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QUANTITATIVE PREPARATION AND GAS CHROMATOGRAPHY OF SHORT AND MEDIUM CHAIN FATTY ACID BENZYL ESTERS (C_1–C_{12})

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SUMMARY

The benzyl esters of short and medium chain fatty acids (C_1–C_{12}) were prepared with N,N'-dicyclohexyl-O-benzylisourea or with phenyldiazomethane. The extent of esterification was checked by using ^{14}C-labelled fatty acids. The fatty acid benzyl esters were separated gas chromatographically on both SE-30 and EGSS-X columns. For quantification, the relative response for equal weights of fatty acids was determined. The mild esterification with phenyldiazomethane allows this method to be used for the assay of short and medium chain free fatty acids in biological materials.

INTRODUCTION

For long chain fatty acids, the gas chromatographic analysis of their methyl esters is generally used. However, for short and medium chain fatty acids, a comparable standard method is lacking because of preparative difficulties. The extraction of the polar short chain fatty acids from aqueous media with organic solvents is laborious because of the volatility and the unfavourable distribution coefficients. A satisfactory recovery rate requires several extractions. The conversion of the acids into their salts during alkaline extraction and subsequent steam distillation of the isolated acids may result in artefacts being formed by the simultaneous hydrolysis of labile fatty acid esters.

Despite continuous improvements[1–6], the gas–liquid chromatography (GLC) of low-boiling free fatty acids (FFA) is complicated by strong adsorption to the stationary phases, the tendency for dimerisation, tailing, the limited durability of the strongly polar packing material, the appearance of ghost peaks and difficulties in handling the volatile components for quantitative determinations[4,7–9]. Conversion of the carboxylic acids into derivatives that are better resolved by GLC was therefore more suitable. In the GLC of fatty acid derivatives, the fatty acid methyl esters were the most frequently used compounds[10–13]. Some workers also described the separation of low-molecular-weight carboxylic acids as their *n*-propyl esters[14], *n*-butyl esters[15], *p*-bromophenacyl and *p*-phenylphenacyl esters[16], *p*-substituted benzyl esters[17] or as anilides[18] and toluidines[19] (see also ref. 20). The following three requirements in particular must be fulfilled by a method for derivative formation and gas chromatographic analysis of short and medium chain FFA: (1) less volatile and stable carboxylic acid derivatives; (2) quantitative derivatisation; and (3) gas chromatographic separation on commonly used columns. For the detection of FFA in the presence of fatty acid

esters, *i.e.*, for application to biological materials, the conditions of preparation should be mild so as to prevent transesterification.

This paper describes the preparation and gas chromatographic analysis of the benzyl esters of short and medium chain FFA.

The carboxylic acids were benzylated by two different methods involving the use of (1). N,N'-dicyclohexyl-O-benzylisourea and (2) phenyldiazomethane. As VOWINKEL[21] has reported, carboxylic acids can be esterified by O-alkylisoureas to give very high yields. In an analogous manner to the methylation of carboxylic acids by diazomethane, the reaction with phenyldiazomethane produces carboxylic acid benzyl esters.

MATERIALS

Carboxylic acids (C$_3$–C$_{12}$) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., formic and acetic acids from Merck, Darmstadt, G.F.R., and isobutyric, 2-methyl-*n*-butyric and 4-methyl-*n*-valeric acids from Fluka AG, Buchs, Switzerland. All solvents (reagent grade) and chemicals were purchased from Merck. Sodium [1-^{14}C] acetate (61 mCi/mmole) and *n*-[1-^{14}C] decanoic acid (19.1 mCi/mmole) were obtained from The Radiochemical Centre, Amersham, Great Britain. Radioactivity was measured with a Tri-Carb 3320 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill., U.S.A.); BRAY solution[22] was used as the scintillation liquid. The gas–liquid chromatograph (Hewlett-Packard Co., Palo Alto, Calif., U.S.A., Model 402) was equipped with a flame ionization detector and a Hewlett-Packard recorder, Model 7127A, and integrator, Model 3370B. Glass columns (6 ft. long × 3.5 mm I.D.) were packed with 3.0 % SE 30 or 3 % EGSS-X on Gas-Chrom Q, 100–120 mesh, obtained from Applied Science Laboratories Inc., State College, Pa., U.S.A.

