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Comprehensive NMR Analysis of Compositional Changes of Black Garlic during Thermal Processing

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Supporting Information

ABSTRACT: Black garlic is a processed food product obtained by subjecting whole raw garlic to thermal processing that causes chemical reactions, such as the Maillard reaction, which change the composition of the garlic. In this paper, we report a nuclear magnetic resonance (NMR)-based comprehensive analysis of raw garlic and black garlic extracts to determine the compositional changes resulting from thermal processing. ^{1}H NMR spectra with a detailed signal assignment showed that 38 components were altered by thermal processing of raw garlic. For example, the contents of 11 L-amino acids increased during the first step of thermal processing over 5 days and then decreased. Multivariate data analysis revealed changes in the contents of fructose, glucose, acetic acid, formic acid, pyroglutamic acid, cycloalliin, and 5-(hydroxymethyl)furfural (5-HMF). Our results provide comprehensive information on changes in NMR-detectable components during thermal processing of whole garlic.

KEYWORDS: *black garlic, thermal process, Maillard reaction, NMR, multivariate statistical analysis*

INTRODUCTION

Garlic (*Allium sativum* L.) is widely used as a spice in many parts of the world, such as Asia, Europe, and the United States, because of its unique flavor and taste. Garlic has been reported to exert many health benefits, including antimicrobial,[1] anticancer,[2] and antioxidant[3] activities, and to improve the effects of hypoglycemic activity.[4] Among the chemical compounds of garlic, organosulfur compounds such as allicin are primarily responsible for its flavor,[5] and the derivatives of these compounds are responsible for its health benefits.[3,6]

Black garlic is a newly processed food that is made by subjecting whole raw garlic to thermal processing at approximately 70−80 °C under controlled humidity conditions for 1−3 months without any additives.[3] This processed food has a fruity taste and is edible without any cooking processes. The thermal processing induces many chemical reactions, such as the enzymatic browning reaction and Maillard reaction, which causes the color to change from white and yellow to dark brown. Compared with raw garlic, black garlic has a less pungent odor because of a decrease in allicin content,[7] whereas *S*-allylcysteine (SAC) contents have been reported to be increased.[7,8] SAC is a water-soluble sulfur compound with high antioxidant activity and shows at least a 5-fold increase in black garlic compared with raw garlic.[8] In addition, increments in the content of polyphenols and flavonoids[9] along with increments in antitumor effects in vitro[2] and immunostimulatory activity[10] are observed in black garlic after thermal processing. There are other reports that showed the potential health benefits of black garlic by chemical analysis,[11,12] cell culture experiments,[13,14] and animal experiments.[15,16]

Nuclear magnetic resonance (NMR) is a useful tool for studying the physicochemical state of foods and does not require the separation of their constituents, which may change the condition of the food samples; therefore, this technique has been applied in food science because of its simplicity and because it is a direct method to obtain comprehensive information about food products.[17−19] In addition, nondestructive NMR is commonly used to study the metabolome of many types of food, such as potato,[17] coffee,[20] mango juice,[21] and milk.[22,23] Although NMR is less sensitive than other analytical techniques, such as absorption spectroscopy and mass spectrometry combined with liquid or gas chromatography, it remains an indispensable and useful technique when combined with multivariate data analysis to provide a comprehensive observation of complex mixtures. Furthermore, the application of quantitative NMR (qNMR) was also reported on food due to the enormous potential in metabolomics profiling,[24] resulting from a feature of NMR techniques, that is, precise compound identification and quantification. NMR techniques are useful for monitoring composition changes during food processing such as fermentation, thermal processing, and ripening.[21,23−25] With untargeted multivariate statistical methods, such as principal component analysis (PCA), different origins,[25] cultivars,[21] processes,[23] or fermentations[24] in foods are clearly distinguished by the ^{1}H NMR spectra.

To date, metabolite changes in black garlic during thermal processing have not been reported. These changes could be detected by the one-dimensional (1D) and two-dimensional (2D) NMR spectra of black garlic with different degrees of thermal processing. Multivariate analyses are also used to obtain

