STUDIES ON SULFONATE-TYPE DERIVATIZING REAGENTS FOR CHROMATOGRAPHY

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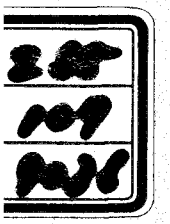
YUTA YASAKA



STUDIES ON SULFONATE-TYPE DERIVATIZING REAGENTS FOR CHROMATOGRAPHY

(クロマトグラフィー用のスルホン酸エステル型誘導体化試薬に関する研究)

1989

YUTA YASAKA

Preface

The works in this thesis were carried out under the guidance of Professor Toshiyuki Shono at Faculty of Engineering, Osaka University.

The object of this thesis is to develop new sulfonate-type derivatizing reagents for the determination of inorganic and organic anions by chromatography.

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List of Publications

The contents of this thesis are composed of the following papers.

1. Derivatization of Inorganic Anions with Pentafluorobenzyl Methanesulfonate for Gas Chromatography
   M. Tanaka, H. Takigawa, Y. Yasaka, K. Funazo, H-L. Wu and T. Shono,
   J.Chromatogr., **404**, 175, (1987).
2. A New Polymeric Pentafluorobenzylating Reagent for Gas Chromatography
   Y. Yasaka, T. Nagasaka, M. Tanaka, K. Funazo, H-L. Wu and T. Shono
   Anal. Sci., **3**, 579, (1987).
3. Polymeric Pentafluorobenzylating Reagent for Gas Chromatography of Inorganic Anions with Electron-Capture Detection
   M. Tanaka, Y. Yasaka, M. Kamino, K. Funazo, H-L. Wu and T. Shono
   J. Chromatogr., **438**, 253, (1988).
4. Polystyrenesulfonates as Polymeric Derivatizing Reagents for Fatty Acids in Liquid Chromatography
   Y. Yasaka, M. Tanaka, T. Matsumoto, K. Funazo and T. Shono
   Anal. Sci., **5**, 611, (1989).
5. Polymeric Pentafluorobenzylating Reagent for Gas Chromatography of Inorganic Anions
   Y. Yasaka, M. Tanaka, K. Funazo and T. Shono
   Chem. Express, **4**, 701, (1989).

6. Poly[2-(1-naphthyl)ethyl p-styrenesulfonate] as Polymeric Derivatizing Reagent for Liquid Chromatography of Fatty Acids
M. Tanaka, Y. Yasaka, K. Funazo and T. Shono
Fresenius Z. Anal. Chem., **335**, 311, (1989).

7. New Ultraviolet Labelling Agents for High-Performance Liquid Chromatographic Determination of Monocarboxylic Acids
K. Funazo, M. Tanaka, Y. Yasaka, H. Takigawa and T. Shono
J. Chromatogr., **481**, 211, (1989).

8. 2-(Phthalimino)ethyl Trifluoromethanesulfonate as a Highly Reactive UV-labeling Agent for Carboxylic Acids in High Performance Liquid Chromatography
Y. Yasaka, M. Tanaka, T. Matsumoto, J. Katakawa, T. Tetsumi and T. Shono
Anal. Sci., in press.

9. Polymeric Pentafluorobenzylating Reagent for Gas Chromatography of Carboxylic Acids
M. Tanaka, Y. Yasaka, H-L. Wu and T. Shono
Fresenius Z. Anal. Chem., submitted for publication.

10. 2-(2,3-Naphthalimino)ethyl Trifluoromethanesulfonate as a Highly Reactive UV and Fluorescent Labeling Agent for Liquid Chromatographic Determination of Carboxylic Acids
Y. Yasaka, M. Tanaka, T. Tetsumi, J. Katakawa and T. Shono
J. Chromatogr., in press.

11. S-(+)-[1-Methyl-2-(2,3-naphthalimino)]ethyl Trifluoromethanesulfonate a Fluorescence Chiral Derivatizing Reagent for Carboxylic Acids in High Performance Liquid Chromatography
Y. Yasaka, M. Tanaka and T. Shono, in preparation.

Contents

General Introduction

Chromatography, in particular gas and liquid chromatography, is one of the most useful analytical techniques and widely used not only in chemistry and biochemistry but also in pharmacology, toxicology, clinical chemistry, environmental sciences, and many other fields. This widespread use seems to be attributed to remarkable progress of column and detector technologies in recent years. Unfortunately, there have been still remained many substances which are not fit on direct chromatography in spite of their analytical importance. Chemical derivatization techniques have been frequently used to convert such substances to adequate ones for chromatographic analyses. As for improving of detectability, there have been already reported many methods based on the reagents with both a variety of tagging groups and of reactivities.

In this study, the author has developed new sulfonate-type derivatizing reagents for use in gas and liquid chromatograpy. This type reagents possess superiority, which has indeed motivated the author to study the reagents, over conventional ones on easy control of reactivity because degree of the reactivities may be changed by replacing the sulfonic moiety of a sulfonate, such as mesyl, tosyl, trifyl, etc.

In chapter I, were described pentafluorobenzylating reagents for both inorganic anions and carboxylic acids. Pentafluorobenzyl methanesulfonate was synthesized and applied to the determination of inorganic anions (chapter I-1). In chapter

I-2, were described polymer-type pentafluorobenzylating reagents and their superiorities over the monomeric reagents in gas chromatographic determination of both inorganic anions and carboxylic acids. Poly(pentafluorobenzyl p-styrenesulfonate) was used for the pentafluorobenzylation of inorganic anions in biphasic reaction system (chapter I-2-1). The author has also investigated the application of pentafluorobenzylation of inorganic anions and carboxylic acids in water miscible organic solvents (chapter I-2-2).

In chapter II, were presented new sulfonate-type labeling reagents for carboxylic acids in HPLC determination. Three kinds of tosylate-type UV-labeling reagents were synthesized and applied to the determination of carboxylic acids (chapter II-1). In chapter II-2, The advantages of use of polimeric reagents was again indicated by the applications of them to the derivatization of carboxylic acids in their HPLC determination. In chapter II-3, was presented highly reactive labeling reagent based on triflate. In chapter II-3-1 and II-3-2 , were described triflate-type ultraviolet and fluorescence labeling reagents, respectively. Chiral triflate-type reagent was developed for diastereomeric separation of carboxylic acid enantiomers (chapter II-3-3).

Chapter I. Derivatizing Reagents for Gas Chromatography

Chemical derivatization in gas chromatography (GC) has become a widely used means of analysis of both organic and inorganic analytes. Such techniques have also been developed for the GC determination of inorganic anions, although most have been applied to organic analytes[1-4]. The derivatization usually imparts volatility to otherwise nonvolatile inorganic anions. In some instances, a detector-oriented tag can be introduced into the resulting derivatives at the same time. Electron capture detection (ECD) is a highly sensitive and selective detection technique for specific analytes with good electron affinity, and many halogen-containing reagents for organic analytes have been developed for this purpose[5]. Among them, pentafluorophenyl-containing reagents have been widely used to derivatize a wide range of organic compounds because the resulting derivatives, containing pentafluorophenyl moiety, are generally stable and volatile with good GC properties and provide a high response to ECD. More than ten such reagents have been reported, including pentafluorobenzyl bromide, pentafluorophenyldialkylchlorosilanes[6] and pentafluorobenzenesulfonyl chloride[7].

Because of the above-mentioned advantages of pentafluorophenyl derivatives, the author have been also interested in penta-fluorobenzylation of inorganic anions for GC.

In this chapter, were presented gas chromatographic determinations of inorganic anions and carboxylic acids with new pentafluorobenzylating agents.

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I-1. Pentafluorobenzyl Methanesulfonate as a Pentafluorobenzylating Reagent for Inorganic Anions

INTRODUCTION

Techniques of analytical derivatization have been developed for determining inorganic anions by gas chromatography (GC). Such derivatization imparts volatility to otherwise nonvolatile inorganic anions. In some cases, a detector-oriented tag is introduced into the molecules at the same time. Electron-capture detection (ECD) is an extremely sensitive detection technique, and several derivatizing reagents for organic analytes have been developed for GC-ECD[1]. Among them, pentafluorophenyl-containing reagents, in particular, have been used to derivatize a broad spectrum of organic compounds, because the resulting pentafluorophenyl derivatives are generally easy to prepare, are volatile with good GC properties and provide a high ECD response.

The author have been interested in the simultaneous determination by GC-ECD of inorganic anions at relatively low concentrations after their pentafluorobenzylation. For this purpose, pentafluorobenzyl p-toluenesulphonate (PFB-OTs) was synthesized[2] and is now commercially available. Bromide, iodide, thiocyanate and nitrite at relatively low concentrations in water were pentafluorobenzylated with PFB-OTs in dichloromethane, using tetra-n-amylammonium chloride as a phase-transfer catalyst. These anions in real samples were successfully determined by GC-ECD[3]. Besides this biphasic reaction system, it is also of

interest to pentafluorobenzylate anions in a homogeneous mixture using a water-miscible organic solvent, because in the case of a homogeneous reaction system there is no need to replace dichloromethane by an other solvent suitable for ECD and to use a phase-transfer catalyst. Unfortunately, however, the solubility of PFB-OTs in organic solvents is not necessarily sufficient, though PFB-OTs is highly soluble in chlorinated hydrocarbons such as dichloromethane, chloroform, etc. Therefore, we have searched for a new, more soluble pentafluorobenzylating agent.

In this research, a new reagent, pentafluorobenzyl methanesulphonate (PFB-OMs), has been synthesized,and its applicability to the simultaneous derivatization of inorganic anions has been investigated in both biphasic and homogeneous reaction systems, using flame ionization detection (FID) and/or ECD.

EXPERIMENTAL

Apparatus

A Yanaco G-180 gas chromatograph equipped with a dual FID system (Kyoto, Japan) and a Shimadzu GC-4BM gas chromatograph equipped with a ^{63}Ni ECD unit (Kyoto, Japan) were used together with stainless-steel coiled tubes (3 mm I.D.) as separation columns. Nitrogen was employed as the carrier gas at a constant flow-rate of 30 ml/min. The detector and injection port temperatures were maintained at 230 ^{o}C for FID and at 200 ^{o}C for ECD. Other GC conditions for the biphasic reaction system are summarized in Table I. A Shimadzu Chromatopac C-R1B data

processor was used as the recorder and integrator.

Reagents

Commercial grade tetra-n-amylammonium chloride (TAAC) was obtained from Wako (Osaka, Japan). The new derivatizing reagent, PFB-OMs, was synthesized by dropwise addition of triethylamine (6.0 g) to a mixture of methanesulphonyl chloride (6.2 g) and penta-fluorobenzyl alcohol (10.0 g). After being isolated as usual, crude PFB-OMs was twice recrystallized from hexane. It was identified by mass spectrometry (m/e = 276: molecular ion peak, and m/e = 181: characteristic base peak corresponding to $C_6F_5CH_2^+$) and infrared spectroscopy (1350 and 1175 cm^{-1}: antisymmetric and symmetric vibrations, respectively, of the SO_2 group). The inorganic anions were used as their sodium or potassium salts, of analytical reagent grade. All other chemicals were also of analytical reagent grade. Solutions of the anion reference standard and TAAC were prepared by dissolving these chemicals in distilled, deionized water.

Derivatization procedures

A. For a biphasic reaction system. A 0.20-ml volume of 10 mM TAAC aqueous solution was added to a 10-ml brown coloured test tube containing 1.0 ml of an aqueous solution of each anion reference standard. Phosphate buffer solution (pH 7.0), and 0.10 ml of internal standard (40 mM) and 1.0 ml of PFB-OMs (50 mM) in dichloromethane were added. The reaction tube was sealed tightly

with a screw cap and shaken at room temperature for 30 min. After the separation of the dichloromethane layer, an aliquot (0.5 µl) of it was injected into the gas chromatograph for FID.

For ECD, this procedure was modified as follows. To 1.0 ml of the anion reference standard solution were added 0.10 ml of 4 mM TAAC aqueous solution, 0.10 ml of a buffer solution (pH 7), and 1.0 ml of 1.25 mM PFB-OMs in dichloromethane. At the end of the reaction, 0.2 ml of the organic layer was separated from the aqueous layer. The dichloromethane solution was evaporated carefully under a nitrogen stream for 2 min at room temperature, and then 1.0 ml of tert.-butyl methyl ether containing 55 µM internal standard was added to the residue. An aliquot (0.2 µl) of this solution was subjected to GC-ECD.

B. For a homogeneous reaction system. A 1.0-ml volume of PFB-OMs (50 mM) in acetone or methanol was added to a 10-ml brown-coloured test tube containing 0.40 ml of aqueous reference standard solution. A buffer solution (pH 7.0, 0.1 ml) and 0.10 ml of 40 mM 1,3,5-trichlorobenzene (internal standard) in acetone or methanol were added. The reaction mixture was shaken at 28 ^{o}C for a fixed time. An aliquot (0.5 µl) of the reaction solution was subjected to GC-FID. The separation column (2 m X 3 mm I.D.) packed with 10 % SE-30 was maintained at 120 ^{o}C for Br^- and I^-, at 150 ^{o}C for SCN^- or at 180 ^{o}C for S^{2-}.

RESULTS AND DISCUSSION

Pentafluorobenzylation of inorganic anions in a biphasic reaction system (for FID)

TABLE I

GC CONDITIONS FOR BIPHASIC REACTION SYSTEM

Internal standards: TRCB = 1,3,5-trichlorobenzene; DBB = p-dibromobenzene; IB = iodobenzene; TRBB = 1,3,5-tribromobenzene; BCB = p-bromochlorobenzene; TECB = 1,2,4,5-tetrachlorobenzene; TEBB = 1,2,4,5-tetrabromobenzene.

Anion	FID			ECD		
	Liquid phase	Column temp. (°C)	Internal standard	Liquid phase	Column temp. (°C)	Internal standard
Br^-	10% SE-30 (2 m)	130	TRCB	5% OV-225 (3 m) + 5% OV-210 (1 m)	130	BCB
I^-	10% SE-30 (2 m)	130	TRCB	5% OV-225 (3 m) + 5% OV-210 (1 m)	130	TRCB
CN^-	3% OV-17 (4 m)	140	DBB			
SCN^-	10% SE-30 (2 m)	170	TRCB	5% OV-225 (3 m) + 5% OV-210 (1 m)	180	TECB
NO_2^-	5% DC-550 (2 m)	120	IB	5% OV-225 (3 m) + 5% OV-210 (1 m)	150	TRCB
NO_3^-	5% DC-550 (2 m)	155	DBB			
S^{2-}	10% SE-30 (2 m)	180	TRBB	10% SE-30 (1.5 m)	160	TEBB

The ability of PFB-OMs to pentafluorobenzylate inorganic anions was examined in a biphasic reaction system consisting of each anion in water and PFB-OMs in dichloromethane, by using TAAC as the phase-transfer catalyst. In the same manner as reaction with PFB-OTs[2], bromide, iodide, cyanide, thiocyanate, nitrite, nitrate and sulphide could be converted into their PFB derivatives i.e., pentafluorobenzyl bromide, pentafluorobenzyl iodide, pentafluorobenzyl cyanide, pentafluorobenzyl thiocyanate, α-nitro-2,3,4,5,6-pentafluorotoluene, pentafluorobenzyl nitrate and bis(pentafluorobenzyl) sulphide, respectively. Each derivative was identified by comparing its retention time and mass spectrum with those of an authentic sample.

In order to establish the optimum derivatization conditions for pentafluorobenzylation of each anion (4.0 μmol) several parameters affecting the derivatization of the anions were investigated, including the concentrations of PFB-OMs in dichloromethane and of TAAC in water, pH of the buffer solution added and reaction time. From the results, the optimum derivatization conditions described in the Experimental section were adopted. Table II gives the pentafluorobenzylation yields of the anions under these conditions using both PFB-OMs and PFB-OTs[2]. It is apparent that PFB-OMs is more reactive than PFB-OTs.

The applicability of the method using PFB-OMs to the determination of the anions was evaluated. Ten different concentrations of the reference standard solutions containing each anion were quantitated to construct a calibration graph, plotting the concentration of each anion in aqueous solution

TABLE II

PENTAFLUOROBENZYLATION YIELDS OF ANIONS IN BIPHASIC REACTION SYSTEM

Concentration of each anion is 4.0 μmol/ml.

Anion	Pentafluorobenzylation yield (%)	
	PFB-OMs	PFB-OTs
Br^-	95.1	82.0
I^-	99.0	87.0
CN^-	73.0	56.2
SCN^-	90.8	83.4
NO_2^-	43.4	39.4
NO_3^-	86.6	68.4
S^{2-}	99.8	60.7

versus the ratio of the peak area of the corresponding PFB derivative to that of the internal standard. Good linear relationships were obtained, the linear regression equations for which are given in Table III together with the correlation coefficients and the concentration ranges.

The optimum pH for pentafluorobenzylation of the seven anions is 7.0. Consequently, it is possible to pentafluorobenzylate the anions simultaneously (Fig. 1). The resolution of the seven PFB derivatives could be attained on a composite column packed with 5 % OV-225 (3 m) and 5 % OV-210 (1 m), with temperature programming.

TABLE III

REGRESSION ANALYSIS FOR BIPHASIC REACTION SYSTEM (FID)

Y = peak-area ratio of PFB derivative of anion to internal standard; X = concentration of anion (mM).

Anion	Concentration range (mM)	Regression equation	Correlation coefficient
Br^-	0.40 - 4.0	Y = 0.2887X - 0.0136	0.9989
I^-	0.40 - 4.0	Y = 0.2716X + 0.0146	0.9992
CN^-	0.40 - 4.0	Y = 0.2191X - 0.0138	0.9987
SCN^-	0.40 - 4.0	Y = 0.2647X - 0.0246	0.9983
NO_2^-	0.80 - 8.0	Y = 0.1267X - 0.0146	0.9991
NO_3^-	0.40 - 4.0	Y = 0.1739X - 0.0007	0.9995
S^{2-}	0.40 - 4.0	Y = 0.2996X + 0.0010	0.9998

Pentafluorobenzylation in a homogeneous reaction system

Compared with PFB-OTs, PFB-OMs is more reactive in the biphasic reaction system and is more soluble in common, water-miscible organic solvents. Therefore, an attempt was made to pentafluorobenzylate inorganic anions in a homogeneous reaction system using a water-miscible organic solvent, instead of the biphasic reaction system. Acetone and methanol were used as the solvents. Only three inorganic anions listed in Table IV exhibited constant pentafluorobenzylation yields within 3 h under the optimum derivatization conditions described in the

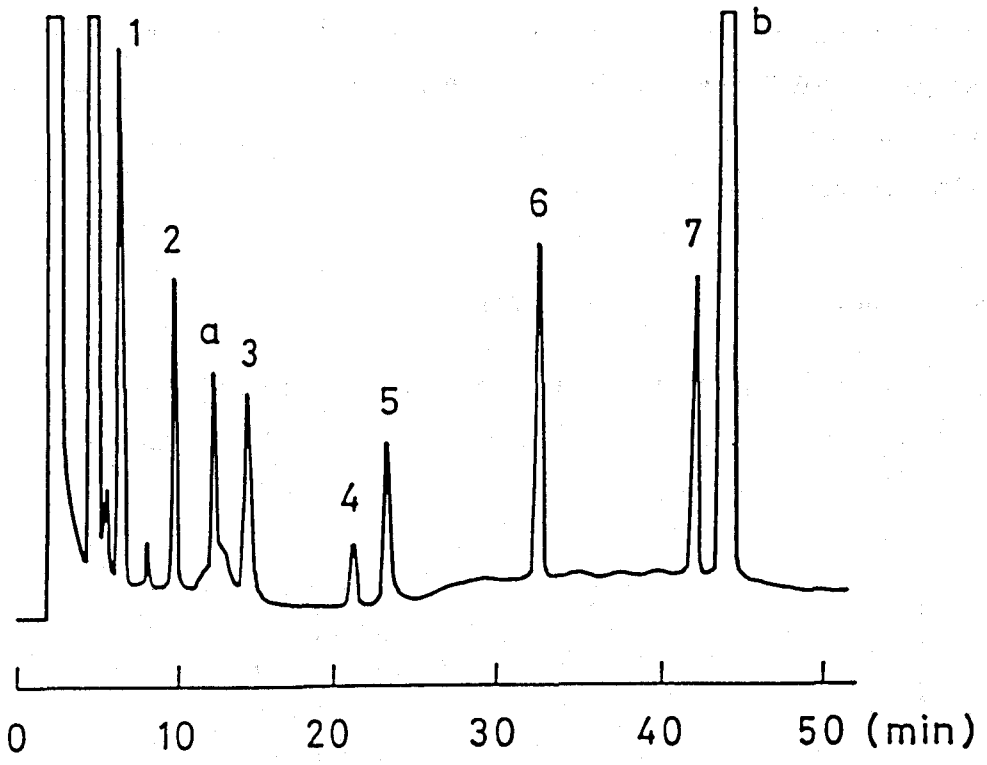

Fig.1 Gas chromatograms for simultaneous pentafluorobenzylation of seven inorganic anions. Column temperature: isothermal at 120 ℃ for 20 min and then programmed at 5 ℃/min to 180 ℃. Peals: 1 = pentafluorobenzyl bromide; 2 = pentafluorobenzyl iodide; 3 = pentafluorobenzyl nitrate; 4 = α -nitro-2,3,4,5,6-pentafluorotoluene; 5 = pentafluorobenzyl cyanide; 6 = pentafluorobenzyl thicyanate; 7 = bis(pentafluorobenzyl) sulphide; a = pentafluorobenzyl alchol; b = PFB-OMs.

experimental section. Sulphide also gave a constant yield of 61.4 % in the aqueous acetone system, but the linearity of its calibration graph was not satisfactory for the determination. Fig. 2 shows the gas chromatograms for the three anions simultaneously derivatized in the aqueous acetone and aqueous methanol systems.

