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# Simple and Sensitive Analysis of Long-Chain Free Fatty Acids in Milk by Fluorogenic Derivatization and High-Performance Liquid Chromatography

CHI-YU LU, HSIN-LUNG WU,* SU-HWEI CHEN, HWANG-SHANG KOU, AND SHOU-MEI WU

Graduate Institute of Pharmaceutical Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan 807

A highly sensitive high-performance liquid chromatography (HPLC) method is described for the simultaneous determination of some important saturated and unsaturated fatty acids in milk, including lauric (dodecanoic), myristic (tetradecanoic), palmitic (hexadecanoic), stearic (octadecanoic), palmitoleic (hexadecenoic), oleic (octadecenoic), and linoleic acids (octadecadienoic acids). The fatty acids were fluorogenically derivatized with 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate (NOEPES) as their naphthoxyethyl derivatives. The resulting derivatives were separated by isocratic HPLC and monitored with a fluorometric detector ($\lambda$ex = 235 nm, $\lambda$em = 350 nm). The fatty acids in milk were extracted with toluene, and the extract with the fatty acids was directly derivatized with NOEPES without solvent replacement. Determination of long-chain free fatty acids in milk is feasible by a standard addition method. A small amount of milk product, 10 $\mu$L, is sufficient for the analysis.

**KEYWORDS: Long-chain free fatty acids; milk products; HPLC; fluorimetry**

## INTRODUCTION

Fatty acids in food play an important role in human health. A diet high in saturated fatty acids has been correlated with chronic diseases of the cardiovascular system (*1*−*6*). In contrast, a diet with suitable amounts of unsaturated fatty acids may have a good effect on the prevention of chronic heart disease. Therefore, the analysis and the content of fatty acids in food are essential for dietary and health purposes.

Free fatty acids do not contain responsive groups such as chromophores or fluorophores for direct monitoring at trace levels by UV or fluorimetric detection. Fluorescence-oriented derivatizations coupled with liquid chromatography are well-documented (*7*, *8*) and widely used in the analysis of fatty acids or carboxylic acids in various samples.

We have developed several fluorescent reagents (*9*−*11*), and in this study, 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate (NOEPES) was used for derivatizing fatty acids in milk for HPLC with fluorimetric detection. This method is simple and sensitive for the analysis of long-chain free fatty acids in milk.

## MATERIALS AND METHODS

**Chemicals and Solutions.** Dodecanoic (C12:0), tetradecanoic (C14:0), hexadecanoic (C16:0), octadecanoic (C18:0), hexadecenoic (C16:1), octadecenoic (C18:1), octadecadienoic (C18:2), and heptadecanoic acids (C17:0, an internal standard, IS, for analytical calibration) were from Sigma (St. Louis, MO). 18-Crown-6 ether (18-crown-6) and 1-(4-(2-phenyl ethyl)benzyl) naphthalene (an additional IS for stability test) were from TCI (Tokyo, Japan). Other reagents were of analytical reagent grade. Reagent solutions of NOEPES (synthesized at our laboratory) (*10*) and 18-crown-6 were prepared in toluene; solutions of sulfuric acid and phosphoric acid were prepared in water. Liquid milk samples all from domestic companies were purchased at local retailers in Kaohsiung (Taiwan).

**Liquid Chromatographic Conditions.** A Waters LC system with a U6K injector, a model 717 plus autosampler, a model 510 pump, a model 474 scanning fluorescence detector, and a model 746 integrator was used. A Symmetry $C_8$ column (150 × 3.9 mm i.d.; 5 $\mu$m) (Waters) and a mixed solvent of methanol−water (92:8, v/v) at a flow rate of 0.7 mL/min were used. The column eluate was fluorimetrically monitored ($\lambda$ex = 235 nm, $\lambda$em = 350 nm).

**Extraction and Derivatization of Long-Chain Free Fatty Acids from Milk.** Milk samples in liquid form were all from food companies in Taiwan. A modified method (*12*) was used for the extraction of long-chain free fatty acids in milk and in spiked milk (as standard addition samples). Namely, a 10 $\mu$L aliquot of milk in each 10 mL test tube was added with and without reference long-chain fatty acids plus IS (190 $\mu$L). Long-chain fatty acids were spiked to milk at four varied concentrations each with an IS at 15.0 $\mu$M (as long-chain fatty acids IS). The long-chain fatty acid IS (C12:0, C14:0, C16:0, C18:0, C16:1, C18:1, C18:2, and IS of C17:0) was prepared by dissolving the reference standards and IS in a mixed solvent of 2-propanol-phosphoric acid (2 M) (5:1, v/v). To the standard addition samples and nonspiked sample (10 $\mu$L milk plus 190 $\mu$L of the mixed solvent only), 200 $\mu$L of toluene was added. After the sample was vortexed for 30 s, 600 $\mu$L of water and 200 $\mu$L of additional toluene were added successively and vortexed for 2 min. The mixture was centrifuged at 1800*g* for 5 min. A 200 $\mu$L aliquot of the toluene layer was taken for the following derivatization.

