Communications to the Editor

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APPLICATION OF 1-NAPHTHYLAMINE TO LABELING OF FATTY ACID FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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The use of 1-naphthylamine, a primary amine, for labeling reagents of fatty acids was examined. When fatty acids were allowed to react with thionyl chloride, high yield of acid chloride derivatives was obtained. The reaction of the acid chlorides with NA gave NA derivatives which showed strong UV absorption around 280 nm. Several sorts of fatty acids in the mixture were labeled by the present method, and they were analyzed by high-performance liquid chromatography.

KEYWORDS ——— high-performance liquid chromatography; fatty acid; thionyl chloride; 1-naphthylamine; preparation of acid chloride

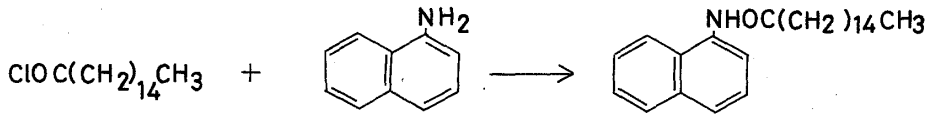
There are many reports on the labeling of fatty acids with UV or fluorescent reagents with the aid of high-performance liquid chromatography (HPLC) for purpose of highly sensitive analysis of the fatty acids which do not have intense ultraviolet adsorption.[1] Many of these reagents are developed to react specifically with carboxylic acids and their potassium salts. Thus, a considerably limited number of these reagents can be utilized.

We proposed the utilization of the amines which had not been used widely for the UV and fluorescent labeling of fatty acids. Carboxylic acids increase their reactivity with amines by leading them into acid chlorides, and amine derivatives are formed within a brief period of time.[2] In the present study utilizing the above-mentioned property, we considered the reaction of fatty acids with labeling reagents having primary amines after leading fatty acids into acid chlorides.

We utilized the above-mentioned property based upon the reaction of the primary amine 1-naphthylamine(NA) with acid chlorides which were derived from fatty acids. As an example, palmitoyl chloride (PT·Cl), an acid chloride of palmitic acid (PT), was selected as a starting material. PT·Cl (0.36 mmol), NA (0.36 mmol), and triethylamine (TEA, 0.36 mmol) as a reaction catalyst were added to 2 ml of benzene, and the reaction was allowed to take place at 80°C for 1 h. After the end of the reaction, the solvent was distilled off and the residual solid matter was recrystallized with ethanol. To determine the chemical structure of the obtained crystal, ^{1}H-NMR, MS, and IR spectrum were measured after drying.

The results were; ^{1}H-NMR($CDCl_3$)δ:0.90(3H, t, J=5Hz, -C<u>H</u>$_3$), 1.29(24H, br -(C<u>H</u>$_2$)$_{12}$-), 1.73(2H, m, -COCH$_2$C<u>H</u>$_2$-), 2.13-2.70(2H, m, -COC<u>H</u>$_2$CH$_2$-), 7.30-8.13(7H, m, aromatic); MS m/z:381 (M^+); IR$\nu_{max}^{CHCl_3}$ cm^{-1}: 1680 (<u>CO</u>NH). From these results,

it was confirmed that this crystal was N-palmitoyl-1-naphtylamine (PT·NA) which was formed by the linkage of NA at the carboxylic radical of the PT.



palmitoyl chloride (PT·Cl) | 1-naphthylamine (NA) | N-palmitoyl-1-naphtylamine (PT·NA)

Measurement of the UV spectrum of PT·NA showed a strong absorption spectrum around 280-290 nm which is not observed in the case of methanol solution of NA (Fig. 1).