The preceding biphasic-method is more practicable than this homogeneous method, considering the advantages of the former:

more anions can be pentafluorobenzylated, in higher yields and with shorter reaction times. These results are probably due to the activation of the anions by the ion-pair formation in the biphasic reaction system.

Determination of anions with ECD

Dichloromethane was the most suitable solvent for pentafluorobenzylation of the anions by the biphasic method,

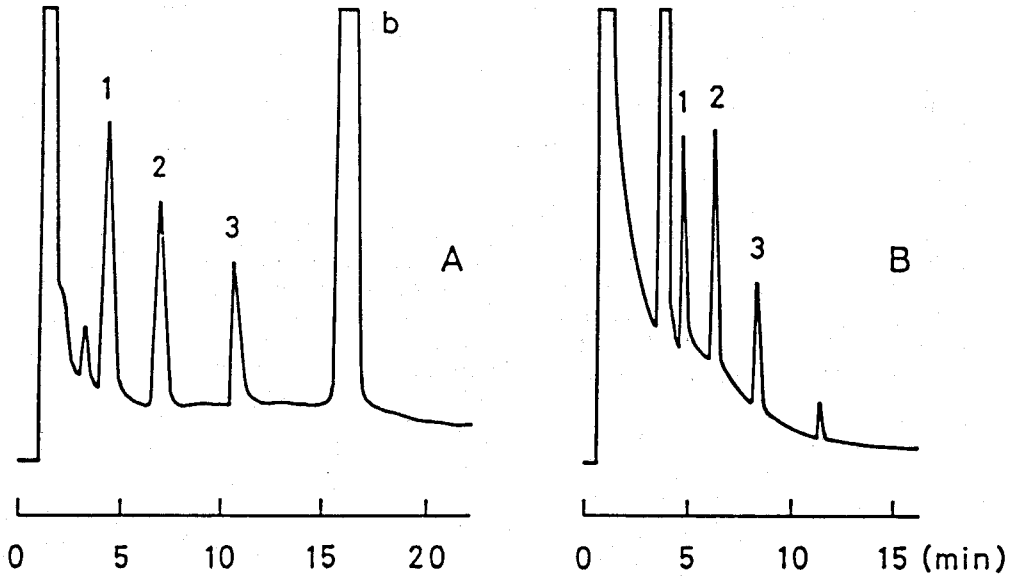

Fig.2 Gas chromatograms for simultaneous pentafluorobenzylation of three inorganic anions in aqueous acetone (A) and in aqueous methanol (B). Column temperatures: (A) isothermal at 120 ℃ for 5 min, then progra mmed at 10 ℃/min to 140 ℃, held for 6 min and programmed again at 10 ℃/min to 190 ℃; (B) programmed from 100 ℃ to 160 ℃ at 10 ℃/min prntafluorobenzyl iodide; 3 = pentafluorobenzyl thiocyanate; b = PFB-OMs.

TABLE IV

PENTAFLUOROBENZYLATION YIELDS AND REGRESSION ANALYSIS FOR HOMOGENEOUS REACTION SYSTEM

Concentration ranges of anions are 1.0 - 10 mM. Y and X as in Table III.

Solvent	Anion	Pentafluorobenzylation yield (%)	Regression equation	Correlation coefficient
Acetone	Br^-	78.8	Y = 0.1106X + 0.0261	0.9976
	I^-	89.4	Y = 0.1314X - 0.0231	0.9994
	SCN^-	64.7	Y = 0.0963X - 0.0132	0.9991
Methanol	Br^-	76.2	Y = 0.1077X + 0.0220	0.9979
	I^-	87.6	Y = 0.1310X - 0.0217	0.9989
	SCN^-	76.7	Y = 0.1156X - 0.0214	0.9988

though apparently unaccepted for ECD. As a Compromise, therefore, dichloromethane was used for the derivatization reaction and then was replaced by tert.-butyl methyl ether for ECD, as described in the Experimental section. Among the seven anions at lower concentrations, bromide, iodide, thiocyanate, nitrite and sulphide gave peaks for their PFB derivatives, after the optimization of the reaction and GC conditions. Table V gives the results of the regression analyses and indicates the applicability of the method to the determination of these five anions.

TABLE V

REGRESSION ANALYSIS FOR BIPHASIC REACTION SYSTEM (ECD)

X = concentration of anion (μM); Y as in Table III.

Anion	Concentration range (μM)	Regression equation	Correlation coefficient
Br^-	0.5 - 5.0	Y = 0.0945X + 0.2058	0.9972
I^-	2.5 - 25	Y = 0.1802X + 0.1275	0.9964
SCN^-	5.0 - 50	Y = 0.0854X - 0.1403	0.9987
NO_2^-	7.0 - 70	Y = 0.0210X + 0.0420	0.9966
S^{2-}	10 - 100	Y = 0.0033X + 0.1824	0.9975

Fig. 3 shows a typical gas chromatogram for the determination of iodide with ECD.

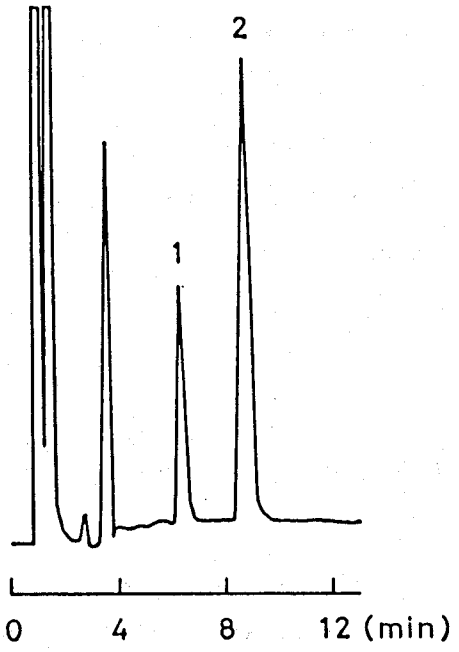

Fig.3 Typical gas chromatogram for iodide determination with ECD.
Peaks: 1 = 1,3,5-trichlorobenzene; 2 = pentafluorobenzyl iodide.

SUMMARY

A new derivatizing reagent, pentafluorobenzyl methanesulphonate, has been synthesized to determine inorganic anions by gas chromatography. Bromide, iodide, cyanide, thiocyanate, nitrite, nitrate and sulphide in water at mM levels could be simultaneously pentafluorobenzylated in dichloromethane, using tetra-n-amylammonium chloride as a phase- transfer catalyst. The resulting derivatives were subsequently determined by gas chromatography with flame ionization detection. In water at μM

levels, except for cyanide and nitrate, the anions could also be derivatized to their pentafluorobenzyl derivatives in the biphasic reaction system and be detected by gas chromatography with electron-capture detection. In addition to the biphasic reaction method, bromide, iodide and thiocyanate were simultaneously pentafluorobenzylated in a homogeneous reaction system of aqueous acetone or aqueous methanol, without the use of the phase-transfer catalyst.

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I-2 Polymeric Sulfonate-Type Derivatizing Reagents for Gas Chromatography

Polymeric reagents have some well-recognized advantages over conventional, low molecular weight reagents. The most obvious advantage of polymeric reagents is their easy separability from low molecular weight compounds by a simple operation such as precipitation and/or filtration. In chapter I-1, the author described pentafluorobenzylation of inorganic anions with pentafluorobenzyl methanesulfonate. After the derivatization, the excess reagent could not be removed from the reacted solutions by simple operations. Therefore, the author was obliged to inject the reagent together with the derivatives into a gas chromatograph. This resulted in the large background. In order to reduce it, an attempt was made to introduce a polymeric reagent and to remove it from the reaction mixtures by a simple operations of precipitation or of filtration.

In this section, the author described polymeric reagents, poly(pentafluorobenzyl p-styrenesulfonate) and Copoly(pentafluorobenzyl p-styrenesulfonate-Divinylbenzene), and their applications to the GC determination of inorganic anions and of carboxylic acids.

I-2-1. Poly(pentafluorobenzyl p-styrenesulfonate) as a Pentafluorobenzylating Reagent for Inorganic Anions

INTRODUCTION

Pentafluorobenzyl bromide(PFB-Br)[1-4] and Pentafluorobenzyl p-toluenesulfonate(PFB-OTs)[5] have been already reported as pentafluorobenzylating reagents for inorganic anions. In the previous section, pentafluorobenzyl methanesulfonate (PFB-OMs)[6] were synthesized and used to pentafluorobenzylate several inorganic anions simultaneously. However, these anions could be determined only down to about 100 ng/ml levels, which are still about 1 or 2 orders of magnitude higher than the lowest detection levels usually expected with ECD. This low sensitivity is considered to result from the injection of a large excess of unreacted PFB-OTs or PFB-OMs into the GC column together with the pentafluorobenzyl derivatives of interest. Therefore, in order to increase the sensitivity, no unreacted reagents should be injected into the column. It is generally difficult and time-consuming to remove chromatographically the excess reagents from solutions containing the pentafluorobenzyl derivatives. Even if it is possible, the precision of the analytical results could be poor owing due to the inconsistent recovery or the loss of the pentafluorobenzyl derivatives. An attempt was described in this section to introduce a polymeric pentafluorobenzylating reagent, because of its easy removal from the reacted solutions by a simple operation such as reprecipitation and/or filtration.

In this research, a polymeric pentafluorobenzylating reagent, poly(pentafluorobenzyl p-styrenesulfonate)(HP-PFB) was synthesized by polymerization of pentafluorobenzyl p-styrenesulfonate and its applicability to the determination of inorganic anions was investigated.

EXPERIMENTAL

Apparatus

A Yanaco G-2800 EN gas chromatograph equipped with an ECD unit of non-radioactive type (Kyoto, Japan) was used. The column was a coiled glass tube (3 m X 3 mm i.d.) packed with 5 % OV-225 (1.9 m) and 5 % OV-210 (1.1 m) on Uniport HP (60-80 mesh) in series. The injection port and detector temperatures were maintained at 200 ^{o}C. The column temperature was varied with each inorganic anion and is given in Table I. Helium (>99.99 %) was used as the carrier gas at a constant flow rate of 30 ml/min. The peak areas were computed by a Shimadzu Chromatopac C-R3A data processor (Shimadzu,Kyoto, Japan).

Chemicals

Commercial grade tetra-n-amylammonium chloride (TAAC) was obtained from Wako (Osaka, Japan). The inorganic anions were used as their sodium or potassium salts, of analytical-reagent grade. All other chemicals were also of analytical-reagent grade. Solutions of inorganic anions and TAAC were prepared by

dissolving them in distilled, deionized water. Michaelis (pH 2-3), McIlvaine (pH 4-8) and Kolthoff (pH 9-12) solutions were prepared.

Preparation of HP-PFB

To a solution of 10.0 g of pentafluorobenzyl alcohol and 20.0 g of p-styrenesulfonyl chloride in 50 ml of tetrahydrofuran were added 20 ml of 5 M sodium hydroxide solution with stirring, keeping the temperature of the reaction mixture at 10-15 ^{o}C. After stirring for a further 4 h, 100 ml of dichloromethane were added. The dichloromethane layer was washed with water and then dried over anhydrous magnesium sulfate. Pentafluorobenzyl p-styrenesulfonate, isolated in the usual way was recrystallized from 1-propanol. Anal. Calcd for $C_{15}H_9SO_3F_5$: C, 49.45; H, 2.47. Found: C, 49.41; H, 2.46. IR: 650 cm^{-1} ($-CH=CH_2$), 1180 and 1360 cm^{-1} ($-O-SO_2-$). MS: m/z=364 (M^+), m/z=181 ($C_6F_5CH_2^+$).

HP-PFB was obtained by radical homopolymerization of 1.5 g of the monomer in a sealed glass tube containing 0.02 g of 2,2'-azobisisobutyronitrile in 8 ml of acetone. After standing for 20 h at 60 ^{o}C, HP-PFB was isolated by precipitation in diethyl ether, purified at least twice by reprecipitation from the same solvent, and then dried in vacuo for 12 h. Anal. Calcd for $(C_{15}H_9SO_3F_5)_n$: C, 49.45; H, 2.47. Found: C, 49.41; H, 2.40.

Derivatization procedures

In a brown-colored test tube with a screw cap (ca. 10 ml) were placed 1.0 ml of an aqueous solution of each anion reference

standard, 1.0 ml of a dichloromethane solution of HP-PFB, 0.20 ml of TAAC aqueous solution and 0.10 ml of a buffer solution. The reaction tube was sealed tightly with the screw cap and shaken at room temperature for a fixed reaction time. After the separation of the dichloromethane layer, 0.5 ml of its aliquot was transferred into another test tube with a pipet. The dichloromethane solution was evaporated carefully under a gentle stream of nitrogen for 2 min at room temperature, and then to the residue was added 1.0 ml of tert-butyl methyl ether containing an internal standard. A 0.5-μl aliquot of the tert-butyl methyl

TABLE I

COLUMN TEMPERATURES AND INTERNAL STANDARDS

Internal standards: BCB = p-bromochlorobenzene; DBB = p-dibromobenzene; TBB = 1,3,5-tribromobenzene; TEBB = 1,2,4,5-tetrabromobenzene.

Anion	Column temperature (°C)	Internal standard (M)
Br^-	100	BCB (8.0×10^{-6})
I^-	105	BCB (8.0×10^{-6})
CN^-	135	TBB (1.6×10^{-7})
SCN^-	150	TBB (2.0×10^{-7})
NO_2^-	140	DBB (2.0×10^{-6})
NO_3^-	120	BCB (8.0×10^{-6})
S^{2-}	180	TEBB (2.0×10^{-7})

ether solution was injected into the gas chromatograph under the conditions indicated in Table I.

RESULTS AND DISCUSSION

Pentafluorobenzylation of inorganic anions with HP-PFB

The homopolymerization product of pentafluorobenzyl p-styrenesulfonate, HP-PFB ([η] = 0.28 in N,N-dimethylformamide), showed no vinyl absorption at 650 cm^{-1}, and was insoluble in diethyl ether or benzene, which can dissolve the starting monomer.

Bromide, iodide, cyanide, thiocyanate, nitrite, nitrate and sulfide at μM levels could be derivatized to pentafluorobenzyl bromide, pentafluorobenzyl iodide, pentafluorobenzyl cyanide, pentafluorobenzyl thiocyanate, α-nitro-2,3,4,5,6-pentafluorotoluene, pentafluorobenzyl nitrate and bis(pentafluorobenzyl) sulfide, respectively. The effects of HP-PFB and TAAC concentrations, the pH of the buffer solution added and reaction time on the derivatization were examined by using the peak area ratio of the derivative to the internal standard, in order to establish the optimum pentafluorobenzylation conditions for each inorganic anion except for nitrate.

Effects of HP-PFB and TAAC concentrations. Fig. 1 shows the effect of HP-PFB concentration (1.0×10^{-5}-5.0×10^{-3} M at monomeric-unit concentration) on the peak area ratio. To ensure a constant formation of each anion derivative, each optimum concentration of HP-PFB chosen in Table II is higher than the

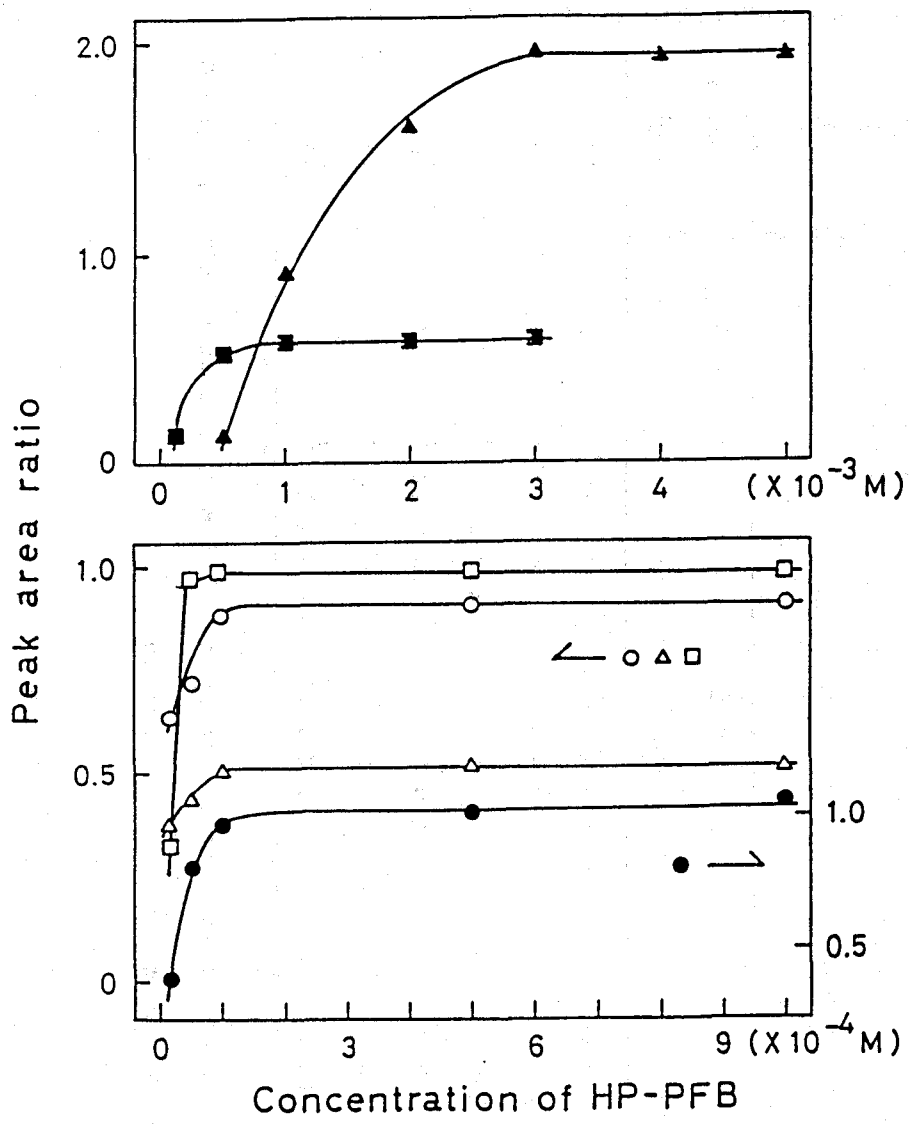

Fig.1 Effect of HP-PFB concentration on pentafluorobenzylation of inorganic anions : (△) bromide, (○) iodide,(▲) cyanide, (□) thiocyanate, (●) nitrite, (■) sulfide.

TABLE II

OPTIMUM PENTAFLUOROBENZYLATION CONDITIONS

Anion		Concentration (M)		pH of	Reaction
concentration (ng/ml)		HP-PFB	TAAC	buffer*	time (min)
Br^-	64.0	5.0×10^{-4}	1.0×10^{-3}	7	10
I^-	127	1.0×10^{-3}	4.0×10^{-4}	7	10
CN^-	130	5.0×10^{-3}	4.0×10^{-3}	11	10
SCN^-	58.0	1.0×10^{-3}	4.0×10^{-4}	7	10
NO_2^-	128	1.0×10^{-3}	1.0×10^{-3}	7	30
S^{2-}	64.0	1.0×10^{-3}	1.0×10^{-3}	7	30

*pH=7, 0.2 M disodium hydrogen phosphate-0.1 M citric acid; pH=11, 0.05 M sodium carbonate-0.05 M sodium borate.

lowest value giving the constant formation in Fig. 1 (double for cyanide and sulfide; five times for bromide; ten times for iodide, thiocyanate and nitrite).

Similarly, the effect of TAAC concentration (1.0×10^{-4}-4.0×10^{-3} M) on the peak area ratio was determined. The optimum TAAC concentrations for the derivatization of the anions are also given in Table II. The use of higher concentrations of HP-PFB and TAAC than those listed in Table II only resulted in an increase in the peaks from the reagent blank.

