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Advantage of LC-MS Metabolomics Methodology Targeting Hydrophilic Compounds in the Studies of Fermented Food Samples

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The utility of a liquid chromatography mass spectrometry (LC-MS) method, using a pentafluorophenylpropyl (PFPP) bonded silica, was demonstrated in a metabolomics study of fermented food samples. Our LC-MS method was applied to Japanese fermented food (miso) of different stages of ripeness. The data acquired were evaluated by principal component analysis (PCA). The score plots indicated that the miso samples could be approximately classified into three groups, based on the stage of miso ripeness. The loading plots indicated that the ions responsible for group separation included not only amino acids and citric acid but also Amadori compounds. On the other hand, the miso samples were also analyzed by a conventional LC-MS method using an octadecyl (C_{18}) column for comparison. The group separation of score plots from the conventional method was less clear than that from our method. The advantage of our LC-MS method is due to the different retention properties of the PFPP column and the C_{18} column with hydrophilic compounds. Our LC-MS method will be useful for the metabolic profiling of fermented food samples.

KEYWORDS: High-performance liquid chromatography mass spectrometry; hydrophilic compounds; principal component analysis; Amadori compound

INTRODUCTION

A variety of metabolic profiling tools have been widely used for the development of metabolomics studies. The primary tool employed for metabolic profiling is nuclear magnetic resonance (NMR) spectroscopy (*1*). NMR is characterized by high resolution and relatively easy sample preparation. In addition to NMR, mass spectrometry (MS) (*2, 3*) has also proven to be useful for metabolic profiling. It provides both sensitive detection and metabolite identification and can be easily coupled to a chromatography step prior to detection.

The combination of MS with gas chromatography (GC) (*4*−*6*) and capillary electrophoresis (CE) (*7*−*10*) is widely used for metabolic profiling studies. GC-MS and GC-MS/MS enable the separation of a variety of hydrophilic and hydrophobic compounds. However, GC requires a derivatization approach in the sample preparation, and it is often tedious and time-consuming. CE-MS is suitable for the separation of hydrophilic compounds without derivatization, but its sensitivity is very poor.

Recently, liquid chromatography mass spectrometry (LC-MS) has been used for metabolic profiling (*11*−*16*). It is a robust, sensitive, and selective technique, and it also has become a popular technique for quantitative and qualitative analyses. Many liquid chromatography mass spectrometry (LC-MS) applications have been reported for analyses of hydrophobic metabolites, using a reversed phase octadecyl (C_{18}) column with volatile mobile phases (*11*−*14*). However, little attention has been paid to the analysis of hydrophilic compounds, such as amino acids, organic acids, and nucleic acids. The analysis of hydrophilic compounds is important in the fields of food science, biochemistry, and fermentation, because hydrophilic compounds are related to taste, color, and flavor in foods, as well as biochemical mechanisms of organisms, such as humans and fungi. Hydrophilic compounds are poorly retained on the C_{18} column (*15*), which hampers their separation, and ion suppression reduces the signal response.

A volatile ion-pairing reagent can be added to improve the separation of hydrophilic compounds (*16*). It can change the retention time and thereby decrease the ion suppression of the matrix. However, the formation of strong ion pairs might result in depression of the signal response in electrospray MS due to the formation of a neutral ion pair.

Hydrophilic interaction chromatography (HILIC) is an alternative method for the separation of hydrophilic compounds (*17*). It also offers unique advantages for the MS detection of hydrophilic compounds. The higher organic content of the eluate in HILIC provides efficient evaporation of the solvent, and as a result, it confers enhanced sensitivity. The combination of MS with HILIC is suitable for LC-MS analysis. However, it

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Table 1. Miso Sample List

sample no.	name	supplier	color class	color difference	fermentation period
1	edo miso	masakiya	red	17	over 4 months
2	echigo kogingyo	masakiya	red	18	over 10 months
3	yamagata ckunohosomichi	masakiya	red	20	over 11 months
4	sendai kuradashi	masakiya	red	23	12 months
5	aizyu kuradashi	masakiya	red	25	12 months
6	shinsyu ninenshikomi	masakiya	red	26	over 24 months
7	hokkaido koitten	masakiya	red	28	over 5 months
8	shinshu aka	masakiya	red	28	over 12 months
9	echigo cyozyukusei	masakiya	red	29	over 10 months
10	sado tennen	masakiya	red	30	over 4 months
11	nanbu shirozukuri	masakiya	brown	34	over 6 months
12	daigen akane	daigen	brown	36	over 6 months
13	genmai	masakiya	brown	36	over 10 months
14	shinsyu daigingyo	masakiya	brown	39	over 3 months
15	tsugaru yuki	masakiya	brown	45	over 3 months
16	shinsyu suri	masakiya	brown	45	3 months
17	shinsyu tokusen	masakiya	brown	46	over 3 months
18	kyoto shirokoshi	masakiya	white	52	2 weeks
19	kyoto shirotsubu	masakiya	white	55	2 weeks
20	tokusen shiro	onojin	white	55	no data
21	daigen bekko	daigen	white	55	no data
22	fucyu	masakiya	white	57	1 week
23	marumiso	marumikoji	white	58	no data
24	bibainouta	marumikoji	white	59	no data

was recently found that a microdialysis sample had to be diluted at least 10-fold with acetonitrile to obtain a reasonable peak shape (*18*).

