NMR-Based Metabolic Profiling of Rice Wines by F_2-Selective Total Correlation Spectra

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ABSTRACT: In this study, we performed NMR-based metabolic profiling of major rice wines (Japanese sake, Chinese Shaoxing wine, and Korean makgeolli). In the ^{1}H NMR spectra, the rice wines showed broad resonances in the region of about 7.9−9.0 ppm. These resonances showed many and complex correlations with approximately 0.5−4.5 ppm in the F_2-selective TOCSY (total correlation spectroscopy) spectra, and these correlations were attributed mainly to peptides. These spectral patterns were characteristic of individual rice wines, and the combination of F_2-selective TOCSY spectra and principal component analysis enabled us to classify the rice wine species. Furthermore, it also provided information about raw materials, namely, what type of koji (rice koji or wheat koji) was used. These spectra may be useful as a new "fingerprint" for quality control or food authentication.

KEYWORDS: NMR, TOCSY, selective excitation, metabolic profiling, rice wine, peptide

■ INTRODUCTION

The metabolic analysis of foods has been widely applied to food science. Since the metabolic components of foods are affected by growth conditions and food processing, metabolic analysis has been used to assess food authenticity (such as country of origin, botanical origin, and adulteration) and is reported to be a useful tool for food science.[1,2] NMR has two primary advantages: (i) sample preparation is simple and easy, which minimizes changes in chemical composition and the loss of minor components during sample preparation, and (ii) various organic chemical species (e.g., sugars, lipids, amino acids, and organic acids) are detected simultaneously. Moreover, NMR spectra may provide information about the interaction between molecules.[3] Therefore, many NMR-based metabolic analyses have been performed.[4−16]

Minor components in a food frequently play an important role in its characteristics. However, many foods contain dominant major components (e.g., sugars, lipids, ethanol, and acetic acid). The signals of these major components may make it difficult to detect the minor components due to the problem of dynamic range. One of the most efficient approaches to solving the problem is band-selective excitation to remove strong signals, allowing the detection of minor components without separation.[17] In a previous study, we developed F_2-selective 2D NMR spectroscopy, which excites a limited range of the spectrum along the F_2 axis, and which can provide high-quality 2D NMR spectra of minor components in foods.[18] Suppression methods such as WET[19,20] (water suppression enhanced through the T_1 effect) are also effective for detecting minor components in food without separation.[21,22]

Fermentation is one of the most important processes in the food industry, and various fermented foods are produced all over the world. Thus far, various fermented foods, such as wine,[11] beer,[12] vinegar,[13] cheese,[14,15] and soy sauce,[16] have been subjected to NMR-based metabolic analysis for establishing food authenticity and examining the time-dependent

composition changes during fermentation or aging. These fermented foods contain a dominant component, such as ethanol, acetic acid, and lipids; therefore, band-selective excitation is considered a useful tool for the analysis of minor components in fermented foods. However, metabolic analysis with band-selective excitation has not been thoroughly investigated.

Rice wines are fermented alcohol beverages made from rice and are mainly manufactured in Asian countries. In the brewing process of rice wine, rice starch is converted to glucose by koji, a grain (e.g., rice, wheat) cultivated with mold (e.g., *Aspergillus oryzae*), and glucose is converted to ethanol by yeast.

In this study, we measured low-field region (6.0−10.5 ppm) NMR spectra of rice wines with band-selective excitation to analyze their minor components. F_2-selective 2D NMR spectroscopy provided high-quality 2D NMR spectra of minor components, including exchangeable amine protons and various minor metabolites that were identified. The combination of the F_2-selective total correlation spectroscopy (TOCSY) spectra of the peptide region (F_2 axis, 7.9−9.0 ppm; F_1 axis, 0.5−4.5 ppm) with an unsupervised principal component analysis (PCA) provides a good model for classifying rice wines.

■ MATERIALS AND METHODS

Materials and Sample Preparation. All rice wine samples (seven samples of Japanese sake, three samples of Chinese Shaoxing wine, and six samples of Korean makgeolli) were of different brands and were obtained from a local market. These samples were stored at 4 °C until NMR analysis. After 50 mL of a sample was transferred into a 50 mL glass beaker, the pH value was adjusted to 4.5 using 6 M HCl or 4 M NaOH. To remove solid content, Korean makgeolli was centrifuged at

12 000 rpm for 5 min at 4 °C, and the supernatant was used for the NMR analysis. For the NMR analysis, D_2O (99.9%, 70 μL; Shoko Co., Ltd., Tokyo, Japan), containing 2 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS; Wako Pure Chemical Industry, Ltd., Osaka, Japan) as a chemical shift reference, was added to the sample (630 μL). The mixture (700 μL) was then transferred to a 5 mm NMR tube.

In the measurement of a free amino acid solution, the sample composition was as follows: 100 mM amino acid (glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, glutamic acid, asparagine, glutamine, lysine, arginine, histidine, phenylalanine, tyrosine, methionine, cysteine, tryptophan, proline, γ-aminobutyric acid (GABA), ornithine, and citrulline; Wako Pure Chemical Industry), 15% (v/v) ethanol (99.5%, Wako Pure Chemical Industry), 10% D_2O, and 0.2 mM DSS. The pH was adjusted to 4.5. For tyrosine, tryptophan, glutamic acid, and aspartic acid, the sample was centrifuged at 12 000 rpm for 5 min at 20 °C to remove the insoluble matter. These samples were transferred to a 5 mm NMR tube.