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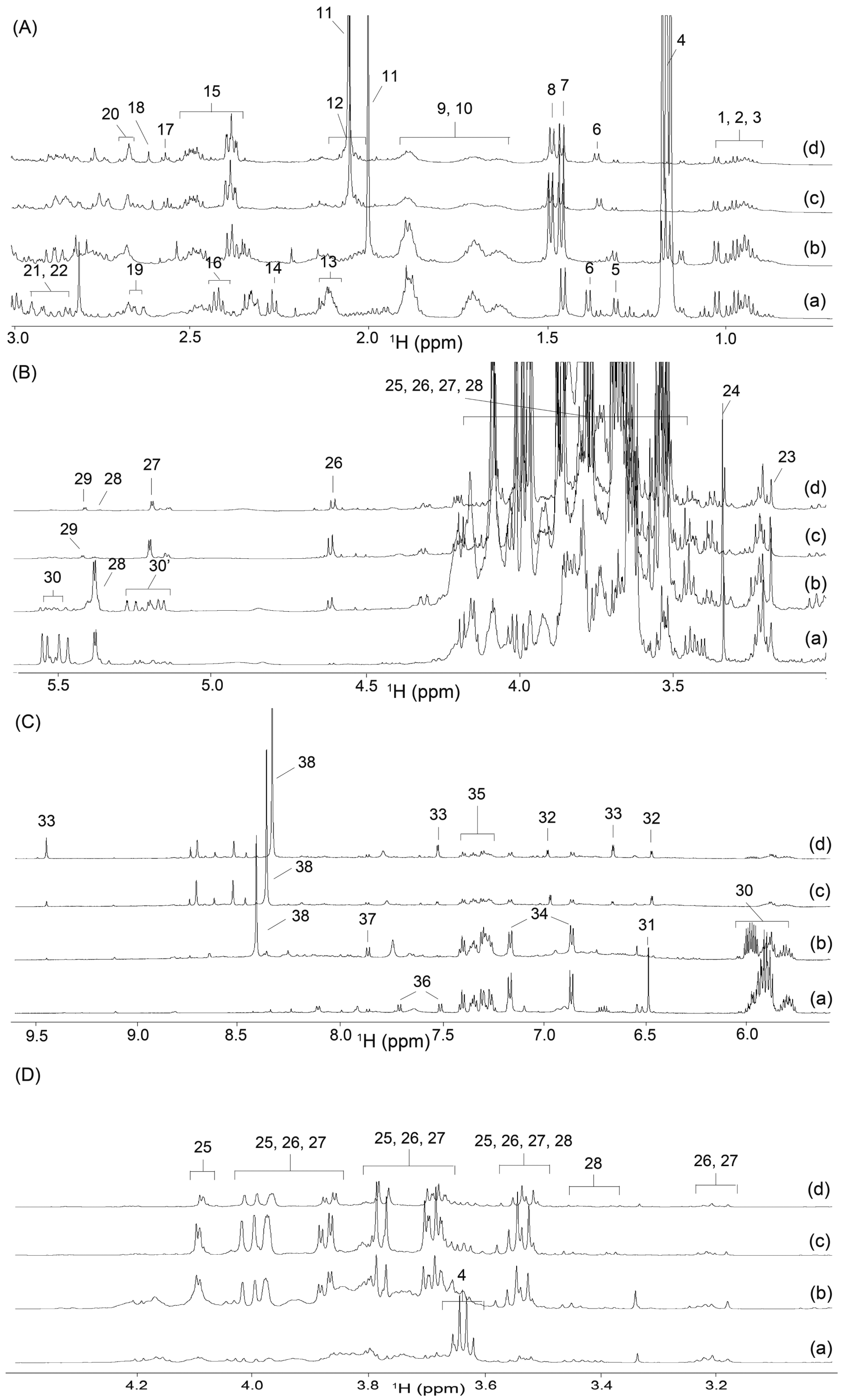

Figure 1. (A, B) High-field, (C) low-field, and (D) sugar regions of the ^{1}H NMR spectra of the (a) raw garlic extract and (b) 5 day, (c) 25 day, and (d) over 90 day black garlic extracts with the following signal assignments: 1, valine; 2, leucine; 3, isoleucine; 4, ethanol; 5, threonine; 6, lactic acid; 7, alanine; 8, cycloalliin; 9, lysine; 10, arginine; 11, acetic acid; 12, proline; 13, glutamine; 14, γ-aminobutyric acid (GABA); 15, pyroglutamic acid; 16, glutamic acid; 17, 3-hydroxypropionic acid; 18, succinic acid; 19, malic acid; 20, citric acid; 21, asparagine; 22, aspartic acid; 23, choline; 24, methanol; 25, fructose; 26, β-glucose; 27,α-glucose; 28, sucrose; 29, glucosamine; 30, 30′, allicin and other allyl organosulfur compounds; 31, fumaric acid; 32, 5-(hydroxymethyl)-2-furoic acid; 33, 5-(hydroxymethyl)furfural; 34, tyrosine; 35, phenylalanine; 36, tryptophan; 37, uridine; 38, formic acid.

comprehensive information regarding the compositional changes of black garlic from the NMR spectra. In this study, we report a ^{1}H NMR-based comprehensive analysis of raw garlic and black garlic extracts. The chemical compositional changes resulting from thermal processing are characterized using 1D ^{1}H NMR spectra, the signal assignment information is provided by analyses of 2D NMR spectra, and the resulting PCA is provided as score and loading plots.

