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# NEW ULTRAVIOLET LABELLING AGENTS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MONOCARBOXYLIC ACIDS

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## SUMMARY

New UV-labelling 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*-toluenesulphonates. Each has been prepared by reaction of *p*-toluenesulphonyl chloride with *p*-nitrobenzyl alcohol, 3,5-dinitrobenzyl alcohol or N-(hydroxyethyl)phtalimide, respectively, in the presence of sodium hydroxide, and they are stable in the 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-labelling with each reagent, 18-crown-6 was used as the catalyst. The effects of the reaction solvent, reaction temperature and time and the concentrations of each reagent and the catalyst were also examined.

## INTRODUCTION

By the introduction of ion chromatography[1,2], 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 diffucult to determine trace amounts of organic anions by ion chromatography because of their low electric conductivities. In the HPLC determination of organic anions a labelling technique has usually been used for enhancement of the sensitivity of UV or fluorescence detection, and various labelling 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

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1-*p*-nitrobenzyl-3-*p*-tolyltriazene[8]) and halomethyl compounds (such as substituted phenacyl bromide (*p*-bromo- and *m*-methoxyphenacyl bromide)[9–11], 9-chloromethylantracene[12], N-chloromethyl-4-substituted phthalimide[13–14] and 7-acetoxy- and 7-methoxy-4-bromomethylcoumarins[15]). To our knowledge, only one report has been published with regard to sulphonate-type labelling agents for HPLC[16]. In this method, carboxylic acids were treated with 4′-bromophenacyl trifluoromethanesulphonate and derivatized to *p*-bromophenacyl esters which were subsequently determined by HPLC with UV detection.

Previously, we developed a derivatizing technique for gas chromatography with a new agent, pentafluorobenzyl *p*-toluenesulphonate[17–19], which is now commercially available. This reagent is designed to introduce the pentafluorobenzyl moiety into the analyte molecules in order to enhance not only their volatility but also their sensitivity.

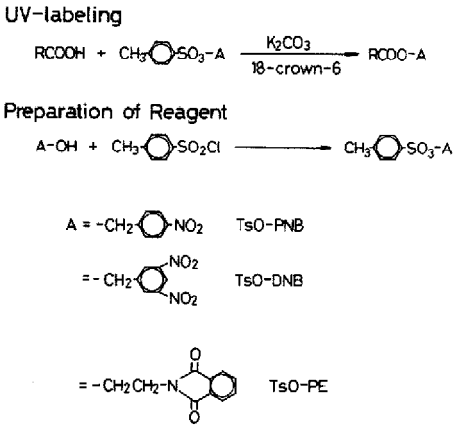
In this work, three *p*-toluenesulphonate-type UV-labelling agents for HPLC have been synthesized; *p*-nitrobenzyl *p*-toluenesulphonate (TsO-PNB), 3,5-dinitrobenzyl *p*-toluenesulphonate (TsO-DNB) and 2-(phthalimino)ethyl *p*-toluenesulphonate (TsO-PE). Their applicabilities to UV labelling and the HPLC determination of monocarboxylic acids have been examined, as shown in Scheme 1.

## EXPERIMENTAL

### *Apparatus*

When using TsO-PNB or TsO-DNB 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 × 4.6 mm I.D., particle size 5 μm), was obtained from Yamamura Chemical Labs. (Kyoto, Japan).

When using TsO-PE, the system consisted of a Model 880-PU pump (Japan




Scheme 1. Preparation of *p*-toluenesulphonate UV-labelling agents.

Spectroscopic, Tokyo, Japan), a Model 7125 syringe loading sample injector (Rheodyne, Cotati, CA, U.S.A.) and a Model 875-UV variable-wavelength UV absorption detector (Japan Spectroscopic) operating at 222 nm. The separation column (15 cm × 4.6 mm I.D.) packed with Tosoh (Tokyo, Japan) ODS-80TM (particle size 5 μm) was used together with a precolumn (3 cm × 4.6 mm I.D.) containing Chemco (Osaka, Japan) Nucleosil 50B anion exchanger, to remove *p*-toluenesulphonic 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.