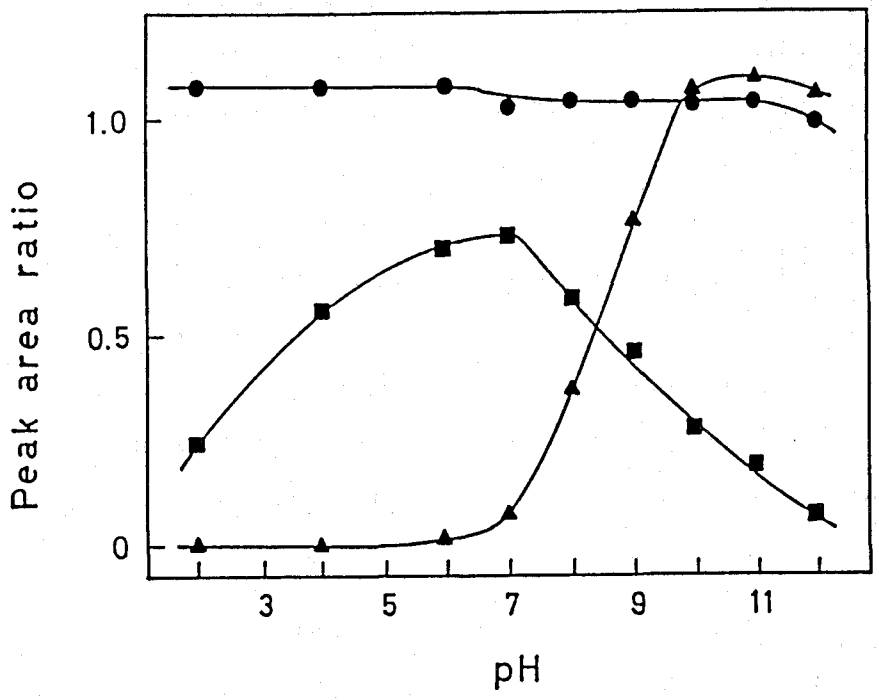

Fig.2 Effect of pH of buffer solutions on pentafluorobenzylation of inorganic anios : (▲) cyanide, (●) thiocyanate, (■) nitrite.

Effects of pH and reaction time. The derivatization of the anions was performed at various pH values (2-12). Fig. 2 shows the results for cyanide, nitrite and thiocyanate. The peak area ratios of the first two anions were dependent on the pH of the buffer added: the optimum pH values were 11 and 7 for cyanide and nitrite, respectively. On the other hand, thiocyanate, and also as bromide, iodide and sulfide, did not exhibit such a clear pH dependence. Therefore, the buffer at pH 7 was used for the pentafluorobenzylation of the five anions except for cyanide (pH 11).

The effect of reaction time on the derivatization at the optimum pH was also examined in the range 10-90 min at room temperature. The results indicated that the reaction times needed were short and they were set as listed in Table II.

It is well known that the detector temperature considerably affects the ECD response. Therefore, the effect of detector temperature was tested up to 300 ^{o}C for iodide, cyanide or nitrite. A small increment in the detector response was observed on increasing the temperature, but the fluctuation of the baseline was also elevated. As a compromise, a detector temperature of 200 ^{o}C was employed.

Analytical calibration. After the optimum pentafluorobenzylation conditions for each inorganic anion had been established as in Table II, the quantitative application of the method to the determination of the anions at ng/ml levels was evaluated. Each calibration graph was constructed by plotting the peak area ratio versus the anion concentration in aqueous

TABLE III

CALIBRATION CURVES FOR INORGANIC ANIONS

Y = peak-area ratio of PFB derivative of anion to internal standard; X = concentration of anion (ng/ml).

Anion	Concentration range (ng/ml)	Regression equation	Correlation coefficient
Br^-	6.4 - 64.0	Y = 0.0215X + 0.0348	0.9979
I^-	12.7 - 127	Y = 0.0077X + 0.0004	0.9999
CN^-	13.0 - 130	Y = 0.0050X - 0.0144	0.9991
SCN^-	5.8 - 58.0	Y = 0.0243X - 0.0094	0.9992
NO_2^-	18.4 - 184	Y = 0.0050X + 0.0105	0.9985
S^{2-}	1.3 - 13.0	Y = 0.0625X - 0.0008	0.9992

solution. Table III gives the results of the regression analyses and indicates good applicability of the method to the determination. The molar ratio of the monomer unit in HP-PFB to the anion establishes the upper limit of the working range. Under the optimum HP-PFB concentration employed in this work (Table II), the derivatization yield was constant up to anion concentrations of 320, 1270, 260, 580, 1280 and 130 ng/ml for bromide, iodide, cyanide, thiocyanate, nitrite and sulfide, respectively, and thereafter began to decrease.

As described at the beginning of this section, nitrate also gave the pentafluorobenzyl derivative. However, good linear relationship was not observed for nitrate, because its peak area gradually increased even up to the second day after the addition of tert-butyl methyl ether to the residue obtained by evaporating the dichloromethane solution. This is probably due to the slow

reaction between the unreacted ion-pair of nitrate initially extracted into the dichloromethane layer and the precipitated HP-PFB formed on the addition of the ether.

Among the solvents tested, dichloromethane was the most suitable one for pentafluorobenzylation of the anions in the biphasic reaction system. Unfortunately, it is apparently unfavorable for ECD, owing to high solvent response. As a compromise, dichloromethane was used for the derivatization reaction and was then replaced with tert-butyl methyl ether for ECD, as described under the experimental section. Because HP-PFB is insoluble in tert-butyl methyl ether, no injection of the pentafluorobenzylating reagent into the gas chromatograph can occur with this solvent replacement, which results in an effectively high sensitivity of the method using HP-PFB as described below. Typical gas chromatograms for the six anions are shown in Fig. 3, together with those for the blank.

It is interesting and of value to compare the results obtained with different pentafluorobenzylating reagents for inorganic anions. Table IV summarizes the determination ranges of the anions with three reagents, PFB-Br, PFB-OTs and HP-PFB. Considering both the number of anions derivatized and their determination ranges, the superiority of the method using HP-PFB over the others is apparent.

The PFB-OTs method is much less sensitive than the HP-PFB method, in spite of their similar biphasic reaction systems; the determination ranges for the PFB-OTs method are too high compared with those usually expected for ECD. This is ascribed

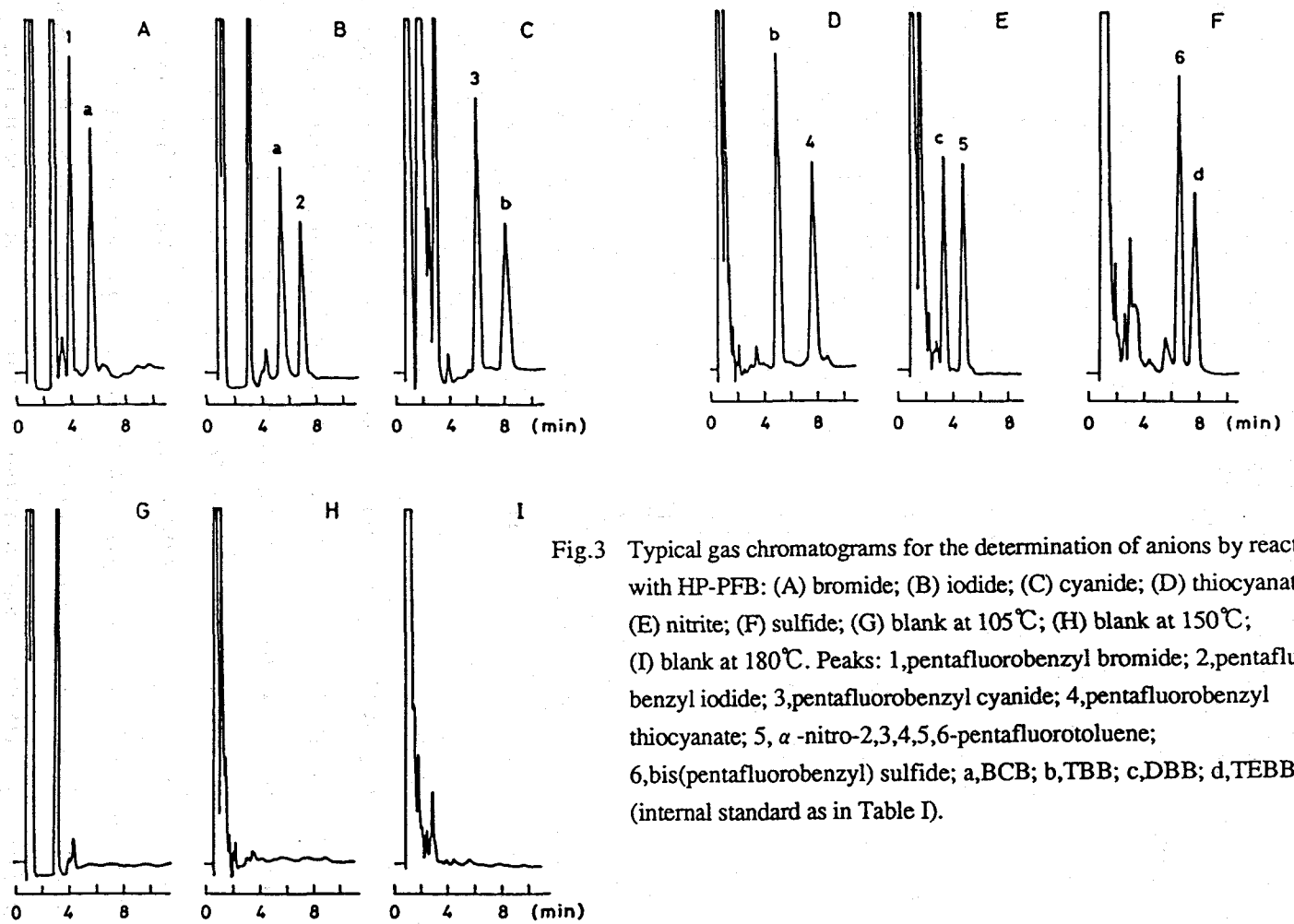

Fig.3 Typical gas chromatograms for the determination of anions by reaction with HP-PFB: (A) bromide; (B) iodide; (C) cyanide; (D) thiocyanate; (E) nitrite; (F) sulfide; (G) blank at 105℃; (H) blank at 150℃; (I) blank at 180℃. Peaks: 1,pentafluorobenzyl bromide; 2,pentafluorobenzyl iodide; 3,pentafluorobenzyl cyanide; 4,pentafluorobenzyl thiocyanate; 5, α -nitro-2,3,4,5,6-pentafluorotoluene; 6,bis(pentafluorobenzyl) sulfide; a,BCB; b,TBB; c,DBB; d,TEBB (internal standard as in Table I).

to the reagent background contribution as follows. First, there was no great difference in the reactivity between PFB-OTs and HP-PFB. Next, in an experiment the tert-butyl methylether solution of the reagent blank from HP-PFB containing no analyte anion was injected into the gas chromatograph with the ECD unit, the detector response returned normally to its original level after a sharp rising due to the solvent. In contrast, this did not the case with PFB-OTs. The rise of the baseline level with PFB-OTs means a decrease in the sensitivity of the PFB-OTs method. Fig. 4

Fig.4

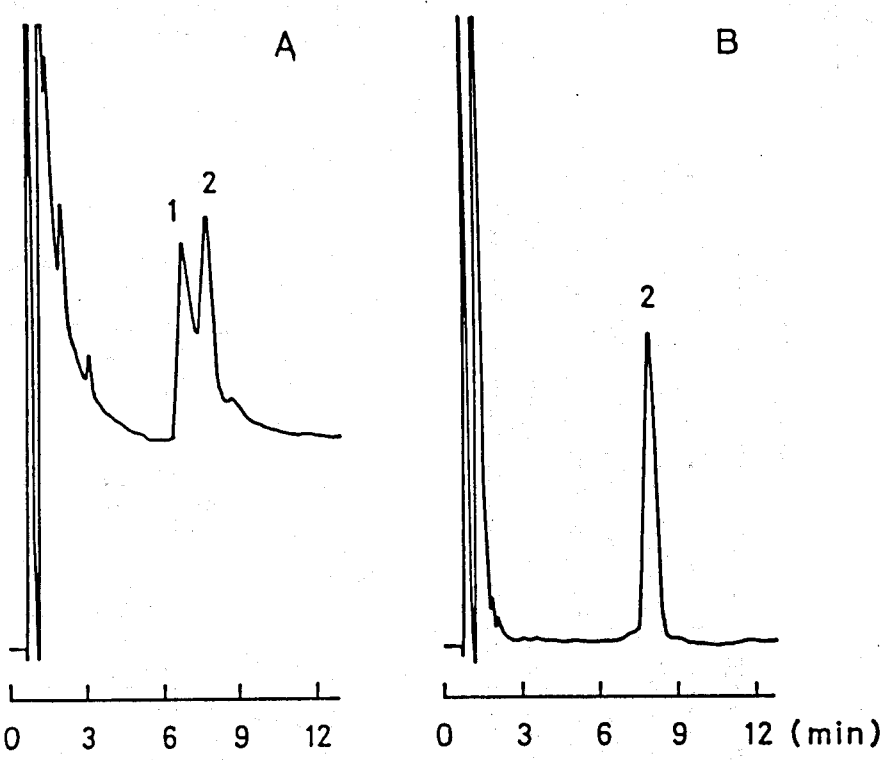

Fig.4 Gas chromatograms for the determination of thiocyanate at 580 ng/ml: (A) $2x10^{-3}$ M PFB-OTs, $4x10^{-4}$ M TAAC; (B) $2x10^{-3}$ M HP-PFB, $4x10^{-4}$ M TAAC. Peaks: 1,unknown; 2,pentafluorobenzyl thiocyanate.

TABLE IV INTERCOMPARISON OF PENTAFLUOROBENZYLATING REAGENTS FOR INORGANIC ANIONS

Anion	Determination range (ng/ml)		
	PFB-Br*	PFB-OTs**	HP-PFB**
Br^-		150 - 1500	6.4 - 64.0
I^-	50 - 500	400 - 4000	12.7 - 127
CN^-	10 - 400		13.0 - 130
SCN^-		900 - 9000	5.8 - 58.0
NO_2^-	20 - 400	600 - 6000	18.4 - 184
S^{2-}	8 - 650		1.3 - 13.0

*Derivatization in aqueous acetone.

**Derivatization in the biphasic reaction system.

illustrates typical results for the determination of 580 ng/ml of thiocyanate and clearly shows the superiority of the HP-PFB method over the PFB-OTs method. This result is due to the fact that there is no injection of HP-PFB into the gas chromatograph.

SUMMARY

Poly(pentafluorobenzyl p-styrenesulfonate) has been synthesized as a pentafluorobenzylating reagent for inorganic anions. Bromide, iodide, cyanide, thiocyanate, nitrite and sulfide in water were readily converted into their pentafluorobenzyl derivatives by reaction with the reagent dissolved in dichloromethane in the presence of tetra-n-amylammonium chloride as phase transfer catalyst. The replacement of dichloromethane with tert-butyl methyl ether can precipitate the reagent and prevent its interference. The injection of the derivatives into a gas chromatograph equipped with an electron capture detector

without introducing the derivatizing reagent can be achieved, only by injecting the supernatant solutions. The use of the polymeric reagent has greatly facilitated this process and resulted in the sensitive determination of the inorganic anions at ng/ml levels.

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I-2-2. Copoly(pentafluorobenzyl p-styrenesulfonate-divinylbenzene) as a Pentafluorobenzylating Reagent for Inorganic Anions and Carboxylic Acids

INTRODUCTION

In chapter I-2-1, the author developed a method using a polymeric pentafluorobenzylating reagent, poly(pentafluorobenzyl p-styrenesulfonate)[1]. In that method, the anions in water were pentafluorobenzylated by reaction with the polymeric reagent dissolved in dichloromethane in the presence of tetra-n-amylammonium chloride as phase-transfer catalyst. After the reaction, the dichloromethane solution was evaporated, and then to the residue was added tert-butyl methyl ether, which does not dissolve the polymeric reagent. This step prevented the reagent from being injected into the gas chromatograph and increased the sensitivity as expected. However, this solvent replacement must be performed carefully under constant conditions, in order to obtain the good precision of the analytical results. Therefore, the author attempted to introduce an insoluble polymeric pentafluorobenzylating reagent, because it is quite easily removed from the reacted solution by a simple operation of centrifugation.

In this research, the author synthesized an insoluble polymeric pentafluorobenzylating reagent, copoly(pentafluorobenzyl p-styrenesulfonate-divinylbenzene) (CP-PFB), and investigated its applicability to the determination of inorganic anions or lower saturated carboxylic acids as their

pentafluorobenzyl derivatives by GC-ECD.

EXPERIMENTAL

Apparatus

A Yanaco-2800 EN gas chromatograph equipped with a non-radioactive ECD unit (Kyoto, Japan) was used. For the determination of inorganic anions, the column was a coiled glass tube (3 m X 3 mm i.d.) packed with 5 % OV-225 (1.9 m) and 5 % OV-210 (1.1 m) on Uniport HP (60-80 mesh) in series; the injection port and detector temperatures were maintained at 200 ^{o}C. For the determination of carboxylic acids, a glass column (4 m X 3 mm i.d.) packed with 3 % OV-17 on Uniport HP (60-80 mesh) was used; the injection port and detector temperatures were 230 and 250 ^{o}C, respectively. The column temperature was varied with each analyte as given in Table I. Helium was used as the carrier gas at a constant flow-rate of 30 ml/min. The peak areas were computed by a Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan).

Chemicals

The inorganic anions were used as their sodium or potassium salts, of analytical reagent grade. All other chemicals were also of analytical reagent grade.

Preparation of CP-PFB

CP-PFB was obtained by radical copolymerization of 1.0 g of pentafluorobenzyl p-styrenesulfonate and 0.1 g of 55 % divinylbenzene in a three-necked flask containing 0.05 g of benzoyl peroxide in 40 ml of toluene and 0.05 g of poly(vinyl alcohol) in 60 ml of water. After 10 h at 90 ^{o}C under stirring (300 r.p.m.), CP-PFB was collected by filtration, washed thoroughly with acetone, water and acetone, and finally dried in vacuo for 12 h.

Derivatization procedure

In a brown test-tube with a screw-cap (ca. 10 ml) were placed 0.08 ml of aqueous reference standard solution of inorganic anion, 0.02 ml of buffer solution, 1.0 ml of acetone containing an internal standard and ca. 20 mg of CP-PFB. The reaction tube was sealed tightly with the screw-cap and shaken for a fixed reaction time. Then anhydrous magnesium sulfate was added to the reaction mixture, and it was centrifuged. A 0.2-µl aliquot of the resulting supernatant solution was injected into the gas chromatograph under the conditions indicated in Table 1.

In the same tube, 1 ml of acetone solution of carboxylic acid, 0.1 ml of acetone containing an internal standard and ca. 10 mg of CP-PFB were stirred in the presence of ca. 5 mg of anhydrous KF. In derivatizing butyric acid, 0.1 ml of 1.0 X 10^{-4} M 18-crown-6 and ca. 10 mg of anhydrous K_2CO_3 were used in place of anhydrous KF. After centrifugation, a 0.5-µl aliquot of the supernatant solution was injected similarly.

TABLE I

COLUMN TEMPERATURES AND INTERNAL STANDARDS

Internal standards: BCB = p-bromochlorobenzene; TBB = 1,3,5-tribromobenzene; TEBB = 1,2,4,5-tetrabromobenzene; TECB = 1,2,3,4-tetrachlorobenzene; DBB = p-dibromobenzene.

Analyte	Column temperature (°C)	Internal standard	Concentration (M)
Br^-	100	BCB	5.0×10^{-6}
I^-	105	BCB	5.0×10^{-6}
SCN^-	150	TBB	2.0×10^{-7}
S^{2-}	180	TEBB	2.0×10^{-7}
n-Butyric acid (n-C_4)	160	TECB	8.0×10^{-5}
n-Valeric acid (n-C_5)	170	DBB	4.0×10^{-5}
Isovaleric acid (i-C_5)	170	TECB	8.0×10^{-5}
n-Caproic acid (n-C_6)	170	TECB	8.0×10^{-5}
n-Caprylic acid (n-C_8)	190	TBB	1.0×10^{-5}

RESULTS AND DISCUSSION

Pentafluorobenzylation of inorganic anions

As mentioned in chapter I-2-1, in the case of the biphasic reaction system of dichloromethane-water using poly(pentafluorobenzyl p-styrenesulfonate) and tetra-n-amylammonium chloride, the replacement of dichloromethane with tert-butyl methyl ether, a solvent accepted for ECD, is inevitable, in addition to precipitating the polymeric reagent dissolved in dichloromethane. For simplification of the analytical procedure, it is of great interest and advantage to use a water-miscible organic solvent amenable to ECD, together with a solid, pentafluorobenzylating reagent in the reaction medium. The reason is clear that there is no need to replace the solvent nor to precipitate the reagent. Therefore, the author attempted to pentafluorobenzylate inorganic anions dissolved in aqueous acetone with CP-PFB suspended in it, by no use of a phase-transfer catalyst such as tetra-n-amylammonium chloride. Among anions tested, bromide, iodide, thiocyanate and sulfide could be readily derivatized to pentafluorobenzyl bromide, pentafluorobenzyl iodide, pentafluorobenzyl thiocyanate and bis(pentafluorobenzyl) sulfide, respectively. Consequently, the derivatization of these four anions was optimized. The evaluation of the effects of the pH of the buffer solution added, reaction time and reaction temperature was based on the peak-area ratio of each anion derivative to the respective internal standard.

