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Use of NMR-Based Metabolomics To Chemically Characterize the Roasting Process of Chicory Root

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Supporting Information

ABSTRACT: Roasted chicory root (*Cichorium intybus*) has been widely accepted as the most important coffee substitute. In this study, a nuclear magnetic resonance (NMR)-based comprehensive analysis was performed to monitor the substantial changes in the composition of chicory root during the roasting process. A detailed signal assignment of dried raw and roasted chicory roots was carried out using ^{1}H, ^{13}C, ^{1}H−^{1}H DQF-COSY, ^{1}H−^{13}C edited-HSQC, ^{1}H−^{13}C CT-HMBC, and ^{1}H−^{13}C HSQC-TOCSY NMR spectra. On the basis of the signal assignments, 36 NMR-visible components were monitored simultaneously during roasting. Inulins, sucrose, and most of the amino acids were largely degraded during the roasting process, whereas monosaccharides decreased at the beginning and then increased until the dark roasting stage. Acetamide, 5-hydroxymethylfurfural, di-D-fructose dianhydride, and norfuraneol were newly formed during roasting. Furthermore, a principal component analysis score plot indicated that similar chemical composition profiles could be achieved by roasting the chicory root either at a higher firepower for a shorter time or at a lower firepower for a longer time.

KEYWORDS: *NMR, chicory root, roast, inulin, coffee substitute*

INTRODUCTION

Roasted chicory root (*Cichorium intybus*) has been widely accepted as the most important coffee substitute.[1] For several generations in central and southern Europe, a strong market for coffee substitutes based upon mixtures of coffee, chicory, barley, rye, wheat, and sugar beets has existed.[2] Because roasted chicory root and coffee share a similar taste, attention has been focused on the chemical changes in chicory root during roasting, especially in comparison with those that occur in coffee roasting.[1,3,4] It is well recognized that inulin is a major ingredient of chicory root. Recently, interest in the health benefits of chicory root has increased because of the wide variety of biological activities of inulin, such as prebiotic activity,[5] enhancement of calcium absorption,[6] and anti-tumorigenesis in mice.[7] A few studies of the chemical characterization of chicory root ingredients, particularly inulin, during the roasting process have been carried out.[8−10] However, there are few studies that have investigated the chemical changes of chicory root during the roasting process in an unseparated natural mixture. In this regard, limited knowledge exists concerning the actual chemical changes of roasted chicory root.

Nuclear magnetic resonance (NMR) spectroscopy is an ideal nondestructive approach to address this problem; NMR has been widely applied in food science to achieve the direct and comprehensive observation and quality control of food.[11−20] While monitoring chemical changes during food processing, NMR tracks all NMR-visible compounds and produces information-rich NMR spectral fingerprints. In addition, NMR spectra contain both qualitative and quantitative information, so the assignment of NMR spectra enables a better understanding of the quality status and characteristics of food as a complex mixture. NMR spectroscopy has been applied to obtain the metabolite profiles of chicory leaf,[21] but there is no report of the use of NMR to investigate the chemical changes of chicory root during the roasting process.

The present study has monitored the substantial changes in the composition of chicory root occurring in the real thermal food processing in our daily life, but not the process of the model experiments under laboratory conditions, using NMR-based metabolomics. The main purposes of this study include the following: (1) the observation and detailed assignment of the NMR spectra derived from raw chicory root and roasted chicory root at different stages of roasting and (2) the chemical tracking and characterization of the changes in NMR-visible components during the roasting process.

MATERIALS AND METHODS

Plant Materials. Chicory samples were grown and kindly provided by Salad Cosmo Co., Ltd. (Gifu, Japan). The chicory root pulp was cut (~3 mm), dried, and then roasted using a direct-fired roaster, to achieve different roasting degrees. Detailed information about the roasting time and temperature is summarized in Table 1. All samples were prepared in triplicate (n = 3).

NMR Samples. Dried raw and roasted chicory roots (2 g) were incubated at 95 °C in a closed plastic tube with D_2O (5 mL, 99.7%; Shoko Co., Ltd., Tokyo, Japan) for 1 h, to focus on only the water-soluble compounds that might be contained in the extract. The extracts were cooled on ice for 15 min; then, they were centrifuged at 5000g at 4 °C for 5 min, and the pH value was measured. The supernatants (600 μL) were placed in new tubes. 4,4-Dimethyl-4-silapentane-1-sulfonic

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Table 1. Roasted Chicory Root Samples (n = 3)

sample name[a]	roasting time (min)	temperature (°C)	pH[b]
R	0		5.5
T1	11	118	5.3
T2	22	142	4.6
T3	34	160	4.2
T4	45	167	4.1
C1	13	100	5.2
C2	26	136	4.6
C3	40	168	4.0
C4	55	173	3.9

[a]R, dried raw chicory root; T, chicory root roasted at a strong firepower for short time used for making a chicory tea beverage; C, chicory root roasted at a medium firepower for long time used for making a chicory coffee beverage. [b]Average value of triplicate determinations.

acid sodium salt-d_6 (DSS-d_6; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as an internal standard at a final concentration of approximately 0.42 mM, and its chemical shift was set to 0 ppm. The chicory root extracts were then transferred into 5 mm NMR tubes.

