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Determination of higher fatty acids in oils by high-performance liquid chromatography with electrochemical detection

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Abstract

A system of high-performance liquid chromatography with electrochemical detection was developed for the separation and determination of higher fatty acids. An octadecylsilica (ODS) column was used as the stationary phase and an ethanol–acetonitrile (10:90) mixture as the mobile phase. The eluate was mixed with a quinone solution which was composed of 6 m*M* 2-methyl-1,4-naphthoquinone and 76 m*M* $LiClO_4$ in ethanol–acetonitrile (10:90) through a mixing coil. Peak height for higher fatty acids measured at −415 mV vs. saturated calomel electrode (SCE) was linear against the amount of acid between 20 and 1200 pmol. Free fatty acids in various oil samples were determined by this method, which was found not only sensitive and reproducible but also a simple means for separating and determining free fatty acids in oils.

Keywords: Electrochemical detection; Fatty acids

1. Introduction

Many attempts have been made for the separation and determination of fatty acids by gas chromatography (GC) [1–3] and high-performance liquid chromatography (HPLC) [4–13]. In current GC analysis commonly employed today, free fatty acids are generally converted to their methyl esters and then injected into a capillary GC. Methylation and GC conditions, such as programmed-temperature, split-injection, as well as type of capillary column, carrier gas, and detector, are all important determinants of high accuracy and precision. The main advantage of HPLC with fluorescent detection (HPLC-FL) of fatty acids is high sensitivity. However, owing to the weak absorption and fluorescent properties of fatty acids, derivatization with a strong chromophore or fluorophore prior the column separation is required for sensitive detection in HPLC. Many derivatizing (labelling) reagents have been developed for this purpose [4–13]. A catalyst in some cases is required for complete derivatization owing to the poor reactivity of carboxyl groups [4]. For the derivatization of fatty acids with such reagents, the amount of reagent, reaction temperature and time are critical for high reaction efficiency and avoiding any side product formation. Water is often an incompatible environment for derivatization reactions and to find a solution to this problem, examination was made of the use of aqueous micellar systems for derivatization [5]. Although fluorimetric detection is quite sensitive, fluorescent intensity is liable to vary owing to the presence of substances in complicated samples unless there is a clean-up procedure for their elimination. It is thus highly desirable to develop a simple and rapid method that requires no such procedure.

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The authors developed a new method for determining free fatty acid content in fats and oils [14,15], based on the reduction of protonated quinone: fatty acid present in an ethanol solution containing 2-methyl-1,4-naphthoquinone (vitamin K_3, VK_3) produces a reduction peak on the voltammogram at a potential more positive than the reduction potential of VK_3 itself, and the peak height is proportional to the acid concentration. The method was applied to electrochemical detection (ECD) in flow injection analysis and total amounts of free fatty acids in various fat and oil samples could be determined simply and rapidly [16]. Since fatty acid content is monitored by measuring the peak height of a flow signal, this method should also be applicable to ECD in liquid chromatography for fatty acid determination.

A HPLC with ECD (HPLC-ECD) was evaluated as a simple method for determining higher fatty acids without derivatization.

2. Experimental

2.1. Reagents

Palmitic acid (99.5%), stearic acid (99.5%), oleic acid (99%) and linoleic acid (99%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Standard acid solutions were prepared by dissolving them in an ethanol–acetonitrile (10:90) mixture.

The following oils were commercially obtained: camellia oil (Shiseido Seiyaku, Tokyo, Japan), corn oil and rapeseed oil (Hayashi Chemicals, Tokyo, Japan), olive oil (Miyazawa Yakuhin, Tokyo, Japan), and soy bean oil (Kanto Chemical, Tokyo, Japan).

An ethanol–acetonitrile (10:90) mixture and one containing 6 m*M* VK_3 and 76 m*M* $LiClO_4$ were used as a mobile phase, (MP), and quinone solution, respectively. $LiClO_4$ was the supporting electrolyte to decrease the electrochemical cell resistance.

