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Analysis of underivatized C_{12}–C_{18} fatty acids by reversed-phase ion-pair high-performance liquid chromatography with conductivity detection

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ABSTRACT

A rapid, simple and precise reversed-phase ion-pair high-performance liquid chromatographic method is described for the separation and determination of underivatized fatty acids (C_{12}–C_{18}) using a conductivity detector. Baseline separation of eight fatty acid standards was achieved on an octadecylsilyl column using isocratic elution with a methanol–5 m*M* tetrabutylammonium mobile phase. The method was successfully applied to the determination both of an anionic surfactant and of free fatty acids extracted from etiolated wheat shoots. The detection limit of margaric acid was *ca.* 2 ng at a signal-to-noise ratio of 3.

INTRODUCTION

The determination of free fatty acids has become important in many fields [1,2]. Although many laboratories have traditionally resolved fatty acids by gas chromatography [3], the determination of fatty acids by high-performance liquid chromatography (HPLC) seems to have become established [4–8]. Most of the available methods need derivatization for satisfactory separation and sensitivity, however, because fatty acids lack fluorescent or strongly UV-absorbing groups. Consequently, numerous types of precolumn labelling agents for HPLC have been developed [9–15].

The development of methods for the determination of underivatized long-chain fatty acids is a challenge. Separation of underivatized fatty acids has frequently been performed on octadecylsilyl (ODS) reversed-phase columns [2,16–20] using various mobile phases such as methanol–water–acetic acid [2], tetrahydrofuran–water [16] and acetonitrile–aqueous phosphoric acid [17,19,20]. Detection has been effected with refractive index [1], UV [2,17], capacitance/conductance [16] and electrokinetic detectors [18], a post-column ion-pair extraction and detection system [19], a differential thermal lens [20] and a chemiluminescence detector [21]. However, each method has drawbacks such as low sensitivity, poor resolution and long analysis time.

We have developed a rapid, simple and precise reversed-phase ion-pair HPLC method with conductivity detection for the separation and determination of underivatized fatty acids. Good separation of eight fatty acid standards was achieved by the use of a reversed-phase column, an isocratic eluent consisting of methanol–5 m*M* tetrabutylammonium (TBA) and a conductivity detector.

EXPERIMENTAL

Reagents and chemicals

Analytical-reagent-grade methanol and myristic acid were purchased from Wako (Osaka, Japan). PIC reagent A (a phosphate-buffered solution of TBA) was obtained from Waters Assoc. (Milford, MA, USA). Lauric, oleic and linoleic acids were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), margaric acid from Aldrich (Milwaukee, WI, USA) and linolenic, palmitic and stearic acids from Sigma (St. Louis, MO, USA). All fatty acids were of

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the highest purity available. Lunac O-P, an anionic surfactant, was supplied by Kao (Tokyo, Japan).

Extraction of plant lipids

Wheat (*Triticum aestivum* L. *cv.* Shirasagi) seeds were sown on moistened vermiculite, germinated and grown in the dark at 25°C for 5 days. A portion (300 mg fresh weight) of shoots were cut into 1–2-cm pieces, transferred into a 10-ml vial containing 5 ml of distilled water, incubated for a further 14 h as above and homogenized in a total of 2 ml of methanol with a mortar and pestle in dim light. The fresh weight after the 14-h incubation was 330 mg. The homogenate was transferred into a 10-ml tube with a Teflon cap. Exactly 1 ml of chloroform was added and mixed well with the methanolic extract, followed by the addition of 7 ml of distilled water. The mixture was centrifuged at 2000 *g* for 5 min at 25°C. An aliquot (0.3 ml) of the chloroform layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 30 μl of 75% aqueous methanol and an 8-μl portion was injected onto the column for fatty acid determination.

HPLC analysis

The HPLC system consisted of a CCPD pump, an IC-8010 CM conductivity detector (both from Tosoh, Tokyo, Japan) and a SIC Chromatocorder 12 (System Instruments, Tokyo, Japan). A Rheodyne Model (Cotati, CA, USA) 7125 sample injector equipped with a 20-μl loop was used for sample injection. A 150 × 4.0 mm I.D. column packed with ODS, 5-μm particle size Hitachi Gel 3056 (Hitachi Instruments Service, Tokyo, Japan) was used. The mobile phase was methanol 5 m*M* TBA (pH 7.5) (75:25, v/v) which was prepared by diluting one bottle of PIC reagent A solution with 1000 ml of distilled water, passed through a 0.45-μm filter (Fuji Photo Film, Tokyo, Japan) before use. The flow-rate was 0.8 ml/min unless stated otherwise, the column temperature being 50°C. The conductivity detector was set at sensitivity 0.01 and range 200 μS/cm. The background conductivity was 114 μS/cm. The attenuation was fixed at 8, except when the detection limit of margaric acid was estimated at 1. The minimum width, minimum height, "twice time" (the period in which the peak width is doubled) and chart speed were set at 0.1 min, 8 μV, 0 min and 2 mm/min, respectively.