NMR Spectroscopic Analysis. NMR experiments were performed at 20 °C using a Varian Unity INOVA-500 spectrometer for the ^{1}H, ^{13}C, ^{1}H−^{1}H DQF-COSY, ^{1}H−^{13}C edited-HSQC, ^{1}H−^{13}C CT-HMBC, and ^{1}H−^{13}C HSQC-TOCSY spectra.[22,23]

The ^{1}H NMR spectra were measured at 500 MHz, and the HDO signal was suppressed using the presaturation method. The acquisition parameters were as follows: number of data points, 128K; spectral width, 8000 Hz; acquisition time, 8.0 s; delay time, 5.0 s; and number of scans, 64.

The ^{13}C NMR spectra were measured at 125.65 MHz. Dioxane was used as an external standard, and its chemical shift was set to 67.5 ppm. The parameters of the ^{13}C NMR spectrum were as follows: number of data points, 32K; spectral width, 31,447 Hz; acquisition time, 1.042 s; delay time, 2.0 s; and number of scans, 40,000.

The ^{1}H−^{1}H DQF-COSY spectra were obtained by suppressing the HDO signal using the presaturation method, and the acquisition parameters were as follows: number of data points, 1024 and 512 (^{1}H, ^{1}H); spectral width, 5195 Hz (^{1}H, ^{1}H); acquisition time, 0.197 s; delay time, 2.0 s; dummy scan, 32; and number of scans, 8.

The ^{1}H−^{13}C edited-HSQC spectra were observed in the phase-sensitive mode. The acquisition parameters were as follows: number of data points, 833 for ^{1}H and 256 for ^{13}C; spectral width, 5195 Hz for ^{1}H and 25,141 Hz for ^{13}C; acquisition time, 0.160 s; delay time, 1.5 s; dummy scan, 8; and number of scans, 16.

The ^{1}H−^{13}C CT-HMBC spectra were measured in the absolute mode using the following parameters: number of data points, 1490 for ^{1}H and 768 for ^{13}C; spectral width, 5751.3 Hz for ^{1}H and 30,165.9 Hz