In our previous studies, we established a novel technique for analyses of underivatized hydrophilic compounds by LC-MS, using a pentafluorophenylpropyl (PFPP) column with a volatile buffer. The advantage of this novel technique can simultaneously separate underivatized hydrophilic compounds, such as amino acids, organic acids, and nucleic acids, in a single run (*19*). This LC-MS method could be applied for targeted analysis of hydrophilic compounds in soy sauces. Furthermore, we also demonstrated the applicability of this LC-MS method for the nontargeted metabolic profiling of hydrophilic compounds in several kinds of soy sauces with principal component analysis (PCA). They could be classified by the manufacturing methods, and the components that corresponded to the difference could be identified. This LC-MS method has high potential for the comprehensive analysis of hydrophilic compounds.

In this study, we compared our LC-MS method using a PFPP column to the conventional LC-MS method using a C_{18} column. We will describe how our LC-MS method was successfully employed for the metabolic profiling of hydrophilic compounds in a fermented food sample.

MATERIALS AND METHODS

Materials. HPLC grade acetonitrile and HPLC grade formic acid were purchased from Junsei Chemical (Tokyo, Japan) and Wako Chemicals (Osaka, Japan), respectively. Amino acids were purchased from Sigma-Aldrich (St. Louis, MO). Amino acid standard solutions (types AN-II and B) were purchased from Wako Chemicals (Osaka, Japan). Citric acid, pyroglutamic acid, and methylsuccinic acid were purchased from Wako Chemicals (Osaka, Japan) and Tokyo Chemical Industrial (Tokyo, Japan), respectively. Distilled water was prepared with a Milli-Q system (Millipore, Bedford, MA). Fructosyl-leucine and fructosyl-phenylalanine were synthesized. A total of 24 kinds of miso samples, made from soybeans by fermentation, were obtained from Japanese suppliers (**Table 1**).

Sample Preparation. A total of 24 kinds of miso samples were stored at 4 °C prior to sample preparation. A 300 mg portion of miso was suspended in 10 mL of distilled water, and then the solution was centrifuged at 553 g for 1 min. The supernatant solution was filtered through a Millipore Ultrafree-MC 5000 cutoff centrifugal filter unite (Tokyo, Japan) at 2765 g for 25 min and then was injected onto an analytical column.

For the identification of unknown compounds, 10 mg portions of amino acids, citric acid, fructosyl-leucine, and fructosyl-phenylalanine were each dissolved in 1 mL of distilled water. These solutions were diluted 1000-fold with distilled water and then were injected onto the PFPP column.

For the quantitative determination, a mixture of amino acid standard solutions (types AN-II/B, 1/1) was diluted 12.5-fold with distilled water and then injected onto a cation-exchange column. Citric acid and pyroglutamic acid were prepared at 260 μmol/L and 390 μmol/L, respectively. Methylsuccinic acid was prepared at a 43 μmol/L concentration in distilled water, as an internal standard. All samples were finally diluted 1:1 with an aqueous solution of the internal standard and then injected onto the PFPP column.

LC-MS Apparatus. For the nontargeted metabolic profiling, LC-MS was performed using two LC-10ADvp pumps (Shimadzu, Kyoto, Japan), a SIL-10ADvp autosampler (Shimadzu, Kyoto, Japan), a CTO-10 ACvp column oven (Shimadzu, Kyoto, Japan), and a Q-Tof Premier mass spectrometer (Micromass, Manchester, U.K.). For the quantitative determination of citric acid and the pyroglutamic acid analysis, LC-MS was performed using a Shimadzu LCMS-2010A system (Kyoto, Japan), two LC-10ADvp pumps, a SIL-HTc autosampler, a CTO-10 ACvp column oven, and a LCMS-2010 quadrupole mass spectrometer.

HPLC Conditions. A Discovery HS-F5 column (250 mm × 4.6 mm i.d., 5 μm), in which the stationary phase was PFPP bonded silica, from Supelco (Bellefonte, PA), was used. The eluting solvent A consisted of 0.1% aqueous formic acid, and the eluting solvent B was acetonitrile. The flow rate was 0.3 mL/min, and the injection volume was 5 μL. Separations were carried out according to ref *19*.