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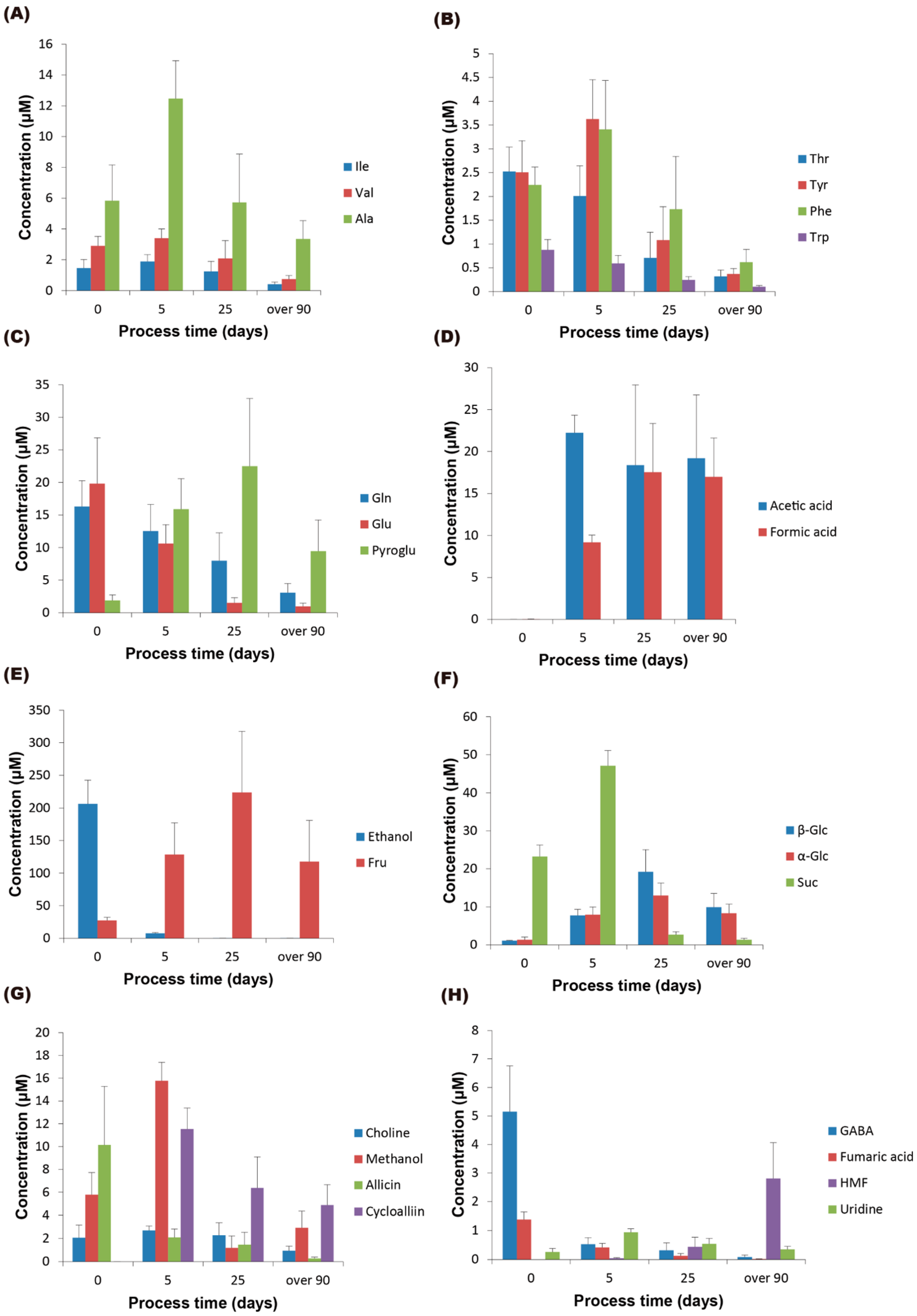

Figure 2. Changes in black garlic extract content during thermal processing: (A–C) amino acids, (D) organic acids, (E, F) ethanol and sugars, (G, H) other compounds. Abbreviations: Pyroglu, pyroglutamic acid; Fru, fructose; α-Glc, α-glucose; β-Glc, β-glucose; Suc, sucrose; GABA, γ-aminobutyric acid; HMF, 5-(hydroxymethyl)furfural. The L-amino acids are represented by the universal three-letter codes. The integral value of the DSS signal (trimethylsilyl protons) was set to unity. The integral values of the signals from the individual constituents were normalized to the value of a proton and then used to calculate the concentration. The data are the mean ± standard deviation (SD; n = 3).

MATERIALS AND METHODS

Materials. The raw garlic and black garlic samples were prepared by Salad Cosmo, Co., Ltd. (Japan) and stored at −30 °C until use. The garlic samples were divided into four groups including raw garlic and black garlic with three different degrees of thermal processing as follows: 5 day black garlic, which represents the early step of thermal processing, i.e., thermal processing of raw garlic at 75 °C for 5 days; 25 day black garlic, which represents the late step of thermal processing, consisting of subsequent thermal processing of the 5 day black garlic at 67 °C for 20 days; over 90 day black garlic, which represents the state offered for sale and is stored at room temperature for approximately 2 months after thermal processing of 25 day black garlic at 35 °C for 14 days.

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NMR Samples. Samples of raw garlic and three types of black garlic (seven samples for each group) were peeled and sliced. The garlic samples (1 g) were soaked in 1 mL of 10% dimethyl sulfoxide-d_6 (DMSO-d_6; Cambridge Isotope Laboratories, Inc.)/90% D_2O (99.7%; Shoko Co., Ltd., Tokyo, Japan) for 1 h at room temperature. The extracts were centrifuged at 10000*g* at 4 °C for 10 min. The supernatants were removed and centrifuged again under the same conditions. Then 5 μL of 178 μM 4,4-dimethyl-4-silapentane-1-sulfonic acid, sodium salt (DSS; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the supernatants (600 μL) as an internal reference (final concentration of 1.47 μM),[21] with a chemical shift that was set to 0 ppm, and the solution was transferred into 5 mm NMR tubes.

