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Nondestructive Quantification of Organic Compounds in Whole Milk without Pretreatment by Two-Dimensional NMR Spectroscopy

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In this study, various organic compounds in commercial whole milk were quantified simultaneously by ^{1}H 1D and ^{1}H−^{13}C HSQC 2D NMR spectra without any pretreatment. 2D NMR spectroscopy was applied to quantification of milk compounds for the first time. Milk fat content was easily determined to be 3.6 ± 0.1%, and the lactose content was 47.8 ± 1.0 mg/mL by ^{1}H NMR spectra. From ^{1}H−^{13}C HSQC spectra, the concentrations of citrate, *N*-acetylcarbohydrates, and trimethylamine were determined to be 3.2 ± 0.2, 2.9 ± 0.1, and 4.0 ± 0.6 mM, respectively. The latter two compounds were quantified in milk for the first time. Butyric acid, total monounsaturated fatty acids, and total polyunsaturated fatty acids of triacylglycerols were 6.2 ± 0.5, 9.1 ± 0.9, and 2.9 ± 0.3 mM, respectively. The fatty acid compositions (mol %) of triacylglycerols were then calculated and were observed to be in good agreement with reference values. The results indicated that ^{1}H 1D and ^{1}H−^{13}C HSQC 2D NMR spectroscopy is useful for the rapid and nondestructive determination of various compounds in milk.

KEYWORDS: ^{1}H NMR; ^{1}H-^{13}C HSQC NMR; milk; quantification; saturated fatty acid chain; unsaturated fatty acid chain; N-acetylcarbohydrates; trimethylamine

INTRODUCTION

Quality control of milk is an important issue for the dairy industry because milk contents fluctuate easily because of various factors such as season, breed, nutrition, or milking habits (*1*). Furthermore, milk is a kind of complex biological fluid with several constituents (triacylglycerols, phospholipids, lactose, proteins, and so on) with different characteristics. For this reason, it is extremely difficult to simultaneously quantify various milk compounds. In conventional quantification methods, triacylglycerols, phospholipids, and the other respective compounds in milk are extracted and separated and then are quantified by gas chromatography (GC), high-performance liquid chromatography (HPLC), or thin layer chromatography (TLC) (*2*−*5*). Infrared (IR) spectroscopy is also applied for milk quality control as a nondestructive method. However, only the contents of macronutrients such as lactose, total fats, and total proteins can be roughly determined by the IR method (*6*−*9*). A detailed and widely applicable method has not yet been established for the simultaneous and nondestructive quantification of the compounds in milk.

NMR spectroscopy is a powerful tool for the simultaneous and nondestructive identification of each compound in a complex mixture (*10*). Since the amplitude or area of the NMR signal is proportional to the concentration of the corresponding molecule, the use of NMR has attracted much attention with regard to quantifying constituents in mixtures in the past decade (*11*−*14*). As for the quantification of milk compounds by NMR, some research using ^{13}C 1D NMR has already been reported (*15*, *16*).

In a previous study, we successfully observed and assigned the 2D NMR signals of the respective compounds in milk in a nondestructive and simultaneous way (*17*). In the present study, we applied the assignment results to measure the absolute concentrations of various milk compounds by using 1D and 2D NMR spectra without any pretreatment. The concentrations of fat and lactose in commercial milk have been easily determined by the ^{1}H NMR spectra of milk. For the compounds with overlapping or weak signals in the ^{1}H NMR spectra, we have successfully applied ^{1}H−^{13}C HSQC to quantify milk compounds. This is the first report of using 2D NMR for the quantification of food constituents.

MATERIALS AND METHODS

Materials and Sample Preparation. $CDCl_3$ (99%) was purchased from Isotec Inc. (Tokyo, Japan); 1,1,2,2- tetrachloroethane ($CHCl_2$-$CHCl_2$) was from Wako Pure Chemical Co. Ltd (Osaka Tokyo, Japan), and chromium(III) acetylacetonate ($Cr(AcAc)_3$) was from Kanto Chemical Co. Ltd (Tokyo, Japan). Sodium citrate, acetylglucosamine, tributyrin, triolein, trilinolein, and lecithin were purchased from Wako Pure Chemical Co. Ltd. Commercial whole milk, nonfat milk, and high-fat milk were purchased at a local supermarket. Every sample of milk had been homogenized and treated at an ultrahigh temperature.