A Waters Symmetry C_{18} column (100 mm × 2.1 mm i.d., 3.5 μm) from Waters (Milford, MA) was used. The eluting solvent A consisted of 0.1% aqueous formic acid, and the eluting solvent B was acetonitrile. The flow rate was 0.5 mL/min, and the injection volume was 5 μL. Separations were carried out according to ref *11*.

MS Conditions. For the nontargeted metabolic profiling, a quadrupole time-of-flight (Q-TOF) mass spectrometer with an electrospray interface was operated, in either the positive or negative ion mode. The capillary voltage was maintained at 3.0 kV in the positive ion mode and at 2.8 kV in the negative ion mode. Other interface settings had the same value in the positive and negative ion modes. The voltages of the sample cone, the collision, and the multichannel plate detector were set to 20 V, 4.0 V, and 2200 V, respectively. The flow rates of the nebulizer gas and the cone gas were adjusted to 500 L/h and 50 L/h, respectively. The desolvation and the source temperature were 120 and 80 °C, respectively. The mass spectrometric data were collected in full-scan ion mode from *m*/*z* 50 to 650. The acquisition duration per spectrum was set to 1.0 s, with a delay of 0.1 s. The MassLynx program (version 4.0 sp1, Micromass, Manchester, U.K.) was used for data collection and analysis. The eluate from the column was split, and 0.2 mL/min of it was introduced into the mass spectrometer.

For the quantitative determination of citric acid and pyroglutamic acid, a quadrupole mass spectrometer with an electrospray interface was operated at unit mass resolution, in the negative ion mode. The capillary and curved desolvation line (CDL) voltages of the interface were set to 3.0 kV and 25 V, respectively. Other interface settings were as follows: drying gas flow rate, 0.2 MPa; block heater temperature, 260 °C; CDL temperature, 200 °C; and nebulizing gas flow rate, 900 L/h. Data were collected in the selected ion monitoring mode at *m*/*z* 191 and *m*/*z* 128, with window widths of 0.4 amu. The LCMS solution was used for data collection and analysis. The eluate from the column was introduced into the mass spectrometer.

Amino Acid Analysis. For the analysis of the amino acids, an L-8800 Amino Acid Analyzer (Hitachi High-Technologies Co., Tokyo, Japan) was used for the quantitative determination of amino acids in miso samples. The analytical column (60 mm × 4.6 mm i.d., 3 μm) and the guard column (5.0 mm × 4.0 mm i.d., 5 μm) consisted of polystyrene cross-linked by divinylbenzene, with sulfone groups as the active exchange sites. A 10 μL aliquot of the sample solution was injected. The biological amino acid analysis program was used.

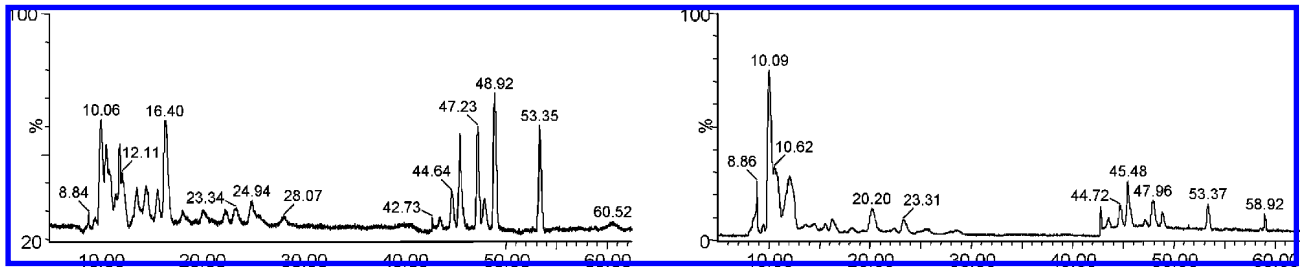

Figure 1. Typical total ion chromatograms of miso samples with 18 of color difference in the positive mode (left) and the negative mode (right). The data were obtained using a PFPP column. Chromatographic conditions are described in the MATERIALS AND METHODS.

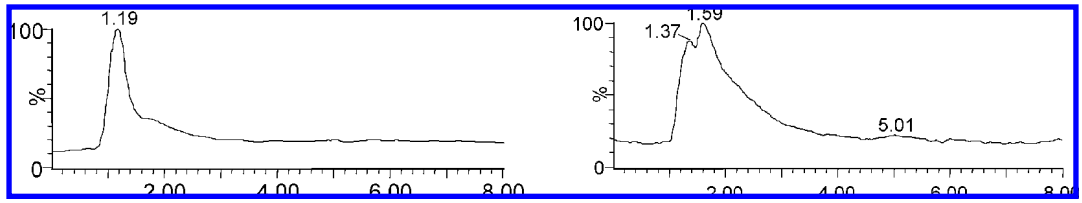

Figure 2. Typical total ion chromatograms of miso samples with 18 of color difference in the positive mode (left) and the negative mode (right). The data were obtained using a C_{18} column. Chromatographic conditions are described in the MATERIALS AND METHODS.