NMR Spectroscopy. Quantitative ^{1}H NMR spectra were measured at 20 °C with a 600 MHz Agilent Unity INOVA-600 spectrometer equipped with a cryogenic probe. The signal of water was suppressed by the presaturation method, and the parameters of the measurements were as follows: number of data points, 64K; spectral width, 7764 Hz; acquisition time, 4.22 s; delay time, 30.0 s; number of scans, 32. The delay time was determined by the spin–lattice relaxation time (T_1) measurement, as used in a previous study.[20]

The ^{13}C NMR spectra were measured at 20 °C and 125.65 MHz on an Agilent Unity INOVA-500 spectrometer equipped with a 5 mm broad-band probe, and the parameters for ^{13}C NMR spectra were as follows: number of data points, 64K; spectral width, 31446 Hz; acquisition time, 1.04 s; delay time, 2.0 s; number of scans, 28128.

All of the 2D NMR spectra were measured at 20 °C on an Agilent Unity INOVA-500 spectrometer equipped with a 5 mm triple-resonance probe. For ^{1}H–^{1}H double-quantum-filtered correlation spectroscopy (DQF-COSY), the signal of water was suppressed by the presaturation method, and the acquisition parameters were as follows: number of data points, 2048 in F_2 (^{1}H) and 1024 in F_1 (^{1}H); spectral width, 5195 Hz in F_2 and F_1; acquisition time, 0.197 s; number of scans, 72. ^{1}H–^{13}C heteronuclear single-quantum coherence (HSQC) in the phase-sensitive mode was measured with the following parameters: number of data points, 2048 for ^{1}H and 512 for ^{13}C; spectral width, 5195 Hz for ^{1}H and 25141 Hz for ^{13}C; acquisition time, 0.160 s; delay time, 1.5 s; number of scans, 64. ^{1}H–^{13}C constant-time heteronuclear multiple-bond correlation (CT-HMBC) was obtained in the absolute mode with the following parameters: number of data points, 2048 for ^{1}H and 512 for ^{13}C; spectral width, 5751 Hz for ^{1}H and 30166 Hz for ^{13}C; acquisition time, 0.259 s; delay time, 2.0 s; number of scans, 512.

NMR Signal Assignments and Data Processing. The free-induction decay (FID) data were processed using ACD/NMR Workbook Suite V2012 (Advanced Chemistry Development, Inc., Canada). The signal assignments were performed as follows: (1) Certain signals in the 1D NMR spectra of the raw garlic extracts were assigned by comparison with the published data.[5,26] (2) For the assignments of the unreported signals in the 1D NMR spectra, we predicted candidate compounds using the Biological Magnetic Resonance Bank (BMRB)[27] and previous reports for the compounds of black garlic.[6,9] (3) We confirmed that the signals were correctly assigned to the candidate compounds using the 2D NMR spectra. The correlations between the carbons and their attached protons were confirmed by the ^{1}H–^{13}C HSQC spectra, and the ^{1}H–^{13}C CT-HMBC spectra were used to identify two- or three-bond coupling between the carbons and protons to detect linkages to the quaternary carbons. Couplings between two protons up to three bonds away were confirmed by the ^{1}H–^{1}H DQF-COSY spectrum. When the signals in the 1D NMR spectra were not distinguished or were undetectable in the 2D NMR spectra because of heavy overlaps or low sensitivity, respectively, spiking experiments[20] were performed in which analytical-grade chemicals were added to the NMR samples of the raw garlic or black garlic extracts to confirm the existence of the components. To observe the changes in metabolites in the garlic resulting from thermal processing, the integral values of the signals were calculated by the ACD/NMR Workbook Suite V2012. The integral value of the DSS signal at 0 ppm (the trimethylsilyl protons) was set to unity as an internal standard.

Multivariate Statistical Analysis. The ^{1}H NMR spectra were reduced into 0.04 ppm spectral buckets, the region of D_2O (4.8–5.1 ppm) was removed, and the ^{1}H NMR spectra from 0.8 to 9.5 ppm were then normalized. For the multivariate statistical analysis, the resulting data sets were imported into SIMCA-P, version 13 (Umetrics, Umeå, Sweden). PCA was performed to examine the variation in the data sets as an unsupervised classification method. The modeled variation in the score plot was defined with a 95% confidence interval, and the spectral data were Pareto scaled (Par). The goodness of fit (R^2x) and the predictability (Q^2) were described for the quality of the model.

