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Review

High-performance liquid chromatographic determination of mono-, poly- and hydroxycarboxylic acids in foods and beverages as their 2-nitrophenylhydrazides

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Abstract

The application of direct derivatization in conjunction with high-performance liquid chromatography (HPLC) is described for the determination of both free and total carboxylic acids in foods and beverages. The method is based on the reaction of the carboxylic acids with 2-nitrophenylhydrazine hydrochloride, without complicated isolation steps, which produces their non-volatile hydrazine derivatives. The HPLC of a series of carboxylic acid groups was performed isocratically with short retention times. The analytical results showed good recovery and reproducibility using each internal standard. Due to its excellent selectivity and sensitivity, the present method can serve as a useful tool for routine determinations of mono-, poly- and hydroxycarboxylic acids in foods and beverages.

Keywords: Reviews; Direct derivatization, LC; Food analysis; Milk; Fats; Oils; Fruit juices; Wine; Beer; 2-Nitrophenylhydrazine hydrochloride; Carboxylic acids; Hydroxycarboxylic acids; Fatty acids

Contents

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1. Introduction

The development of liquid chromatographic methods for the routine simultaneous identification and quantification of a variety of carboxylic acids, such as mono-, poly- and hydroxycarboxylic acids, is desirable for use in various fields. Although several chromatographic methods, e.g., ion-exchange and ion-exclusion chromatography, solvophobic chromatography, ion-pair chromatography and reversed-phase chromatography, have been extensively studied for the determination of carboxylic acids, their determination is still an object of research. In the chromatographic methods, carboxylic acids are commonly monitored by refractive index or ultraviolet detection at about 210 nm. This is due to the weak chromophoric properties of the carboxyl group, giving relatively poor sensitivity and selectivity of detection.

To achieve more sensitive and selective detection with high-performance liquid chromatography (HPLC), pre-column derivatization methods have been developed [1–42]. However, most of these methods did not consider quantitative aspects, were not always successful in the series of separations of mono-, poly- and/or hydroxycarboxylic acid derivatives and also need a fairly long analysis time and/or a rigorous sample clean-up procedure. The main problem in the chromatographic methods is that the quantitative isolation of those carboxylic acids is required prior to suitable derivatization. Common isolation procedures involve the use of potassium hydroxide-silicic acid [43], anion-exchange [44] and alumina columns [45]. These procedures may result in loss of sample or in hydrolysis of the endogenous carboxylic acids such as glycerides owing to the relatively long contact with the strong alkali used in the isolation procedures. For determination of esterified carboxylic acids, in addition, extraction is required after saponification to obtain the free carboxylic acids prior to derivatization. Extraction is often tedious and can cause problems with recovery and analytical reliability.

It is therefore desirable to establish a more convenient HPLC method that is rapid and easy to use, involves minimum sample preparation and is suitable for routine analysis.

We have developed the utility of the reagent 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) for the derivatization of mono-, poly- and hydroxycarboxylic acids, and their separation and quantitation by HPLC in various fields [46–62]. The present review demonstrates the direct derivatization of carboxylic acids with 2-NPH·HCl in various foods and beverages without any pre-treatment and/or extraction steps, and the determinations of the acid hydrazides using a reversed-phase HPLC method with simple isocratic elution systems.

2. Derivatization

2.1. Reagent solutions

2-NPH·HCl (Tokyo Kasei Kogyo, Tokyo, Japan) solutions (0.02 *M*) were prepared by dissolving the reagent in water, 0.1 *M* hydrochloric acid–ethanol (1:1, v/v) and 0.3 *M* hydrochloric acid–ethanol (1:1, v/v). A 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC·HCl) (Sigma, St. Louis, MO, USA) solution (0.25 *M*) was prepared by dissolving the reagent in a solution of pyridine (3%, v/v) in ethanol. A potassium hydroxide solution (10%, w/v) in methanol–water (1:1, v/v) and a potassium hydroxide (0.4 *M*)–ethanol (1:1, v/v) solution were prepared. All the reagent solutions were stable for at least 3 months when kept below 5°C, and were also commercially available from Yamamura Chemical Laboratories (Kyoto, Japan). All other chemicals were of analytical-reagent grade, unless stated otherwise.

2.2. Derivatization procedure

Carboxylic acids were dissolved in water, aqueous ethanol or ethanol in various concentrations. To 100 μl of each sample solution, 200 μl of ethanol (if necessary internal standards were added), 200 μl of 2-NPH·HCl in water solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 60°C for 20 min. After the addition of 100 μl of 15% (w/v) potassium hydroxide solution, the mixture was further heated at 60°C for 15 min and then cooled. An aliquot (1–20 μl) of the resulting hydrazide mixture was injected directly into the chromatograph.

2.3. Derivatization conditions

Aqueous, aqueous ethanolic and ethanolic solutions of carboxylic acids react sensitively with 2-NPH·HCl using 1-EDC·HCl as a coupling agent to give non-volatile acid hydrazides [46]. In order to ensure the maximum derivatization of the carboxylic acids, the reaction conditions were investigated with *n*-valeric and myristic acids. Temperature is a very important factor in optimizing the derivatization rate [46]. Investigation of the effect of the temperature on the formation of the acids hydrazides showed that the derivatization rate gradually increased with increasing temperature, but the produced derivatives slightly decreased with reproducible quantitative yields. The peak heights for all carboxylic acids become constant at 3 min and thereafter at 80°C, which suggested that the derivatization was maximal in this period. Using an optimum reaction time of 5 min, the carboxylic acids were converted into their hydrazides without any deterioration [55,56,59,60].

Fig. 1 shows the relationship between the peak heights of the acid hydrazides and the concentration of 2-NPH·HCl. The peak height increased with increase in concentration of 2-NPH·HCl. A 0.02 *M* solution was preferred for the HPLC analyses because a more concentrated solution of the reagent gave impurity peaks on the chromatogram [46].

The effect of the 1-EDC·HCl concentration on the peak height is shown in Fig. 2. Relatively higher peak heights were obtained in the concentration range 0.2–0.3 *M* without affecting the impurity

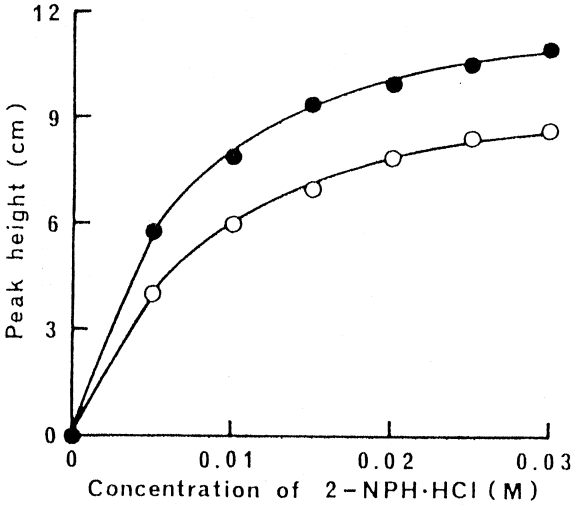

Fig. 1. Effect of concentration of 2-NPH·HCl on colour formation. A 0.8 μmol amount of each acid was treated by the derivatization procedure using various concentrations of 2-NPH·HCl. An aliquot of 2 μl of the reaction mixture was injected into the chromatograph and was detected at 400 nm with $1 \cdot 10^{-2}$ units absorbance range. ●, *n*-Valeric acid; ○, myristic acid.

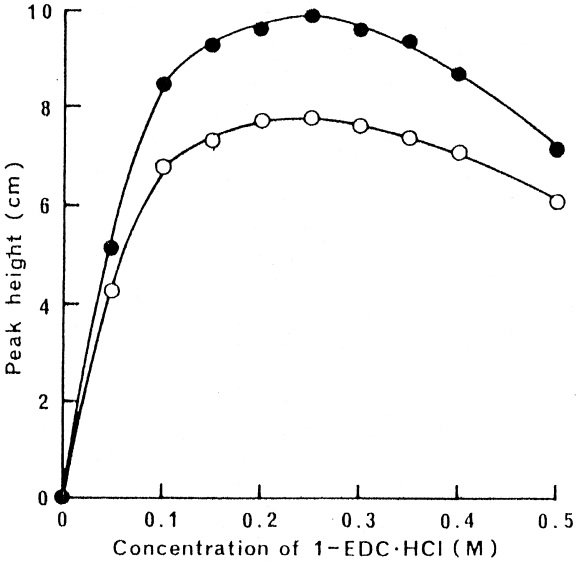

Fig. 2. Effect of concentration of 1-EDC·HCl on colour formation. A 0.8 μmol amount of each acid was treated by the derivatization procedure using various concentrations of 1-EDC·HCl. An aliquot 2 μl of the reaction mixture was injected on into the chromatograph and was detected at 400 nm with $1 \cdot 10^{-2}$ units absorbance range. ●, *n*-Valeric acid; ○, myristic acid.