2.2. Apparatus

The system of HPLC-ECD is shown in Fig. 1 and consists of a degassor (Model DG-980-50, Jasco, Tokyo, Japan), pumps P_1 and P_2 (Model PU-980, Jasco), sample injector (Model 7125, Rheodyne, Cotati, CA, USA), octadecylsilica (ODS) column (LiChrospher 100 RP-18, 250 mm×4 mm I.D., 5 μm, Cica-Merck, Tokyo, Japan), electrochemical cell (Model EC-840, Jasco), potentiostat (Model 311B, Huso Electrochemical System, Kawasaki, Japan) and recorder (Model 807-IT, Jasco). The electrochemical cell was made from a glassy carbon working electrode, saturated calomel electrode (SCE) reference electrode and stainless-steel auxiliary electrode. Flow lines were made from stainless-steel tubing (0.5 mm I.D.) and polytetrafluoroethylene tubing (0.5 mm I.D.) covered with aluminum foil to shield the light.

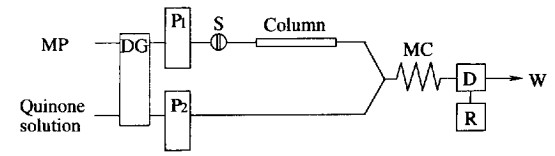

Fig. 1. HPLC system: MP, mobile phase, ethanol–acetonitrile mixture; quinone solution, 6 m*M* VK_3+76 m*M* $LiClO_4$ in ethanol–acetonitrile mixture; DG, degassor; P_1, P_2, pumps; S, sample injector (20 μl); C, column (LiChrospher 100 RP-18, 250 mm×4 mm I.D., 5 μm); MC, mixing coil (50 cm); D, electrochemical detector, electrochemical cell and potentiostat; R, recorder.

2.3. Procedure

To prepare a sample solution for injection into the separation column, the sample oil was mixed in the proper amount with an ethanol–acetonitrile (10:90) mixture. When the oil was not soluble in the ethanol–acetonitrile (10:90) mixture, the mixture was centrifuged after mixing, and the supernatant was used as a sample solution.

A 20 μl sample aliquot and standard acid solutions were injected into an ODS column maintained at room temperature. The deaerated MP and the quinone solution were made to flow at 1.1 ml/min with P_1 and P_2. The detection potential for monitoring free fatty acid was maintained at −415 mV vs. SCE. Each fatty acid in the sample solution was determined based on signal peak height.

3. Results and discussion

3.1. Voltammetric reduction of VK_3 in the presence of fatty acids

Protonation of quinone at the electrode surface occurs prior to its electron transfer. Protonated quinone is reduced at a potential less negative than that of quinone to give a new peak on the voltammogram of quinone [17,18]. Higher fatty acids, such as palmitic acid, in an ethanol solution containing VK_3 and $LiClO_4$ were previously found to give rise to a peak of protonated VK_3 on the voltammogram of VK_3 at a potential less negative than the reduction potential of VK_3; VK_3 itself gave a clear reduction peak at −720 mV vs. SCE, and a peak of protonated VK_3 was noted at −320 mV vs. SCE after adding palmitic acid to the solution. The peak height was proportional to added acid concentration [16].

The half-peak potential of a peak of protonated VK_3 was previously shown to shift to a more negative potential accompanied by an increase in pK_a of the added acid [17]. However, half-peak potentials of prepeaks for different higher fatty acids were essentially the same, since acid strength was nearly the same. Each fatty acid could thus be detected at that applied potential that would give the reduction current of protonated quinone.

The reduction potential of protonated quinone was less negative than that of dissolved oxygen. The half-peak potential of the first reduction wave of oxygen in ethanol containing 38 m*M* $LiClO_4$ was −730 mV vs. SCE. However, there may have been background current due to the dissolved oxygen, since the oxygen was reduced at potentials more negative than −300 mV vs. SCE.

