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Liquid chromatographic determination of free and total fatty acids in milk and milk products as their 2-nitrophenylhydrazides

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method with direct derivatization is described for the determination of both free fatty acids and total fatty acids in milk and milk products. The method is based on the reaction of these acids with 2-nitrophenylhydrazine hydrochloride with and without saponification of the samples, and there are no sample work-up steps. The derivatized fatty acids were separated into two groups, short-chain fatty acids ($C_{2:0}$–$C_{6:0}$) and long-chain fatty acids ($C_{8:0}$–$C_{22:6}$), as their hydrazides, by a simple solvent extraction. The HPLC of each acid group was performed isocratically with short retention times on a YMC-FA column. The results showed good recovery and reproducibility using 2-ethylbutyric acid and margaric acid as internal standards for the two acid groups. The method is simple, rapid and reliable and has advantages with regard to resolution, analysis time and sensitivity over previous methods.

INTRODUCTION

Free fatty acids (FFA) 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 [1]. The determination of total fatty acids (TFA), *i.e.*, the sum of FFA and esterified fatty acids (EFA) in milk and milk products, is necessary to investigate whether they are contaminated with other fats and oils.

Although gas–liquid chromatography (GLC) is a popular means for the determination of FA, several inherent advantages of high-performance liquid chromatography (HPLC) make it more convenient. The main problem in these chromatographic methods is that the quantitative isolation of FFA is required prior to suitable derivatization. Common isolation procedures involve the use of potassium hydroxide–silicic acid [2], anion-exchange [3] and alumina columns [4]. These procedures may result in loss of sample or in hydrolysis of the endogenous EFA such as glycerides owing to the relatively long contact with the strong alkali used in the isolation procedures.

We have previously described a direct derivatization method with 2-nitrophenylhydrazine hydrochloride (2-NPH · HCl) for the determination of FFA in serum and of TFA in fats and oils without the need for any isolation procedures [5–7]. The aims of this study were to improve the method for the direct derivatization of both FFA and TFA in milk and milk products and to determine FA hydrazides such as short-chain FA (SCFA) ($C_{2:0}$–$C_{6:0}$) and long-chain FA (LCFA) ($C_{8:0}$–$C_{22:6}$) hydrazides using isocratic HPLC.

EXPERIMENTAL

Reagent solutions

All FA solutions in ethanol were obtained from Yamamura Chemical Labs. (Kyoto, Japan). 2-NPH · HCl (Tokyo Kasei Kogyo, Tokyo, Japan) solutions (0.02 *M*) were prepared by dissolving the reagent in 0.1 *M* hydrochloric acid–ethanol (1:1, v/v) and 0.3 *M* hydrochloric acid-ethanol (1:1, v/v) for determining the FFA and TFA, respectively. A 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC · HCl) (Sigma, St. Louis, MO, U.S.A.) 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. These solutions were also commercially available from the Yamamura Chemical Labs. All other chemicals were of analytical-reagent grade, unless stated otherwise. As the samples, commercially available milk and milk products wer used.

Determination of FFA 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 were added. To each sample, 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 solution and 200 μl of 1-EDC · HCl solution were successively 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 LCFFA hydrazides were extracted with 5 ml of *n*-hexane. About a 3-ml portion of the residual aqueous layer was taken and the 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 filtered through a YMC Top-Filter (pore size 0.45 μm) (Yamamura Chemical Labs.). An aliquot of 10–20 μl was injected into the chromatograph.

Determination of TFA 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 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 treated in the same way as in the determination of FFA 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.

HPLC analysis

Chromatographic analyses were carried out using a Shimadzu (Kyoto, Japan) LC-6A liquid chromatograph equipped with an ERC-3310 on-line degasser (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV–visible detector. The detector was set at 400 nm and the detector signals were recorded on a Rikadenki (Tokyo, Japan) multi-pen recorder. The column temperature was kept constant at 35°C using a Shimadzu GTO-6A column oven. The column consisted of a BBC-4-C_8 guard column (particle size 5 μm, 10 × 4 mm I.D.) and a YMC-FA (C_8) main column (particle size 5 μm, 250 × 6 mm I.D.), packed at Yamamura Chemical Labs.

