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Time-dependent changes of fruit metabolites studied by ^{1}H NMR

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Abstract The browning phenomenon of fruits can be easily observed when fruits or vegetables (apples, pears, bananas, potatoes, etc.) are cut with a knife and the part turns brown. When this browning occurs, changes in taste, color, and nutrients usually are introduced. The cause of this browning phenomenon has been well studied for a long time, but these studies have mainly focused on preventing deterioration of processed foods during food processing or storage. Resultantly, there are few studies on how much changes in nutrients (saccharides, amino acids, fats, water-soluble low molecular weight ammonium ions, etc.) are caused by browning.

The purpose of this study is to determine the change in nutrients during browning using apple as a model fruit. We conducted a comparative study on how much the nutrient fluctuations differ depending on the presence or absence of pretreatment such as the application of heat. All analysis was conducted using ^{1}H NMR. The ANOVA analysis showed that the concentrations of 4 amino acids (alanine, asparagine, isoleucine, and valine), 3 types of sugars (fructose, glucose, and xylose), 1 type of organic acid (lactate) and choline were significantly increased in samples showing browning. In addition, the groups before and after browning were clearly separated using multivariate statistical analysis methods (PCA, PLS-DA), which was greatly contributed by two sugar components (fructose and glucose) present in high concentrations in apples.

Keywords Fruit browning, Metabolomics, NMR

Introduction

As consumers' preference for fresh agricultural products increases, the demand for them is also steadily increasing. These fresh produce are susceptible to oxidative browning reactions because they undergo many processing steps such as washing, sorting and peeling during the production stage. Also, browning, which is more likely to occur due to tissue damage that may occur during post-harvest processing, eventually leads to deterioration of food quality (change in color, loss of texture, and loss of flavor). This is the reason why research to suppress this browning phenomenon is still in progress.[1-4]

In the paper, “Study on the commercialization of fresh sliced pears through the development of anti-browning agents”, it was found an effective blending ratio for preventing browning of pears and increasing sweetness by conducting a research on the anti-browning effect and consumer preference using oxidized starch, citric acid, and sucralose.[1] The study “Anti-browning effect of Citrus peel extract on antioxidant and apple slices” describes the anti-browning effect. As a result of observing the degree of browning by immersing apple slices in

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extracts of oranges, tangerines, lemons, and grapefruits, the most excellent browning inhibitory effect was found in 0.1% of the extract, and the lemon peel extract showed the best effect among the four types of peels. This study suggested the possibility of using lemon peel extract as a natural browning inhibitor.[3]

This study was aimed to determine the change in nutrients during browning using apple as a model fruit. Apples contain a lot of polyphenols that have antioxidant properties. Polyphenols are abundant in plants, and representative polyphenol compounds include placyanidins in apples, catechins in green tea, resveratrol in grapes, and cacao in chocolate. The main effects of these compounds are antioxidant activity, fat burning activity, and antibacterial activity. Among them, the representative effect is the antioxidant action, which plays a role in removing free radicals.[5] It has been reported that the antioxidant polyphenols contained in apples increase the synthesis of short-chain fatty acids, thereby reducing the incidence of colorectal cancer.[6] In addition, pectin, a dietary fiber contained in apple peel, is known to help prevent colorectal cancer by increasing the formation of butyrate, a type of fatty acid.[5] A research team at Cornell University in the United States observed breast cancer-induced mice by dividing them into a group to which apple extract was administered and a group to which they did not. As a result, it was found that the rate of adenocarcinoma, a type of breast cancer, decreased in proportion to the amount of apple extract administered in the group to which the apple extract was supplied compared to the group to which the apple extract was not administered. From the results of this experiment, it can be seen that phenolic compounds and flavonoids, antioxidant substances in apples, have an inhibitory effect on tumor growth.[7]

