JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comprehensive Analytical Method for the Determination of Hydrophilic Metabolites by High-Performance Liquid Chromatography and Mass Spectrometry

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A method for the comprehensive analysis of hydrophilic metabolites, based on a combination of high-performance liquid chromatography and mass spectrometry, is described. We evaluated three types of stationary phases to achieve the separation of highly hydrophilic metabolites. Good chromatographic retention and separation of these metabolites were achieved on a pentafluorophenylpropyl-bonded silica column with gradient elution, using 0.1% aqueous formic acid and acetonitrile as the mobile phase. The optimized conditions allowed the comprehensive determination of the standard 49 kinds of amino acids, 6 kinds of amines, 45 kinds of organic acids, 18 kinds of nucleic bases, 5 kinds of nucleosides, and 14 kinds of nucleotides, and then the linearity, dynamic range, detection limit, and precision of the retention time and the peak area were validated. We applied this method for the targeted analysis of the components in soy sauce. The results from the quantitative determination of amino acids were compared to those obtained with an amino acid analyzer, and the accuracy was in the range between 85 and 119%. The accuracy of other detected components was confirmed to be 105−133% by the recovery rate after the addition of standard compounds. We also applied the method for the nontargeted metabolic profiling of the components in several kinds of soy sauces with the principal component analysis. They were classified by the manufacturing methods, and the components that corresponded to the differences were identified. This method could be useful for the targeted analysis of hydrophilic metabolites as well as their nontargeted metabolic profiling.

KEYWORDS: High-performance liquid chromatography mass spectrometry; hydrophilic metabolites; targeted analysis; nontargeted metabolic profiling; pentafluorophenylpropyl-bonded silica column; metabolomics

INTRODUCTION

Metabolomics studies are a rapidly growing area of scientific research. The assessment of low molecular weight, endogenous metabolites, and their intermediates is an important tool for understanding biological regulation and control in living organisms and human nutrition. Especially highly hydrophilic metabolites, such as amino acids, organic acids, nucleic acids, and sugar phosphates, are important in many fields, such as biochemistry, fermentation, food science, and drug discovery. Since these compounds have various functional groups, such as amino, carboxyl, hydroxyl, thiol, and phosphate groups, and have different polarities and electrical charges, comprehensive analysis is difficult in a single condition.

In metabolomics studies, two methodologies (i.e., targeted analysis and nontargeted metabolic profiling) are presented for analyzing metabolites. Targeted analysis means the quantitative determination of a selected number of predefined metabolites (*1*). Nontargeted metabolic profiling means a comprehensive analysis, and the data acquired are evaluated by statistical analyses such as a principal components analysis (PCA) (*2*−*6*). This indicates changes in the metabolic profile of a living system following a toxic insult or due to a disease and also identifies the components that correspond to the change. For the two methodology types, several analytical methods have been developed.

Combinations of mass spectrometry (MS) with a separation technique, such as gas chromatography (GC), capillary electrophoresis (CE), and high-performance liquid chromatography (HPLC), have also been widely used for analyses of hydrophilic metabolites. MS provides high sensitivity and selectivity in complex matrixes and can identify the unknown metabolite.

GC enables the separation of many classes of hydrophilic compounds as well as hydrophobic ones and their identification by molecular masses and fragmentation (*7*). However, almost

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10.1021/jf061955p CCC: $37.00
Published on Web 01/09/2007

all compounds, especially hydrophilic ones, have to be derivatized to increase their volatility before a gas chromatography mass spectrometry (GC−MS) analysis. A derivatization approach is often not acceptable, because of the time-consuming sample preparation and the restriction to compounds including a specific functional group.

CE, which separates molecules based on their charge under an electrical field, is suitable for the separation of hydrophilic compounds. The capillary electrophoresis mass spectrometry (CE−MS) methods have been reported for many classes of hydrophilic compounds, such as amino acids, organic acids, sugar phosphates, and nucleotides (*8*−*10*). CE−MS has the advantage that most hydrophilic compounds can be analyzed without derivatization. However, the sensitivity of CE is very poor.

HPLC has become the dominant analytical tool for analyses of metabolites and pharmaceuticals. HPLC with a revesed-phase column is suitable for routine analyses, because it has the advantages of robust operation and usability. HPLC with an octadecyl (C18)-bonded silica stationary phase is the most versatile separation method for analyses of not only small molecules but also large ones such as proteins. Many liquid chromatography mass spectrometry (LC−MS) applications, using the combination of a C18 column with volatile mobile phases, have been reported for analyte detection and determination. However, hydrophilic compounds are poorly retained on a C18 column. Specific derivatization methods of the functional groups have been developed to increase their hydrophobicity (*11*−*15*). For this reason, most of the reported metabolomics studies by LC−MS focused mainly on hydrophobic and relatively hydrophilic compounds that were retained on a C18 column. Ion-exchange chromatography is preferred over reversed-phase chromatography for the retention and separation of hydrophilic compounds without derivatization. However, nonvolatile aqueous solvents are used for the mobile phase of ion-exchange chromatography because of their strong ionic strength, and the eluent cannot be introduced directly into a mass spectrometer without a special interface for desalting between HPLC and MS (*16*). Therefore, we developed a novel online desalting system that could facilitate LC−MS metabolomics studies by focusing on highly hydrophilic compounds.

In this article, we describe a novel LC−MS method that we developed for the comprehensive determination of hydrophilic compounds, by investigating several types of reversed-phased columns. We analyzed highly hydrophilic compounds, such as amino acids, amines, organic acids, nucleic bases, nucleosides, and nucleotides without derivatization. This method was optimized and then validated. Furthermore, the suitability of this method for metabolomics studies was demonstrated by analyzing soy sauce in targeted analysis and nontargeted metabolic profiling with PCA.

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, amines, organic acids, nucleic bases, nucleosides, and nucleotides were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), Tokyo Chemical Industrial (Tokyo, Japan), and Wako Chemicals (Osaka, Japan). Distilled water was prepared with a Milli-Q system (Millipore, Bedford, MA). Ten kinds of soy sauces were obtained from Japanese suppliers. Valine-d_8, in which all of the unexchangeable hydrogen atoms were substituted with deuterium, was purchased from Isotec (Miamisburg, OH). Methylsuccinic acid was purchased from Wako Chemicals (Osaka, Japan).

