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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBOXYLIC ACIDS USING 4-BROMOMETHYL-7-ACETOXYCOUMARIN AS FLUORESCENCE REAGENT

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SUMMARY

A system for the high-performance liquid chromatography of carboxylic acids using 4-bromomethyl-7-acetoxycoumarin (Br-Mac) as the fluorescence reagent is described. Br-Mac reacts with carboxylic acids to give the ester derivatives, which are separated using reversed-phase liquid chromatography. The eluate from the column is mixed with an alkaline solution. The labelled carboxylic acids are hydrolysed to the fluorescent coumarin derivatives, which are introduced into a flow-through fluorimeter. In this system, the fluorescent hydrolysate, equimolar to a carboxylic acid, is detected and this fluorophor is common to every carboxylic acid. Only a slight variation is found in the peak areas for various carboxylic acids. A gradient elution technique is effectively used in this system because the fluorescence quantum yield of the fluorescent hydrolysate is not affected by the constitution of the mobile phase. Low femtomole levels of carboxylic acids can be detected.

INTRODUCTION

In general, non-aromatic organic acids do not produce strong fluorescence or absorption in the ultraviolet or visible regions. Therefore, derivatization with suitable labelling reagents has been used for the sensitive determination of carboxylic acids.

Recently 4-bromomethyl-7-methoxycoumarin (Br-Mmc)[1–3], 9,10-diaminophenanthrene[4] and 9-anthryldiazomethane[5,6] have been reported as fluorescence reagents for labelling carboxylic acids. When these reagents are used as pre-column labelling reagents for carboxylic acids, it is generally presumed that the smaller the

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molecular size of the labelling reagent, the better the separation of the labelled carboxylic acids would be. Therefore, Br-Mmc seems to be the most suitable of the above reagents for the analysis of carboxylic acids by high-performance liquid chromatography (HPLC).

The fluorescence quantum yields of carboxylic acids labelled with Br-Mmc are, however, severely influenced by the solvent environment[7]. 9,10-Diaminophenanthrene is also subject to the same solvent effect[4]. It is therefore considered that a gradient elution technique could not be used effectively for the HPLC separation of carboxylic acids labelled with these reagents.

Further, a wide variation of the fluorescence intensity of the carboxylic acid Mmc esters was found with equimolar amounts of different carboxylic acids[7]. Accordingly, various kinds of carboxylic acids could not be determined sensitively with Br-Mmc.

In this paper, a fluorimetric HPLC method for carboxylic acids using Br-Mac, which does not suffer from the above disadvantages, as the pre-column labeling reagent is described.

EXPERIMENTAL

Reagents and chemicals

All reagents were of analytical-reagent grade. The carboxylic acids and acetone were obtained from Wako (Osaka, Japan). Acetonitrile for use as the mobile phase was purchased from Kanto Chemicals (Tokyo, Japan) and was distilled prior to use. Dibenzo-18-crown-6 was purchased from Aldrich (Milwaukee, WI, U.S.A.) and ODS-6013 (10 μm) from Kyowa Seimitsu (Tokyo, Japan). Redistilled water was used throughout.

The coumarin derivatives related to Br-Mac were prepared according to the method of Baker *et al.*[8] with some modifications.

Preparation of Br-Mac

Br-Mac was prepared according to the following method. β-Methylumbelliferone (50 g) was refluxed with acetic anhydride (100 ml) for 1 h. After cooling, the resulting mixture was poured into cold water (500 ml). The solid product (4-methyl-7-acetoxycoumarin) was filtered, dried and recrystallized from ethanol. A mixture of 4-methyl-7-acetoxycoumarin (10 g), N-bromosuccinimide (9 g), a small amount of α,α′-azobisisobutyronitrile and carbon tetrachloride (100 ml) were refluxed for 20 h and, after cooling, the solvent was removed *in vacuo*. The residue was washed with water, filtered and dried. Recrystallization from ethyl acetate and cyclohexane gave pure Br-Mac, m.p. 184–185°C. The purity of the product was checked by HPLC and the structure of Br-Mac was confirmed by elemental analysis and infrared, ultraviolet and nuclear magnetic resonance spectroscopy.