3.2. HPLC-ECD

In consideration of the above, HPLC-ECD of fatty acids was carried out. Reversed-phase separation of higher fatty acids was done using an ODS column and a MP of an ethanol–acetonitrile mixture. The dissolved oxygen in MP and the quinone solution was removed by the degassor. A 20 μl aliquot of solution containing fatty acids was injected into the column; the eluate was mixed with the quinone solution and the fatty acids was detected with ECD. Examination was made of how the ratio of acetonitrile to ethanol in the MP influenced the separation and sensitivity for acid determination. In Fig. 2, the retention time (A) and the peak heights (B) of signals for 200 pmol linoleic, oleic, palmitic and stearic acid were plotted against the ratio of the two solvents, in

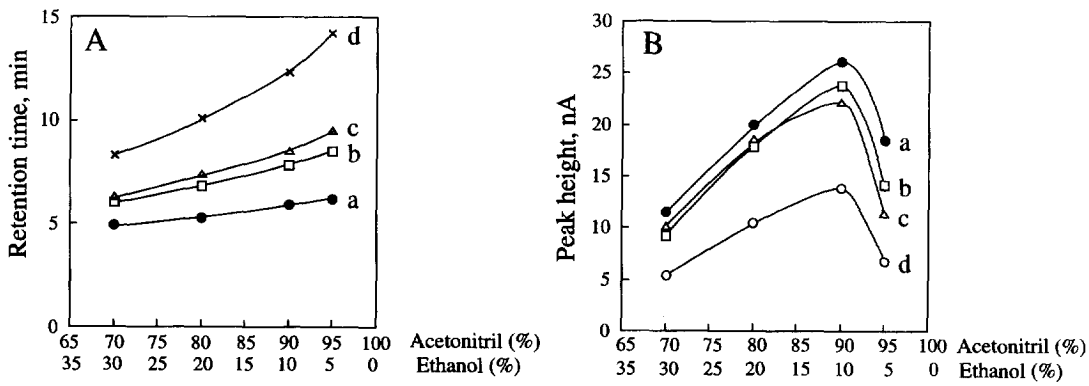

Fig. 2. Retention time (A) and peak height (B) as functions of solvent ratio of the mobile phase. (a) Linoleic acid, (b) oleic acid, (c) palmitic acid, (d) stearic acid. Amount of acid=200 pmol. HPLC conditions: quinone solution, 6 m*M* VK_3+76 m*M* $LiClO_4$ in ethanol–acetonitrile mixture; sample volume, 20 μl; column, LiChrospher 100 RP-18 (250 mm×4 mm I.D., 5 μm); column temperature, room temperature; flow-rate, 1.1 ml/min; applied potential, −415 mV vs. SCE.

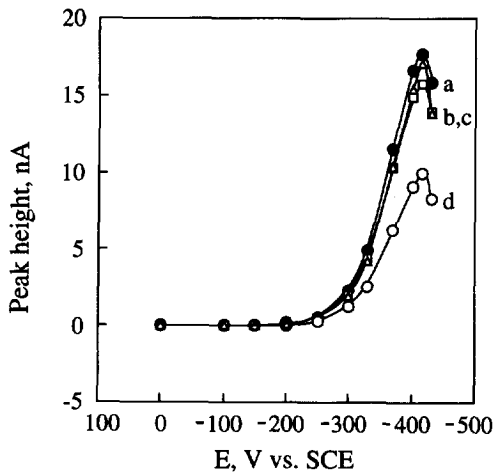

Fig. 3. Hydrodynamic voltammograms of standard acids. (a) Linoleic acid, (b) oleic acid, (c) palmitic acid, (d) stearic acid. Amount of acid=200 pmol. HPLC conditions: mobile phase, ethanol–acetonitrile (10:90); quinone solution, 6 m*M* VK_3+76 m*M* $LiClO_4$ in ethanol–acetonitrile (10:90); other HPLC conditions are as in Fig. 2.

which the larger the content of acetonitrile, the greater was the separation of the acid peaks. Peak height was maximum at about 90% acetonitrile for all fatty acids examined. The (10:90) ethanol–acetonitrile mixture was concluded to be the most suitable MP.

Hydrodynamic voltammograms for 200 pmol linoleic, oleic, palmitic and stearic acid under the present experimental conditions are shown in Fig. 3 and based on which, the potential for fatty acid detection was determined as −415 mV vs. SCE.

A typical chromatogram for a mixture of standard linoleic, oleic, palmitic and stearic acid appears in Fig. 4A. The acids were well separated within 15 min. Peak height was linear against acid amount injected in the range of 20–1200 pmol (Table 1). Linoleic, oleic, palmitic and stearic acid at 200 pmol were determined ten times with relative standard deviations (R.S.D.) of 1.3, 1.2, 0.96 and 0.79%, respectively. The detection limit (signal-to-noise ratio=2) of acids per injection were 20 pmol.