Apples contain about 85-90% moisture, and the main ingredient is about 13% sugar. The sugar composition shows various distributions depending on the variety, but the three sugars, fructose, glucose, and sucrose, show the highest content at about 50%, 20%, and 25%, respectively. Although there is a difference in the amino acid content depending on the variety of apple, it is less than other fruits, about 50 to 90 mg per 100 g of pulp. It has the highest content of aspartic acid and tryptophan, and contains most of the essential amino acids such as alanine, glutamic acid, isoleucine, and leucine. In addition, apples contain several polyphenol components such as 5-hydroxymethyl furfural, chlorogenic acid, caffeic acid, epicatechin, and quercetin that act as antioxidants. In particular, many of these ingredients are contained in the peel.[6] The total amount of organic acid shows a distinct difference depending on the variety. It is mainly composed of malic acid, and other citric acid, ginaic acid, succinic acid, fumaric acid, maleic acid, etc. are included.[8]

Apple browning is classified into enzymatic and non-enzymatic effects. The enzymatic effect is browning by polyphenol oxidase and browning by tyrosinase in enzymatic browning reactions that require enzymes, substrates and oxygen. Non-enzymatic browning reactions are usually divided into three categories: Maillard reaction, caramelization reaction, and oxidation reaction of vitamin C. However, non-enzymatic browning of food usually occurs as a complex reaction rather than a single reaction due to a number of complex components.

In this study, the browning phenomenon of apples according to temperature and time was observed, and NMR measurement and analysis were performed to confirm the change of nutrients according to the browning phenomenon.

Experimental Methods

Observation of apple browning phenomenon and extraction of nutrients- Five slices (1.5 g) of the same amounts of apples were weighed. After storage (15 hours) at room temperature and 4°C, respectively, the state before and after browning was observed. Each sample was pulverized using a pulverizer. Nutrient components of each sample were extracted with methanol/chloroform/distilled water (2:2:1). In the case of browning samples (samples stored at room temperature or refrigerated for 15 hours), the weight

was re-weighed before grinding because moisture evaporated. After addition of 200 µl of methanol with grinding, 100 µl of chloroform was added and grinded again. 100 µl of chloroform and 100 µl of distilled water were more added for resuspension. After 1 hour incubation at 4°C, centrifugation was performed at 13,000 rpm for 20 minutes. After transferring the upper layer (hydrophilic) from the two separated layers to a new tube, the solvent was removed using a centrifugal vacuum evaporator.

NMR sample preparation- A sample from which the solvent has been removed was obtained using a centrifugal vacuum evaporator, dissolved in the buffer (100 mM sodium phosphate buffer, pH 7.0) containing TSP, a standard material, and then sealed in an NMR tube. To analyze the metabolites, 1D Carr-Purcell-Meiboom-Gill (CPMG) NMR spectra (cpmgpr1d) were obtained at 298 K on a Bruker ASCENDⅢ 600 spectrometer equipped with a cryoprobe.[9] The CPMG pulse sequence generated spectra edited by T2 relaxation times, reducing broad resonances from high molecular weight compounds, improving the observation of low molecular weight metabolites. The water signal was removed by a presaturation method using low-power irradiation on the water resonance. ^{1}H-NMR spectrum for each sample consisted of 128 scans with following parameters: spectral width = 12019.2 Hz, spectral size = 65,536 points, pulse width (90) = 13.2 µs and relaxation delay (RD) = 2.0 s. Each free induction decay (FID) was zero-filled to 64,000 points and transformed with line broadening (LB) = 0.3 Hz.

Spectral processing and metabolite identification- Initially, ^{1}H NMR spectra were manually phased and baseline corrected using Bruker Topspin 3.2 software (Bruker GmbH, Karlsruhe, Germany) and referenced to TSP at 0.0 ppm. The post-processing baseline correction was conducted with Mnova (Mestrelab Research, Santiago, Spain). In each spectrum, the algorithm of multipoint baseline correction was used for building the baseline model. Because the NMR spectral bins of each spectrum can be easily influenced by small change of pH and/or ionic strength and the broad bin size consisting of more than one resonance often makes the result difficult to interpret, the peak alignment using segment and pair-wise peak alignment by Mnova was applied before binning. In addition, a variable bin size ranging from 0.005 ppm to 0.09 ppm was used so that each single bin contains single metabolic information as much as possible. The ^{1}H NMR spectra were segmented into variable-sized spectral regions (bins) between 0.94 and 8.48 ppm. The chemical shift region of 4.69-5.20 ppm containing residual water was excluded. The lipid or protein contaminated region (1.10–1.33, 1.52–1.68, 1.78–1.90, and 5.28–5.70 ppm) was also removed from the spectra to clarify the contribution of metabolites.[10] The integrated bins were used as the variables for statistics. The assignment of bins was achieved using Chenomx NMR suite 7.7 (Chenomx Inc., Edmonton, Canada).[11]