Apparatus

The apparatus shown in Fig. 1 was constructed. All parts were obtained from Japan Spectroscopic Co. (Tokyo, Japan). For the separation of the labelled carboxylic acids, a TRI ROTAR I high-performance liquid chromatograph equipped with a Model GP-A30 solvent programmer was used. HPLC separations were carried

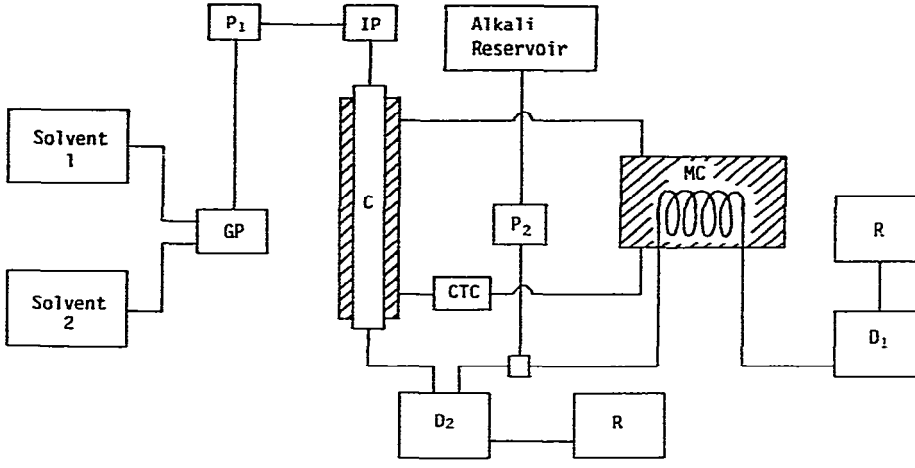


Fig. 1. Schematic diagram of the apparatus in the flow system. GP = Gradient programmer; P_1,P_2 = pumps; IP = inject port; C = column; CTC = constant-temperature circulator (50°C); MC = mixing coil; D_1 = spectrofluorimeter; D_2 = UV detector (connected only in the study of Br-Mac reactivity); R = recorder.

out with a stainless-steel column (250 × 2.1 mm I.D.) packed with ODS-6013 (10 μm) by a balanced-density slurry packing method. The temperature of the column and the mixing coil was maintained at 50°C. The operating conditions for HPLC are shown in Fig. 5. A Model FP-110 fluorescence spectrofluorimeter (excitation 365 nm, emission 460 nm) and a Model RC-225 strip-chart recorder were used. In the examination on the reactivity of BR-Mac with carboxylic acids, a UVIDEC 100 UV detector monitoring absorbance at 280 nm was introduced before mixing with an alkaline solution using a Model LCP 150 liquid chromatographic pump, and hydrolysis was performed through a coil made of 10 m × 0.5 mm I.D. stainless-steel tubing.

The fluorescence intensity of the coumarin derivatives related to Br-Mac was measured with a Model RF-510 spectrofluorimeter (Shimazu Seisakusho, Kyoto, Japan).

Derivatization

Each carboxylic acid (0.1–20 nmol), a 2.5-fold molar excess of Br-Mac and an equimolar amount of dibenzo-18-crown-6 were dissolved in 50 μl of acetone and the mixture was placed in a glass ampoule containing about 3 mg of a finely powdered mixture of potassium hydrogen carbonate and sodium sulphate (1:1). The ampoule was sealed and heated for 30 min at 50°C in the dark. After cooling, an aliquot of the resulting solution was injected on to the column.