Effect of pH. The derivatization of the anions was performed at various pH values (2-12). The peak-area ratios of the bromide,

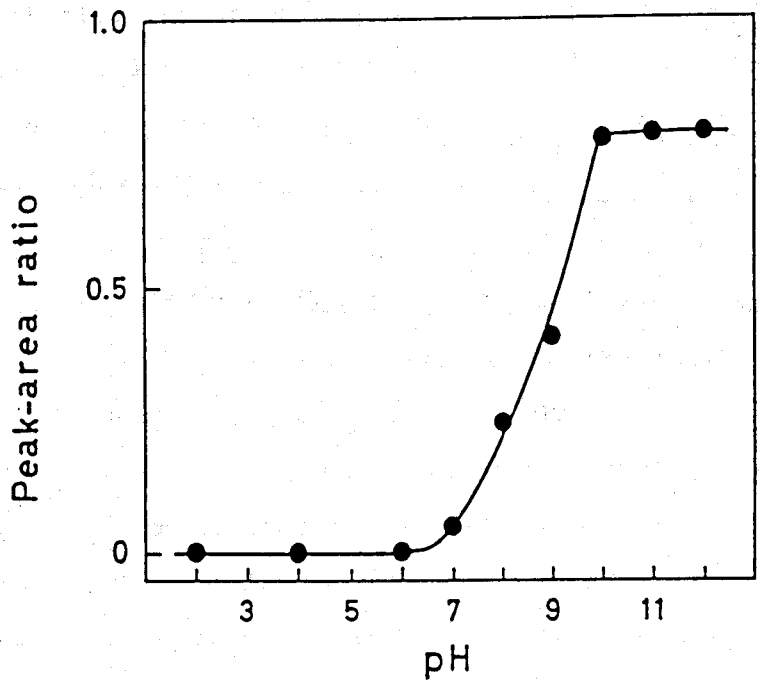

Fig.1 Effect of pH of buffer solution on pentafluorobenzylation of sulfide.

iodide and thiocyanate derivatives were independent of the pH of the buffer added. On the other hand, as shown in Fig. 1, sulfide exhibited a clear pH dependence: no derivative was obtained at pH below 6, and its formation became constant at pH above 10. Therefore, buffer at pH 12 was used for the pentafluorobenzylation of sulfide and that at pH 7 for the other anions.

Effects of reaction time and reaction temperature. The pentafluorobenzylation at the optimum pH fixed as above was carried out for reaction times between 10-90 min in the temperature range of 30-50 °C in steps of 10 °C. The results

obtained are shown in Fig. 2. Bromide, iodide and sulfide gave the constant peak-area ratios by derivatizing even for 10 min at each temperature. On the contrary, this is not the case with thiocyanate. Its derivatization proceeded considerably slow at

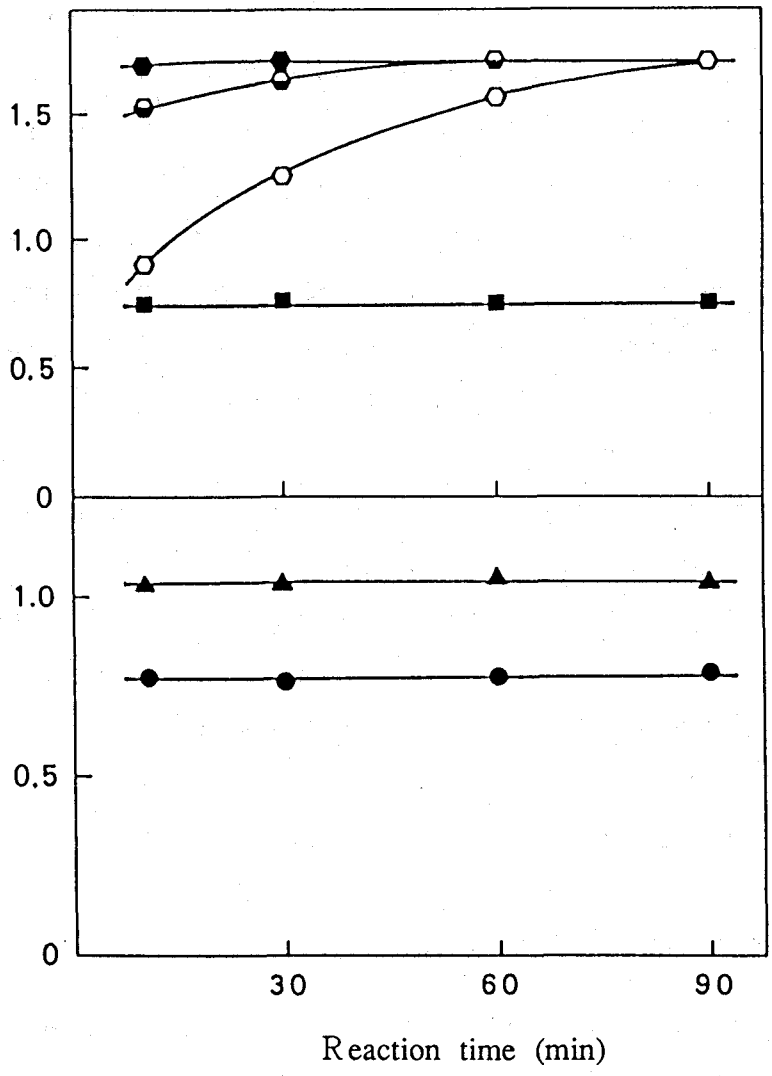

Fig.2 Effects of reaction time and reation temperature on pentafluorobenzylation of sulfide (●), bromide (▲), and iodide (■), at 30 - 50 ℃, and thiocyanate at 30 ℃ (⬡), 40 ℃ (⬙) and 50 ℃ (⬢).

30 °C. Therefore, the reaction temperature had to be raised to 50 °C, in order to obtain a constant formation of the derivative within a short time. The optimum pentafluorobenzylation conditions thus fixed are listed in Table II.

Analytical calibration. Six different concentrations of reference standard solutions containing each inorganic anion were evaluated, to examine the quantitative applicability of the method to the determination of the anions at ng/ml levels. Each calibration curve was constructed by plotting the peak-area ratio versus the anion concentration in the solution. Table III gives the results of the regression analyses and indicates good applicability of the method to the determination.

After the derivatization reaction, anhydrous magnesium sulfate was added to the reaction mixture lest water in it be introduced to the ECD cell. Then the mixture was centrifuged to settle suspended CP-PFB and inject the supernatant solution into the gas chromatograph. It is apparent that this process is much simpler, compared with that of the solvent replacement for the biphasic reaction system. Typical gas chromatograms for the four anions are shown in Fig. 3.

Pentafluorobenzylation of carboxylic acids

CP-PFB was also applied to the determination of carboxylic acids in addition to the inorganic anions. n-Butyric (n-C_4), n-valeric (n-C_5), isovaleric (i-C_5), n-caproic (n-C_6) and n-caprylic (n-C_8) acids were examined as model analytes.

TABLE II

OPTIMUM PENTAFLUOROBENZYLATION CONDITIONS

Analyte	Analyte concentration (ng/ml)	pH of buffer*	Reaction time (min)	Reaction temterature (°C)
Br^-	160	7	10	30
I^-	250	7	10	30
SCN^-	290	7	30	50
S^{2-}	130	12	10	30
$n\text{-}C_4$	88	-	30	50
$n\text{-}C_5$	103	-	30	50
$i\text{-}C_5$	103	-	40	50
$n\text{-}C_6$	116	-	40	50
$n\text{-}C_8$	144	-	40	50

* pH 7, 0.2 M disodium hydrogen phosphate-0.1 M citric acid; pH 12, 0.1 M disodium hydrogen phosphate-0.1 M sodium hydroxide.

TABLE III

CALIBRATION CURVES FOR ANALYTES

Analyte	Concentration range (ng/ml)	Regression equation*	Correlation coefficient
Br^-	16.0 - 160	Y = 0.6453X + 0.0039	0.9980
I^-	25.0 - 250	Y = 0.3002X + 0.0074	0.9994
SCN^-	29.0 - 290	Y = 0.5034X + 0.0121	0.9990
S^{2-}	13.0 - 130	Y = 0.7878X - 0.0044	0.9988
$n\text{-}C_4$	9.0 - 90	Y = 0.0101X - 0.0276	0.9982
$n\text{-}C_5$	10.0 - 100	Y = 0.0129X + 0.0901	0.9996
$i\text{-}C_5$	10.0 - 100	Y = 0.0131X + 0.0352	0.9990
$n\text{-}C_6$	12.0 - 120	Y = 0.0097X + 0.1006	0.9989
$n\text{-}C_8$	13.0 - 130	Y = 0.0068X + 0.0262	0.9995

* Y = peak-area ratio of pentafluorobenzyl derivative of analyte to internal standard; X = concentration of analyte (ng/ml).

The derivatization was performed under various reaction conditions. The optimized pentafluorobenzylation conditions fixed for these acids are also given in Table II. The acids were derivatized nearly quantitatively in the presence of potassium fluoride under these conditions. In this case, the peak of the $n\text{-}C_4$ derivative overlapped that from the blank. Therefore, $n\text{-}C_4$ acid was pentafluorobenzylated in the presence of both potassium carbonate and 18-crown-6, in place of potassium fluoride, as described in the Experimental section. This potassium carbonate-18-crown-6 pair used widely gave almost the same derivatization

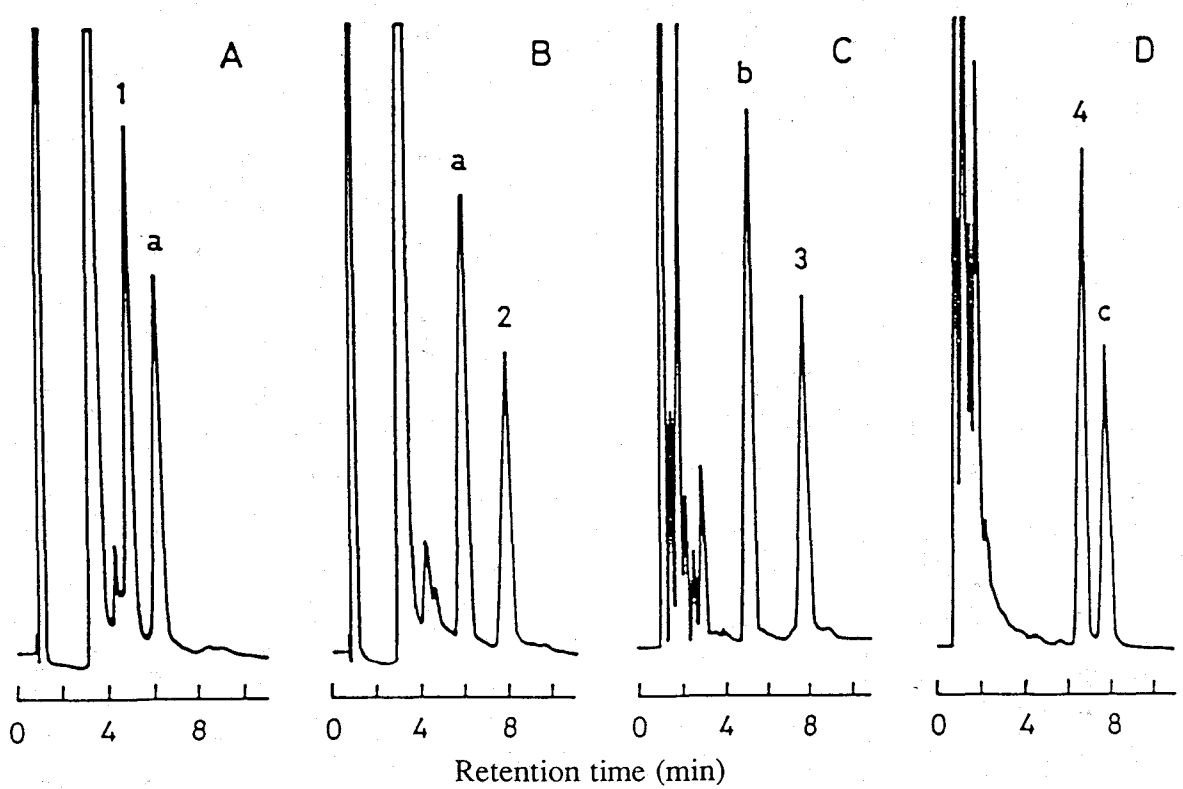

Fig.3 Typical gas chromatograms for the determination of anios: A, bromide; B, iodide; C, thiocyanate; D,sulfide. Peaks: 1, pentafluorobenzyl bromide; 2, pentafluorobenzyl iodide; 3, pentafluorobenzyl thiocyanate; 4, bis(pentafluorobenzyl) sulfide; a, p-bromochlorobenzene; b, 1,3,5-tribromobenzene; c, 1,2,4,5-tetrabromobenzene.

yield for each acid, compared with potassium fluoride. The derivatization reactions, however, proceeded more speedily in the presence of potassium fluoride. Calibration curves for the acids were constructed. The results of the regression analyses are given in Table III, and it shows good applicability of the method to the determination. Fig. 4 shows typical gas chromatogram for the derivatives of five carboxylic acids reacted simultaneously in the presence of potassium fluoride or potassium carbonate-18-crown-6. As mentioned above, it is apparent that the peak of the n-C_4 acid derivative overlaps the blank peak (marked with an arrow in Fig. 4A).

SUMMARY

Copoly(pentafluorobenzyl p-styrenesulfonate-divinylbenzene) was synthesized as an insoluble pentafluorobenzylating reagent for inorganic anions and carboxylic acids. Bromide, iodide, thiocyanate and sulfide were readily converted into their pentafluorobenzyl derivatives with the copolymeric reagent suspended in aqueous acetone. n-Butyric, n-valeric, isovaleric, n-caproic and n-caprylic acids were also derivatized to their pentafluorobenzyl esters in acetone. The injection of the derivative into a gas chromatograph with an electron capture detector, without introducing the derivatizing reagent, can be achieved only by injecting the supernatant solutions aftercentrifuging the reaction mixtures. This process using the insoluble polymeric reagent has resulted in the sensitive determination of the inorganic anions or the carboxylic acids at ng/ml levels.

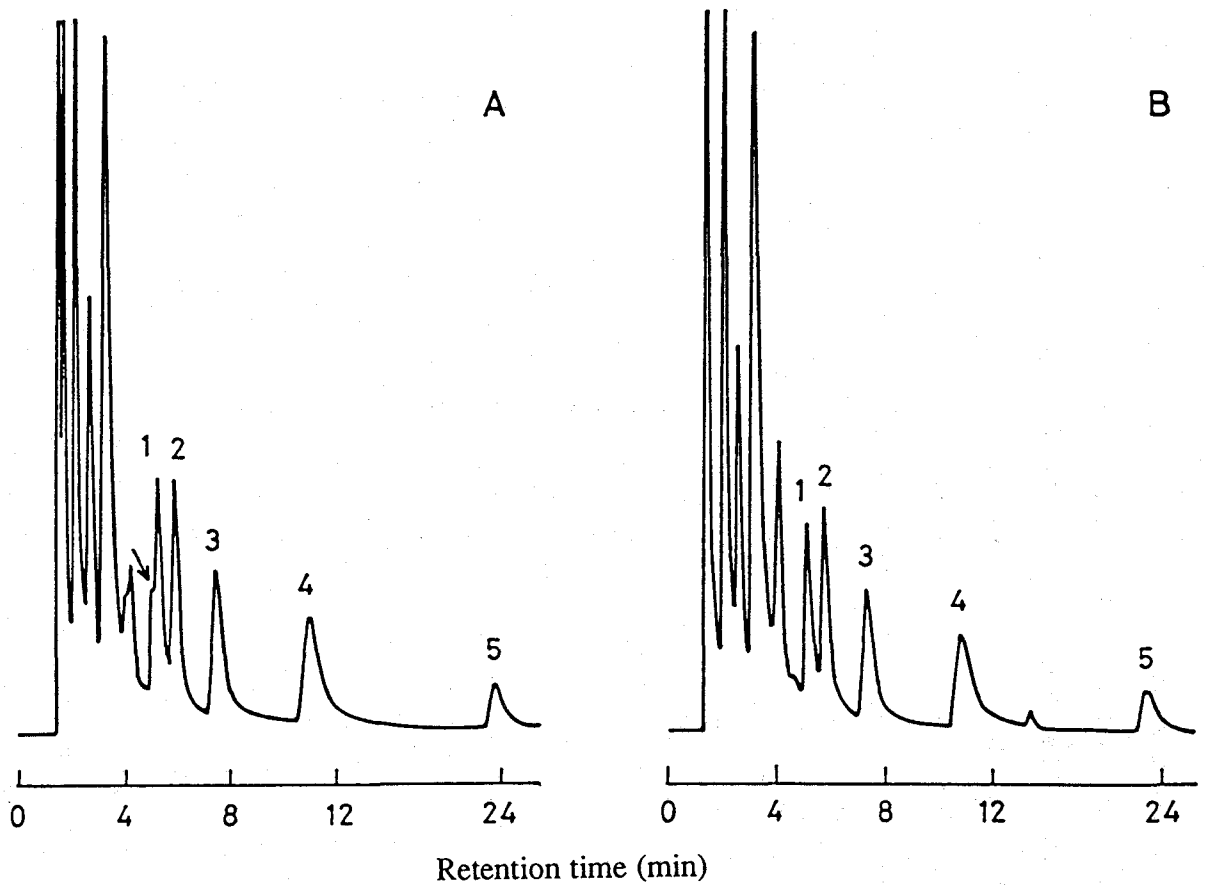

Fig.4 Gas chromatograms for pentafluorobenzyl derivatives of carboxylic acids simultaneously reacted at 50 ℃ for 40 min in the presence of potassium fluoride (A) and potassium carbonate-18-crown-6 (B). Peaks; 1, n-C_4; 2, i-C_5; 3, n-C_5; 4, n-C_6; 5, n-C_8.

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Chapter II Derivatizing Reagents for Liquid Chromatography

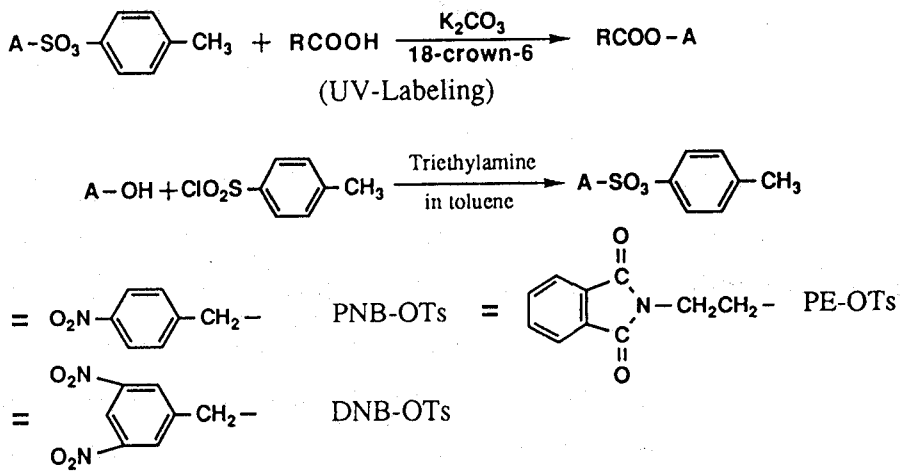
One of the major disadvantages of HPLC is a lack of detectors, particularly universal detectors, which can match the sensitivity of GC detectors. The best HPLC detectors currently available are photometric and fluorimetric detectors. It seems logical, therefore, to solve the immediate detection problems at least partially by UV- and fluorescence- derivatization techniques. By labeling poorly detectable compounds with suitable chromophores, fluorophores or other activity enhancing groups, the detectability can be improved, in some cases, to exceed the level of GC. The selectivity that can be, at the same time, gained by using labeling reagents of varied reactivity and perhaps also with favorable spectral qualities. This becomes important in the analysis of a complex matrix such as encountered in polluted water samples, biological specimens, pharmaceutical preparations, etc. The derivatization steps can also serve as a clean up procedure. In this chapter, was presented new sulfonate-type (tosylate-, polymeric sulfonate- and triflate-type) derivatizing reagents for UV- and fluorescence-labeling of carboxylic acids in HPLC.

II-1. p-Toluenesulfonate-Type Ultraviolet Labeling Reagents for Carboxylic Acids

INTRODUCTION

By the introduction of ion chromatography[1,2], the analyses using high-performance liquid chromatography (HPLC) have been widely extended. Ion chromatography has unmatched ability to determine trace inorganic anions[3], since a conductivity detector is used. However, it is difficult to determine trace amounts of organic anions by ion chromatography because of their low electric conductivities. In the HPLC determination of organic anions, a labeling technique has usually been used for enhancement of the sensitivity of UV or fluorescence detection, and various labeling agents have been developed[4,5]. For carboxylic acids, frequently used were O-(p-nitrobenzyl)-N,N'-diisopropylisourea[6], tolyltriazene derivatives (such as 1-benzyl-[7] and 1-p-nitrobenzyl-3-p-tolyltriazene[8]), and halomethyl compounds (such as substituted phenacyl bromide (p-bromo- and m-methoxyphenacyl bromide)[9-11],9-chloromethylanthracene[12], N-chloromethyl-4-substituted phthalimide[13,14], and 7-acetoxy and 7-methoxy-4-bromomethylcoumarins[15]).

In this work, three p-toluenesulfonate-type UV-labeling agents for HPLC have been synthesized; p-nitrobenzyl p-toluenesulfonate (PNB-OTs), 3,5-dinitrobenzyl p-toluenesulfonate (DNB-OTs) and 2-(phthalimino)ethyl p-toluenesulfonate (PE-OTs). Their applicabilities to UV-labeling and the HPLC determination of monocarboxylic acids have been examined, as shown in Scheme I.



