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Fast Determination of Multiple-Reaction Intermediates for Long-Chain Dicarboxylic Acid Biotransformation by Gas Chromatography-Flame Ionization Detector

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For the analysis of multiple-reaction intermediates for long-chain dicarboxylic acid biotransformation, simple and reproducible methods of extraction and derivatization were developed on the basis of gas chromatography with flame ionization detector (GC-FID) instead of mass spectrometry. In the derivatization step, change of the ratio of pyridine to MSTFA from 1:3 to 9:1 resulted in higher peak intensity (p = 0.021) and reproducibility (0.6%CV) when analyzing 32 g/l ricinoleic acid (RA). Extraction of RA and ω-hydroxyundec-9-enoic acid with water containing 100 mM Tween 80 showed 90.4–99.9% relative extraction efficiency and 2–7%CV compared with those with hydrophobic ethyl acetate. In conclusion, reduction of the pyridine content and change of the extraction solvent to water with Tween 80 provided compatible derivatization and extraction methods to GC-FID-based analysis of long-chain carboxylic acids.

Keywords: Long-chain dicarboxylic acid, simple extraction, ricinoleic acid, ω-hydroxyundec-9-enoic acid, gas chromatography with flame ionization detector

Long-chain ω-hydroxycarboxylic acids and ω-dicarboxylic acids are used in the production of a variety of chemical products, including nylons and other polyamides [8]. The production of those compounds depends mainly on chemical processes under harsh reaction conditions, which cause serious environmental problems [19]. Recent development of the long-chain dicarboxylic acids biotransformation from natural long-chain fatty acids has been proposed, where multiple enzymatic synthesis was chosen in recombinant *Escherichia coli* transformed with three genes encoding aldehyde dehydrogenase, Baeyer–Villiger monooxygenase (BVMO), and esterase [17]. Accordingly, the optimization and development for the biotransformation processes have been greatly extended to production of various types of hydroxy unsaturated fatty acid [9, 14]. Meanwhile, the development of an efficient analytical method is essential for the reliable and rapid quantification of substrates, intermediates, and products preferentially in laboratories with less analytical expertise. Although mass spectrometry (MS) is one of the powerful analytical platforms, gas chromatography with flame ionization detector (GC-FID) has been widely accepted in broad application to biochemical, clinical, and microbiological research [2, 13]. The GC-FID system is also a cost-effective and robust platform for particularly analyzing various types of hydrocarbons, including hydroxy fatty acids [4, 18]. To date, the long-chain carboxylic acids and their intermediates have been analyzed by MS-based instrumental analysis [6, 7, 17]. In order to facilitate long-chain carboxylic acids analysis, in this study, we developed sample preparation methods of extraction and derivatization, and evaluated instrumental procedures using GC-FID on behalf of MS-based analytical methods.

We first tested whether all reactants and products can be

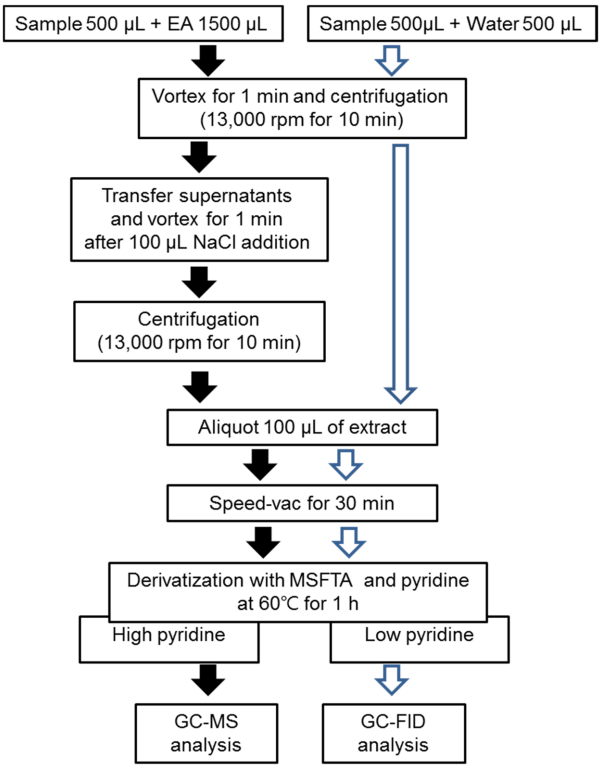

Fig. 1. Experimental design and workflow.
Black arrows indicate the previous extraction strategy and white arrows present the simple method designed in this study.