In the measurement of a tryptone solution, the sample preparation was as follows: 630 μL of 10% tryptone (Nacalai Tesque Inc., Tokyo, Japan) aqueous solution was mixed with 70 μL of D_2O (containing 2 mM DSS), and the mixture (700 μL) was transferred to a 5 mm NMR tube.

NMR Spectroscopy. All NMR spectra were obtained at 20 °C on a Varian Unity INOVA-500 spectrometer. The water signal was suppressed by presaturation during the relaxation delay.

Selective excitation was achieved using double pulsed field gradient spin echo (DPFGSE)[23] and the refocusing band-selective 180° pulse with a uniform response and phase (Re-Burp).[24] The pulse lengths of Re-Burp covering 6.0−10.5 and 6.5−10.5 ppm were 2.0 ($\gamma H_1/2\pi$ = 3.2 kHz) and 2.2 ($\gamma H_1/2\pi$ = 3.2 kHz) ms, respectively. The F_2-selective 2D NMR spectra were observed according to the method previously described,[18] with excitation along the F_2 axis. The DPFGSE is incorporated just before free induction decay (FID).

The acquisition parameters of the ^1H NMR spectra were as follows: spectral width, 8000 Hz; number of data points, 32K; number of scans, 256; acquisition time, 2.048 s; delay time, 2.0 s. The acquisition parameters of TOCSY were as follows: spectral width, 6000 Hz; number of t_2 data points, 4096; number of t_1 data points, 512; number of scans, 64; acquisition time, 0.341 s; delay time, 2.0 s; field strength of the MLEV-17 spin-lock pulse, 7.1 kHz; length of the trim pulse, 2 ms; mixing time, 80 ms. The acquisition parameters of double-quantum-filtered correlation spectroscopy (DQF-COSY) were as follows: spectral width, 6000 Hz; number of t_2 data points, 4096; number of t_1 data points, 512; number of scans, 64; acquisition time, 0.341 s; delay time, 2.0 s. The acquisition parameters of nuclear Overhauser effect spectroscopy (NOESY) were as follows: spectral width, 6000 Hz; number of t_2 data points, 4096; number of t_1 data points, 512; number of scans, 96; acquisition time, 0.341 s; delay time, 2.0 s; mixing time, 800 ms.

NMR Data Processing and Multivariate Analysis. Sixteen samples of rice wines described above were used in the multivariate analysis. For the ^1H NMR spectra, the FIDs were zero-filled to 32K spectral data points prior to Fourier transformation, and an exponential broadening of 0.5 Hz was applied to the FIDs. For the F_2-selective TOCSY spectra, the FIDs were zero-filled to 512 (F_1) × 4096 (F_2) spectral data points prior to Fourier transformation, and exponential line broadenings of 23.44 Hz (F_1) and 2.92 Hz (F_2) were applied. All ^1H NMR spectra and F_2-selective TOCSY spectra were manually phased, baseline corrected, and referenced to DSS at 0.00 ppm. For ^1H NMR spectra, each ^1H NMR spectrum was divided into regions having an equal bin size of 0.04 ppm in the range of 6.5−10.5 ppm, and the area within each bin was integrated using MestRe Nova version 5.3.0 (MestReC, Santiago de Compostela, Spain). Each F_2-selective TOCSY spectrum was divided into 14 regions having an equal bin size of 0.08 ppm in the range of 7.89−9.01 ppm along the F_2 axis and divided into 57 regions having an equal bin size of 0.07 ppm in the range of 0.50−4.49 ppm along the F_1 axis. Each box having a constant size of 0.08 × 0.07 ppm was integrated using MestRe Nova version 5.3.0.

The integrated values of each spectrum were normalized to a constant sum. Each 14 × 57 two-dimensional F_2-selective TOCSY data matrix was arranged to 1 × 798 one-dimensional data before the application of multivariate analysis. These one-dimensional data were imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden). Unsupervised PCA was carried out to classify the spectra of different rice wine species and to evaluate the multivariate analysis data using selective ^1H NMR and F_2-selective TOCSY spectra.

NMR Signal Assignment of Rice Wines. The signals observed in 1D and 2D NMR spectra were compared with the data given in several online databases for metabolomics, such as BMRB,[25] HMDB,[26] and MMCD,[27] and several candidate compounds were picked out. Finally, signal assignment was accomplished by spiking authentic standard compounds (Wako Pure Chemical Industry or Nacalai Tesque) into a rice wine and comparing the spectra of the resulting mixtures with those of the plain rice wine.

