; 0.1 a.u.f.s.

dimer formed from pentafluorobenzyl bromide during the reaction period. Since both unsaturated and shorter PFB esters elute after PFB 18:0, these impurities do not affect the desired separation. Also included is a plot of log retention time against number of double bonds (Fig. 1B). The resulting straight line suggests that retention due to a double bond is independent of a neighboring double bond, even though the two or three double bonds are only separated by single methylene groups. Further, the abscissa could perhaps be better labelled as number of pairs of electrons, the resulting data points therefore corresponding to 1, 2, 3 and 4 pairs. This would suggest that the π electrons in the carbonyl group of the ester linkage make a similar contribution to adsorption to the π electrons of the double bonds, but that the PFB group makes no contribution.

The separation of the straight-chain saturated PFB esters is shown in Fig. 2A. Although the longer-chain esters elute very close together there is some separation which increases as chain lengths shorten, suggesting that this method could be used for separating short-chain fatty acids (say $\leqslant C_6$). Fig. 2B shows a plot of log (retention time) against log (number of carbon atoms). Except for PFB acetate, the plot forms two straight lines with an abrupt change of slope at C_{10}.

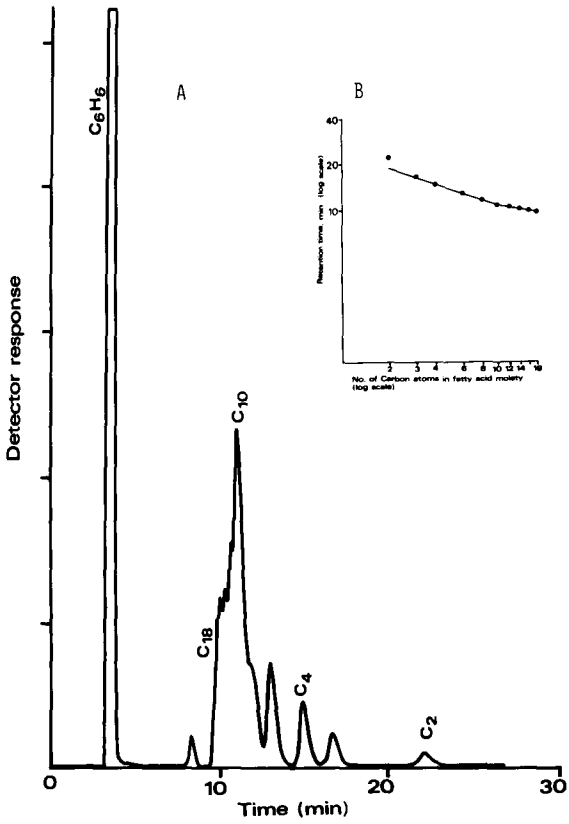


Fig. 2. (A) Normal-phase semi-preparative HPLC separation of the PFB esters of the straight-chain saturated fatty acids: C_2, C_3, C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, approx. 2.5 mg total. (B) Log/log plot of retention data. Conditions as for Fig. 1; 1.0 a.u.f.s.

Since the proportion of water or other hydrogen bonding compounds in the eluting solvent in these normal-phase systems is of over-riding importance in the determination of retention times, we feel that the interactions occurring at the silica gel surface are best described in terms of Scott's [12] model for that surface. This implies that the surface of the column packing is covered with a bilayer of water surmounted by a monolayer of dichloromethane. Displacement of the dichloromethane by the carbonyl of PFB esters or by double bonds would then lead to separation by double-bond number. Further, the relative displacement of dichloromethane by the carbonyl group in the homologous saturated PFB esters might well be reduced with increasing chain length due to the inductive effect. One would surmise that the increase in the inductive effect for each additional carbon atom would be logarithmic and Fig. 2B suggests that this is indeed so, up to C_{10}. The change in slope beyond C_{10} suggests that some additional interaction between the PFB esters and the silica gel surface comes into play. Does this imply that molecules longer than C_{10} can loop around so that the terminal portion of the alkyl chain can also independently displace a dichloromethane molecule? We feel that this system could provide some interesting data for those who are interested in the physical chemistry of interactions at the silica gel surface.

