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# GAS CHROMATOGRAPHY OF FREE FATTY ACIDS AFTER TREATMENT WITH IODINE AND IODINE TRICHLORIDE SOLUTION

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A modified gas chromatographic procedure is described for the separation of mixtures of saturated and unsaturated higher fatty acids having the same carbon number. On a 2% OV-17 column, the chromatogram of mixtures of stearic acid (18:0) and oleic acid (18:1) treated with iodine and iodine trichloride ($I_2$-$ICl_3$) in acetic acid at 20°C for 1 h showed better separation than that of those non-treated acids. The reaction product of oleic acid and $I_2$-$ICl_3$ solution was identified as stearolic acid by gas-liquid chromatography and mass spectrometry. The proposed method was also applied to the separation of palmitic (16:0) and palmitoleic acid (16:1).

Polyesters, polar liquid phases, such as ethylene glycol succinate (EGS), ethylene glycol adipate (EGA) or diethylene glycol succinate (DEGS), have been used as the stationary phase in gas chromatography (GC) mainly for the separation of the saturated and the unsaturated fatty acids having the same carbon number. Liquid phases of silicone oil are superior to polyesters in heat resistance, so that the reproducible result can be obtained and also the silicone liquid phase can be applied for the continuous fatty acid determination for a long period of time at higher temperature. However, the effectiveness of silicone liquid phase for separating saturated and unsaturated fatty acids is inferior to DEGS.

Several methods have been reported for the separation of saturated and unsaturated fatty acids. Non-polar liquid phases, such as Silar 10 C,[1),2)] 10 CP[3)] or SP-2330[4)], have been utilized by combination with programming temperature for most of the reports. Vine has shown the effectiveness of SE-30 support-coated open-tubular capillary column (50 m x 0.5 mm).[5)]

Through the investigation of the fatty acid separation by the addition of halogen atom to the unsaturated acid, the existence of the different derivative from halogen adducts was observed by using iodine-iodine trichloride reagent, which is used as the determination of iodine value in Japan Pharmacopeia X.

In the present study, gas chromatographic separation of saturated and unsaturated fatty acids having the same carbon number was investigated by means of silicone column for the liquid phase. By treatment of an unsaturated free fatty acid with $I_2$-$ICl_3$ reagent, the retention time of the fatty acid was delayed

considerably, and consequently it was possible to separate the saturated fatty acid from the unsaturated fatty one by OV-17 column.

## EXPERIMENTAL

Materials. Palmitic acid (16:0), palmitoleic acid (16:1) and stearic acid (18:0, ST) were purchased from P-L, Biochemicals, Inc. (Milwaukee, Wis. U.S.A.), oleic acid (18:1, OL) from Sigma Chemical Co. (St. Louis, Mo. U.S.A.), and margaric acid (17:0) from Nakarai Chemicals, Ltd. (Nakakyo-ku, Kyoto).

An iodine and iodine trichloride ($I_2$-$ICl_3$) solution was prepared by mixing 0.87 g of iodine and 0.79 g of iodine trichloride in an acetic acid solvent, and was made to a total volume of 100 ml with acetic acid.

Apparatus. JGC-20 KFP (JEOL Co. Ltd., Tokyo) gas chromatograph, equipped with flame ionization detector, was used in this study. The samples were analyzed on a glass column (2 m x 2 mm, I.D.) packed with 2% OV-17 on Gas Chrom Q (80/100 mesh). The detector temperature was maintained at 300°C, and the column oven was kept at 240°C. Carrier gas was nitrogen with a flow rate at 21 ml/min. Infrared spectra were recorded on a Jasco (Japan Spectroscopic Co. Ltd.) IR A-3 spectrophotometer. Mass spectra were determined on a Hitachi RMU-7L spectrometer. Thin-layer chromatography (TLC) was carried out using Merck pre-coated TLC plates with Kieselgel 60 and by using petr.ether-ether-AcOH (80:20:1) as a developing solvent.