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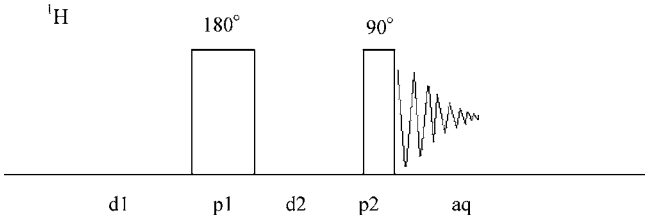

Figure 1. NMR pulse sequence of the partial relaxation FT method. d1, d2, p1, p2, and aq indicate the delay time, pulse interval time, duration of 180° pulse, duration of 90° pulse, and acquisition time, respectively. The horizontal axis is not indicated in a linear scale.

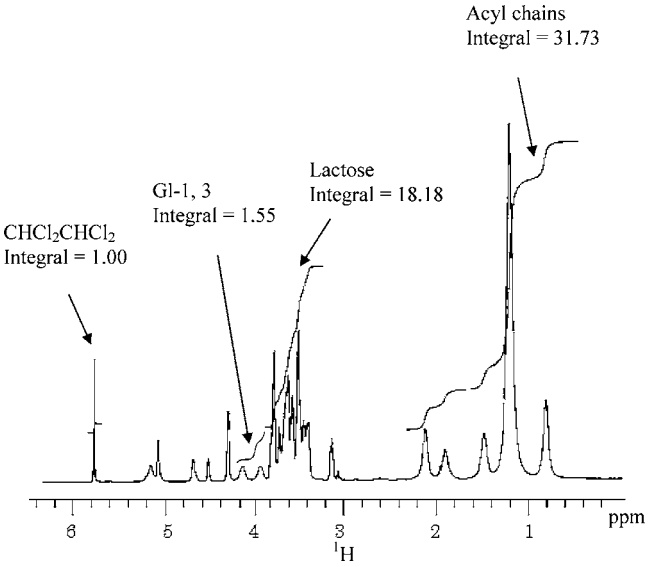

Figure 2. Quantitative ^{1}H NMR spectrum of commercial whole milk. Signals from 0.78 to 2.12 ppm are due to acyl chains of triacylglycerols. Signals from 3.4 to 3.9 ppm are due to lactose. The assignments of signals are reported (*17*).

^{1}H NMR Measurements for Quantification of Lactose and Fat in Milk. Milk was introduced into a 5 mm tube with a capillary tube for a concentration standard. The ^{1}H NMR experiments were performed on a JEOL JNM-α 500 NMR spectrometer at 20 °C. The signal of H_2O was suppressed by the presaturation method. H_2O was used as an internal reference of chemical shift (δ), the value of which was 4.65 ppm. The number of data points was 16k, the acquisition time was 3.28 s, and the number of scans was 16 (*18*). To quantify the constituents of milk and lactose solution, the delay time was determined by the partial relaxation Fourier transform (FT) method.

Determination of Delay Time by the Partial Relaxation FT Method. Delay time (d1) greatly affects the total NMR measuring time as well as the accuracy of quantification. For quantitative measurements, d1 is generally evaluated according to eqs 1 and 2, where T1 is the longitudinal relaxation time, aq is the acquisition time, and T_{null} is the pulse interval time (d2) when the observed NMR signals are null with the pulse sequence shown in **Figure 1** (*18*).

$$d1 \gtrsim 5 \times T1 - aq \quad (1)$$

$$T1 = 1.44 \times T_{null} \quad (2)$$

The Standard Used in Quantitative NMR Experiments. When measuring the NMR spectra of milk, the capillary containing $CHCl_2$-$CHCl_2$ was inserted into NMR sample tubes as the concentration standard. The signal area of $CHCl_2CHCl_2$ was measured and compared with those of the compounds in milk to determine the absolute concentrations of those compounds. The area of the $CHCl_2CHCl_2$ signal was standardized to represent the area of the signals from the milk compounds.