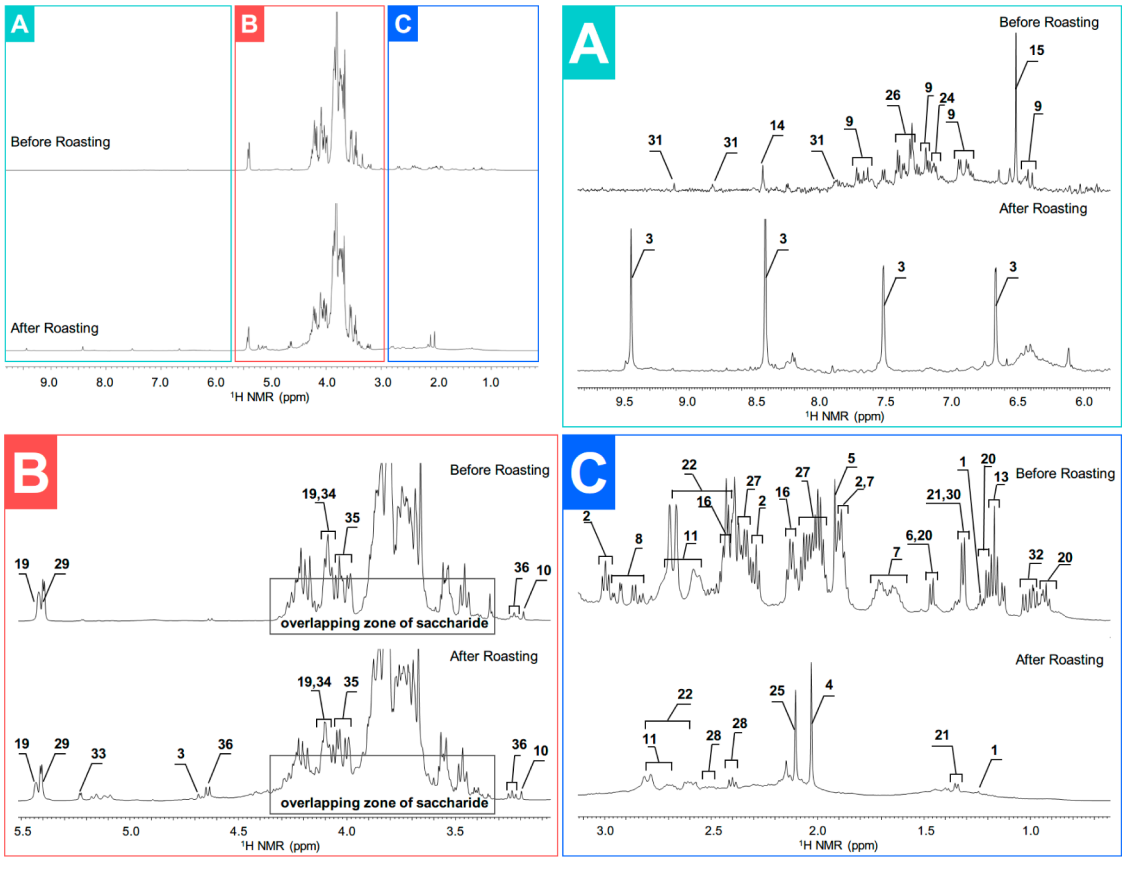

Figure 1. ^{1}H spectra of dried raw (top, sample R) or roasted (bottom, sample C4) chicory root. ^{1}H NMR spectra (upper left) are divided into (A) low-field, (B) middle-field, and (C) high-field regions. The scales of the Y axes are different among spectra and panels. **1**, 2-methyl-2-propanol; **2**, 4-amino-*N*-butyric acid (GABA); **3**, 5-hydroxymethylfurfural (5-HMF); **4**, acetamide; **5**, acetate; **6**, alanine; **7**, arginine; **8**, asparagine; **9**, chicoric acid; **10**, choline; **11**, citrate; **12**, di-D-fructose dianhydride (α-D-Fru*f*-1,2′:2,1′-β-D-Fru*f*; DFA I); **13**, ethanol; **14**, formate; **15**, fumarate; **16**, glutamine; **17**, terminal fructose unit of m-type inulin (inulin-Fm); **18**, chain fructose unit of m- and n-type inulin (inulin-Fm, n); **19**, terminal glucose unit of n-type inulin (inulin-GFn); **20**, isoleucine; **21**, lactate; **22**, malate; **23**, methanol; **24**, monocaffeoyltartate; **25**, norfuraneol (4-hydroxy-5-methyl-2*H*-furan-3-one; NF); **26**, phenylalanine; **27**, proline; **28**, pyroglutamate; **29**, sucrose; **30**, threonine; **31**, trigonelline; **32**, valine; **33**, α-D-glucopyranose; **34**, β-D-fructofuranose; **35**, β-D-fructopyranose; **36**, β-D-glucopyranose.