Methylesterification of fatty acids. Less than 1.0 mg of fatty acid was esterified with 1 ml of diazomethane-saturated ethereal solution. After standing for 15 min, excess of diazomethane was evaporated under a stream of nitrogen. The esterified materials thus obtained were dissolved in acetone for GC analysis.

Recovery tests of ST and OL added to rabbit plasma. To 1 ml of rabbit plasma, 1 ml of heptane solution containing 20-100 μg of ST and 40-200 μg of OL was added, and the combined mixtures were shaken well by an automatic mixer. In a glass-stoppered centrifuge tube (17 x 105 mm), the heptane solution containing ST and OL was suspended in 1.0 ml of water. The suspension was shaken with 2 ml of iso-PrOH-heptane-1N $H_2SO_4$ (40:10:1) as reported by Dole.[6] The mixture, to which 2 ml each of heptane and of water were added, was shaken again for 3 min. then centrifuged at 1000 rpm for 5 min. The organic layer was transferred to another centrifuge tube. The remaining aqueous layer was subjected to two more extraction with 3 ml of heptane by 3 min shaking, and was centrifuged at 1000 rpm for 5 min. To combined organic layers were added 2 ml of 0.05% $H_2SO_4$ and the mixture was shaken for 3 min. After centrifugation of the mixtures at 1000 rpm for 5 min, the heptane layer was evaporated to dryness in vacuo, then dried over silica gel in a vacuum desiccator for 30 min. The dried materials were dissolved in a small volume of $CCl_4$, and the solution were subjected to GC analysis using margaric acid as an internal standard and by the calibration curves of ST and OL.

## RESULTS AND DISCUSSION

Silicone columns of non-polar stationary phases generally can not separate the important pair of ST (18:0) and OL (18:1), which are components of fats and oils, while capillary column[7),8)] or polar stationary phases[1),8)] provide better

separation.

Methylesters of stearic (ST) and oleic acid (OL) appeared at the same retention time on a 2% OV-17 column (Fig.1). ST and the derivative of OL which were treated with $I_2$-$ICl_3$, however, had different retention times; ST, 2.9 min, OL, 10.7 min. This indicates that OL might be converted into another substance by the $I_2$-$ICl_3$ reagent treatment, and this is referred to as "OL derivative" in the present paper.

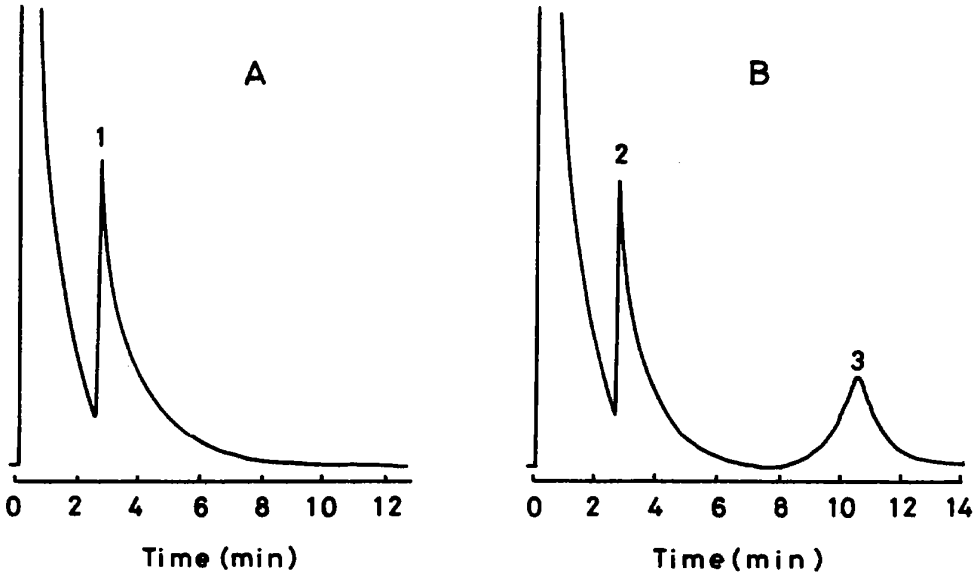



Fig.1. Gas chromatograms of $I_2$-$ICl_3$ reagent-treated ST and OL.
A: Before treatment of $I_2$-$ICl_3$, B: After treatment of $I_2$-$ICl_3$
Peak 1: ST and OL, Peak 2: ST, Peak 3: OL derivative