RESULTS

^{1}H and ^{13}C NMR Spectra of Raw Garlic and Black Garlic Extracts. Figure 1 shows a representative 1D ^{1}H NMR spectrum of the raw garlic extract, 5 day, 25 day, and over 90 day black garlic extracts. On the basis of the 2D NMR analysis and the spiking experiments, 38 components were identified in the raw garlic and black garlic extracts (Table S1 in the Supporting Information). Among these components, 27 constituents of the raw garlic extracts, i.e., sucrose, α-glucose, β-glucose, fructose, L-alanine, L-valine, L-leucine, L-isoleucine, L-threonine, L-lysine, L-arginine, L-glutamine, L-glutamic acid, L-proline, L-asparagine, L-aspartic acid, L-tyrosine, L-phenylalanine, L-tryptophan, γ-aminobutyric acid (GABA), methanol, ethanol, citric acid, malic acid, fumaric acid, choline, and allyl organosulfur compounds such as allicin, have also been reported in previous studies on Italian raw garlic,[5] and all of these components were observed in the ^{1}H NMR spectrum of the raw garlic extract. In addition, uridine and lactic acid were detected in the raw garlic extract for the first time in this study. The following nine components were produced after thermal processing and were identified only in the black garlic extracts: pyroglutamic acid, cycloalliin, formic acid, acetic acid, succinic acid, 3-hydroxypropionic acid, glucosamine, 5-(hydroxymethyl)furfural (5-HMF), and 5-(hydroxymethyl)-2-furoic acid (5-HMFA). Four of the components observed in the raw garlic, i.e., GABA, L-tryptophan, ethanol, and fumaric acid, disappeared in the spectra of the black garlic extracts.

Changes in Garlic Components Resulting from Thermal Processing. To investigate the changes in the garlic components resulting from thermal processing, the contents of the garlic components were determined from the integral values of the ^{1}H signals corresponding to the individual components. Among the detectable components, 25 were quantitatively analyzed because their signals were separately observed (Figure 2).

Among the 17 detected amino acids, the contents of L-alanine, L-valine, L-leucine, L-isoleucine, L-lysine, L-arginine, L-proline, L-asparagine, L-aspartic acid, L-tyrosine, and L-phenylalanine increased in the 5 day black garlic extracts after the early step of the thermal processing and then decreased in the 25 and over 90 day black garlic extracts (Figure 2). Among the other detected amino acids, the contents of L-threonine, L-glutamine, and L-glutamic acid decreased with the progression of thermal processing. The pyroglutamic acid content increased in the 5 and 25 day black garlic extracts but decreased in the over 90 day black garlic extracts. The signals of GABA and L-tryptophan in the 1D ^{1}H NMR spectra nearly disappeared in the 5 day black garlic extract and showed a trace amount in the 25 and over 90 day black garlic extracts (Figure 1).

Among the four detected sugars, α-glucose, β-glucose, and fructose showed changes similar to those of pyroglutamic acid with the progression of thermal processing (Figure 2). The content of these sugars increased in the 5 and 25 day black garlic extracts and then decreased in the over 90 day black garlic extract. However, the sucrose content showed a 2.0-fold increase in the 5

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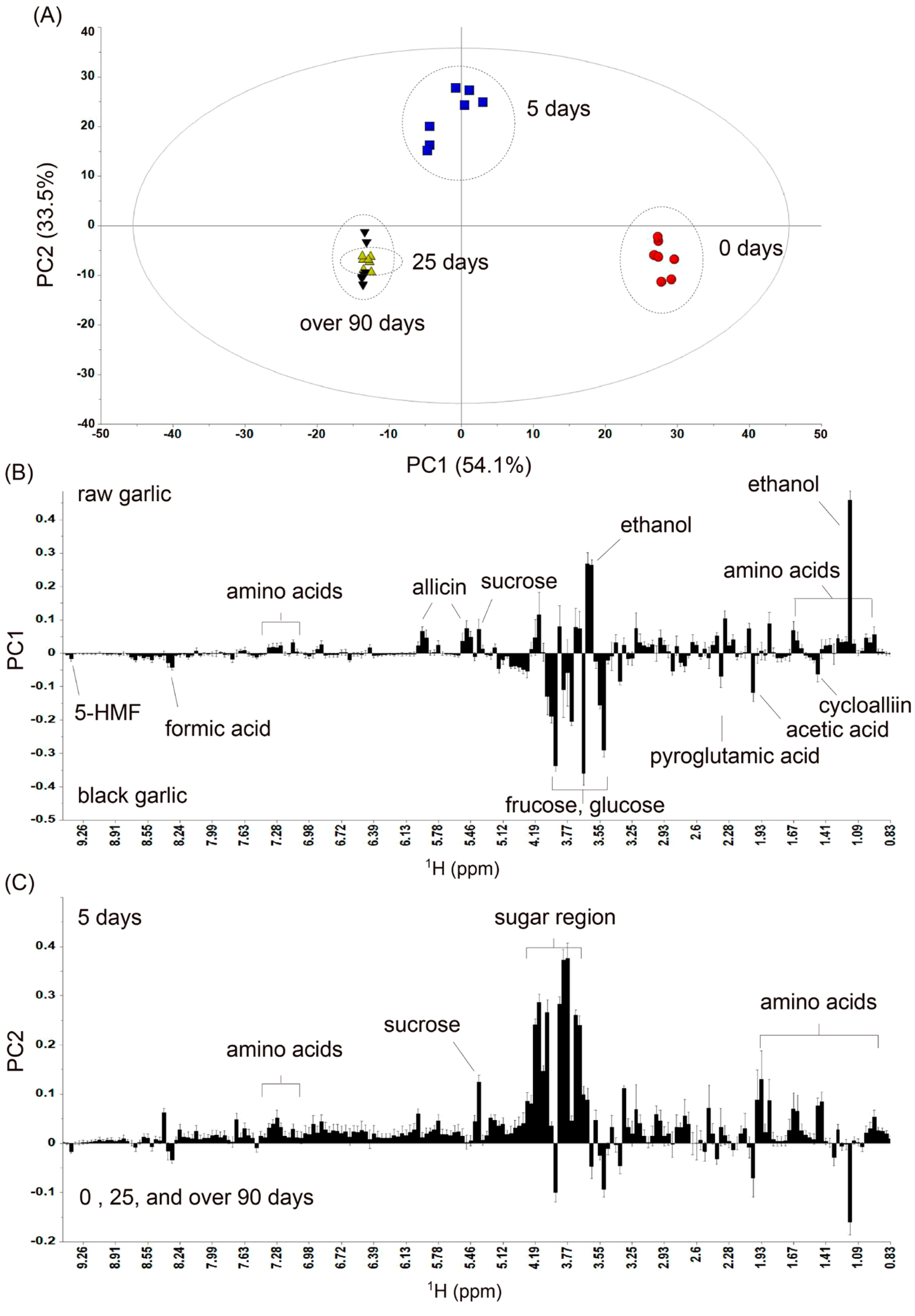