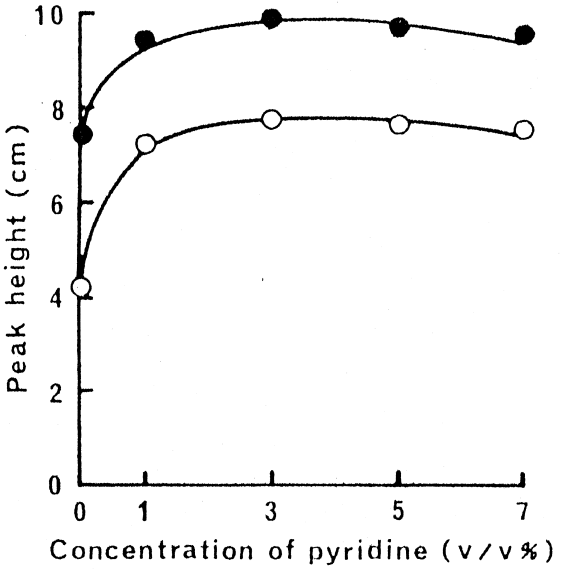

Fig. 3. Effect of concentration of pyridine on colour formation. A 0.8 μmol amount of each acid was treated by the derivatization procedure using various concentrations of pyridine. An aliquot of 2 μl of the reaction mixture was injected into the chromatograph and was detected at 400 nm with $1 \cdot 10^{-2}$ units absorbance range. ●, *n*-Valeric acid; ○, myristic acid.

peaks and 0.25 *M* was selected in subsequent studies [46].

The influence of the concentration of pyridine on the peak heights of the *n*-valeric and myristic derivatives is shown in Fig. 3. The peak heights were almost constant over the range of pyridine concentrations investigated. These experiments indicated that the optimum concentration of pyridine was 3% (v/v) [46].

A concentration of 10% (w/v) potassium hydroxide was needed in order to eliminate the interference in the chromatogram due to the excess of the reagents and the reaction by-products that might be formed during the coupling reaction [46]. In this derivatization process, polycarboxylic acids converted to their monohydrazine derivatives, i.e., acidic acid compounds due to the residual carboxyl group [47,53,54,60].

The absorption curves of acetic acid hydrazide in solutions of various pH are shown in Fig. 4. The hydrazide ionizes at high pH (>12) to give an intense violet colour. At low pH (<8.5), however, the absorption maximum of the hydrazide shifts considerably towards the blue region. On the other

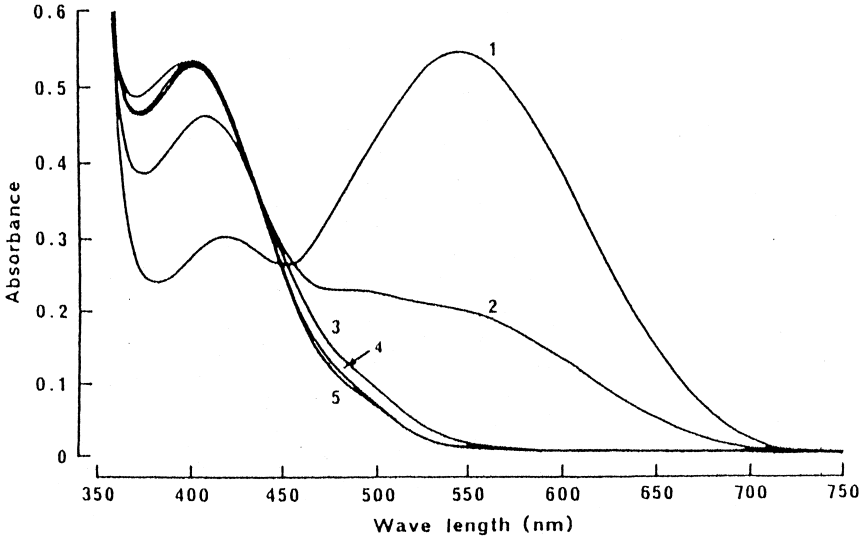

Fig. 4. Absorption spectra of solutions of acetic acid 2-nitrophenylhydrazine of various pH. A 0.5 μmol amount of acetic acid was treated by the derivatization procedure. The reaction mixture was adjusted with 3 *M* HCl to the desired pH and was measured from 350 to 750 nm. pH: 1=13.85; 2=10.5; 3=8.5; 4=6.5; 5=4.5.

hand, with the reversed-phase HPLC column the pH of the eluent was restricted to the range 2–8, and the elution system of mixtures of methanol, acetonitrile and water was chosen to maintain the pH at 4–7. All of the carboxylic acid hydrazides gave absorption maxima at 400 nm in the acidic medium, and were detectable photometrically by monitoring at this wavelength. These derivatives also showed strong absorption in the UV region, with maximal absorption at around 230 nm, and were monitored with a UV detector. An excess of the reagents and the reaction by-products did not interfere with the HPLC analyses in the visible range, because they did not absorb visible radiation at 400 nm and were eluted before any of the carboxylic acid hydrazides. The advantage of using visible detection is that the chromatograms are simpler and more selective, in spite of approximately four-fold lower sensitively than when UV detection is used [46,51].

This derivatization procedure with slightly modification is used to the direct derivatization of various carboxylic acids, such as mono-, poly- and hydroxycarboxylic acids, in foods and beverages without conventional isolation steps [48,55,58–60]. The major problems arising in the direct derivatization of carboxylic acids in foods and beverages are the presence of protein-bound acids and the pH of the reaction mixture. Deproteinization was easily accomplished by the ethanol in the reaction mixture, and the protein-bound acids could be converted into their hydrazides, because the amount of ethanol (ca. 71.4%, v/v) was sufficient to denature and precipitate the protein [50,51,55]. Another problem is that the pH of the reaction mixture was slightly increased by basic substances occurring the foods and beverages, resulting in a decrease of the yields of the carboxylic acid hydrazides. Therefore, 2-NPH·HCl was dissolved in 100 m*M* hydrochloric acid–ethanol (1:1, v/v) instead of water to obtain maximum yields of the hydrazides. The carboxylic acid profile was unaffected by the amount of hydrochloric acid [50,51,55]. These experiments established that the carboxylic acids in foods and beverages could be directly converted into their hydrazides in high yields. The carboxylic acids derivatized with 2-NPH·HCl were classified for each purpose into the two groups, short-chain fatty acids (SCFAs) and long-chain fatty acids (LCFAs) [50,51,55].

2.4. Assay of free fatty acids in milk and milk products

For samples 100 μl of milk, about 20 mg of butter and cheese and about 50 mg of condensed milk, ice cream and yogurt were measured exactly. To the milk product samples 100 μl of water was added. To each sample solution, 200 μl of ethanol containing 20 nmol of 2-ethylbutyric acid and 20 nmol of margaric acid as internal standards, 200 μl of 2-NPH·HCl in 0.1 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. The resulting hydrazide mixture was neutralized by adding 4 ml of 1/30 *M* phosphate buffer (pH 6.4)–0.5 *M* hydrochloric acid (7:1, v/v) and the long-chain free fatty acid (LCFFA) hydrazides were extracted with 5 ml of *n*-hexane. About a 3 ml portion of the residual aqueous layer was taken and the short-chain free fatty acid (SCFFA) hydrazides were extracted twice with 4 ml of diethyl ether. The *n*-hexane layer and the combined ether layer were evaporated with a stream of nitrogen at room temperature. Each residue was dissolved in 200 μl of methanol and an aliquot of 10–20 μl was injected into the chromatograph.

2.5. Assay of total fatty acids in milk and milk products

For samples, 10 μl of milk, about 1 mg of butter and cheese, about 2 mg of condensed milk and ice cream and about 10 mg of yogurt were measured exactly. Each sample was dissolved in 200 μl of ethanol containing 400 nmol of 2-ethylbutyric acid and 200 nmol of margaric acid as internal standards and was saponified with 100 μl of 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) solution at 80°C for 20 min. To the saponified sample, 200 μl of 2-NPH·HCl in 0.3 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCI solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. The resulting hydrazide mixture was treated in the same way as in the de-

termination of free fatty acids (FFAs) in milk and milk products.

The *n*-hexane layer and combined ether layer were evaporated with a stream of nitrogen at room temperature. Each residue was then dissolved in 200 μl of methanol and an aliquot of 2–10 μl was injected into the chromatograph.

2.6. Assay of long-chain total fatty acids in fats and oils

About 1 mg of fat and oil samples were measured exactly. Each sample was dissolved in 200 μl of ethanol containing 400 nmol margaric acid as internal standard and saponified with 100 μl of 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) at 80°C for 20 min. To the saponified sample, 200 μl of 2-NPH·HCl in 0.3 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution was added. The mixture was heated at 80°C for 5 min. After addition of 200 μl of 10% (w/v) potassium hydroxide, the mixture was further heated at 80°C for 5 min and then cooled. The hydrazide mixture (5–10 μl) was injected into the chromatograph.

