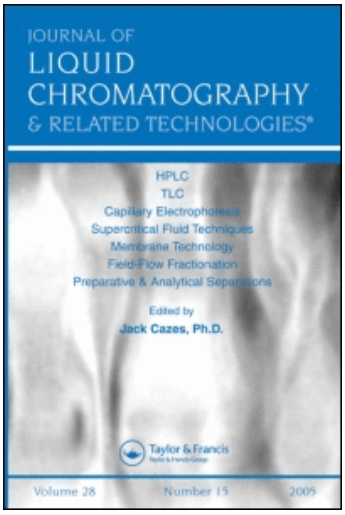

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Simultaneous Separation and Sensitive Measurement of Free Fatty Acids in Ancient Pottery by High Performance Liquid Chromatography

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SIMULTANEOUS SEPARATION AND SENSITIVE MEASUREMENT OF FREE FATTY ACIDS IN ANCIENT POTTERY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic method for the analysis of the free fatty acid composition in ancient pottery with fluorescence detection was examined. Free fatty acids extracted from pottery were derivatized with 9-anthryldiazomethane and were analyzed using methanol-water as the mobile phase. Thirteen kinds of fatty acid were identified and well separated. Concentrations of individual fatty acids were estimated from an internal standard, *p*-butyl benzoic acid. The results correlated well with those from other quantitative analysis. These results indicate that the high-performance liquid chromatographic analysis of fatty acids is a reliable method for measuring individual fatty acids in ancient pottery.

INTRODUCTION

Information on the contents of ancient pottery was up to now obtained by occasional findings of residues recognizable by archaeologists[1-3]. Recently the use of lipid identification for food analysis considerably broadened the possibilities for pottery residues. The purpose of the study of lipid residues absorbed and retained in the fabric of ancient pottery is to offer a methodology that can corroborate the circumstantial

evidence[4,5]. Accordingly, an easy, unambiguous, and rapid analysis for fatty acids is important from the archaeological point of view.

Most laboratories typically have relied upon gas chromatography (GC) or GC-mass spectrometric (GC-MS) methods for the analysis of fatty acids in ancient materials[6-8]. However, GC is necessary to esterify the fatty acids before gas chromatography analysis and this has limited sensitivities. Although the GC-MS method is very sensitive, the method has not been used for the identification of the free acids. The analysis by high-performance liquid chromatography (HPLC) has been gradually used for the simultaneous measurement of free fatty acids, since fluorescence derivatization reagents for free fatty acids, such as 9-anthryldiazomethane (ADAM), have been developed[9-12]. The fluorometric HPLC methods are sensitive, and require a smaller sample volume.

In this study, we have examined the optimal conditions for the analysis of the fatty acid composition in ancient pottery by HPLC with fluorescence detection. We also used the method to analyze fatty acid compositions of the ancient pottery collected from Sogo and Tokuzen C site (the Late Yayoi period, c.a.1900 B.P.), Ishikawa Prefecture, Japan.

EXPERIMENTAL

Materials

Standard fatty acids (myristic acid [$C_{14:0}$], palmitic acid [$C_{16:0}$], palmitoleic acid [$C_{16:1}$], stearic acid [$C_{18:0}$], oleic acid [$C_{18:1}$], linoleic acid [$C_{18:2}$], arachidic acid [$C_{20:0}$], 5-eicosenoic acid [$C_{20:1}$], heneicosanoic acid [$C_{21:0}$], behenic acid [$C_{22:0}$], erucic acid [$C_{22:1}$], tricosanoic acid [$C_{23:0}$], and lignoceric acid [$C_{24:0}$]) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). ADAM was purchased from Funakoshi Co. Ltd. (Tokyo, Japan), and *p*-butyl benzoic acid as an internal standard was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals and reagents, purchased from commercial sources, were of analytical grade and were used without further purification.

Apparatus

The chromatographic system (Shimadzu Co., Kyoto, Japan) consisted of a SIL-6B digital solvent programmer and pumps (an LC-6A and an LC-9A) , an RF-550 variable-wavelength fluorescence spectrophotometer set at an excitation maximum of 365 nm and an emission maximum of 412 nm, a CTO-6A column oven set at 40 °C, and a Chromatopac C-R6A data processor.