Statistical analysis- The statistical analysis was carried out using the SIMCA 15 (Umetrics, Umea, Sweden).[12] The spectra were classified into controls and clinically diagnosed case subjects (diabetic neuropathy and diabetes). The integrated bins were normalized using probabilistic quotient normalization (PQN) algorithm to facilitate comparison of samples. In order to provide a reasonable balance of contributions from high and low amplitude signals, the spectral integrals (bins) were scaled by the procedure called pareto-scaling: each variable is mean-centered and divided by the square root of the standard deviation.[13, 14]

The multivariate analysis was performed as follows. To clarify the separation between groups, bin data were processed using a supervised pattern recognition method, orthogonal partial least squares discriminant analysis (OPLS-DA).[15,16]

The efficiency and reliability of OPLS-DA models was validated using 500-random permutation test.[17] The quality of the models is described by R2 and Q2 values. R2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q2 is defined as the proportion of variance in the data predictable by the model and

Table 1. Quantitation of Apple Nutrients obtained by NMR

Nutrients	Apple concentration before browning (mM)		Apples after browning (stored at room temperature, mM)		Apples after browning (stored at 4°C)	
	Average	S.D	Average	S.D	Average	S.D
Acetate	0.150	0.0202	0.137	0.0901	0.151	0.0609
Alanine	0.098	0.0181	0.217	0.0494	0.304	0.0291
Asparagine	0.962	0.3072	1.967	0.3504	2.113	0.5903
Aspartate	0.713	0.1716	0.819	0.0834	1.037	0.1124
Betaine	0.296	0.1150	0.227	0.0622	0.254	0.0904
Choline	0.028	0.0069	0.075	0.0032	0.068	0.0075
Fructose	266.541	15.2457	350.445	3.8506	332.794	27.1426
Galactose	0.366	0.1379	0.319	0.0860	0.413	0.1161
Glucose	110.135	8.6038	136.562	3.1720	138.923	11.2690
Isobutyrate	0.005	0.0014	0.007	0.0007	0.007	0.0005
Isoleucine	0.017	0.0018	0.024	0.0025	0.029	0.0035
Lactate	0.844	0.0506	1.143	0.0458	1.092	0.1265
Leucine	0.003	0.0011	0.005	0.0010	0.004	0.0009
Malate	16.824	2.0524	17.290	0.3038	21.479	1.9403
Pyruvate	0.154	0.0450	0.163	0.0250	0.187	0.0345
Sucrose	12.143	2.9077	17.893	2.1807	14.853	3.4388
Tartrate	1.719	0.2121	1.698	0.0724	2.328	0.4266
Valine	0.014	0.0029	0.022	0.0044	0.028	0.0041
Xylose	3.023	0.1553	4.483	0.1845	4.256	0.3506

indicates predictability.[18] An observed p-value of 0.05 was used to identify statistically significant group separation. The OPLS-DA models were further characterized by their p-values obtained from CV-ANOVA (Analysis Of Variance testing of Cross-Validated predictive residuals) implemented in SIMCA 15.[19]

The potential biomarker candidates were selected based on the contribution to classification between groups based on values of variable importance in project (VIP) of all variables and the results of univariate analysis. The VIP value of each variable in the model was calculated to indicate its contribution the separation. A higher VIP value represents a stronger contribution to classification between groups.[20]

Results and Discussion

Qualitative/quantitative analysis of nutrients in apples before and after browning- Apple nutrients extracted from five samples of 1.5 g each under three conditions (before browning, stored at room temperature, and stored in refrigeration) were measured at 600 MHz NMR and these spectra were analyzed to qualitatively analyze 19 types of apple nutrients. The concentration (mM) of each nutrient was analyzed based on the TSP, and the mean and standard deviation of each nutrient were also

indicated (Table 1). 6 types of organic acids (acetate, isobutyrate, lactate, malate, pyruvate, and tartrate), 6 types of amino acids (alanine, asparagine, aspartate, isoleucine, leucine, and valne), 5 types of sugars (fructose, galactose, glucose, sucrose, and xylose), choline, and betaine were identified and their concentrations were analyzed successfully.