2.7. Assay of mono-, poly- and hydroxycarboxylic acids in beverages

For samples, 50 μl of wines, 25 μl of fruit juices, 100 μl of beer and 50 μl of Japanese ''sake'' were exactly measured and each sample, with the exception of beer, was diluted with water to 100 μl. To each sample solution, 200 μl of ethanol containing 400 nmol of 3-methylglutaric acid as internal standard, 200 μl of 2-NPH·HCl in 0.1 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of 10% (w/v) potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. The hydrazide mixture (5–10 μl) was injected into the chromatograph.

3. Chromatographic analysis

3.1. Instrumentation

Chromatographic analyses were carried out using a Shimadzu LC-6A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an on-line degasser ERC-3310 (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV–visible detector. The detector signals were recorded on a Rikadenki multi-pen recorder (Tokyo, Japan). The column temperature was kept constant at 30–50°C using a Shimadzu GTO-6A column oven. All columns were packed at Yamamura Chemical Laboratories.

3.2. HPLC conditions

The separation of 10 SCFA hydrazides was achieved on a YMC-FA (C_8) main column (particle size 5 μm, 250×6 mm I.D.) with a BBC-4-C_8 guard column (particle size 5 μm, 10×4 mm I.D.). The eluent was maintained at about pH 4–5 by adding 0.1 *M* hydrochloric acid and was filtered through a Nuclepore filter (pore size 2 μm) (Nomura Micro Science, Osaka, Japan).

The separation of 29 LCFA hydrazides was achieved on a J'sphere ODS-M 80 main column (particle size 4 μm, 250×4.6 mm I.D.) with a guard cartridge (J'sphere ODS-M 80). The eluent was maintained at about pH 4–5 by adding 0.1 *M* hydrochloric acid and was filtered through a Fluoropore filter (pore size 0.45 μm) (Sumitomo Electric, Osaka, Japan).

The separation of 10 carboxylic acid hydrazides was carried out on a J'sphere ODS-M 80 main column (particle size 4 μm, 250×6 mm I.D.) with a BBC-5-C_8 guard column (particle size 5 μm, 10×5 mm I.D.). The pH was adjusted to the desired value by mixing 0.005 *M* KH_2PO_4–acetonitrile–methanol with 0.005 *M* Na_2PO_4–acetonitrile–methanol and then dissolving counter-ions at a concentration of 0.005 *M*. The counter-ions studied were tetramethylammonium, tetraethylammonium and tetra-*n*-propylammonium (TMA, TEA and TPA, respectively) as their bromides. The eluent was filtered through a Nucleopore filter (pore size 0.2 μm) (Nomura Micro Science).

3.3. HPLC separation

Chromatographic separations of fatty acid derivatives are usually performed on reversed-phase columns with isocratic or gradient elution systems

comprising acetonitrile, methanol, and water in various proportions. The elution volumes of the fatty acid derivatives are affected principally by the number of carbon atoms and the number of unsaturated bonds in the fatty acid chains [48,50,55,56]. Acetonitrile and methanol have different effects on the two parameters. Therefore, the conditions for HPLC separations of the SCFA and LCFA hydrazides were investigated using the YMC-FA (C_8) column and/or the J'sphere ODS-M 80 column with different isocratic eluents consisting of acetonitrile, methanol and water in various proportions.

Fig. 5 shows a typical separation of 10 SCFA hydrazides ($C_{2:0}$–$C_{6:0}$) including iso isomers and

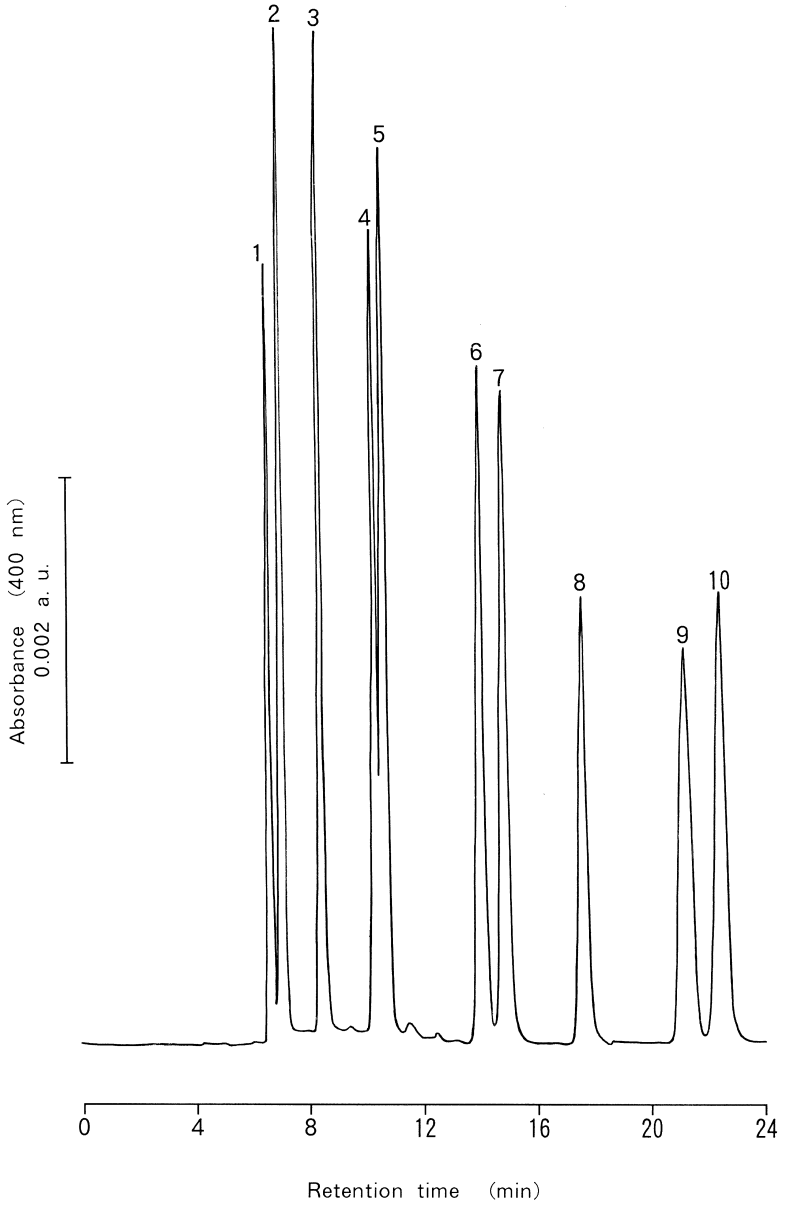

Fig. 5. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 10 fatty acids obtained with visible detection. Peaks: 1=lactic; 2=acetic; 3=propionic; 4=isobutyric; 5=*n*-butyric; 6=isovaleric; 7=*n*-valeric; 8=2-ethylbutyric (internal standard); 9=isocaproic; 10=*n*-caproic acid hydrazide. Each peak corresponds to 150 pmol.

lactic acid hydrazide by HPLC with acetonitrile–methanol–water (30:20:50, v/v/v) as the eluent at a flow-rate of 1.2 ml/min. The column temperature was maintained at 30°C. The iso isomers were eluted faster than the normal isomers [46,50,55].

Fig. 6 shows a chromatogram of a mixture of saturated and mono- and polyunsaturated LCFA hydrazides ($C_{8:0}$–$C_{22:6}$), including *cis–trans* isomers, obtained by using acetonitrile–water (86:14, v/v) as the eluent at a flow-rate of 2.0 ml/min. The retention times increased with increasing chain length for the LCFA hydrazides and inversely with the degree of unsaturation for the unsaturated LCFA hydrazides. The *trans* isomers were eluted after the corresponding *cis* isomers [55,56,59,61]. The chromatographic behaviour of the hydrazine derivatives agree with those of other FA derivatives, and lead to the occurrence of several pairs of fatty acids which are difficult to separate.

However, resolution of double-bond positional isomers, such as γ-linolenic (*n*-6) and α-linolenic acid (*n*-3) hydrazides and *n*-9, *n*-12, and *n*-15 eicosenoic acid hydrazides, is not always achieved. Our previous works [55,56,59,61] showed that acetonitrile has a significant effect on the two factors affecting the elution volumes of fatty acid hydrazides. By increasing the proportion of acetonitrile in the eluent, γ-linolenic (*n*-6) and myristic acid hydrazides were resolved, as were docosahexaenoic (*n*-3) and palmitoleic acid (*n*-7) hydrazides. This change, however, also resulted in decreased resolutions of myristoleic (*n*-5) and octadecatetraenoic acid (*n*-3) hydrazides and of palmitic and docosatetraenoic acid (*n*-6) hydrazides and of oleic (*n*-9) and elaidic acid (*n*-9) hydrazides. Column temperature also has a significant effect on resolution. Increasing the column temperature from 30°C to 50°C leads to inversion in selectivity. Therefore, increasing column temperature caused greater resolutions of the latter three critical pairs but loss of resolutions of the former two critical pairs. For this study, column temperature was set at 50°C to shorten analysis time and yet achieve good resolution. However, resolution of eicosatrienoic (*n*-3) and dihomo-γ-linolenic acid (*n*-6) hydrazides could not be achieved. Separation of 29 LCFA hydrazides is performed within only 22 min by elution in the isocratic mode, which is a distinct advantage over gradient elution techniques [59,61]. The same separation has hitherto been achieved only with capillary gas chromatography.