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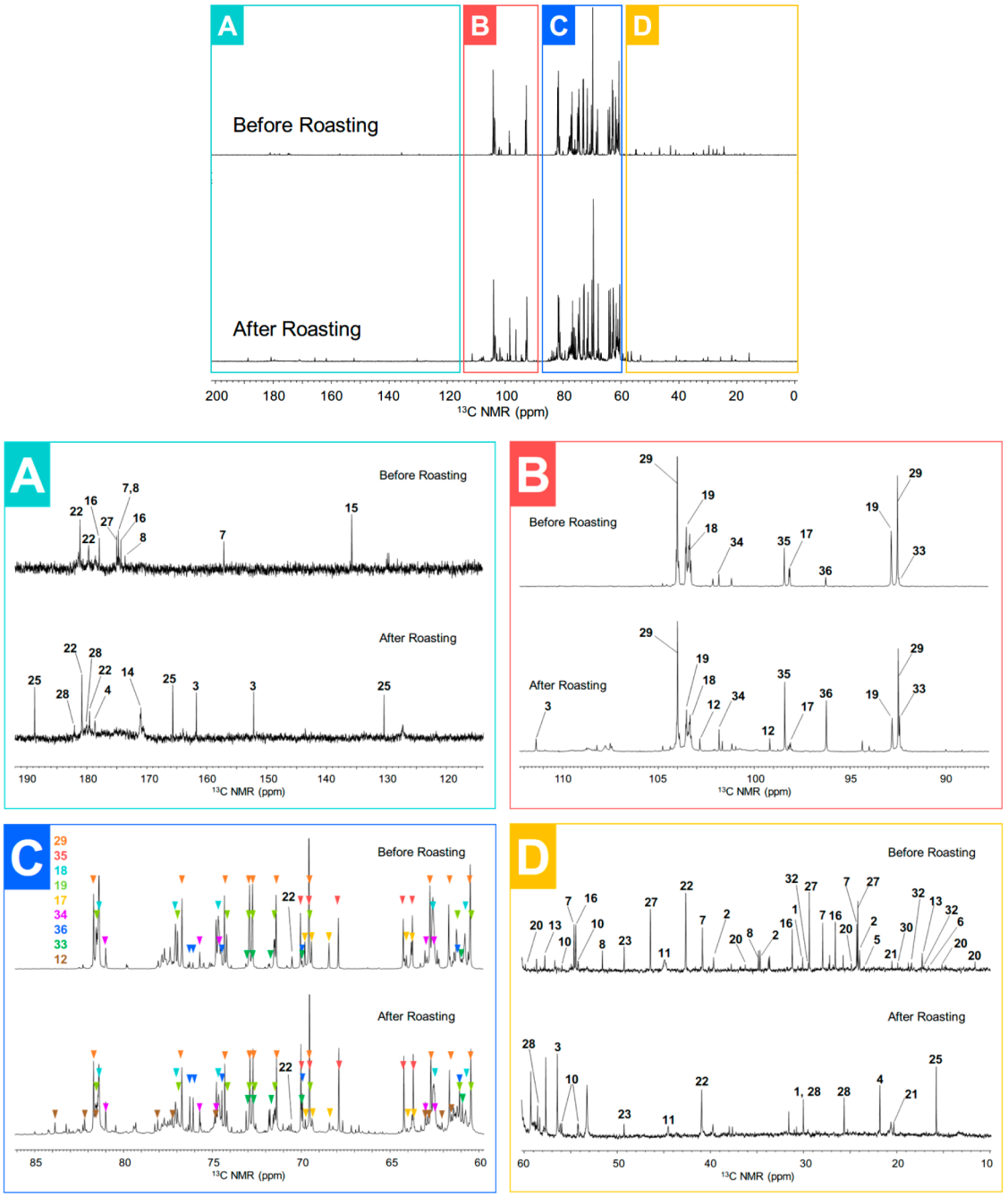

Figure 2. ^{13}C spectra of dried raw (top, sample R) or roasted (bottom, sample C4) chicory root. ^{13}C NMR spectra (top) are divided into four regions according to the range of the chemical shift: (A) 114−192 ppm, (B) 88−112 ppm, (C) 60−86 ppm, and (D) 10−60 ppm. The scales of the *Y* axes are different among spectra and panels. **1**, 2-methyl-2-propanol; **2**, 4-amino-*N*-butyric acid (GABA); **3**, 5-hydroxymethylfurfural (5-HMF); **4**, acetamide; **5**, acetate; **6**, alanine; **7**, arginine; **8**, asparagine; **9**, chicoric acid; **10**, choline; **11**, citrate; **12**, di-D-fructose dianhydride (*α*-D-Fru*f*-1,2′:2,1′-*β*-D-Fru*f*; DFA I); **13**, ethanol; **14**, formate; **15**, fumarate; **16**, glutamine; **17**, terminal fructose unit of m-type inulin (inulin-Fm); **18**, chain fructose unit of m- and n-type inulin (inulin-Fm, n); **19**, terminal glucose unit of n-type inulin (inulin-GFn); **20**, isoleucine; **21**, lactate; **22**, malate; **23**, methanol; **24**, monocaffeoyltartrate; **25**, norfuraneol (4-hydroxy-5-methyl-2*H*-furan-3-one; NF); **26**, phenylalanine; **27**, proline; **28**, pyroglutamate; **29**, sucrose; **30**, threonine; **31**, trigonelline; **32**, valine; **33**, *α*-D-glucopyranose; **34**, *β*-D-fructofuranose; **35**, *β*-D-fructopyranose; **36**, *β*-D-glucopyranose.

for ^{13}C; acquisition time, 0.259 s; delay time, 2.0 s; dummy scan, 4; and number of scans, 40.

The ^{1}H−^{13}C HSQC-TOCSY spectra were obtained in the phase-sensitive mode, and the parameters were as follows: number of data points, 2048 for ^{1}H and 512 for ^{13}C; spectral width, 4998 Hz for ^{1}H and 14,454 Hz for ^{13}C; acquisition time, 0.410 s; delay time, 1.5 s; dummy scan, 8; and number of scans, 32.