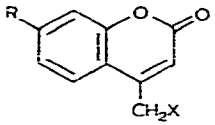
RESULTS AND DISCUSSION

Since Dünges and co-workers[1,2] used Br-Mmc as the fluorescence labelling reagent for various carboxylic acids, HPLC methods for organic acids using this reagent have been examined[8–13]. Recently other fluorescence reagents such as 9,10-

diaminophenanthrene[4] and 9-anthryldiazomethane[5,6] have been reported. In comparison with these reagents for pre-column labelling, Br-Mmc might be more suitable for the separation of labelled carboxylic acids, because it has the smallest molecular size. The fluorescence quantum yields of the carboxylic acids labelled with Br-Mmc are, however, subject to the solvent effect[7]. It has also been shown that 9,10-diaminophenanthrene had the same property[4]. Therefore, a gradient elution technique could not be used effectively with these reagents. In addition, it was reported that the fluorescence intensity of the carboxylic acid Mmc esters depended on the kind of carboxylic acid residues[7]. In this instance, various kinds of carboxylic acids could not be detected with high sensitivity using Br-Mmc. An initial effort was directed to overcoming these problems and to develop a fluorescence reagent without the defects mentioned above.

TABLE I

RELATIVE FLUORESCENCE INTENSITIES OF COUMARIN DERIVATIVES



1 μmol of each compound was dissolved in 10 ml of the solution shown and the fluorescence intensity was measured at the wavelength of the excitation and emission maxima. The results are given in arbitrary units.

X	*R*	*Acetonitrile–water (20:80)*	*Acetonitrile–water (50:50)*	*Acetonitrile–water (80:20)*	*Acetonitrile–0.1 M borate buffer (pH 11.0) (50:50)*	*Acetonitrile–0.1 M acetate buffer (pH 4.0) (50:50)*
H	OH	$1.2 \cdot 10^2$	$9.5 \cdot 10$	$4.0 \cdot 10^2$	$3.8 \cdot 10$	
H	$OCOCH_3$	1.0	0.5	0.2	$3.0 \cdot 10^2$	1.0
H	OCH_3	$1.8 \cdot 10^2$	$9.0 \cdot 10$	$3.4 \cdot 10$	$7.6 \cdot 10$	$8.6 \cdot 10$
Br	OCH_3	Precipitate	2.6	1.7	1.4	3.9
Br	$OCOCH_3$	0.5	0.4	0.3	$8.6 \cdot 10$	0.5
COOH	$OCOCH_3$	11.0	8.0	3.0	$3.8 \cdot 10^2$	8.7
COOH	CH_3	2.0	1.1	0.6	1.3	1.3

Table I gives the relative fluorescence intensities of several coumarin derivatives with different substituent groups at the 4- or 7-position in various solvents. Each fluorescence intensity was measured at the wavelength of the excitation and emission maxima. A substituent group at the 7-position in coumarin greatly affects the fluorescence quantum yield, whereas one in the 4-position has hardly any effect. The presence of an electron-donating group such as a hydroxy group tends to enhance the fluorescence intensity of the 7-hydroxy derivative, which is increased by making the pH of its solution more alkaline. The coumarin derivatives with acetoxy groups in the 7-position show strong fluorescence only in alkaline solutions. This phenomenon seems to be due to the hydrolysis of the acetoxy group to a hydroxy group. Based on these results, Br-Mac was adopted as the pre-column labelling reagent for carboxylic acids.

The reaction of Br-Mac with a carboxylic acid and the principle of the detection system are shown in Fig. 2 compared with those for Br-Mmc. With all of the fluorescence reagents reported previously, the reagent is allowed to react with carbox-