Reversed-phase ion-pair chromatography (RP-IPC), in which a hydrophobic stationary phase and an aqueous buffer containing a low concentration of counter-ion are used, facilitates the separation of both ionized and non-ionized compounds under the same chromatographic conditions. Our previous works [47,53,54,60] showed that mono-, poly- and hydroxycarboxylic acids were selectively separated as their 2-nitrophenylhydrazides on RP-IPC by the influence of pH, the polarity of mobile phase, and the size of counter-ion.

A pH of 7 was chosen to convert the acidic acid hydrazides into their ionized forms. A small-size ion-pair reagent should be selected to not dominate the chromatographic behaviour of the ion-pair reagent. The eluents containing quaternary alkyl ammonium as their bromide compounds ranging from TMA to TPA were prepared by mixing known volumes of acetonitrile, methanol and aqueous phosphate buffers. When TPA was used as the counter-ion, large retention volumes were observed with a significant loss of resolution. TMA and TEA gave similar resolution, but TEA was selected as the optimum counter-ion since it yielded a higher capacity factor with no loss in resolution. The column temperature has a significant effect on the separation of various pairs of carboxylic acids and an increase from 30°C to 50°C leads to an inversion in the selectivity [60]. By increasing the temperature separation of L-pyroglutamic and lactic and acetic acid hydrazides could be achieved, but this change also resulted in a decreased separation of tartaric and malic and succinic acid hydrazides. Vice versa, decreasing the temperature caused greater separation of these acidic acid hydrazides, but resulted in a loss of separation between lactic and acetic acids hydrazides.

Fig. 7 shows a typical chromatogram of 10 mono-, poly- and hydroxycarboxylic acid hydrazides by RP-IPC analysis with phosphate buffer–acetonitrile–methanol (80:10:10, v/v) containing 0.005 *M* TEA as the isocratic eluent at a flow-rate of 2.0 ml/min. The column temperature was maintained at 35°C. In the chromatogram, two peaks appeared for both citric and malic acid hydrazides: these are attributed to stereochemical isomers of the derivatives [60].

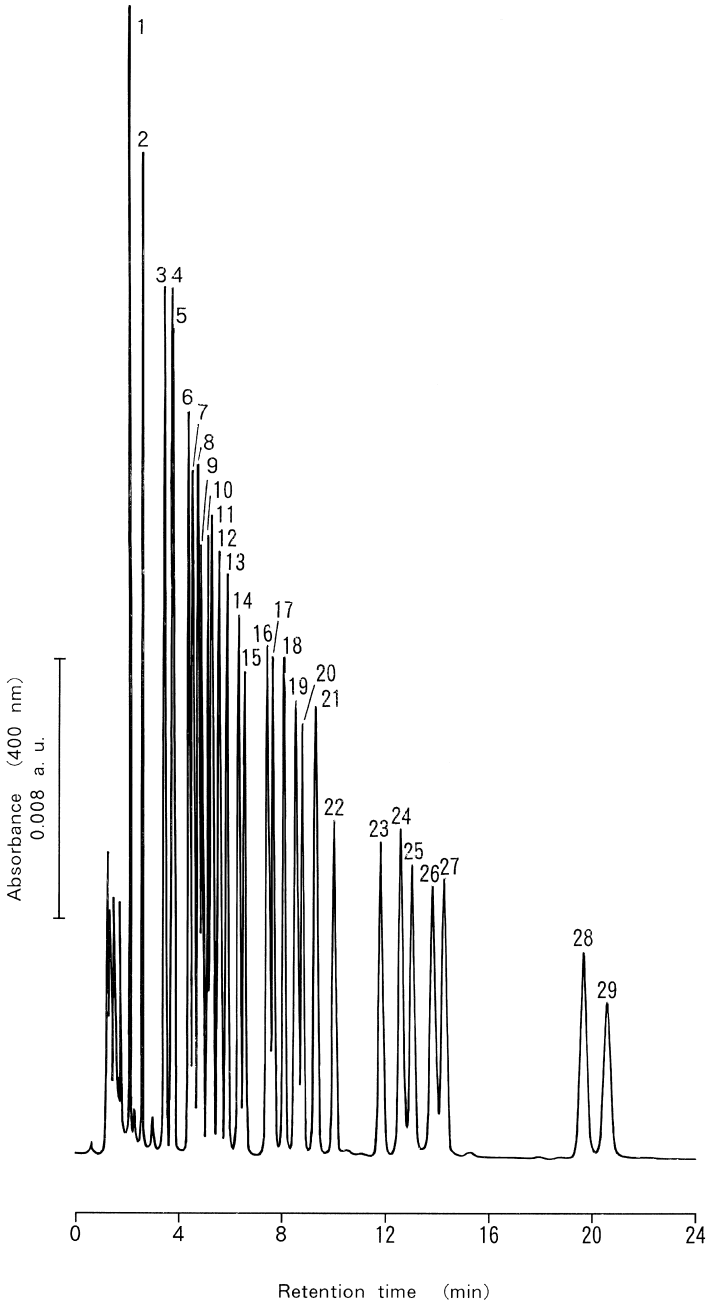

Fig. 6. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 29 fatty acids obtained with visible detection. Peaks: 1=caprylic ($C_{8:0}$); 2=capric ($C_{10:0}$); 3=lauric ($C_{12:0}$); 4=myristoleic ($C_{14:1}$, *n*-5); 5=octadecatetraenoic ($C_{18:4}$); 6=eicosapentaenoic ($C_{20:5}$, *n*-3); 7=α-linolenic ($C_{18:3}$, *n*-3); 8=γ-linolenic ($C_{18:3}$, *n*-6); 9=myristic ($C_{14:0}$); 10=docosahexaenoic ($C_{22:6}$, *n*-3); 11=palmitoleic ($C_{16:1}$, *n*-7); 12=arachidonic ($C_{20:4}$, *n*-6); 13=linoleic ($C_{18:2,cis,cis}$, *n*-6); 14=linoelaidic ($C_{18:2,trans,trans}$, *n*-6); 15=eicosatrienoic ($C_{20:3}$, *n*-3) and dihomo-γ-linolenic ($C_{20:3}$, *n*-6); 16=palmitic ($C_{16:0}$); 17=docosatetraenoic ($C_{22:4}$, *n*-6); 18=oleic ($C_{18:1,cis}$, *n*-9); 19=elaidic ($C_{18:1,trans}$, *n*-9); 20=eicosadienoic ($C_{20:2}$, *n*-6); 21=margaric ($C_{17:0}$) (internal standard); 22=docosatrienoic ($C_{22:3}$, *n*-3); 23=stearic ($C_{18:0}$); 24=eicosenoic ($C_{20:1}$, *n*-9); 25=eicosenoic ($C_{20:1}$, *n*-12); 26=docosadienoic ($C_{22:2}$, *n*-6); 27=eicosenoic ($C_{20:1}$, *n*-15); 28=arachidic ($C_{20:0}$); 29=erucic ($C_{22:1}$, *n*-9) acid hydrazide. Each peak corresponds to 150 pmol.

