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Nondestructive Observation of Bovine Milk by NMR Spectroscopy: Analysis of Existing States of Compounds and Detection of New Compounds

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In this study were successfully observed the one- (^{1}H, ^{13}C) and two-dimensional (^{1}H−^{13}C, ^{1}H−^{15}N, ^{1}H−^{31}P) NMR spectra of milk directly without any pretreatment. The signals of each NMR spectrum were assigned, and their existing states were also analyzed. Lactose existed in a free state in milk. The signals due to the butyric acid chain can be assigned among the other fatty acid chains. Monounsaturated fatty acid (oleic acid chains) and polyunsaturated fatty acid chains (linoleic and linolenic acid) were assigned by their characteristic signals. The signals from citrate, *N*-acetylcarbohydrates, and lecithin could be observed directly in the ^{1}H−^{13}C HSQC NMR spectra; the assignment of their signals was made through the ^{1}H−^{13}C, ^{1}H−^{15}N, and ^{1}H−^{31}P HMBC spectra of extracted milk. Signals from creatine and *N*-acetylcarbohydrates were detected for the first time.

KEYWORDS: ^{1}H NMR; ^{13}C NMR; ^{31}P NMR; 2D-NMR; milk; saturated fatty acid chain; unsaturated fatty acid chain; creatine; *N*-acetylcarbohydrates; lecithin

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy has been widely applied in organic chemistry and biochemistry to identify organic compounds and to structurally analyze biopolymers (*1*). Recently, NMR spectroscopy has been shown to be a very effective and versatile tool for food scientists because it is nondestructive, selective, and capable of simultaneous detection of compounds in complex mixtures (*2*). NMR spectroscopy has, therefore, been applied to the analysis of oils, juice, drugs, and coffee (*3*−*5*). In the observations that have been conducted, NMR experiments have been made under "no water" conditions. NMR experiments have always required the removal of H_2O and the dissolution in D_2O. This may cause the loss of volatile or low molecular weight substances.

Other analytical techniques such as chromatography and mass spectrometry have been successfully applied to milk (*6*). All of these methods require specific extractions from milk, which cause problems such as the loss or change of volatile or sensitive compounds and the denaturation of proteins, as well as measurements that require a long time to complete and are complicated to perform. ^{1}H NMR spectroscopy has not been applied directly to milk for two reasons: Milk is overwhelmingly made up of water, which means that the signal from water is so large that it possibly overlaps most of the signals in the ^{1}H NMR spectra from other components; milk is a complicated emulsion, and therefore it is difficult to obtain a good, sensitive, NMR spectrum. As a result, although there have been several reports on the NMR measurements of milk (*7*−*12*), almost all of the NMR experiments that have been conducted to date have been made by ^{13}C and ^{31}P NMR and required pretreatment of the milk, which consisted of the extraction of triacylglycerols, removal of fat and metal ions, or adjustment of the pH (*7*−*9*, *11*, *12*). The ^{1}H NMR spectrum of milk has been obtained with the spin−echo pulse sequence, where 0.2 mM $MnCl_2$ was added to milk and the signals were not assigned (*10*). Recently, with the development of NMR machines and the improvement in measurement techniques, it has become possible to apply ^{1}H and ^{13}C one- (1D) and two-dimensional (2D) NMR spectra directly to milk without any additive or pretreatment.

In this study we tested the potential of NMR spectroscopy as a tool for the analysis of milk and investigated the number of constituents that could be observed in the NMR spectra of milk. Milk was analyzed through 1D and 2D NMR experiments. This is the first report on the observation of the NMR spectra of milk without any pretreatment.

MATERIALS AND METHODS

Materials and Sample Preparation. D_2O (99.7%) was purchased from Shoko Co. Ltd. (Tokyo, Japan) and $CDCl_3$ (99%) from Isotec Inc. (Tokyo, Japan). Ultrahigh-temperature pasteurized, homogenized whole milk was purchased at a local supermarket. For most of the measurements, 0.1 mL of D_2O was added to 0.9 mL of milk, to make it easier to adjust the lock and shims system so that better spectra could be easily obtained. The sample was then placed in a 5 mm NMR tube. The volume of the sample was ∼0.65 mL. Whole milk without any

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additive was also examined by NMR to confirm that D_2O has no effect on the ^1H and ^{13}C NMR spectra of milk.