The separations of the SCFA and LCFA hydrazides were achieved isocratically using acetonitrile–methanol–water as the eluent at a flow-rate of 1.2 ml/min. The pH of the eluents was maintained at about 4.5 by adding 0.1 *M* hydrochloric acid. The acetonitrile–methanol–water (30:20:50, v/v/v) eluent for the determination of SCFA was filtered through a Nuclepore filter (pore size 2 μm) (Nomura Micro Science, Osaka, Japan). The acetonitrile–methanol–water (75:11:14, v/v/v) eluent for the determination of LCFA was filtered through a Fluoropore filter (pore size 0.45 μm) (Sumitomo Electric, Osaka, Japan).

RESULTS AND DISCUSSION

Sample preparation

The major problems arising in the direct derivatization of FA in saponified and unsaponified milk and milk products are the presence of protein-bound acids and the pH of the reaction mixture. The protein-bound acids in milk and milk products were deproteinized by the ethanol contained in the reaction mixture. The reaction mixture was maintained at a constant pH by the addition of hydrochloric acid because an increase in the pH of the reaction mixture decreased the yields of the hydrazides [5,6]. The experiments established that the FFA and EFA in milk and milk products could be converted into their hydrazides in high yields.

The SCFA in milk and milk products are important from the standpoint of flavour and as a criterion of quality deterioration, whereas the LCFA are of widespread interest in human health and nutrition [8,9]. Therefore, the FA derivatized with 2-NPH · HCl were classified for each purpose into the two groups, SCFA and LCFA, according to previously described methods [5,6] with a slight modification.

Chromatographic conditions

The two principal parameters for the elution volumes of FA hydrazides were the number of carbon atoms and the number of unsaturated bonds in the FA chain [7]. 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 a YMC-FA column and different eluents consisting of acetonitrile, methanol and water in various proportions.

Fig. 1 shows a typical separation of SCFA hydrazides ($C_{2:0}$–$C_{6:0}$) including *iso*