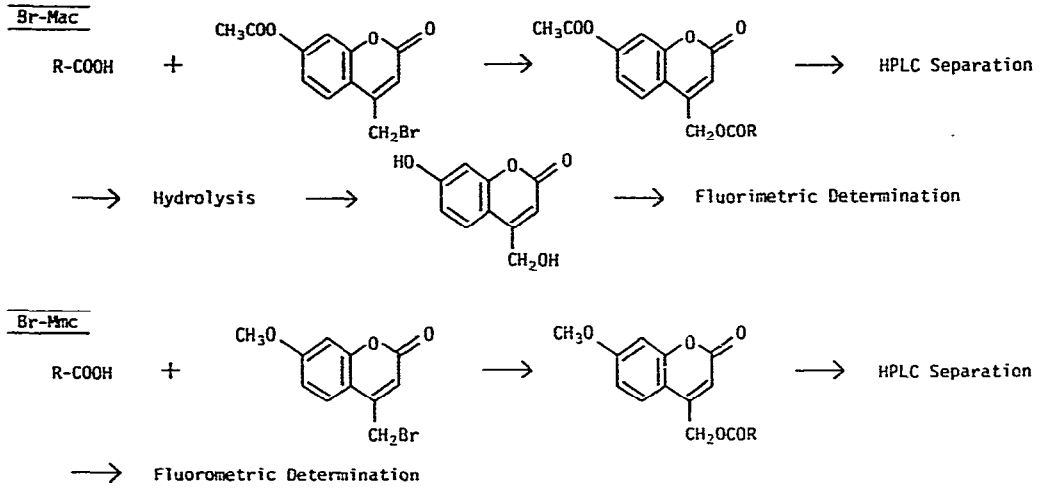


Fig. 2. Schemes for the reactions of carboxylic acids with Br-Mac and Br-Mmc and the analytical systems.

ylic acids first and, after column separation, each carboxylic acid labelled with the fluorophore is detected. Therefore, the fluorescence quantum yields tend to be influenced by the carboxylic acid residues. The fluorescence intensity of carboxylic acids labelled with Br-Mmc varies with the kind of carboxylic acid[7] and therefore different carboxylic acids could not be detected with similar sensitivities.

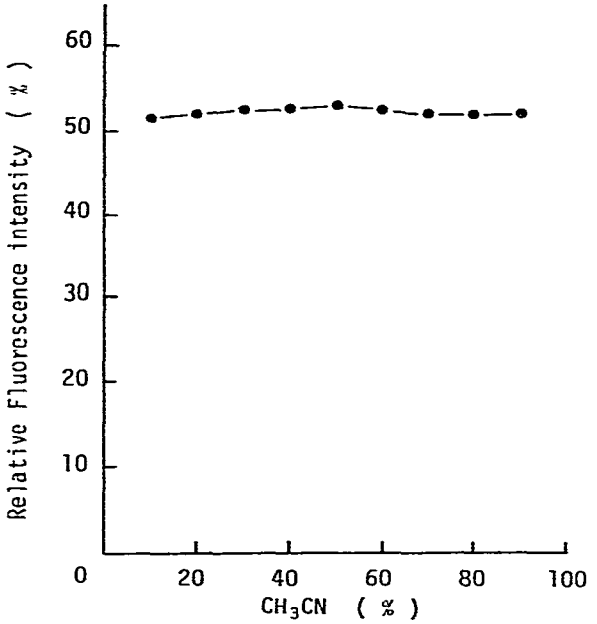


Fig. 3. Variation of the fluorescence intensity of the 7-hydroxycoumarin derivative with the concentration of acetonitrile in borate buffer. 4-Methyl-7-acetoxycoumarin was dissolved in acetonitrile (1 μmol/ml). To 1 ml of this solution, 5 ml of aqueous acetonitrile solution and 0.1 *M* borate buffer (pH 11.0) were added to give a total volume of 10 ml. After heating for 30 min, the fluorescence intensity was measured at the wavelength of the excitation and emission maxima.