■ RESULTS AND DISCUSSION

^1H NMR Spectrum of Japanese Sake. Figure 1a shows the ^1H NMR spectrum of Japanese sake. The spectrum is
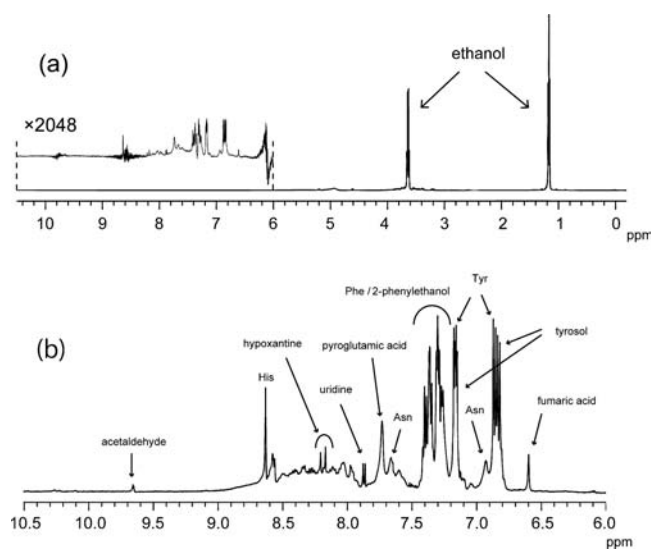

Figure 1. ^1H NMR (500 MHz) spectra (10% D_2O, pH 4.5) of Japanese sake: (a) nonselective full ^1H NMR spectrum with a vertical expansion, (b) expansion of the selective ^1H NMR spectrum from 6.0 to 10.5 ppm as observed by the selective excitation method (range of excitation 6.0−10.5 ppm).

dominated by ethanol, whose signals were observed at 1.17 and 3.64 ppm, and a few signals were observed in the low-field region (6.0−10.5 ppm). One of the reasons for such an observation was considered to be the problem of dynamic range caused by the strong ethanol signals. To remove the ethanol signals, we performed band-selective excitation experiments in the low-field region. This improved the dynamic range problem, and many signals of the minor components were newly detected (Figure 1b). In the range of approximately 7−8 ppm, several signals, which may be due to tyrosine or phenylalanine, were observed. It was thought that the broader signals in the range of approximately 7.9−9.0 ppm may have been caused by backbone amide protons of the peptide, but further interpretation with only the ^1H NMR spectrum was difficult.

Signal Assignment of Japanese Sake Using the F_2-Selective 2D NMR Spectrum. Figure 2a shows the F_2-selective TOCSY spectrum of Japanese sake (range of
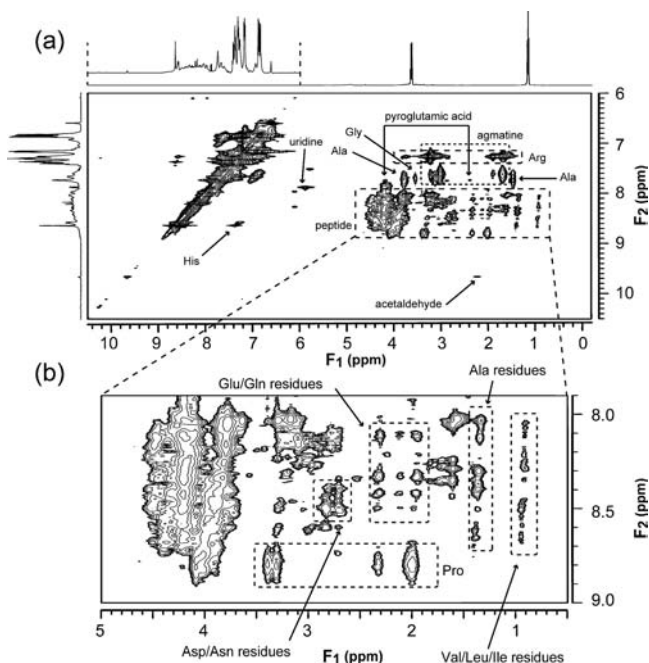
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Figure 2. F_2-selective TOCSY (500 MHz) spectra (10% D$_2$O, pH 4.5) of Japanese sake: (a) expansion of the F_2-selective TOCSY spectrum (F_1 = −0.2 to +10.5 ppm, F_2 = 6.0–10.5 ppm), (b) expansion of the F_2-selective TOCSY spectrum (F_1 = 0.5–5.0 ppm, F_2 = 7.9–9.0 ppm). The range of excitation and the number of scans were 6.0–10.5 ppm and 64, respectively.

Table 1. ^{1}H NMR Chemical Shifts of Japanese Sake (10% D$_2$O, pH 4.5) Identified by Authentic Standards Using Selective ^{1}H NMR and F_2-Selective 2D NMR Spectra