Two-dimensional HMBC NMR spectra of extracted milk were measured to assign minor signals of whole milk. Skim milk was obtained by removing the fatty acid layer after centrifugation three times at 6000 rpm for 30 min (*13*). Then 2-fold volumes of ethanol (99.5%) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), were added to skim milk to denature proteins, and after the mixture was shaken by vortex for 5 min, it was centrifuged for 30 min at 15000 rpm. Then the supernatant was vacuum-dried and dissolved in D_2O (99.7%). With these treatments, most of the fats and proteins were removed from milk. The extracted milk prepared in this way was used for the 2D HMBC NMR experiments.

NMR Spectroscopy. All of the NMR experiments were made at room temperature on a JEOL JNM-α 500 NMR spectrometer. The ^1H NMR spectra of whole milk were measured at 500 MHz. The signal of H_2O was suppressed by the presaturation method. The H_2O was used as an external reference, and its chemical shift was 4.65 ppm. The number of data points was 16K; the acquisition time was 3.28 s, the delay time, 2.0 s, and the number of scans, 16. The ^{13}C NMR spectra of whole milk were recorded at 125.65 MHz. Dioxane was used as an external reference, and its chemical shift was 67.4 ppm. The number of data points was 16K; an acquisition time of 0.54 s and a delay time of 2.0 s gave a total repetition time of 2.54 s, and 1000 scans were accumulated with proton decoupling.

The $^1H-^{13}C$ PFG-HSQC spectra of whole milk were performed with the phase sensitive mode. The acquisition parameters were as follows: number of data points, 1024 for ^1H and 256 for ^{13}C; spectral width, 8000 Hz (^1H) and 20169 Hz (^{13}C); digital resolution, 7.81 Hz in F2 (^1H) and 78.79 Hz in F1 (^{13}C); acquisition time, 0.128 s; delay time, 1.5 s; number of scans, 64; number of dummy scans, 16. For the 2D HMBC NMR experiments, the total measurement time required for the $^1H-^{13}C$ operation was 7.5 h.

The $^1H-^{13}C$ FG-HMBC spectra of extracted milk were acquired with the absolute mode. The acquisition parameters were as follows: number of scans, 64; dummy scans, 16; number of data points, 512 in F2 (^1H) and 512 in F1 (^{13}C); spectral width, 3016.59 Hz in F2 (^1H) and 25113.01 Hz in F1 (^{13}C); digital resolution, 5.89 Hz in F2 and 49.05 Hz in F1; acquisition time, 0.1697 s; delay time, 1.8 s; HMBC delay time, 60 ms.

The $^1H-^{31}P$ FG-HMBC spectra of extracted milk were performed with the absolute mode. Potassium phosphate was used as an external reference, and its chemical shift of ^{31}P was 0 ppm. The acquisition parameters were as follows: number of scans, 16; number of dummy scans, 2; number of data points, 512 in F2 (^1H) and 512 in F1 (^{31}P); spectral width, 2514.46 Hz in F2 (^1H) and 10000 Hz in F1 (^{31}P); digital resolution, 4.91 Hz in F2 and 19.53 Hz in F1; acquisition time, 0.2036 s; delay time, 1.4 s; HMBC delay time, 60 ms.

The $^1H-^{15}N$ FG-HMBC spectra of extracted milk were acquired with the absolute mode. Ammonia was used as an external reference, and its chemical shift of ^{15}N was 0 ppm. The acquisition parameters were as follows: number of scans, 256; number of dummy scans, 16; number of data points, 512 in F2 (^1H) and 256 in F1 (^{15}N); spectral width, 3077.87 Hz in F2 (^1H) and 20408.16 Hz in F1 (^{15}N); digital resolution, 6.01 Hz in F2 and 79.72 Hz in F1; acquisition time, 0.1663 s; delay time, 1.8 s; HMBC delay time, 60 ms.

Assignment of NMR Signals. At first, the NMR spectra of milk were analyzed by referring to the published data of chemical shifts for most compounds of milk (*6*, *14*, *15*). The ^1H and ^{13}C signals of lactose and fats were assigned in such a way. Although the chemical shifts of ^1H and ^{13}C signals of whole milk are a little different from published data, due to the different solvent conditions, the correlations in 2D NMR spectra would not change. We, therefore, assigned signals in 1D ^1H and ^{13}C NMR spectra tentatively and then demonstrated the accuracy of assignments in various kinds of 2D spectra. The signals due to trace compounds could be also detected in the 2D NMR spectra. To assign the signals of trace compounds in whole milk, we used the correlations of signals in 2D HMBC spectra. Finally, the assignments were confirmed by adding authentic compounds to whole milk.