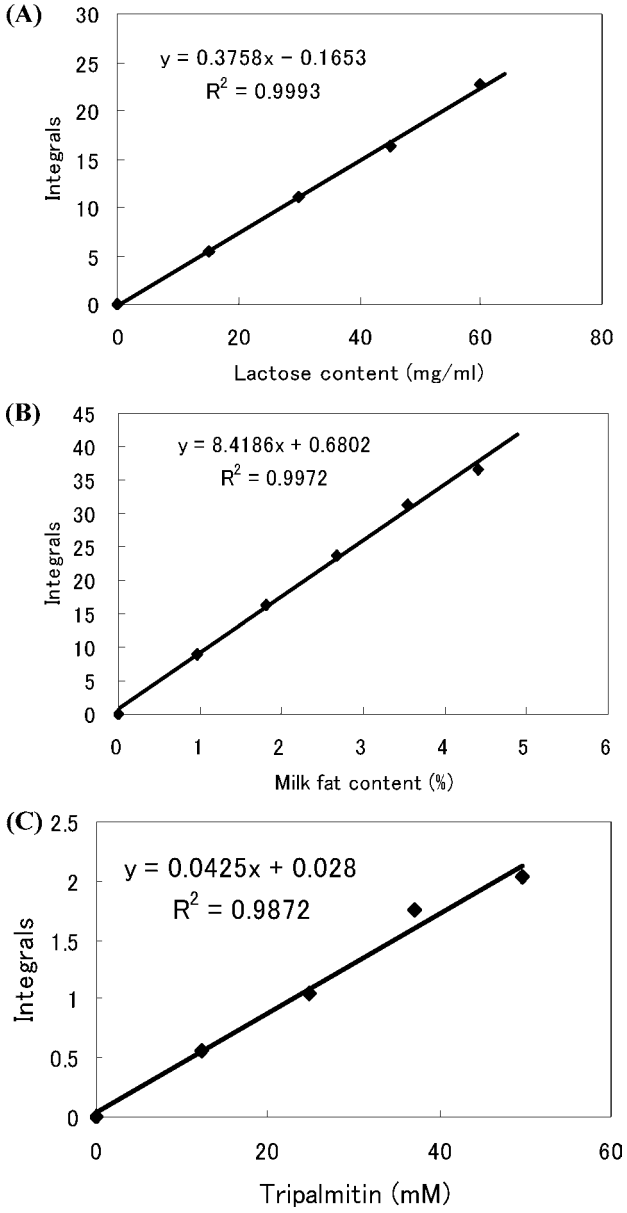

Figure 3. The working curves of (**A**) lactose, (**B**) milk fat, and (**C**) Gl-1, 3.

So it could be used as a concentration standard, the null signal interval time (T_{null}) of $CHCl_2CHCl_2$ in $CDCl_3$ was measured using the partial relaxation FT method (**Figure 1**). T_{null} of $CHCl_2CHCl_2$ in $CDCl_3$ was 1.4 s. When an NMR relaxation reagent $Cr(AcAc)_3$ was added to a concentration of 1 mg/mL, T_{null} of $CHCl_2CHCl_2$ was reduced to 0.28 s. Therefore, a chloroform-*d* ($CDCl_3$) solution of 20% (v/v) $CHCl_2$-$CHCl_2$ containing 1 mg/mL $Cr(AcAc)_3$ placed in a capillary tube was used as a concentration standard.