As the retention time of "OL derivative" is different from that of OL and the derivative also shows different behavior in TLC, it is assumed that OL and "OL derivative" are completely different substances. Thus, the structure of the methyl ester of "OL derivative" which was derived from OL and an $I_2$-$ICl_3$ solution was determined by both GC-mass spectrometry (MS) and infrared spectrometry (IR). The parent peak of the MS spectrum is at m/e 294. Fragmentations undergo subsequent cleavage, giving rise to ions of m/e 262, 210, 178, 150, 87, 81, 74 and 67, and the fragment patterns show the difference between methylesters of OL and OL derivatives.[9] We can conclude that "OL derivative", in which a double bond has been changed to a triple bond by dehydrohalogenation, corresponds to the structure of stearolic acid, as shown in Scheme I. Methyl ester of "OL derivative" was examined by IR spectrum. The absorption band at 3020 $cm^{-1}$ to the =C-H streching vibration of OL was not observed in "OL derivative". In addition, the absorption bands at 2800-3100 $cm^{-1}$ are consistent with the spectrum of methyl stearolate[10] (Fig.2). It is concluded that the peaks of ST and OL were easily separated with

the silicone liquid phase column by treatment of $I_2$-$ICl_3$ solution.

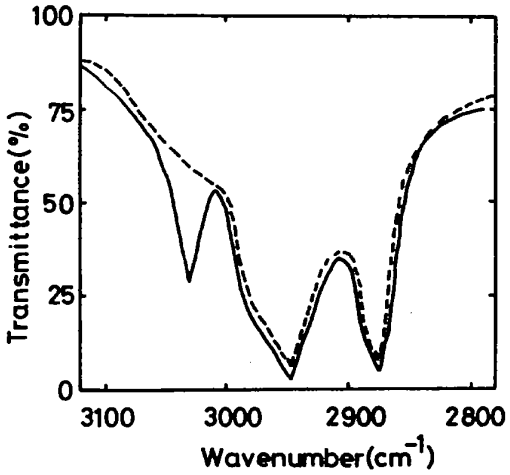
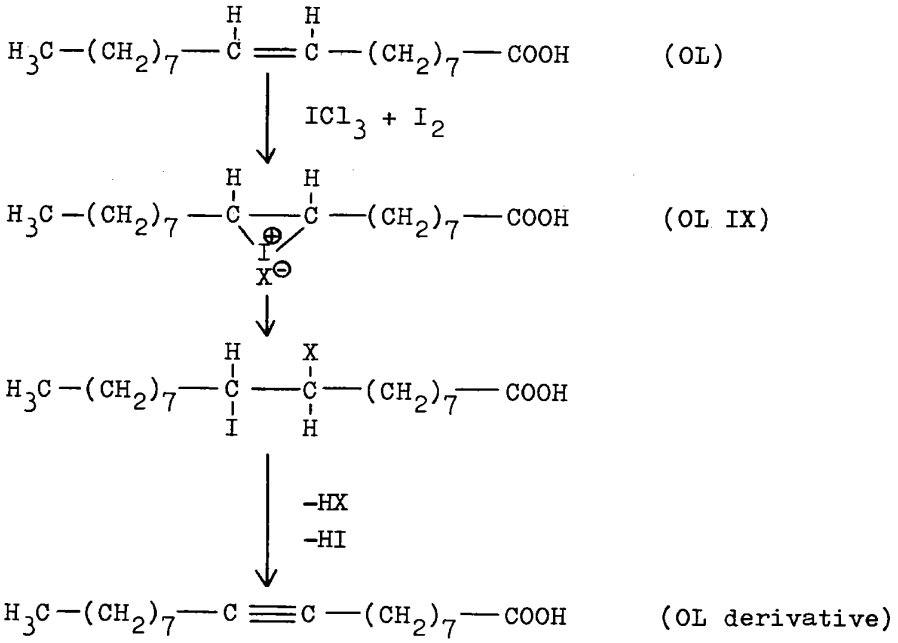



Fig.2. Infrared spectra of methyl esters of OL and "OL derivative" (methyl stearolate) in the C-H streching region. The solid line and dotted line show methyl esters of OL and "OL derivative", respectively.