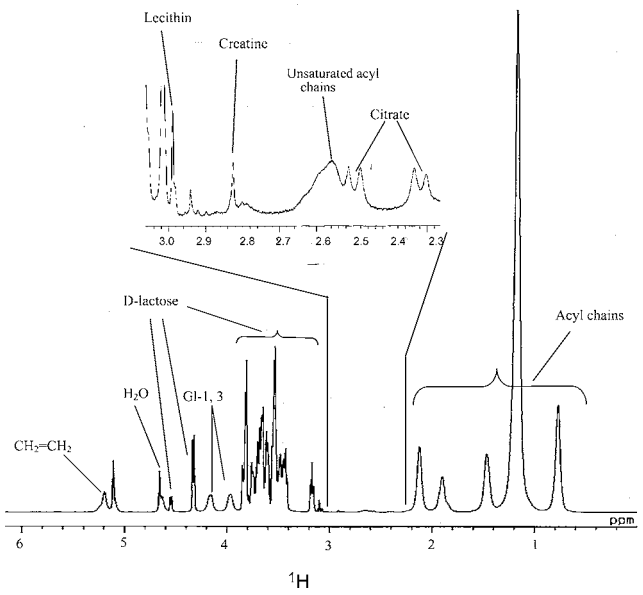

Figure 1. ^1H NMR spectrum of milk.

RESULTS AND DISCUSSION

The ^1H and ^{13}C NMR spectra of milk were obtained both in the absence and in the presence of 10% D_2O, which indicated no effect on the NMR spectra from the addition of D_2O (data not shown). However, the addition of 10% D_2O to the milk made it easier to adjust the lock and shims system during the NMR experiments. As a result, a small amount (10%) of D_2O was added to the milk for the NMR measurements. The ^1H and ^{13}C NMR spectra are shown in **Figures 1** and **2**, respectively.

^1H NMR Spectrum of Whole Milk. The ^1H NMR spectrum of milk is shown in **Figure 1**. Note that the signal caused by H_2O at 4.65 ppm was successfully suppressed. Three main regions of the ^1H NMR spectrum were characterized. A high-field (low-frequency) region between 0.6 and 2.2 ppm was assigned to the signals due to acyl chains of milk fats, and a mid-low-field region between 3.1 and 5.1 ppm was assigned to the signals due to lactose, on the basis of the contents and their chemical shifts. Attention should also be paid to the region of weak signals between 2.2 and 3.1 ppm, because the signals at this region are due to trace compounds of milk. This region was enlarged as the subspectrum in **Figure 1**. The ^1H signals of glycerol were observed at 3.98 and 4.18 ppm, and those of the double bond due to unsaturated milk fats were at 5.20 ppm, as assigned by comparing the chemical shifts of the observed signals with those available in the references. The assignment of the ^1H NMR spectrum is summarized in **Table 1**.

Very small signals were observed in the region from 5.5 to 9.0 ppm, which may be due to the amide protons of proteins. These signals were very weak and heavily overlapped, which could be considered to be due to the following reasons: (1) The molecular weights of proteins are so large that their molar concentrations are rather low. (2) Caseins are the main milk proteins, and their signals would be very broad and extremely low in peak height because they may exist as large complexes in milk. (3) In the ^1H NMR spectrum of whole milk, the signal of H_2O is suppressed with the presaturation pulse, which would decrease the resonance intensity of the broad signals dramatically by spin diffusion while leaving unaffected the sharp signals (*16*).

In the ^1H NMR spectrum, the signals due to lactose were observed to be narrow and sensitive, whereas the signals due to milk fats were broad, because lactose is readily soluble in milk, but milk fats consist of various acyl chains and are