RESULTS AND DISCUSSION

TBA has conventionally been used to separate organic anions as ion pairs [22–24]. A mobile phase consisting of methanol–5 m*M* TBA (75:25, v/v) efficiently separated C_{12}–C_{18} fatty acid standards (Fig. 1). Methanol–water (75:25, v/v) mixture gave a poor separation with rapid elution of the fatty acids. The retention time increased with increase in the concentration of TBA in the mobile phase. At 5 m*M* TBA the lauric acid (C12:0) and myristic acid (C14:0) peaks were segregated from the in-between system peak, and the myristic acid peak was adequately separated from the linolenic acid (C18:3) peak. At 7.5 and 10 m*M* TBA the lauric acid peak overlapped the system peak (the range was raised to 500 μS/cm only for 10 m*M* TBA having an elevated background beyond the initial setting of 200 μS/cm). Lauric acid was eluted after the system peak

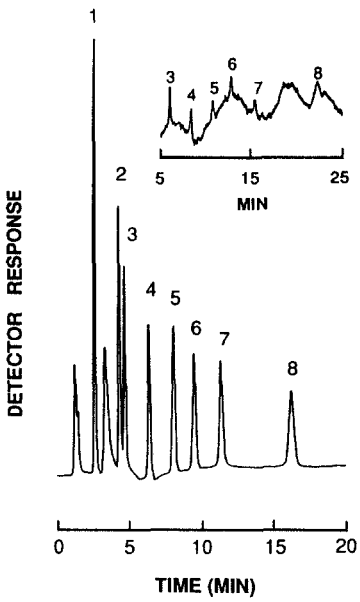

Fig. 1. Separation of free fatty acids by HPLC with conductivity detection. Mobile phase, methanol–5 m*M* TBA (pH 7.5) (75:25, v/v); flow-rate, 0.8 ml/min; oven temperature, 50°C. Detector settings: sensitivity, 0.01; range, 200 μS/cm. A 312.5-ng portion of each acid was injected. The inset shows the detection limits obtained by injecting 2 μl of 1 ppm fatty acid standards and setting the attenuation at 1 and the flow-rate at 0.6 ml/min. Peaks: 1 = lauric (C12:0) 2 = myristic (C14:0); 3 = linolenic (C18:3); 4 = linoleic (C18:2); 5 = palmitic (C16:0); 6 = oleic (C18:1); 7 = margaric (C17:0); 8 = stearic acid (C18:0).

with methanol–5 m*M* TBA (70:30, v/v), but not with methanol–5 m*M* TBA (72.5:27.5, v/v). The 70:30 eluent perfectly segregated myristic and linolenic acid, but required a longer analysis time, *e.g.*, 42.5 min for stearic acid (C18:0), whereas methanol–10 m*M* TBA (72.5:27.5, v/v) eluent yielded an almost identical chromatogram with a shorter analysis time, *e.g.*, 32.5 min for stearic acid.

As has been observed with underivatized fatty acids and their esters [17, 25], the retention time increased with increase in the number of carbon atoms and decreased with increase in the number of double bonds in the fatty acid chain (Fig. 1). The present method completely separated palmitic acid (C16:0) from oleic acid (C18:1), although the discrimination of these acids has often met with difficulty previously [1, 10, 19, 26]. Margaric acid (C17:0) was perfectly separated from the other fatty acid standards and can be used as an internal standard for fatty acid determinations.

The conductivity detector has widely been used for the detection of free fatty acids [16], ionic species and organic acids [27] and carboxylic acids and non-ionic substances [28]. The present conductimetric detection of fatty acid standards was more satisfactory with regard to both resolution and sensitivity than in a previous method [16] in which saturated C_8–C_{22} fatty acids were separated using an ODS column and tetrahydrofuran–water (45:55, v/v) as the mobile phase. The detection limit reported was 0.1 μg for capric acid (C10:0), which is higher than our detection limit given below.