Scheme I Preparation of tosylate-type labeling agents.

EXPERIMENTAL

Apparatus.

When using PNB-OTs or DNB-OTs as the reagent, the HPLC system comprised a Model KHD-W-52 pump, a Model KHG-250L pressure gauge, a Model KHP-UI-130 high-pressure universal injector (Kyowa Seimitsu, Tokyo, Japan), a special damper (Gasukuro Kogyo, Tokyo, Japan), and a Model UVD-2 fixed-wavelength (254 nm) UV absorption detector (Shimadzu, Kyoto, Japan). The separation column, YMC A-302 ODS (15 cm X 4.6 mm i.d.) 5 μm particle size), was obtained from Yamamura Chemical Laboratories (Kyoto, Japan).

When using PE-OTs, the system consisted of a Model 880-PU pump (Japan Spectroscopic Co., Ltd., Tokyo, Japan), a Model 7125

syringe loading sample injector (Rheodyne, Cotati, California, U.S.A.) and a Model 875-UV variable-wavelength UV absorption detector (Japan Spectroscopic Co., Ltd.) operating at 222 nm. The separation column (15 cm X 4.6 mm i.d.) packed with Tosoh (Tokyo, Japan) ODS-80TM (5 μm particle size) was used together with a precolumn (3 cm X 4.6 mm i.d.) containing Chemco (Osaka, Japan) Nucleosil 5SB anion exchanger, to remove p-toluenesulfonic acid and other by-products interfering with the detection at 222 nm.

In both cases, the mobile phase was acetonitrile at a constant flow rate of 0.5 ml/min. A Shimadzu Chromatopac C-R6A data processor was used as the recorder and integrator.

A Hitachi RMU-6E mass spectrometer was employed with an ionization source temperature of 200 ^{o}C and an acceleration energy of 1.8 kV.

Reagents

Analytical reagent grade 18-crown-6 was obtained from Aldrich (Milwaukee, Wisconsin, U.S.A.), and monocarboxylic acids used were of analytical reagent grade from Wako (Osaka, Japan) and Tokyo Kasei (Tokyo, Japan). Acetonitrile was distilled before use for the labeling-reaction solvent, and it was further filtered with a Millipore FH-0.5 μm membrane filter (Bedford, Massachusetts, U.S.A.) for the mobile phase.

Syntheses of UV-labeling agents.

Each of the three new UV-labeling agents was prepared from

reaction between p-toluenesulfonyl chloride and corresponding alcohol by modifying the literature method[20] as follows.

PNB-OTs. p-Nitrobenzyl alcohol (10 g), p-toluenesulfonyl chloride (20 g) and tetra-n-butylammonium hydrogensulfate (1 g) were dissolved in 300 ml of toluene, and the toluene solution was stirred in a water-bath at 10 °C. Then 5 M NaOH (25 ml) was added carefully lest the temperature of the reaction mixture should exceed 15 °C. After the addition, stirring was continued for 5 h, keeping the temperature below 15 °C. After the filtration of the precipitate liberated during the reaction, the toluene layer was separated from the water layer, washed three times with 200 ml of water, dried on anhydrous magnesium sulfate and evaporated. By recrystallizing the resulting solid from methanol, PNB-OTs was obtained as white needles (Yield : 40%).

DNB-OTs. 3,5-Dinitrobenzyl alcohol (1.58 g) and p-toluenesulfonyl chloride (2.2 g) were dissolved in 1,4-dioxane (15 ml), and then 40% NaOH (4 g) was added slowly to the 1,4-dioxane solution stirred in an ice-bath. After stirring for 7 h, the reacted solution was poured into 500 ml of cold water. The resulting solid was collected on a glass filter (3G5), washed with methanol and then recrystallized from ligroin. DNB-OTs was obtained as yellow needles (Yield : 80%).

PE-OTs. Two pyridine solutions were prepared by dissolving 2 g of N-(hydroxyethyl)phthalimide and 2.4 g p-toluenesulfonyl chloride in 10 ml of pyridine, respectively. The p-toluenesulfonyl chloride solution was added dropwise to the N-(hydroxyethyl)phthalimide solution which was maintained at -10 °C

with a mixture of ice and sodium chloride. After stirring for 8 h, pyridine hydrochloride was removed by filtration. The filtrate was diluted in chloroform and washed with water. The chloroform solution dried on anhydrous magnesium sulfate was placed on a column of silica gel. PE-OTs eluted with chloroform was recrystallized from ethanol and obtained as white needles (Yield : 75%).

The three p-toluenesulfonates thus synthesized were identified by mass and infrared spectrometries.

Procedure

The recommended procedure for UV-labeling of monocarboxylic acids with each of the three reagents was as follows. A brown test-tube with a screw cap (ca. 10 ml) was used as the reaction vessel in order to protect the contents from the light. To 1.00 ml of a reference standard solution of monocarboxylic acids was added a solution (1.5 ml) containing the reagent and 18-crown-6 as the catalyst. As the solvent of the reference standard and the reagent solutions, acetone, acetonitrile and propionitrile were used in the UV-labeling with PNB-OTs, DNB-OTs and PE-OTs, respectively (Table I). The concentrations of the agent and 18-crown-6 in the solution were dependent on the labeling agent as shown in Table I. Then a small amount of anhydrous potassium carbonate was added, and the mixture was stirred at a fixed temperature for a fixed time. After the reaction period, the reacted solution was filtered with a Minisart SRP 15 disposable filter holder in which 0.45 μm pore size hydrophobic membrane

filter was fitted (Sartorius, Gottingen, West Germany). An aliquot (10 µl) of the filtrate was injected into the high performance liquid chromatograph. The optimum reaction temperature and reaction time for each reagent are given in Table I.

RESULTS AND DISCUSSION

Myristic acid was selected as the model monocarboxylic acid, and it was UV-labeled with each of the three reagents according to the procedure described in the Experimental section. In each case the HPLC peak corresponding to the p-nitrobenzyl (PNB), 3,5-dinitrobenzyl (DNB) or 2-(phthalimino)ethyl (PE) derivative was observed. The authentic sample of each derivative was synthesized by scaling up the reaction system and isolated. By mass spectrometry of the authentic samples, the PNB, DNB and

Table I Optimum conditions and calibration

	PNB-OTs	DNB-OTs	PE-OTs
Solvent	Acetone	Acetonitrile	Propionitrile
Reaction temperature (℃)	Room temp.	50	85
Reaction time (min)	30	20	60
Reagent concentration (mM)	15	5	20
18-Crown-6 concentration (mM)	2.5	2.5	not used
Wavelength of UV detection (nm)	232 (254)[a]	272 (254)[a]	222
Determination level (μ M)[b]	25-250	10-100	1.0-10.0
Correlation coefficient of calibration graph[b]	0.9989	0.9991	0.9988

[a] In this work,the 254nm fixed-wavelength UV detector was used.
[b] Obtained for myristic acid.

PEderivatives were identified as PNB, DNB and PE esters of myristic acid, respectively.

Optimum derivatization conditions

The derivative of myristic acid labeled with each reagent should be detected at a wavelength corresponding to the absorption maximum of the derivative. In order to determine the wavelength, the reacted solution with each agent was detected at different wavelengths (Fig. 1). The peaks of the PE derivative are much larger than those of the PNB and DNB derivatives. In Fig. 1, therefore, the peak areas of the PE derivative are shown separately from those of other two derivatives; i.e., the ordinate of Fig. 1 is shown by assigning the maximum peak areas of PE and DNB derivatives as 100. The wavelength which gives the maximum peak area of the PE derivative is 218 nm, and the peak

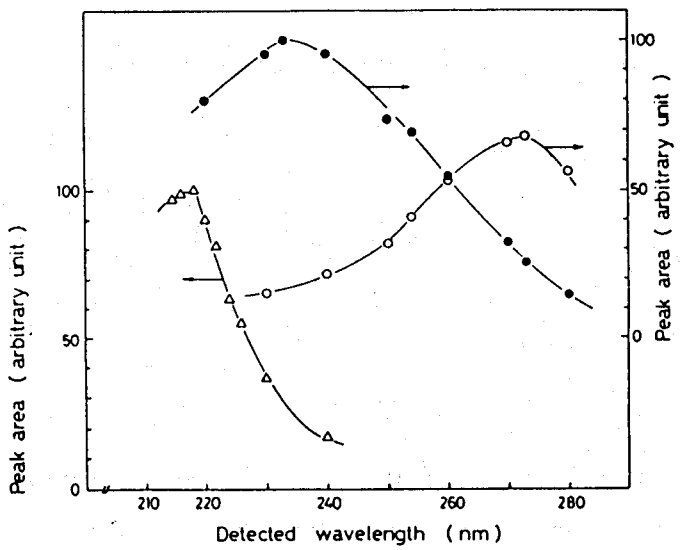
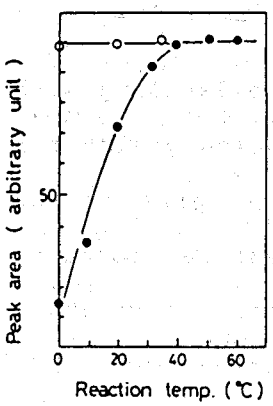


Fig.1 Effect of wavelength on the peak area of PNB (○), DNB (●) and PE (△) derivatives of myristic acid. The peak area on the ordinate is shown by assigning the maximum peak area of DNB and PE derivatives as 100.

area detected at 254 nm is much less than the maximum peak area. For the PNB and DNB derivatives, on the other hand, the wavelengths are 273 and 233 nm, respectively, and the peak areas of the derivatives detected at 254 nm are relatively high. In this work, an fixed-wavelength(254 nm) UV detector was used for the analysis of PNB or DNB derivatives. The variable-wavelength UV detector was used at 222 nm for the analysis of PE derivatives, because the noise at 222 nm is much less than that at 218 nm.

The UV-labeling of myristic acid was performed in several organic solvents frequently used. From the results, acetone gives the highest peak area of the derivative for the labeling with PNB-OTs, even if the labeling is carried out at room temperature. For the labeling with DNB-OTs, on the other hand, the yield is the highest at 50 ^{o}C in acetonitrile. For PE-OTs, the best results were obtained using propionitrile as the solvent at 85 ^{o}C.

The effect of reaction temperature was studied with the optimum labeling system and solvent. Fig.2 shows the results for the labeling with DNB-OTs, together with PNB-OTs. In Fig.2 or 3, the peak area on the ordinate is exhibited by assigning the maximum peak area of the derivative labeled with each reagent as 100. The peak area of the DNB derivative increases with increasing reaction temperature to a constant value beyond 40 ^{o}C, while that of the PNB derivative is constant, independent of the reaction temperature. Therefore, the reaction temperature was fixed at 50 ^{o}C for the labeling with DNB-OTs and at room temperature for that with PNB-OTs. From the result of the



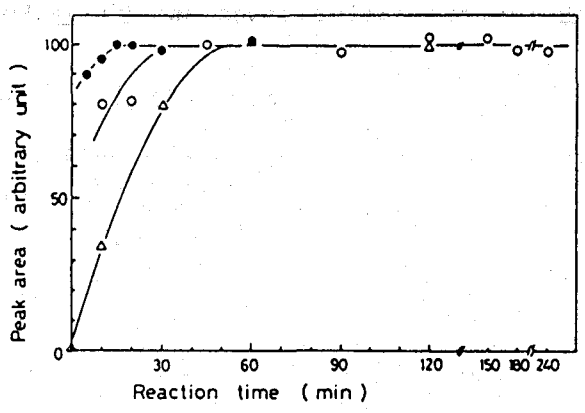


Fig. 2 Effect of reaction temperature on the labelling with PNB-OTs (○) and DNB-OTs (●).

Fig. 3 Effect of reaction time on the labelling with PNB-OTs (○), DNB-OTs (●) and PE-OTs (△).

similar measurement, the labeling with PE-OTs was performed at 85 ^{o}C.

The author has also tested the effect of reaction time on the labeling. From these results shown in Fig. 3, the reaction times were fixed as given in Table I.

Next, the effect of the concentration of the reagent in the solution added to the reference standard solution was examined. From the results, the optimum concentrations of PNB-OTs, DNB-OTs and PE-OTs is 15, 5 and 30 mM, respectively. Furthermore, the effect of the catalyst, 18-crown-6, concentration in that solution on the labeling was examined. To perform the labeling with PNB-OTs or DNB-OTs, 2.0 mM 18-crown-6 solution is necessary, while the derivative peak area does not vary regardless of the presence of 18-crown-6 in the labeling with PE-OTs. Therefore, the optimum concentrations of the catalyst were fixed as given in Table I.

Analytical calibrations and chromatograms

After the optimum reaction conditions for the labeling with each reagent had been established, the derivatization yields for myristic acid were evaluated as follows. The peak area of the derivative of myristic acid labeled with each agent was compared with that of the standard solution of the authentic derivative mentioned above. The yields of the labeling reactions with PNB-OTs, DNB-OTs and PE-OTs are 105, 94 and 94 %, respectively.

The calibration graph was constructed by analyzing ten reference standard solutions of myristic acid with each agent and by plotting the concentration of myristic acid vs. the peak area of the derivative. Three straight lines passing through the origin were obtained with the correlation coefficients nearly equal to 1, as shown in Table I. The determination levels are primarily dependent on the strength of the absorption of the derivatives labeled with the agents. When the detection of PNB and DNB derivatives is carried out at the optimum wavelength (PNB : 272 nm, DNB : 232 nm), the determination levels should be reduced down to about half of those given in Table I. The sensitivity of the labeling method with PE-OTs is very high, but the detection at 222 nm is apt to be obstructed by many compounds.

Fig. 4 shows the chromatograms obtained for the determination of several monocarboxylic acids. The separation of the peaks of the derivatives of lower monocarboxylic acids from that of the reagent is somewhat improved by using the acetonitrile solution containing small quantities of water as the

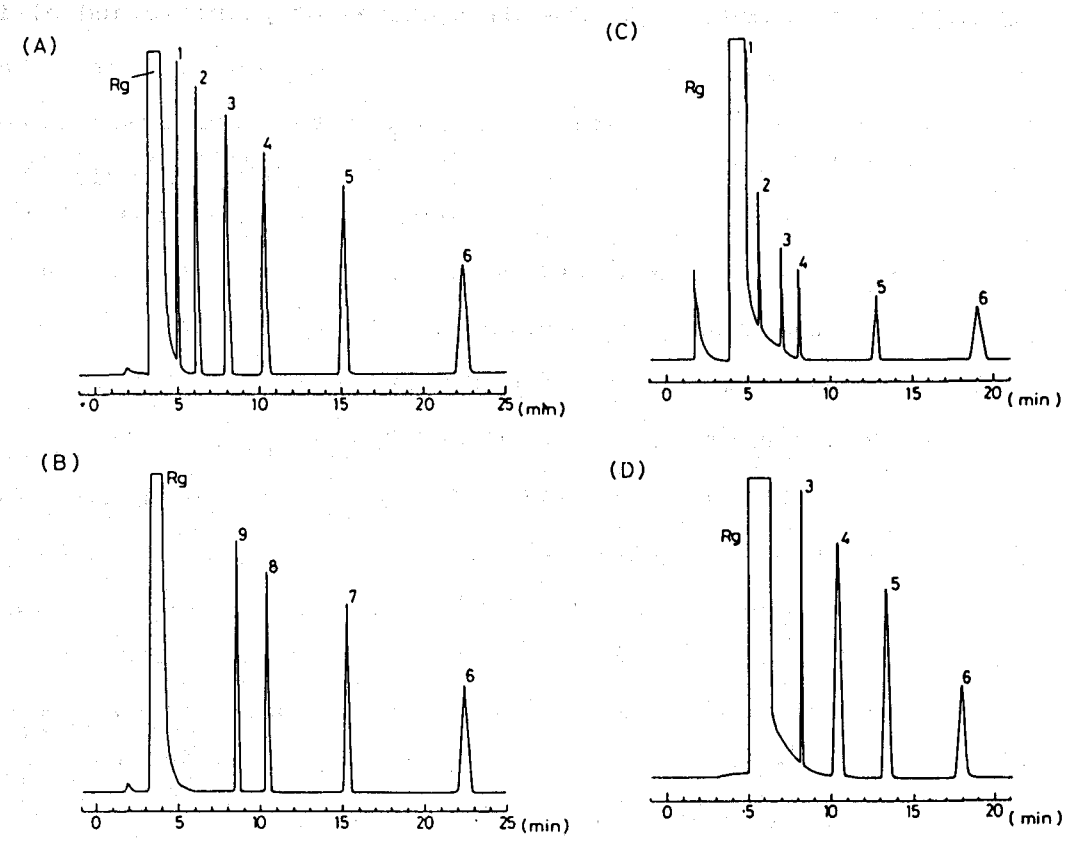


Fig. 4 Chromatograms of a mixture of monocarboxylic acids labelled with PNB-OTs (A,B), DNB-OTs (C) and PE-OTs (D). Peaks: Rg = reagent; 1 = caprylic acid; 2 = capric acid; 3 = lauric aid; 4 = myristic acid; 5 = palmitic acid; 6 = stearic acid; 7 = oleic acid; 8 = linolic acid and 9 = linolenic acid.

mobile phase. With these reagents, unsaturated monocarboxylic acids can also be labeled, together with saturated ones. Fig. 4B shows the chromatogram obtained when three unsaturated monocarboxylic acids (carbon number : 18) were labeled with PNB-OTs. The retention times for linolenic, linolic and oleic acids are close to those for lauric, myristic and palmitic acids, respectively. Under these HPLC conditions, the separation of the PNB derivatives of myristic and linolic acids is impossible, while the derivatives of lauric and linolenic acids can be

separated almost entirely. The derivatives of palmitic and oleic acids can be separated slightly under these conditions. The separation of the derivatives of saturated and unsaturated monocarboxylic acids will be further studied by changing the mobile phase and/or the separation column.

SUMMARY

New UV-labeling agents have been synthesized, which are designed to convert monocarboxylic acids into their highly UV-absorbing derivatives for enhancement of the sensitivities of UV detection in high performance liquid chromatography. The reagents are p-nitrobenzyl, 3,5-dinitrobenzyl and 2-(phthalimino)ethyl p-toluenesulfonates. Each has been prepared by reaction of p-toluenesulfonyl chloride with p-nitrobenzyl alcohol, 3,5-dinitrobenzyl alcohol or N-hydroxyethylphthalimide, respectively, in the presence of sodium hydroxide, and they are stable in solid state for at least 6 months. Monocarboxylic acids were derivatized to their p-nitrobenzyl, 3,5-dinitrobenzyl or 2-(phthalimino)ethyl esters with each of the above reagents, respectively, Then determined by high-performance liquid chromatography with UV detection. In the UV-labeling with each reagent, 18-crown-6 was used as the catalyst. The effects of reaction solvent, reaction temperature and time and the concentrations of each reagent and the catalyst were also examined.

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II-2 Polymeric Sulfonate-Type Labeling Reagents for Carboxylic Acids

INTRODUCTION

Polymeric reagents have certain advantages over conventional, low molecular weight reagents.[1,2] One of them is that polymeric reagents can easily be separated from the derivatives of interest, by a simple operation. As mentioned in chapter I-2, a polymeric pentafluorobenzylating reagent, poly(pentafluorobenzyl p-styrenesulfonate), could be readily removed from the reaction mixtures by precipitation after the derivatization of inorganic anions. This resulted in highly sensitive determination of inorganic anions by gas chromatography with electron capture detection.

In chapter II-1, the author described p-toluenesulfonate-type reagents bearing tagging groups for UV detection in liquid chromatography (LC) of fatty acids.[4] These low molecular weight reagents could not be removed from the reacted solutions by simple operations. Consequently, the author was obliged to co-inject the excess reagents with the derivatives into a liquid chromatograph, which gave a large background. In order to reduce it, an attempt was made to introduce the polymeric reagents and then to remove them later from the reaction mixtures by precipitation.

In this research, three polymeric reagents were synthesized: poly(4-nitrobenzyl p-styrenesulfonate) (PS-NB), poly[2-

(phthalimino)ethyl p-styrenesulfonate] (PS-PE) and poly[2-(1-naphthyl)ethyl p-styrenesulfonate] (PS-NE), and investigated their applications to the LC determination of long chain, saturated fatty acids (even-numbered C_8-C_{18} acids) as models.

EXPERIMENTAL

Preparation of polymeric derivatizing reagents

4-Nitrobenzyl, 2-(phthalimino)ethyl or 2-(1-naphthyl)ethyl p-styrenesulfonate was synthesized by reaction of p-styrenesulfonyl chloride with 4-nitrobenzyl alcohol in dioxane, 2-(phthalimino)ethanol in pyridine or 2-(1-naphthyl)ethanol in benzene respectively, and identified by IR, MS and elemental analysis.

PS-NB was obtained by radical polymerization of 4-nitrobenzyl p-styrenesulfonate (5 g) in a sealed tube containing 2,2'-azobisisobutyronitrile (0.06 g) in acetone (25 ml). After keeping the tube for 24 h at 60 °C, PS-NB was isolated by precipitation in diethyl ether.