NMR Data Processing. The free induction decay (FID) data were processed using MestRe Nova software (version 8.0.1; MestReC, Santiago de Compostela, Spain). Signal assignments were made according to a previously described method based on two-dimensional NMR correlations[12,24] and were confirmed by a spiking method.[25,26] To investigate the changes in the chemical composition with time, the integral values of the signals (applied only for the spectra of R and C1−4 during deep roasting conditions, which showed strong compositional changes, including those produced by light roasting conditions) were calculated automatically, relative to those due to choline (3.19 ppm for

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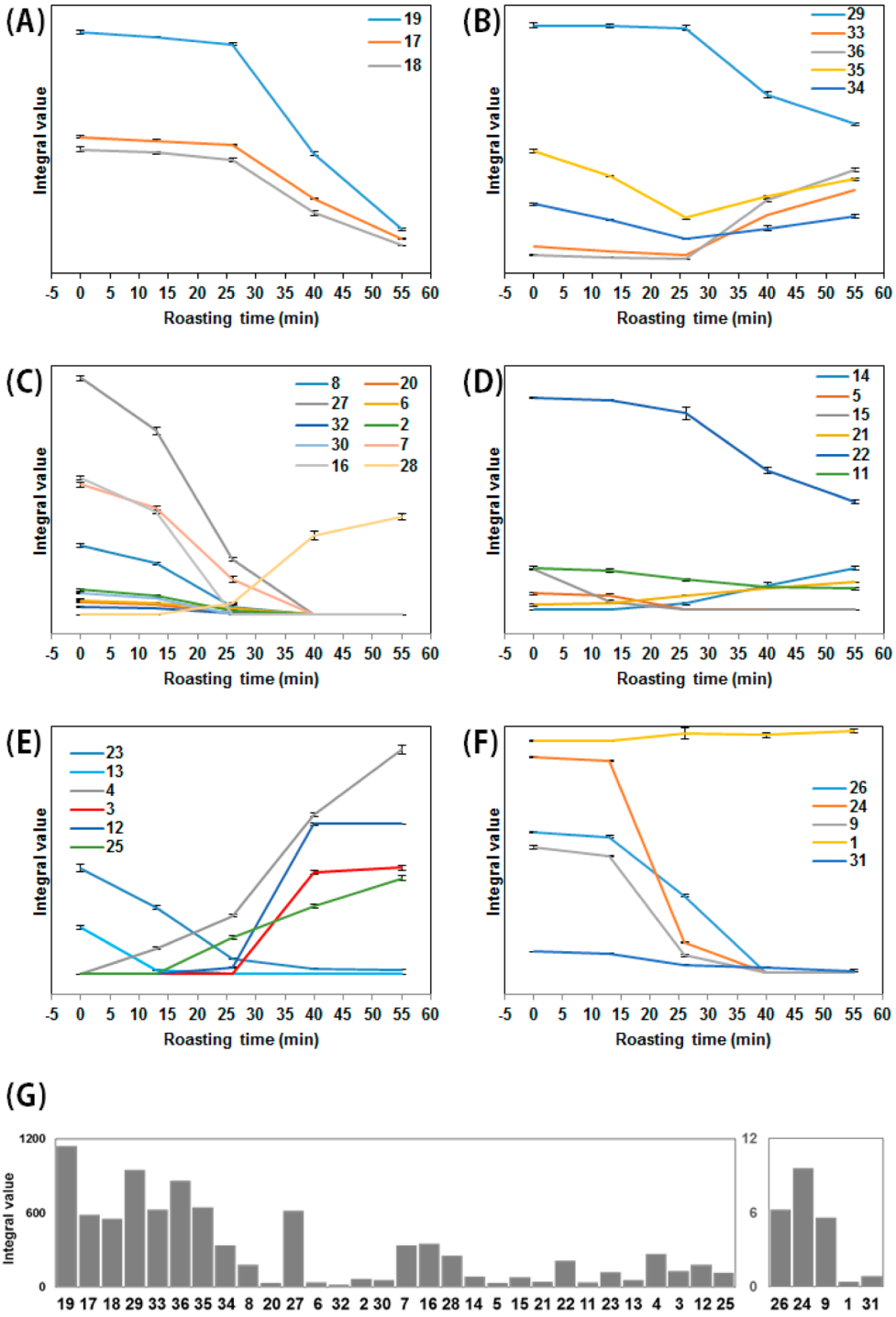