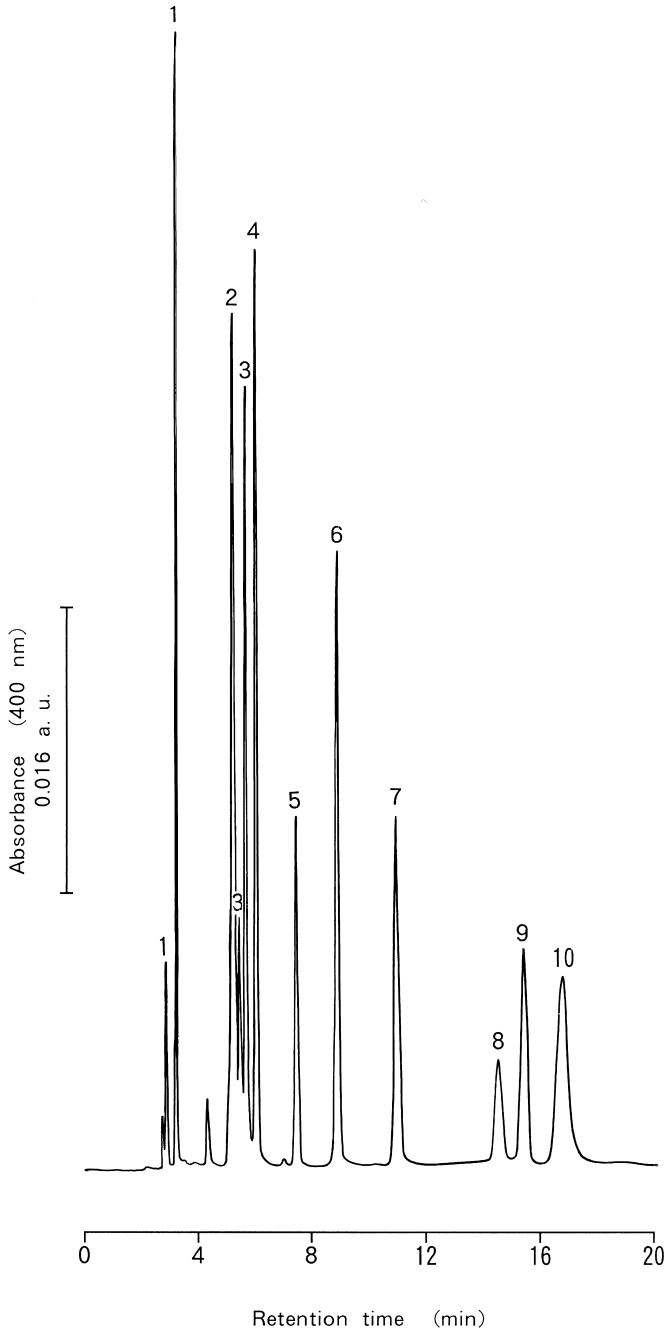

Fig. 7. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of mono-, poly- and hydroxycarboxylic acids obtained with visible detection. Peaks: 1=citric; 2=tartaric; 3=malic; 4=succinic; 5=fumalic; 6=3-methylglutaric (internal standard); 7=glycolic; 8=L-pyroglutamic; 9=lactic; 10=acetic acid hydrazide. Each peak corresponds to 1 nmol.

4. Quantitative analysis

4.1. Calculation

Calibration curves were constructed by derivatizing increasing amounts of carboxylic acids in the presence of internal standard and analysing as described above. The calibration test was replicated five times. From the chromatograms obtained, the relationships between the peak-height ratios of the acid hydrazides to that of internal standard and the concentrations of the acids were calculated by the least-squares method. Previous works [48,55,59,60] demonstrated that the calibration curves of individual carboxylic acids were linear over a wide concentration range with good correlation coefficients (0.999–1.000). The limits of detection, based on a signal-to-noise ratio of 2, were 500 fmol–4 pmol per injection.

4.2. Recovery and precision

The recovery and reproducibility for the assay of free fatty acids (FFAs) and total fatty acids (TFAs) in milk and milk products were investigated six times in butter by adding known mixtures of the SCFAs and LCFAs. In this experiment, the recoveries of FFAs were in the range 95.2–104.3% [relative standard deviations (RSDs)=1.1–4.2%], when the following amounts of fatty acids were added to 20 mg of the butter: $C_{4:0}$, $C_{6:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$=20 nmol; others=5 nmol. The recoveries of TFAs were in the range 96.4–103.3% (RSD=0.5–3.9%), when the following amounts of fatty acids were added to 1 mg of the butter: $C_{4:0}$, $C_{6:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$=200 nmol; others=50 nmol. The inter-assay precision was evaluated by assaying nine times the same butter sample for FFAs and TFAs. The RSDs ranged from 0.4 to 4.5% and from 0.6 to 3.7% for the FFAs and TFAs, respectively.

In order to examine the recovery and reproducibility for the assay of LCTFAs in fats and oils known amounts (50 and 200 nmol) of the fatty acid mixtures were added to triglycerides (TGs) from pig liver (1 mg). Each aliquot was analysed by nine separate measurements. The recoveries of fatty acids were in the range 97.3–102.8% (RSD=0.5–2.4%) and 97.9–103.1% (RSD=0.6–2.9%), respectively, for 50 and 200 nmol of added fatty acids. The intra-assay precision was evaluated by assaying the same TGs six times. The inter-assay precision was determined by analyzing spiked TGs on different days over 1 week (n=6). The intra- and inter-assay precisions ranged from 0.4 to 2.0% and from 0.5 to 2.6%, respectively.

To determine the precision for the assay of mono-, poly- and hydroxycarboxylic acids in beverages known amounts (50 and 200 nmol) of the carboxylic acid mixture was added to pooled red wine (50 μl). Each aliquot was analysed nine times. The recoveries of the carboxylic acids were 97.5–103.9% (RSD=0.9–3.1%) and 98.7–102.8% (RSD=0.8–3.7%) for 50 and 200 nmol, respectively. The intra-assay precision was evaluated by assaying the same red wine six times. The inter-assay precision was determined by analyzing spiked red wine on different days over 1 week (n=6). The intra- and inter-assay precisions ranged from 0.7 to 2.8% and from 0.5 to 3.7%, respectively. These results indicate that the method has a satisfactory precision and reproducibility for the determination of various carboxylic acids in foods and beverages [55,59,60].

5. Application

5.1. Determination of free fatty acids and total fatty acids in milk and milk products

FFAs in milk and milk products contribute to their desirable flavour, but when present in excessive amounts can impart a rancid flavour. Elevated FFA levels are generally caused by the natural milk lipase and/or by the heat-stable bacterial lipase [63]. The determination of TFAs, i.e., the sum of FFAs and esterified fatty acids (EFAs) in milk and milk products, is necessary to investigate whether they are contaminated with other fats and oils.

The FFA and TFA profiles of SCFAs in butter are shown in Figs. 8 and 9, respectively. In the HPLC of SCFFAs and SCTFAs some unknown peaks were found on the chromatograms, but the hydrazides

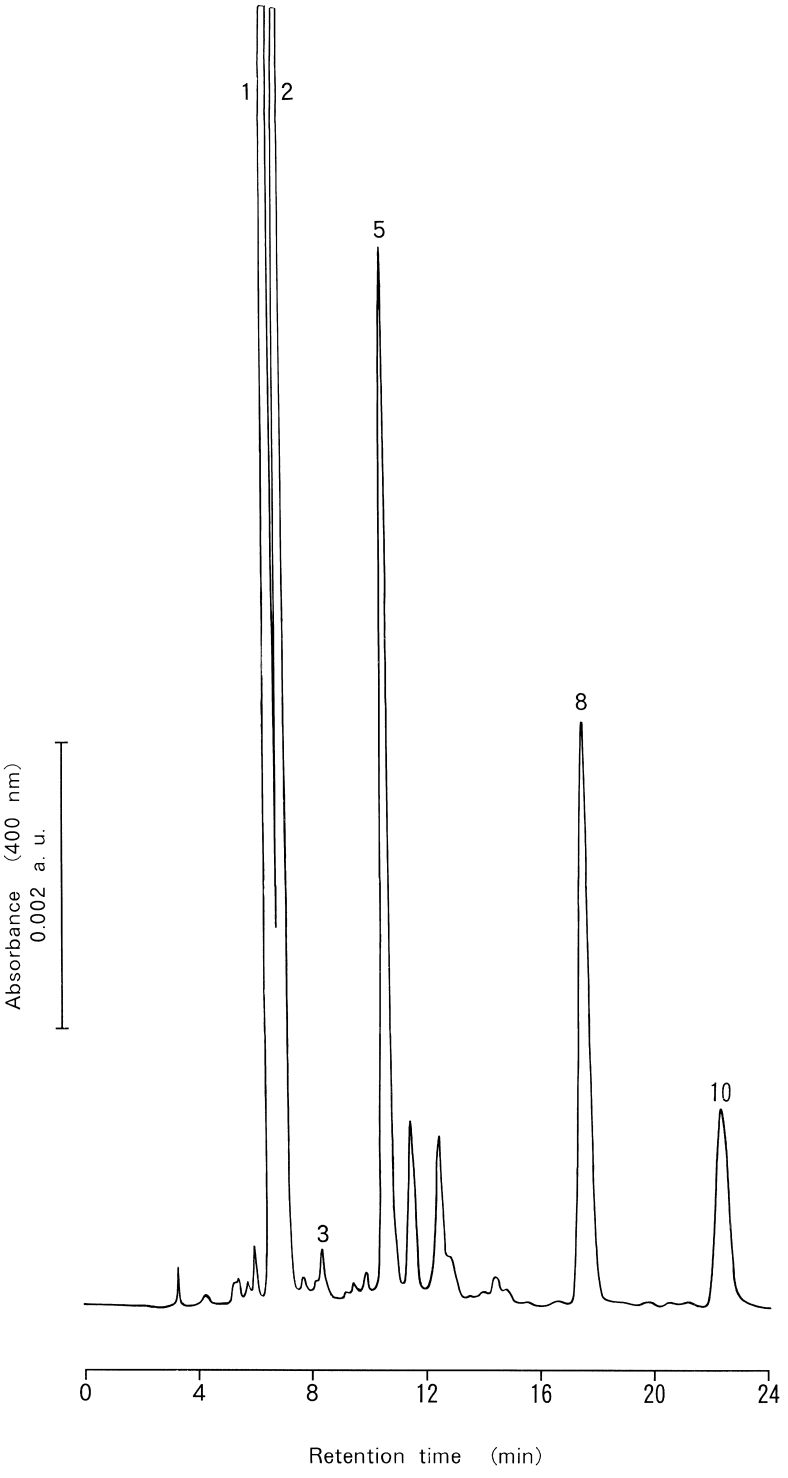

Fig. 8. Short-chain free fatty acid profile of butter. Each peak number corresponds to that in Fig. 5.