Figure 3. (A) PCA score plot, (B) loading plot for PC1, and (C) loading plot for PC2 for the extracts of raw garlic (0 days) and black garlic with different degrees of thermal processing (5, 25, and over 90 days). The PCA model with seven components was computed, and the goodness of fit (R^2x) was 54.1% and 33.5% for PC1 and PC2, respectively. The goodness of predictability was indicated by a Q^2 value of 0.95.

day black garlic extract compared with the raw garlic extract and significantly decreased in the 25 day black garlic extracts.

Among the observed organic acids, the ^{1}H signal of acetic acid was not observed in the raw garlic extract (Figure 1a), but it appeared in the 5 day black garlic extracts, and the content did not significantly change in the 25 and over 90 day black garlic extracts (Figure 2). The ^{1}H signal of formic acid also appeared in the 5 day black garlic extract and showed a small increase in the 25 and over 90 day black garlic extracts. The chemical shifts of acetic acid and formic acid changed in the ^{1}H NMR spectra during thermal processing (Figure 1), possibly as a result of changes in pH. However, the fumaric acid content decreased with the progression of thermal processing, and fumaric acid was not detected in the over 90 day black garlic extract. Succinic acid and 3-hydroxypropionic acid were detected only in the black garlic extracts (Figure 1). The contents of both of these

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components increased in the 5 and 25 day black garlic extracts and then decreased in the over 90 day black garlic extracts.

Ethanol showed strong signals in the ^{1}H NMR spectra in the raw garlic extract but disappeared after thermal processing (Figures 1 and 2). Methanol showed a 2.7-fold increase in the 5 day black garlic extract compared with the raw garlic extract, and its content was significantly decreased in the 25 day black garlic extracts (Figure 2). However, a slight increase of methanol content was observed in the over 90 day black garlic extracts.

Among the other five detected components, the content of choline increased in the 5 day black garlic extracts and then decreased in the 25 and over 90 day black garlic extracts. The multiple signals at approximately 5.5 ppm in the ^{1}H NMR spectra of the raw garlic extracts were assigned to the allyl protons of allicin (Figure 1), and the content of allicin decreased during the thermal process (Figure 2). The multiplet signals at approximately 5.2 ppm were assigned to the allyl protons of organosulfur compounds. These signals increased in the 5 day black garlic extract and then decreased in the 25 day black garlic extract (Figure 1). Other compounds in the black garlic, such as cycloalliin, 5-HMF, and 5-HMFA, were detected in the 5 day black garlic extract (Figure 1). The content of cycloalliin decreased in the 25 and over 90 day black garlic extracts, whereas that of 5-HMF and 5-HMFA increased in the 25 day black garlic extracts. At the later thermal processing step of the over 90 day black garlic extracts, the content of 5-HMF was increased by more than 6-fold compared with that in the 25 day black garlic extracts.

Variations in Black Garlic Constituents Resulting from Thermal Processing. To determine the overall compositional changes in black garlic during thermal processing, PCA was performed on the quantitative ^{1}H NMR spectral data for all of the garlic extracts. As shown in Figure 3A, the raw garlic and 5 and 25 day black garlic extracts were clearly distinguished from one another on the PCA score plot with high statistical values of R^2x (54.1% for PC1 and 33.5% for PC2) and Q^2 (0.95). However, the over 90 day black garlic extracts were not distinguished from the 25 day black garlic extracts, indicating that the overall composition of the 25 day black garlic extracts was similar to that of the over 90 day extracts. The PCA loading plots demonstrated that the components in the raw garlic extracts and the black garlic extracts were different (Figure 3B). The PC1 negative loading indicated that the black garlic extracts contained higher amounts of fructose, glucose, acetic acid, formic acid, cycloalliin, pyroglutamic acid, and 5-HMF than the raw garlic extracts. On the basis of the PC1 positive loading, the raw garlic extract contained higher amounts of ethanol, amino acids, sucrose, and allicin than the black garlic extracts. However, the PC2 loading plot showed that the 5 day black garlic extract was chemically different from the raw garlic, 25 day black garlic, and over 90 day black garlic extracts. The differences were primarily observed in the amino acid and sugar contents.