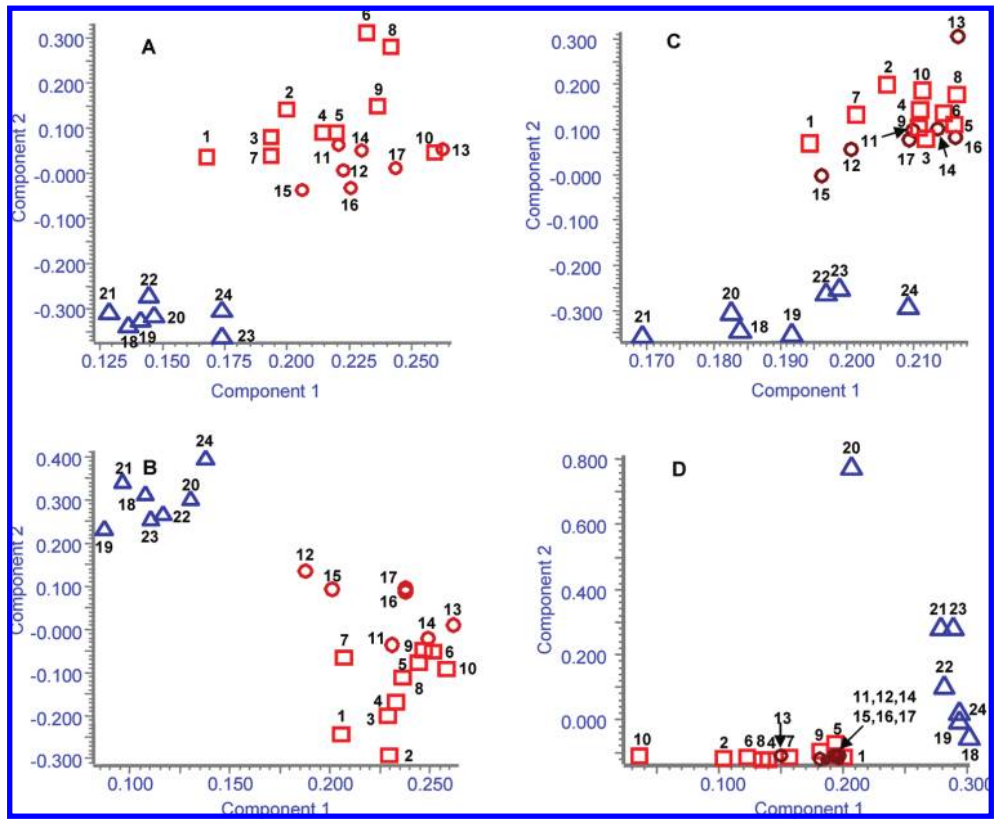

Figure 3. Score plot obtained following the LC-MS data on a PFPP column with miso samples in the positive mode (**A**) and in the negative mode (**B**), following LC-MS data on a C_{18} column in the positive mode (**C**) and in the negative mode (**D**). Color difference: (red □) 17−30; (brown ○) 34−46; (blue △) 52−59.

Color Difference Measurement of Miso Samples. A Spectro Color Meter SE200 (Nippon Denshoku Industries CO., Ltd., Tokyo, Japan) was used for the measurement of color differences in miso samples. The values were acquired by analyzing two preparations of each sample.

Statistical Analysis. The raw data from LC-MS, obtained using the Q-Tof Premier mass spectrometer, were analyzed by the Micromass MarkerLynx Applications Manager program, version 1.0 (Micromass, Manchester, U.K.). After all of the detected peaks were subjected to noise-reduction in both the LC and MS domains, the analytical peaks were processed by the software. A list of peak intensities with retention times and *m*/*z* data pairs was generated. The intensity of each peak was normalized by the sum of all of the peak intensities. This process was repeated for each LC-MS run, and then PCA was carried out.

RESULTS AND DISCUSSION

Ripeness Stages of the Miso Samples. Miso, which is a traditional Japanese food produced by fermenting soybeans, was chosen as a model sample. The samples used in this study were at various stages of ripeness, with fermentation periods from 1 week to over 24 months. Young miso with a short fermentation period is light colored (high color difference), while the ripe