An Hitachi RMU-6E mass spectrometer was employed with an ionization source temperature of 200°C and an acceleration energy of 1.8 kV.

*Reagents*

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

*Syntheses of UV-labelling agents*

Each of the three new UV-labelling agents was prepared from reactions between *p*-toluenesulphonyl chloride and corresponding alcohols by modifying the literature method[20] as follows.

*TsO-PNB.* *p*-Nitrobenzyl alcohol (10 g), *p*-toluenesulphonyl chloride (20 g) and tetra-*n*-butylammonium hydrogensulphate (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 sulphate and evaporated. By recrystallizing the resulting solid from methanol, TsO-PNB was obtained as white needles (yield: 40%).

*TsO-DNB.* 3,5-Dinitrobenzyl alcohol (1.58 g) and *p*-toluenesulphonyl chloride (2.2 g) were dissolved in 1,4-dioxane (15 ml), and then 40% NaOH (4 g) was added slowly to the 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. TsO-DNB was obtained as yellow needles (yield: 80%).

*TsO-PE.* Two pyridine solutions were prepared by dissolving 2 g of N-(hydroxyethyl)phthalimide and 2.4 g *p*-toluenesulphonyl chloride in 10 ml of pyridine, respectively. The *p*-toluenesulphonyl 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 sulphate was placed on a

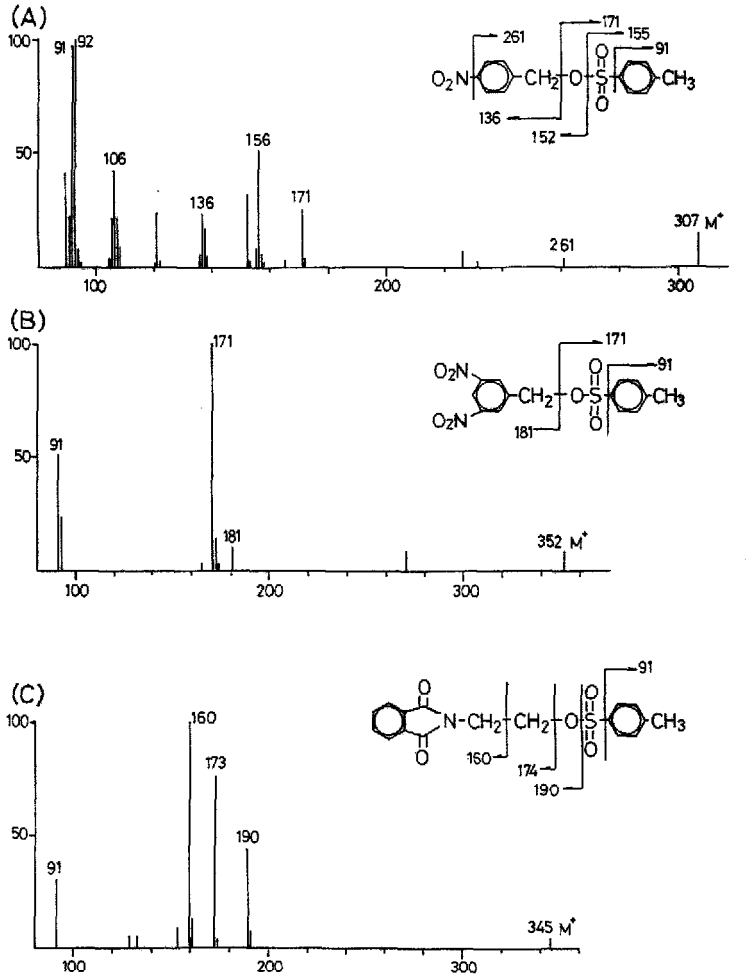



Fig. 1. Mass spectra of TsO-PNB (A), TsO-DNB (B) and TsO-PE (C).

column (5 cm × 5 cm I.D.) of silica gel (C-300, 200–300 mesh) obtained from Wako. TsO-PE eluted with chloroform was recrystallized from ethanol and obtained as white needles (yield : 75%).

