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Rapid Quantitative Analysis of Metabolites in Kimchi Using LC-Q-Orbitrap MS

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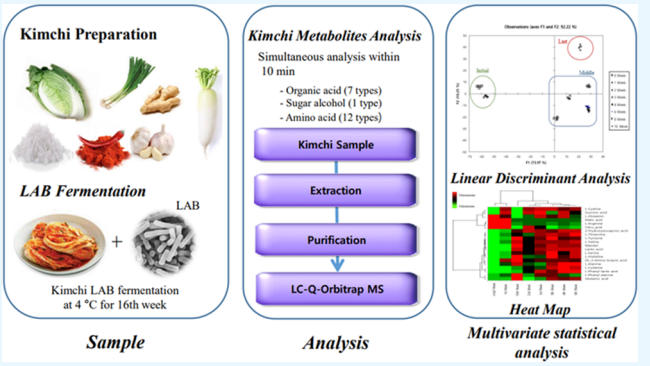
ABSTRACT: Kimchi is a traditional Korean salted spontaneous lactic acid bacteria (LAB)-fermented food made using various vegetables. Organic acids, free sugars, and amino acids are key metabolites produced during LAB fermentation that determine the taste and quality of kimchi. However, each metabolite is typically analyzed using an independent analytical method, which is time-consuming and expensive. Therefore, in this study, we developed a method based on LC-Q-Orbitrap MS using which 20 types of representative fermented kimchi metabolites were selected and simultaneously analyzed within 10 min. The established method was validated, and its detection and quantification limits, linearity, precision, and accuracy were found to satisfy the Association of Official Agricultural Chemists (AOAC) validation guidelines. The 20 metabolites were simultaneously extracted from kimchi with different degrees of fermentation and quantitatively analyzed using LC-Q-Orbitrap MS. These results were analyzed using linear discriminant analysis and heat mapping, and the metabolites were grouped into early, middle, and late stages of fermentation. Malic acid (6.518−7.701 mMol) was only present in the initial stage of fermentation, and L-phenylalanine rapidly increased from the middle stage (2.180 mMol) to late stage (4.770 mMol). Lactic acid, which is representative of the sour taste of kimchi, was detected in the middle stage and increased rapidly up to 74.452 mMol in the late stage. In summary, in this study, 20 major kimchi metabolites were accurately analyzed within 10 min and grouped based on the degree of fermentation. Therefore, the method established in this study accurately and rapidly provides information on kimchi consumption and fermentation that could be highly valuable to the kimchi industry and kimchi consumers.



1. INTRODUCTION

Kimchi is a well-known traditional Korean fermented health food that has been certified as a CODEX standard.[1] The main ingredient in kimchi is cabbage, and other ingredients include radish, green onion, garlic, ginger, fermented shrimp sauce, and chili powder. Kimchi contains several nutrients owing to its ingredients and metabolites formed during fermentation. During the fermentation process, which is mainly mediated by lactic acid bacteria (LAB), namely *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weisella*, a unique flavor is imparted through proteolysis and amino acid catabolism.[2] Furthermore, kimchi confers several health benefits because of its antioxidant,[3] antiobesity,[4] antidiabetic,[5] and anticancer effects.[6]

The major metabolites produced during the fermentation of kimchi by LAB are organic acids such as phenylacetic acid (PLA), γ-aminobutyric acid (GABA), hydroxyisocaproic acid (HICA), and methionine. These substances have functions such as antibacterial,[7] increasing the human growth hormone levels,[8] anti-inflammatory,[9] and improving immune function.[10] Futhermore, the kimchi LAB fermentation process produces a unique taste that is sour, sweet, and umami in harmony and directly related to its quality.[11] Organic acids have a strong influence on the sour taste in kimchi.[12] Mannitol, the representative sugar alcohol of kimchi, imparts a cool, sweet taste to foods, and its sweetness is about half that of sucrose.[13] Additionally, various amino acids affect the umami and flavor of kimchi.[14]

The characteristics of the metabolites in kimchi differ depending on the degree of fermentation.[11,12] Therefore, to evaluate the quality of kimchi, its components, including organic acids, amino acids, free sugars, and sugar alcohols, need to be quantitatively analyzed.

The commonly used equipment for analyzing organic acids, free sugars, and amino acids are a high-performance liquid chromatography−diode array detector (HPLC−DAD), high-

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Table 1. List of Metabolite Compounds and Mass Spectrometry Data

compound name	chemical formula	retention time (min)	molecular weight (g/mol)	adduct	precursor ions (m/z)	NCE[a]	fragments (m/z)[b]
			Organic Acid				
malic acid	$C_4H_6O_5$	3.4	134.1	(M − H)−	133.0143	10	71.0139, 115.0037
lactic acid	$C_3H_6O_3$	3.61	90.1	(M − H)−	89.0245	20	73.0116
succinic acid	$C_4H_6O_4$	6.18	118.1	(M − H)−	117.0194	10	73.0295, 99.0085
citric acid	$C_6H_8O_7$	5.59	192.1	(M − H)−	191.0203	10	87.0087, 111.0088
2-hydroxyisocaproic acid	$C_6H_{12}O_3$	9.41	132.2	(M − H)−	131.0705	12	69.0365, 85.0671
3-phenyl lactic acid	$C_9H_{10}O_3$	9.51	166.2	(M − H)−	165.056	10	72.9931, 119.0502, 147.0452
			Sugar Alcohol				
mannitol	$C_6H_{14}O_6$	2.17	182.2	(M − H)−	181.0699	10	71.0118, 89.0223, 101.0223
			Amino Acid				
L-alanine	$C_3H_7NO_2$	2.31	89.1	(M + H)+	90.05592	15	57.9359,67.9057, 72.9057
L-arginine	$C_6H_{14}N_4O_2$	2.29	174.2	(M + H)+	175.1195	18	60.0567, 70.06618, 116.0714, 130.0981
L-cystine	$C_6H_{12}N_2O_4S_2$	2.31	240.3	(M + H)+	241.0319	13	74.0246, 120.0120, 151.9840, 195.0266
L-cysteine	$C_3H_7NO_2S$	2.32	121.2	(M + H)+	122.0278	10	58.9961, 76.0226, 86.9909, 105.0014
L-glutamine	$C_5H_{10}N_2O_3$	2.32	146.1	(M + H)+	147.0761	10	130.0497
L-serine	$C_3H_7NO_3$	2.38	105.1	(M + H)+	106.0507	10	60.0455, 88.0403
L-threonine	$C_4H_9NO_3$	2.37	119.1	(M + H)+	120.0662	10	56.0506, 74.0611, 102.0558
L-valine	$C_5H_{11}NO_2$	2.94	117.1	(M + H)+	118.0869	10	72.0818
L-phenylalanine	$C_9H_{11}NO_2$	7.48	165.2	(M + H)+	166.0867	20	79.0551, 93.0707, 103.0549, 120.0813
L-tyrosine	$C_9H_{11}NO_3$	6.83	181.2	(M + H)+	182.0819	13	123.0446, 136.0761, 147.0444, 165.0551
L-histidine	$C_6H_9N_3O_2$	2.34	155.2	(M + H)+	156.0774	15	83.0613, 95.0613, 110.0721
D-glutamic acid	$C_5H_9NO_4$	2.32	147.1	(M + H)+	148.0609	10	84.0454, 102.0559, 130.0506
DL-2-aminobutyric acid	$C_4H_9NO_2$	2.31	103.1	(M + H)+	104.0715	15	69.0346, 87.0450
(IS) L-proline-1-^{13}C	$^{13}CC_4H_9O_2N$	2.66	116.1	(M + H)+	117.0739	10	70.0657