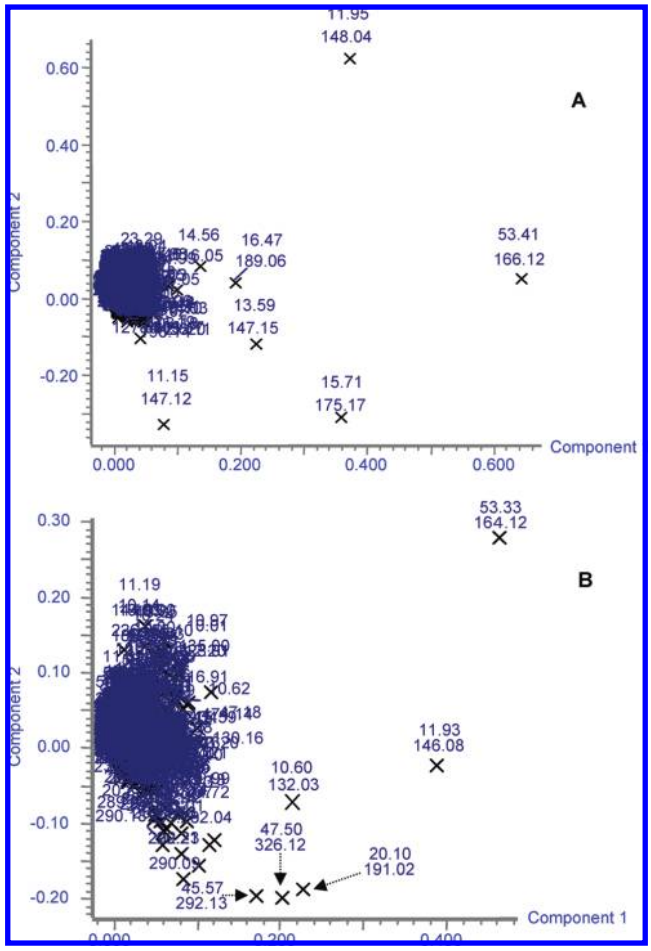

Figure 4. Loading plot obtained following the LC-MS data on the PFPP column in the positive (**A**) and negative mode (**B**).

Table 2. Assignments of the Ions Responsible for Group Separation from Loading Plots

positive ion mode			negative ion mode		
RT	*m/z*	identified compd	RT	*m/z*	identified compd
12.0	148.0614	glutamate	11.9	146.0464	glutamate
53.4	166.0849	phenylalanine	53.3	164.0717	phenylalanine
15.7	175.1179	arginine	20.1	191.0201	citric acid
11.2	147.0757	glutamine	48.0	326.1223	fructosyl-phenylalanine
13.6	147.1133	lysine	45.5	292.1382	fructosyl-leucine
16.5	189.0958	not identified	10.6	132.0320	aspartate

varieties are brownish-red colored (low color difference). Therefore, the stage of ripeness can be represented by the color difference. The results of the color difference measurements of each miso sample are shown in **Table 1**.

Comparison of Separation Properties between the LC-MS Methods Using a PFPP Column and a C_{18} Column. **Figures 1** and **2** show typical total ion chromatograms (TIC) of miso samples with 18 of color difference in the positive (left) and negative (right) modes, respectively. **Figure 1** was obtained using a PFPP column, and **Figure 2** was obtained using a C_{18} column. The TIC of **Figure 1** on the PFPP column show more peaks than those of **Figure 2** on the C_{18} column, in spite of the polarity. This is because the hydrophilic compounds in the miso samples are retained on the PFPP column, while they are poorly retained on the C_{18} column. A PFPP column is superior to a C_{18} column for the separation of hydrophilic compounds.

PCA Obtained from LC-MS Data. The data were divided into subsets for analyses to assess the differences in the miso samples. PCA was applied to the entire data set of 24 kinds of miso samples. The data shown in **Figure 3A** and **B** are score plots obtained following the LC-MS data on the PFPP column in the positive and negative ion modes, respectively. The data shown in **Figure 3C** and **D** are also score plots obtained following the LC-MS data on the C_{18} column in the positive and negative ion modes, respectively. The group separations of the score plots (**Figure 3A** and **B**) were clearer than those of the score plots (**Figure 3C** and **D**) in both the positive and negative ion modes, respectively. The score plots (**Figure 3A** and **B**) obtained following the LC-MS data on the PFPP column were symbolized according to the category by color classes of miso samples represented in **Table 1**. The results indicated that the miso samples could be approximately classified into three groups, based on the stage of miso ripeness (low (17−30), middle (34−46), and high color difference (52−59)).

Structure Determination of Key Compounds To Distinguish the Stage of Miso Ripeness. We focused on the loading plots (**Figure 4A** and **B**) obtained following the LC-MS data on the PFPP column, because of the clearer group separation. The data acquisition was performed using a Q-TOF mass spectrometer for nontargeted metabolic profiling. It provides not only enhanced resolution with an exact mass but also MS/MS analysis. It is one of the best mass spectrometers for the identification of the ions responsible for group separation by PCA. The mass spectrometric data were acquired in both the positive and negative ion modes. This is because the hydrophilic compounds within the miso samples have different ionization efficiencies in the positive and negative modes.