Table 1. Assignment of ^{1}H and ^{13}C NMR Signals of Compounds in Milk

compound	chemical shift (δ) ^{1}H	^{13}C	assignment[a]
D-lactose	3.69–3.82	60.83	CH_2OH-α6
	3.69–3.83	60.94	CH_2OH-β6
	3.64	61.92	CH_2OH-6′
	3.80	69.44	CH-4′
	3.81	70.90	CH-2
	3.43	71.84	CH-2′
	3.52	72.06	CH-3
	3.71	72.28	CH-5
	3.53	73.43	CH-3′
	3.15	74.74	CH-2
	3.51	75.22	CH-3
	3.49	75.58	CH-5
	3.60	76.15	CH-5′
	3.53	79.06	CH-β4
	3.53	79.20	CH-α4
	5.03	92.68	CH-α P
	4.48	96.61	CH-β P
	4.27	103.67	CH-1′
acyl chains of fatty acids	0.78	14.63	CH_3-ω1
	1.19	23.34	CH_2-ω2
	1.47	25.45	CH_2-Δ3
	1.02–1.38	29.81–30.50	CH_2 (ω4–n)
	1.02–1.38	32.64	CH_2-ω3
	2.17	34.40	CH_2-Δ2
		172.15	COO^- (Gl-2)
		172.43	COO^- (Gl-1,3)
	0.78	14.46	butyric CH_3-ω1
	1.47	18.81	butyric CH_2-ω2
	2.17	36.10	butyric CH_2-ω3
	1.89	27.78	oleate CH_2-ω8,11
	5.20	130.20	oleate HC=CH-ω9,10
	2.68	25.52	linoleate CH_2-ω8
glycerol backbone of fats	3.98, 4.18	62.52	Gl-1, 3
	5.12	69.64	Gl-2

[a] The symbol "ω" indicates the position from the methyl group end; the symbol "Δ" indicates the position from the ester group end.

suspended in milk as fat globules. We also analyzed the ^{1}H NMR spectra of both nonhomogenized and homogenized milks. Compared with the spectrum of the homogenized milk, the signals caused by the nonhomogenized milk were broader and less sensitive (data not shown), because the nonhomogenized milk contained very large fat globules. As a result, the broadening of the signals and the sensitive level of the signals reflected the size of the fat globules in the milk. However, the ^{1}H NMR spectrum of the milk was very crowded, and many signals overlapped (for example, in the regions containing acyl chains and lactose). Furthermore, the signals between 2.2 and 3.1 ppm were so weak that they could not be assigned by the ^{1}H NMR spectra alone. For further assignment of the overlapped and weak signals, therefore, we measured the ^{13}C and ^{1}H−^{13}C HSQC spectra.

^{13}C NMR Spectrum of Whole Milk: Assignment of the Signals of Lactose and Fats. The ^{13}C NMR spectrum of milk is shown in **Figure 2**. The signals were narrow and did not overlap, which allowed us to analyze the spectrum. The details of the signal assignments are summarized in **Table 1**. The signals due to proteins were not observed in the ^{13}C NMR spectrum for the same reasons as for the ^{1}H NMR spectrum.

Lactose was assigned after being compared with the published data on chemical shifts (*14*). The assignment was confirmed by the ^{1}H NMR (**Figure 1**) and ^{1}H−^{13}C HSQC spectra (**Figure 3**) of milk. Lactose was concluded to exist in the free state in milk, because its signals in milk are not different from the standard data of the authentic lactose solution (*14*).

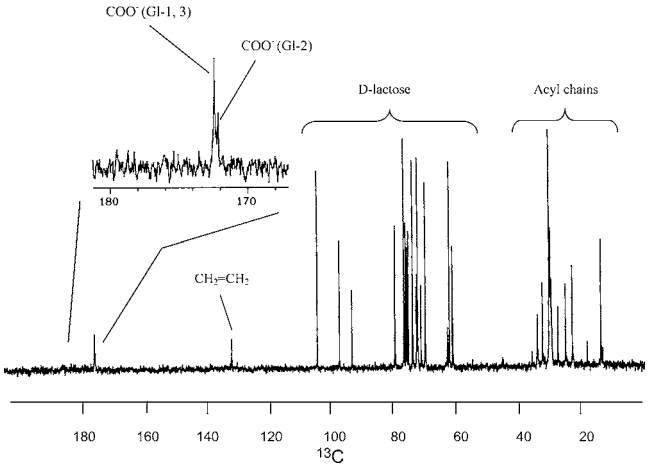

Figure 2. ^{13}C NMR spectrum of milk.