[a]NCE: normalized collision energy. [b]Fragments, m/z: the product ion with the highest intensity is underlined.

performance liquid chromatography−refractive index detector (HPLC-RID), and amino acid analyzer, respectively. Metabolites such as amino acids and organic acids are analyzed by liquid chromatography−tandem mass spectrometry (LC-MS/MS). These methods include a preprocessing step, employ a complex instrument, and involve substantial time and effort.[15] Recently, simultaneous quantitative analysis of water-soluble substances, such as amino acids using LC-MS/MS,[16,17] has been reported to reduce the analytical time and cost.

In this study, simultaneous analysis of amino acids, organic acids, and sugar alcohols, which are water-soluble kimchi metabolites, was performed and validated, and the metabolites were profiled in different fermentation stages of kimchi using multivariate statistical analyses.

2. RESULTS AND DISCUSSION

2.1. Optimization of Analytical Methods and Quality Assurance. *2.1.1. Optimization of UPLC-HESI-Q-Orbitap MS Condition.* The ESI-Q-Orbitrap MS condition was optimized as follows. Individual standard solutions were injected directly to optimize the MS/MS parameters. The precusor ions of organic acids and sugar alcohol were selected with high sensitivity in $(M - H)^-$. However, amino acids showed high sensitivity in $(M + H)^+$. Also, the collision energy that generates fragments with high sensitivity was confirmed. All substances were detected within 10 min (Table 1).

Optimization of LC conditions for the separation of analytes was performed as follows. The main column was a Hypersil GOLD C18 column (150 mm × 2.1 mm, i.d. with 3 μm particle diameter, Thermo Fisher Scientific, Waltham, MA), and for the separation efficiency of polar substances, an Accucore aQ polar endcapped column (100 mm × 2.1 mm, 2.6 μm particle diameter, Thermo Fisher Scientific, Waltham, MA) was used by connecting it in front of the main column in parallel to increase the separation efficiency of polar substances. Formic acid in water (0.1% [v/v]) and formic acid in acetonitrile (0.1% [v/v]) were used as the mobile phase, and the flow rate was 0.3 mL/min. Despite having the same retention time, the metabolites were separated through different precursor ions and fragments.[18]

2.1.2. Optimization of Extraction Condition. For simultaneous analysis of various substances, the extraction buffer is important and critical. The solvent buffer is able to dissolve all of the metabolites and is analyzed with high sensitivity using LC-MS/MS. All of the target substances in this study were polar substances; three polar extraction solvents such as distilled water (solvent A), water/methanol mixture (80/20, v/v) (solvent B), and 0.1% formic acid in water/methanol mixture (80/20, v/v) (solvent C) were tested, and the recovery results are shown in Table 2. For the test, extraction conditions were shaking for 5 min and ultrasonic extraction for 20 min. The extraction solvent had to sufficiently dissolve the metabolite in a kimchi sample during the extraction process.[19]

In this study, the recovery rate after the addition of six types of organic acids, one type of sugar alcohol, and 12 types of different amino acids was analyzed. The percentage of the

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Table 2. Average Recovery and Relative Standard Deviation (RSD, %) of Metabolites in Kimchi Using Different Extraction Solvents

compound name	solvent A[a]	solvent B[b]	solvent C[c]
	Organic Acid		
malic acid	63.7 ± 4.7[d]	89.1 ± 3.4	90.7 ± 3.4
lactic acid	77.9 ± 3.5	84.0 ± 5.1	90.4 ± 3.5
succinic acid	87.7 ± 2.9	87.1 ± 2.7	89.3 ± 3.1
citric acid	83.5 ± 3.0	87.6 ± 3.3	90.0 ± 3.5
2-hydroxyisocaproic acid	78.6 ± 3.8	86.4 ± 6.9	87.6 ± 3.6
3-phenyl lactic acid	71.5 ± 6.2	84.8 ± 3.2	88.0 ± 4.9
	Sugar Alcohol		
mannitol	71.1 ± 4.8	80.3 ± 3.4	81.4 ± 3.4
	Amino Acid		
L-alanine	89.0 ± 6.3	86.5 ± 3.4	88.2 ± 3.7
L-arginine	62.2 ± 3.3	86.9 ± 3.5	87.8 ± 3.2
L-cystine	65.8 ± 6.6	83.9 ± 3.4	88.5 ± 3.5
L-cysteine	69.6 ± 3.5	87.9 ± 3.1	90.1 ± 3.4
L-glutamine	85.2 ± 6.3	90.6 ± 3.3	88.2 ± 3.4
L-serine	82.6 ± 6.5	84.2 ± 4.9	85.7 ± 5.1
L-threonine	81.5 ± 3.4	84.8 ± 4.8	87.4 ± 5.0
L-valine	81.8 ± 3.4	82.9 ± 3.4	89.6 ± 3.7
L-phenylalanine	60.9 ± 3.5	74.7 ± 4.9	83.6 ± 3.2
L-tyrosine	70.8 ± 3.2	76.9 ± 3.5	82.8 ± 3.4
L-histidine	89.7 ± 3.3	88.9 ± 3.2	87.4 ± 3.0
D-glutamic acid	75.2 ± 3.3	79.5 ± 5.1	90.0 ± 3.5
DL-2-aminobutyric acid	83.1 ± 3.6	85.1 ± 7.0	85.0 ± 3.3