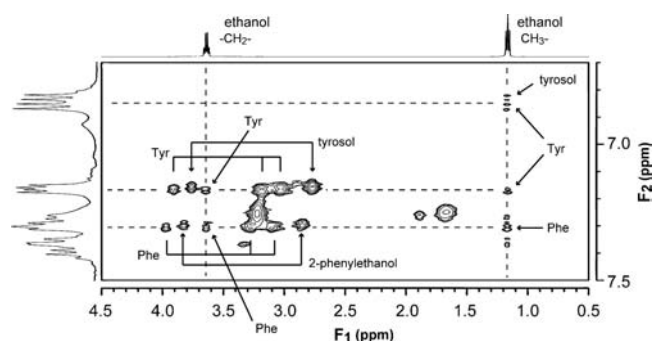| compound	chemical shift (ppm)
acetaldehyde	2.24 (CH$_3$), 9.66 (CHO)
agmatine (1-amino-4-guanidinobutane)	1.70 (C2-CH$_2$, C3-CH$_2$), 3.03 (C1-CH$_2$), 3.23 (C4-CH$_2$), 7.26 (C4-NH), 7.59 (C1-NH$_2$)
alanine	1.47 (β-CH$_3$), 3.78 (α-CH), 7.72 (α-NH$_2$)
arginine	1.67 (γ-CH$_3$), 1.90 (β-CH$_2$), 3.23 (δ-CH$_2$), 3.75 (α-CH), 7.27 (δ-NH)
asparagine	6.94 (NH$_2$CO−), 7.67 (NH$_2$CO−)
fumaric acid	6.60 (−CH=)
glysine	3.54 (α-CH$_2$), 7.69 (α-NH$_2$)
histidine	7.38 (C4-CH, ring), 8.64 (C2-CH, ring)
hypoxantine	8.18 (C2-CH), 8.21 (C8-CH)
tyrosine	3.04 (β-CH$_2$), 3.19 (β-CH$_2$), 3.91 (α-CH), 6.87 (C3-CH, C5-CH, ring), 7.17 (C2-CH, C6-CH, ring)
tyrosol (2-(4-hydroxyphenyl)ethanol)	2.76 (C2-CH$_2$), 3.75 (C1-CH$_2$), 6.83 (C3-CH′, C5-CH′, ring), 7.16 (C2-CH′, C6-CH″, ring)
phenylalanine	3.10 (β-CH$_2$), 3.27 (β-CH$_2$), 3.97 (α-CH), 7.30 (C2-CH, C6-CH, ring), 7.36 (C4-CH, ring), 7.40 (C3-CH, C5-CH, ring)
2-phenylethanol	2.85 (C2-CH$_2$), 3.83 (C1-CH$_2$), 7.29 (C2-CH′, C6-CH′, ring), 7.35 (C4-CH′, ring)
proline	2.01 (β-CH$_2$, γ-CH$_2$), 2.32 (β-CH$_2$), 3.35 (δ-CH$_2$), 8.80 (α-NH)
pyroglutamic acid	2.06 (β-CH$_2$), 2.39 (γ-CH$_2$), 2.49 (β-CH$_2$), 4.18 (α-CH), 7.74 (α-NH)
uridine	4.19 (C3-CH, ribose), 4.31 (C2-CH, ribose), 5.88 (C5-CH, uracil), 5.89 (C1-CH, ribose), 7.88 (C6-CH, uracil),

Figure 3. Expansion of the 500 MHz F_2-selective NOESY spectrum (10% D$_2$O, pH 4.5) of Japanese sake. The range of excitation and the number of scans were 6.0–10.5 ppm and 96, respectively.

excitation 6.0–10.5 ppm). A high-quality selective TOCSY spectrum of minor components was obtained without the removal of ethanol. In the range of approximately 0.5–4.5 ppm of the F_1 axis, many cross-peaks were observed, showing complex spectral patterns (Figure 2b). Using the F_2-selective 2D NMR method, high-quality selective DQF-COSY and NOESY spectra were also obtained, and the signal assignment of minor components was efficiently carried out (Table 1).

In the region of 6.0–7.5 ppm of the ^{1}H NMR spectrum, the signals from aromatic protons of tyrosine, tyrosol, phenylalanine, and 2-phenylethanol were dominant (Figure 1b). Although the signal assignment of phenylalanine and 2-phenylethanol was difficult due to the overlap of the cross-peaks in the F_2-selective TOCSY and DQF-COSY spectra, the cross-peaks were clearly separated in the F_2-selective NOESY spectrum (Figure 3), and signal assignment was accomplished. Moreover, in the F_2-selective NOESY spectrum, several cross-peaks were observed between the CH$_3$ group of ethanol at 1.17 ppm and the aromatic ring protons of tyrosine, tyrosol, and phenylalanine. Moreover, cross-peaks were also observed between the CH$_2$ group of ethanol and the aromatic ring of tyrosine and phenylalanine (Figure 3). The reproducibility of this observation was confirmed, and this phenomenon was also observed with another brand of Japanese sake. In addition, the same phenomenon (nuclear Overhauser effect, NOE) of the interaction was observed with free phenylalanine in 15% (v/v) ethanol at pH 4.5 (data not shown). Therefore, the NOESY cross-peaks between ethanol and these aromatic compounds do not seem to be experimental artifacts. These results indicate that hydrophobic aromatic compounds may form complexes with ethanol in Japanese sake.

In the region of 7.5–7.9 ppm of the ^{1}H NMR spectrum, the signals were assigned mainly to amine protons of amino acids (alanine, glycine, asparagine, pyroglutamic acid) and polyamine (agmatine) (Figure 1b). These signals were broad and overlapped each other; therefore, it was difficult to assign the signals with only the ^{1}H NMR spectrum. These signals were separated in the F_2-selective TOCSY spectrum, and the signal assignments were facilitated. Although water presaturation reduced the signal intensity of the amine protons, various cross-peaks derived from amine protons were observed under conditions containing sufficient amount of light water (e.g., the addition of only 10% heavy water for the field lock). In contrast, only a few cross-peaks derived from amine protons were observed in the F_2-selective DQF-COSY and the NOESY spectra. Therefore, TOCSY seems to be useful for detecting amine protons.