Sample Preparation. Amino acids, amines, organic acids nucleic bases, nucleosides, and nucleotides were prepared at a concentration of 100 μmol/L in either distilled water, 0.1% aqueous formic acid, or a 50% acetonitrile aqueous solution. Valine-d_8 was the internal standard in the positive mode, and methylsuccinic acid was the internal standard in the negative mode. Val-d_8 and methylsuccinic acid were prepared at concentrations of 20 and 100 μmol/L in distilled water, respectively. Soy sauces were diluted to 100-fold or 1000-fold with distilled water and then were filtered through a Millipore 5000 cutoff centrifugal filter. All samples were finally diluted 1:1 with an aqueous solution of the internal standard and then injected onto the analytical column.

HPLC Columns. We used a Discovery HS-F5 column (250 mm × 4.6 mm i.d., 5 μm), in which the stationary phase was pentafluorophenylpropyl (PFPP)-bonded silica, from Supelco (Bellefonte, PA); an Inertsil Phe-3 column (250 mm × 4.6 mm i.d., 5 μm) in which the stationary phase was phenyl-bonded silica, from GL Science (Tokyo, Japan); and a Develosil RPAQUEOUS-AR-5 column (250 mm × 4.6 mm i.d, 5 μm), in which the stationary phase was triacontyl (C30)-bonded silica, from Nomura Chemicals (Seto, Japan).

LC−MS Apparatus. For the targeted analysis, LC−MS was carried out using an Agilent 1100 Series LC/MSD Quadrupole SL mass spectrometer (Agilent Technologies, Waldbronn, Germany). For the nontargeted metabolic profiling, LC−MS was carried out using a Shimadzu LC system (Kyoto, Japan) and a Q-Tof-2 mass spectrometer (Micromass, Manchester, UK).

HPLC Conditions. The eluting solvent A consisted of 0.1% aqueous formic acid, and the eluting solvent B was acetonitrile. A gradient elution was adopted for all of the chromatographic separations, using the three kinds of columns described above. After a 15-min isocratic run at 100% of the eluting solvent A, the ratio of eluting solvent B was linearly increased to 30% from 15 to 35 min, and to 50% from 35 to 40 min. The composition of 50% of the eluting solvent B was maintained for 22 min. The column was washed with 100% of the eluting solvent B for 10 min, and the column equilibration was carried out with 100% of the eluting solvent A for 20.5 min. A 5-μL aliquot of the sample solution was injected onto the column. The flow rate was 0.3 mL/min, and the column temperature was maintained at 40 °C throughout the analysis. The column holdup time was estimated by monitoring the first MS signal disturbance upon an injection (*17*). The holdup time of each column was approximately 6.1 min.

MS Conditions. For the targeted analysis, a quadrupole mass spectrometer with an electrospray interface was operated at unit mass resolution in either the positive or negative mode. The fragmentor and capillary voltages of the interface were set at 70 and 3500 V. Other interface settings were as follows: drying gas flow rate, 12 L/min; vaporizing temperature, 400 °C; and nebulizing pressure, 35 psi. The same conditions were used in both the positive and negative ion modes. Amino acids, amines, nucleic bases, and nucleosides were detected in the positive ion mode, and organic acids and nucleotides were detected in the negative ion mode. Data were collected in the selected ion monitoring mode with window widths of 0.3 amu. To detect 137 components and two internal standards, we prepared five sets of methods including individual 30 ion channels. These methods were applied to both experiments of validation using standards and application to matrix sample. The Chemstation program (revision 08. 04, Agilent Technologies, Waldbronn, Germany) was used for data collection and analysis. The effluent from a column was introduced into the mass spectrometer. For the nontargeted metabolic profiling, a quadrupole time-of-flight mass spectrometer with an electrospray interface was operated in a positive ion mode. The capillary voltage was maintained at 3.0 kV. The voltages of the sample cone, the collision, and the multichannel plate detector were set to 20, 4.0, and 2200 V, respectively. The flow rates of the nebulizer gas and the cone gas were adjusted to 500 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 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, UK) was used for data collection and analysis. The effluent from a column was split, and an aliquot comprising two-thirds of it was introduced into the mass spectrometer.