^{1}H−^{13}C HSQC NMR Measurements for Quantification of Milk Components Other than Lactose and Fat. The quantitative ^{1}H−^{13}C HSQC experiments were carried out in the phase-sensitive mode on a Varian Unity INOVA 500 NMR spectrometer equipped with a Narolac *z*-axis gradient probe at 20 °C. The 2D NMR signals measured were processed using NMRPipe (*19*), and integral volumes were calculated using Sparky (*20*) by Lorentzian fit. The acquisition parameters were as follows: number of data points, 1024 for ^{1}H and 256 for ^{13}C; spectral width, 8000 Hz (^{1}H) and 20169 Hz (^{13}C); digital resolution, 7.81 Hz in F2 (^{1}H) and 78.79 Hz in F1 (^{13}C); and an acquisition time of 0.128 s. For quantification, the delay time (d1) was determined by the partial relaxation FT method, and $CHCl_2CHCl_2$ in a capillary was used as an external concentration standard.

Statistics. All measurements were performed in triplicate ($n = 3$), and data were reported as mean ± SD.

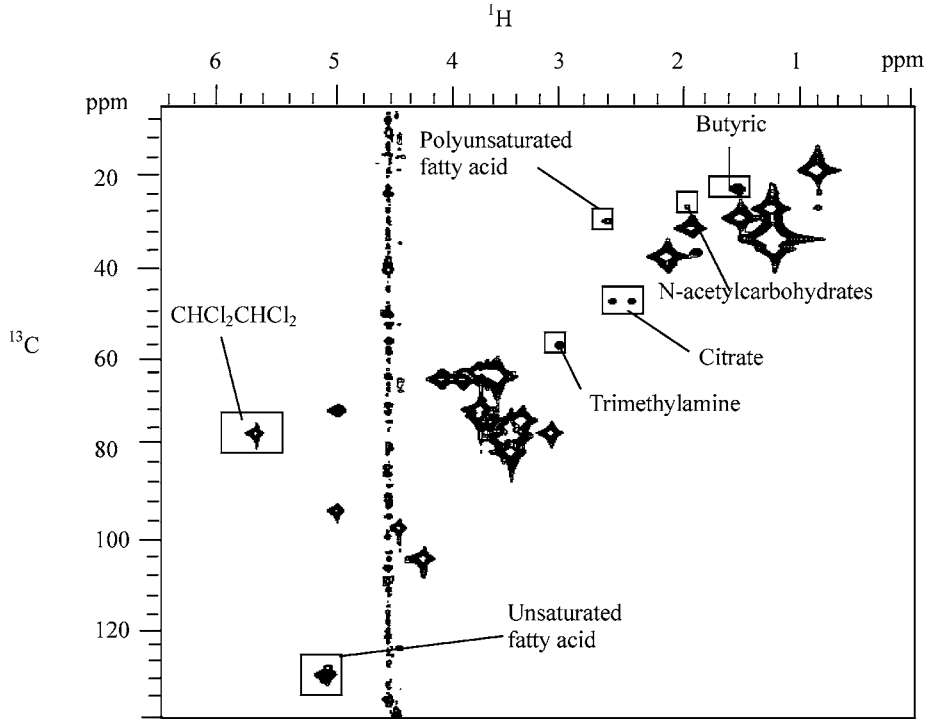

Figure 4. Quantitative ^{1}H–^{13}C HSQC spectrum of commercial milk. The assignments of signals are reported (*17*).

RESULTS AND DISCUSSION

Quantitative Determination of Lactose and Fat in Milk by ^{1}H NMR Spectroscopy. We measured ^{1}H NMR spectra of milk to determine the quantity of lactose, triacylglycerols, and fat without any additive to conveniently calculate the concentration. The ^{1}H signals selected for this study were in the ranges of $\delta = 0.78-2.12$ ppm and $\delta = 3.40-3.90$ ppm, which were due to acyl chains of triacylglycerols and lactose, respectively (**Figure 2**).