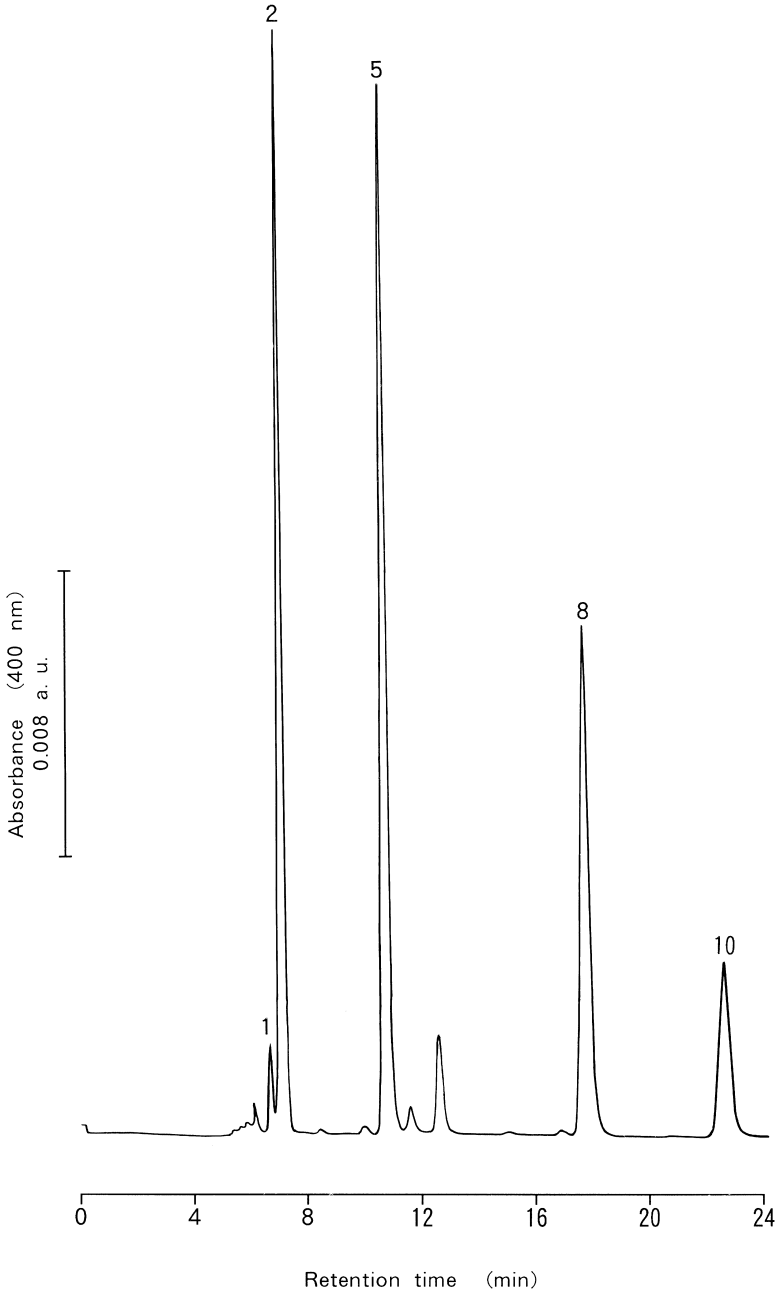

Fig. 9. Short-chain total fatty acid profile of butter. Each peak number corresponds to that in Fig. 5.

were separated from the unknown peaks, with the exception of acetic acid hydrazide. All the fatty acids in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards, because the fatty acids derived from milk and milk products varied from C_4 to C_{20} [43–45,64–67].

Tables 1 and 2 give the amounts of SCFFA and

Table 1
Amounts of SCFFAs in milk and milk products

SCFFA	Amount of SCFFA (nmol/g)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
Lactic	459.10[b]	733.75	5.39[c]	80.03[c]	649.50	64.26[c]
n-Butyric	162.89[b]	128.17	873.95	532.21	51.39	112.49
n-Caproic	39.20[b]	41.60	328.00	168.00	18.80	40.00

[a] Mean results ($n=3$).
[b] nmol/ml.
[c] μmol/g.

Table 2
Amounts of SCTFAs in milk and milk products

SCTFA	Amount of SCTFA (nmol/g)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
Lactic	15.29[b]	28.43	38.55	194.13	85.67	88.63
n-Butyric	15.55[b]	31.78	484.99	235.58	20.45	9.20
n-Caproic	5.29[b]	10.60	165.09	81.04	24.58	2.95

[a] Mean results ($n=3$).
[b] μmol/ml.

Table 3
LCFFA compositions of milk and milk products

LCFFA	LCFFA compositions (mol %)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
$C_{8:0}$	3.69	3.76	1.48	1.78	1.57	5.05
$C_{10:0}$	5.77	5.59	3.58	3.90	4.75	5.71
$C_{12:0}$	5.69	5.56	5.78	6.99	5.07	6.05
$C_{14:0}$	12.16	12.07	14.27	11.81	10.68	14.18
$C_{14:1}$	1.34	1.39	1.36	1.00	0.79	1.12
$C_{16:0}$	28.32	26.64	31.88	26.68	28.57	31.43
$C_{16:1}$	1.93	2.15	2.08	1.76	1.52	1.44
$C_{18:0}$	9.34	8.83	9.91	13.99	22.64	9.34
$C_{18:1,cis}$	25.15	25.97	22.71	16.79	19.69	20.54
$C_{18:2,cis,cis}$ (*n*-6)	4.87	5.91	5.03	12.16	3.34	3.70
$C_{18:3}$ (*n*-3)	0.53	0.65	0.58	1.45	1.38	0.44
$C_{20:2}$ (*n*-6)	0.57	0.53	0.48			
$C_{20:3}$ (*n*-6)	0.24	0.21	0.20	0.20		
$C_{20:4}$ (*n*-6)	0.40	0.74	0.66	1.49		1.00
Total (μmol/g)	1.013[b]	1.680	9.554	6.899	1.281	0.560

[a] Mean results ($n=3$).
[b] μmol/ml.

SCTFA in milk and milk products. The SCFFAs, butyric acid ($C_{4:0}$) and caproic acid ($C_{6:0}$) are largely responsible for the rancid flavour, and the total $C_{4:0}/C_{6:0}$ ratio is very significant for determining the criterion of contamination. Tables 1 and 2 indicate that there is a large difference between the free and total lactic acid estimates. One possible explanation for the difference is the decomposition of lactose that

Table 4
LCTFA compositions of milk and milk products

LCTFA	LCTFA compositions (mol %)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
$C_{8:0}$	3.43	3.20	3.76	3.19	4.18	3.31
$C_{10\ 0}$	6.85	5.65	7.04	6.01	7.45	5.68
$C_{12:0}$	5.81	5.31	6.51	6.29	6.54	5.22
$C_{14:0}$	16.78	16.31	18.04	16.78	16.06	15.70
$C_{14:1}$	1.29	1.49	1.59	1.22	1.11	1.27
$C_{16:0}$	30.44	29.98	31.62	29.81	28.18	30.73
$C_{16:1}$	1.50	1.82	1.57	1.45	1.37	1.58
$C_{18:0}$	11.16	10.42	9.45	11.90	13.76	11.80
$C_{18:1,cis}$	17.23	20.15	15.33	18.12	16.34	18.65
$C_{18:2,cis,cis}$ (*n*-6)	3.90	3.80	3.38	3.35	2.64	4.15
$C_{18:3}$ (*n*-3)	0.39	0.34	0.34	0.69	1.11	0.29
$C_{20:2}$ (*n*-6)	0.14	0.19	0.19	0.10	0.12	0.30
$C_{20:3}$ (*n*-6)	0.24	0.31	0.24	0.24	0.23	0.32
$C_{20:4}$ (*n*-6)	0.93	1.03	0.94	0.85	0.91	1.00
Total (mmol/g)	0.140[b]	0.334	2.300	0.938	0.454	0.088

[a] Mean results ($n=3$).
[b] mmol/ml.