The three *p*-toluenesulphonates thus synthesized were identified by mass and infrared spectrometry. Fig. 1 shows their mass spectra, and the infrared spectrum of TsO-PNB is given in Fig. 2.

*Procedure*

The recommended procedure for UV-labelling 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 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

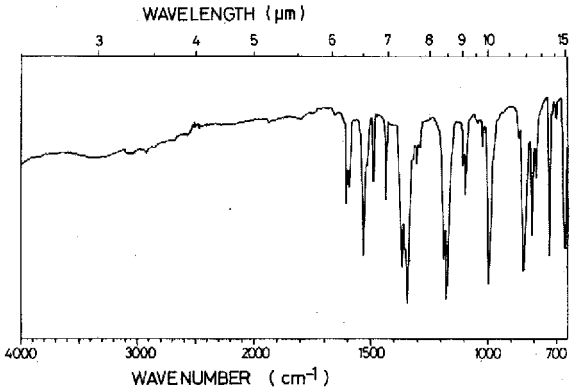



Fig. 2. Infrared spectrum of TsO-PNB.

reference standard and the reagent solutions, acetone, acetonitrile and propionitrile were used in the UV-labelling with TsO-PNB, TsO-DNB and TsO-PE, respectively (Table I). The concentrations of the agent and 18-crown-6 in the solution were dependent on the labelling 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 an 0.45-$\mu$m pore size hydrophobic membrane filter was fitted (Sartorius, Göttingen, F.R.G.). An aliquot (10 $\mu$l) 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

### *Preparation of reagents, and UV-labelling*

Fig. 1 shows the mass spectra of TsO-PNB, TsO-DNB and TsO-PE synthe-

TABLE I

OPTIMUM CONDITIONS AND CALIBRATION

| | *TsO-PNB* | *TsO-DNB* | *TsO-PE* |
|---|---|---|---|
| Reaction solvent | Acetone | Acetonitrile | Propionitrile |
| Reaction temperature (°C) | Room temp. | 50 | 85 |
| Reaction time (min) | 30 | 20 | 60 |
| Reagent concentration (m*M*) | 15 | 5 | 20 |
| 18-Crown-6 concentration (m*M*) | 2.5 | 2.5 | Not used |
| Wavelength of UV detection (nm) | 232 (254)[a] | 272 (254)[a] | 222 |
| Determination level ($\mu M$)[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 fixed-wavelength (254 nm) UV detector was used.
[b] These values were obtained for myristic acid.

sized. The mass peak at 307, 352 or 345 corresponds to the parent ion of TsO-PNB, TsO-DNB or TsO-PE, respectively, and other peaks are equivalent to the fragment ions of the *p*-toluenesulphonates. Fig. 2 shows the infrared spectrum of TsO-PNB. Two bands at 1180 and 1350 $cm^{-1}$ are characteristic of the symmetric and antisymmetric vibrations of $S(=O)_2$, respectively. The other two reagents give similar infrared spectra in this region.

Myristic acid was selected as the model monocarboxylic acid, and it was UV-labelled 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 PE derivatives were identified as PNB, DNB and PE esters of myristic acid, respectively.

*Optimum derivatization conditions*

The derivative of myristic acid labelled 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 reagent was detected at different wavelengths (Fig. 3). The peaks of the PE derivative are much larger than those of the PNB and DNB derivatives. In Fig. 3, therefore, the peak areas of the PE derivative are shown separately from those of the other two derivatives; *i.e.*, the ordinate of Fig. 3 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 area detected at 254 nm is much less than the maximum peak area. For the PNB and DNB derivatives, on the other hand, the