Mean comparison analysis using ANOVA, a univariate statistical analysis method- As a result of ANOVA, a univariate statistical analysis method, the quantitative results of browning samples before and after browning were statistically significant (*p*-value less than 0.05) in the groups before and after browning (Table 2). 4 types of amino acids (alanine, asparagine, isoleucine, and valine), 3 types of sugars (fructose, glucose, and xylose), lactate and chloline, which are organic acids, were all increased in browned apple samples after storage at room temperature and refrigeration for 15 hours compared to before browning. There was no significant change in nutritional content between the room temperature browning sample and the refrigerated browning sample.

The change in the component is shown in Figure 1 as a Box-whisker plot. Each box indicates the 25th to the 75th percentile and includes half of the total, and the line within the box indicates the median (50th percentile).

The whiskers above and below the box represent the minimum and maximum values excluding outliers, and the extreme values are circled. Compared to before browning, the nine nutrients showed an increase in both room temperature and refrigerated storage for 15 hours, and there was no significant change between the samples stored at 25°C and 4°C. There are reports that when browning occurs, nutrients such as sugars, phenolic compounds, and vitamin C are reduced. These studies are the results of browning events that occurred over a period of as short as 9 days and as long as 6 months.[21-23] We set a storage time of 15 hours for browning, and visual browning was sufficiently observable with this amount of time. However, it seems that the time is insufficient for the browning of nutrients such as sugars, amino acids, and organic acids to occur. Interestingly, leaving it for a day or so seems to help increase some of the ingredients. According to the results of our experiment, the four amino acids tended to increase after browning occurred after 15 hours. In order to check where this result really comes from, three apples were browned again at room temperature for 15 hours, and then an experiment was conducted to compare the amount of protein. The amount of protein was measured by Brad-Ford protein quantification method. A concentration standard curve was created using the

Table 2. ANOVA result before/after browning (room temperature, refrigerated storage)

Nutrients	Comparison between the nutrient before and after browning (stored at room temperature) *p*-value	Comparison between the nutrient before and after browning (stored at 4°C) *p*-value	Comparison between the nutrients after browning (stored at room temperature and 4°C) *p*-value
alanine	0.046	0.002	0.413
asparagine	0.043	0.017	1.000
isoleucine	0.048	0.002	0.312
valine	0.034	0.004	0.867
fructose	0.040	0.027	1.000
glucose	0.037	0.012	1.000
xylose	0.022	0.027	1.000
lactate	0.040	0.027	1.000
choline	0.006	0.028	1.000