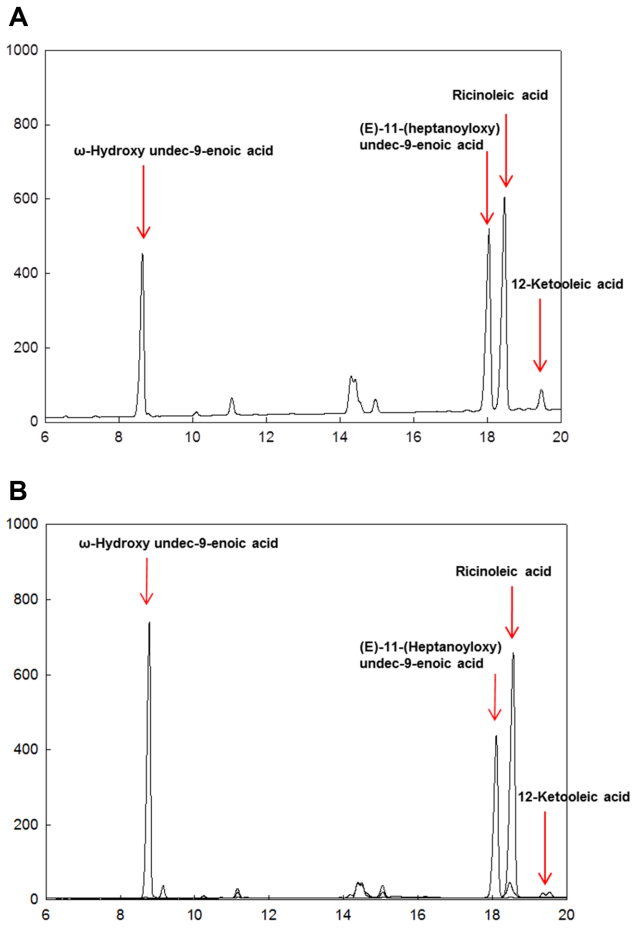

Fig. 2. GC-FID profiles for (**A**) the biotransformation of ricinoleic acid to ω-hydroxyundec-9-enoic acid and of (**B**) the standard compounds.
Each compound was detected at the corresponding elution times of 8.7, 18.1, 18.7, or 19.6 min.

determined by a simple GC-FID method. According to the previous method [17], the cells of *E. coli* BL21(DE3)/pACYC-*adh-kt2440*/pCOLA-*pfel* were reacted in Riesenberg medium [10] with ricinoleic acid and Tween 80 at pH 8.0. After 20 h of incubation at 37°C, the reaction mixture was subjected to the analysis of ricinoleic acid and its derivatives by GC-FID (Fig. 1). The reaction mixture was mixed with pure ethyl acetate at a volume ratio of 1:2, and the upper space was collected and completely dried in a rotary vacuum evaporator (N-BIOTEK, Korea). For chemical derivatization, the pellets were treated with a derivatization mixture of pyridine and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) at a volume ratio of 3:1. The derivatized extract was injected into an injector port at 260°C with a split mode (10:1), and volatilized samples were separated using a gas chromatograph (6890 model; Agilent Co., Santa Clara, CA, USA) equipped with a capillary column (30 m long, 0.25 mm i.d., DB5; Agilent Co., USA) and analyzed by a FID (Agilent Co., USA). The temperature of the column oven was programmed as follows: starting at 150°C, 150°C to 250°C at 4°C/min, and 250°C for 27.25 min. Ricinoleic acid (RA) was purchased from TCI Co. (Japan). The standards for ω-hydroxyundec-9-enoic acid (ω-HUA) and (*E*)-11-(heptanoyloxy)undec-9-enoic acid were given by Kolong Bioscience Co. (Kyounggi, Korea) and Prof. Jin-Byoung Park (Ewha Womans University, Korea), of which the purities were about 96% and 62%, respectively. 12-Ketooleic acid was identified by the variation of its peak area according to the ricinoleic acid consumption and enoic acids production, because its standard was not commercially available. The resultant chromatogram showed successful detection of four distinctive peaks, which were eluted at 8.7, 18.1, 18.7, and 19.6 min of retention time, respectively (Figs. 2A and 2B). To clearly confirm the identities of the chemicals, the same samples were analyzed by a GC-MS, the methods of which followed a previous report [17]. As shown in Fig. 3, the peaks of the four compounds were identical to the GC-MS profiles