PS-PE and PS-NE were also obtained by similarly polymerizing 2-(phthalimino)ethyl p-styrenesulfonate and 2-(1-naphthyl)ethyl p-styrenesulfonate respectively in N,N-dimethylformamide at 60 °C for 22 h, and by precipitating with methanol and with diethyl ether, respectively.

Chemicals and apparatus

The chemicals used in this study were obtained from Wako,

Tokyo Kasei and Aldrich.

The LC system utilized comprised a JASCO 880-PU pump and two detectors in series, JASCO 875-UV detector operating at 254 nm for the 4-nitrobenzyl derivatives and 221 nm for the 2-(phthalimino)ethyl ones, and a Hitachi F1000 fluorescence spectrophotometer operating at 332 nm emission and 275 nm excitation for 2-(1-naphthyl)ethyl derivatives. TSKgel ODS-80TM (5 μm) column (4.6 mm i.d.X15 cm) was used with an eluent (acetonitrile) at a flow rate of 0.5 ml/min. The peak areas were measured with a Shimadzu Chromatopac C-R6A.

Derivatization procedures

In a brown-colored tube with a screw cap (ca. 10 ml) were placed acetone solutions of the fatty acid (1.0 ml), $1.0X10^{-2}$ M PS-NB (1.0 ml) and $1.0X10^{-2}$ M 18-crown-6 (0.5 ml), and anhydrous potassium carbonate (ca. 30 mg). After the mixture was stirred for 30 min at 47 ^{o}C, 2 ml was pipetted. The solvent was removed by rotary evaporation, and then to the residue was added 2 ml of tert-butyl methyl ether-ethyl acetate (1:1). PS-NB precipitate was filtered off through a membrane filter, and an aliquot (10 μl) of the filtrate was injected into the liquid chromatograph.

The acid was also derivatized in dioxane for 2 h at 85 ^{o}C in the same manner as above, except for concentrations of PS-PE ($1.5X10^{-2}$ M) and 18-crown-6 ($5.0X10^{-3}$ M). The reaction mixture was treated as described above, but using methanol in place of tert-butyl methyl ether-ethyl acetate (1:1). For the derivatization with PS-NE, the following conditions were different for that

with PS-PE: in concentrations of PS-NE($5x10^{-3}$ M) and 18-crown-6($5x10^{-4}$), reaction time(90 min) and precipitant(ethyl acetate).

RESULTS AND DISCUSSION

The author has already reported the availability of the p-Toluenesulfonate-type reagents for 4-nitrobenzylation, 3,5-dinitrobenzylation and 2-(phthalimino)ethylation of several fatty acids for their LC determination.[4] In order to overcome their above-mentioned disadvantages, an attempt was made to prepare their polystyrenesulfonate-type reagents. the author could obtain three polymeric ones, PS-NB,PS-PE and PS-NE.

In order to establish the optimum derivatization conditions for formation of the acids' derivatives, several parameters (solvent, concentrations of the reagents and 18-crown-6, temperature, time and precipitant) which affect their formation were evaluated for C_{14} acid ($2.0X10^{-4}$ M for PS-NB and $1.0X10^{-5}$ M for PS-PE and PS-NE). The results as optimized are given in the experimental section. Under these conditions, the other acids were also derivatized quantitatively with PS-NB, PS-PE and PS-NE. The quantitative application of the methods to the determination of the acids was examined. The results indicated good linearity for the determination of each acid at five different concentrations. The linear regression equations obtained for C_{14} acid, for instance, were $y=0.0650x+0.0010$ with a correlation coefficient $r=0.9994$ ($2.0X10^{-5}$-$2.0X10^{-4}$ M) for PS-NB, $y=1.2031x-0.0998$ with $r=0.9997$ ($1.0X10^{-6}$-$1.0X10^{-5}$ M) for PS-PE,

and $y = 0.9187x + 0.7199$ with $r = 0.9998$ (1.0×10^{-7} - 5.0×10^{-6} M) where y and x are the peak area and the concentration of the acid (10^{-6} M), respectively. The smallest r-value was 0.9990 for the 2-(phthalimino)ethylation of C_{16} acid. Figure 1 compares the typical chromatogram of the derivatives of the acids simultaneously treated with PS-NB to that with 4-nitrobenzyl p-toluenesulfonate (NB-OTs). Similar chromatograms with PS-PE and 2-(phthalimino)ethyl p-toluenesulfonate (PE-OTs), and with PS-NE and 2-(1-naphthyl)ethyl p-toluenesulfonate (NE-OTs) are shown in Fig. 2 and Fig. 3, respectively. It is apparent that each polymeric reagent is superior to its corresponding p-toluenesulfonate-type one, as expected: the large background tailing can be greatly suppressed by precipitating a large excess

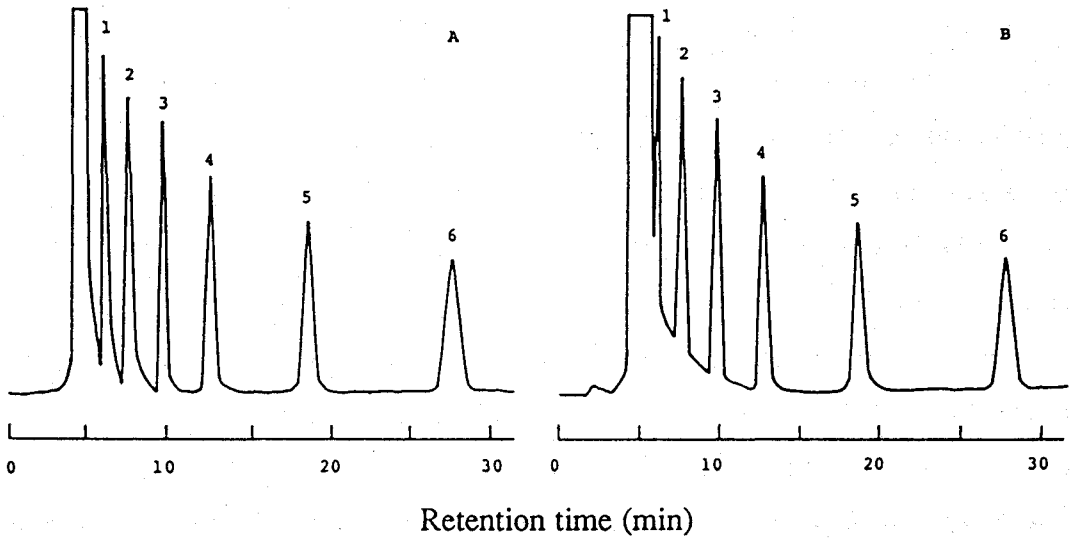

Fig.1 Liquid chromatograms for a mixture of the derivatives of the acids, with PS-NB (A) and NB-OTs (B). Peaks: 1, C_8; 2, C_{10}; 3, C_{12}; 4, C_{14}; 5, C_{16}; 6, C_{18} acids.

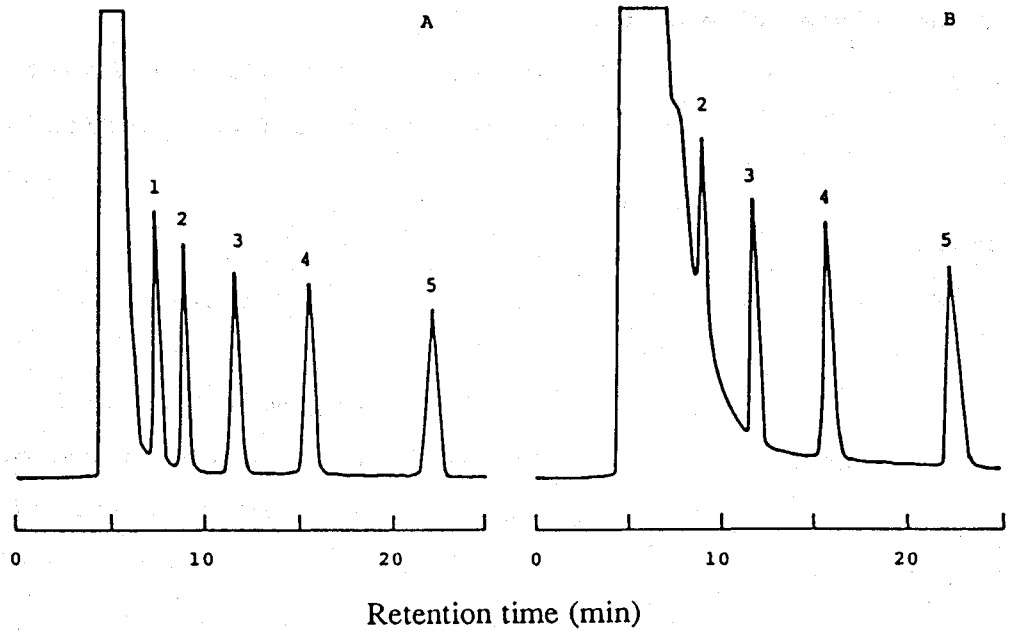

Fig.2 Liquid chromatograms for a mixture of the derivatives of the acids, with PS-PE (A) and PE-OTs (B). Peaks: 1, C_{10}; 2, C_{12}; 3, C_{14}; 4, C_{16}; 5, C_{18} acids.

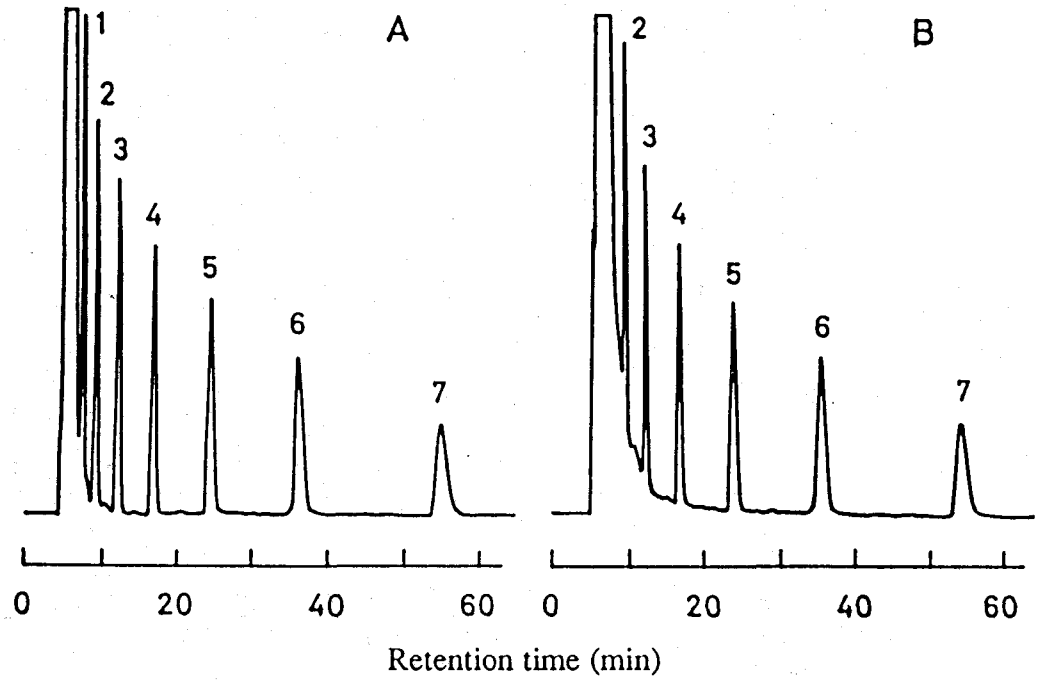

Fig.3 Liquid chromatograms for a mixture of the derivatives of the acids, with PS-NE (A) and NE-OTs (B). Peaks: 1, C_6; 2, C_8; 3, C_{10}; 4, C_{12}; 5, C_{14}; 6, C_{16}; 7, C_{18} acids.

of the polymeric reagents.

Finally, it is of great interest to introduce a variety of tagging groups giving high detector-response and/or high reactivity.

SUMMARY

The use of polymeric reagent for derivatization in HPLC as well as that in GC has remarkably facilitated the procedure of removing excess reagent from reaction mixture after derivatization reactions. The background based on excess reagents was, as a result, clearly decreased by using polymeric reagents instead of monomeric ones.

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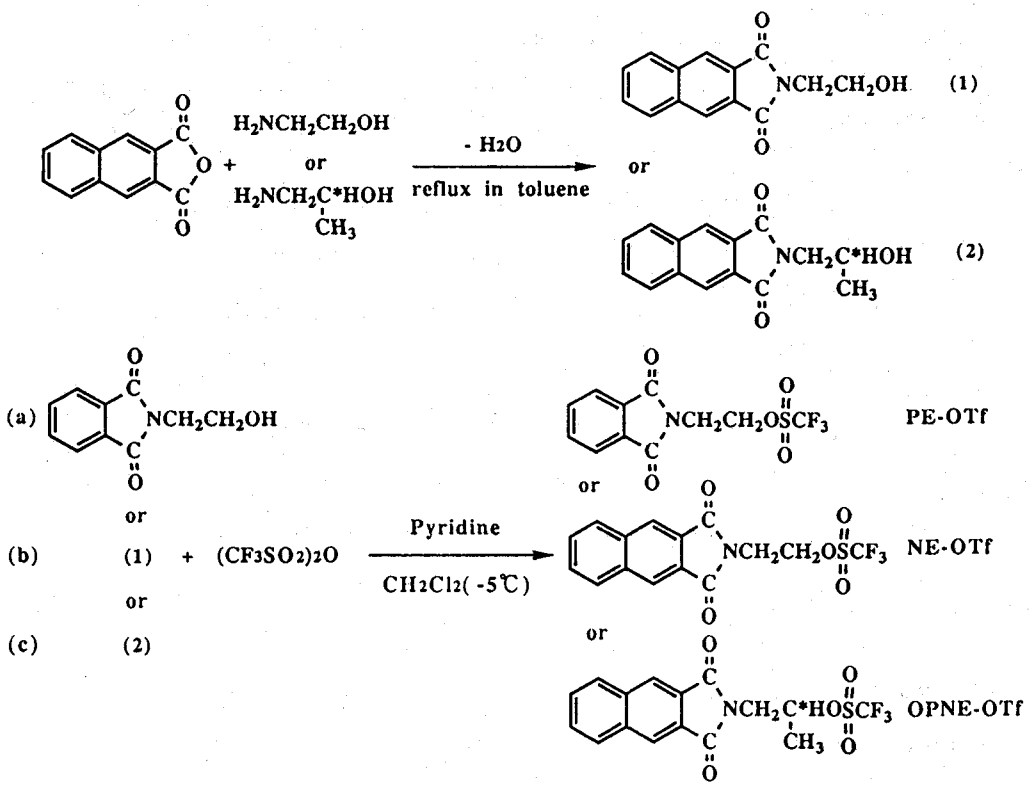
II-3. Triflate-Type Derivatizing Reagents for Carboxylic Acids

Carboxylic acids distribute widely in nature and play important roles as nutritional substrates and metabolites in living bodies. The carboxyl functional group is only weakly chromophoric, thus, carboxylates with no other adequately chromophoric structural feature must be derivatized for the sensitive detection of them by high performance liquid chromatography . For the functional groups with which carboxylic acids are derivatized to the corresponding derivatives, frequently used are halogenomethyl or halogenoacyl compounds such as N-chloromethyl phthalimide, 9-chloromethyl anthracene and p-bromophenacyl bromide. The reactions of carboxylic acids with these reagents require longer reaction time and/or elevated temperatures for complete derivatization of the acids because of poor reactivity of carboxyl groups. Longer reaction times at elevated temperatures seem to be unfavorable not only for the simplicity in derivatization procedures but for the determination of thermolabile substances such as α-ketocarboxylic acids. On the other hand, powerful alkylating abilities of perfluoromethanesulfonates are well know in the field of organic synthesis. Ingalls et al. have thus developed 4'-bromophenacyl trifluoromethanesulfonate[6], as a highly reactive UV-labeling agent for carboxylic acids in HPLC determination. This reagent can derivatize the acids completely to the corresponding derivatives within 5 min in acetonitrile at room temperature, in the presence of N,N-diisopropylethylamine. In this chapter, the syntheses of new triflate-type derivatizing reagents (Scheme I)

and the applications for the determination of carboxylic acids with these reagents are described.



Scheme I Preparation of triflate-type reagents.

II-3-1. 2-(Phthalimino)ethyl Trifluoromethanesulfonate as a UV-labeling Reagent for Carboxylic Acids

INTRODUCTION

Many kinds of UV-labeling agents have been developed for the determination of carboxylic acids by high performance liquid chromatography. Halogenomethyl or halogenoacyl compounds, such as N-chloromethyl phthalimide,[1] 9-chloromethylanthracene[2] and p-bromophenacyl bromide[3] have been extensively examined. The derivatization reactions of carboxylic acids with these reagents require, even if crown catalysts are present, higher temperatures for a complete derivatization of the acids within a reasonable time. To orvercome this disadvantage mentioned above, 2-(Phthalimino)-ethyl trifluoromethanesulfonate (PE-OTf) was developed as a highly reactive UV-labeling agent for carboxylic acids in HPLC.

EXPERIMENTAL

Apparatus

HPLC system. The HPLC system consisted of a JASCO 880-PU pump, a Rheodyne model-7125 injector valve, a JAI model 3702-UV detector operating at 219 nm and a Shimadzu Chromatopac C-R6A integrator. Two types of analytical columns were used, a Chemcosorb 5C8 (5μm, 150x4.6 mm i.d., Chemco, Japan) for the analysis of mouse-brain samples because of shortening separation

times, and a Wakosil 5C18-200T (5μm, 150x4.6 mm i.d., Wako, Japan) for all of the other experiments.

Others. A Hitachi model 304 spectrophotometer was used for measuring the UV spectra. The melting point was measured with a Yanaco melting-point apparatus and was uncorrected. A Hitachi centrifuge 05P-21 and a handy micro homogenizer NS-310E (Nichion Medical Instruments Co. Japan) were used in the preparation of mouse-brain samples. An Erma ERC-3510 degasser was utilized for continuous degassing of a mobile phase.

Reagents and materials

HPLC grade CH_3CN was purchased from Wako (Osaka, Japan) and used in the preparation of chromatographic mobile phases. Water was purified through a Milli-Q water purification unit (Millipore, U.S.A.). All other chemicals were of reagent grade. Eppendorf Safe-Lock microcentrifuge tubes (2.0 ml) were used as reaction tubes. Male ddY(7-8 weeks old) mice were purchased from Shimizu Laboratory Supplies, Co.(Kyoto, Japan). Authentic 2-(phthalimino)ethyl ester of myristic acid was prepared by a reaction between N-hydroxyethylphthalimide and myristoyl chloride according to a literature method[5] and identified by mass spectrometry, ir spectrophotometry and elemental analysis. This ester was used as an external reference standard in optimization studies of the derivatization reaction conditions.

Synthesis of 2-(phthalimino)ethyl trifluoromethanesulfonate

PE-OTf was synthesized according to a method developed by Vedejs et al. for the preparation of trifluoromethanesulfonate esters of alcohols.[6] Thus, to a solution of trifluoromethanesulfonic anhydride (5 g, 0.018 mol) in dichloromethane (50 ml) was carefully added a mixture of N-hydroxyethylphthalimide (3 g, 0.016 mol) and pyridine (1.3 g, 0.016 mol) in dichloromethane (70 ml) at -5 °C (ice-NaCl bath). After the addition, stirring was continued for 2 hours, while maintaining a temperature of -5 °C. The resulting solution was washed with cold deionized water and then dried over anhydrous magnesium sulfate. After evaporating dichloromethane, a crude product was recrystallized twice from n-hexane. PE-OTf was obtained as transparent fine needles: yield 60% ; mp 79 °C; Anal. calcd for $C_{11}H_8NSO_5F_3$, 40.87% C, 2.49% H, 4.33% N, found: 40.93% C, 2.61% H, 4.39% N; IR : 1200 and 1400 cm^{-1} ($-O-SO_2-$); MS: m/z=324 (MH^+).

Derivatization procedure

A typical derivatization procedure was as follows: To 0.5 ml of a test solution of fatty acids (10^{-5} M) in acetonitrile placed in a reaction tube were added 0.1 ml of 18-crown-6 (10^{-3} M) in acetonitrile and ca. 30 mg of anhydrous K_2CO_3. After vortexing the tube slightly, 0.1 ml of PE-OTf (10^{-3} M) in acetonitrile was poured into it. The mixture was vortexed for 10 min at room temperature (20-25 °C). The resulting solution was left standing for 30 s and an aliquot (10 µl) of the supernatant was directly injected into the chromatograph.

Preparation of mice brain samples

To the cerebrum dissected from a mouse was added margalic acid (0.1 ml) as an internal standard, which was homogenized in methanol (3 ml) and centrifuged for 10 min (5000 g). The supernatant was removed and the pellet was rehomogenized in methanol. This extraction process was repeated 3 times in total. The supernatant were combined and evaporated in vacuo; then, the residue was dissolved in acetonitrile (0.2 ml), followed by the above-mentioned derivatization procedure.

RESULTS AND DISCUSSION

Optimization of derivatization reaction conditions

In order to evaluate a derivatization reaction for use in analysis I have investigated some parameters which affect the rate of reaction and the derivatization yield, such as the reaction time and the amounts of the reagent and the catalyst. Myristic acid was chosen due to an adequate retention time of its derivative, as a model monocarboxylic acid in the following studies.