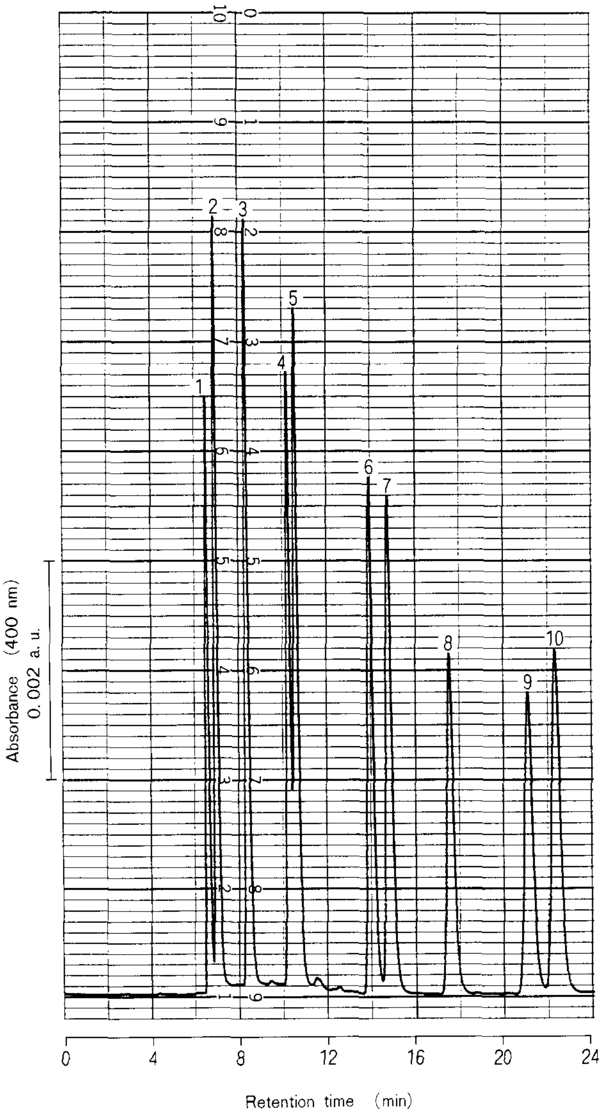

Fig. 1. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of ten fatty acids obtained with visible detection. Peaks: 1 = lactic; 2 = acetic; 3 = propionic; 4 = isobutyric; 5 = *n*-butyric; 6 = isovaleric; 7 = *n*-valeric; 8 = 2-ethylbutyric (internal standard); 9 = isocaproic; 10 = *n*-caproic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

isomers and lactic acid hydrazide by HPLC with acetonitrile–methanol–water (30:20:50, v/v/v) as the eluent and detection in the visible range. The *iso* isomers were eluted faster than the normal isomers. Fig. 2 shows a chromatogram of a mixture of