To determine the quantity of lactose, we first measured ^{1}H NMR of lactose dissolved in D_2O at concentrations of 15, 30, 45, and 60 mg/mL for creating the working curve of lactose. T_{null} of lactose in D_2O was determined as 1.5 s by using the partial relaxation method, and the delay time of the lactose D_2O solutions was set to be 5.0 s. By normalizing the integral of the ^{1}H signal due to $CHCl_2CHCl_2$ (defined as 1.00), the integrals of the ^{1}H NMR signals due to lactose were calculated to create the working curve of lactose (**Figure 3A**). The commercial whole milk was then measured with ^{1}H NMR, which gave the lactose content in commercial whole milk by comparison of their integrals with the working curve. Thus, lactose content was determined to be 47.8 ± 1.0 mg/mL. This value agrees well with literature values (*1*).

In the case of fat, we prepared milk samples containing various fat concentrations from 0.96 to 4.4% by mixing nonfat milk (0.1%) and high-fat milk (4.4%), measured ^{1}H NMR with a delay time of 2.0 s, and created the working curve of acyl chains to determine the fat content of commercial whole milk (**Figure 3B**). Thus, the fat content of commercial ordinary whole milk was determined to be 3.6 ± 0.1%. The value agreed well with that determined by the milk company using Rose-Gottlieb method (*21*). The working curve of different concentrations of tripalmitin was created, with the absolute average molar concentration (mM) of total triacylglycerols in milk being determined in the same way as that of lactose (**Figure 3C**). The lactose and fat contents in milk are the main parameters during quality control of milk in the dairy industry. Our experiments indicate that ^{1}H NMR spectroscopy can accurately and easily detect the main compounds in milk within just a few minutes.

^{1}H−^{13}C HSQC NMR Spectra of Milk. The ^{1}H NMR spectrum of milk was very crowded, and many signals overlapped. To quantify more compounds in milk, our strategy was to apply 2D ^{1}H−^{13}C HSQC spectra (**Figure 4**), which has never before been tested. During the quantitative 2D NMR experiments, 10% D_2O was added to milk, which helped us to easily adjust the NMR lock and shim system and to obtain good ^{1}H−^{13}C HSQC spectra (*17*). Considering the addition of D_2O to milk, concentrations of milk compounds were compensated during the data processing.

Because ^{1}H−^{13}C 2D NMR spectra have never been applied to quantification of milk constituents, we had to verify whether volumes of 2D signals could be proportional to the concentration of the corresponding molecule. Since the delay times of standard chemicals for quantification were quite long for the 2D measurements, 1 mg/mL $Cr(AcAc)_3$ was added to the solution, which made the delay time (T_{null}) shorter. For example, T_{null} of triolein dissolved in $CDCl_3$ was 1.1 s, while by adding 1 mg/mL $Cr(AcAc)_3$, T_{null} was reduced to 0.5 s. As a result, we successfully shortened the delay time and set it to 2.0 s for the ^{1}H−^{13}C HSQC experiments.

We measured the ^{1}H−^{13}C HSQC spectra of standard chemicals such as sodium citrate, acetylglucosamine, tributyrin, triolein, trilinolein, and lecithin, and we plotted their working curves of known concentrations (**Figure 5**). The obtained working curves successfully indicated that the volumes of ^{1}H−^{13}C HSQC signals are perfectly proportional to the concentrations, although the sensitivity enhancement mode of HSQC measurements could not be applied for the quantification. As a result, ^{1}H−^{13}C HSQC can be easily employed for quantification of milk compounds.

The concentrations of citrate, *N*-acetylcarbohydrates, trimethylamine, butyric, total monounsaturated fatty acids, and total polyunsaturated fatty acids in milk are summarized in **Tables**