HPLC conditions

Separation was done on two reversed-phase columns of Nucleosil $5C_8$ (Chemco Scientific Co. Ltd., Osaka, Japan; particle size 5 μm, 150 x 4.6 mm I.D.) connected tandem. Mobile phase solvents were methanol and distilled water. The following gradient program was used: segment 1: isocratic at 85 % methanol and 15 % water for 5 min; segment 2: a linear gradient from the initial conditions to 100 % methanol in 45 min; segment 3: isocratic at 100 % methanol for 10 min. Columns were re-equilibrated to initial conditions (segment 1) for 10 min between each run. Flow rate was 1.1 ml/min at 40 °C. The solvent program was started simultaneously with the injection. A sample volume of 10 μl was injected in all the studies. Measurement of fatty acids was based on peak areas calculated electronically by the data processor.

Derivatization procedure

ADAM stock solution (1 mg/ml) was prepared daily by dissolving the crystals in acetone, followed by addition of methanol to reach the final acetone-methanol concentration of 1:4 (v/v). ADAM crystals remain stable at room temperature (25 ± 2 °C) during weighing but should be protected from light and returned to the freezer as soon as possible. The orange ADAM solution (50 μl) was added to the extracted sample (50 μl) in a test tube, mixed well, transferred to an autosampler injection vial using a glass pipette, and allowed to derivatize in the dark at room temperature for 1 h. A 10 μl aliquot of this solution was injected directly into the chromatograph.

Calibration curves

Triplicate measurements of the respective spiked sample having a known concentration of authentic fatty acids were made and calibration curves of ADAM-derivatized fatty acids were obtained. The peak area were plotted against the concentrations and the calculations were made by the least-squares method.

Recovery and precision

The recovery of the fatty acids were assessed by 5 replicate analyses with a spiked sample of 7.5 μg/ml and was calculated as the percentage of the measured concentration to the known concentration sample. The within-day and between-day precisions were examined. For the latter, spiked samples were assayed once a day for 7 successive days.

Free fatty acid extraction

The ancient pottery was collected from Sogo and Tokuzen C site (the Late Yayoi period, c.a.1900 B.P.), Ishikawa Prefecture, Japan. The process of extracting the fatty acids from the pottery was as follows. To avoid as many contaminants as possible, the interior surfaces of the samples were first scraped lightly with a chemistry spatula. Then, with the same spatula, powdered samples were scraped from the interior surface about a two centimeter diameter area. Usually about 1-2 mm of the thickness of the sample was removed. The fine powder was then mixed with approximately 30 ml of chloroform-methanol (2:1, v/v). The mixture was ultrasonicated for 30 min and filtered through a 0.5 μm membrane filter (SJFH; Nihon Millipore Kogyo K.K., Yonezawa, Japan). The organic layer was evaporated to dryness *in vacuo* and the residue was dissolved in 1 ml of methanol containing 10 μg/ml *p*-butyl benzoic acid as the internal standard because it is not present in foods. The samples were filtered through a 0.5 μm membrane filter before derivatization of ADAM.