METHODS

Preparation of N,N'-dicyclohexyl-O-benzylisourea (DBI)

DBI was synthesized from dicyclohexylcarbodiimide and benzyl alcohol with copper(I) chloride as catalyst according to the method of VOWINKEL[23]:

$$C_6H_{11}-N=C=N-C_6H_{11} + C_6H_5CH_2OH \xrightarrow{CuCl} C_6H_{11}-N=\underset{\underset{OCH_2C_6H_5}{|}}{C}-NH-C_6H_{11}$$

After distillation *in vacuo* at 130° and 10^{-4} mm Hg, DBI caused no interfering peaks in the appropriate gas chromatographic range. If the pressure was too high during distillation, DBI could be cleaved into the original reactants, dicyclohexylcarbodiimide and benzyl alcohol. DBI was stable for several weeks at −15°.

Preparation of benzyl esters. Reaction of carboxylic acids with DBI occurred according to the following scheme:

$$C_6H_{11}-N=\underset{\underset{OCH_2C_6H_5}{|}}{C}-NH-C_6H_{11} + RCO_2H \longrightarrow RCO_2CH_2C_6H_5 + C_6H_{11}-NH-\underset{\underset{O}{||}}{C}-NH-C_6H_{11}$$

A solution of the carboxylic acids in benzene (1 g per 100 ml) was allowed to reflux for 1 h with a slight excess of DBI. After cooling to room temperature and sedimen-

tation of the dicyclohexylurea, 0.5–1.0 μl of supernatant liquid was injected without further purification into the gas–liquid chromatograph.

Phenyldiazomethane (PDM)

Of all the methods previously described, the preparation of PDM via N-nitroso-N-benzyl-N''-nitroguanidine should yield the purest diazo product[24]. PDM synthesized by this method produced numerous interfering gas chromatographic peaks after reaction with carboxylic acids. Furthermore, as this skin-irritating nitroso compound is stable over a long period only in the dark at low temperatures, an alternative method of synthesis of PDM as described by OVERBERGER AND ANSELME[25] was used. By using sodium nitrite–glacial acetic acid, the nitroso group was introduced into the easily accessible N-benzyl-p-toluenesulphonamide[26] to form N-nitroso-N-benzyl-p-toluenesulphonamide. This compound was obtained in a high yield and is harmless to skin, stable at room temperature and not decomposed by light. It was cleaved with sodium methylate under alkaline conditions. PDM separated as a red oil. The diazo product had to be further purified for gas chromatography. In contrast to diazomethane, PDM is stable and can be kept in n-pentane for several weeks at −20°. The well known explosive properties of diazomethane are considerably reduced in PDM because of resonance stability due to the vicinal aromatic substituent. PDM can be distilled *in vacuo* at room temperature.

Preparation of PDM*. Over a period of 1 h, 14.5 g of N-nitroso-N-benzyl-p-toluenesulphonamide was gradually added to a mixture of 3 g of sodium methylate, 15 ml of methanol and 90 ml of diethyl ether, stirring the reactants vigorously at 0°. The reaction mixture was refluxed for 20 min, taking care to exclude water, then evaporated *in vacuo* at 25° in a rotary vacuum evaporator, and the residue was dissolved in 100 ml of n-pentane and the solution filtered. The filtrate was cooled to − 20°, during which a liquid phase separated that solidified after a few minutes. The supernatant n-pentane solution was decanted and then reduced in volume to *ca*. 20 ml. While stirring, this concentrated PDM solution was distilled *in vacuo* (0.1 mm Hg) at room temperature into a low-temperature condenser (−30° or below). The solution of PDM in n-pentane was kept at −20° in a smooth-surfaced flask sealed with a polyethylene stopper. Benzene is unsuitable as a solvent for PDM as phenylcycloheptatriene might be formed**.

Preparation of benzyl esters. PDM in n-pentane was added to a 1 % (w/v) solution of carboxylic acids in n-pentane at room temperature until the red colour of PDM persisted. Esterification was observed to occur by the disappearance of the colour and the evolution of nitrogen. After 10–15 min***, the excess of PDM was destroyed by adding a 1% ethereal phosphoric acid solution. Without further purification, 0.5–1.0 μl of the decoloured solution was injected into the gas–liquid chromatograph.

Quantitation of benzylation

A defined activity (10^5–10^7 d.p.m.) of a ^{14}C-labelled acid was added to 5 ml of a 1 % solution of carboxylic acids. After reaction with an excess of benzylating

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* For lower demands in purity, the procedure described by OVERBERGER AND ANSELME[25] can be used. The distillation described above is recommended, however.