Scheme I

This $I_2$-$ICl_3$ reagent treatment was then applied to the separation of palmitic (16:0, PL) and palmitoleic acid (16:1, PT). The methyl esters of PL and PT have the same retention times when 2% OV-17 column of GC was used. The PL which was treated with $I_2$-$ICl_3$ solution showed the same retention time as that of PL methyl ester, but $I_2$-$ICl_3$ treatment for PT had the different retention time, so that $I_2$-$ICl_3$ treatment could separate PL from PT using OV-17 column.

The proposed technique was also found to be useful for the GC determination of saturated and unsaturated fatty acid mixtures having the same carbon number. The liquid phase is so stable for heat that reproducible retention and response can constantly be obtained by GC.

The preliminary recovery tests of ST and OL which were added to rabbit plasma were also investigated. As shown in Table 1, the recovery of ST which was added to 1 ml of rabbit plasma was recovered between 99.5-105.3% and that of OL added to 1 ml of plasma was between 97.9-100.7%.

Although the application of $I_2$-$ICl_3$ reagent to ST and OL was of primary concern in these studies, the applicability of the method for the separation of PL (16:0) and PT (16:1) was confirmed.

Table 1 Recovery test of ST and OL added to rabbit plasma

| Plasma (ml) | ST | | | OL | | |
|---|---|---|---|---|---|---|
| | Added (μg) | Recovery* (μg) | (%) | Added (μg) | Recovery* (μg) | (%) |
| 1.0 | 20 | 21.1 ± 0.3 | 105.3 ± 1.4 | 40 | 39.1 ± 1.4 | 97.9 ± 3.6 |
| 1.0 | 40 | 40.7 ± 0.3 | 101.1 ± 0.8 | 80 | 80.0 ± 1.4 | 100.0 ± 1.8 |
| 1.0 | 60 | 60.1 ± 0.2 | 100.2 ± 0.4 | 120 | 120.8 ± 0.0 | 100.7 ± 0.0 |
| 1.0 | 80 | 80.3 ± 0.2 | 100.3 ± 0.3 | 160 | 158.5 ± 4.0 | 99.0 ± 2.5 |
| 1.0 | 100 | 99.5 ± 0.5 | 99.5 ± 0.5 | 200 | 199.1 ± 1.4 | 99.6 ± 0.7 |

*Mean ± S.D., n=3

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## References

1) E.S. Van Vleet, J.G. Quinn: J. Chromatogr., 151, 396 (1978).
2) J. MacGee, M.G. Williams: J. Chromatogr., 205, 281 (1981).
3) M. Höckel, W. Dünges, A. Holzer, P. Brockerhoff, G.H. Rathgen: J. Chromatogr., 221, 205 (1980).
4) G.J. Haan, S.V. Der Heide, B.G. Wolthers: J. Chromatogr., 162, 261 (1979).
5) J. Vine: J. Chromatogr., 196, 415 (1980).
6) V.P. Dole: J. Clin. Invest., 35, 150 (1956).
7) C. Litchfield, R. Reiser, A.F. Isbell: J. Am. Oil Chemists' Soc., 40, 302 (1963).
8) T. Murata, S. Takahashi, T. Takeda: Anal. Chem., 47, 573 (1975).
9) G. Odham, E. Stenhagen: "Biochemical Applications of Mass Spectrometry", Edited by G.R. Wallaer, p.211 (1972), (Wiley-Interscience, New York).
10) Y. Hirabayashi, N. Kato, M. Mizuta, H. Ishihara: Bull. Chem. Soc. Jpn., 44 2733 (1971).

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gas chromatography of fatty acids; saturated and unsaturated fatty acids; iodine and iodine trichloride; silicone liquid phase.

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