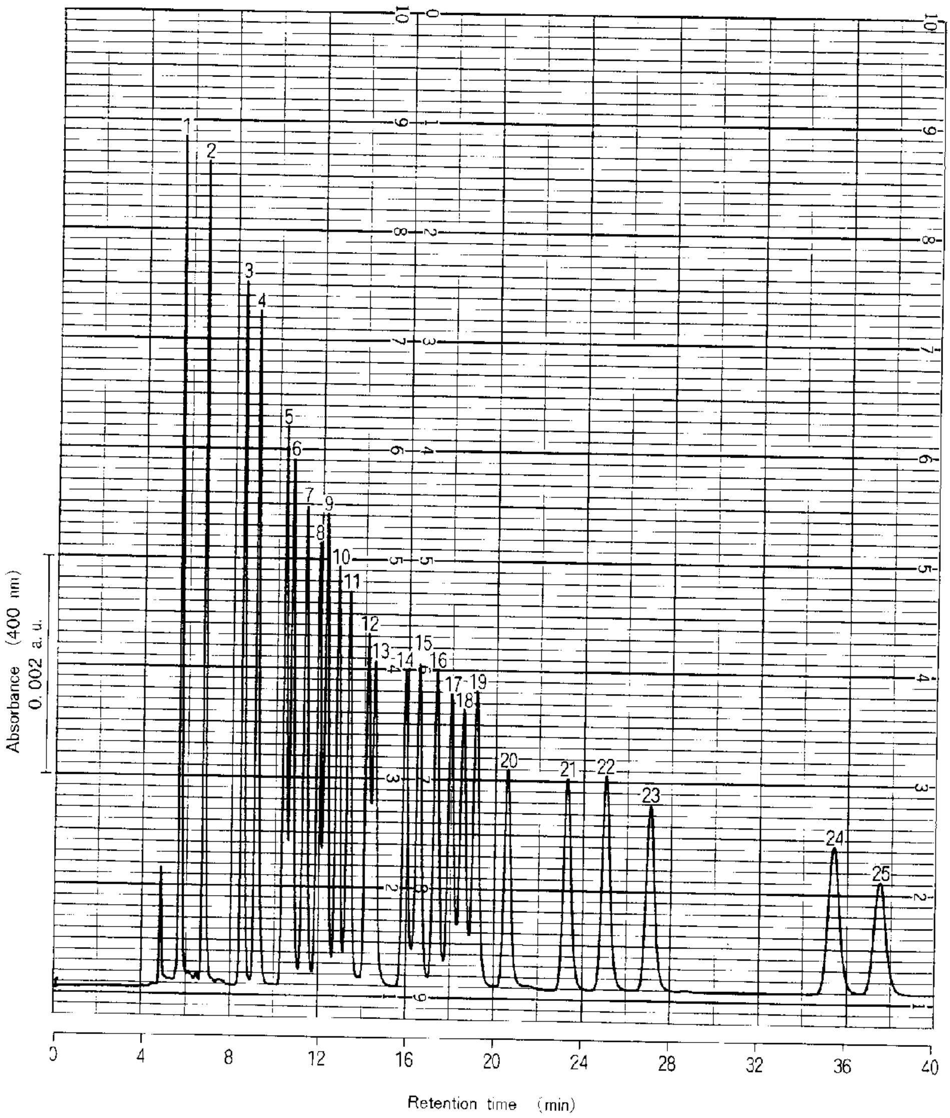

Fig. 2. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 25 fatty acids obtained with visible detection. Peaks: 1 = caprylic ($C_{8:0}$); 2 = capric ($C_{10:0}$); 3 = lauric ($C_{12:0}$); 4 = myristoleic ($C_{14:1}$); 5 = eicosapentaenoic ($C_{20:5}$); 6 = linolenic ($C_{18:3}$);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

saturated and mono- and polyunsaturated LCFA hydrazides ($C_{8:0}$–$C_{22:6}$), including *cis–trans* isomers, obtained by using acetonitrile–methanol–water (75:11:14, v/v/v) as the eluent and detection in the visible range. 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. These chromatographic behaviours of the hydrazine derivatives agree with those of other FA derivatives [10–14], and lead to the occurance of several pairs of FA which are difficult to separate.

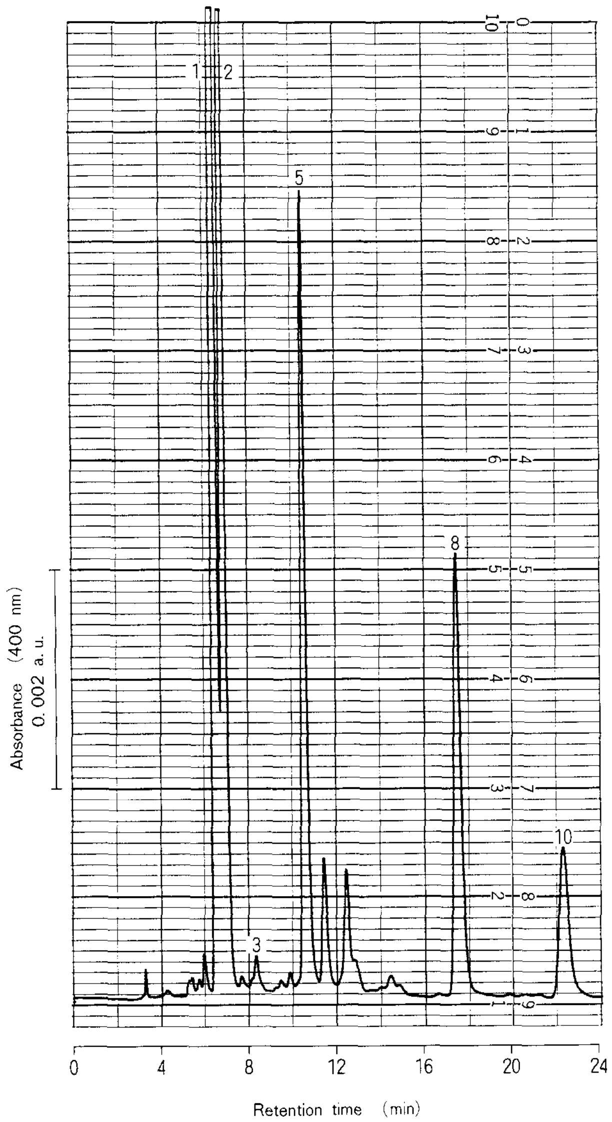

Fig. 3. Short-chain free fatty acid profile of butter. Peaks as in Fig. 1.

However, the excellent resolution of critical pairs and triplets, such as eicosapentaenoic ($C_{20:5}$) and linolenic ($C_{18:3}$) acids, docosahexaenoic ($C_{22:6}$) and palmitoleic ($C_{16:1}$) acids, arachidonic ($C_{20:4}$) and linoleic ($C_{18:2}$) acids and palmitic ($C_{16:0}$), docosatetraenoic ($C_{22:4}$) and oleic ($C_{18:1}$) acids could be accomplished by elution in the isocratic mode, which is a distinct advantage over gradient elution techniques [10–14].