[a]Solvent A: water. [b]Solvent B: water/methanol (80/20, v/v). [c]Solvent C: 0.1% formic acid in water/methanol (80/20, v/v). [d]Values are mean ± standard deviations of three (n = 3) measurements of 1 Mmol of standard solution added to the sample.

recovery of the six types of organic acids using water, water/methanol (80/20, v/v), and 0.1% formic acid in water/methanol (80/20, v/v) was 63.7−87.7%, 24.0−89.1%, and 89.3−90.7%, respectively. The recovery of mannitol, a sugar alcohol, was 71.1%, 80.3%, and 81.4% for solvents A, B, and C, respectively. In the case of 12 types of amino acids, 60.9−89.7% was confirmed in solvent A, whereas 74.7−90.6% and 82.8−90.4% in solvents B and C, respectively.

Solvent B showed a relatively higher recovery rate than solvent A owing to better spraying efficiency in LC-MS/MS.[20] Furthermore, solvent C showed higher recovery than solvent B because of the number of potential charge centers and hydrogen-bonding acceptor capacity by formic acid leading to high ionization efficiency.[21] Therefore, solvent C was selected as the optimal extraction buffer for various metabolites extracted and simultaneous analysis by LC-MS/MS.

2.1.3. Validation of Analytical Methods and Quality Assurance. Table 3 shows the quality parameters evaluated for kimchi metabolite analysis using the optimized method in LC-MS/MS. All metabolites were detected within 9.51 min: organic acids at 3.40−9.51 min, sugar alcohol at 2.17 min, and amino acids at 2.29−7.48 min. The equation of the standard materials was dependent on the sensitivity of each metabolite. As determined from the calibration curve, all of the correlation coefficients (R2) were over 0.990. The correlation coefficients were over 0.9931, 0.9937, and 0.9907 for organic acids, sugar alcohol, and amino acid, respectively. All CV% values of metabolites were under 10%; for organic acids, sugar alcohols, and amino acids, they were less than 9.51, 2.17, and 7.8%, respectively. The LOD values were in the range of 3.7−44.2 μmol for organic acids, 25.9 μmol for sugar alcohol, and 2.3−122.6 μmol for amino acids. Also, the LOQ values were in the range of 11.1−132.6 μmol for organic acids, 77.7 μmol for sugar alcohol, and 6.9−367.9 μmol for amino acids. Recovery experiments were performed by adding three different concentrations (0.1 mmol, 1 mM, and 10 mM) of each metabolite standard solution to three different fermented states (initial week, 4th week, and 16th week) of the kimchi sample.

Table 3. Method Validation Parameters of the Instrument (UHPLC-Q-Orbitrap-MS) for Metabolite Determination

compound name	LOD (mMol)	LOQ (mMol)	CV %	linearity	retention time (min)	equation
			Organic Acid			
malic acid	0.026	0.078	6.6	0.9963	3.4	$Y = 171.26X + 150.64$
lactic acid	0.044	0.133	4.1	0.9977	3.61	$Y = 246.75X - 2023.8$
succinic acid	0.014	0.043	3.9	0.9931	6.18	$Y = 319.62X + 337.94$
citric acid	0.004	0.011	5.8	0.9982	5.59	$Y = 388.21X - 1357.9$
2-hydroxyisocaproic acid	0.014	0.043	6.5	0.998	9.41	$Y = 281.33X - 674.9$
3-phenyl lactic acid	0.017	0.05	5.4	0.9918	9.51	$Y = 8644.5X - 225.13$
			Sugar Alcohol			
mannitol	0.026	0.078	5.6	0.9937	2.17	$Y = 1080.8X + 1820.3$
			Amino Acid			
L-alanine	0.004	0.011	1.9	0.9954	2.31	$Y = 777.6X + 1347$
L-arginine	0.009	0.026	4.1	0.9984	2.29	$Y = 684.48X + 28.979$
L-cystine	0.007	0.021	7.6	0.9969	2.31	$Y = 4.338X + 36.534$
L-cysteine	0.002	0.007	5.6	0.9907	2.32	$Y = 70.076X + 26.143$
L-glutamine	0.034	0.103	4.7	0.9905	2.32	$Y = 221.62X + 2496.5$
L-serine	0.008	0.024	7.8	0.9913	2.38	$Y = 99.212X + 292.27$
L-threonine	0.011	0.034	4.5	0.9925	2.37	$Y = 295.01X - 21.246$
L-valine	0.051	0.153	7.0	0.9983	2.94	$Y = 2730.1X + 3904.2$
L-phenylalanine	0.005	0.015	6.6	0.9993	7.48	$Y = 67.271X + 190.58$
L-tyrosine	0.006	0.019	3.4	0.9986	6.83	$Y = 381.98X + 1157.9$
L-histidine	0.123	0.368	4.3	0.9961	2.34	$Y = 67.58X + 138.28$
D-glutamic acid	0.035	0.106	3.5	0.9944	2.32	$Y = 1169X + 823.68$
DL-2-aminobutyric acid	0.065	0.193	5.2	0.9931	2.31	$Y = 5002.8X - 2043.3$