Figure 3. Relative changes in chicory root components during the roasting process. (A) Inulins: **17**, terminal fructose unit of m-type inulin (inulin-Fm); **18**, chain fructose unit of m- and n-type inulin (inulin-Fm, n); **19**, terminal glucose unit of n-type inulin (inulin-GFn). (B) Other sugars: **29**, sucrose; **33**, α-D-glucopyranose; **34**, β-D-fructofuranose; **35**, β-D-fructopyranose; **36**, β-D-glucopyranose. (C) Amino acids: **2**, 4-amino-*N*-butyric acid (GABA); **6**, alanine; **7**, arginine; **8**, asparagine; **16**, glutamine; **20**, isoleucine; **27**, proline; **28**, pyroglutamate; **30**, threonine; **32**, valine. (D) Organic acids: **5**, acetate; **11**, citrate; **14**, formate; **15**, fumarate; **21**, lactate; **22**, malate. (E) Others: **3**, 5-hydroxymethylfurfural (5-HMF); **4**, acetamide; **12**, di-D-fructose dianhydride (α-D-Fru*f*-1,2′:2,1′-β-D-Fru*f*; DFA I); **13**, ethanol; **23**, methanol; **25**, norfuraneol (4-hydroxy-5-methyl-2*H*-furan-3-one; NF). (F) Minor components: **1**, 2-methyl-2-propanol; **9**, chicoric acid; **24**, monocaffeoyltartrate; **26**, phenylalanine; **31**, trigonelline. The scales of the *Y* axes are different among panels. (G) Total amount of integral value change (difference between the maximum and minimum integral values due to each chicory root component during roasting). Each label shows the same compound as panels A–F.

^{1}H; 54.18 ppm for ^{13}C), which is thermostable during roasting and was set to a constant.[17]

In each NMR spectrum of the chicory root extracts during the roasting process, the relative integral values of the signals due to the same proton or carbon atoms were calculated. Changes in the concentrations of chicory components with time were determined by changes in the relative integral values at different degrees of roasting.

Multivariate Statistical Analysis. The ^{1}H NMR spectra were reduced into 0.04 ppm spectral buckets, and all spectra were aligned using the Correlation Optimized Warping (COW) method; then, they

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were normalized using the total as 100. The resulting data sets were imported into SIMCA-P version 13.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis. The mean center was applied for all multivariate analyses. A principal component analysis (PCA), an unsupervised pattern recognition method, was performed to examine the intrinsic variations in the data set. Hotelling's T2 region, shown as an ellipse in the score plots, defined the 95% confidence interval of the modeled variation. The quality of the model was described by the Rx^2 and Q^2 values. Rx^2 was defined as the proportion of the variance in the data explained by the model and indicated the goodness of fit. Q^2 was defined as the proportion of variance in the data predictable by the model and indicated predictability.

RESULTS

^{1}H and ^{13}C NMR Spectra of the Dried and Roasted Chicory Root Extracts. Figures 1 and 2 show the ^{1}H and ^{13}C NMR spectra of the chicory root extracts before (sample R) and after the roasting process at a relatively high temperature (sample C4). The ^{1}H NMR spectra have a high sensitivity for components present in trace amounts, such as trigonelline, chicory acid, and monocaffeoyltartrate, but intense overlapping occurred in the region from 3.3 to 4.3 ppm, which was dominated by several types of saccharides. In contrast, the ^{13}C NMR spectra have superior resolution, especially for the carbohydrate regions from 60 to 105 ppm, but do not resolve minor components. Different 2D NMR spectra were used to assign 36 NMR-visible components, including 2-methyl-2-propanol, 4-amino-*N*-butyric acid (GABA), 5-hydroxymethylfurfural (5-HMF), acetamide, acetate, alanine, arginine, asparagine, chicoric acid, choline, citrate, di-D-fructose dianhydride (*α*-D-Fruf-1,2′:2,1′-*β*-D-Fruf; DFA I), ethanol, formate, fumarate, glutamine, the terminal fructose unit of m-type inulin (inulin-Fm), the fructose unit of m- and n-type inulin (inulin-Fm, n), the terminal glucose unit of n-type inulin (inulin-GFn), isoleucine, lactate, malate, methanol, monocaffeoyltartrate, norfuraneol (4-hydroxy-5-methyl-2*H*-furan-3-one; NF), phenylalanine, proline, pyroglutamate, sucrose, threonine, trigonelline, valine, *α*-D-glucopyranose, *β*-D-fructofuranose, *β*-D-fructopyranose, and *β*-D-glucopyranose. The structures, NMR correlations, and chemical shifts of the assigned compounds are shown in Figure S1 of the Supporting Information. The 2D NMR spectra of ^{1}H−^{13}C edited-HSQC and ^{1}H−^{13}C HSQC-TOCSY (spectra not shown) were indispensable in the assignment of signals that badly overlapped, such as the signals due to hexatomic rings in *α*-D-glucopyranose, *β*-D-fructopyranose, *β*-D-glucopyranose, inulin-GFn, and sucrose.