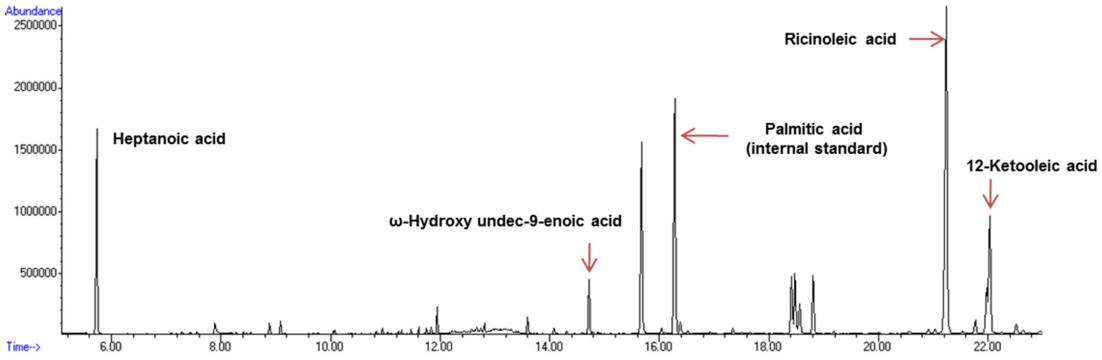

Fig. 3. GC-MS profile of the standard compounds.

previously reported [17]. Finally, GC-FID was able to detect all compounds of interest and was used for further design of sample preparation methods.

To shorten and modify some steps for GC-FID analysis, methods for extraction and subsequent derivatization of samples were investigated, and all steps for long-chain carboxylic acid analysis used in this study are illustrated in Fig. 1. For statistical evaluation, two criteria were applied as follows: reproducibility was estimated by the percentage of coefficient of variation (%CV), which was calculated by dividing the standard deviation by the average value [1, 12]. Extraction efficiency was inferred from the area of a single and dominant peak of each compound [15]. The Student *t*-test was performed to statistically compare the means of extraction efficiency in independent samples, and a *p*-value less than 0.05 was considered statistically significant. First, the composition of the derivatization mixture previously reported [5, 7] was changed, in which the amount of pyridine was reduced and MSTFA was increased. When the ratio of MSFTA to pyridine was set to be 9:1, the result showed statistically consistent difference from that using the MSTFA and pyridine mixture previously formulated at a volume ratio of 1:3 [5, 7] (Table 1). The modified regime presented higher peak intensities ($p = 0.021$) and reproducibility (0.6%CV) when 32 g/l RA was extracted. Following these experiments, standard calibration curves were determined in a range of 4 to 64 g/l of RA. The measured detector response over a series of concentrations of the target analytes was plotted (Figs. 4A–4C). By linear regression between the peak areas and the concentrations, the r^2 value was found to be approximately 0.999 in this range. Generally, a lower amount of pyridine gives better peak shapes for early eluting compounds, particularly when splitless injections or lower ratios of split mode are carried out [3], and also the composition has been preferentially applied to MS analysis [11, 12, 16]. Thus, the ratio of MSFTA to pyridine was set to be 9:1.

The extraction procedure was further examined by comparing two extraction solvents of ethyl acetate and water with 100 mM Tween 80. RA as the substrate for the long-chain carboxylic acid production can be dissolved in hydrophilic culture broth with Tween 80 detergent, and thus a polar solvent such as water may provide higher compatibility for the RA extraction. The extraction efficacy was evaluated at 4–64 g/l of RA, where the extraction volume was downsized to 1.0 ml, and the enrichment step with NaCl treatment in the previous report [6, 17] was skipped. As shown in Table 1, the statistical evaluation demonstrated that the simplified method was not significantly different compared with the previous method with ethyl acetate [6, 7, 17] in which the relative extraction efficiency and %CV resulted in 90.4% and 7%, respectively. The method using water without NaCl treatment facilitated the extraction procedure by avoiding extra steps. Likewise, a high value of squared correlation coefficient was obtained over the ranges of 4–64 g/l of RA ($r^2 = 0.999$) (Fig. 4C).

Following the same criteria, the derivatization, extraction, and linear regression were comparably applied for the

Table 1. Effects of extraction solvents and derivatization methods on ricinoleic acid analysis.

Chemical	Extract solvent	Derivatization mixture (MSTFA:pyridine)	Peak area	%CV
Ricinoleic acid	Ethyl acetate	1:3	18,457	2.59
		9:1	19,508	0.64
	Water with Tween 80	9:1	17,644	7.15