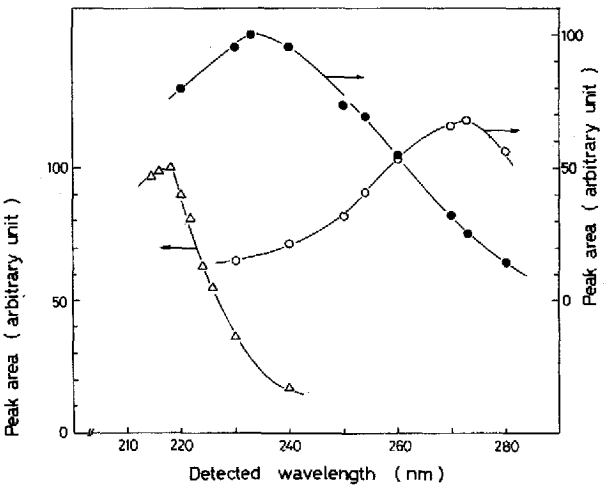
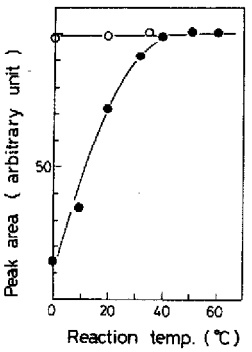



Fig. 3. Effect of wavelength on the peak area of PNB (○), DNB (●) and PE (△) derivatives of myristic acid. The peak area on the ordinate is shown by assigning the maximum peak areas of DNB and PE derivatives as 100.



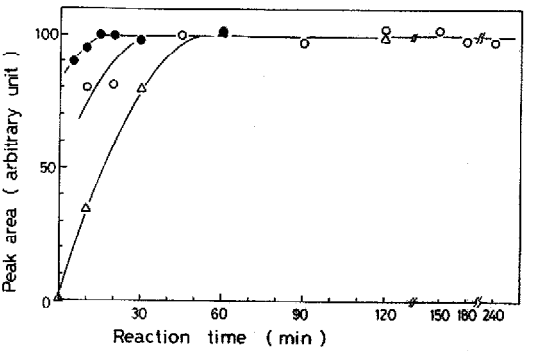




Fig. 4. Effect of reaction temperature on the labelling with TsO-PNB (○) and TsO-DNB (●).

Fig. 5. Effect of reaction time on the labelling with TsO-PNB (○), TsO-DNB (●) and TsO-PE (△).

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-labelling 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 labelling with TsO-PNB, even if the labelling is carried out at room temperature. For the labelling with TsO-DNB, on the other hand, the yield is highest at 50°C in acetonitrile. For TsO-PE, the best results were obtained using propionitrile as the solvent at 85°C.

The effect of the reaction temperature was studied with the optimum labelling system and solvent. Fig. 4 shows the results for the labelling with TsO-DNB, together with TsO-PNB. In Fig. 4 or 5, the peak area on the ordinate is exhibited by assigning the maximum peak area of the derivative labelled with each reagent as 100. The peak area of the DNB derivative increases with increasing reaction temperature to a constant value beyond 40°C, while that of the PNB derivative is constant, independent of the reaction temperature. Therefore, the reaction temperature was fixed at 50°C for the labelling with TsO-DNB and at room temperature for that with TsO-PNB. From the results of similar measurements, the labelling with TsO-PE was performed at 85°C.

We have also tested the effect of the reaction time on the labelling. From these results, shown in Fig. 5, 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 TsO-PNB, TsO-DNB and TsO-PE are 15, 5 and 20 m*M*, respectively. Furthermore, the effect of the catalyst, 18-crown-6, concentration in that solution on the labelling was examined. To perform the labelling with TsO-PNB or TsO-DNB, 2.5 m*M* 18-crown-6 solution is necessary, while the derivative peak area does not vary regardless of the presence of 18-crown-6 in the labelling with TsO-PE. Therefore, the optimum concentrations of the catalyst were fixed as given in Table I.

*Analytical calibrations and chromatograms*

After the optimum reaction conditons for the labelling 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 labelled with each agent was compared with that of the standard solution of the authentic derivative mentioned above. The yields of the labelling reactions with TsO-PNB, TsO-DNB and TsO-PE are 105, 94 and 94%, respectively.