Figure 1 shows the reaction progress curves for myristic acid (10^{-5} M) with PE-OTf. The derivatization yields became constants in 10 min at room temperature with a 10-fold excess of PE-OTf, reagardless of the presence of 18-crown-6 as a catalyst; without the catalyst, however, it didn't reach completion: 10 min was chosen in the following experiments. In order to optimize the amounts of PE-OTf and 18-crown-6, the reactions were carried out

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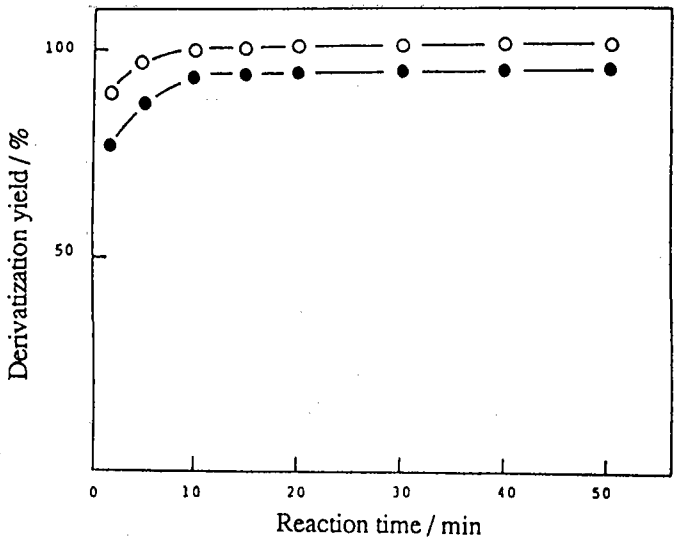

Fig. 1 Reaction time curves of myristic acid (10^{-5} M) with PE-OTf (10^{-4} M). ○: with 18-crown-6 (10^{-4} M); ●: without 18-crown-6

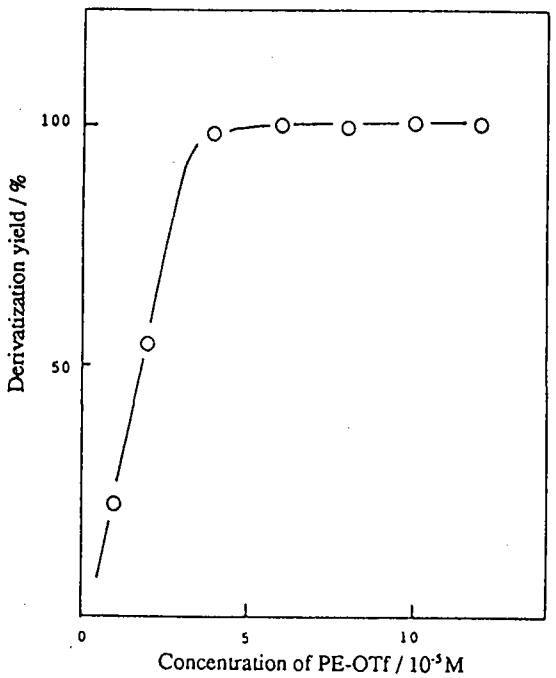

Fig. 2 Effect of PE-OTf concentration on the derivatization of myristic acid (10^{-5} M). Concentration of 18-crown-6 = 10^{-4} M.

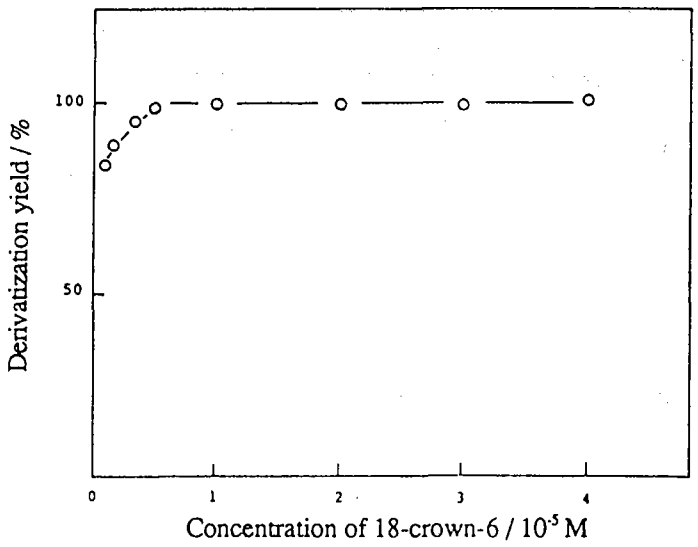

Fig.3 Effect of 18-crown-6 concentration on the derivatization of myristic acid (10^{-5} M). Concentration of PE-OTf = 10^{-4} M.

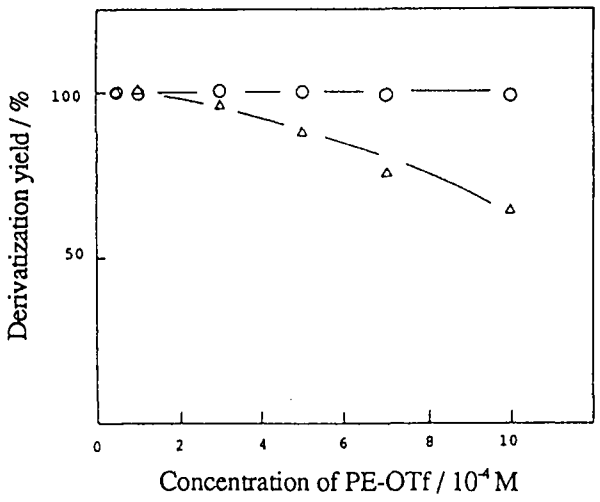

Fig.4 Effect of relative concentration of PE-OTf to 18-crown-6 on the derivatization of myristic acid (10^{-5} M). ○: 18-crown-6 concentration = PE-OTf concentration; △: 18-crown-6 = 10^{-4} M.

with various equivalent ratios of them to the acid (Figs. 2 and 3). The reaction proceeded to completion with a 5-fold excess of PE-OTf in the presence of a 10-fold excess of 18-crown-6.

The effect of the equivalent ratio of PE-OTf to 18-crown-6 on the derivatization yield was also examined, as shown in Fig. 4. An excess amount of PE-OTf to 18-crown-6 obviously brought about a decrease in the yield. On the other hand, in the case of maintaining the ratio at one, a complete derivatization yield was obtained, even when a 100-fold excess of PE-OTf to the acid was used in the derivatization reaction. It is therefore essential for this derivatization system to use even or excess amounts of 18-crown-6 to PE-OTf.

The quantitative application of the method to the determination of saturated fatty acids (C_6-C_{16}) was examined. The results indicated good linearity for determining each acid at six different concentrations (1.0×10^{-6}-10^{-5} M). The linear regression equation obtained for C_{12} acid, for instance, was $y=0.376x-0.0928$ with a correlation coefficient $r=0.999$, where y and x are the peak area and the concentration of the acid, respectively, and the relative standard deviation was 1.53% (5×10^{-6} M, n=7). A typical chromatogram of the derivatives of the acids is shown in Fig. 5.

Determination of carboxylic acids in mouse brain

As an application of this reagent to the analysis of biological samples, three carboxylic acids (palmitic , oleic and stearic acids) in mouse brain were determined according to

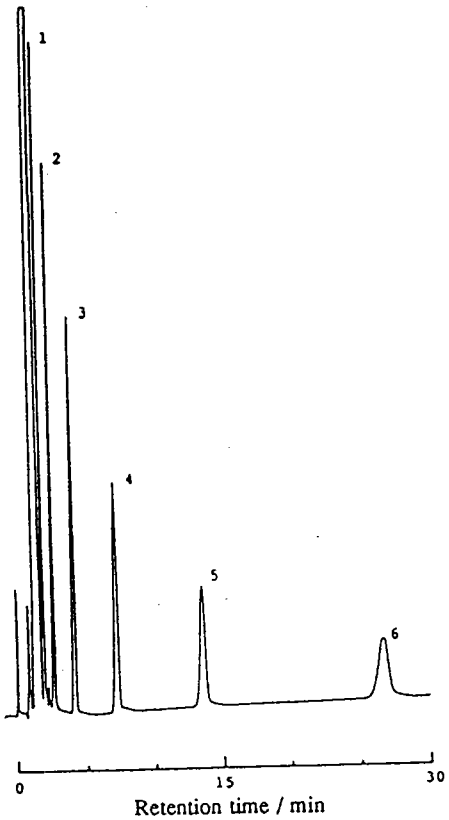

Fig. 5 Typical chromatogram of six fatty acid derivatives. Column, Wakosil 5C18-200T; mobile phase, $CH_3CN : H_2O = 8 : 2$; flow rate, 1.2 ml/min. Peaks: 1 = n-caproic acid; 2 = n-caprylic acid; 3 = n-capric acid; 4 = lauric acid; 5 = myristic acid; 6 = palmitic acid.

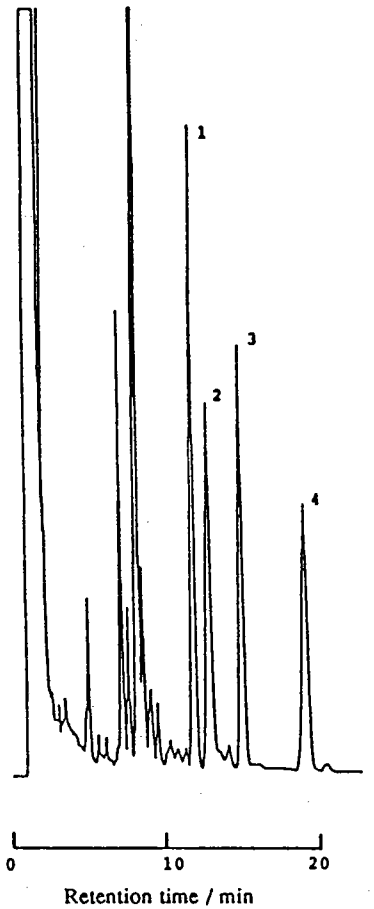

Fig. 6 Chromatogram for the determination of carboxylic acids in mouse brain. Column, Chemcosorb 5C8; mobile phase, $CH_3CN : H_2O = 85 : 15$; flow rate ,1.0 ml / min. Peaks: 1 = palmitic acid; 2 = oleic acid; 3 = margaric acid (internal standard); 4 = stearic acid.

the procedure in the Experimental section. The recoveries of these acids and the margaric acid in the extraction process were 94% for palmitic acid, 92% for oleic acid, 96% for stearic acid and 95% for margaric acid, respectively. These acids were well separated from each other on the C-8 column with an eluent of CH_3CN/H_2O within 30 min (Fig. 6). The peak identification was made by comparing their retention times with one of the reference standards. The obtained values for the cerebrum (0.31 g wet weight) with five replicated analyses were 45±3 nmol/g of palmitic acid, 58±16 nmol/g of oleic acid, 61±3 nmol/g of stearic acid, respectively.

Stability of the reagent

PE-OTf was stable for at least one year in a refrigerator. The stability of PE-OTf in an acetonitrile solution (10^{-2} M) was examined by periodically chromatographing the solution; though 40% of the initial peak height was lost in a single day at room temperature, it is possible to keep the loss at 5% after 6 days by keeping the solution below -20 ^{o}C.

SUMMARY

PE-OTf could easily be synthesized by a one-step reaction from commercially available starting materials. The labeling reaction proceeds rapidly to completion only by mixing the reagents at room temperature, which makes it possible to label the thermolabile carboxylic acids without isomerization and

decomposition. The resulting derivatives possess good chromatographic properties and strong UV-absorptivity (λ_{max}=219 nm, ε=42000): 200 fmol of detection limits(S/N=3).

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II-3-2 2-(2,3-Naphthalimino)ethyl Triflate as a Fluorescence Labeling Reagent for Carboxylic Acids

INTRODUCTION

Of the detection methods presently available for HPLC, fluorometric detection is one of the most selective and sensitive methods. Several fluorescent labeling agents, 4-bromomethyl-7-methoxycoumarin[1], 1-bromoacetylpyrene[2], 9-aminophenanthrene[3], 9-anthryldiazomethane[4] and 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone[5] have been developed for the determination of carboxylic acids by HPLC. The reactions of carboxylic acids with these reagents require longer reaction time and/or elevated temperatures for complete derivatization of the acids because of poor reactivity of carboxyl groups. It was shown in chapter II-3-1 that the triflate bearing 2-(phthalimino)ethyl moiety was easily synthesized and reacted with carboxylic acids at room temperature. The author has developed 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (NE-OTf) in order to expand a triflate-type reagent for fluorescence labeling of carboxylic acids.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a JASCO 880-PU pump, a Rheodyne model-7125 injector valve, a JAI model 3702-UV detector operating at 259 nm or a Hitachi F-1000 fluorescence spectrophotometer

operating at 394 nm emission and 259 nm excitation, and a Shimadzu Chromatopac C-R6A integrator. Two types of analytical columns were used, a Chemcosorb 5C8 (5μm, 150 x 4.6 mm i.d.) for the analysis of mouse brain samples, and a Wakosil 5C18-200T (5μm, 150 x 4.6 mm i.d.) for all the other experiments. A Hitachi 850 fluorescence spectrophotometer and a Hitachi 304 spectrophotometer were used for the measurements of fluorescence and ultraviolet spectra. Melting point was measured with a Yanaco melting point apparatus and was uncorrected. A Hitachi centrifuge 05P-21 and a handy micro homogenizer NS-310E (Nichion Medical Instruments Co, Japan) were used in the preparation of mouse brain samples. An Erma ERC-3510 degasser was utilized for continuous degassing of a mobile phase.

Reagents and materials

2,3-Naphthalenedicarboxylic anhydride was obtained from Tokyo Kasei Co. (Tokyo, Japan). HPLC grade CH_3OH was purchased from Wako Pure Chemical Co. (Osaka, Japan) and used in the preparation of chromatographic mobile phases. All the other chemicals were of special grade. Water was purified with a Milli-Q water purification unit (Millipore, U.S.A.). Eppendorf Safe-Lock microcentrifuge tubes(2.0 ml) were used as reaction tubes. Male ddy(7-8 weeks old) mice were purchased from Shimizu Laboratory Supplies, Co.(Kyoto, Japan). Authentic 2-(2,3-naphthalimino)ethyl ester of myristic acid was prepared by reaction between N-(2-hydroxyethyl)-2,3-naphthalimide and myristoyl chloride according to the literature method[7] and

identified by mass spectrometry, ir spectrophotometry and elemental analysis. This ester was used in spectrophotometric studies and in optimization studies of derivatization reaction conditions as an external reference standard.

Synthesis of NE-OTf

NE-OTf was prepared by two synthetic steps from commercially available precursors. N-(Hydroxyethyl)-2,3-naphthalimide was synthesized by modifying the literature method[8]. Thus, in a 500 ml flask fitted with a water separator and a reflux condenser were placed 9.9g (0.05 mol) of 2,3-naphthalenedicarboxylic anhydride, 3.1g (0.051 mol) of 2-aminoethanol and 300 ml of dry toluene. The mixture was heated for 3h with a vigorous reflux on an oil bath. After cooling, the solid product was then filtered off on a G-3 glass filter and was washed with three 50 ml portions of cold water. Recrystallization from ethanol gave transparent needles of the naphthalimide: yield 85%; mp 192-195 ^{o}C; Anal. calcd for $C_{14}H_{11}NO_3$, 69.71% C, 4.56% H, 5.81% N, found: 69.89% C, 4.54% H, 5.75% N.

To a solution of trifluoromethanesulfonic anhydride (5 g, 0.018 mol) in dichloromethane (100 ml) was dropwise added a mixture of pyridine (1.4 g, 0.018 mol) and the naphthalimide (3.9 g, 0.016 mol) suspended in warm dichloromethane (100 ml) carefully at a rate of keeping the temperature of the reaction mixture below -5 ^{o}C; the addition required about 1 h. After the addition, stirring was continued for 2 h. The resulting solution was washed three times with cold deionized water and then dried

over anhydrous magnesium sulfate. After removing dichloromethane under reduced pressure, a crude product was recrystallized twice from a mixture of dichloromethane and tetrachloromethane. NE-OTf was obtained as transparent flakes: yield 53%; mp 138-140 ^{o}C; Anal. calcd for $C_{15}H_{10}NSO_5F_3$, 48.26% C, 2.68% H, 3.75% N, found: 48.06% c, 2.68% H, 3.74% N; IR: 1200 and 1400 cm^{-1} (-O-SO_2-); MS: m/z=374 (MH^+).

Derivatization procedure

A typical derivatization procedure was as follows. To 0.5 ml of a test solution of fatty acids in acetonitrile placed in a reaction tube were added 0.1 ml of 18-crown-6 (10^{-3} M) in acetonitrile and ψα. 5 mg of anhydrous KF. After vortexing the tube slightly, 0.1 ml of NE-OTf (10^{-3} M) in acetonitrile was combined with it. The mixture was vortexed for 10 min at room temperature. The resulting solution was standing for 30 s and an aliquot (10 ml) of the supernatant was directly injected into the chromatograph.

Preparation of mice brain samples

To the cerebrum dissected from a mouse was added margaric acid (0.1 ml) as an internal standard, and this sample was homogenized in methanol (3 ml) and centrifuged for 10 min (5000 g). The supernatant was removed and the residual pellet was rehomogenized in methanol. This extraction process was repeated 3 times in total. The supernatant were combined and evaporated

in vacuo and then the residue was dissolved in acetonitrile (0.2 ml), followed by the above-mentioned derivatization procedure.

RESULTS AND DISCUSSION

It is well known that trifluoromethanesulfonate possesses an excellent alkylating ability on nucleophilic species. But as far as I know, there has been published only one report which intended to use 4'-bromophenacyl trifluoromethanesulfonate as a UV-labeling agent for the determination of carboxylic acids by HPLC. This unforeseen situation might be due to the difficulties in the synthesis and the isolation of trifluoromethanesulfonate bearing HPLC-detector oriented moeties. I have found that NE-OTf was easily synthesized from commercially available materials and stable at room temperature.

Fluorescence properties of carboxylic acid derivatives

Figure 1 shows the excitation and fluorescence spectra of 2-(2,3-naphthalimino)ethyl ester of myristic acid (NE-C_{14} ester) in methanol-water (9:1). The excitation and the emission maximum were at 259 nm and 394 nm, respectively. The effect of water concentration on the fluorescence intensity was examined. As shown in Fig. 2, the fluorescence intensity of NE-C_{14} ester in aqueous methanol was almost constant at water concentrations of 0 - 30% (v/v), but was slightly decreased with increasing the water concentration over 30% of water.

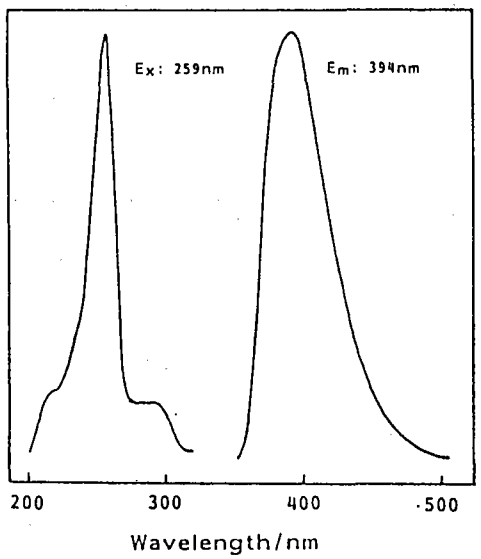

Fig. 1 Fluorescence spectra of 2-(2,3-naphthalimino)ethyl myristate in methanol-water (9:1).

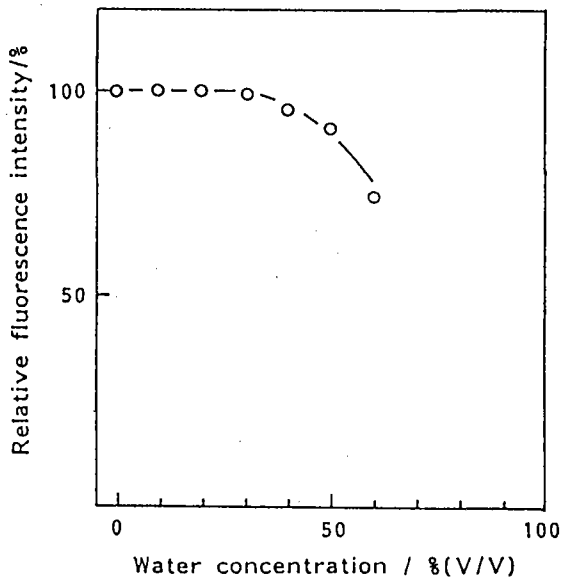

Fig. 2 Effect of water concentration on the fluorescence intensity of NE-C_{14} ester in aqueous methanol.