Quantitative analysis

To construct calibration graphs for quantification, increasing amounts of mixtures of the SCFA and LCFA were derivatized in the presence of 2-ethylbutyric acid and margaric acid as internal standards and analysed. The relationships between the relative peak heights and the amounts of the SCFA were linear at least over the range

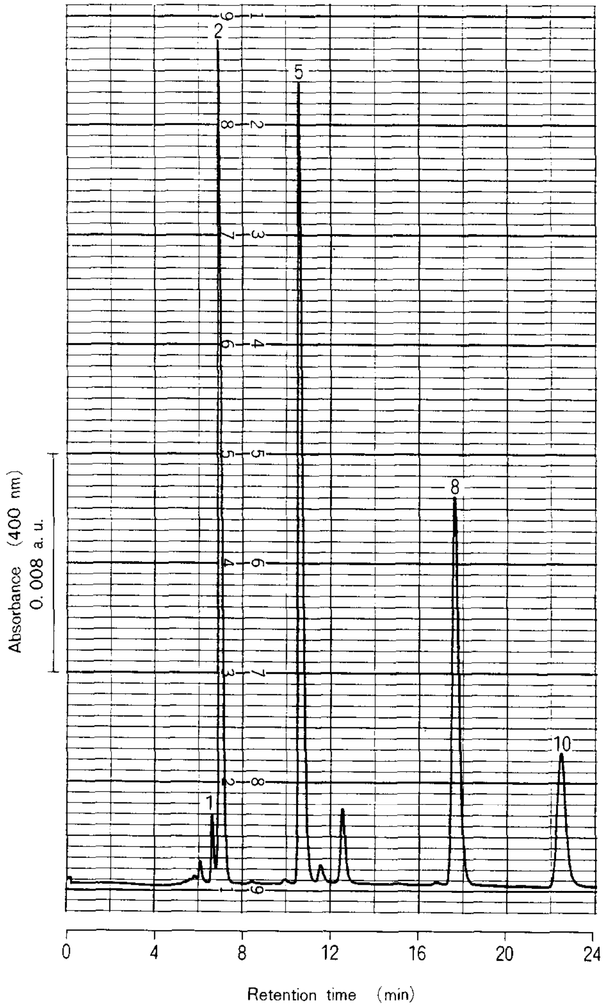

Fig. 4. Short-chain total fatty acid profile of butter. Peaks as in Fig. 1.

2 pmol–5 nmol per injection with correlation coefficients of 0.999–1.000. The calibration graphs for the LCFA were linear over a wide concentration range, 1 pmol–5 nmol per injection, with correlation coefficients of 0.999–1.000. The limits of detection, based on a signal-to-noise ratio of 2, were 1–2 pmol and 500 fmol–1 pmol per injection for the SCFA and LCFA, respectively.

Recovery and reproducibility were investigated six times in butter by adding known mixtures of the SCFA and LCFA. In this experiment, the recoveries of FFA were in the range 95.2–104.3% [coefficients of variation (C.V.) = 1.1–4.2%] when the