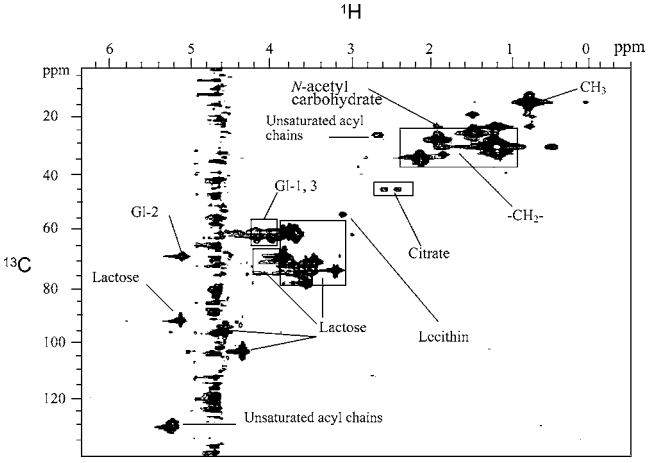

Figure 3. ^{1}H–^{13}C HSQC NMR spectrum of milk.

There are many kinds of acyl chains in triacylglycerols. The signals were assigned by the comparison of the ^{13}C NMR spectrum of milk with the published data (*14*, *15*). The signals of the ω1, ω2, and ω3 carbons of fatty acid acyl chains were observed to overlap at 14.63, 23.34, and 32.64 ppm, respectively, whereas the ω1, ω2, and ω3 carbons of the butyric group were observed at 14.46, 18.81, and 36.10 ppm, respectively. Among short-chain fatty acids, butyric acid is an important flavor component of milk. The ^{13}C signals of the other methylene groups from ω4 to ωn neighboring the carbonyl end or double bond were observed at 29.81−30.50 ppm. The ^{13}C signals at 25.45 and 34.40 ppm were, respectively, assigned to Δ3 and Δ2 of the fatty acid acyl chains. From these signals, it was not possible to distinguish whether the acyl chains came from a saturated or unsaturated fatty acid, except for the butyric group in the triacylglycerols, and the number of carbons could also not be identified. The ^{13}C signals at 130.20 and 27.78 ppm were assigned to the double-bond carbons and the methylene carbons connecting the double bonds, respectively. Because the largest quantity of unsaturated acids in milk is supplied by oleic acid (*6*), it has been suggested that these two signals are mainly contributed by oleic acid. The signal at 172.15 ppm is caused by the carbonyl group bonding to the 2-position of glycerol, and the signal at 172.43 ppm is caused by the carbonyl group bonding to the 1,3-positions of glycerol (*5*). The signals due to the carbonyl group were enlarged in the subspectrum of the ^{13}C NMR spectrum in **Figure 2**.

The signal at 62.52 ppm was assigned to the C_1 and C_3 carbons of the glycerol moiety of fats. The signal at 69.64 ppm

was assigned to the C_2 carbon of the glycerol moiety, although it overlapped with the lactose signals.

Compared with the ^{13}C NMR spectrum of triacylglycerols extracted from milk (*11*, *12*), our ^{13}C NMR spectrum of whole milk appeared to be somewhat different because fats exist in milk as the fat globules, whereas extracted fats exist in the free state in organic solvent.

$^1H-^{13}C$ HSQC Spectrum of Whole Milk. Figure 3 shows the $^1H-^{13}C$ HSQC spectrum of whole milk without extraction. The noise from H_2O was not completely suppressed, despite presaturation. The $^1H-^{13}C$ HSQC spectrum supplied useful information on the signals, due to minor constituents that overlapped in the 1D ^1H NMR spectra.

The ^1H signals at 2.2−3.1 ppm showed correlations to the ^{13}C signals at 25.0−55.0 ppm. Through consultation of the databases (*14*, *15*), the ^1H resonance at 2.68 ppm, which showed a cross-peak with the ^{13}C resonance at 25.52 ppm, was assigned to be the methylene group next to the double bond of polyunsaturated acyl chains. This signal was concluded to be caused by the linoleate and linolenate chains, because they are the main polyunsaturated fatty acids in milk. The ^1H resonances at 2.40 and 2.54 ppm are suggested to be due to 2,4-CH_2 of citric acid, because they were correlated to the ^{13}C signal at 45.15 ppm in the HSQC spectrum. The ^1H signals at 1.95 and 3.10 ppm showed correlations to the ^{13}C signals at 23.1 and 54.90 ppm, respectively. The signals supplied too little information to be assigned, so we measured the HMBC spectra of extracted milk.

$^1H-^{13}C$ HMBC Spectrum of Extracted Milk. HMBC spectroscopy is particularly useful because it connects protons with carbons via two or three bond couplings that can supply more information about connectivities. We attempted to employ HMBC experiments of market milk without any pretreatment at first, but the HMBC spectrum could not be obtained. Extracted milk was, therefore, prepared by removing water and fat.