Quantification of fatty acids was based on peak-area calculations, and the linearity of the method was assessed. The correlation coefficient of the regression line for each fatty acid standard ranged from 0.9995 (C16:0) to > 0.9999 (all other acids examined except lauric and myristic acids) between 43.7 ng (C16:0) and 2.66 μg (C18:3). The reproducibility of peak areas was good as shown by the small relative standard deviations (R.S.D.) of 0.75% (the largest R.S.D, obtained with 665 ng of linolenic

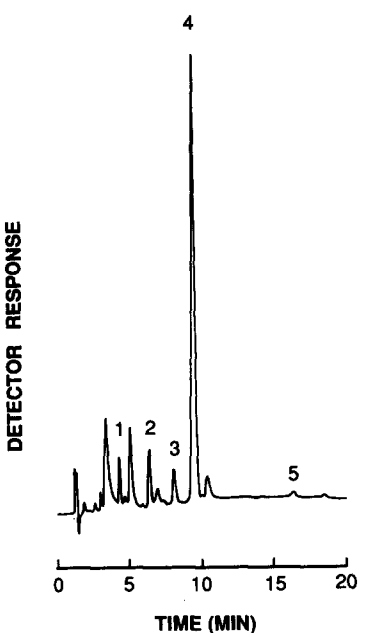

Fig. 2. Chromatogram of the anionic surfactant Lunac O-P. Lunac O-P was diluted with mobile phase to 1 mg/ml and a 2-μl portion was analysed as in Fig. 1. Fatty acids were tentatively assigned to individual peaks by comparing the retention times with those of the authentic standards in Fig. 1. Peaks: 1 = C14:0; 2 = C18:2; 3 = C16:0, 4 = C18:1; 5 = C18:0.

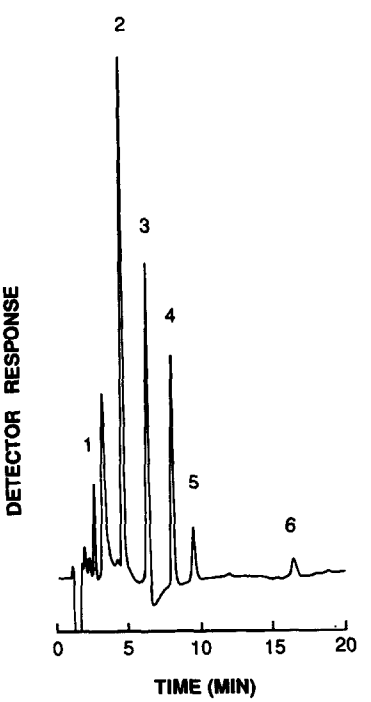

Fig. 3. HPLC profiles of lipoidal extracts from wheat shoots. Lipid fraction of dark-grown wheat shoots was analysed as in Fig. 1. See text for lipid extraction. Tentative peak identifications were made by comparing the retention times with those in Fig. 1. Peaks: 1 = C12:0; 2 = C18:3; 3 = C18:2; 4 = C16:0; 5 = C18:1; 6 = C18:0.

acid) ($n = 5$). The limit of detection at a signal-to-noise ratio of 3:1 was *ca.* 2 ng, based on the detectability of 1 ppm of margaric acid (2-μl injection) (Fig. 1).

The method has been successfully applied to the determination of the fatty acid contents in an anionic surfactant and in higher plant tissues, as shown in Figs. 2 and 3, respectively. Most pesticides contain surfactants as spreaders, emulsifiers or compatibility agents [29]. Therefore, exact information on the composition of a surfactant is important for pesticide formulations. Fig. 2 shows the exact compositional profile of the anionic surfactant Lunac O-P, which is reported by the manufacturer to contain mainly oleic acid, revealing that small amounts of C14:0, C18:2 and C16:0 are present. Fig. 3 shows that the main plant fatty acids were detected without interference from other lipoidal components such as carotenoids. The fatty acid profile obtained seems not too far from the galactolipid fatty acid composition of wheat shoots [30], although the linolenic acid ratio shown in Fig. 3 was low probably because of the difference between the plant tissues analysed.

The method described has several advantages: no need for derivatization, isocratic elution, good reproducibility, fairly high sensitivity and rapidity.

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