A 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 correlation coefficients nearly equal to 1, as shown in Table I. The determination levels are primarily dependent on the strengths of the absorption of the derivatives labelled 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 labelling method with TsO-PE is very high, but the detection at 222 nm is apt to be obstructed by many compounds.

Fig. 6 shows the chromatograms obtained for the determination of several

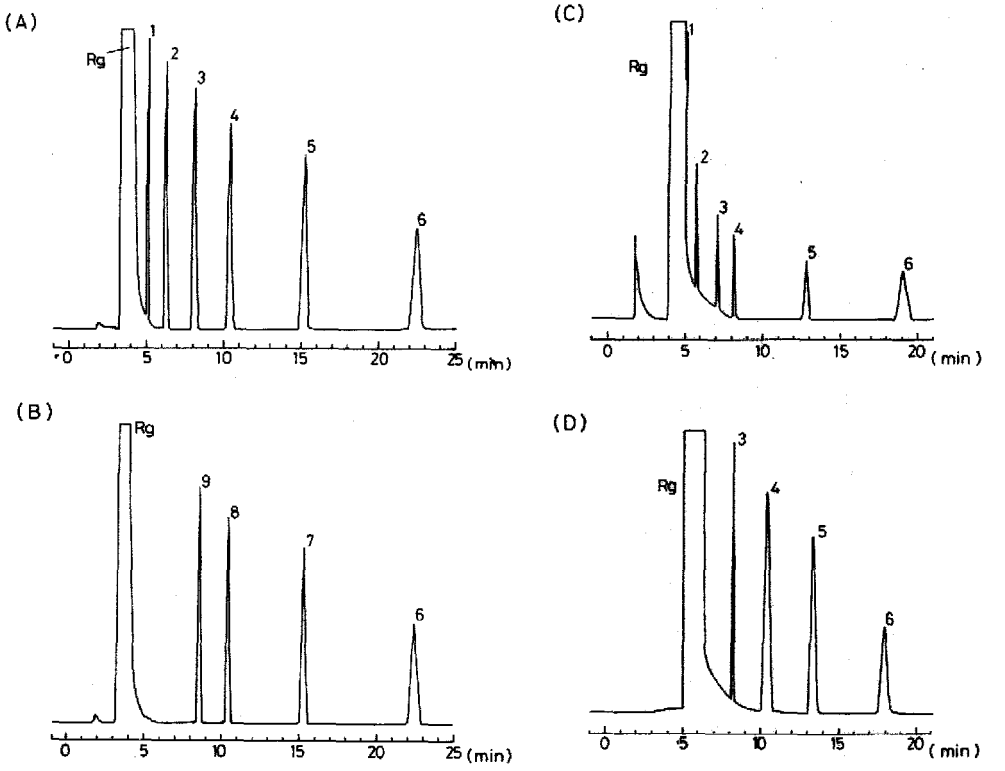



Fig. 6. Chromatograms of a mixture of monocarboxylic acids labelled with TsO-PNB (A,B), TsO-DNB (C) and TsO-PE (D). Peaks: Rg = reagent; 1 = caprylic acid; 2 = capric acid; 3 = lauric acid; 4 = myristic acid; 5 = palmitic acid; 6 = stearic acid; 7 = oleic acid; 8 = linolic acid and 9 = linolenic acid.

monocarboxylic acids. The separation of the peaks of the derivatives of lower monocarboxylic acids from that of the reagent is somewhat improved by using acetonitrile solution containing small quantities of water as the mobile phase. With these reagents, unsaturated monocarboxylic acids can also be labelled, together with saturated ones. Fig. 6B shows the chromatogram obtained when three unsaturated monocarboxylic acids (carbon number: 18) were labelled with TsO-PNB. 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.

For the HPLC determination of monocarboxylic acids, three *p*-toluenesulphonate-type UV-labelling agents have been developed, which bear tagging groups for UV detection. Various tagging groups will also be introduced for fluorescence detection in a similar fashion. Further work is now in progress to develop a polystyrene-type polymeric UV-labelling agent, in order to facilitate the separation of the reagent from the derivatives, and to avoid the coinjection of the reagent and the contamination of the separation column.

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