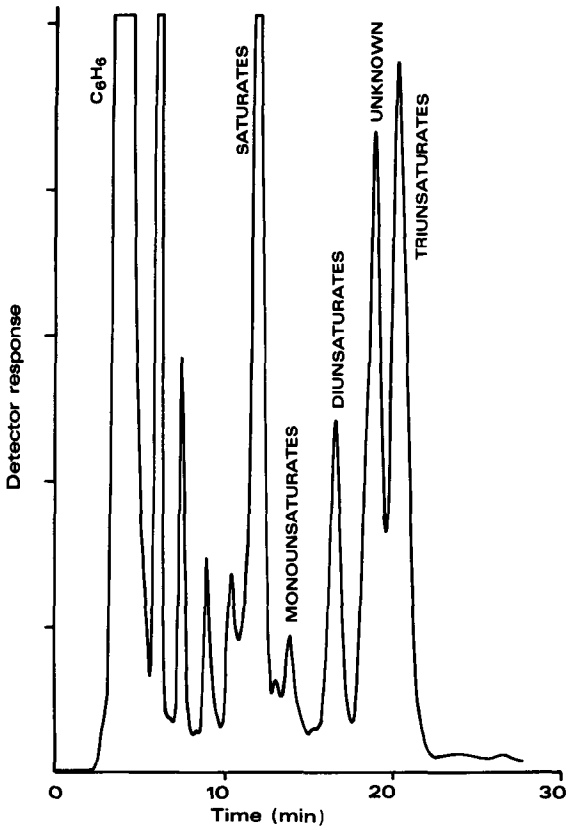


Fig. 3. Normal-phase HPLC separation of the PFB esters from a barley leaf extract. Total injected equivalent to approx. 1.8 g fresh weight leaves. PFB esters were synthesised from a saponified extract using the method given in the text. Conditions as for Fig. 1.

Fig. 3 shows the separation of the PFB esters obtained from a barley leaf extract. The four peaks containing saturates, monounsaturates, diunsaturates and triunsaturates are well separated from each other and can be isolated and separately applied to a reversed-phase column for separation on the basis of chain length. Such a separation, for the saturates isolated as in Fig. 3, is shown in Fig. 4. Taken together Figs. 3 and 4 give a typical pattern for fatty acids from barley leaves [13].

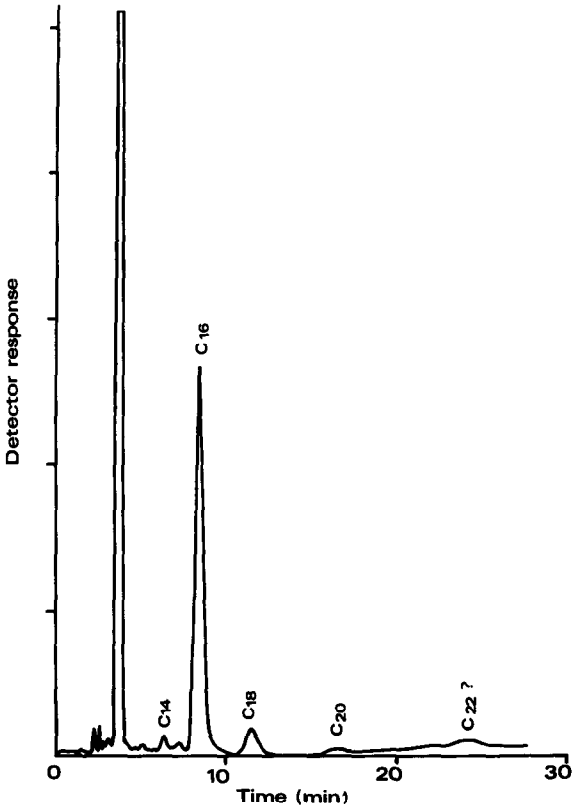
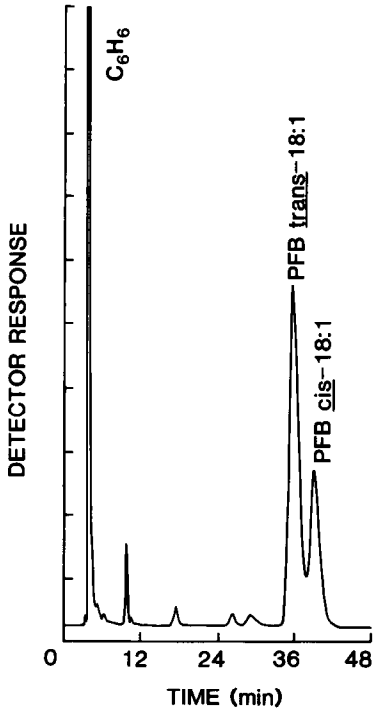


Fig. 4. Reversed-phase separation of the saturated fraction of the PFB esters from a barley leaf extract. C_{18} μBondapak semi-preparative column; solvent: methanol—water (19:1); total injected equivalent to approx. 0.2 g fresh weight leaves; other conditions as for Fig. 1.