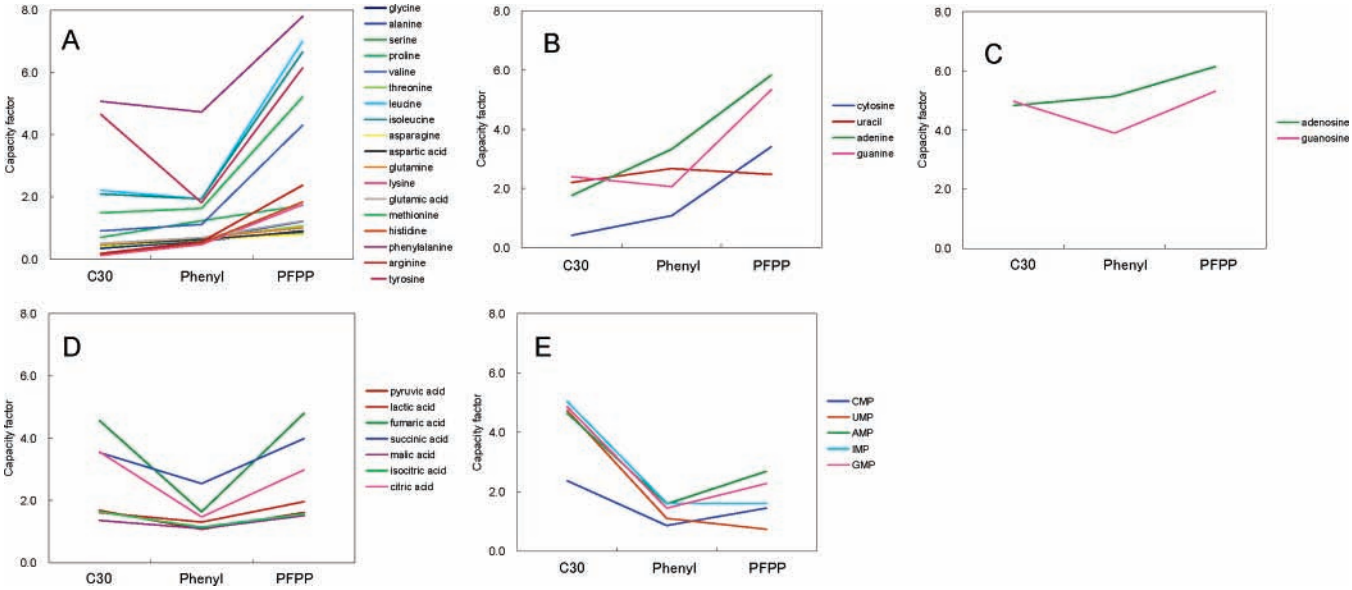


Figure 1. Comparison of capacity factor values obtained from (**A**) amino acids, (**B**) nucleic bases, (**C**) nucleosides, (**D**) organic acids, and (**E**) nucleotides on the C30, phenyl, and PFPP-bonded silica columns.

Amino Acid Analysis. An L-8800 amino acid analyzer (Hitachi High-Technologies Co., Tokyo, Japan) was also used for the quantitative analysis of amino acids in the sampled soy sauces to verify the accuracy of the targeted analysis by our method. 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.

Method Validation. The method validation was achieved by using three different standard solutions. The standard amino acid solution consisted of serine, proline, aspartic acid, glycine, threonine, alanine, glutamic acid, lysine, histidine, arginine, valine, methionine, tyrosine, isoleucine, leucine, and phenylalanine. The standard organic acid solution consisted of pyruvic acid, lactic acid, fumaric acid, succinic acid, malic acid, isocitric acid, and citric acid. The standard nucleic base and nucleoside solution consisted of cytosine, uracil, adenine, and guanosine. Each solution was prepared at concentrations of 0.01, 0.1, 0.5, 1.0, 5, 10, 50, and 100 μmol/L for the evaluation of linearity. Blanks were also prepared for each curve but were not included in the regression analysis. The precisions of the peak area and the retention time were evaluated by analyzing three replicates of 10 μM standard solutions. The detection limit of each compound was determined at a signal-to-noise ratio of three. The values were also obtained by analyzing three replicates. To evaluate the accuracy, we compared the concentrations of amino acids in soy sauce, measured by our LC−MS method, with those obtained with an amino acid analyzer. For other detected compounds, such as organic acids and nucleic bases, the recovery rates were calculated by adding known amounts of the standard solution to the soy sauce.

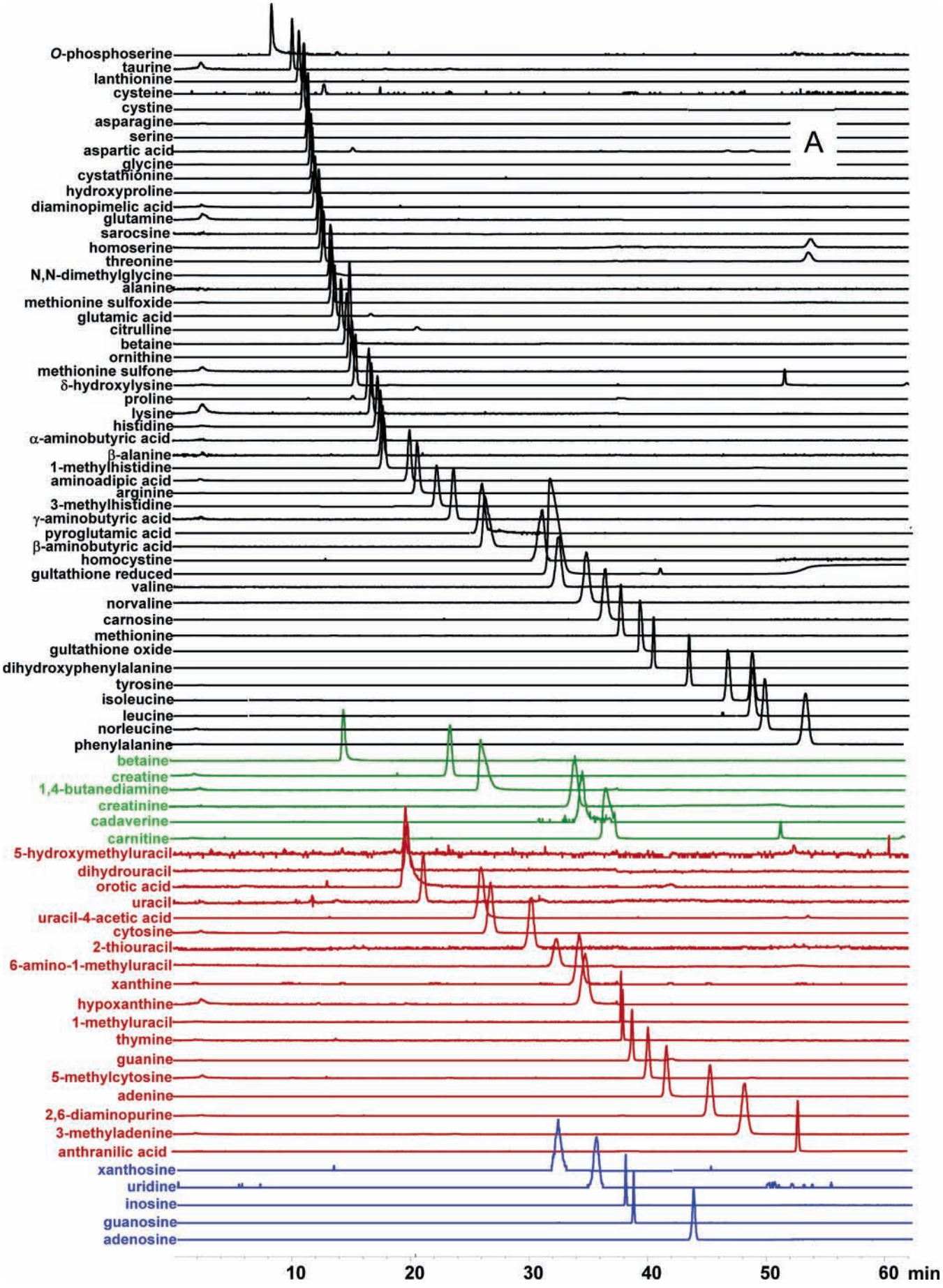
Statistical Analysis. The raw data from LC−MS, using the Q-Tof-2 mass spectrometer, were analyzed by the Micromass MarkerLynx Applications Manager program, version 1.0 (Micromass, Manchester, UK). After all of the detected peaks were subjected to noise reduction in both the HPLC and MS domains, a list 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