DISCUSSION

Amino Acids. The ^{1}H NMR spectra showed that most of the amino acid content of garlic, except for that of GABA, L-tryptophan, L-threonine, L-glutamic acid, L-glutamine, and pyroglutamic acid, changed in a similar manner during thermal processing as follows: the content increased at the early step of thermal processing up to 5 days and decreased during the later thermal processing. The increased content of the amino acids, including L-alanine, L-valine, L-leucine, L-isoleucine, L-lysine, L-arginine, L-proline, L-asparagine, L-aspartic acid, L-tyrosine, and L-phenylalanine, in the 5 day black garlic most likely resulted from the degradation of proteins or peptides, which may result from enzymatic hydrolysis or nonenzymatic hydrolysis, such as pyrolysis. In addition, the pH value decreased from 7.1 for the raw garlic extract to 5.3 for the 5 day black garlic extract as thermal processing progressed, which may have led to further enzymatic hydrolysis of proteins under acidic conditions. However, additional data are required to verify that the increase in the amino acid content of garlic after 5 days of thermal processing is derived from proteins. In the 25 day black garlic extracts, the decrease in amino acids may have been caused by reactions with reducing sugars, such as the Maillard reaction. The amounts of glucose and fructose increased in the 25 day black garlic extracts, providing good conditions for the Maillard reaction, which frequently occurs during thermal treatment of foods such as coffee and chocolate.[28]

The decreased level of L-tryptophan in the black garlic extracts likely resulted from consumption of L-tryptophan in the chemical reaction to produce carboline, which has been reported in aged garlic extracts.[29] During this reaction, L-tryptophan reacts with an aldehyde or an α-oxo acid, such as pyruvic acid, which is produced from the Maillard reaction or from alliin metabolism, via Pictet−Spengler condensation to form tetrahydro-β-carboline derivatives (THβCs).[29,30] The THβCs exert high antioxidant activity in short-term-fermented black garlic and aged garlic extracts but were not detected in the ^{1}H NMR spectra in our study, likely because of the extraction method used. The THβCs in the aged garlic were extracted by ethanol or a relatively high ratio of an organic solvent in water solution.[29,30]

Levels of pyroglutamic acid increased in the 5 and 25 day black garlic extracts, most likely through conversion from glutamic acid or glutamine by enzymatic or nonenzymatic reactions.[31] The decrease in the contents of glutamic acid and glutamine supports their conversion to pyroglutamic acid during thermal processing. Pyroglutamic acid is a cyclic lactam of glutamic acid that is an agonist of glutamic acid and is beneficial for the prevention of amnesia or memory loss in rats.[32]

Sugars and Organic Acids. In the 5 day black garlic extracts, the sugars content, especially that of sucrose, glucose, and fructose, increased, possibly through the degradation of polysaccharides. The polysaccharides in garlic have been reported to include fructan and galactan.[33,34] The remarkable increase in fructose levels in the 5 and 25 day black garlic extracts may also have resulted from degradation of the abundant fructan in the garlic.[35] Degradation of the polysaccharides could occur under acidic and high-temperature conditions,[36] which have been well studied in food products such as coffee beans after thermal processing.[37,38] In plants, degradation of cell wall polysaccharides causes tissue softening; therefore, the texture of black garlic is more gumlike than that of raw garlic. The decrease in pH and the heat treatment during thermal processing could promote further hydrolysis of sucrose to glucose and fructose, which may also be supported by the increase in glucose and fructose contents accompanying the reduced sucrose levels in the 25 day black garlic extracts.

Signals of organic acids, such as acetic acid, formic acid, succinic acid, and 3-hydroxypropionic acid, were identified in the 1D ^{1}H NMR spectra and the 2D NMR spectra of the black garlic extracts. The contents of acetic acid were significantly increased in the 5 day black garlic extracts, as demonstrated by the differences between the raw garlic extract and the black garlic extracts in the PC1 loading plot. This change in acetic acid content may result from sugar fragmentation.[39,40] During

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thermal processing, the production of short-chain carboxylic acid results from α-dicarbonyl and β-dicarbonyl cleavage of hexose or pentose.[39−41] These reactions may also produce formic acid, succinic acid, and 3-hydroxypropionic acid.[42] Fumaric acid was detected in the raw garlic extracts, but its content decreased after thermal processing, most likely because it was converted to other metabolites. Malic acid and citric acid were detectable in the 1D and 2D NMR spectra, but their signals in the ^{1}H NMR spectra were highly overlapped with other signals, which made it difficult to determine the integral values. The increased organic acid content reduced the pH of the solution, which thus affected the degradation of the proteins, peptides, and polysaccharides. In black garlic, the increased organic acid and sugar (fructose and glucose) contents most likely contribute to sourness and sweetness, respectively, which result in the fruity taste. The over 90 day black garlic may show better taste than 25 day black garlic due to the texture change after further thermal processing.