A 200 $\mu$L aliquot of the toluene extract was transferred to a 10 mL screw-capped test tube containing 300 $\mu$L of NOEPES (12 mM) in

* Corresponding author Tel: 886-7-312-1101 ext. 2316. Fax: 886-7-315-9597. E-mail: m555001@cc.kmu.edu.tw.

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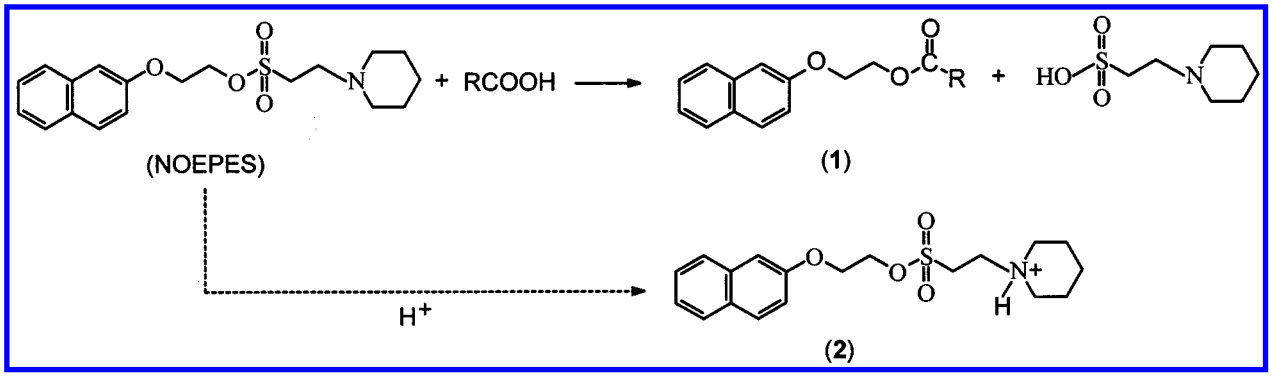


**Figure 1.** Derivatization of long-chain fatty acids (RCOOH) with NOEPES (solid arrow) and protonation of excess NOEPES (dashed arrow) for removal.

toluene, 100 $\mu$L of 18-crown-6 in toluene (20 mM), and about 10 mg of potassium carbonate. The reactants were shaken at 95 °C for 30 min. After the solution was cooled, 300 $\mu$L of the solution was transferred to a test tube and washed with 1.0 mL of 1.0 M $H_2SO_4$ by vortexing for 30 s. A 100 $\mu$L aliquot of the toluene layer was diluted with an equal volume of methanol for compatibility with the mobile phase. The resulting sample solution was used for HPLC analysis.

## RESULTS AND DISCUSSION

Figure 1 shows a reaction scheme for the derivatization of fatty acids with NOEPES and the removal of excess NOEPES after derivatization, leading to a cleaner chromatogram (Figure 2) that was obtained by derivatizing a synthetic mixture of long-chain fatty acids used in this study. The derivatives of long-chain fatty acids in Figure 2 were identified by fast atom bombardment mass spectroscopy (FABMS) of the derivatives obtained by scaling up the amount of long-chain fatty acids used for derivatization. The thin-layer chromatography (TLC)-separated derivatives were analyzed by using a MS VG Quattro 5002 mass spectrometer with FAB mode (nitrobenzyl alcohol as matrix) and an acceleration energy of 10 kV.

The mass spectra obtained exhibited molecular ions ($M^+$) each with a significant ion corresponding to loss of a naphthoxy fragment (M-143) from the derivatives of saturated fatty acids and quasi-molecular ions ($MH^+$) each with a significant ion at MH-143 from the derivatives of unsaturated fatty acids; i.e., *m/z* 370 and 227 (C12:0), *m/z* 398 and 255 (C14:0), *m/z* 426 and 283 (C16:0), *m/z* 440 and 297 (C17:0), *m/z* 454 and 311 (C18:0), *m/z* 425 and 281 (C16:1), *m/z* 453 and 309 (C18:1), and *m/z* 451 and 307 (C18:2), indicating the formation of related ester derivatives of the long-chain fatty acids.