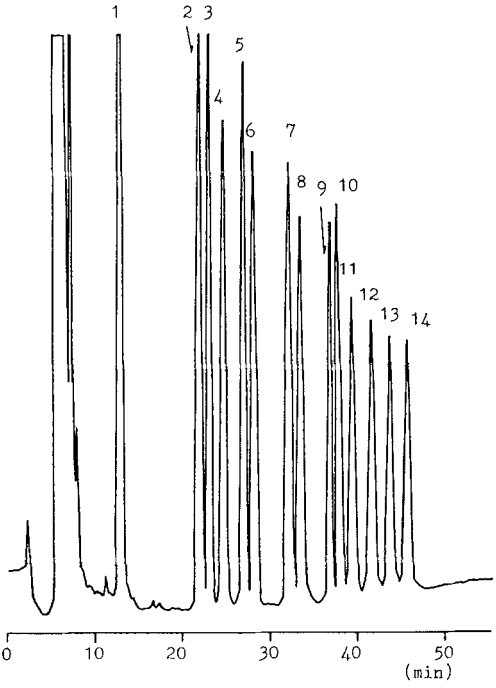

FIGURE 1: Chromatogram of ADAM derivates of authentic fatty acids. Peaks: 1=*p*-butyl benzoic acid [I.S.]; 2=myristic acid [$C_{14:0}$]; 3=palmitoleic acid [$C_{16:1}$]; 4=linoleic acid [$C_{18:2}$]; 5=palmitic acid [$C_{16:0}$]; 6=oleic acid [$C_{18:1}$]; 7=stearic acid [$C_{18:0}$]; 8=5-eicosenoic acid [$C_{20:1}$]; 9=arachidic acid [$C_{20:0}$]; 10=erucic acid [$C_{22:1}$]; 11=heneicosanoic acid [$C_{21:0}$]; 12=behenic acid [$C_{22:0}$]; 13=tricosanoic acid [$C_{23:0}$]; 14=lignoceric acid [$C_{24:0}$].The identification of each peak component was done by comparison with retention times of known individual standards.

RESULTS AND DISCUSSION

Chromatogram

Fluorometric HPLC methods with ADAM reagent has been used for analysis of fatty acids in serum or plasma[11,12]. Trials looking for the optimum conditions were made, and we found that methanol-water was the best mobile phase to separate thirteen fatty acids from other peaks. The column temperature was set at 40 °C in order to

TABLE 1: Retention times of ADAM-derivatized fatty acids

Fatty acid	Retention time (min)	Fatty acid	Retention time (min)
I.S.	13.13	$C_{20:1}$	34.31
$C_{14:0}$	22.56	$C_{20:0}$	37.78
$C_{16:1}$	23.63	$C_{22:1}$	38.59
$C_{18:2}$	25.37	$C_{21:0}$	40.41
$C_{16:0}$	27.85	$C_{22:0}$	42.70
$C_{18:1}$	28.74	$C_{23:0}$	44.87
$C_{18:0}$	33.10	$C_{24:0}$	46.96

decrease the operating pressure. Chromatograms of the derivatized authentic fatty acids are shown in FIGURE 1. ADAM-derivatized fatty acids were separated in ca. 60 min under these conditions (TABLE 1). The contour chromatograms of each fatty acid had single, sharp peaks. The peak areas were consistent with the respective known concentrations of fatty acids in the sample.

Calibration

The relation of the fatty acid concentration versus peak height showed good linearity within the range of 5 - 20 μg/ml. Each regression equation and the correlation coefficient are shown in TABLE 2. These respective curves showed good linearity but the slope and the intercept at the vertical axis were different. Long-chain fatty acids were tend to smaller slope. The minimum detectable amounts of the authentic fatty acids were 15 - 20 pmol.

Recovery and precision

The recovery and precision studies on this method are shown in TABLE 3. The recovery of each fatty acid seemed to be complete. The within-day precisions varied less than 2 % and the between-day precisions were within approximately 8 % expressed as

TABLE 2: The regression equation and the correlation coeffeicient of ADAM-derivatized fatty acids

Fatty acid	a	b	r
$C_{14:0}$	0.031	-0.073	0.994
$C_{16:1}$	0.030	-0.067	0.995
$C_{18:2}$	0.025	-0.054	0.995
$C_{16:0}$	0.024	-0.062	0.989
$C_{18:1}$	0.025	-0.050	0.997
$C_{18:0}$	0.020	-0.041	0.989
$C_{20:1}$	0.024	-0.046	0.996
$C_{20:0}$	0.018	-0.081	0.994
$C_{22:1}$	0.017	-0.072	0.993
$C_{21:0}$	0.015	-0.030	0.986
$C_{22:0}$	0.013	-0.026	0.979
$C_{23:0}$	0.012	-0.024	0.974
$C_{24:0}$	0.011	-0.023	0.967

The regression equation and the correlation coefficient are expressed as Y (I.S. ratio) = a · X (concentration; mg/ml) + b and r, respectively.

the coefficients of variation. These results suggested the applicability of the method to the measurement of fatty acids in pottery.