On the other hand, Br-Mac reacts with carboxylic acids to give the ester derivatives, followed by HPLC separation. After separation, each compound is hydrolysed by addition of an alkaline solution, and then the resulting fluorescent compound is introduced into a spectrofluorimeter. Therefore, the fluorescence intensity is hardly influenced by the kind of carboxylic acid because not only the fluorescent hydrolysate, equimolar to a carboxylic acid, is detected, but also this fluorophore is common to all carboxylic acids. Further, the alkaline hydrolysis produces a much stronger fluorescence of the hydrolysate. For these reasons, highly sensitive detection of different carboxylic acids by HPLC using Br-Mac could be expected.

Fig. 3 shows the relationship between the fluorescence intensity of 4-methyl-7-hydroxycoumarin and the concentration of acetonitrile in alkaline solution. The fluorescence intensity was almost constant over the range of acetonitrile concentrations investigated. This result indicates that the detection system using Br-Mac might not be affected by changes in the acetonitrile concentration. Thus, a gradient elution technique could be used effectively in this system.

The results of the investigation of the hydrolysis time necessary for hydrolysis of the acetoxy group to a hydroxy group are shown in Fig. 4. To an aqueous acetonitrile solution of 4-carboxymethyl-7-acetoxycoumarin was added borate buffer (0.1 *M*, pH 11.0) and the mixture was pumped to the spectrofluorimeter. The fluorescence intensity became constant after 1–2 min, which suggests that the hydrolysis of carboxylic acid Mac esters proceeds to completion in this period. Therefore, a coil of length 10 m is sufficient to hydrolyse the Mac esters when tubing of 0.5 mm I.D. was used at a flow-rate of 1–2 ml/min.

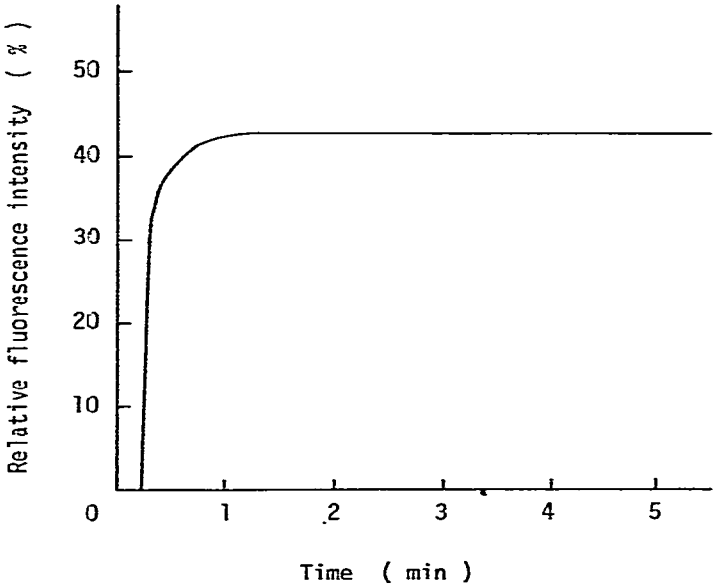


Fig. 4. Hydrolysis rate of 4-carboxymethyl-7-acetoxycoumarin. To 2 ml of 4-carboxymethyl-7-acetoxycoumarin in acetonitrile (0.5 mmol/ml), 18 ml of acetonitrile and 10 ml of water were added. This solution was mixed with 0.1 *M* borate buffer (pH 11.0) and pumped through the spectrofluorimeter, keeping the temperature at 50°C.