Detection of Citrate. The $^1H-^{13}C$ HMBC spectrum of extracted milk is shown in **Figure 4A**. In the spectrum, the ^1H signals at 2.40 and 2.54 ppm showed a correlation with the ^{13}C signal at 45.15 ppm, which was also the case in the $^1H-^{13}C$ HSQC spectrum (**Figure 3**). Both of these protons also connected to the ^{13}C signals at 77.35, 179.97, and 182.50 ppm in the $^1H-^{13}C$ HMBC spectrum (**Figure 4A**). These data suggest that they are from ionized citric acid, as judged by the chemical structure of citric acid (**Figure 4B**) (*15*). Because the pH of milk was ~6.8, the ionic state of citrate could be predicted from its pK_a, and the chemical shifts of signals coincide with those reported for citrate ion. Citrate is the main organic acid in milk and provides the weak acidic flavor, as well as its heat stability in milk (*6*). It has been reported that citrate binds to calcium ion and acts as a component of casein micelles (*6*). However, from the NMR spectra, the line widths of the signals due to the citrate ion in the milk were narrow and showed little difference from those of citrate dissolved in water. Thus, we think that in milk, citrate is mobile to a considerable degree. The results of the assignment of the signals from citrate ion are summarized in **Table 2**.

Detection of Creatine. In the $^1H-^{13}C$ HMBC spectrum of extracted milk, a new ^1H signal at 2.88 ppm was observed (**Figure 4A**). This ^1H resonance was correlated to the ^{13}C signal at 55 and 158 ppm in the $^1H-^{13}C$ HMBC spectrum and to the ^{15}N signal at 78 ppm in the $^1H-^{15}N$ HMBC spectrum (**Figure 5A**). The other ^1H signal at 3.79 ppm was correlated to the ^{13}C signals at 38, 158, and 176 ppm in the $^1H-^{13}C$ HMBC spectrum.

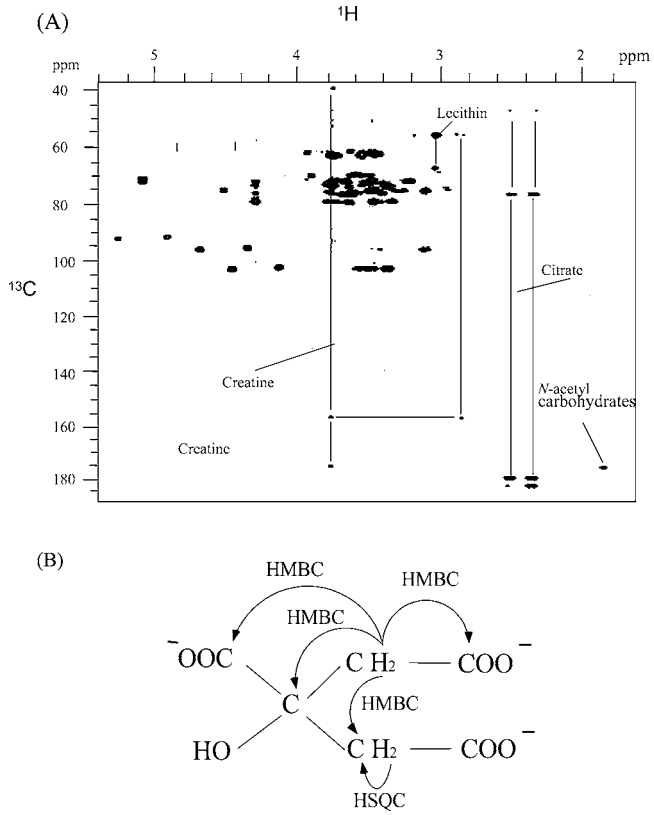

Figure 4. (**A**) $^1H-^{13}C$ HMBC NMR spectrum of the extracted milk. (**B**) Chemical structure and the expected NMR correlation of citrate. The cross-peaks can be observed among CH_2 with C and COO^- in the $^1H-^{13}C$ HMBC spectrum and between C and H of CH_2 in the $^1H-^{13}C$ HSQC spectrum.