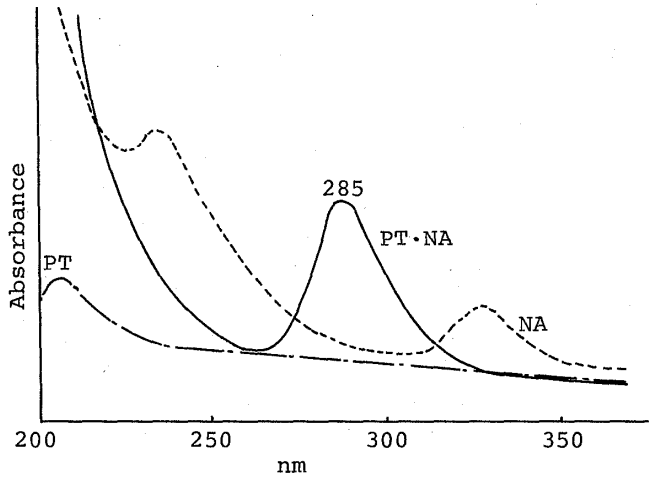


Fig. 1. Absorption Spectra of PT·NA, NA, and PT in Methanol

PT·NA : N-palmitoyl-1-naphthylamine
NA : 1-naphthylamine
PT : palmitic acid

Fatty acids were found to react easily with NA having primary amine when they were led into acid chloride, and the reaction product showed absorption at the UV area. Therefore, UV-labeling of free fatty acids utilizing this reaction was examined.

Firstly, the method to make acid chloride derivatives of fatty acids was examined. Many methods of obtaining acid chloride derivatives have been reported.[3] In particular, Hoffman et al.[4] prepared the acid chlorides of higher fatty acids using triphenyl phosphine.

In the present study, thionyl chloride ($SOCl_2$) was tested as the reagent for preparing acid chlorides. When using $SOCl_2$, the SO_2 and HCl produced along with acid chloride can easily be separated, and the excess $SOCl_2$ can be distilled off because of its low boiling point (79°C).[3a]

In a reaction vial of 2 ml volume, 0.1 ml of $SOCl_2$ was added to 0.1 ml benzene solution containing 0.78 μmol PT, and the mixture was shaken sufficiently; the amounts of the reaction products, acid chlorides (PT·Cl), were compared by changing the reaction temperature and reaction time (Fig. 2). PT·Cl thus produced was changed into NA derivatives, and chrysene was added as an internal standard;

thereafter, the solution was injected into HPLC. In the obtained chromatographic chart, the peak height was measured, and the amount of PT·Cl was determined on the calibration curve made beforehand.

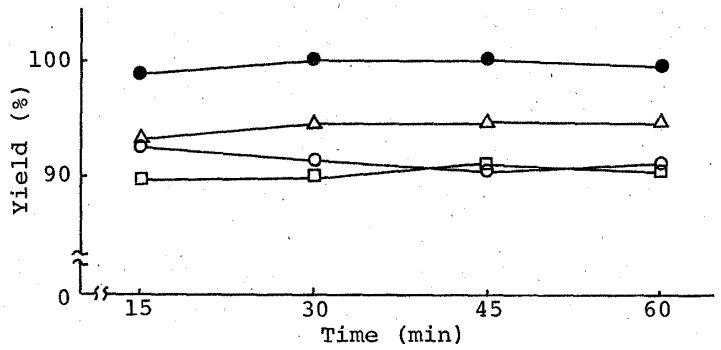


Fig. 2. Effect of Reaction Temperature on Yields of Acid Chlorides from PT

□—□ : 30°C, △—△ : 50°C, ●—● : 70°C, ○—○ : 90°C

When the reaction temperature was increased to 30, 50, and 70°C, the amount of the produced PT·Cl increased, and the yield of PT·Cl production was close to 99-100% at 70°C. However, when the temperature was raised to 90°C, production of PT·Cl decreased. Therefore, the optimum condition for producing acid chlorides of fatty acids by the reaction with $SOCl_2$ was determined to be 70°C and 30 min.