Changes of Chicory Root Components during the Roasting Process. To investigate the changes in the composition of chicory root during the roasting process, the total and step-by-step integral value changes were summarized for all of the components identified (Figure 3). The evolution of the components in Figure 3A−E was obtained from the ^{13}C signal integral value for better signal separation; the other components in Figure 3F were undetected in the ^{13}C spectra because they were present in trace amounts or their overlaps were traced using the ^{1}H signal integral value. Although the ^{13}C spectra produced in the present study are not quantitative, a relative change in a particular signal can be considered as the change during roasting because all of the spectra were produced under the same conditions.

As shown in Figure 3, two types of inulins (i.e., the Fm-type and the GFn-type) experienced severe degradation during the roasting process. A stable decrease was observed in the initial stage at temperatures lower than 136 °C; subsequently, a sharp decrease was observed in all three of the assigned inulin units after 26 min. Sucrose was similar to inulin. The monosaccharides *α*-D-glucopyranose, *β*-D-fructofuranose, *β*-D-fructopyranose, and *β*-D-glucopyranose decreased with the roasting time from the start to 26 min; then, they increased until the dark roasting stage was reached at 55 min. All of the amino acids detected in the raw chicory root decreased during the roasting process. Pyroglutamate was not found in raw chicory root but was produced during the roasting process, and the amount continuously increased. Malate, citrate, acetate, and fumarate decreased during the roasting process, but formate and lactate increased. Methanol and ethanol in the raw chicory root decreased during roasting, and acetamide, 5-HMF, DFA I, and NF were formed during roasting. 5-HMF and DFA I sharply increased beginning at 26 min, and acetamide increased from the beginning to the end of roasting, whereas the rate of NF formation increased after 13 min. Chicory acids, phenylalanine, and trigonelline decreased during roasting, but 2-methyl-2-propanol remained stable during the entire roasting process.

Characterization of the Degree of Chicory Root Roasting by Principal Component Analysis. To investigate the overall changes in the composition under different roasting conditions, PCA was performed on the data for all chicory root extracts at different degrees of roasting. Figure 4 indicates that

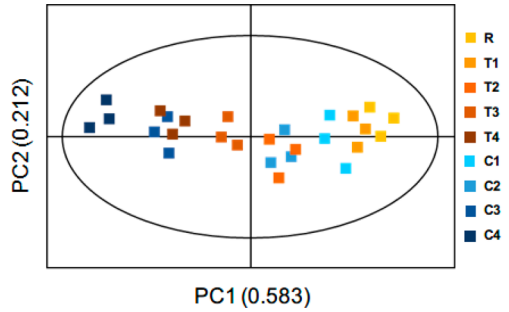

Figure 4. PCA score plot derived from the ^{1}H NMR spectra of chicory root extracts at different degrees of roasting. The fitness and predictability of the models are indicated by the Rx^2 values of 58.3 and 21.2% for PC1 and PC2, respectively, and a Q^2 value of 0.95.

chicory root extracts at each roasting degree were clearly distinguished from the others by the PCA score plots, which had high statistical values for Rx^2 (58.3 and 21.2% of PC1 and PC2, respectively) and Q^2 (0.95). The PCA score plot demonstrated that similar chemical composition profiles could be achieved by roasting the chicory root either at a higher firepower for a shorter time or at a lower firepower for a longer time.

DISCUSSION

The present study has monitored the substantial changes in the composition of chicory root during the roasting process using NMR-based metabolomics. Distinguished from other studies that were focused on a limited number of simple components, NMR spectroscopy enables a thorough characterization of complex chemical reactants and products during food processing due to its remarkable advantages of characterization of the principal ingredients of complex mixtures. A detailed and complete assignment of 1D and 2D NMR spectra of chicory root was performed in this study. Furthermore, the chemical transformation of each assigned component during the roasting process was followed by tracking the specific signal of each particular component.