** After completion of this paper PDM was prepared in high purity and yield by oxidation of benzaldehyde hydrazone with mercury(II) oxide (see ref. 24, p. 568).

*** If the reaction is carried out at 0°, the excess of PDM should act for at least 30 min.

reagent, the mixture, to which one drop of a 1 % phenolphthalein solution had been added, was extracted with 2 ml of a 1 % aqueous sodium carbonate solution. The two phases were separated by centrifugation and the ^{14}C activities of both were measured. As a blank, an equal amount of unesterified [^{14}C]carboxylic acids was extracted from benzene in the same manner as above, and the activity was counted in both phases. The reaction rate could be calculated from the distribution of the activity in both phases.

Gas chromatography of benzyl esters

Equal flow-rates were used for all separations: helium 42 ml/min (inlet pressure 60 p.s.i.g.); synthetic air 370 ml/min; hydrogen 36 ml/min. Under isothermal conditions, the temperatures of the injection port and the flame ionization detector exceeded the column temperature by 50°; for temperature programming, the temperatures of the injection port and the flame ionization detector were adjusted to be 20° above the final temperature. The stationary phases used and the oven temperatures applied in the GLC separation of the carboxylic acid benzyl esters are shown in Table I.

TABLE I

COLUMN TEMPERATURES AND STATIONARY PHASES FOR SEPARATION OF CARBOXYLIC ACID BENZYL ESTERS

Carboxylic acid benzyl esters	Column temperature (°C)	
	SE-30	EGSS-X
C_1–C_6	110 (Fig. 1)	130 (Fig. 2)
C_7–C_{12}	170 (Fig. 3)	170
C_1–C_{12}	100–200 3°/min (Fig. 4)	

For the determination of the relative response for equal weights of fatty acids, peak areas were measured by electronic integration.

RESULTS

Rate of esterification

Quantitative benzylation of short and medium chain FFA was carried out with DBI and PDM according to the described methods. After a single extraction with a 1 % sodium carbonate solution, 99–100 % of the FFA as benzyl esters was recovered in the organic phase of the reaction mixture. The benzylation with PDM indicated that the reaction velocity was dependent on the acidity of the carboxylic acids. As acetic acid reacted vigorously, with the evolution of nitrogen, the reaction velocity of the higher homologues decreased gradually. To ensure that the carboxylic acids are quantitatively converted, even at low concentrations and during short reaction periods, it is advisable to use an excess of PDM. For benzylation with DBI, a slight excess above an equimolar amount is sufficient to ensure a quantitative reaction.

Gas–liquid chromatography

Fig. 1 illustrates the isothermal (110°) separation of the benzyl esters of the following acids on an SE-30 column: formic acid (C_1), acetic acid (C_2), propionic acid (C_3), isobutyric acid $(i\text{-}C_4)$, n-butyric acid $(n\text{-}C_4)$, 2-methyl-n-butyric acid $(i\text{-}C_5)$, n-valeric acid $(n\text{-}C_5)$, 4-methyl-n-valeric acid $(i\text{-}C_6)$ and n-caproic acid $(n\text{-}C_6)$.
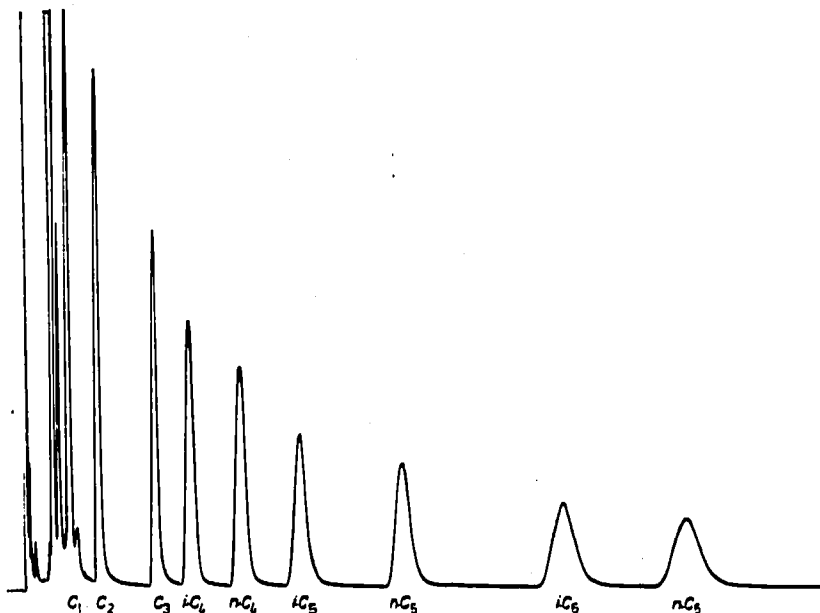
Fig. 1. Chromatogram of carboxylic acid benzyl esters $(C_1\text{-}C_6)$ on an SE-30 column at 110°.