Table 5
Fatty acid compositions of fats and oils[a]

Fatty acid	Fatty acid composition (mol %)								
	Vegetable						Animal		Fish,
	Coconut	Olive	Soybeen	Corn	Safflower	Margarine	Beef tallow	Lard	Sardine
$C_{8:0}$	6.30								
$C_{10:0}$	5.28						0.08	0.18	
$C_{12:0}$	50.06					0.74	0.13	0.16	1.03
$C_{14:0}$	20.51					2.83	2.82	2.89	11.57
$C_{14:1}$ (*n*-5)							0.35	0.27	3.92
$C_{16:0}$	7.93	11.85	8.91	12.52	10.20	35.39	31.77	28.96	19.17
$C_{16:1}$ (*n*-7)	0.63						1.71	3.35	10.21
$C_{18:0}$	2.68	4.28	3.72	1.80	2.25	7.29	27.79	13.88	2.70
$C_{18:1,cis}$ (*n*-9)	5.31	75.58	23.73	25.30	11.69	39.61	29.92	37.14	11.64
$C_{18:1,trans}$ (*n*-9)							0.86	0.76	
$C_{18:2,cis,cis}$ (*n*-6)	1.93	7.01	54.24	58.72	74.12	13.42	3.04	10.85	3.48
$C_{18:3}$ (*n*-3)	0.65	7.71	1.66	1.74	0.08	0.54	0.30	0.90	
$C_{18:3}$ (*n*-6)		1.69							
$C_{18:4}$ (*n*-3)									0.12
$C_{20:1}$ (*n*-9)					0.64		0.57	2.34	
$C_{20:2}$ (*n*-6)						0.42	0.42	0.30	
$C_{20:3}$ (*n*-3 and 6)						0.57	0.45		
$C_{20:4}$ (*n*-6)									0.82
$C_{20:5}$ (*n*-3)									16.17
$C_{22:1}$ (*n*-9)									4.11
$C_{22:2}$ (*n*-6)									0.28
$C_{22:6}$ (*n*-3)									11.24
Total (mmol/g)	5.897	2.281	2.462	2.482	2.637	2.006	2.682	2.770	2.495

[a] Data are expressed as the mean ($n=3$).

may be present in the samples by heating with potassium hydroxide. Other differences between the SCFFAs and SCTFAs were accounted for the EFAs. The fatty acid compositions of LCFFAs and LCTFAs in the samples are given in Tables 3 and 4. In all the samples, the chain length of the LCFAs extends from C_8 to C_{20}, and the amounts of LCTFAs were at least 100-times higher than those of LCFFAs. The individual levels of SCFFAs and LCFFAs in the butter sample were consistent with those reported by Wood and Lindsay [67].

5.2. Determination of long-chain total fatty acids in fats and oils

The biological effects of routinely consumed fats and oils are of wide interest because of their impact on human health and nutrition [68,69]. In particular, the ratio of *n*-3 polyunsaturated fatty acids to *n*-6 polyunsaturated fatty acids (*n*-3/*n*-6) seems to be associated with atherosclerosis and breast and colon cancers [70–74].

The method was used to quantitate LCTFAs in some common edible fats and oils (Figs. 10 and 11). The chromatograms, monitored by visible absorbance, showed very clean backgrounds. Thus, LCFAs in samples were easily identified by comparison of retention times of their hydrazides with those of standards. The peak labelled ''X'' in Fig. 11 is still unknown. This fatty acid may be an isomer of eicosenoic acid (*n*-11), on the basis of the retention behaviour of the other positional isomers (*n*-9, *n*-12, and *n*-15). Table 5 compares the major TFAs of vegetable and fish oils and animal fats. Vegetable oils differ among themselves in the percentage of C_{16} to C_{18} fatty acids, with the exception of coconut oil. In animal fats, fatty acid chain lengths extend from C_{10} to C_{20}. Sardine oil has similar fatty acids as other edible fats and oils and also contains longer chain *n*-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexanoic acid. Table 5 indicates that the principal polyunsaturated fatty acids of sardine oil are in the *n*-3 family.

The data obtained by the present method were in good agreement with those of other reports [11,48,74].

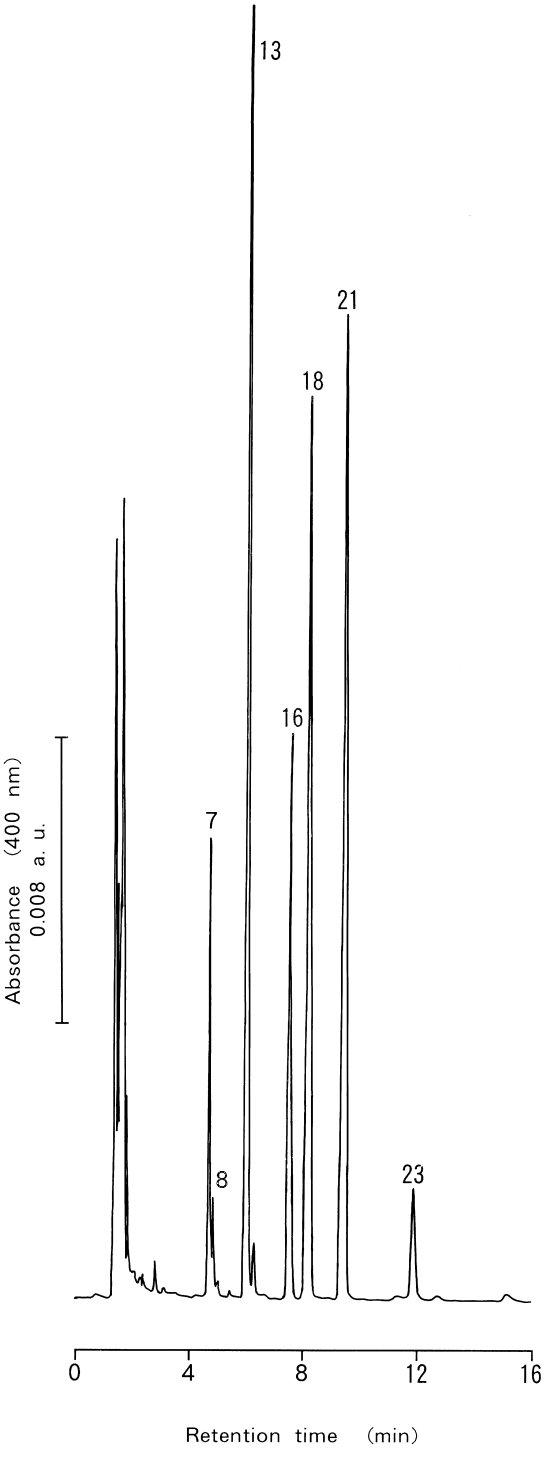

Fig. 10. Long-chain total fatty acid profile of soybeen oil. Each peak number corresponds to that in Fig. 6.

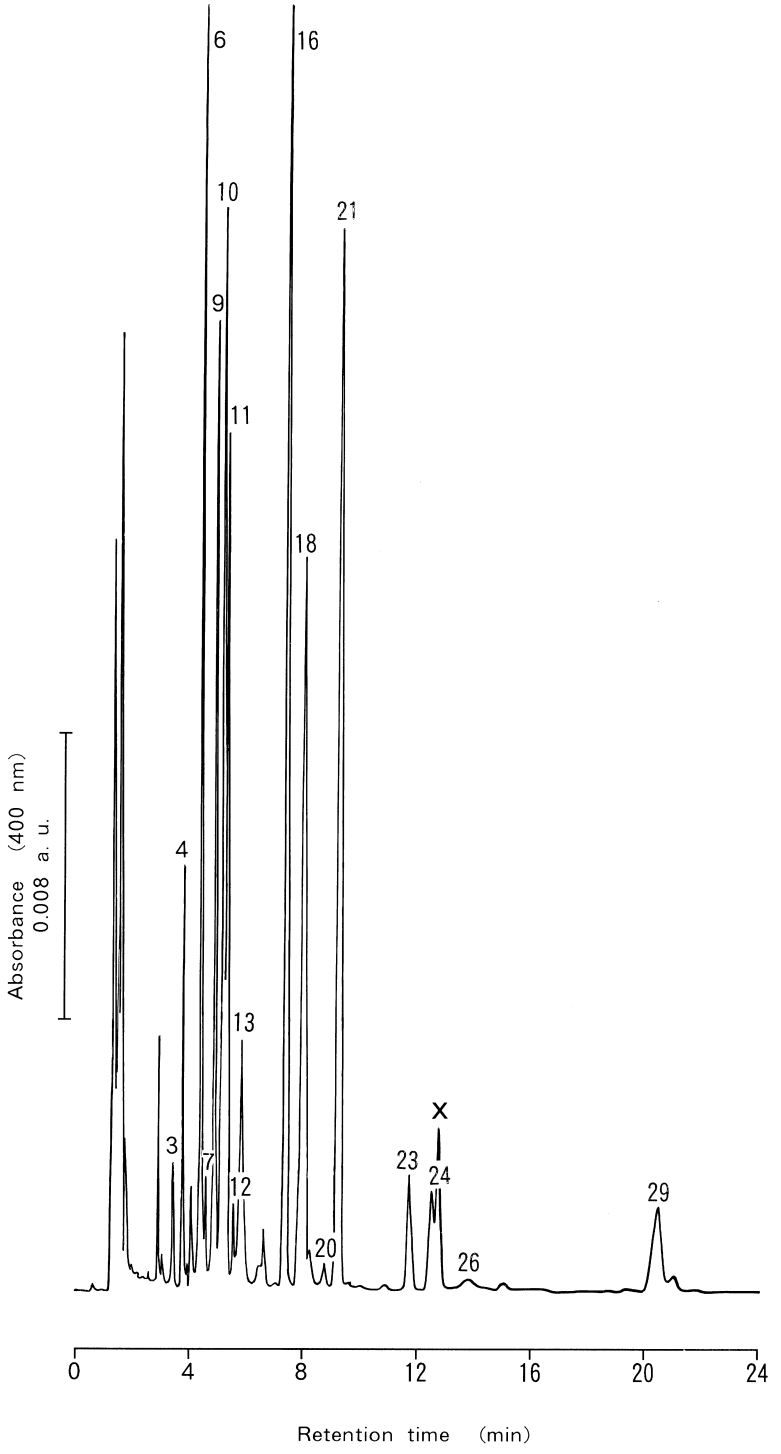

Fig. 11. Long-chain total fatty acid profile of sardine oil. Each peak number corresponds to that in Fig. 6.