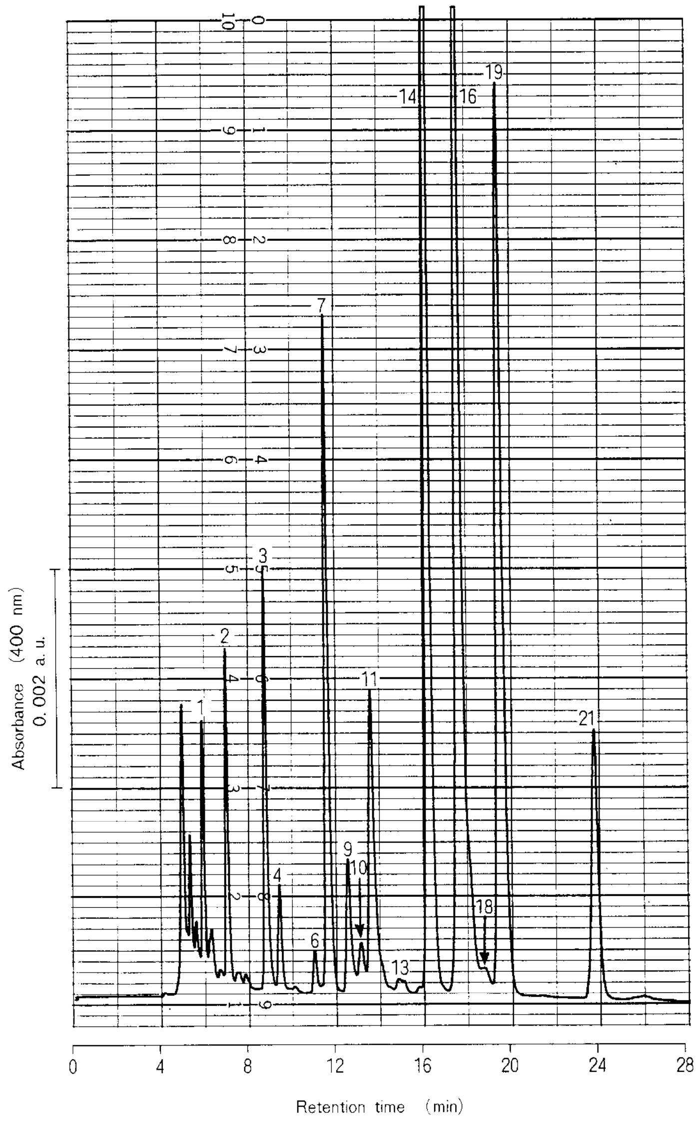

Fig. 5. Long-chain free fatty acid profile of butter. Peaks as in Fig. 2.

following amounts of FA were added: $C_{4:0}$, $C_{6:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ = 20 nmol; others = 5 nmol. The recoveries of TFA were in the range 96.4–103.3% (R.S.D 0.5–3.9%) when the following amounts of FA were added: $C_{4:0}$, $C_{6:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ = 200 nmol; others = 50 nmol.

The within-run precision was evaluated by assaying the same butter sample nine times for FFA and TFA. The precision (C.V.) ranged from 0.4 to 4.5% and from 0.6 to 3.7% for the FFA and TFA, respectively. These results indicate that the method has a satisfactory precision for the determination of FFA and TFA in milk and milk products.