Other Compounds. Ethanol showed extremely strong signals in the 1D and 2D NMR spectra of the raw garlic extracts and was dominant in the PC1 positive loading. However, the ethanol contents were significantly decreased in the 5 day black garlic extracts and not detected in the 25 and over 90 day black garlic extracts. Because ethanol is a volatile compound, it may have been vaporized during the thermal treatment. Moreover, ethanol may be converted to acetaldehyde, which then reacts with L-tryptophan via Pictet−Spengler condensation to form THβCs.[29] The methanol signal in the ^{1}H NMR spectra increased in the 5 day black garlic extracts and then decreased during additional thermal processing. The changes in methanol content were unclear because similar changes have been reported only in plant growth.[43,44] In addition, the decrease in methanol content in the 25 day black garlic extracts may have occurred through evaporation, similar to the results observed for ethanol, because methanol is also volatile.

The content of allicin, a metabolite largely responsible for the strong odor of garlic, decreased with the progression of thermal processing in black garlic, as detected in the ^{1}H NMR spectra. Allicin could be converted from alliin by the enzymatic reaction of alliinase in raw garlic under conditions above pH 3.6.[7] In our experimental conditions, the pH values of raw garlic and 5 day black garlic extracts were around 7.1 and 5.3, respectively, and the pH value of over 90 day black garlic extracts was around 4.1. Therefore, allicin could be generated under these conditions. Although allicin is an unstable sulfur compound and it could be changed to other sulfur compounds by any processing, it was still detected in ^{1}H NMR spectra of raw and black garlic extracts with our extraction conditions and those described in the previous report.[5] The decrease in the allicin content results in a "smoother" taste of black garlic. However, the increased signal at approximately 5.2 ppm in the 5 day black garlic extracts suggests that other allyl organosulfur compounds may be produced from allicin at this stage. The 1D ^{13}C NMR and 2D NMR spectra showed that the signals were not from allicin but may represent other allyl organosulfur compounds described in previous studies, such as diallyl disulfide or other allicin derivatives.[1,5,7] As an important organosulfur compound in garlic, allicin has been studied for its health potential, including its antibacterial, antifungal, and antiviral activities.[45] However, allicin and other organosulfur compounds in garlic are eliminated by processing, such as long-time extraction and thermal processing.[7] Thus, allicin could not be the primary functional factor underlying the health benefits of black garlic. Processed garlic products, including aged garlic extract and black garlic, also showed higher antioxidant activity along with other potential health benefits compared with raw garlic.[46] These effects resulted from the formation of other functional compounds, such as SAC and THβCs. We have tried to assign the signals of major organosulfur compounds γ-glutamyl-*S*-alk(en)ylcysteines and *S*-alk(en)ylcysteine sulfoxides in garlic, but the existence of a cysteine structure, which is a methylene group linked to a sulfur atom ($-S-CH_2-$), was not detected in our NMR data. Signals of cycloalliin, a sulfur imino acid, appeared in the ^{1}H NMR spectra of the 5 day black garlic extracts. Although this organosulfur compound has been reported to exist in *Allium* species, such as onions and garlic,[47] it was not detected in the raw garlic extracts in our study or in a previous study[5] by NMR. Cycloalliin has been reported to have many biological effects, such as serum triacylglycerol-lowering effects[47] in rats and cancer risk reduction.[48] A previous report also indicated that cycloalliin could be generated from isoalliin by storage of raw garlic.[49] Because isoalliin is produced by γ-glutamyl transpeptidase and oxidase, the increased cycloalliin contents in black garlic extracts may result from thermal processing that enhances the enzymatic activity. Therefore, the increased amount of cycloalliin in the black garlic extracts may contribute to their higher functionality after thermal processing when compared with that of raw garlic.

In the thermal processing of foods, the Maillard reaction and caramelization products including 5-HMF and 5-HMFA[13,50] were also detected in the ^{1}H NMR spectra of the black garlic extracts. The increased contents of 5-HMF and 5-HMFA in the 25 and over 90 day black garlic extracts indicated that further thermal processing may have a positive correlation with the amount of 5-HMF in black garlic. 5-HMF and 5-HMFA were generated from fructose and glucose, which in turn were produced by sugar decomposition in the black garlic during the thermal treatments.

In summary, we characterized the compositional changes of black garlic through thermal processing using ^{1}H NMR spectra combined with multivariate statistical analysis. The PCA score plot indicated differences between the raw garlic extracts and black garlic extracts, although the 25 day black garlic extract was not clearly distinguished from the over 90 day black garlic extract. However, the amount of 5-HMF was different in the 25 day extract compared with the over 90 day black garlic extract as determined by detailed signal assignments. In total, 38 NMR-visible components in the raw garlic and black garlic extracts were identified by the present nondestructive NMR method, and the changes in the content of these compounds, including amino acids, sugars, organic acids, and other metabolites, were successfully monitored throughout the thermal processing. Thermal processing of garlic is important not only for generating a functional food but also for improving the taste of garlic as a processed food product. The insights into the changes in metabolite levels during thermal processing in the present study shed light on the changes in color, flavor, and taste of food products resulting from the Maillard reaction, browning reaction, and other reactions caused by thermal treatments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed signal assignment data for the NMR experiments of the raw and black garlic extracts. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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