TABLE II

REACTIVITY OF 4-BROMOMETHYL-7-ACETOXYCOUMARIN WITH DIFFERENT CARBOXYLIC ACIDS

Carboxylic acid	*Reactivity**	*Carboxylic acid*	*Reactivity**
Aliphatic carboxylic acids:		Adipic acid	+
Saturated monocarboxylic acids:		Hydroxycarboxylic acids:	
Formic acid	+	Lactic acid	+
Acetic acid	+	Malic acid	+
Propionic acid	+	Citric acid	−
Butyric acid	+	Ketocarboxylic acids:	
Isovaleric acid	+	Pyruvic acid	−
Caproic acid	+	α-Ketoglutaric acid	−
Caprylic acid	+	Trichloroacetic acid	−
Capric acid	+	*Aromatic carboxylic acids:*	
Lauric acid	+	Benzoic acid	+
Palmitic acid	+	Phthalic acid	+
Stearic acid	+	Salicylic acid	+
Arachidic acid	+	*p*-Hydroxyphenylpropionic acid	+
Unsaturated monocarboxylic acids:		*p*-Aminobenzoic acid	+
Oleic acid	+	*o*-Aminobenzoic acid	+
Linoleic acid	+	Picolinic acid	+
Dicarboxylic acids:			
Succinic acid	+	Barbital	+

* + indicate carboxylic acids that give peaks with both UV and fluorescence detection; − indicate carboxylic acids that give neither a UV nor a fluorescence response.

In order to investigate the reactivity of Br-Mac with various carboxylic acids, each carboxylic acid listed in Table II was allowed to react with Br-Mac under the conditions described under *Derivatization*. The resulting solution was subjected to the chromatographic investigation. From the data shown in Table II, it is considered that the reactivity of Br-Mac is very similar to that of Br-Mmc.

Fig. 5 shows the separation of fatty acid Mac esters (C_4–C_{20}) using a gradient elution technique. A graph of peak area *versus* the carbon number of the fatty acids is shown in Fig. 6. Only a slight decrease in the peak areas occurred when the carbon number increased, which indicates that with Br-Mac the fluorescence intensity is hardly related to the kind of carboxylic acid, unlike Br-Mmc, and remains almost constant in spite of the variation of the acetonitrile concentration by using a gradient elution technique. As expected in the study using the compound related to Br-Mac, it was found that the fluorescence quantum yields of the hydrolysates of carboxylic acid Mac derivatives were hardly affected by the constitution of the mobile phase.

To ascertain the detection limit, the sample used in the experiment in Fig. 5 was diluted successively with acetone and injected on to the column. Fig. 7 shows a chromatogram in which each peak corresponds to 200 fmol of a fatty acid. Considering the signal-to-noise ratio, the detection limit is about 10 fmol. A further improvement in the sensitivity might be possible with optimization of the reaction and HPLC conditions.

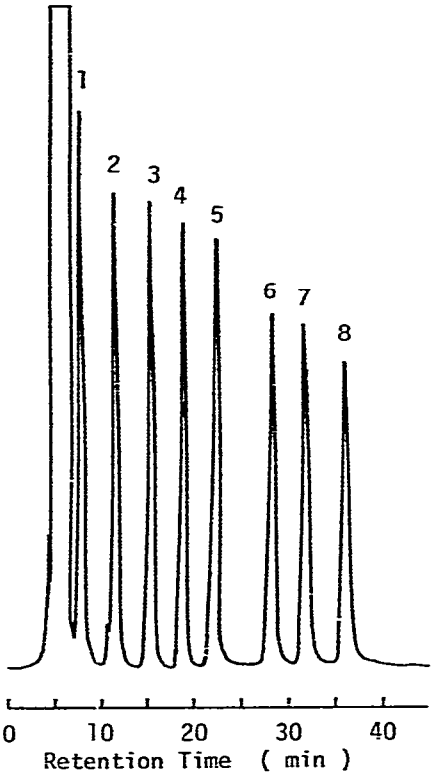


Fig. 5. High-performance liquid chromatogram of the Br-Mac derivatives of linear saturated fatty acids. 1 = $C_{4:0}$; 2 = $CC_{6:0}$; 3 = $CC_{8:0}$; 4 = $CC_{10:0}$; 5 = $CC_{12:0}$; 6 = $C_{16:0}$; 7 = $CC_{18:0}$; 8 = $CC_{20:0}$. Operating conditions: column, 250 × 2.1 mm ODS-6013 (10 μm); column and mixing coil temperature, 50 C; mobile phase, aqueous acetonitrile solution 40 % (0)–90 % (99). The gradient was prepared by use of a Model GP-A30 solvent programmer (Convex 1, 32 min); flow-rate, 0.8 ml/min; alkali flow-rate, 0.4 ml/min; detector, spectrofluorimeter (excitation 365 nm, emission 460 nm).