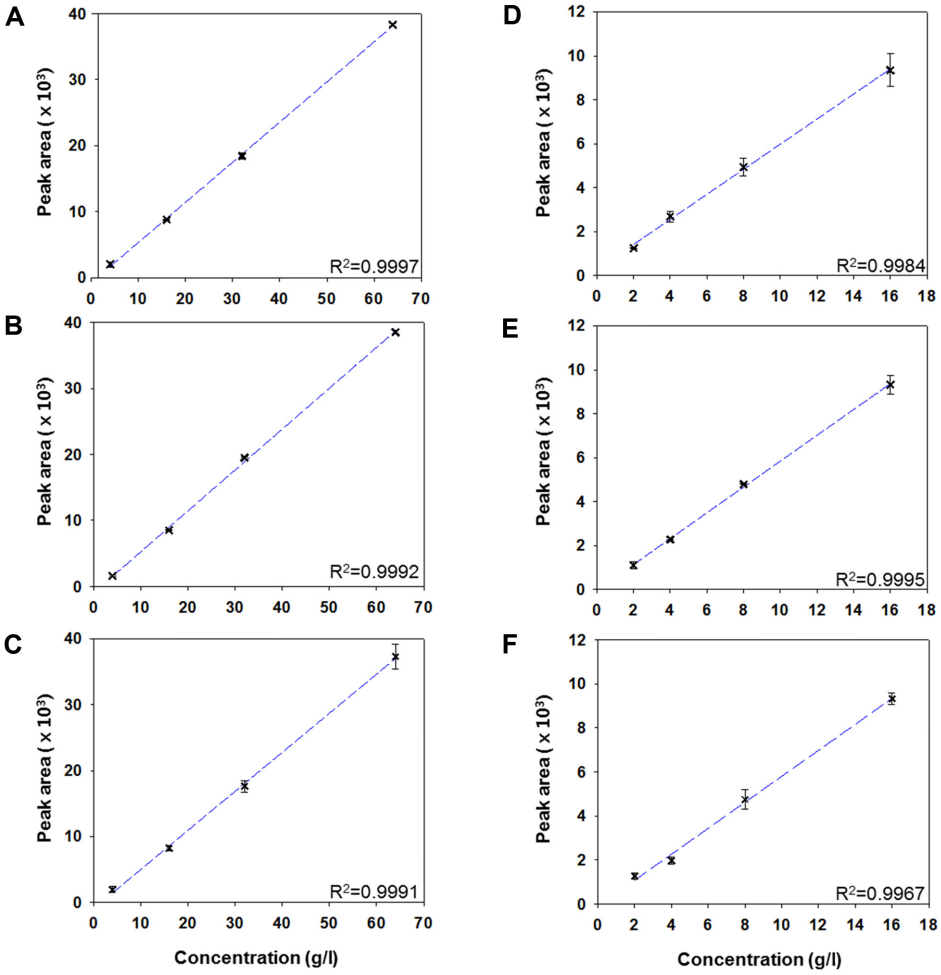

Fig. 4. Linear-fit standard curves for ricinoleic acid (**A**–**C**) and ω-hydroxyundec-9-enoic acid (**D**–**F**).
For derivatization and extraction of the samples, the ratios of MSFTA to pyridine and solvents were chosen as follows: (**A**, **D**) 1:3, ethyl acetate; (**B**, **E**) 9:1, ethyl acetate; (**C**, **F**) 9:1, water with Tween 80.

analysis of the final product of ω-hydroxyundec-9-enoic acid (ω-HUA). When the peak area for 16 g/l ω-HUA was quantitatively compared, the modified derivatization method showed statistically improved performance for analytical precision while the peak intensities were comparable between the two derivatization regimes (Table 2). Subsequently, the response linearity was evaluated over the concentrations of 4–32 g/l, which was the expected range of ω-HUA concentration for the bioconversion of RA in this experimental schema. The strong linear relationship between the response factor and the concentrations was inferred from the coefficient of determination (r^2 = 0.997) (Figs. 4D and 4E). Last, the simplified extraction using water with 100 mM Tween 80 was tested following the same procedure as the RA extraction. The modified extraction showed comparable performance, in which the relative extraction efficiency and %CV were 99.9% and 2.8%, respectively (Table 2), and a high coefficient of determination (r^2 = 0.999) was obtained

Table 2. Effects of extraction solvents and derivatization methods on ω-hydroxyundec-9-enoic acid analysis.

Chemical	Extraction solvents	Derivatization mixture (MSTFA:pyridine)	Peak area	%CV
ω-Hydroxyundec-9-enoic acid	Ethyl acetate	1:3	9,018	7.76
		9:1	9,076	1.98
	Water with Tween 80	9:1	9,327	2.81

over the range of 4–32 g/l of ω-HUA (Fig. 4F).

In conclusion, the GC-FID analysis is a reliable, easy-to-use, and rapid way for the measurement of various types of hydrocarbons, including hydroxy unsaturated fatty acids, proposed here. In particular the method modified by reduction of the pyridine content, removal of the enrichment step, and water based-extraction demonstrated sufficient sensitivity, reproducibility, and linearity in combination with the rapid extraction and derivatization of RA and ω-HUA, a substrate and a product of the fatty acid biotransformation process, respectively. In addition, the modified extraction method using water with Tween 80 provides high applicability, where it prevents phase separation between the extraction solvent and culture broth, and sample preparation is streamlined without compromising the data quality. The further development of GC-FID coupled to reliable and simple sample preparation for targeted compounds will facilitate the analysis of various types of fatty acids and their derivatives.

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