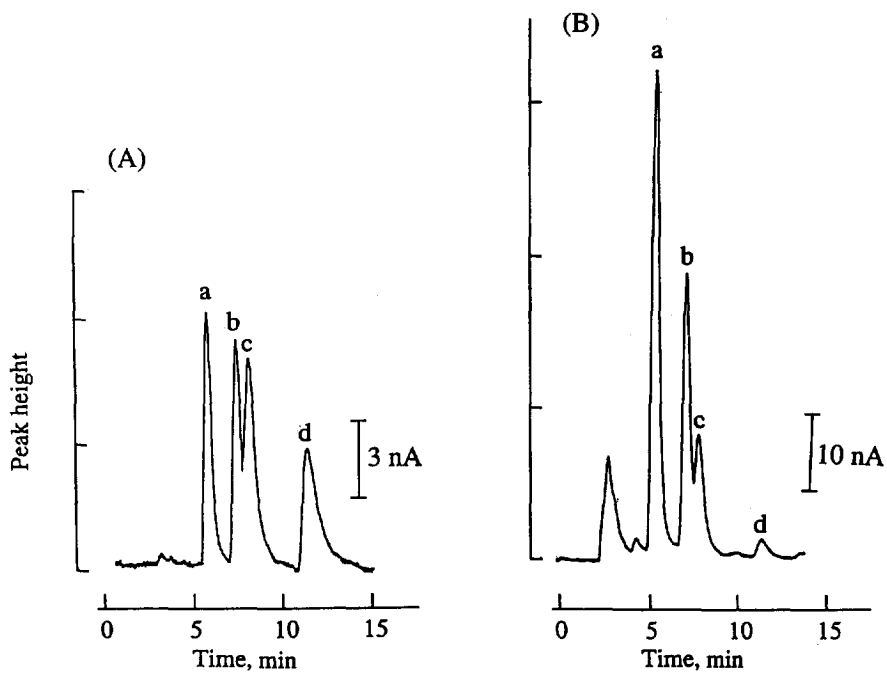

Fig. 4. Typical chromatograms of (A) a standard mixture containing (a) linoleic acid (200 pmol), (b) oleic acid (200 pmol), (c) palmitic acid (200 pmol), (d) stearic acid (200 pmol); (B) corn oil. HPLC conditions: applied potential, −415 mV vs. SCE; other HPLC conditions are as in Fig. 3.

Table 1
Regression lines of higher fatty acids obtained for standard acid mixture

Compound	Amount range (pmol)	Regression line	Correlation coefficient, (r)
Palmitic acid	20–1400	$y=1.085x-0.006406$	0.9988
Stearic acid	20–1800	$y=0.7762x-0.1403$	0.9995
Oleic acid	20–1400	$y=1.080x+0.02491$	0.9987
Linoleic acid	20–1200	$y=1.145x+0.06833$	0.9989

3.3. Determination of higher fatty acids in oils

The analysis of real sample, olive oil, camellia oil, corn oil, rapeseed oil and soy bean oil was performed to assess the effectiveness of the present method. Chromatograms were readily obtained following the injection of simply prepared sample solutions. A typical chromatogram for corn oil is shown in Fig. 4B. The peak height ratio for the free fatty acids was palmitic acid:stearic acid:oleic acid: linoleic acid at 5:1:13:25, this being essentially the same as the constituent ratio reported for fatty acids in corn oil [19]. The contents of free higher fatty acids in oil determined are listed in Table 2. R.S.D. was from 0.8 to 2.8%.

For recovery assessment, a standard higher fatty acid mixture was added to each sample oil (olive, camellia, corn, rapeseed and soy bean oils) and then analyzed by the present method. The recoveries were satisfactory for the acids examined, as shown in Table 2.

A comparison with HPLC with fluorescent detection (HPLC-FL) indicated the present HPLC-ECD method made possible the simple determination of higher fatty acids within a short time. R.S.D. by FL is less than 5%, even when using an internal standard, while by the present method for oil samples, R.S.D. is less than 3% (Table 3).