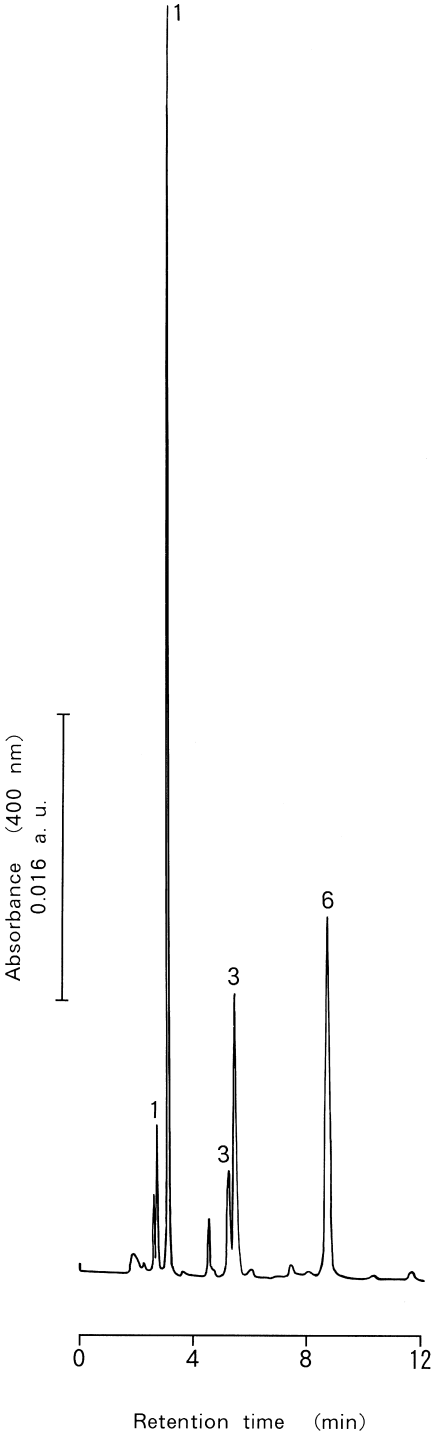

Fig. 12. Carboxylic acid profile of orange juice. Each peak number corresponds to that in Fig. 7.

5.3. Determination of mono-, poly- and hydroxycarboxylic acids in beverages

The nature and concentration of carboxylic acids, such as mono-, poly- and hydroxycarboxylic acids, in foods and beverages are of wide interest with respect to quality control. The present method was tested for the identification and determination of carboxylic acids in some beverages. The carboxylic acid profiles of typical orange juice and white wine are shown in Figs. 12 and 13, respectively. The chromatograms monitored by visible absorbance showed very clean backgrounds, and thus the carboxylic acids in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards.

The results of the determination of the major carboxylic acids in the beverages tested are listed in Table 6. According to the literature [38,41], apple and orange juices contain citric (0.25–8.47 g/l) and malic (0.20–7.20 g/l) acids. In wine, citric (0.10–3.80 g/l), tartaric (0.80–2.50 g/l), malic (0.06–3.73 g/l), succinic (0.10–0.76 g/l), lactic (0.10–4.50 g/l) and acetic (0.20–0.80 g/l) acids were found [38,40,41,75–77]. The values observed by us are compatible with those literature values.

6. Conclusions

Mono-, poly- and hydroxycarboxylic acids in foods and beverages can be directly converted into their hydrazine derivatives which absorb visible radiation. The advantage of using visible detection is that the chromatograms are simpler and more selective. This method also allows the direct derivatization of esterified carboxylic acids after saponification of the samples, and there are no work-up steps involving evaporation of solvent or aqueous washes where any carboxylic acids could be lost. The HPLC analyses described here permit the isocratic separations of those carboxylic acids in the samples with good accuracy, precision and sensitivity owing to the minimum sample preparation required. Despite its simplicity and speed the present method enables a remarkably long column life-time and therefore the method is particularly suitable for routine determinations of carboxylic acids in foods and beverages.

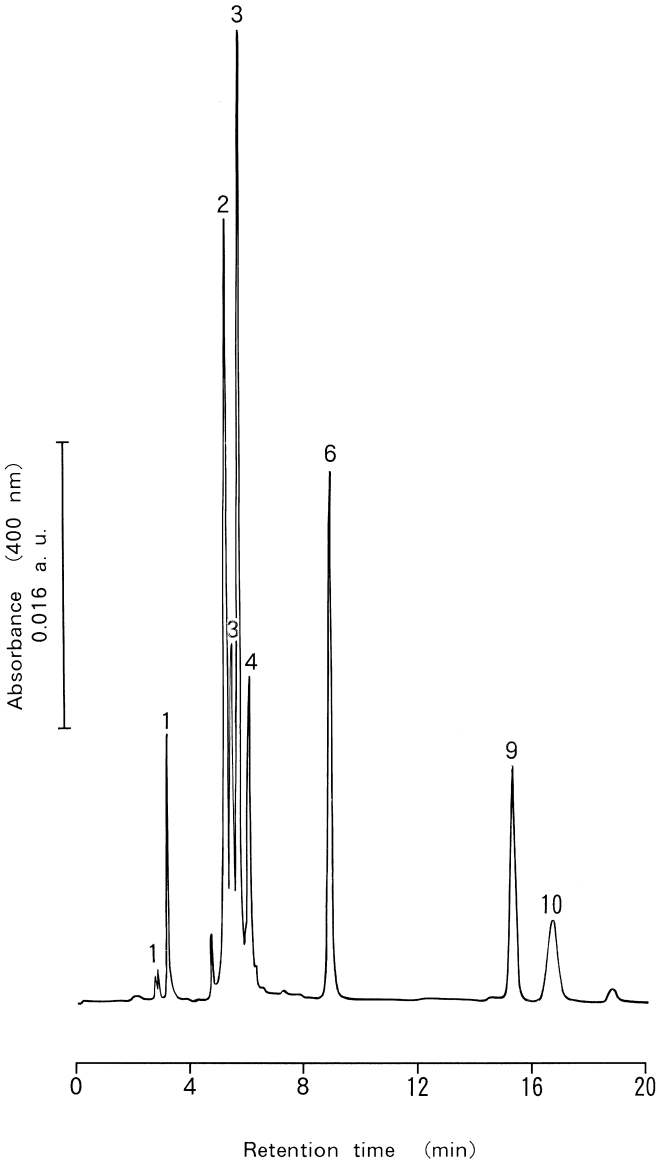

Fig. 13. Carboxylic acid profile of white wine. Each peak number corresponds to that in Fig. 7.

Table 6
Determination of major carboxylic acids in beverages by the present method[a]

Beverage	Carboxylic acid (g/l)						
	Citric	Tartaric	Malic	Succinic	L-Pyroglutaric	Lactic	Acetic
Apple juice	0.52±0.01	N.D.	2.37±0.04	N.D.	N.D.	N.D.	N.D.
Orange juice	5.35±0.14	N.D.	1.34±0.03	N.D.	N.D.	N.D.	N.D.
White wine	0.41±0.01	1.28±0.01	1.59±0.02	0.39±0.01	N.D.	0.91±0.03	0.24±0.01
Red wine	0.20±0.01	1.95±0.02	0.29±0.004	0.66±0.02	N.D.	2.99±0.05	0.58±0.01
Beer	0.16±0.01	N.D.	0.05±0.001	0.04±0.001	0.18±0.004	0.08±0.001	0.08±0.002
Japanese ''sake''	0.14±0.004	N.D.	0.07±0.002	0.17±0.003	0.27±0.01	1.19±0.03	0.03±0.001

[a] Data are expressed as the mean±SD (n=3).
N.D.: Not detectable.

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