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Table 4. Spiked Recovery Data (%) of Metabolites in Different Fermentation States of Kimchi

compound name	initial week kimchi			4th week fermented kimchi			16th week fermented kimchi		
	low[a]	medium[b]	high[c]	low	medium	high	low	medium	high
				Organic Acid					
malic acid	78.0 ± 4.3	87.4 ± 5.6	85.2 ± 5.6	82.4 ± 4.5	104.7 ± 5.1	92.2 ± 5.8	84.4 ± 5.5	112.2 ± 6.4	93.0 ± 5.8
lactic acid	89.9 ± 5.7	95.2 ± 4.9	88.6 ± 5.7	75.3 ± 3.3	83.5 ± 5.5	85.2 ± 5.6	73.2 ± 5.2	87.3 ± 5.6	88.3 ± 5.6
succinic acid	82.3 ± 4.5	85.1 ± 3.6	87.1 ± 4.6	92.4 ± 5.8	81.6 ± 5.4	94.4 ± 4.8	83.0 ± 5.5	91.2 ± 5.7	87.1 ± 5.6
citric acid	73.3 ± 5.2	81.6 ± 5.4	89.1 ± 5.7	81.3 ± 5.4	82.9 ± 4.5	88.6 ± 5.7	78.2 ± 4.3	80.8 ± 4.4	87.0 ± 5.6
2-hydroxyisocaproic acid	102.3 ± 6.1	104.9 ± 4.1	93.5 ± 5.8	86.6 ± 4.6	81.9 ± 5.5	87.8 ± 5.6	82.0 ± 5.5	88.7 ± 5.7	81.5 ± 5.4
3-phenyl lactic acid	79.3 ± 3.4	78.8 ± 5.4	86.1 ± 4.6	77.1 ± 5.3	76.2 ± 5.3	82.5 ± 3.5	74.8 ± 4.2	85.2 ± 5.6	87.8 ± 5.6
				Sugar Alcohol					
mannitol	81.0 ± 4.4	87.4 ± 5.6	85.2 ± 5.6	82.4 ± 5.5	82.7 ± 5.5	85.2 ± 4.6	74.4 ± 5.2	82.2 ± 5.5	93.0 ± 5.8
				Amino Acid					
L-alanine	81.0 ± 3.4	86.8 ± 5.6	97.7 ± 5.9	83.7 ± 5.5	85.6 ± 4.6	87.6 ± 5.6	79.8 ± 5.4	88.0 ± 5.6	94.3 ± 5.8
L-arginine	82.5 ± 5.5	91.0 ± 5.7	93.3 ± 3.8	80.7 ± 5.4	87.0 ± 5.6	83.5 ± 5.5	81.0 ± 4.4	96.4 ± 3.9	89.5 ± 4.7
L-cystine	92.6 ± 3.8	85.9 ± 5.6	89.6 ± 5.7	79.2 ± 5.4	73.2 ± 5.2	79.8 ± 5.4	82.8 ± 4.5	86.0 ± 5.6	84.0 ± 5.5
L-cysteine	89.9 ± 5.7	76.5 ± 5.3	86.5 ± 5.6	73.3 ± 4.2	83.9 ± 5.5	81.8 ± 4.5	77.0 ± 5.3	88.4 ± 4.7	92.4 ± 5.8
L-glutamine	81.6 ± 3.4	82.7 ± 5.5	87.2 ± 5.6	74.0 ± 5.2	81.0 ± 5.4	78.5 ± 5.4	80.9 ± 5.4	79.9 ± 5.4	83.0 ± 5.5
L-serine	91.4 ± 3.7	86.2 ± 4.6	95.6 ± 4.9	87.6 ± 5.6	78.7 ± 4.4	82.1 ± 4.5	86.3 ± 4.6	90.6 ± 4.7	82.9 ± 5.5
L-threonine	87.5 ± 5.6	84.0 ± 4.5	90.0 ± 4.7	83.0 ± 4.5	84.0 ± 4.5	96.6 ± 4.9	88.5 ± 5.7	87.5 ± 4.6	92.9 ± 4.8
L-valine	87.9 ± 5.6	87.8 ± 5.6	95.8 ± 5.9	89.0 ± 3.7	80.5 ± 3.4	82.6 ± 5.5	91.7 ± 5.8	91.2 ± 5.7	83.6 ± 4.5
L-phenylalanine	89.4 ± 5.7	87.1 ± 4.6	85.3 ± 5.6	86.8 ± 5.6	84.6 ± 3.5	88.9 ± 5.7	89.9 ± 5.7	88.3 ± 3.6	87.2 ± 4.6
L-tyrosine	79.7 ± 5.4	83.3 ± 5.5	94.9 ± 4.8	73.2 ± 3.2	92.6 ± 5.8	96.1 ± 3.9	78.1 ± 5.3	84.5 ± 4.5	94.3 ± 5.8
L-histidine	82.4 ± 5.5	79.5 ± 5.4	83.4 ± 5.5	83.3 ± 4.5	84.3 ± 4.5	82.4 ± 4.5	94.9 ± 3.8	82.1 ± 5.5	91.6 ± 5.7
D-glutamic acid	83.3 ± 5.5	95.1 ± 4.9	89.1 ± 5.7	82.7 ± 3.5	82.3 ± 5.5	82.7 ± 4.5	80.9 ± 5.4	86.8 ± 5.6	88.1 ± 5.6
DL-2-aminobutyric acid	92.8 ± 5.8	108.2 ± 4.2	83.9 ± 5.5	81.1 ± 3.4	78.8 ± 5.4	83.3 ± 5.5	85.5 ± 5.6	92.4 ± 5.8	83.2 ± 5.5

[a]0.1 mM standard solution added to the sample. [b]1 mM standard solution added to the sample. [c]10 mM standard solution added to the sample.

This is because the concentrations of metabolites were different, and also, it was varied by the degree of fermentation in kimchi. As a result, the recovery (%) was 73.3−102.3% for the initial week 0.1 mMol spiked sample; 76.5−104.9% for the 1 mMol spiked sample, and 83.9−95.8% for the 10 mMol spiked sample. For the 4th and 16th week kimchi samples, the recovery range of all samples was 73.2−96.6% and 73.2−96.4%, respectively (Table 4). All validation results of the metabolite analysis for each quality parameter fulfill the required criteria of the AOAC.[22]

2.2. Kimchi Metabolite Analysis According to the Degree of Fermentation. *2.2.1. Organic Acid Composition in Kimchi According to Fermentation.* Organic acids, which are the major substances produced during the LAB fermentation of kimchi, directly affect the taste and flavor of kimchi. In this study, malic acid, lactic acid, succinic acid, citric acid, 2-hydroxyisocaproic acid, and 3-phenyl lactic acid were quantitatively analyzed for each stage of kimchi fermentation.[23,24]