The good reproducibility is considered to be due to the simple pretreatment procedure without de-

Table 2
Contents of fatty acids in plant oils and recovery of fatty adds from the oils spiked with acid standards

Oil	Fatty acid	Content ($n=5$)		Recovery ($n=5$)	
		Measured conc. mean (mol/g)	R.S.D. (%)	Concentration added (mol/g)	Recovery (%)
Olive oil	Palmitic acid	3.8×10^{-7}	2.1	3.3×10^{-7}	105±2
	Stearic acid	6.4×10^{-8}	1.4	5.0×10^{-8}	106±1
	Oleic acid	1.4×10^{-6}	1.4	2.0×10^{-6}	96±2
	Linoleic acid	1.8×10^{-7}	2.6	1.7×10^{-7}	105±2
Camellia oil	Palmitic acid	7.2×10^{-6}	2.5	5.0×10^{-6}	97±2
	Stearic acid	1.6×10^{-6}	1.8	2.0×10^{-6}	103±2
	Oleic acid	3.9×10^{-5}	1.6	4.0×10^{-5}	93±1
	Linoleic acid	1.9×10^{-6}	1.4	2.0×10^{-6}	101±1
Corn oil	Palmitic acid	2.3×10^{-7}	1.3	1.7×10^{-6}	109±1
	Stearic acid	4.3×10^{-8}	2.8	5.0×10^{-8}	106±2
	Oleic acid	5.8×10^{-7}	1.4	5.0×10^{-6}	97±2
	Linoleic acid	1.1×10^{-6}	2.5	1.0×10^{-6}	93±2
Rapeseed oil	Palmitic acid	–		–	
	Stearic acid	–		–	
	Oleic acid	1.9×10^{-7}	2.3	2.0×10^{-7}	102±3
	Linoleic acid	5.8×10^{-8}	1.6	5.0×10^{-8}	95±3
Soy bean oil	Palmitic acid	1.9×10^{-7}	2.4	1.0×10^{-7}	91±2
	Stearic acid	4.6×10^{-8}	0.83	5.0×10^{-8}	98±1
	Oleic acid	2.6×10^{-7}	1.5	1.7×10^{-7}	94±2
	Linoleic acid	5.1×10^{-7}	1.1	5.0×10^{-7}	90±2

Table 3
Comparison of determination of free fatty acids by the HPLC with electrochemical detection (HPLC-ECD) and HPLC-fluorescent detection (HPLC-FL)[a]

Method	Linear range of calibration curve	Analysis time[b]	Free fatty acid in camellia oil	Free fatty acid content (μmol/g)	R.S.D. (n=10)
HPLC-ECD	20–1200 pmol	20 min	Palmitic acid	7.2	2.5
			Stearic acid	1.6	1.8
			Linoleic acid	1.9	1.4
HPLC-FL	2–1000 pmol	100 min	Palmitic acid	6.8	4.1
			Stearic acid	1.9	4.8
			Linoleic acid	2.2	4.3

[a] HPLC-FL was conducted using ADAM (9-anthryldiazomethane) as a fluorescent reagent according to [9]. Docosahexiaenoic acid was used as an internal standard.
[b] Time required for pretreatment and determination by HPLC.

rivatization of sample fatty acids. When the oil contained a highly nonpolar constituent, it was not soluble in an ethanol–acetonitrile (10:90) mixture and thus only the supernatant of the oil–ethanol–acetonitrile mixture was injected into the column. Thus, the highly nonpolar constituent hardly came in contact with the working electrode, as well as ODS in the column used in the present method. The working glassy carbon electrode surface remains stable for periods of more than three months for analysis of more than ten samples a day using the HPLC system. The applied potential for monitoring fatty acids is considered so negative as not to cause carbon surface oxidation and organic eluent solvent may prevent the adhesion of hydrophobic contaminants on the electrode surface. These factors also lead to greater reproducibility. A detection limit as low as 20 fmol was possible with HPLC-FL [8–12]. The detection limit was as low as 20 pmol by the present method. Because of the simple procedure without derivatization using labelling agents, the amount of sample required is less and the time for determination is reduced. Measurement can be made only through injection of test solution directly into the HPLC system. The present method with ECD is thus shown quite effective for the determination of higher fatty acids.

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