The benzylation with DBI caused no interfering peaks over the whole range of the separation. In the fraction of benzyl esters synthesized with PDM, peaks of several low-boiling compounds appeared between the solvent peak and the peak of formic acid benzyl ester. These small peaks impeded the quantitative determination of formic acid at 110°. However, formic acid benzyl ester could be resolved completely on an SE-30 column at 90°. Depending on the amount of PDM added, an interfering peak appeared on the SE-30 column at the retention time of heptanoic acid benzyl ester. Gas chromatographic and infrared spectroscopic analysis suggest this peak to be dibenzyl ether. Dibenzyl ether and benzyl alcohol were formed by reaction between PDM and water. Heptanoic acid benzyl ester and dibenzyl ether could be resolved on an EGSS-X column. A complete separation of the benzyl esters of 2-methyl-n-butyric acid and 3-methyl-n-butyric acid could not be achieved on both columns.

Fig. 2 demonstrates the separation of the benzyl esters of formic acid to heptanoic acid on an EGSS-X column at 130°. Although the isomers were incompletely separated under the operating conditions, the peak of formic acid benzyl ester was completely isolated from the solvent and reagent peaks.

Fig. 3 indicates the separation on an SE-30 column of the benzyl esters of the following carboxylic acids: heptanoic acid (n-C_7), octanoic acid (n-C_8), nonanoic acid (n-C_9), decanoic acid (n-C_{10}), undecanoic acid (n-C_{11}) and dodecanoic acid (n-C_{12}).
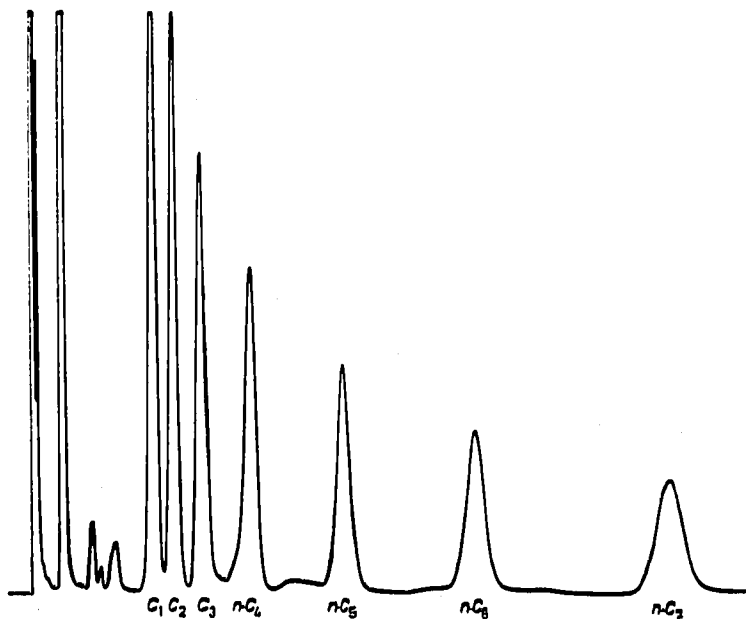
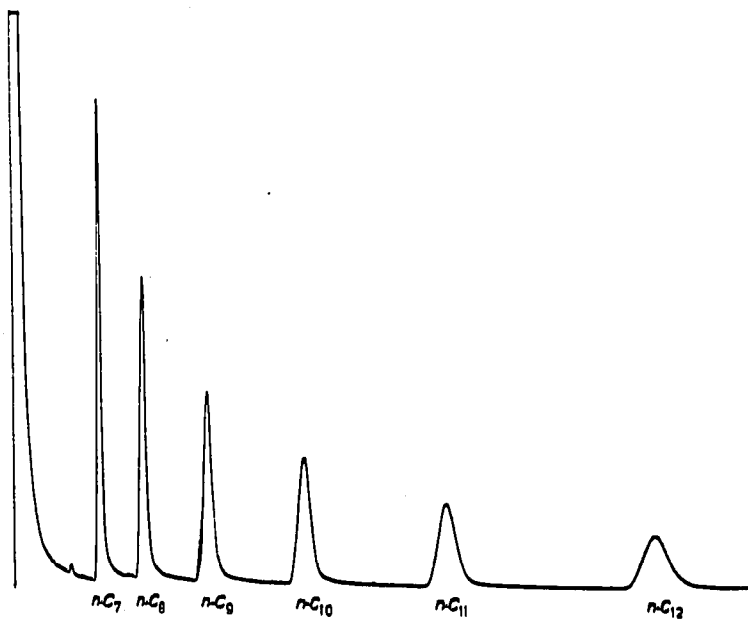Fig. 2. Chromatogram of carboxylic acid benzyl esters (C_1-C_7) on an EGSS-X column at 130°