Separation of Hydrophilic Metabolites. These data were acquired with an Agilent 1100 Series LC/MSD Quadrupole SL mass spectrometer. **Figure 1** provides a comparison of the capacity factors (k') obtained for 18 kinds of amino acids, seven kinds of organic acids, four kinds of nucleic bases, two kinds of nucleosides, and five kinds of nucleotides on the C30, phenyl, and PFPP-bonded silica columns. All of the amino acids, the nucleic bases, except uracil, the nucleosides, and the organic acids, except citric acid, achieved higher retention on the PFPP-bonded silica column than on the C30 and phenyl columns. Uracil and citric acid were also sufficiently retained on the column, and their k' values were 2.46 and 2.97, respectively. Nucleotides were also retained on the PFPP-bonded silica column, although their k' values on the column were poorer than those on the C30 and phenyl-bonded silica columns.

Selective ion chromatograms of 49 kinds of amino acids, 6 kinds of amines, 45 kinds of organic acids, 18 kinds of nucleic bases, 5 kinds of nucleosides, and 14 kinds of nucleotides on a PFPP-bonded silica column as the stationary phase, with gradient elution using 0.1% aqueous formic acid and acetonitrile as the mobile phase in both the positive and negative ion modes, are shown in **Figure 2A**,**B**, respectively. Their retention times are summarized in **Table 1**. The elution order of the metabolites detected in the positive ion mode followed highly hydrophilic amino acids, basic amino acids, amines, nucleic bases, nucleosides, and neutral amino acids. That detected in the negative mode followed short-chain organic acids, nucleotides, long-chain organic acids, and aromatic organic acids.

Method Validation. These data were acquired with an Agilent 1100 Series LC/MSD Quadrupole SL mass spectrometer using standard solutions. The linearity, the precision of the peak areas, and the sensitivity of the developed LC−MS method are summarized in **Table 2**. The correlation factors of all of the compounds detected in the positive mode were more than 0.997, and those detected in the negative mode were more than 0.995. The linear dynamic range of each compound encompassed about 3 or 4 orders of magnitude. The relative standard deviation (RSD) values of the peak areas (10 μmol/L solution) were between 0.3 and 4.0% in the positive mode and were between 1.3 and 8.0% in the negative mode. The variations of the retention times in three replicated runs were within 0.1 min. The detection limits of amino acids were in the range from 2 to 18 nmol/L, at a signal-to-noise ratio of three. Those of organic acids and nucleic bases were in the range from 15 to 39 nmol/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glycine
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glutamine
sarocsine
homoserine
threonine
N,N-dimethylglycine
alanine
methionine sulfoxide
glutamic acid
citrulline
betaine
ornithine
methionine sulfone
δ-hydroxylysine
proline
lysine
histidine
α-aminobutyric acid
β-alanine
1-methylhistidine
aminoadipic acid
arginine
3-methylhistidine
γ-aminobutyric acid
pyroglutamic acid
β-aminobutyric acid
homocystine
gultathione reduced
valine
norvaline
carnosine
methionine
gultathione oxide
dihydroxyphenylalanine
tyrosine
isoleucine
leucine
norleucine
phenylalanine
betaine
creatine
1,4-butanediamine
creatinine
cadaverine
carnitine
5-hydroxymethyluracil
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guanine
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adenine
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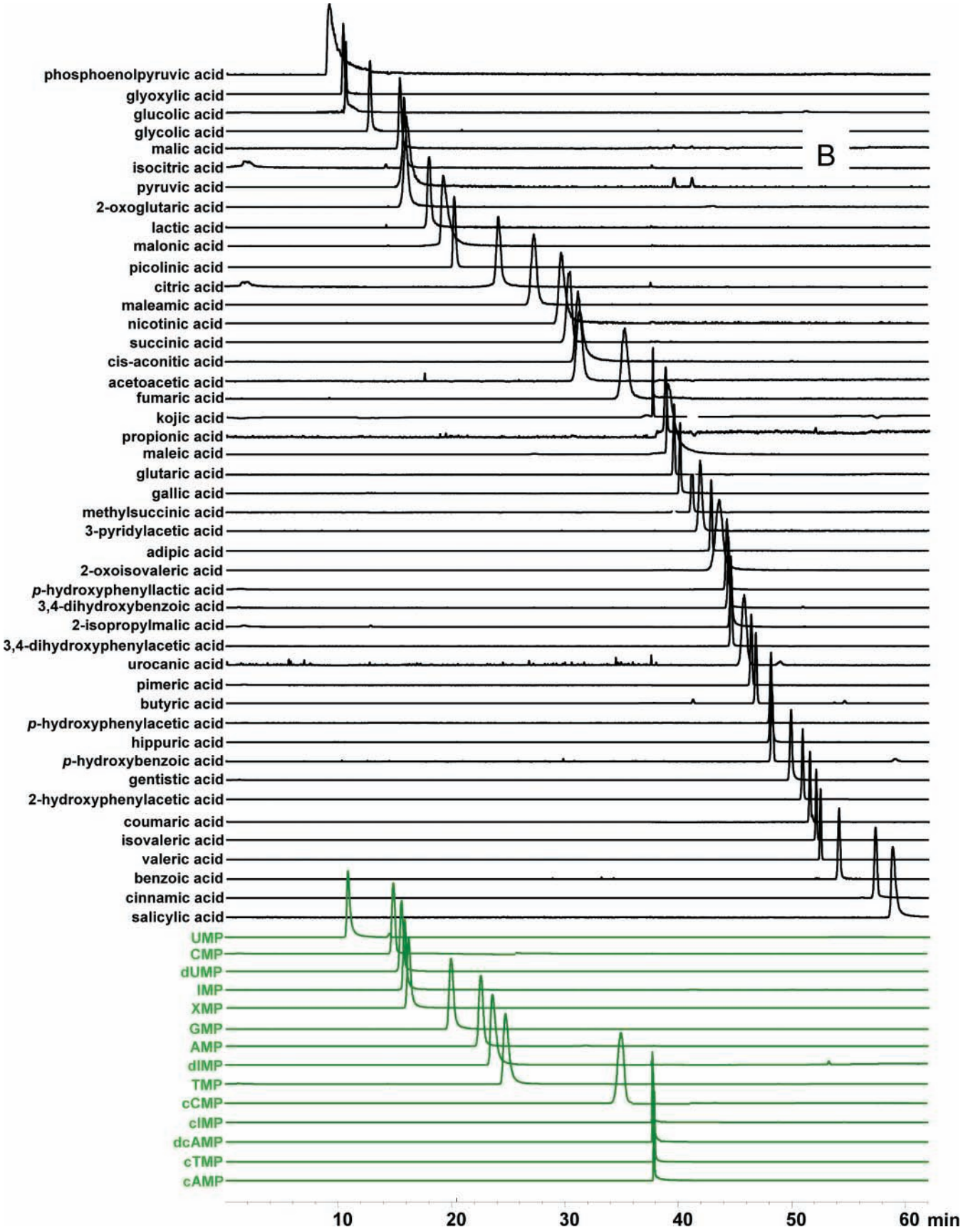