Lactic acid is the major organic acid produced by LAB and has a strong influence on the sour taste of kimchi. It was not detected in the initial week, immediately after kimchi production, but 13.31 mMol was detected in the 1st week, and it increased sharply from 13.31 to 44.548 mMol from 1st to 2nd week.[25] This is a similar pattern to the LAB growth curve. It continued to increase from 4th to 16th weeks. For instance, malic acid and succinic acid have a relatively mild sour taste than lactic acid and are consumed as the LAB fermentation metabolism progresses and tend to decrease gradually.[24] Malic acid, which originates from garlic among the kimchi submaterials,[26] is at 7.701 mMol in the initial week after kimchi production and decreases slightly to 6.518 mMol after the 1st week. However, all of it was consumed during fermentation in the 2nd week. It is estimated that the entire amount is consumed in the section where LAB rapidly proliferates. Citric acid was also detected at a level of 3.153 mMol in the initial stage of kimchi production and slightly decreased to 2.755 mMol in the 1st week and 1.957 mMol in the 2nd week and continued to decrease. It was not detected in the 8th week owing to the entire amount being consumed during the LAB metabolism. Succinic acid with mild sour taste was detected at a level of 0.466 mMol immediately after kimchi production, and there was no increase or decrease during fermentation. It is known that these decreasing substances are consumed by the tricarboxylic acid cycle during the LAB metabolism.[27]

2-Hydroxyisocaproic acid and 3-phenyl lactic acid are known to be functional substances produced via the LAB metabolism of kimchi.[28] 2-Hydroxyisocaproic acid is produced through the leucine degradation pathway, and it was effective in inhibiting the growth of tested Gram-positive and Gram-negative bacteria.[29] HICA was not detected in the initial stage of kimchi fermentation. However, it was detected at a level of 0.241 mMol in the 1st week and detected approximately twice as much in the 2nd week at 0.486 mMol. And in the 4th week, 0.512 mMol was confirmed as the highest level, and it was detected at 0.405 mMol at the 16th week with a slight decrease. Phenyl lactic acid is produced by lactate dehydrogenase using phenylpyruvate as a medium, and it has antimicrobial activity and is a metabolite derived from phenylalanin.[28] In this study, it was not detected in the kimchi manufacturing stage but first detected at a concentration of 0.027 mMol in kimchi in the 2nd week, and it was confirmed that it increased about 2 times till the 6th week and then gradually decreased (Table 5).

2.2.2. Sugar Alcohol Content in Kimchi According to Fermentation. Mannitol, a sugar alcohol, is a metabolite produced by the reduction of fructose in the process of the LAB metabolism and gives a fresh soft sweet taste to kimchi.[30]

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Table 5. Metabolite Concentration (mMol) of Kimchi in Different Fermentation Weeks

	initial kimchi	1st week	2nd week	3rd week	4th week	6th week	8th week	16th week
				Organic Acid				
malic acid	7.701 ± 0.404^{a}[A,B]	6.518 ± 0.361^{b}	D.L.[C]^{c}	D.L.^{c}	D.L.^{c}	D.L.^{c}	D.L.^{c}	D.L.^{c}
lactic acid	0.863 ± 0.051^{g}	13.31 ± 0.609^{f}	44.548 ± 2.365^{e}	59.95 ± 2.535^{d}	63.807 ± 3.368^{c}	65.869 ± 2.812^{bc}	68.502 ± 2.399^{ab}	71.452 ± 4.446^{a}
succinic acid	0.466 ± 0.022^{e}	0.657 ± 0.021^{b}	0.681 ± 0.040^{ab}	0.709 ± 0.030^{a}	0.663 ± 0.037^{b}	0.572 ± 0.039^{c}	0.517 ± 0.02^{d}	0.426 ± 0.021^{f}
citric acid	2.755 ± 0.184^{b}	3.153 ± 0.244^{a}	1.957 ± 0.136^{c}	1.154 ± 0.093^{d}	0.452 ± 0.026^{e}	0.165 ± 0.009^{f}	D.L.^{g}	D.L.^{g}
2-hydroxyisocaproic acid	D.L.^{f}	0.241 ± 0.014^{e}	0.486 ± 0.030^{ab}	0.450 ± 0.033^{bc}	0.512 ± 0.046^{a}	0.490 ± 0.032^{ab}	0.423 ± 0.031^{cd}	0.405 ± 0.028^{d}
3-phenyl lactic acid	D.L.^{f}	D.L.^{f}	0.027 ± 0.003^{d}	0.040 ± 0.002^{b}	0.053 ± 0.004^{a}	0.055 ± 0.005^{a}	0.033 ± 0.003^{c}	0.010 ± 0.001^{e}
				Sugar Alcohol				
mannitol	D.L.^{f}	D.L.^{f}	35.098 ± 2.224^{e}	44.282 ± 2.389^{d}	48.483 ± 2.711^{c}	50.089 ± 2.437^{c}	55.796 ± 3.863^{b}	62.08 ± 3.743^{a}
				Amino Acid				
L-alanine	2.586 ± 0.120^{b}	2.911 ± 0.139^{ab}	2.913 ± 0.212^{ab}	2.849 ± 0.195^{c}	3.177 ± 0.186^{a}	3.165 ± 0.147^{a}	3.063 ± 0.216^{abc}	3.114 ± 0.151^{ab}
L-arginine	2.073 ± 0.115^{a}	2.186 ± 0.116^{a}	0.085 ± 0.004^{b}	0.096 ± 0.007^{b}	0.046 ± 0.003^{c}	D.L.^{d}	D.L.^{d}	D.L.^{d}
L-cystine	6.001 ± 0.401^{c}	8.220 ± 0.500^{ab}	8.146 ± 0.534^{ab}	8.255 ± 0.637^{ab}	7.865 ± 0.494^{b}	8.078 ± 0.593^{ab}	8.624 ± 0.376^{a}	5.444 ± 0.325^{c}
L-cysteine	0.132 ± 0.007^{f}	0.148 ± 0.004^{f}	0.300 ± 0.014^{d}	0.288 ± 0.013^{d}	0.603 ± 0.045^{a}	0.483 ± 0.024^{b}	0.453 ± 0.017^{c}	0.229 ± 0.017^{e}
L-glutamine	66.117 ± 2.202^{a}	63.326 ± 3.128^{ab}	61.198 ± 2.327^{b}	64.671 ± 3.399^{ab}	65.597 ± 4.467^{ab}	61.826 ± 4.054^{ab}	63.153 ± 3.275^{ab}	56.89 ± 2.245^{c}
L-serine	2.590 ± 0.136^{e}	2.922 ± 0.191^{d}	3.248 ± 0.208^{c}	3.394 ± 0.232^{bc}	3.214 ± 0.255^{c}	3.570 ± 0.261^{b}	4.020 ± 0.194^{a}	3.287 ± 0.233^{bc}
L-threonine	0.441 ± 0.033^{e}	0.591 ± 0.046^{d}	0.625 ± 0.036^{cd}	0.739 ± 0.043^{b}	0.725 ± 0.038^{b}	0.809 ± 0.069^{a}	0.784 ± 0.057^{ab}	0.663 ± 0.034^{c}
L-valine	2.140 ± 0.100^{d}	2.729 ± 0.180^{c}	2.906 ± 0.188^{bc}	3.133 ± 0.279^{ab}	3.149 ± 0.284^{ab}	3.26 ± 0.273^{a}	3.184 ± 0.276^{ab}	3.292 ± 0.229^{a}
L-phenylalanine	0.913 ± 0.050^{f}	1.263 ± 0.056^{d}	1.078 ± 0.075^{ef}	2.260 ± 0.142^{b}	1.476 ± 0.133^{c}	1.163 ± 0.068^{de}	2.180 ± 0.118^{b}	4.770 ± 0.259^{a}
L-tyrosine	3.551 ± 0.263^{d}	6.241 ± 0.401^{c}	8.056 ± 0.483^{b}	8.909 ± 0.756^{b}	8.452 ± 0.417^{b}	8.518 ± 0.655^{b}	10.635 ± 0.791^{a}	10.328 ± 0.855^{a}
L-histidine	0.847 ± 0.047^{h}	1.378 ± 0.100^{g}	1.729 ± 0.096^{f}	1.929 ± 0.165^{e}	2.179 ± 0.154^{d}	2.665 ± 0.263^{c}	3.331 ± 0.154^{b}	2.906 ± 0.153^{a}
D-glutamic acid	9.559 ± 0.451^{c}	9.485 ± 0.290^{c}	9.334 ± 0.345^{c}	9.690 ± 0.401^{b}	10.773 ± 0.728^{b}	10.943 ± 0.425^{b}	10.95 ± 0.536^{b}	12.416 ± 0.377^{a}
DL-2-aminobutyric acid	0.242 ± 0.017^{c}	0.322 ± 0.027^{ab}	0.308 ± 0.024^{b}	0.313 ± 0.017^{ab}	0.317 ± 0.015^{ab}	0.314 ± 0.019^{ab}	0.315 ± 0.024^{ab}	0.342 ± 0.013^{a}