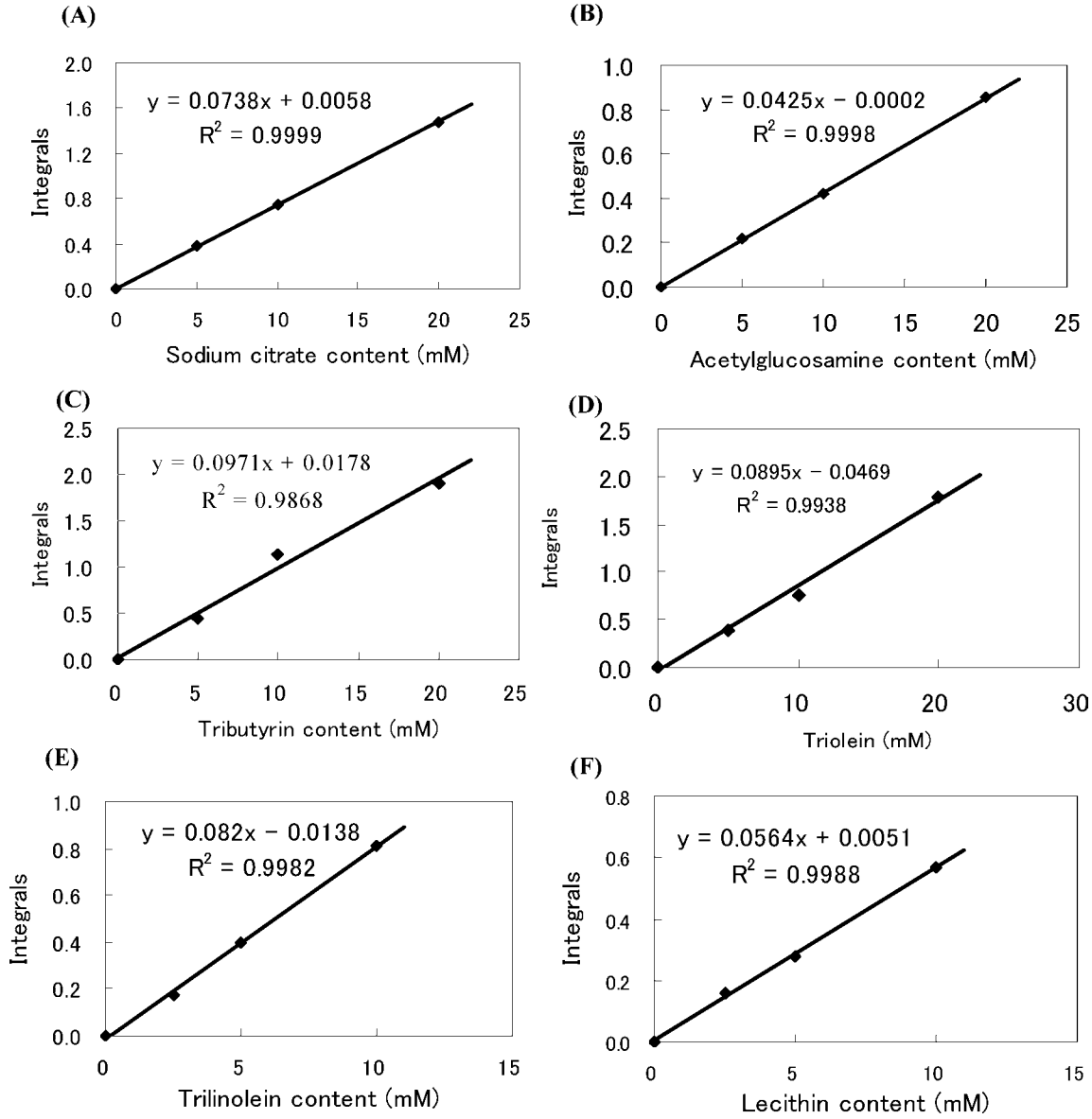

Figure 5. The working curves of (**A**) sodium citrate, (**B**) acetylglucosamine, (**C**) tributyrin, (**D**) triolein, (**E**) trilinolein, and (**F**) lecithin.

Table 1. Concentrations of Compounds Contained in Commercial Milk

compound	chemical shift (δ ppm) used for quantification ^{1}H	^{13}C	concentration	reference values
total fats	0.8–2.1		3.6 ± 0.1%	3.6%[a]
D-lactose	3.4–3.9		47.8 ± 1.0 mg/mL	42∼48 mg/mL[b]
citrate	2.4, 2.6	45.2	3.2 ± 0.2 mM[c] (0.9 ± 0.1 mg/mL)	∼2 mg/mL[b]
N-acetylcarbohydrates	2.0	23.1	2.9 ± 0.1 mM	not reported
trimethylamine	3.1	54.9	4.0 ± 0.6 mM	not reported

[a] The value reported by milk company. [b] The values adapted from Kaminogawa et al. (*1*). [c] The whole citrate exists as a sodium salt.