Table 2. Assignment of NMR Signals of Citrate, Creatine, *N*-Acetylcarbohydrates, and Lecithin in Milk

	chemical shift (δ)				
compound	^1H	^{13}C	^{15}N	^{31}P	assignment
citrate	2.40, 2.54	45.15			CH_2
	2.40, 2.54	77.35			C
	2.40, 2.54	179.97			CH_2–COO^-
	2.40, 2.54	182.50			C–COO^-
creatine	2.88	55.00			CH_2
	2.88	158.00			C
	2.88		78.00		N*
	3.79	38.00			CH_3
	3.79	158.00			C
	3.79	176.00			COOH
N-acetylcarbohydrates	1.95	23.1			CH_3
	1.95	176.00	120		$NHCOCH_3$
lecithin	3.12	54.90			3Me
	3.12	67.50			CH_2-5
	4.22		46.51		CH_2-4
	3.12		46.51		3Me
	4.22			0.6	CH_2-4
	4.18			0.6	CH_2-3
	3.75, 3.83			0.6	CH_2-5

The cross-peaks in the 2D NMR spectra and the chemical shift values of the signals indicated that these signals were caused by creatine from consideration of its chemical structure (**Figure 5B**). The chemical shift values and the narrow line widths of the observed signals compared with the standard data (*15*) led us to the conclusion that creatine exists in a free state, without any interaction with other compounds in milk. The assignments that were obtained are listed in **Table 2**.

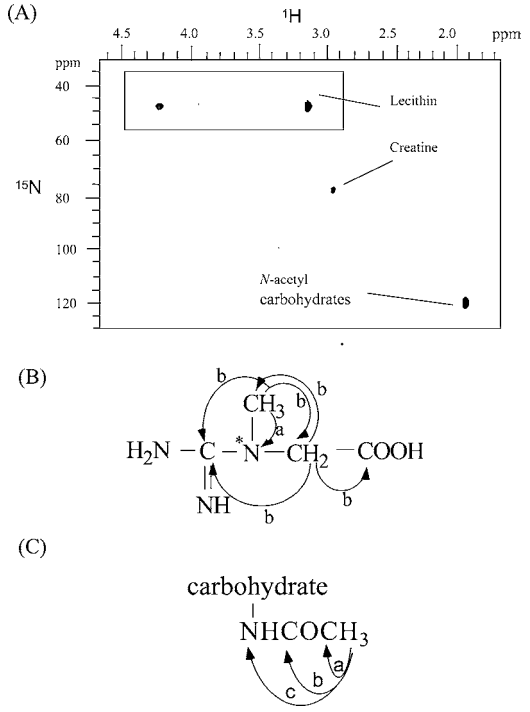

Figure 5. (**A**) ^{1}H–^{15}N HMBC NMR spectrum of the extracted milk. (**B**) Creatine correlations; "a" and "b" indicate the possible correlations in the ^{1}H–^{15}N HMBC and ^{1}H–^{13}C HMBC spectra, respectively. (**C**) *N*-Acetylcarbohydrate correlations; "a", "b", and "c" indicate the possible correlations in the ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC, and ^{1}H–^{15}N HMBC spectra, respectively.

Detection of N-Acetylcarbohydrates. In the ^{1}H−^{13}C HSQC spectrum of milk (**Figure 3**), the ^{1}H signal at 1.95 ppm, which was overlapped with the signals from fatty acid acyl chains in the 1D ^{1}H NMR spectrum, showed a correlation to the ^{13}C signal at 23.1 ppm. This ^{1}H signal was also correlated to the ^{13}C signal at 176 ppm in the ^{1}H−^{13}C HMBC spectrum (**Figure 4A**) and correlated to the ^{15}N signal at 120 ppm in the ^{1}H−^{15}N HMBC spectrum of the extracted milk (**Figure 5A**). After consultation of some databases (*15*, *17*), it was concluded that these signals were due to the acetamido group of *N*-acetylcarbohydrates (**Figure 5C**). To confirm this, a small amount of *N*-acetylcarbohydrate (*N*-acetyllactosamine or *N*-acetylglucosamine) was added to the sample of extracted milk, and then its ^{1}H−^{13}C HMBC spectrum was observed. In the spectrum, the cross-peak between 1.95 ppm in ^{1}H and 176 ppm in ^{13}C became larger, but no new signal appeared. Thus, it was confirmed that *N*-acetylcarbohydrates are contained in milk. *N*-Acetylcarbohydrates are very important because they stimulate the production of bifidus bacteria, which enhances intestinal function (*18*). To our knowledge, this is the first time *N*-acetylcarbohydrates have been detected in commercial milk. The assignment is summarized in **Table 2**.