[A](a–f) Values with different superscript letters within a row differ significantly ($p < 0.05$). [B]Values are mean ± standard deviations of three ($n = 3$) measurements. [C]DL: below detection limit.

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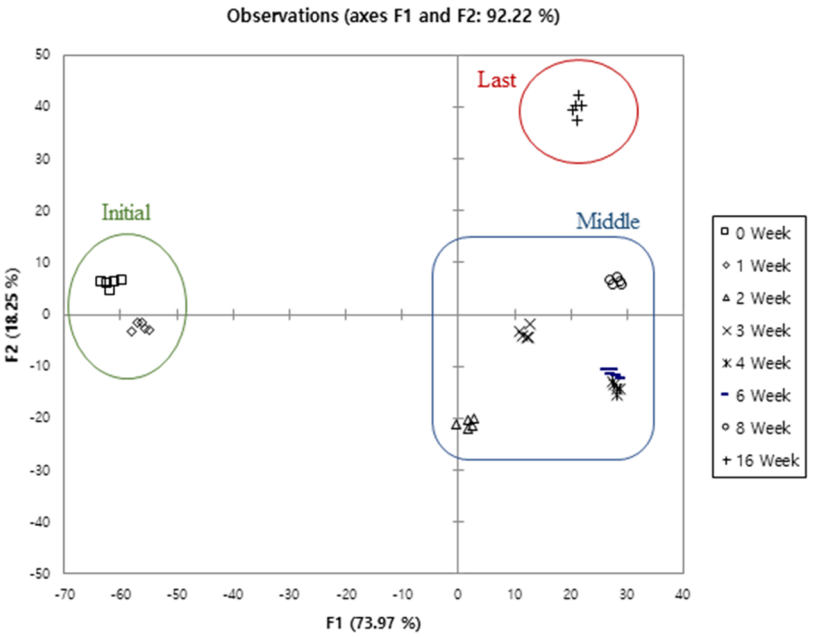

Figure 1. LDA plot of metabolite profiles in kimchi during each LAB fermentation period.

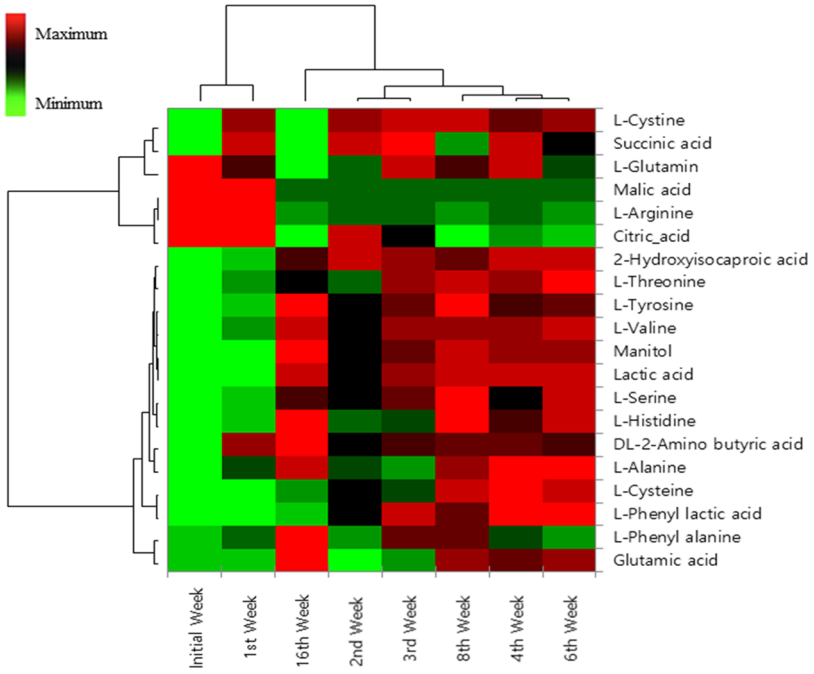

Figure 2. Heat map of metabolite profiles in kimchi during each LAB fermentation period.