The retention times of the separated derivatives of C12:0, C14:0, C16:1, C18:2, C16:0, C18:1, C18:0, and C17:0 correspond to the peaks 1−8, respectively, in Figure 2A. The retention times of the synthesized derivatives were used to identify the long-chain free fatty acids peaks (Figure 2B) from milk samples.

The basic procedure for the derivatization of long-chain fatty acids in this work was similar to that (*13*) used for the derivatization of the fatty acids in plasma. However, the procedure for the extraction of long-chain free fatty acids in milk was simplified by using toluene as both the extracting solvent and the reacting solvent. This avoids the additional time used for the evaporation of one solvent such as *n*-heptane used for the extraction of long-chain free fatty acids and derivatizing the residue obtained in another solvent (toluene) (*13*). The detection limit (signal:noise ratio = 3) of the method for the analysis of reference long-chain fatty acids is about 8 nM. This indicates that the method is simple, sensitive, and time-saving for the trace analysis of the fatty acids.

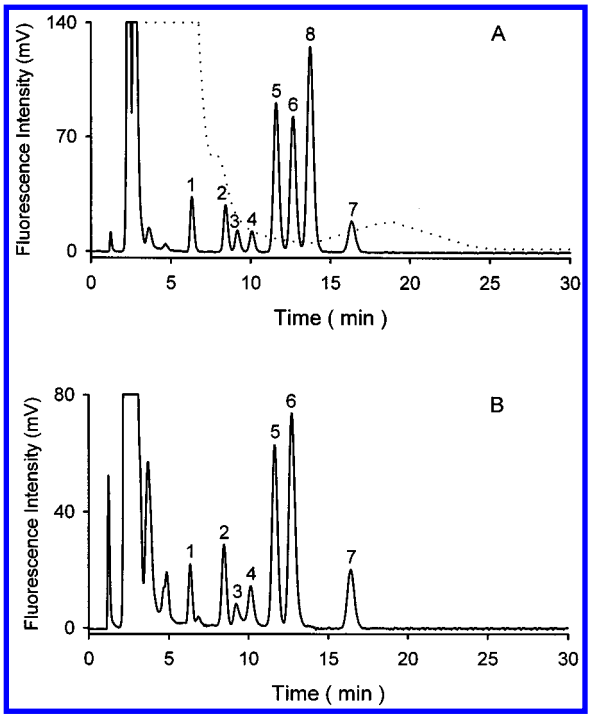


**Figure 2.** (A) LC chromatograms for a mixture of reference long-chain fatty acids (C16:1 and C18:2 each at 2.5 $\mu$M; C12:0, C14:0, and C18:0 each at 5.0 $\mu$M; C16:0 and C18:1 each at 7.5 $\mu$M with IS at 10.0 $\mu$M). Peaks for LCFFA derivatives: 1 = C12:0; 2 = C14:0; 3 = C16:1; 4 = C18:2; 5 = C16:0; 6 = C18:1; 7 = C18:0; and 8 = C17:0 (IS), derivatized with NOEPES with acid treatment (solid line). The reagent blank without acid treatment (dashed line) overlapped with the peaks of long-chain fatty acids. (B) LC chromatograms for the analysis of long-chain free fatty acids in plain milk (from sample 3 in Table 1A). Peak numbering is the same as in A. This milk sample is in the absence of IS, indicating that no other peaks interfere with the IS (C17:0).

The stability of long-chain fatty acid derivatives (esters) was studied using 1-[4-(2-phenyl ethyl)benzyl] naphthalene (a non-ester) as the second IS for evaluating the peak−area ratios of the long-chain fatty acid derivatives to the second IS. No significant change of peak−area ratios was found over 24 h, indicating that the long-chain fatty acid derivatives are sufficiently stable for the time required for LC analysis.

**Analysis of Long-Chain Free Fatty Acids in Milk.** Milk without fatty acids was unavailable as the matrix for the analysis of long-chain free fatty acids in milk; therefore, a standard addition approach (*14*) was used. The applicability of the method to the analysis of long-chain free fatty acids in milk was evaluated by spiking milk with and without long-chain fatty acids. Four different amounts of each long-chain fatty acid