Fig. 3. Chromatogram of carboxylic acid benzyl esters (C_7-C_{12}) on an SE-30 column at 170°.

This mixture of benzyl esters was separated without difficulty on both the SE-30 and EGSS-X columns. No differences concerning the benzylating reagent used were observed.

Fig. 4 illustrates a gas chromatogram of the benzyl esters of the C_1–C_{12} acids on an SE-30 column under temperature-programmed conditions (100–200° at 3°/min).

The relative retention times of benzyl esters are plotted logarithmatically *versus* the carbon numbers of the acids in Fig. 5. Butyric acid and nonanoic acid benzyl esters served as reference substances.
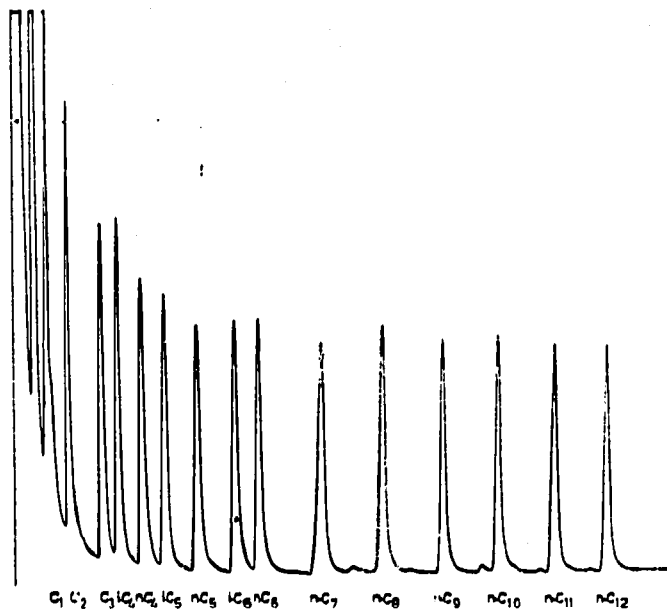
Fig. 4. Chromatogram of carboxylic acid benzyl esters (C_1–C_{12}) on an SE-30 column, temperature programmed from 100 to 200° at 3°/min.
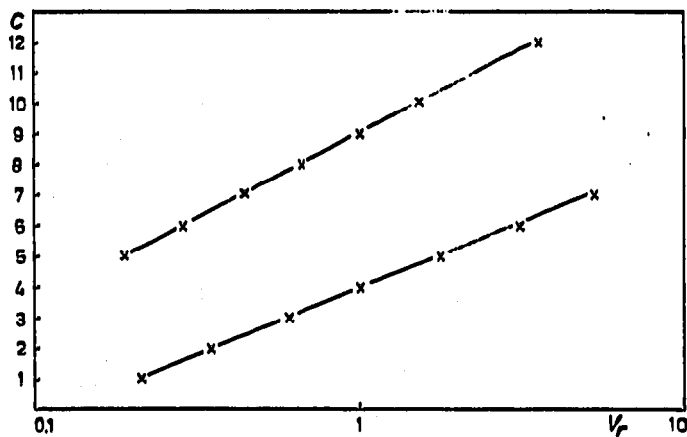
Fig. 5. Relative retention volumes of C_1–C_6 and C_6–C_{12} carboxylic acid benzyl esters on an SE-30 column at 110° and 170°, respectively.