As a result of the analysis in this study, it was confirmed that mannitol was not detected in the initial stage to the first week of fermented kimchi. However, it was explosively generated at a level of 35.098 mMol in the second week. After that, it showed a steadily increasing trend. In the 16th week, it was detected at a concentration of 62.08 mMol, which was close to twice as that in the second week (Table 5).

2.2.3. Amino Acid Composition in Kimchi According to Fermentation. Amino acids are produced or converted by microorganisms through metabolic reactions; the metabolites directly affect the taste of food.[31] D-Glutamic acid contributes to umami (savory taste); L-alanine, S-serine, S-threonine, and L-glutamine contribute to sweet taste; and L-arginine, L-valine, L-phenylalanine, L-tyrosine, and L-histidine contribute to bitter taste.[32]

D-Glutamic acid is a representative substance that contributes to umani flavor in kimchi. Its concentration did not change substantially, ranging from 9.334 mMol in the beginning to 9.690 mMol in the 3rd week of fermentation. However, in the 4th week, it slightly increased to 10.773 mMol and thereafter, gradually increased to 12.416 mMol in the 16th week.

Glutamine, which was detected in the highest concentration, imparted a mild sweet taste. Its concentration of 66.117 mMol in the beginning of kimchi production gradually decreased as fermentation proceeded and was found to be 56.890 mMol in

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the 16th week. However, other amino acids that contribute to sweetness, such as L-alanine, L-serine, and L-threonine, showed a slight increase as fermentation progressed. L-Alanine was initially found to be 2.586 mMol and gradually increased till the 16th week showing an overall increasing trend. L-Serine and L-threonine were initially found at 2.590 and 0.441 mMol, respectively. L-Serine and L-threonine were the highest in the 8th and 6th weeks at 4.020 and 0.809 mMol, respectively. In the 16th week, L-serine and L-threonine were detected at 3.287 and 0.663 mMol, respectively, showing a slightly decreasing pattern after an overall increase.

Among amino acids that contribute to the bitter taste of kimchi, L-tyrosine was present at the highest concentration. This increased rapidly from the initial stage of kimchi (3.551 mMol) to the 3rd week (8.909 mMol) and then gradually increased to 10.328 mMol in the 16th week. L-Phenylalanine and L-histidine also contribute to the bitter taste of kimchi. They were analyzed at 0.913 and 0.847 mMol in the initial stage of kimchi production and gradually increased to 4.770 and 2.906 mMol, respectively, in the 16th week. L-Valine was initially analyzed at 2.140 mMol and slightly increased to 3.292 mMol in the 16th week. Among amino acids that contribute to bitter taste, L-arginine is the only amino acid that originates from garlic among kimchi ingredients. It was initially analyzed at 2.073 mMol but decreased considerably from the 1st to the 2nd week and was too low to be detected in the 6th week (Table 5).

2.3. Multivariate Statistical Analysis. The results of multivariate statistical analysis (LDA, heat map) of kimchi metabolites (organic acids, sugar alcohol, and amino acids) from the initial week to 16th week are shown in Figures 1 and 2.

As a result of LDA (Figure 1), the initial and 1st weeks were identified as the same group with the *X*-axis distributed from −50 to −70 and the *Y*-axis from −10 to 10. The middle stage of LAB-fermented kimchi includes the 3rd−8th weeks; the *X*-axis ranges from 0 to 30 and *Y*-axis from −25 to 10 in this section. The 16th week was independently distributed to the last LAB-fermented kimchi, and at this time, the *X*-axis was found in the range of 20−30 and *Y*-axis in the range of 35−45. Overall, both *X*-axis and *Y*-axis tended to rise from negative to positive values. This is because of the produced metabolites during LAB fermentation in kimchi.

From a heat map analysis (Figure 2), the initial and the 1st weeks were confirmed as the same group. This means that LAB fermentation did not proceed substantially until the 1st week; therefore, the metabolite distribution in kimchi was similar between the initial and 1st weeks. The 2nd and 3rd weeks were grouped; and the 4th, 6th, and 8th weeks were grouped together when the LAB fermentation proceeds. The 16th week was grouped independently. This is directly related to the change in metabolite concentration according to the LAB fermentation of kimchi.

3. CONCLUSIONS

In this study, we developed an analytical method for simultaneous analysis of six types of organic acids, one type of sugar alcohol, and 13 types of amino acids within 10 min using LC-Q-Orbitrap MS. Selectivity was confirmed by checking MS1 and MS2 individually for all metabolites, and as a result of validation, all items satisfied the AOAC guidelines. This method dramatically reduced the analytical time and cost.

During the LAB fermentation of kimchi, lactic acid changed the most in concentration. On the one hand, lactic acid increased rapidly. On the other hand, malic acid and citric acid gradually decreased and disappeared. 2-Hydroxyisocaproic acid and 3-phenyl lactic acid are organic acids produced by LAB fermentation, and mannitol, a sugar alcohol, was also produced by fermentation and showed a tendency to gradually increase. In addition, amino acids that affect various tastes, namely, umami, sweetness, and bitterness, also showed a tendency to increase or decrease as the LAB fermentation progressed.

Based on the metabolite analysis results of kimchi from the initial to 16th weeks, the LAB fermentation did not proceed sufficiently until the 1st week; thus, it was classified into the same group as the initial week, from the 2nd to 8th weeks up to the intermediate group, and independently at the 16th week as a result of multivariate statistical analysis. The conditions that indicate a change from the initial to middle stages are the disappearance of malic acid and an increase in lactic acid, 3-phenyl lactic acid, and mannitol levels. In particular, the sour and sweet tastes of kimchi are influenced by an increase in lactic acid and mannitol in the middle group. Conditions affecting the classification from the middle to last group are high concentrations of lactic acid, mannitol, L-phenylalanine, and D-glutamic acid compared with those in the middle group. The above substances strengthen the sour and umami tastes of kimchi. This result has a direct relationship with the concentration of metabolites that change as the LAB fermentation of kimchi progresses and could provide key information on kimchi selection to kimchi consumers.