In the next step, we examined the conditions for the reaction of acid chloride with NA. Benzene was selected as the solvent, and 0.73 μmol of PT·Cl, 4 μmol of NA and 4 μmol of TEA were added to 0.21 ml of benzene. The mixture solution was allowed to react to at 30, 50, 70, and 90°C for 15, 30, 45 and 60 min. At the end of the reaction, after addition of chrysene (44 nmol) as an internal standard, the solution was injected into HPLC. As is shown in Fig. 3, the yield of PT·NA obtained in each condition was as high as 97-101%. Even when the reaction was made at 30°C for 15 min, about 99% of PT·Cl was changed into PT·NA. Therefore, the optimum condition for the reaction of acid chloride with NA was determined to be 30°C and 15 min.

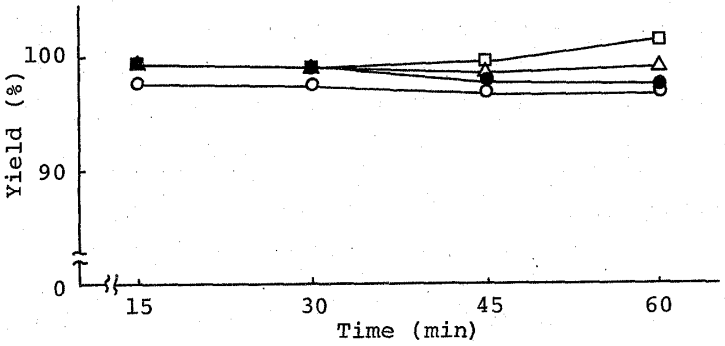


Fig. 3. Effect of Reaction Temperature on Yields of NA Derivative from PT·Cl

□—□ : 30°C, △—△ : 50°C, ●—● : 70°C, ○—○ : 90°C

The method for making NA derivatives which was established in the present study was applied to several other fatty acids. Seven sorts of fatty acids, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{19:0}$, were selected, and their mixture was led to NA derivatives by the present method; thereafter, crysene was added and the solution was injected into HPLC (Fig. 4). The condition of HPLC was as follows. A μBondapak C_{18} (30 X 0.4 cm) column was connected to Hitachi HPLC model 635A. The mobile phase was methanol-water (83:17), which was used at the flow rate of 2.0 ml/min. The detector used was a Hitachi multi-wavelength UV monitor (280 nm).

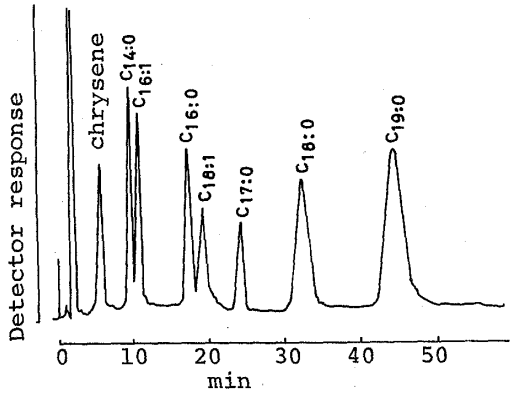


Fig. 4. HPLC of Some NA Derivatized Fatty Acids

As a result, seven sorts of fatty acids were well separated with simple solvent. The limit of detection in the present method was 4 ng (about 10.5 pmol) in the case of PT; the recovery percentage of $C_{14:0}$ (0.36 μg) was found to be 100.7 ± 1.1% (n=4) and the coefficient of variation (CV%) was 1.0%. This means that the present method has about the same sensitivity as that using other UV labeling reagents, and, thus, the present method is considered to be useful as the labeling method for the quantitative microanalysis of fatty acids with HPLC. The present method is a new one which is quite different from previously reported methods for making the derivatives of fatty acids. Moreover, the present method is considered to be applicable for labeling of secondary amines and alcohols in addition to primary amines. In the future, we are planning to utilize this method for the quantitative analysis of fatty acids in biological samples including unsaturated fatty acids.

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