Detection of Lecithin. The ^{1}H signal at 3.10 ppm showed a correlation to the ^{13}C signal at 54.90 ppm in the HSQC spectrum of milk and also showed a correlation to the same ^{13}C signal in the HMBC spectrum of the extracted milk. This proton was also correlated to the ^{13}C signal at 67.50 ppm in the ^{1}H−^{13}C HMBC spectrum. These observations supposed that the signals were due to the trimethylamine group (*15*). This hypothesis was confirmed by the ^{1}H−^{15}N HMBC spectrum (**Figure 5A**), which showed that the ^{1}H signal at 3.12 ppm due to the methyl group and that at 4.22 ppm due to the methylene group were coupled to the ^{15}N signal at 46.51 ppm. A previous paper stated that

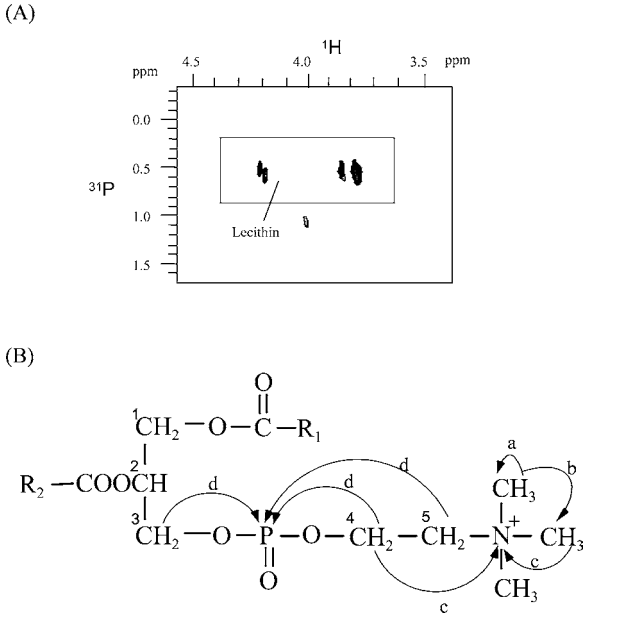

Figure 6. (**A**) ^{1}H–^{31}P HMBC NMR spectrum of the extracted milk. (**B**) Lecithin correlations; "a", "b", "c", and "d" indicate the possible correlations in the ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC, ^{1}H–^{15}N HMBC, and ^{1}H–^{31}P HMBC spectra, respectively.

trimethylamine was detectable in milk but that the quantity of trimethylamine was very small and even showed undetectable levels in half-numbers of milk (*19*). The signals from trimethylamine could not be observed in the milk we analyzed. In milk, the trimethylamine group mainly exists in lecithin (*6*). The ^{1}H−^{31}P HMBC spectrum (**Figure 6A**) supported the hypothesis that we observed the trimethylamine group of lecithin, because the proton resonance at 4.22 ppm showed a cross-peak with the ^{31}P resonance at 0.60 ppm (**Table 2**). The assignment of lecithin was done by detecting the cross-peaks in the ^{1}H−^{13}C HSQC, ^{1}H−^{13}C HMBC, ^{1}H−^{15}N HMBC, and ^{1}H−^{31}P HMBC spectra, as shown in **Figure 6B**. The signals may be due to glycerophosphocholine and some other similar compounds.

In conclusion, ^{1}H, ^{13}C, and ^{1}H−^{13}C HSQC NMR spectra of whole milk could be observed successfully without any additive or pretreatment. We confirmed that 10% D_2O added to milk increases the resolution and S/N ratio of NMR signals more easily. The 2D HMBC spectra of extracted milk have been performed to assign signals. During the analysis of milk, we have found the convenience, sensitivity, and efficiency of NMR spectroscopy. Using NMR, we may distinguish the milk of different kinds of cows and other animals. The quantitative analysis of various compounds of milk would also be feasible. This technology is considered to be applicable to quality control or specification of other types of mixtures such as juice, serum, and urine.

ABBREVIATIONS USED

FG-HMBC, heteronuclear multiple-bond correlation with field gradient; FID, free induction decay; NMR, nuclear magnetic resonance; PFG-HSQC, heteronuclear single-quantum coherence with pulse field gradient; 1D, one-dimensional; 2D, two-dimensional.

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