In the region of 7.9–9.0 ppm of the ^{1}H NMR spectrum, the broad resonance, with a bell-shaped curve, was assigned mainly

Table 2. ^{1}H NMR Chemical Shifts (ppm) of Free Amino Acids (10% D$_2$O, 15% Ethanol (v/v), pH 4.5) Obtained by ^{1}H NMR and F_2-Selective TOCSY Spectra

	α-NH$_2$	side chain NH	α-CH	β-CH	γ-CH	δ-CH	others
Gly	7.69		3.53				
Ala	7.74		3.75	1.46			
Val	7.65		3.58	3.37	0.98		
Leu	7.73		3.70	1.70	1.70	0.95	
Ile	7.65		3.64	1.96	1.00 (met), 1.25, 1.46	0.92	
Ser	7.85		3.81	3.95			
Thr	7.80		3.56	4.24	1.31		
Asp	nd		3.91	2.71, 2.84			
Glu	nd		3.75	2.09	2.42		
Asn	nd	6.94, 7.67 (−CONH$_2$)	3.98	2.83, 2.94			
Gln	7.89	6.90, 7.64 (−CONH$_2$)	3.75	2.12, 2.44			
Lys	7.77	7.60 (ε-NH$_2$)	3.73	1.89	1.46	1.71	3.01 (ε-CH$_2$)
Arg	7.85	6.72 (ω-NH), 7.27 (δ-NH)	3.75	1.90	1.69	3.24	
His	nd	nd (ring NH)	4.01	3.53			ring: 7.39 (C5-CH), 8.64 (C2-CH)
Phe	nd		3.96	3.10, 3.28			ring: 7.31 (C2-CH, C6-CH), 7.36 (C4-CH), 7.40 (C3-CH, C5-CH)
Tyr	nd		3.91	3.03, 3.19			ring: 6.87 (C3-CH, C5-CH), 7.18 (C2-CH, C6-CH)
Met	7.85		3.83	2.63	2.14		2.12 (thio-Met)
Cys	nd		3.96	3.06			
Trp	nd	10.23 (ring NH)	4.02	3.28, 3.47			ring: 7.18 (C6-CH), 7.26 (C5-CH), 7.30 (C2-CH), 7.52 (C7-CH), 7.72 (C4-CH)
Pro	7.95, 8.81		4.09	2.06, 2.33	1.99	3.32, 3.39	
GABA		7.67 (γ-NH$_2$)	2.34	1.90	3.01		
ornithine	7.74	7.71 (γ-NH$_2$)	3.76	1.77	1.92	3.04	
citrulline	7.80	6.39(δ-NH), n.d. (−CO-NH$_2$)	3.72	1.86	1.56	3.12	
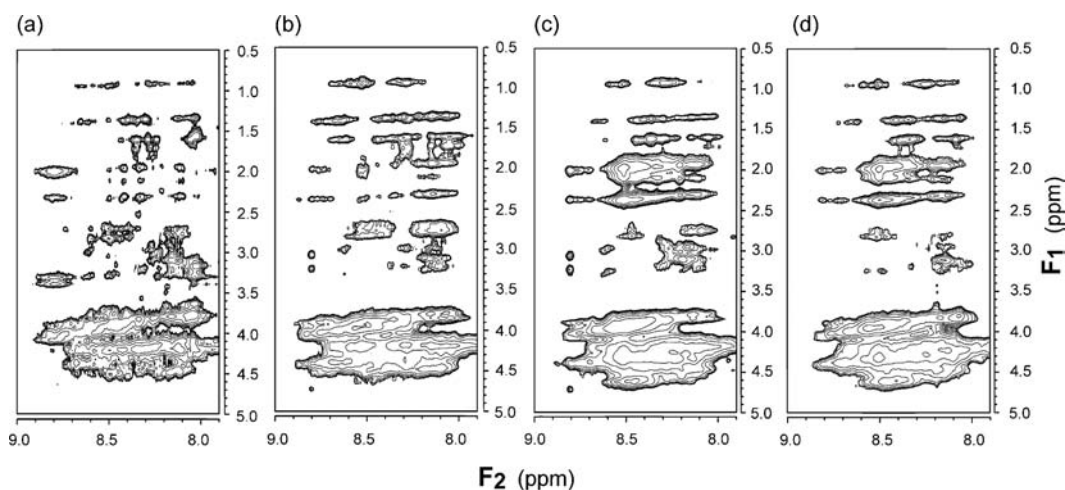
Figure 4. Expansion of the 500 MHz F_2-selective TOCSY spectra (10% D$_2$O, pH 4.5) of the rice wines: (a) Japanese sake (rice koji), (b) Korean makgeolli (rice koji), (c) Korean makgeolli (wheat koji), and (d) Chinese Shaoxing wines (wheat koji). The range of excitation and the number of scans were 6.5−10.5 ppm and 64, respectively.

to the amide group in peptides (Figure 1b). In the F_2-selective TOCSY spectrum, many and complex correlations were observed in the range of approximately 0.5−4.5 ppm of the F_1 axis (Figure 2, peptide region). The correlations in the range of 4.0−4.5 ppm of the F_1 axis are attributable mainly to α-CH of amino acid residues, and those in the range of 0.5−4.0 ppm of the F_1 axis are attributable mainly to the side chains.