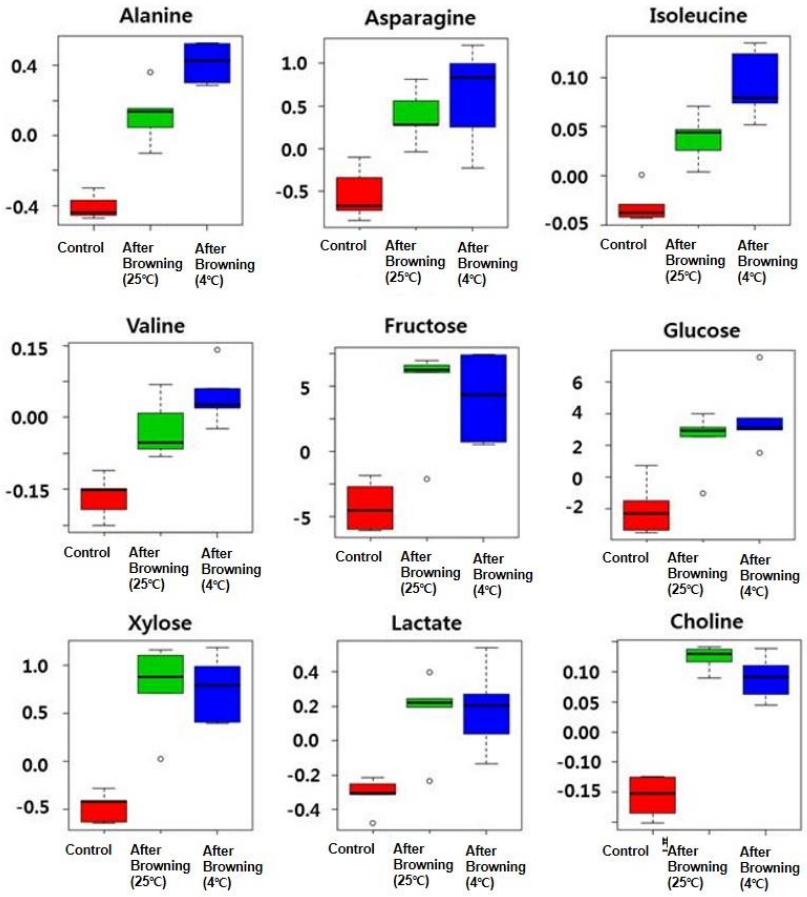

Figure 1. Changes in nutritional composition before and after browning

BSA protein. By measuring the absorbance at 595 nm, a reliable standard curve of protein concentration with a coefficient of determination (R2) of 0.9976 was obtained (data not shwon). The absorbance of the extracts before and after browning was measured at 595 nm wavelength and substituted into the concentration standard curve to calculate the protein concentration (Table 3). As a result of comparing the amount of protein before and after browning, it was confirmed that the protein concentration decreased by 14% on average. Proteins consist of 20 amino acids linked by peptide bonds, and can be broken down into amino acids again by proteolytic enzymes. The decrease in the amount of protein after browning means that the protein is decomposed, and this result partially supports the significant increase in the four amino acids that was shown in the NMR quantitative analysis of our apple nutrients.

Group separation using PCA and PLS-DA, multivariate statistical analysis methods- Through

Table 3. Protein concentration of browned apple extract before browning and after 15 hours at room temperature

	Protein concentration before browning (mg/ml)		Protein concetration after browning (stored at 25℃, mg/ml)	
	Average	S.D	Average	S.D
Three samples	0.150	0.0202	0.137	0.0901

principal component analysis (PCA), the degree of separation of three groups was confirmed before browning (Fig. 2A, red) and after browning (room temperature, Fig. 2A, green) and refrigerated storage (Fig. 2A. blue)).

As a result, it was confirmed that, while a relatively clear degree of separation was seen between the two groups before and after browning, the separation was not seen well between the two groups of room temperature storage and refrigeration storage. Group