Figure 2. Selective ion chromatograms for the standard solution of hydrophilic metabolites in the positive ion mode (**A**) and in the negative mode (**B**). The data in this section were acquired with an Agilent 1100 Series LC/MSD Quadrupole SL mass spectrometer. Details of the conditions are described in the Materials and Methods.

Table 1. Retention Time of Hydrophilic Metabolites with the LC–MS Method Featuring a PFPP-Bonded Stationary Phase

amino acids and related compounds	rt		rt		rt
O-phosphoserine	8.6	alanine	13.4	pyroglutamic acid	25.7
taurine	10.0	methionine sulfoxide	13.4	β-aminobutyric acid	26.3
lanthionine	10.6	glutamic acid	13.6	homocystine	30.9
cystine	10.9	citrulline	14.2	glutathione reduced	31.2
cysteine	12.8	ornithine	14.6	valine	32.3
asparagine	11.3	methionine sulfone	15.1	norvaline	34.8
serine	11.3	δ-hydroxylysine	15.3	carnosine	36.1
aspartic acid	11.5	proline	16.6	methionine	37.8
glycine	11.6	lysine	16.7	glutathione oxide	39.4
cystathionine	11.7	histidine	17.3	dihydroxyphenylalanine	40.5
hydroxyproline	11.7	α-aminobutyric acid	17.5	tyrosine	43.5
diaminopimelic acid	11.9	β-alanine	17.7	isoleucine	46.7
glutamine	12.2	1-methylhistidine	18.0	leucine	48.8
sarcosine	12.3	aminoadipic acid	20.0	norleucine	49.9
homoserine	12.6	arginine	20.6	phenylalanine	53.6
threonine	12.7	3-methylhistidine	22.3		
N,N-dimethylglycine	13.3	γ-aminobutyric acid	23.7		
Organic Acids	rt		rt		rt
phosphoenolpyruvate	9.1	*cis*-aconitic acid	31.1	3,4-dihydroxyphenylacetic acid	44.6
glyoxylic acid	10.4	acetoacetic acid	31.2	urocanic acid	45.6
glucolic acid	10.6	fumaric acid	35.3	pimeric acid	46.3
glycolic acid	12.8	kojic acid	37.6	butyric acid	46.8
malic acid	15.4	propionic acid	38.7	*p*-hydroxyphenylacetic acid	48.1
isocitric acid	15.7	maleic acid	39.1	hippuric acid	48.1
pyruvic acid	15.9	glutaric acid	39.6	*p*-hydroxybenzoic acid	48.2
2-oxoglutaric acid	15.9	gallic acid	40.2	gentisic acid	49.8
lactic acid	18.0	methylsuccinic acid	41.1	2-hydroxyphenylacetic acid	50.9
malonic acid	19.1	3-pyridylacetic acid	41.8	coumaric acid	51.5
picolinic acid	20.0	adipic acid	42.9	isovaleric acid	52.1
citric acid	24.2	2-isovaleric acid	43.5	valeric acid	52.4
maleamic acid	27.3	*p*-hydroxyphenyllactic acid	44.2	benzoic acid	54.1
nicotinic acid	29.6	3,4-dihydroxybenzoic acid	44.3	cinnamic acid	57.3
succinic acid	30.4	2-isopropylmalic acid	44.5	salicylic acid	58.8
Amines	rt		rt		rt
betaine	14.5	1,4-butanediamine	26.2	cadaverine	34.7
creatine	23.6	creatinine	34.2	carnitine	36.8
nucleic bases	rt		rt		rt
5-hydroxymethyluracil	19.6	2-thiouracil	30.3	guanine	38.7
dihydrouracil	19.8	6-amino-1-methyluracil	32.3	5-methylcytosine	40.0
orotic acid	19.8	xanthine	34.3	adenine	41.6
uracil	21.1	hypoxanthine	34.9	2,6-diaminopurine	45.3
uracil-4-acetic acid	26.0	1-methyluracil	37.8	3-methyladenine	48.3
cytosine	26.8	tymine	37.9	anthranilate	52.8
Nucleosides	rt		rt		rt
xanthosine	32.2	inosine	37.9	adenosine	43.5
uridine	35.4	guanosine	38.5		
Nucleotides	rt		rt		rt
UMP	10.5	GMP	19.9	cIMP	37.7
CMP	14.9	AMP	22.5	dcAMP	37.8
dUMP	15.6	dIMP	23.6	cTMP	37.8
IMP	15.8	TMP	24.8	cAMP	37.8
XMP	16.1	cCMP	35.0		

L, and from 3 to 32 nmol/L, respectively. The detection limit of guanosine was 1 nmol/L.