Fig. 6 demonstrates the relative response for equal weights of fatty acids relative to the n-C_5 and n-C_9 acids as internal standards. The deviation of the value for heptanoic acid is due to the proportion of dibenzyl ether present.
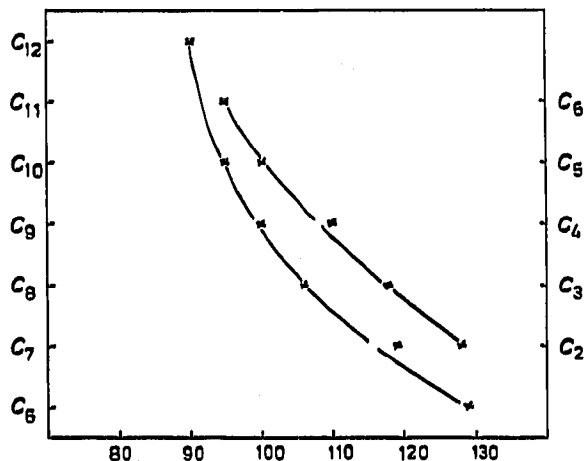

Fig. 6. Mean values ($n = 12$) of relative response for equal weights of fatty acids relative to n-C_5 and n-C_9 acids as internal standards on an SE-30 column at 110° and 170°, respectively.

DISCUSSION

The boiling points of the benzyl esters of short and medium chain fatty acids are in the range from 203°/760 mm Hg for formic acid benzyl ester to 210°/11 mm Hg for dodecanoic acid benzyl ester. Therefore, short chain carboxylic acid benzyl esters can be handled easily with regard to solvent evaporation and silica gel chromatography. For GLC, the boiling range of the benzyl esters of short and medium chain fatty acids, in contrast to their methyl esters, enabled favourable operating conditions (100–200°) to be used. The difficulties arising from the extreme volatility of methyl esters in quantitative GLC are largely avoided by benzyl esters. In contrast to the p-bromo- and p-phenylphenacyl esters used by UMEH[16], the benzyl esters, except for the less stable formic acid benzyl ester, were stable both in solution and alone. The apolar nature of benzyl esters enables them to be quantitatively extracted from aqueous systems without the need for multiple solvent partition, which is required when using methyl-, p-bromo- and p-phenylphenacyl esters. The p-substituted benzyl esters, described by WATSON AND CRESCUOLO[17], are less suitable for GLC owing to the polar substituent. The non-catalyzed esterification with DBI proceeded under relatively mild conditions. The quantitative formation of derivatives is favoured by the insoluble disubstituted urea that is generated during the reaction. Further purification of the benzyl esters is unnecessary as no interfering by-products were detected during GLC. Many solvents, such as benzene, dioxan, tetrahydrofuran and carbon tetrachloride, could be used[21]. In the anilidation of carboxylic acids carried out after their conversion into acid halogenides with $SOCl_2$, UMEH[18] observed a considerable deterioration in the quantitative reaction due to the presence of water in the reaction mixture. Formic acid is destroyed under these drastic conditions. UMEH[19] therefore recommended that formic acid should be converted into

its toluidine derivative for gas chromatographic assay. During esterification with DBI, absolute solvents were unnecessary. The reaction product with water, benzyl alcohol, did not interfere in the gas chromatogram. Formic acid was easily benzylated with DBI.

PDM showed great reactivity towards carboxylic acids. Quantitative conversion into benzyl esters was achieved by using an excess of reagent under extremely mild conditions at 0°. Possible competitive reactions, e.g., 1,3 dipolar cyclo-addition at carbon–carbon double bonds, were considerably slower than esterification. PDM reacted with water to form dibenzyl ether to a moderate extent at 0° only. Water, the presence of which is unavoidable in the preparation of biological materials, therefore did not interfere. For the qualitative and quantitative determination of short and medium chain FFA in combination with fatty acid esters, their conversion into benzyl derivatives with PDM is very suitable. The quantitative extraction of short chain fatty acids from a lipid mixture is impeded by artefacts that are produced during the hydrolysis of fatty acid esters and by the volatility and different polarities of the acids. Their conversion into the water-soluble salts during alkaline aqueous extraction increases the hydrolysis of glycerolipids. The benzylation of carboxylic acids with PDM at the beginning of the preparation avoids these difficulties.

The fatty acid benzyl esters (C_1–C_{12}) were resolved on both SE-30 and EGSS-X columns. Complete separation of selected isomers was achieved on an SE-30 column only. The formic acid benzyl ester was resolved better on an EGSS-X column. Isothermal GLC could be carried out at a temperature of 110° for C_1–C_6 and at 180° for C_7–C_{12} acid esters. All components were resolved within 35 min by using a temperature program of 100–200° at a rate of 3°/min. Dibenzyl ether could not be separated completely from heptanoic acid benzyl ester on an SE-30 column and for the quantitative determination of this acid an EGSS-X column can be used.

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