Similarly, the F_2-selective TOCSY spectrum of a tryptone solution also provided many and complex correlations in the peptide region (data not shown). There was a possibility that some correlations arose from free amino acids; therefore, we measured F_2-selective TOCSY spectra of 23 major amino acids under conditions (15% (v/v) ethanol, pH 4.5) similar to those for Japanese sake and confirmed the chemical shift values
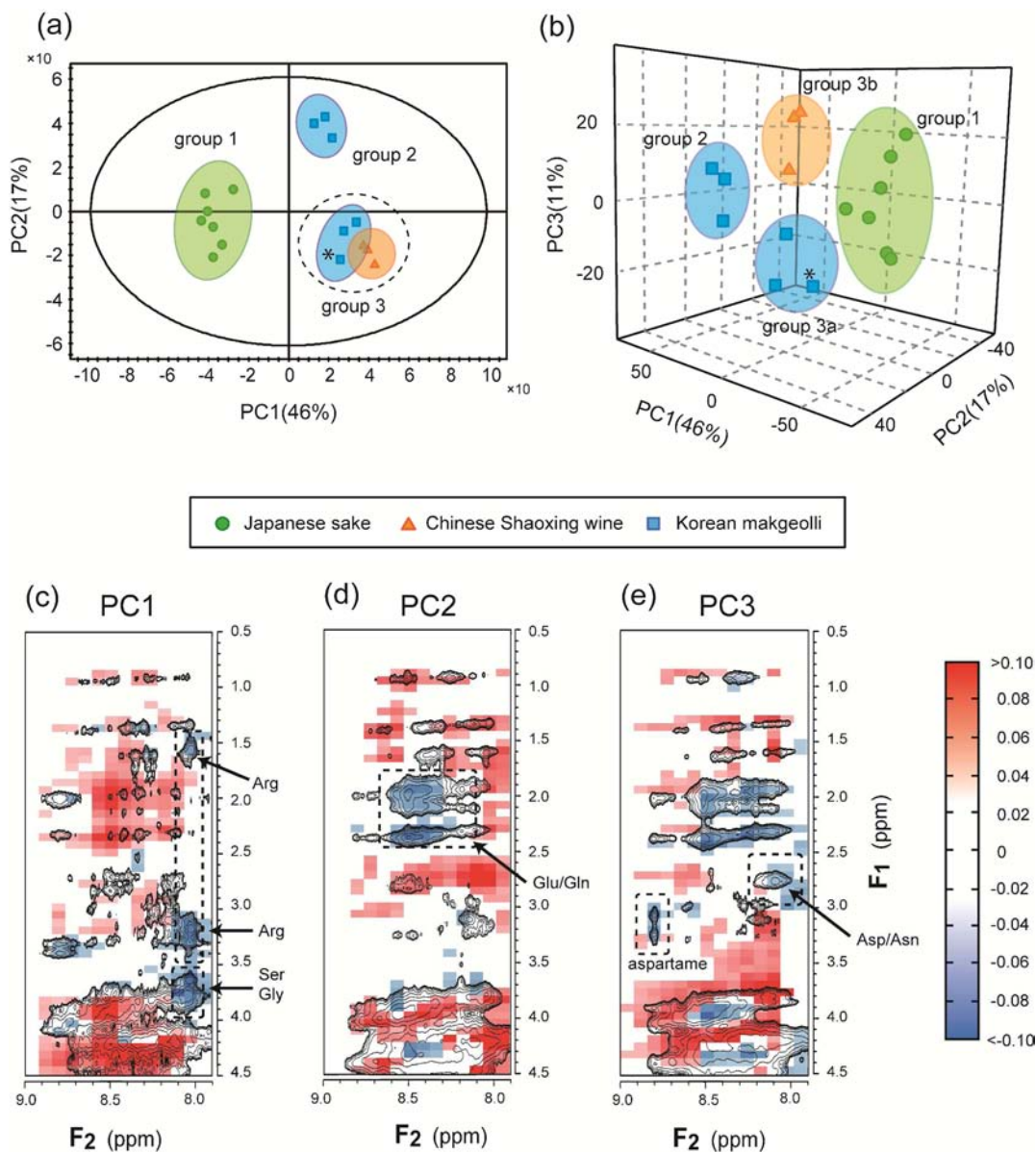
Figure 5. PCA score plots derived from the F_2-selective TOCSY spectra of the rice wines for (a) PC1−PC2 and (b) PC1−PC2−PC3. Korean makgeolli, marked with an asterisk, was not labeled by the type of koji used. PCA loading plots overlaid with their representative F_2-selective TOCSY spectra: (c) loading plot of PC1 and the F_2-selective TOCSY spectrum of the Japanese sake, (d) loading plot of PC2 and the F_2-selective TOCSY spectrum of the Chinese Shaoxing wine, (e) loading plot of PC3 and the F_2-selective TOCSY spectrum of the Korean makgeolli (malted wheat).