Targeted Analysis and Verification of Accuracy in Its Experiment. Targeted analysis of a typical soy sauce was carried out by the developed LC−MS method. These data were acquired with an Agilent 1100 Series LC/MSD Quadrupole SL mass spectrometer. Twenty-four compounds were detected by comparison with these 137 standard samples described above and are listed in **Tables 3** (amino acids) and **4** (others). To verify the accuracy of the quantification, two experiments were performed. One compared the concentrations of amino acids calculated by the developed LC−MS method and the amino acid analyzer. There were no significant differences between them. The amino acid with the most different concentration between the two methods was threonine, with a ratio of 1:1.2. Another way to verify the accuracy is to evaluate the recovery rates of other detected compounds, such as organic acids and bases. Their recovery rates are listed in **Table 4**. They were in the range between 105 and 133%, and the RSD values of the peak areas were in the range between 0.4 and 4.3%.

Nontargeted Metabolic Profiling Coupled to PCA for Soy Sauce. The data in this section were acquired with a LC-Q-

Table 2. Validation Results of the Developed LC–MS Method[a]

compound	correlation factors	range (μM)	RSD of peak area (%)	detection limit (nM)
glycine	0.9996	0.1–50	0.9	4
alanine	0.9994	0.01–50	0.3	6
serine	0.9994	0.1–50	0.3	9
proline	0.9993	0.1–50	0.4	14
valine	1.0000	0.01–50	0.5	2
threonine	0.9994	0.1–50	1.4	14
leucine	0.9996	0.01–50	0.6	3
isoleucine	0.9996	0.01–50	0.8	3
aspartic acid	0.9995	0.1–50	0.9	7
lysine	0.9996	0.1–50	1.7	11
glutamic acid	0.9994	0.1–50	1.0	10
methionine	0.9996	0.1–50	1.7	18
histidine	0.9996	0.1–50	0.3	3
phenylalanine	0.9995	0.01–50	0.9	2
arginine	0.9994	0.1–50	0.4	4
tyrosine	0.9996	0.01–50	1.3	2
cytosine	0.9994	0.1–50	1.6	8
uracil	0.9985	0.1–50	4.0	32
adenine	0.9993	0.01–5	1.2	3
guanosine	0.9977	0.01–5	2.4	1
pyruvic acid	0.9991	0.1–100	7.6	30
lactic acid	0.9977	0.1–100	4.1	39
fumaric acid	0.9969	0.1–100	8.0	30
succinic acid	0.9958	0.1–100	1.7	20
malic acid	0.9960	0.1–50	3.0	25
isocitric acid	0.9950	0.1–100	1.6	27
citric acid	0.9958	0.1–100	1.3	15

[a] The data were acquired with an Agilent 1100 Series LC/MSD Quadrupole SL mass spectrometer.

Table 3. Amino Acid Concentrations in a Soy Sauce Sample, Measured by the Developed LC–MS Method and by an Amino Acid Analyzer

compound	concentration (mM)	
	LC–MS	amino acid analyzer
glycine	34	29
alanine	46	47
serine	41	35
proline	38	33
valine	29	30
threonine	25	21
leucine	40	42
isoleucine	25	25
aspartic acid	50	47
lysine	25	23
glutamic acid	70	82
methionine	7	6
histidine	6	6
phenylalanine	22	20
arginine	19	16
tyrosine	3	3

Tof-2 mass spectrometer. **Figure 3** shows a typical total ion chromatogram of a soy sauce. PCA was performed on the entire data set of 10 kinds of soy sauces, which were analyzed by the developed method. The data shown in **Figures 4** and **5** are a score plot and a loading plot obtained from PCA of soy sauce samples, respectively. The score plot revealed that the soy sauce samples could be clearly divided into two groups.

From the loading plot, the ions with the most influence on the grouping were found to be at *m*/*z* 148.05 and 134.04, which eluted at 12.5 and 11.0 min, respectively. The ions at *m*/*z* 175.11 and 325.11, eluting at 17.4 and 10.2 min, respectively, also influenced the group cluster, although their contributions were much lower.

Table 4. Results of the Spike Recovery Experiment

compound	concentration (mM)	recovery rate (%)	RSD (%)
uracil	2	108	0.6
cytosine	1	127	4.0
pyroglutamic acid	15	105	0.7
guanine	2	125	0.4
malic acid	10	133	4.3
lactic acid	21	105	1.2
citric acid	17	117	0.7
succinic acid	9	126	0.4

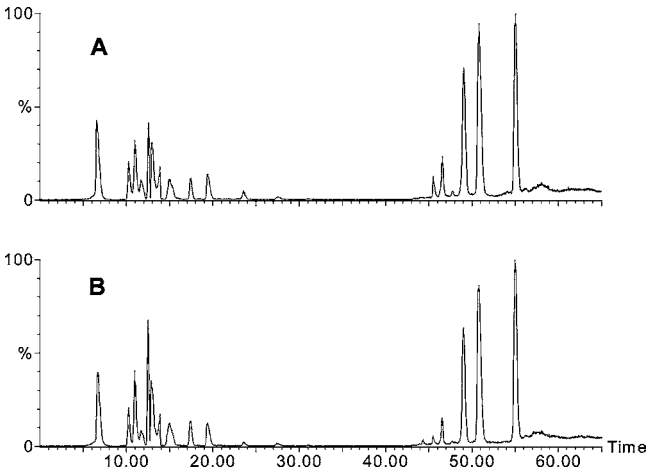

Figure 3. Typical total ion chromatograms in the positive mode of soy sauce derived from a basic fermentation process (**A**), and a mixture of some fermented soy sauces and those with hydrolyzed, defatted soybean meals (**B**). The data in this section were acquired with a LC-Q-Tof-2 mass spectrometer. Chromatographic conditions are described in the Materials and Methods.