Table 2 summarizes the ions responsible for the group separation, obtained from the loading plots. These ions were identified by accurate mass, fragmentation pattern, and retention time. As an example, **Figure 5A** and **C** shows the fragmentation patterns of *m/z* 292.14 eluting at 45.5 min and *m/z* 326.12 eluting at 48.0 min in a negative ion mode, respectively, which were generated by collision-induced dissociation of the mass. Composition formulas of $C_{12}H_{22}NO_7$ and $C_{15}H_{20}NO_7$ were estimated from the accurate masses of *m/z* 292.1382 and *m/z* 326.1223, and the fragmentation pattern and the retention time allowed us to assign the peak as fructosyl-leucine and fructosyl-phenylalanine, respectively (**Figure 5B** and **D**). The major compounds responsible for the differences in miso samples were phenylalanine, glutamate, arginine, lysine, glutamine, aspartate fructosyl-leucine, fructosyl-phenylalanine, and citric acid. Unfortunately, the ion of *m/z* 189.0958, eluting at 16.5 min, could not be identified.

Glutamate and phenylalanine were found in the loading plots of the positive and negative ion modes, respectively. Lysine, arginine, and glutamine were only found in the loading plot of the positive ion mode. Aspartate, citric acid, fructosyl-leucine, and fructosyl-phenylalanine were only found in the loading plot of the negative ion mode.

Quantitative Determination of the Ions Responsible for a Group Separation. Quantitative determination of the identified compounds described above was performed. Amino acids were analyzed by an amino acid analyzer, and citric acid was analyzed by target analysis, using LC-MS. Since authentic standards of fructosyl-leucine and fructosyl-phenylalanine with high purity could not be obtained, these compounds were not analyzed. All of the proteinogenic amino acids except glutamine were increased during fermentation (data not shown). **Figure 6** shows the relationship between their concentrations and the color difference. The concentrations of phenylalanine, glutamate, aspartate, and lysine, but not glutamine, were high in the miso

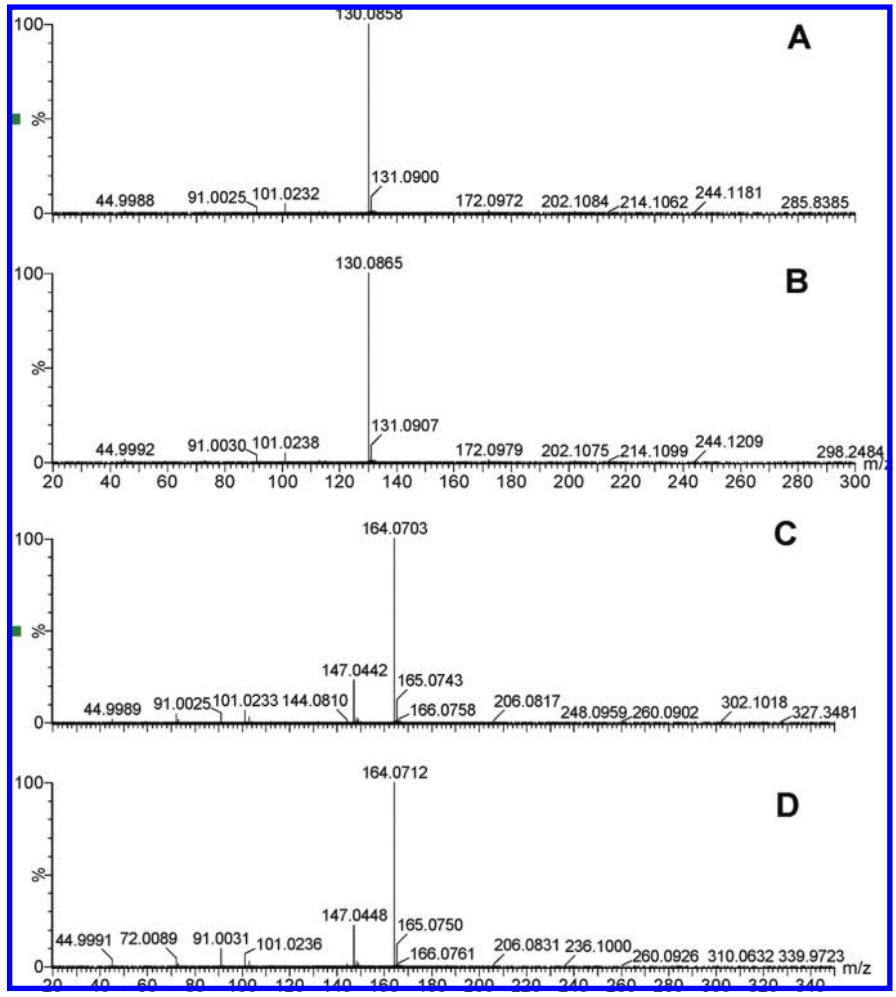

Figure 5. Fragmentation pattern of the ion (*m*/*z* 292) eluting at 45.5 (**A**) and the ion (*m*/*z* 326) eluting at 48.0 min (**C**) in the negative mode, and those of fructosyl-leucine (**B**) and fructosyl-phenylalanine (**D**).