(Table 2). The results showed that free proline provides a cross-peak in the peptide region of the F_2-selective TOCSY spectra but other free amino acids do not. Correct signal assignment of the individual cross-peaks was difficult, but several cross-peaks were estimated to be derived from Val/Leu/Ile (δ = 0.9 ppm), Ala (δ = 1.4 ppm), Glu/Gln (δ = 2.0, 2.3 ppm), and Asp/Asn (δ = 2.7, 2.8 ppm) residues on the basis of the chemical shift values (Figure 2b).[28,29]

The NMR spectra of various brands of Japanese sake were measured, and the peptide region (F_2/F_1 axis 7.9−9.0/0.5−4.5 ppm) of the F_2-selective TOCSY spectra showed spectral patterns similar to one another with good reproducibility. Therefore, it was thought that this pattern may be characteristic of Japanese sake. One of the reasons for the observation of similar spectral patterns in the peptide region among various brands of Japanese sake is that these detected peptides were not

produced by the random digestion of proteins but rather were generated mainly by regular digestion of proteins by protease(s) or peptidase(s). In addition, these results do not conflict with the analysis of the peptides of Japanese sake using an isolation method, which showed that the major amino acid residues in the low molecular weight peptides of Japanese sake are Gly, Glu (or Gln), Asp (or Asn), Ser, and Ala.[30]

F_2-Selective TOCSY Spectra of the Peptide Region of Chinese Shaoxing Wines and Korean Makgeolli. The F_2-selective TOCSY spectra of Chinese Shaoxing wines and Korean makgeolli, both of which are rice wines like Japanese sake, showed patterns quite different from those of Japanese sake in the peptide region (Figure 4). Chinese Shaoxing wines showed spectral patterns similar to one another. The signals in the ranges of 1.8−2.4 ppm in the F_1 axis and 8.2−8.6 ppm in the F_2 axis were characteristic (Figure 4d), and the signals were
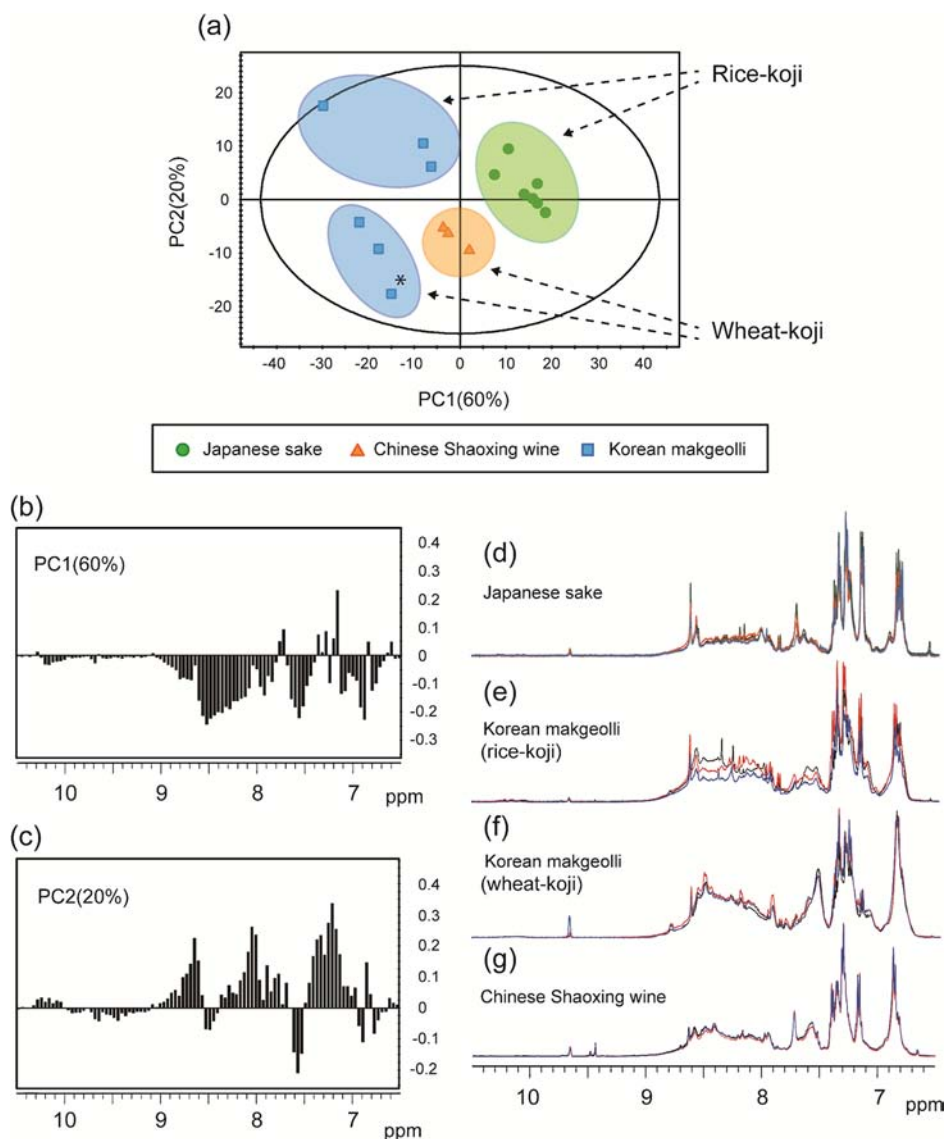
Figure 6. (a) PCA score plots derived from the ^{1}H NMR spectra of the rice wines in the low-field region (6.5−10.5 ppm) using PC1−PC2. Loading plots of (b) PC1 and (c) PC2. Korean makgeolli, marked with an asterisk, was not labeled by the type of koji used. Expansion of the selective 500 MHz ^{1}H NMR spectra of the rice wines (pH 4.5, 10% D_2O) from 6.5 to 10.5 ppm: (d) Japanese sake, (e) Korean makgeolli (rice koji), (f) Korean makgeolli (wheat koji), (g) Chinese Shaoxing wine. The range of excitation and the number of scans were 6.5−10.5 ppm and 256, respectively.

considered to be derived mainly from Glu or Gln residues. On the other hand, Korean makgeolli showed two different spectral patterns: some showed spectral patterns similar to those of Chinese Shaoxing wines (Figure 4c), whereas others showed spectral patterns quite different from those of either the Japanese sake or Chinese Shaoxing wines (Figure 4b). It was thought that these differences in the spectral patterns probably reflect differences in manufacturing processes or raw materials. In addition, the F_2-selective TOCSY spectra showed a good reproducibility similar to that of Japanese sake. These results suggest that the peptide region of the F_2-selective TOCSY spectra may be available as a fingerprint for the discrimination of rice wine species.