Optimization of derivatization reaction conditions

Potassium carbonate is frequently used to convert free carboxylic acids into their carboxylate anions in the derivatization systems using halogenoalkyl type reagents. Our preliminary experiments indicated that the use of the carbonate caused many endogenous peaks, some of which overlapped on the derivative peaks of interest in sensitive detection. Shimada et al.[9] reported that potassium fluoride could be used in place of the carbonate in conversion of free carboxylic acids into the carboxylate anions. I have also examined its use in the present derivatization system and found that appearance of the disturbing peaks was obviously suppressed by using it instead of potassium carbonate. Potassium fluoride was thus used in this derivatization system.

I have investigated some parameters which affect the rate of reaction and the derivatization yield, such as reaction time, and the amounts of the reagent and the catalyst. Myristic acid was chosen, due to an adequate retention time of its derivative, as a model monocarboxylic acid in the following studies. Figure 3 shows the reaction progressing curves for myristic acid (10^{-5} M), both with and without 18-crown-6 as a catalyst. The reaction completed within 10 min at room temperature with a 10-fold excess of NE-OTf in the presence of 18-crown-6. On the other hand, without 18-crown-6, it took 40 min to reach 96% of derivatization yield. From this results a reaction time of 10 min in the presence of 18-crown-6 was adopted in the following experiments. To optimize the amounts of NE-OTf and 18-crown-6,

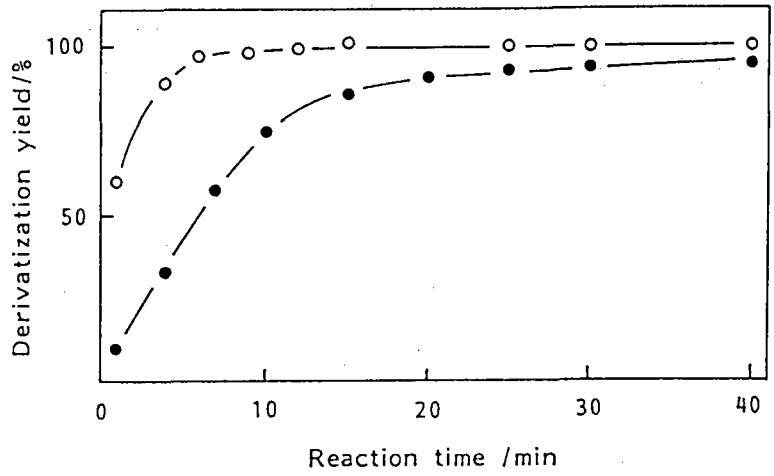

Fig. 3 Reaction progressing graph of myristic acid (10^{-5} M) with NE-OTf (10^{-4} M).
○ : with 18-crown-6 (10^{-4} M); ● : without 18-crown-6

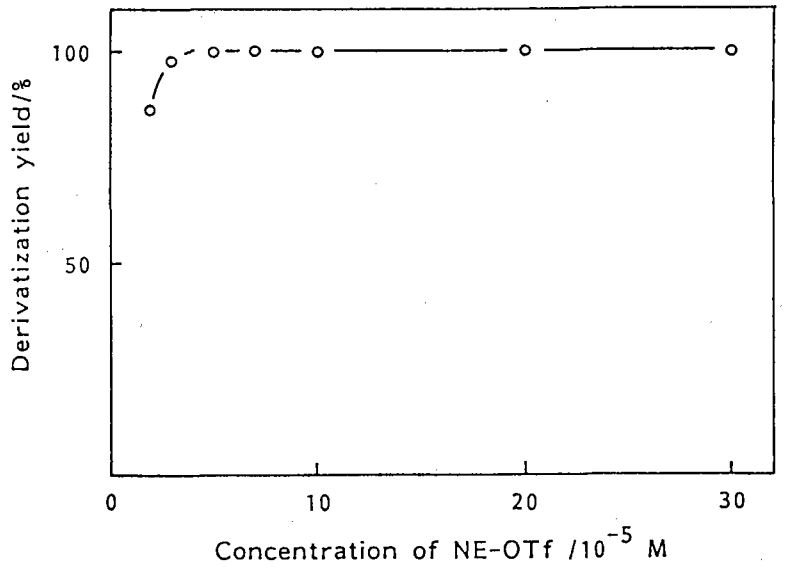

Fig.4 Effect of NE-OTf concentration on the derivatization of myristic acid (10^{-5} M). Concentration of 18-crown-6 = 10^{-4} M.

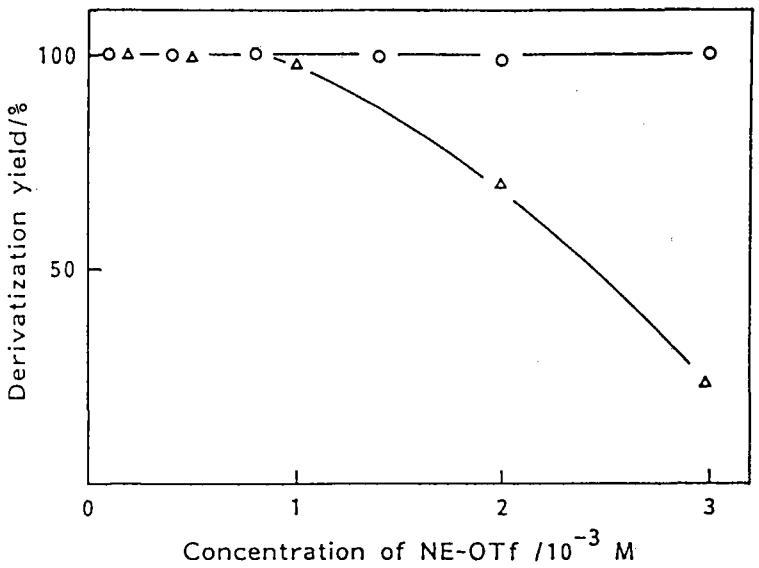

Fig.5 Effect of relative concentration of NE-OTf to 18-crown-6 on the derivatization of myristic acid (10^{-5} M).
○: 18-crown-6 concentration = NE-OTf concentration
△: 18-crown-6 concentration = 10^{-4} M

the reactions were carried out with various equivalent ratios of them to the acid. The reaction proceeded to completion with a 5-fold excess of NE-OTf in the presence of a 10-fold excess 18-crown-6 (Fig. 4). The effect of the equivalent ratios of NE-OTf to 18-crown-6 on the derivatization yield was also examined as shown in Fig.5. A large excess amount of NE-OTf to 18-crown-6 obviously brought about a decrease in the yield. On the other hand, in the case of maintaining the ratio at one, a complete derivatization yield was obtained even when a 30-fold excess of NE-OTf to 18-crown-6 was used in the derivatization reaction. It is therefore essential for this derivatization system to use even

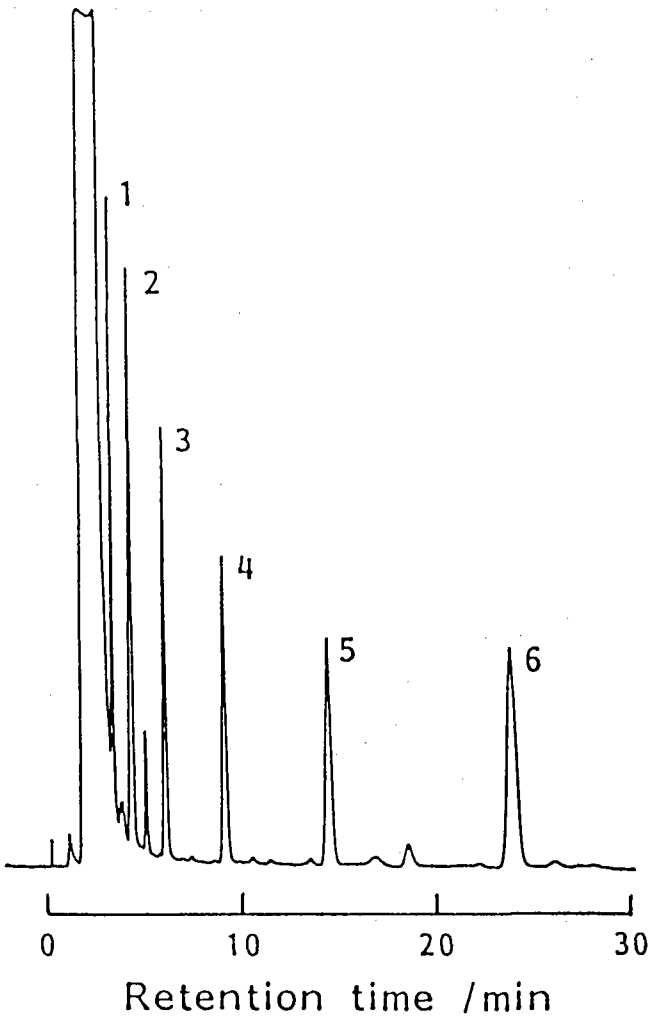

Fig. 6 Typical chromatogram of six fatty acid derivatives Column, Wakosil 5C18-200T; mobile phase, $CH_3OH : H_2O = 9 : 1$; flow rate, 1.0 ml / min. P.eaks: 1 = n-caproic acid; 2 = n-caprylic acid; 3 = n-capric acid; 4 = lauric acid; 5 = myristic acid; 6 = palmitic acid.

or excess amounts of 18-crown-6 to NE-OTf. The quantitative application of the method to the determination of saturated fatty acids (C_6 - C_{16}) was examined. The results indicated good linearity for the determination of each acid at seven different concentrations. The linear regression equations obtained for C_{14} acid, for instance, was y=0.868x + 0.471 with a correlation coefficient r=0.9996 (2×10^{-8} - 1.2×10^{-7} M), where y and x are the peak area and the concentration of the acid, respectively. A typical chromatogram of the derivatives of the acids is shown in

Determination of carboxylic acids in mouse brain

As an application of this reagent to the analyses of real samples, five carboxylic acids listed in Table 1 in mouse brain were simultaneously determined according to the procedure in the Experimental section. The recovery of these acids in the extraction process was estimated from that of spiked margaric acid which did not naturally exist in mouse brain, and was 95%

Table I Determination of five carboxylic acids in mouse brain.

Fatty acid	nmol/g wet	C.V.*
Docosahexaenoic acid	18 ±2	2.0
Arachidonic acid	79 ±5	1.7
Palmitic acid	36 ±3	1.9
Oleic acid	46 ±3	2.1
Stearic acid	76 ±3	2.1

* With seven replicated analyses

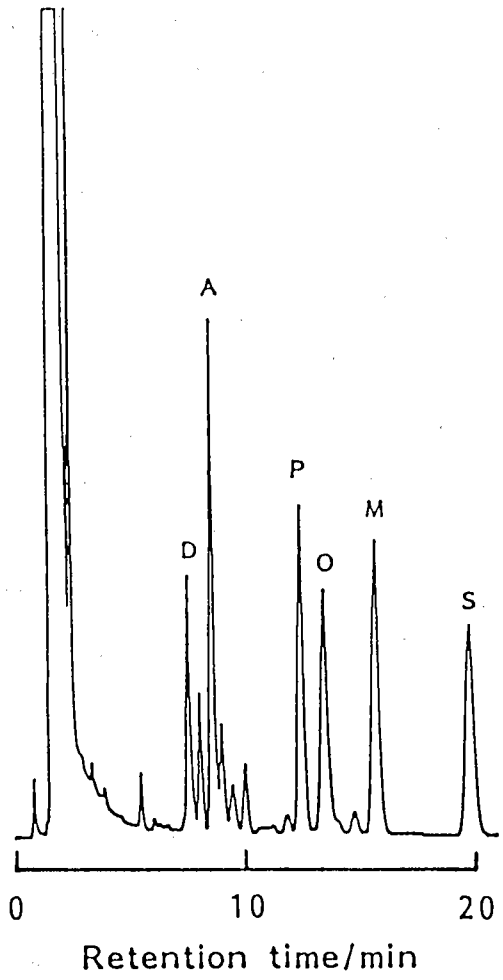

Fig.7 Chromatogram for the determination of carboxylic acids in mouse brain. Column, Chemcosorb 5C8; mobile phase, CH_3OH:H_2O = 87:13; flow rate, 1.0 ml / min. Peaks: D = Docosahexaenoic acid; A = Arachidonic acid; P = Palmitic acid; O = Oleic acid; M = Margaric acid (internal standard); S = Stearic acid.

consistently. These derivatives were well separated each other on the C-8 column with an eluent of CH_3OH/H_2O (87/13) within 20 min (Fig. 7). The obtained values for the cerebrum (0.33 g wet weight) were summarized in Table 1. All acids could be determined with coefficients of variation of ca. 2%.

Stability of the reagent

Solid NE-OTf was stable at least for six months in a light protected desiccator at room temperature. Stability of NE-OTf in acetonitrile (10^{-2} M) was examined by periodically chromatographing the solution, Fifty percentages of the initial peak heights were lost in 3 h at room temperature (32 °C), but it was possible to keep the loss in 8% after 6 days by keeping the solution below -20 °C.

SUMMARY

The use of 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate in the preparation of 2-(2,3-naphthalimino)ethyl ester derivatives of carboxylic acids for ultraviolet and fluorescent detections in high performance liquid chromatography was described. The reagent was easily synthesized by two step operations from 2,3-naphthalenedicarboxylic anhydride and was stable at least for 6 months at room temperature. Reactions of carboxylate potassium salts (10^{-5} M) with a 10-fold equivalent excess amount of the reagent proceeded to completion within 10 min in acetonitrile at room temperature, in the presence of 18-

crown-6 as a catalyst. The derivatization procedure with this reagent was successfully applied to the determination of some carboxylic acids in mouse brain. The detection limits (S/N=3) with ultraviolet and fluorescent detection were 100 fmol (at 259 nm) and 4 fmol (λ_{ex}=259 nm, λ_{em}=394 nm), respectively.

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II-3-3. S-(+)-[1-Methyl-2-(2,3-naphthalimino)]ethyl Trifluoromethanesulfonate as a Fluorescence Chiral Derivatizing Reagent for Carboxylic acids in High Performance Liquid Chromatography

INTRODUCTION

The chiral derivatization method is one of the useful methods for the separation of enantiomeric compounds by high performance liquid chromatography(HPLC); many chiral derivatization reagents have been developed for the optical resolution of various enantiomers.[1-9] In chapter II-3-2 the author developed 2-(2,3-naphthalimino)ethyl Trifluoromethanesulfonate as a derivatizing agent for carboxylic acids in HPLC. This reagent was highly reactive for carboxylic acids, and thus could derivatize these acids at room temperature to the corresponding derivatives of intense fluorescence High reactivity of triflate-type reagent has motivated the author to develop a chiral derivatizing reagent of triflate-type, because such a reagent should derivatize enantiomers to the corresponding diastereomers without racemization.

EXPERIMENTAL

Apparatus

The HPLC system used was the same one described in chapter II-3-2. Analytical column used was a Wakosil 5C18-200T (5μm, 150

x 4.6 mm i.d.). Melting point and specific rotation were measured with a Yanaco melting point apparatus and with a Jasco DIP-181 digital polarimeter, respectively. An Erma ERC-3510 degasser was utilized for continuous degassing of a mobile phase.

Reagents and materials

2,3-Naphthalenedicarboxylic anhydride, S-(+)-1-Amino-2-propanol and DL-α-Methoxyphenylacetic acid were obtained from Tokyo Kasei Co. (Tokyo, Japan). HPLC grade CH_3CN was purchased from Wako Pure Chemical Co. (Osaka, Japan) and used in the preparation of chromatographic mobile phases. All the other chemicals were of special grade. Water was purified with a Milli-Q water purification unit (Millipore, U.S.A.). Eppendorf Safe-Lock microcentrifuge tubes(2.0 ml) were used as reaction tubes.

Synthesis of OPNE-OTf

OPNE-OTf was prepared according to the same manner described in chapter II-3-2. Thus, in a 300 ml flask fitted with a water separator and a reflux condenser were placed 5,2g (0.026 mol) of 2,3-naphthalenedicarboxylic anhydride, 2.0g (0.027 mol) of S-(+)-1-Amino-2-propanol and 200 ml of dry toluene. The mixture was heated for 2h with a vigorous reflux on an oil bath. After cooling, the solid product was then filtered off on a G-3 glass filter and was washed with three 50 ml portions of cold water. Recrystallization from toluene gave transparent needles of S-(+)-1-(2,3-naphthalimino)-2-propanol(NP-OH): yield 75 %; mp 165°C;

$[\alpha]_D$ = +4.9°; Anal. calcd for $C_{15}H_{13}NO_3$, 70.59% C, 5.1% H, 5.49% N, found: 70.32% C, 4.95% H, 5.30% N. MS: m/z=255 (M^+).

To a solution of trifluoromethanesulfonic anhydride (5 g, 0.018 mol) in dichloromethane (100 ml) was dropwise added a mixture of pyridine (1.4 g, 0.018 mol) and NP-OH (4.1 g, 0.016 mol) in warm dichloromethane (100 ml) carefully at a rate of keeping the temperature of the reaction mixture below -5 °C; the addition required about 1 h. After the addition, stirring was continued for 2 h. The resulting solution was washed three times with cold deionized water and then dried over anhydrous magnesium sulfate. After removing dichloromethane under reduced pressure, a crude product was recrystallized twice from dichloromethane OPNE-OTf was obtained as transparent flakes: yield 35 % ; mp 135°C (deco); $[\alpha]_D$ = +18.78°; Anal. calcd for $C_{16}H_{12}NSO_5F_3$, 49.61% C, 3.10% H, 3.62% N, found: 49.36% c, 3.18% H, 3.54% N; IR: 1200 and 1400 cm^{-1} (-O-SO_2-); MS: m/z=387 (M^+).

Derivatization procedure

A typical derivatization procedure was as follows. To 0.5 ml of a test solution of DL-α-Methoxyphenylacetic acid in acetonitrile placed in a reaction tube were added 0.1 ml of 18-crown-6 (10^{-2} M) in acetonitrile and *ca.* 5 mg of anhydrous KF. After vortexing the tube slightly, 0.1 ml of OPNE-OTf (10^{-2} M) in acetonitrile was combined with it. The mixture was vortexed for 60 min at room temperature. The resulting solution was standing for 30 s and an aliquot (10 µl) of the supernatant was directly injected into the chromatograph.

RESULTS AND DISCUSSION

In general, normal-phase HPLC (NP-HPLC) is more suitable for the separation of diastereomeric compounds than RP-HPLC. In this researches, however, the author attempted to separate the diastereomers formed from the reaction of OPNE-OTf and DL-α-Methoxyphenylacetic acid. This method is more favorable than NP-HPLC for the separation of biological samples which will be used in further applications of this reagent. As shown in Fig. 1, a sufficient separation of the diastereomers was attainded by RP-HPLC.

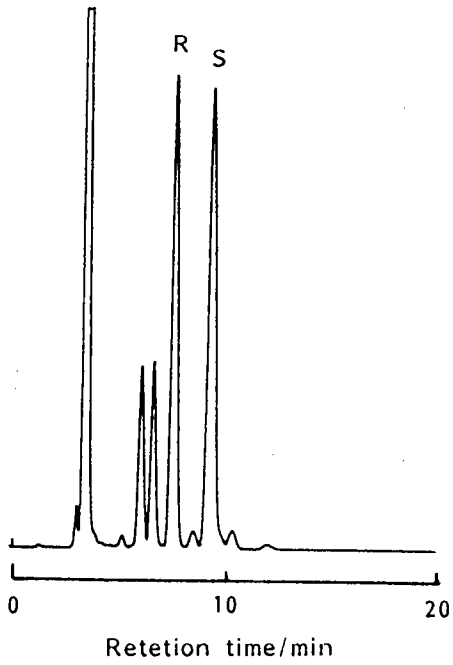

Fig.1 Chromatogram of α -methoxyphenylacetic acid derivatized with OPNE-OTf. Column,Wakosil 5C 18-200 mobile phase, CH_3CN:H_2O=6:4; flow rate 0.6ml/min. Peaks R=R-(-)-enantiomer,S=S-(+)-enantiomer.

SUMMARY

The new fluorescence chiral derivatization reagent developed in this researches is highly reactive and sensitive, and the resulting diastereomers can be well separated by RP-HPLC. This reagent should be useful for the determination of carboxylic acid enantiomers.

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Conclusion

In the present study, efforts have been made to develop new sulfonate-type derivatizing reagents for chromatography.

The main accomplishments of this thesis could be summarized as follows:

1. Pentafluorobenzyl methanesulfonate is more reactive than pentafluorobenzyl p-toluenesulfonate in the pentafluorobenzylation of inorganic anions. Seven inorganic anions, bromide, iodide, cyanide, thiocyanate, nitrite and sulfide in water at mM levels could be simultaneously pentafluorobenzylated and subsequently determined by GC-FID. In water at μM levels, except for cyanide and nitrate, the anions could also be determined by GC-ECD.
2. The procedure of removing excess reagent from reaction mixture was extremely simplified by using polymeric reagents instead of monomeric ones. Base line responses caused with excess reagents, as a result, was clearly decreased by use of polymeric reagents.
3. The reactivities of a series of tosylate-type derivatizing reagents on carboxylic acids were much depend on the label moieties themselves and the reagents bearing the functional group of much more electronegative showed high reactivities for the acids.
4. Triflate-type reagents was highly reactive ones for carboxylic acid. In particularly, 2-(2,3-naphthalimino)-ethyl triflate was the most useful labeling reagent for the acids in light of sensitivity.

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