Figure 6**A** shows the fragment ions eluting at 12.5 min, which were generated by collision-induced dissociation of the mass. A composition formula of $C_5H_{10}NO_4$ was estimated from the accurate mass of *m*/*z* 148.0612, and the fragment pattern allowed us to assign the peak as glutamic acid (**Figure 6B**). The peaks at *m*/*z* 134.0459, eluting at 11.0 min, and *m*/*z* 175.1196, eluting at 17.4 min, were also identified as aspartic acid and arginine, respectively. Unfortunately, the ion of *m*/*z* 325.1138, eluting at 10.2 min, could not be identified.

DISCUSSION

To develop a method for the comprehensive LC−MS analysis of hydrophilic metabolites, including amino acids, amines, organic acids, nucleic bases, nucleosides, and nucleotides, we evaluated three types of stationary phases with characteristic modifications, instead of the most popular stationary phase C18, on which hydrophilic metabolites are poorly retained.

The C30 column has a longer hydrophobic alkyl chain, which provides an advantage for greater hydrophobic interaction than the C18 column. In addition, the C30 stationary phase has higher retention reproducibility under 100% aqueous mobile phase conditions (*18*, *19*). A phenyl-bonded silica column provides not only hydrophobic interactions with analytes but also $\pi-\pi$ interactions. The interaction between the phenyl groups on the stationary phase and aromatic compounds, such as aromatic amino acids and nucleic bases, can contribute to both good retention and more specific selectivity. A PFPP-bonded silica column provides good retention of basic analytes, as compared

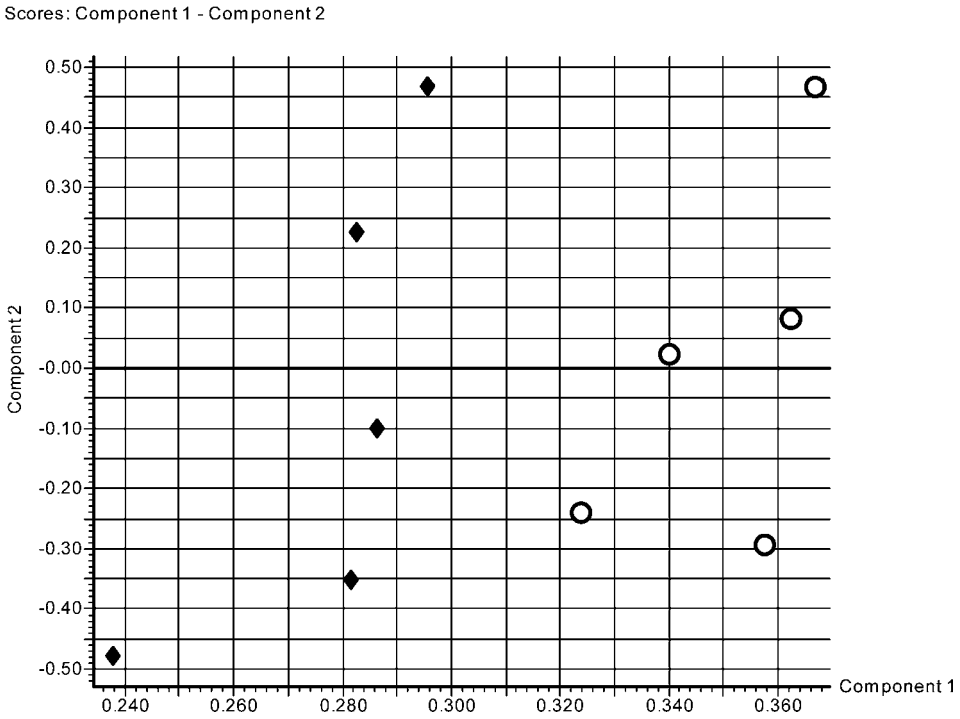

Figure 4. PCA score plot from the LC–MS results of 10 kinds of soy sauces. (◆) Basic fermentation process. (○) Mixture of some fermented soy sauce samples and those with hydrolyzed, defatted soybean meal.

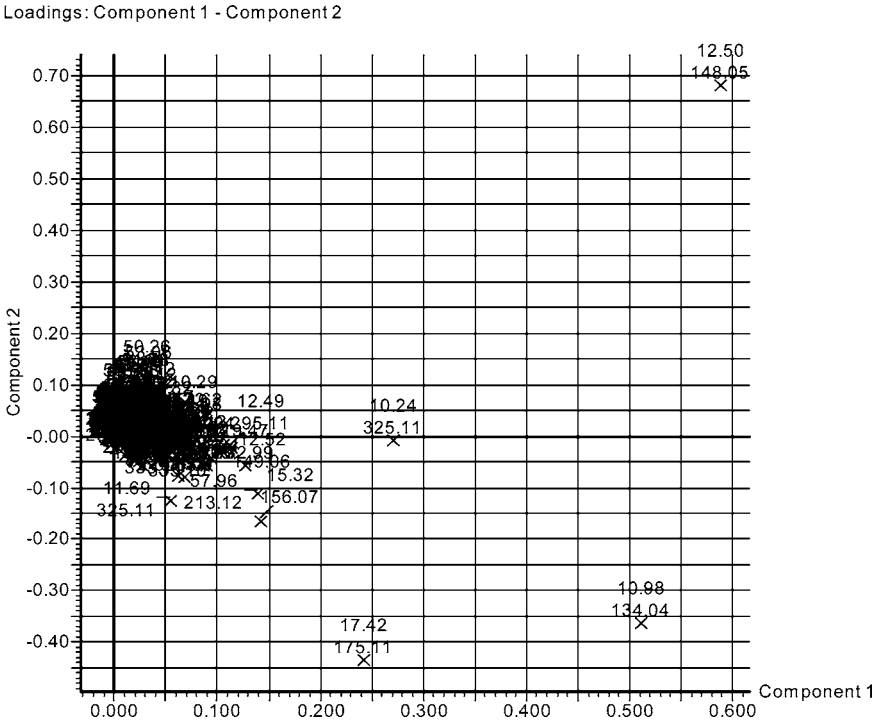

Figure 5. PCA loading plot from the LC–MS results of 10 kinds of soy sauces.

to the popular C18 and octyl-bonded silica stationary phases (*20*). Basic analytes are separated by both the reversed-phase mode, with an alkyl chain, and the ion-exchange mode, with an ionized surface silanol (*21*). In addition, this column provides hydrophobic and $\pi-\pi$ interactions with the analytes. We chose typical intracellular metabolites (i.e., 18 kinds of amino acids, seven kinds of organic acids, four kinds of nucleic bases, two kinds of nucleosides, and five kinds of nucleotides) to evaluate the stationary phases. Valine-d_8 and methylsuccinic acid were chosen as the internal standards for the normalization of peak intensity for each LC−MS run, because they had modest retention times under our chromatographic conditions.