samples with 17−46 of color difference and low in the miso samples with 52−59 of color difference (**Figure 6**). This result is reasonable, because the fermentation period of the miso samples with 17−46 of color difference is longer than that of the miso samples with 52−59 of color difference, and as a result, the soybean proteins in the miso samples with 17−46 of color difference are more degraded to amino acids than those with 52−59 of color difference. It was also observed that amino acids were increased during cheese fermentation (*20*). In contrast, the concentration of glutamine was low in the miso samples with 17−46 of color difference and high in the miso samples with 52−59 of color difference (**Figure 6**). **Figure 7** shows the relationship between glutamine and pyroglutamic acid, which was quantified by target analysis using LC-MS. Pyroglutamic acid increased with a decrease of glutamine. This result is also reasonable, because it is generally known that glutamine is degraded to pyroglutamic acid. However, pyroglutamic acid was not one of the major compounds responsible for group separation. This is because the pyroglutamic acid concentration is relatively low. The concentration of arginine was high in the miso samples with 34−46 of color difference and low in the miso samples with 17−30 and 52−59 of color difference. The behavior of arginine was different from that of the other amino acids. Unfortunately, the reason for this was not clear. In the score plot of **Figure 3**A, the miso sample (sample no. 10) with a color difference of 30 stood out among the group of miso samples with 17−30 of color difference. This was because a large amount of arginine was present in this miso sample (**Figure 6**, arrow). Citric acid also increases during the ripeness stage, and it is supposed to be one of the byproducts during amino acid synthesis by a fungus (*kojikin*) (**Figure 6**). Most of the familiar organic acids, such as formic acid and acetic acid, which are generally produced in the fermentation process, were not detected in this analysis, because these volatile compounds evaporated with the mobile phase during the ionization process of the MS detection.

Utility of the Metabolomics Methodology Using a PFPP Column for Fermented Food Samples. Other compounds responsible for group separation included fructosyl-leucine and fructosyl-phenylalanine (Amadori compounds). Amadori compounds are derived from the Maillard reaction of reducing sugars and amino compounds. The Maillard reaction is known to occur in heated, dried, or stored foods. This reaction is responsible for changes in taste, color, flavor, and nutritive value. **Figure 8** shows the relationship between the total peak area of the observed Amadori compounds (fructosyl-leucine and fructosyl-phenylalanine) and the color difference of the miso samples. The color difference of the miso samples decreased with an increase in the total peak area of the observed Amadori compounds. The result of an inverse correlation revealed that the Amadori compounds were directly related to the color in miso samples. This is also reasonable, because a longer fermentation period would produce more Amadori compounds.