We have also demonstrated (Fig. 5) that PFB *trans*-18:1 elutes prior to PFB *cis*-18:1. The resolution of 1.25, calculated from Fig. 5, is not sufficient to be of great practical value but it does emphasise the point that separations in the normal-phase system depend primarily on interactions of the π-electrons with the silica gel surface and are only slightly influenced by steric effects.

The chromatographic separation of cyclopropanoid fatty acids from their monounsaturated isomers in biological extracts is a difficult problem [14] and to our knowledge has not previously been achieved. Fig. 6 shows the separation of the PFB esters of *cis*-9,10-methylene hexadecanoic (16:CH_2) and heptadec-10-enoic (17:1) acids, using a normal-phase system. A similar resolution can be obtained using a reversed-phase column with methanol—water (19:1).



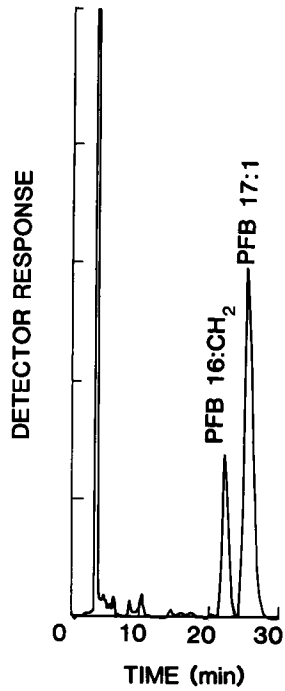


Fig. 5. Normal-phase HPLC separation of PFB *cis*-18:1 and PFB *trans*-18:1. Solvent: dry dichloromethane—hexane half saturated with water (7:93); approx. 0.38 mg PFB *trans*-18:CH_2, 0.22 mg PFB *cis*-18:1; conditions as for Fig. 1; 0.1 a.u.f.s.

Fig. 6. Normal-phase HPLC separation of PFB 16:CH_2 and PFB 17:1. Solvent: dry dichloromethane—hexane half saturated with water (1:9); approx. 0.17 mg PFB 16-CH_2, 0.65 mg PFB 17:1; conditions as for Fig. 1; 0.2 a.u.f.s.

This raises the proposition that it is not so much the chromatographic system that is responsible for the resolution in the separations described here, but the choice of derivative. That is if, for example, a separation on number of double bonds is required it is important that no other functional group in the esters to be used dominates either their adsorptive properties or their distribution coefficients. The result illustrated in Fig. 6 was duplicated with the PFB esters of 19:1 and 18:CH_2. In both cases each fraction was collected and subjected to methane CI GC—MS. The CI mass spectra of each isomeric pair were virtually identical with only minor variations in the intensity of the ions recorded. A similar result was found with the electron-impact (EI) and CI mass spectra of the isomeric methyl ester derivatives [14].

Overall we therefore feel that PFB esters are well suited for semi-preparative isolations of fatty acids. The resolution in the normal-phase separations reported here seems to be primarily due to a lack of any interaction between the PFB group and the stationary phase. Thus the strength of the interaction between the stationary phase and a PFB ester is determined by the structure of the acyl chain, other than a small contribution from the carbonyl group. It appears to us that this is not true for methyl esters and particularly for the highly UV absorbing esters used in the analytical separation [1, 2] of fatty acids. In these

reversed-phase systems, although several esters have been tried: phenacyl [1], *p*-bromophenacyl, *p*-nitrophenacyl, *p*-chlorophenacyl and 2-napthacyl [2], with some large columns: 900 mm × 6.4 mm C_{18} μBondapak [1] or two 300 mm × 3.9 mm C_{18} μBondapak in series or two 300 mm × 3.9 mm fatty acid analysis in series [2], the nett result is to only slightly change the relative retention times of the various fatty acids. Thus in these methods it has taken 3—4 h to resolve some critical pairs, and even these conditions would not have allowed clean isolation of some of the individual fatty acids. However, PFB esters used with a combination of a normal-phase followed by a reversed-phase column (only one of each) can afford the isolation of any desired fatty acid in 20 min on each column. Thus, in order to completely resolve all critical pairs, some sacrifice in sensitivity has to be made so that the complementary normal- and reversed-phase systems can both be utilised. Finally, since the esterifying methyl group of methyl esters appears to have some interaction with the silica in normal-phase separations, it may be of advantage to use PFB esters in the separation of geometrical and positional isomers of fatty acids on silver nitrate impregnated silica [5].

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