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Carbohydrates are important precursors of many aromatic compounds and organic acids.[27−29] Changes in the carbohydrate composition are regarded as major alterations that occur in foods during soaking, roasting, or cooking. Inulin is a naturally occurring storage polysaccharide with a polyfructose backbone.[30] In the present study, a considerable amount of inulin was confirmed in the hot water extracts of chicory roots using NMR. Figure 3A indicates that inulin disappeared during the roasting process. However, a small amount of inulin was still present at the end of the roasting process. Inulin introduced into the intestine can be decomposed by gut bacteria such as *Bifidobacterium*, which promotes the proliferation of probiotics and benefits the optimization of the intestinal environment.[31] In this study, the rapid thermal decomposition of both inulin and sucrose was observed after a peak temperature between 136 and 168 °C was reached during the roasting process; this result is in accordance with previous reports that the thermal decomposition of inulin in water occurs at approximately 150 °C.[9]

Glucose and fructose are intermediate products of inulin and sucrose during roasting. In the early stage of the roasting process, the amount of glucose and fructose tended to decrease, probably because they were decomposed and polymerized and/or the Maillard reaction with ammonia precursors occurred.[9] In contrast, the rapid decomposition of polysaccharides during the later stages of the roasting process resulted in a dramatic increase in the total amount of glucose and fructose. However, fructose is not the final thermal decomposition product of inulin during roasting, as it continues to break down to generate other substances. A considerable number of previous studies that used inulin, fructose, and amino acids as reactants in the roasting process exist.[8,9,32−35] In the present study, the products DFA I[32−34] and NF[35] were also identified in roasted chicory root. This result demonstrated that, different from studies that were focused on a limited number of simple components, the formation of DFA I and NF was the dominant thermal reaction during the roasting process of chicory root. This result exemplifies an important advantage of NMR-based metabolomics, which enables an objective evaluation of the relative proportion of all thermal reactions in the roasting process by quantifying the molar concentration of the products. Roasted chicory root has been widely used as a coffee substitute. The primary carbohydrate component of chicory root is inulin, which is different from green coffee beans, which are far more complex and mainly contain sucrose.[11,17] However, the intermediate products of inulin and sucrose produced during roasting commonly include glucose and fructose. Therefore, it is entirely possible that components that confer a similar aroma, acidity, and bitterness can be produced from glucose and fructose by reactions with other common ingredients under similar roasting conditions.

Similar to the roasting process of coffee beans, most amino acids in chicory root tend to disappear during roasting, probably due to the Maillard reaction that occurs between the amino acids and reducing sugars such as fructose, an intermediate derived from inulin. However, the highly thermostable amino acid pyroglutamic acid continually increases during roasting. One likely explanation is that the self-condensation reaction of pyroglutamic acid, when heated, results in the generation of thermostable ring-like structures.[36] Moreover, similar to the results of coffee beans, citric acid and malic acid decreased, whereas aliphatic acids derived from the decomposition of sugar increased during roasting.[27]

It also should be noted that acetamide, commonly present in tobacco smoke,[37,38] was dramatically increased in roasted chicory root. Indeed, acetamide has been identified as a Maillard reaction product of chitin[39] and a lignocellulosic biomass[40] after thermochemical treatment. The oncogenic potential of acetamide has been demonstrated in rat and mouse models,[38] suggesting a potential health impact of roasted chicory root. It is also of interest that raw chicory root contains a large amount of methanol and ethanol, which almost completely disappeared during roasting via volatilization and/or thermal reactions. However, the content of 2-methyl-2-propanol, which was confirmed with a spiking method,[25,26] tended to be relatively constant during the roasting process. Because 2-methyl-2-propanol is a volatile alcohol, it may be generated at a similar rate to volatilization during roasting. There is a previous report that the content of 2-methyl-2-propanol in honey, a main ingredient of which is fructose, will increase after heat sterilization.[41] Because fructose was markedly generated in the roasted chicory root, it might be a source to continuously produce 2-methyl-2-propanol. In addition, small amounts of active plant ingredients such as chicory acids, phenylalanine, and trigonelline were also decreased during roasting.

In summary, this study monitored the substantial changes in the composition of chicory root during roasting using NMR-based metabolomics. Due to the diversity of food composition, chemical changes during food processing are often more complicated than those of studies that employ single or multiple representative compound(s). Major chemical reactions and their products during the roasting of chicory root were elucidated using a comprehensive analysis of NMR spectra. Unlike other methods analyzing individual compounds by physical separation, NMR analysis not only quantifies each reaction product but can also evaluate the relative proportion of all thermal reactions in a complex mixture during roasting. We believe that advances in NMR probe development and the peak alignment technique will bring about a remarkable contribution of NMR-based metabolomics to the characterization of food processing because of the continual improvements in detection sensitivity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02423.

Chemical structures, NMR correlations, and chemical shifts of the assigned compounds in dried and roasted chicory root extracts (PDF)

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Notes

The authors declare no competing financial interest.

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