4. METHODS

4.1. Reagents. The following standards were used: malic acid (99%), lactic acid (98%), succinic acid (99%), citric acid (98%), 2-hydroxyisocaproic acid (99%), phenyl lactic acid (98%), mannitol (98%), sorbitol (98%), L-asparagine (98%), L-alanine (98%), L-arginine (98%), L-cysteine (99%), L-cystine (98%), L-citrulline (98%), L-glutamine (98%), L-serine (98%), L-theronine (98%), L-valine (98%), L-phenylalanine (98%), L-tyrosine (99%), L-histidine (99%), L-glutamic acid (99%), DL-aminobutyric acid (99%), and L-Proline-1-^{13}C (99%) were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (98% purity) was purchased from Fluka (Buchs, Switzerland). All solvents used for LC-MS grade and purchased from J. T. Baker (Phillipsburg, NJ). Ultrapure water was obtained using Milli-Ro plus and Milli-Q systems (Millipore, MA).

4.2. Preparation of Kimchi. Kimchi cabbage was soaked in 10% (w/v) salt solution for 18 h and washed thrice with water and soaked for 2 h. Then, a seasoning prepared using radish 3% (w/w), green onion 2.5% (w/w), garlic 2% (w/w), ginger 0.8% (w/w), fermented shrimp sauce 1.5% (w/w), chili powder 3% (w/w), glutinous rice paste 0.8% (w/w), and water 4.4% (w/w) was mixed with the salted kimchi cabbage 82% (w/w). The kimchi was packaged in units of 500 g each using a polyethylene film and sealed using a vacuum packaging machine (AZC-070, INTRISE, Ansan, Korea). Then, it was stored in a refrigerator at 4 °C and analyzed every 7 days (1 week). Analyses were performed in five independent kimchi samples.

4.3. Metabolite Extraction and Purification. For extraction solvent optimization, several extraction solvents such as distilled water (solvent A), water/methanol mixture (80/20, v/v) (solvent B), and 0.1% formic acid in water/methanol mixture (80/20, v/v) (solvent C) were selected and

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tested. Approximately 2 g of homogenized kimchi sample was weighed and placed in a polypropylene tube. Subsequently, 20 ml of extraction solvent was added and shaken for 5 min, and ultrasonic extraction was performed for 20 min. The extracted sample was centrifuged at 3200*g* for 10 min. The supernatant was then filtered through a 0.22 μm syringe filter for direct injection in ultra-high-pressure liquid chromatography (UHPLC). The solution was transferred and diluted using an extraction solvent for identification and quantitative analysis.

4.4. Analytical Condition of Metabolites Using UPLC-HESI-Q-Orbitap MS. The UPLC-HESI-MS system consisted of a UHPLC Dionex Ultimate 3000 and a heated electrospray ionization qudropole−orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The system was controlled by Xcalibur 4.5 software (Thermo Fisher Scientific, San Jose). The Q-Orbitrap mass spectrometer was equipped with a heated electrospray ionization (HESI) source. The optimized HESI parameters were as follows: sheath gas flow rate, 30 L/min; auxiliary gas flow rate, 10 L/min; sweep gas flow rate, 1 L/min; spray voltage, 4.30 kV; capillary temperature, 320 °C; S-lens RF level, 50.0; and heater temperature, 200 °C. In SIM mode, the LC/Q-Orbitrap MS settings were as follows: resolution, 70,000; AGC target, 3E06; maximum injection time (IT), 100 ms; and scan range, 80−250 m/z. All quantitative data in this study were acquired using full MS scan mode. In MS2 mode, resolution was set at 17,500 FWHM (m/z 200) for a confirmatory purpose. Metabolites were identified and quantified based on the mass-to-charge ratio of the target compound in full-scan mode and confirmed by MS2 mode. The metabolites were identified and quantified based on m/z values determined in the multiple reaction monitoring (MRM) mode, and the conditions and fragmentation patterns are shown in Table 1. For metabolite separation, the analytical column was used by connecting two different columns in series. The main column was a Hypersil GOLD C_{18} column, and an Accucore aQ polar endcapped column was connected in front of it to increase the separation efficiency of polar substances. Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The following gradient elution was applied: 0−3.0 min, 0% B; 3.0−8.0 min, 45% B; and 8.0−10.0 min, 100% B. The flow rate was 0.3 mL/min. The oven was thermostatted at 40 °C, and the injection volume was 2 μL. The autosampler was thermostatted at 10 °C.

4.5. Method Validation and Quality Assurance. The analytical method was validated by determining the selectivity, standard solution concentration linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and recovery. Selectivity was determined using the chromatographic system, and the quantification of each metabolite was calculated using the ratio of peak area of precursor ion m/z values (analyte peak area versus standard peak area). LODs and LOQs were determined experimentally as 3 and 10 times the standard deviation of the blank divided by the slope of the analytical standard curve, respectively. Linearity was evaluated using metabolite calibration curves and a nonweighted least-squares linear regression analysis method. Standard working solutions were prepared in a concentration range of 0.1−100 μM. Precision was obtained as the correlation coefficient (CV, %) of the relative standard deviation of 10 repeated determinations for each standard solution of metabolite-spiked samples. Accuracy was verified by adding the standard material solutions to the sample at three concentrations (0.1, 1, and 10 mM) and determining the % recovery.

4.6. Data Processing. The results of metabolites are reported as the mean ± standard deviation of triplicate measurements. Significant differences ($p < 0.05$) measured among the means were reported via one-way analysis of variance (ANOVA) and Tukey's honest significant difference test. SPSS Statistical Package for Social Sciences Software Version 20 (IBM, New York) was used for the statistical analyses.[33] The multivariate linear discriminant analysis (LDA) test and heat map were performed using XLSTAT 2020 software (Addinsoft, Paris, France) to determine the distribution and grouping of the sample groups.

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Notes

The authors declare no competing financial interest.

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