Amino acids, nucleosides, almost all organic acids, and almost all nucleic bases showed better retention on the PFPP-bonded silica column than on the C30 and phenyl-bonded silica columns. Since the amino acids acted as basic analytes under 0.1% aqueous formic acid mobile phase conditions, it was conceivable that the amino acids were strongly retained on the PFPP-bonded silica column. Nucleotides were poorly retained on the PFPP-bonded silica column because of the dissociation

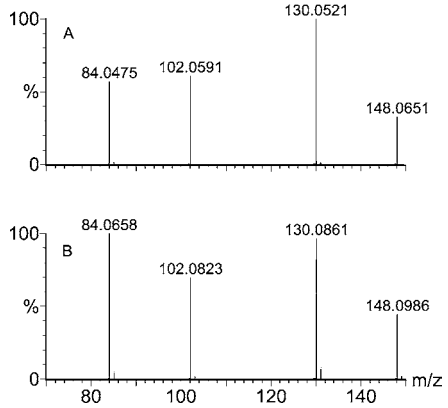

Figure 6. Fragment patterns of the peak at *m*/*z* 148.0612, eluting at 12.5 min (**A**) and glutamic acid (**B**).

of the phosphate group. As their *k*′ values, except for that of uridine monophosphate, were more than 1.4, they were sufficiently retained in the stationary phase. Therefore, we adopted PFPP-bonded silica as the stationary phase, with gradient elution using 0.1% aqueous formic acid and acetonitrile as the mobile phase, for the comprehensive analysis of hydrophilic metabolites.

The precision of the peak areas of all of the compounds, except for uracil, pyruvic acid, lactic acid, and fumaric acid, was within 4.0% and was found to be satisfactory. Uracil, pyruvic acid, lactic acid, and fumaric acid were not sensitively detected in comparison with the other metabolites, and this might be the reason for their poorer precisions.

To evaluate the accuracy and to demonstrate the suitability of this method for analyzing matrix samples, two experiments were carried out using a model sample. One experiment compared the concentrations of amino acids in soy sauce with those determined by a traditional analytical method. The other calculated the recovery rates for standard addition. The results of the two experiments revealed that the accuracy was satisfactory in the metabolomics studies and that the signal intensity in LC−MS was minimally influenced by the sample matrix. This developed method is similar to the CE−MS method, in terms of analyses of amino acids and organic acids without derivatization (*8*, *9*). The method achieved approximately 100-fold higher sensitivity, 4-fold better precision of peak area, 6-fold better precision of retention (migration) time, and 3-fold longer analysis time than the CE−MS method (*8*). Although the analysis time is longer, it is conceivable that this developed LC−MS method is better than the CE−MS method, in terms of sensitivity and precision. Therefore, the developed LC−MS method is quite suitable for the comprehensive analysis of hydrophilic metabolites.

To demonstrate that this method could be applied to nontargeted metabolic profiling, focusing on hydrophilic compounds, the combination of the developed LC−MS method and PCA was applied to several kinds of commercially available soy sauces, as a model of a matrix sample. These soy sauces were divided into two groups, based on differences between the manufacturing methods. One was the soy sauces manufactured by a basic fermentation process, in which all hydrolysis is performed by enzymes from microorganisms. The other was the soy sauces composed of a mixture of some fermented and some hydrolyzed, defatted soybean meal. From the loading plot and the structural analysis by MS, the components that were the most responsible for the differences between the manufacturing processes were found to be glutamic acid and aspartic acid. This result is reasonable because large amounts of amino acids, especially glutamic acid and aspartic acid, are present in hydrolyzed, defatted soybean meal (*22*).

In conclusion, the developed LC−MS method was shown to retain small and hydrophilic compounds without any derivatization. We demonstrated the applicability of the developed LC−MS method to the analysis of at least 137 kinds of compounds containing several classes of metabolites. The usefulness of our method was demonstrated by the targeted analysis and nontargeted metabolic profiling of a matrix sample, soy sauces. We believe that this simple LC−MS method will become the first choice for metabolomics studies for hydrophilic metabolites.

ABBREVIATIONS USED

AMP, adenosine 5′-monophosphate; amu, atomic mass units; C18, octadecyl; C30, triacontyl; cAMP, cyclic AMP; CE−MS, capillary electrophoresis mass spectrometry; CMP, cytidine 5′-monophosphate; cCMP cyclic CMP; cIMP, cyclic IMP; cTMP, cyclic TMP; dcAMP, 2′deoxy cyclic AMP; dIMP, 2′ deoxyinosine 5'-monophosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; GC−MS, gas chromatography mass spectrometry; GMP, guanosine 5′-monophosphate; HPLC, high-performance liquid chromatography; IMP, inosine 5′-monophosphate; MS, mass spectrometry; LC−MS, high-performance liquid chromatography mass spectrometry; *k*′, capacity factors; PCA, principal components analysis; PFPP, pentafluorophenylpropyl; rt, retention time; RSD, relative standard deviation; TMP, thymidine 5′-monophosphate; UMP, uridine 5′-monophosphate; XMP, xanthosine 5′-monophosphate.

ACKNOWLEDGMENT

We thank Shin-ichi Ozawa and Junko Yamazaki, of the Institute of Life Sciences, Ajinomoto Co., Inc., for amino acid measurements with the amino acid analyzer.

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Received for review July 13, 2006. Revised manuscript received November 22, 2006. Accepted November 24, 2006.

JF061955P