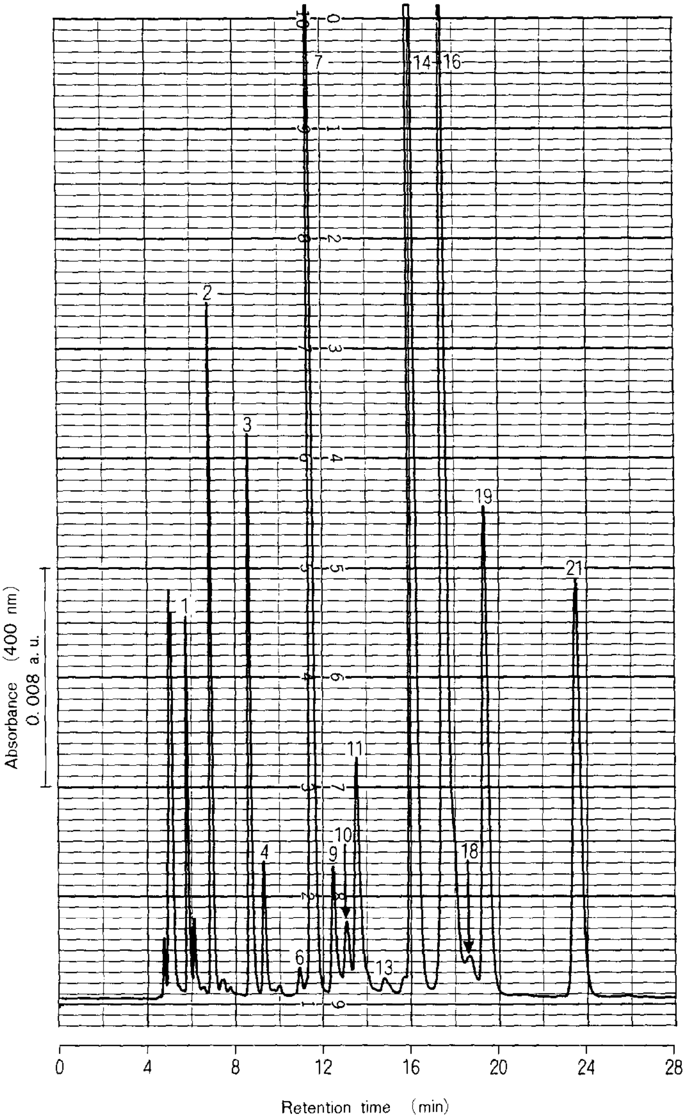

Fig. 6. Long-chain total fatty acid profile of butter. Peaks as in Fig. 2.

Applicability

We applied the method to the determination of FFA and TFA in milk and milk products. The FFA and TFA profiles of SCFA and LCFA in butter are shown in Figs. 3–6. The chromatograms of LCFFA and LCTFA monitored by the visible absorbance showed a very clean background. In the HPLC of SCFFA and SCTFA some unknown peaks were found on the chromatograms, but the hydrazides were completely separated from the unknown peaks, with the exception of acetic acid hydrazide. All the FA in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards, because the FA derived from milk and milkproducts varied from C_4 to C_{20} [2–4, 15–18].

Tables I and II give the amounts of SCFFA and SCTFA in milk and milk products. The SCFFA, 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 I and II 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 which may be present in the samples by heating with potassium hydroxide. Other differences between the SCFFA and SCTFA were accounted for the EFA. The FA compositions of LCFFA and LCTFA in the samples are given in Tables III and IV. In all the samples, the chain length of

TABLE I

AMOUNTS OF SCFFA 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 II

AMOUNTS OF SCTFA 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 III

LCFFA COMPOSITIONS OF MILK AND MILK PRODUCTS

LCFFA	LCFFA composition (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
$C_{18:1c}$	25.15	25.97	22.71	16.79	19.69	20.54
$C_{18:2c,c}(n\text{-}6)$	4.87	5.91	5.03	12.16	3.34	3.70
$C_{18:3}(n\text{-}3)$	0.53	0.65	0.58	1.45	1.38	0.44
$C_{20:2}(n\text{-}6)$	0.57	0.53	0.48			
$C_{20:3}(n\text{-}6)$	0.24	0.21	0.20	0.20		
$C_{20:4}(n\text{-}6)$	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.

TABLE IV

LCTFA COMPOSITIONS OF MILK AND MILK PRODUCTS

LCTFA	LCTFA composition (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
$C_{18:1c}$	17.23	20.15	15.33	18.12	16.34	18.65
$C_{18:2c,c}(n\text{-}6)$	3.90	3.80	3.38	3.35	2.64	4.15
$C_{18:3}(n\text{-}3)$	0.39	0.34	0.34	0.69	1.11	0.29
$C_{20:2}(n\text{-}6)$	0.14	0.19	0.19	0.10	0.12	0.30
$C_{20:3}(n\text{-}6)$	0.24	0.31	0.24	0.24	0.23	0.32
$C_{20:4}(n\text{-}6)$	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.

the LCFA extends from C_8 to C_{22}, and the amounts of LCTFA were at least 100 times higher than those of LCFFA.

The individual levels of SCFFA and LCFFA in the butter sample were consistent with those reported by Wood and Lindsay [18].

CONCLUSION

The FFA in milk and milk products can be reacted directly with 2-NPH · HCl without hydrolytic or oxidative degradation. This method also allows the direct derivatization of TFA after saponification of milk and milk products, and there are no work-up steps involving evaporation of solvent or aqueous washes where SCFA could be lost. The HPLC analyses described here permit the isocratic separations of both SCFA and LCFA in samples with good accuracy, precision and sensitivity owing to the minimum sample preparation required.

The simplicity and rapidity of this method are particularly suitable for routine determinations of FFA and TFA in milk and milk products.

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