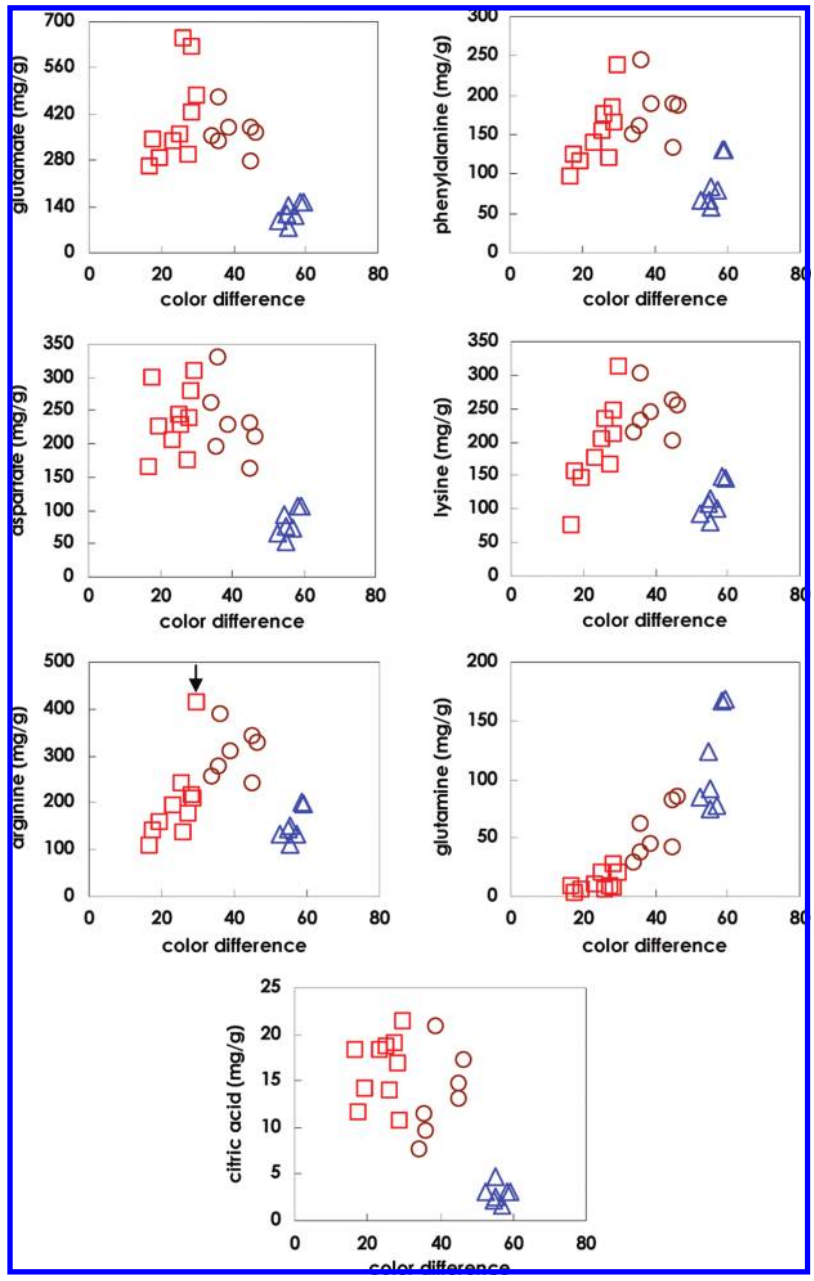

Figure 6. Quantitative determination of amino acids and citric acid in miso samples: (red □) 17−30; (brown ○) 34−46; (blue △) 52−59.

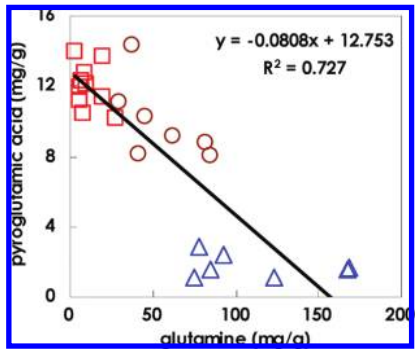

Figure 7. Relationship between glutamine and pyroglutamic acid in miso samples: (red □) 17−30; (brown ○) 34−46; (blue △) 52−59.

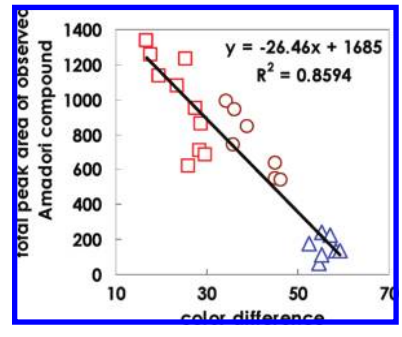

Figure 8. Relationship between the total peak area of observed Amadori compounds and the color difference of miso samples: (red □) 17−30; (brown ○) 34−46; (blue △) 52−59.

To our knowledge, this is the first report describing the use of a PFPP column in the LC-MS method for the analysis of Amadori compounds. We previously reported that a PFPP column could be applied for the analysis of amino acids, organic acids, amines, nucleic bases, nucleosides, and nucleotides (*19*). In food studies, a cation-exchange column, a reversed phase column, an anion-exchange column, an ion exclusion column,

and an amino column have been used for the analysis of amino acids (*21*, *22*), organic acids (*23*, *24*), amines (*25*) nucleosides (*26*), nucleotides (*27*), and Amadori compounds (*28*), respectively. Using a PFPP column, the simultaneous determination of these hydrophilic compounds can be performed, and also the stage of ripeness in a fermented food can be evaluated. It was clear that amino acids, organic acids, and Amadori compounds could be used as markers for proteolytic degradation, fermentation byproducts, and condensation compounds of sugar and amino acids generated by proteolytic degradation at certain ripeness stages. This LC-MS method using a PFPP column will be useful for the analysis of other fermented foods such as wine and cheese.

In conclusion, our LC-MS method using a PFPP column was suitable for the analysis of hydrophilic compounds in miso samples. Hydrophilic compounds were retained on the PFPP column, and as a result, we could successfully demonstrate the differences between miso samples, based on their ripeness stages. This LC-MS method will be useful for the metabolic profiling of hydrophilic compounds in future studies of fermented food samples.

ABBREVIATIONS

CDL, curved desolvation line; CE, capillary electrophoresis; C18, octadecyl; GC, gas chromatography; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; LC-MS, high-performance liquid chromatography mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PCA, principal component analysis; PFPP, pentafluorophenylpropyl; TIC, total ion chromatograms.

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