Multivariate Analysis with F_2-Selective TOCSY Spectra of the Peptide Region. To explore the availability of the F_2-selective TOCSY spectra of the peptide region as a fingerprint, we performed a PCA using the F_2-selective TOCSY spectra (spectral regions: F_2 axis, 7.9−9.0 ppm; F_1 axis, 0.5−4.5 ppm) of three rice wine species (seven samples of Japanese sake,

three samples of Chinese Shaoxing wines, and six samples of Korean makgeolli).

Figure 5a shows the PCA score plot with PC1 and PC2. The samples were clustered into three groups. Group 1 contained Japanese sake, group 2 some Korean makgeolli, and group 3 the other Korean makgeolli and Chinese Shaoxing wines. The Korean makgeolli and Chinese Shaoxing wine of group 3 were separated along PC3 (group 3a and group 3b); as a result, a PCA score plot separating the three rice wine species was obtained (Figure 5b). The reason for the separation of Korean makgeolli into two groups is probably due to the difference in the koji species used as raw materials. The Korean makgeolli belonging to group 2 was labeled "rice koji", and those belonging to group 3a were labeled "wheat koji". Although Korean makgeolli, marked with asterisks in Figure 5a,b, was not labeled by the type of koji used, this makgeoli seems to be brewed with wheat koji, judging by the score plot and the pattern of the F_2-selective TOCSY spectrum. In addition, all of

the brands of the Japanese sake were labeled "rice koji", and the Chinese Shaoxing wines were labeled "wheat koji".

Parts c−e of Figure 5 show the loading plots of PC1, PC2, and PC3 overlaid with the F_2-selective TOCSY spectra. The loading plot of PC1 (Figure 5c) indicates important cross-peaks for characterizing Japanese sake, and these signals are thought to be derived from Arg, Ser, and Gly residues due to the chemical shift values. In addition, the loading plot of PC1 showed that the cross-peaks of free proline (F_1/F_2 = 3.32/8.80 and 3.39/8.80 ppm) are also characteristic signals of Japanese sake. The loading plot of PC2 (Figure 5d) indicates that the signal intensities of the regions of 1.8−2.4 ppm in the F_1 axis and 8.2−8.6 ppm in the F_2 axis are higher for Korean makgeolli and Chinese Shaoxing wines, which were brewed using wheat koji. The signals were thought to be derived mainly from Glu or Gln residues. The loading plot of PC3 (Figure 5e) indicates that the cross-peak at about F_1/F_2 = 2.7/8.1 ppm is important for separating Korean makgeolli from Chinese Shaoxing wines of group 3, and the signals were thought to be derived from Asp or Asn residues. A similar signal was also observed with Korean makgeolli made from rice koji (Figure 4b); this cross-peak may be a characteristic of Korean makgeolli. In addition, the loading plot of PC3 showed that the cross-peaks at F_1/F_2 = 3.07/8.80 and 3.24/8.80 ppm were important for distinguishing Korean makgeolli from Chinese Shaoxing wines of group 3, and the cross-peaks were assigned to aspartame. In this study, aspartame was detected in five out of six Korean makgeolli samples, and these five Korean makgeolli samples were labeled as containing "aspartame". On the other hand, aspartame was not detected in Japanese sake or Chinese Shaoxing wines. These results demonstrate that the F_2-selective TOCSY spectra of the peptide region combined with PCA have the potential to distinguish not only rice wine species, but also the koji species used as raw materials. Furthermore, an increase in the number of samples and the validation of the PCA model would provide a good discrimination model for these rice wines. The selective ^{1}H NMR spectra in the low-field region (6.5−10.5 ppm) combined with PCA could divide these rice wines into four groups with only PC1 and PC2 (Figure 6a). Although the interpretation of the loading plot of 7.9−9.0 ppm (Figure 6b,c) is difficult because of the complex signal overlap of the ^{1}H NMR spectra (Figure 6d−f), it may be available for high-throughput screening.

In conclusion, the F_2-selective 2D NMR method provided high-quality 2D NMR spectra of minor components, including exchangeable amine protons, and various minor metabolites were identified. The broad resonance, with a bell-shaped curve in the region of 7.9−9.0 ppm of the ^{1}H NMR spectra, was assigned mainly to amide groups in peptides, and the F_2-selective TOCSY spectra showed complex and characteristic spectral patterns. The combination of the spectra with an unsupervised PCA provides a good classification model of the rice wines. Furthermore, it also provided information about what type of koji was used as the raw material. Metabolic profiling with F_2-selective TOCSY spectra of the peptide region may be available not only for rice wines but also various foodstuffs as a new "fingerprint" for quality control or food authentication.

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