Analysis of fatty acids

The purpose of the study of lipid residues absorbed and retained in the fabric of ancient pottery is to offer a methodology that can corroborate the circumstantial evidence[13]. Thus, the analysis of fatty acids in ancient sherds is important archaeologically and for food science. At first, the fatty acids in rice grains were measured with this method (TABLE 4). The fatty acids were extracted from rice with a

TABLE 3: Within-day and between-day precisions and the recovery of ADAM-derivatized fatty acids

Fatty acid	Coefficient of variation (%; n=7) Within-day	Between-day	Recovery (%; n=5) [mean ± S.D.]
$C_{14:0}$	1.09	1.81	101.05 ± 0.42
$C_{16:1}$	0.79	2.03	102.45 ± 0.90
$C_{18:2}$	0.84	2.42	100.93 ± 0.65
$C_{16:0}$	2.00	2.84	111.63 ± 1.79
$C_{18:1}$	1.79	3.00	104.73 ± 1.20
$C_{18:0}$	1.75	2.58	107.22 ± 0.40
$C_{20:1}$	1.28	2.24	101.26 ± 1.28
$C_{20:0}$	1.02	3.41	99.27 ± 0.95
$C_{22:1}$	1.05	3.40	99.07 ± 0.99
$C_{21:0}$	0.83	6.31	98.45 ± 1.19
$C_{22:0}$	1.04	7.33	99.28 ± 1.59
$C_{23:0}$	1.14	7.71	99.93 ± 2.10
$C_{24:0}$	1.26	8.12	99.38 ± 2.69

TABLE 4: Fatty acid compornents in rice grains

Fatty acid	Experimental	Reference[14]
$C_{14:0}$	2.05	0.51
$C_{16:0}$	18.51	18.46
$C_{16:1}$	0.81	0.51
$C_{18:0}$	2.83	2.74
$C_{18:1}$	40.30	39.25
$C_{18:2}$	34.67	37.42
$C_{20:0}$	0.39	1.11
$C_{20:1}$	0	0
$C_{21:0}$	0	0
$C_{22:0}$	0.14	0
$C_{22:1}$	0	0
$C_{23:0}$	0.02	0
$C_{24:0}$	0.28	0

Experimental values are the mean of two.

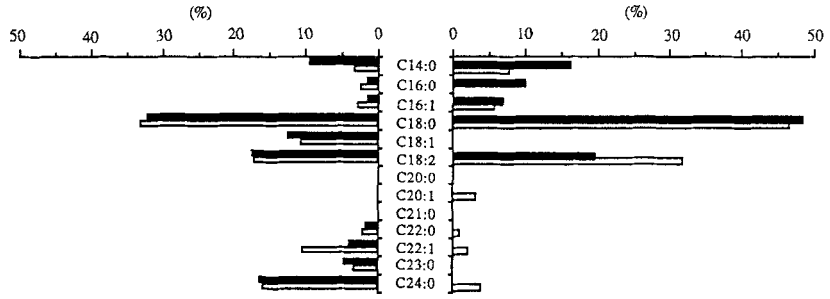

FIGURE 2: The composition of fatty acids of ancient pottery collected from Sogo (right side) and Tokuzen (left side). The inner and the outer surface samples of the ancient pottery indicated opened and closed bars, respectively.

chloroform-methanol solution as described above. These values are in good agreement with those obtained by gas chromatography[14].

Then, the fatty acids in the ancient pottery collected from Sogo and Tokuzen C site were identified and measured. Because the aim of the analysis is to examine whether cooking pots of the Yayoi period, the first farming period in Japan, were differentiated into rice cooking pots and vegetable/meat cooking pots, jar-shaped pots (cooking pots) were selected for the lipid analysis. The total amount of fatty acids was found to be 2.72 (Sogo) and 23.78 (Tokuzen) μg/g in the inner bottom of the pottery but only a trace in the outer surface sample. The composition of fatty acids was very different between the inner and the outer surface samples of pottery (FIGURE 2); while the outer surface sample contained a higher level of $C_{14:0}$ than inner, fatty acids longer than $C_{20:0}$ in the inner surface sample were significant. Thus, fatty acids characteristically found in the inner surface of the ancient pottery may have originated from food stored or cooked at the time, although these fatty acids might be retained until now as the result of influence by circumstances and degradation of other fatty acids. When data of fatty acid composition retained in the ancient samples are accumulated and analyzed[13], we may be able to deduce more about the life style at the time.

In conclusion, the HPLC method as presented here enables to identify fatty acids using a smaller sample volume and without complicate pretreatment. In addition, the analytical procedure outlined in this study is a simple and rapid method, and may be applicable to routine use for ancient evidence.

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