CONCLUSION

Compared with HPLC using other fluorescence labelling reagents, the present system with Br-Mac has several advantages. The fluorescence intensity depends neither on the kind of carboxylic acid nor on the concentration of acetonitrile in the mobile phase. In this system, a gradient elution technique can be effectively applied. Low femtomole levels of carboxylic acids can be detected. Methods for the determination of the organic acids involved in inborn errors of metabolism, bile acids and prostaglandins are at present under intensive investigation.

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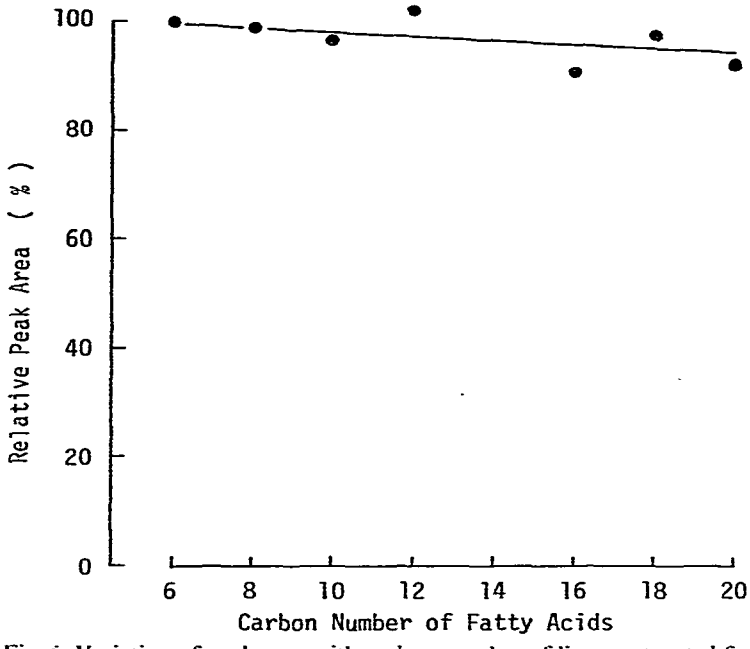

Fig. 6. Variation of peak area with carbon number of linear saturated fatty acids.

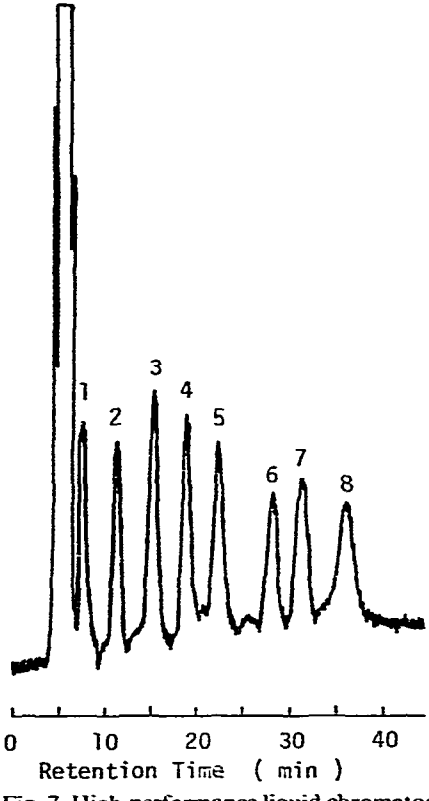

Fig. 7. High-performance liquid chromatogram of Br-Mac derivatives of linear saturated fatty acids. Each peak corresponds to 200 fmol of a fatty acid. Operating conditions and peaks as in Fig. 5.

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