Table 2. Concentrations of Fatty Acids of Triacylglycerols Contained in Commercial Milk

compound	chemical shift (δ ppm) used for quantific ation ^{1}H	^{13}C	concentration	composition (% mol)	composition[a] (% mol)
total fatty acids	4.0, 4.2		35.9 ± 0.7 mM		
butyric acid	1.5	18.8	6.2 ± 0.5 mM	17.2	11.8
total monounsaturated fatty acids	5.2	130.2	9.1 ± 0.9 mM	25.2	26.6
total polyunsaturated fatty acids	2.7	25.5	2.9 ± 0.3 mM	7.9	2.5
total saturated fatty acids			∼24.0 mM	66.8	70.1

[a] Reference values reported by Christie and Clapperton using gas chromatography.[22]

1 and **2**. Citrate provides the weak acidic flavor of milk. Its concentration was 3.2 ± 0.2 mM. *N*-Acetylcarbohydrates act as the stimulant of bifidus bacteria, and their concentrations were 2.9 ± 0.1 mM. Studies of *N*-acetylcarbohydrates in commercial milk have rarely been reported because of its extremely low concentration. In this study, we quantified *N*-acetylcarbohydrates in commercial milk for the first time. Considering the various functions of *N*-acetylcarbohydrates with regard to human health, the quantification of *N*-acetylcarbohydrates in commercial milk is important to evaluating the nutrition of milk. Many milk

compounds such as lecithin, sphingomyelin, glycerophosphocholine, or other similar compounds contain a trimethylamine group, and its content was determined to be 4.0 ± 0.6 mM. Among the short-chain fatty acids of triacylglycerols, butyric acid has a high-level content (6.2 ± 0.5 mM). As for the unsaturated fatty acids of triacylglycerols, a high-level content of monounsaturated fatty acids (9.1 ± 0.9 mM) was determined, while the content of polyunsaturated fatty acids was very low (2.9 ± 0.3 mM). Saturated fatty acid concentrations were determined to be approximately 24.0 mM. The fatty acid composition (mol %) of triacylglycerols was then calculated and compared to the reference values in **Table 2**. Compared to gas chromatography data (*22*), the fatty acid content determined by $^{1}H-^{13}C$ HSQC 2D NMR spectra seems to have the same tendency. In contrast, the unsaturated fatty acid content determined by NMR spectroscopy is higher than that obtained by gas chromatography. This difference likely has two explanations: first, the milk compounds were quantified by NMR spectra without any pretreatment, while the gas chromatography data were obtained after the abstraction of milk fats; second, the contents of milk compounds usually fluctuate because of many factors.

To increase the quantitative accuracy of milk compounds using two-dimensional NMR spectra, all of the samples were applied for NMR measurements in the same way three times, and every NMR spectrum was used for the quantitative experiment alone. Concentrations of all compounds were the average values of the parallel experiments and their standard variations were calculated, which indicated that this new quantitative method is reproducible and precise.

In conclusion, the present results indicate that ^{1}H 1D and $^{1}H-^{13}C$ HSQC 2D NMR spectra are efficient for determining the concentrations of various organic compounds in milk. In particular, $^{1}H-^{13}C$ HSQC 2D NMR spectra were first applied to the quantification of milk components even at low concentrations. NMR will likely be widely applied to quality control and constituent analysis in various fields of food industry.

ABBREVIATIONS USED

FID, free induction decay; GC, gas chromatography; Gl-1, 3, glycerol-1, 3; HPLC, high-performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; PFG-HSQC, pulse field gradient-heteronuclear single quantum coherence; TLC, thin layer chromatography.

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