**Table 1.** Analysis of Long-Chain Free Fatty Acids in Liquid Milk

(A) Plain milk

| milk sample[a] | long-chain free fatty acids ($\mu$M)[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | C12:0 | C14:0 | C16:0 | C18:0 | C16:1 | C18:1 | C18:2 |
| 1 | 92.5 | 105.8 | 280.8 | 150.5 | 27.1 | 461.7 | 75.8 |
| 2 | 25.3 | 51.3 | 150.2 | 100.5 | 16.6 | 280.3 | 62.4 |
| 3 | 63.5 | 115.5 | 236.0 | 148.6 | 30.1 | 380.4 | 84.6 |
| 4 | 28.3 | 54.2 | 189.6 | 158.5 | 10.6 | 194.6 | 43.1 |
| 5 | 51.6 | 103.0 | 215.5 | 124.8 | 37.2 | 360.7 | 73.2 |
| 6 | 37.8 | 93.0 | 228.5 | 107.0 | 32.1 | 336.1 | 68.7 |
| 7 | 40.5 | 69.0 | 245.0 | 119.9 | 31.3 | 256.3 | 67.7 |
| mean | 48.5 | 84.5 | 220.8 | 130.0 | 26.4 | 324.3 | 67.9 |
| SD | 23.4 | 26.1 | 41.8 | 22.8 | 9.4 | 88.4 | 13.0 |

(B) Low-fat milk

| milk sample[a] | long-chain free fatty acids ($\mu$M)[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | C12:0 | C14:0 | C16:0 | C18:0 | C16:1 | C18:1 | C18:2 |
| 1 | 15.9 | 54.4 | 122.1 | 64.5 | 15.4 | 195.5 | 42.3 |
| 2 | 11.8 | 37.7 | 102.2 | 67.8 | 11.3 | 149.6 | 37.0 |
| 3 | 31.9 | 74.2 | 161.3 | 84.6 | 24.2 | 255.2 | 58.0 |
| 4 | 14.7 | 32.3 | 116.8 | 84.8 | 5.1 | 96.6 | 31.5 |
| 5 | 20.2 | 63.2 | 138.6 | 79.4 | 18.9 | 206.2 | 45.5 |
| 6 | 22.8 | 67.1 | 159.8 | 88.3 | 21.9 | 290.3 | 61.6 |
| 7 | 18.5 | 45.3 | 117.2 | 78.1 | 13.8 | 159.6 | 41.6 |
| mean | 19.4 | 53.5 | 131.1 | 78.2 | 15.8 | 193.3 | 45.4 |
| SD | 6.6 | 15.7 | 22.8 | 9.0 | 6.5 | 65.6 | 10.9 |

[a] Seven different milk samples (1–7) in liquid form were all from food companies in Taiwan. [b] The fatty acid concentrations ($\mu$M) were averages of three replicate analyses.

within a suitable range reflecting their levels in milk samples were prepared as follows for the analysis of long-chain free fatty acids in milk: C16:1 and C18:2 at 1−25 $\mu$M; C12:0, C14:0, and C18:0 at 2−50 $\mu$M; C16:0 and C18:1 at 8−200 $\mu$M. The linearity between the peak−area ratios ($y$) and long-chain free fatty acids ($x$, $\mu$M) was examined. Correlation coefficients obtained are all over 0.999 for each long-chain free fatty acid studied. The precision based on known amount of long-chain free fatty acids in milk cannot be evaluated because of the coexistence of the endogenous long-chain free fatty acids in milk. Therefore, the precisions (relative standard deviations, RSDs) based on the slope and intercept of the related regression equation for intraday ($n = 5$) and interday ($n = 5$) analysis were studied on plain milk and low-fat milk. These showed that the RSDs for the slope and intercept are all below 6.0 and 6.4%, respectively.

The method was applied to the analysis of long-chain free fatty acids in dairy products, low-fat milk and plain milk. A typical chromatogram for the analysis of long-chain free fatty acids in plain milk is shown in Figure 2B (without addition of IS), indicating that no peaks from milk interfere with the IS. Analysis of a reagent blank also showed no interference with the separation of long-chain free fatty acid derivatives. The analytical results of long-chain free fatty acids in milk are shown in Table 1A,B, establishing that the free fatty acid content in low-fat milk is lower than that in plain milk and the RSDs ($n = 3$) for each free fatty acid content in milk samples 1−7 of A and B in Table 1 are all below 6.5%.

In conclusion, a highly sensitive method was established for the trace analysis of long-chain free fatty acids in milk by fluorogenic derivatization and LC. Separation of the long-chain free fatty acids is attainable by simple isocratic LC on a reversed column. Only a small amount of milk (10 $\mu$L) is required for the long-chain free fatty acids analysis. This method could be useful for the analysis of long-chain free fatty acids in animal tissue samples for the study of the free fatty acid content or fatty acid-related diseases in veterinary sciences, especially in cases where the sample source is limited, as from a small animal.

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