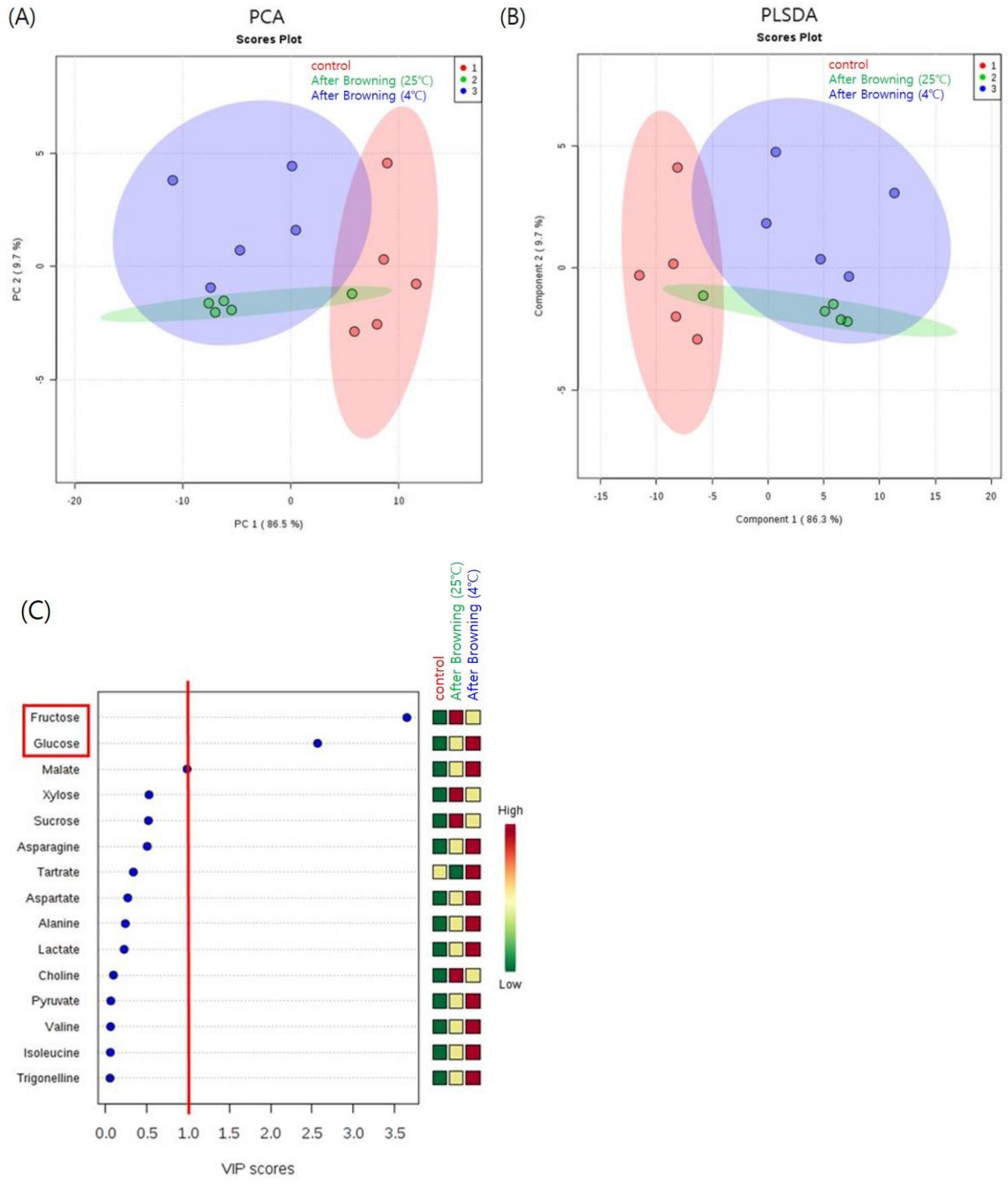

Figure 2. PCA and PLS-DA analysis of groups before and after browning (room temperature and refrigerated storage) and VIP scores of PLS-DA model.

differences were analyzed by partial least squares plate variation (PLS-DA), which is a supervised method that proceeds with group information given. The degree of separation of three groups was checked before browning (Fig. 2B. red), after browning (room temperature (Fig. 2B. green), and refrigerated storage (Fig. 2B. blue)). Similar to the PCA results, it was confirmed that a relatively lear degree of separation was observed between the two groups before and after browning, whereas the separation was not seen well between the two groups of room temperature storage and refrigeration storage. As in the univariate analysis, no significant nutritional component was found between the room temperature and refrigerated browning groups, both the PCA and PLS-DA multivariate analyzes showed that the room temperature and refrigerated browning groups were not well separated from each other.

As a result of checking the VIP score of the PLS-DA model, which indicates the value contributing to the group separation, in order to find out which nutrient components actually contribute to the separation of these groups (greater than 1), they exist in high concentrations in apples such as fructose and glucose. It was confirmed that the groups before and after browning were clearly distinguished by the saccharides. In the case of malate, it was excluded because it had a value of 0.98, but it seems to contribute a lot to group separation compared to other components other than fructose and glucose. (Fig. 2C).

Concluding Remarks

It is known that most of the browning of apples is caused by enzymatic reactions.[24] When exposed to oxygen in the air, it causes a browning reaction by polyphenol oxidase in the apple flesh. In this experiment, the browning phenomenon was observed by storing apples under conditions of varying temperature (room temperature and refrigeration) and for time (15 hours) through NMR measurement and analysis. Nineteen species of nutrients including amino acids, sugars, and organic acids were identified and their concentrations were analyzed. Univariate and multivariate statistical analysis was performed using the nutrient concentration values analyzed in this way, and it was confirmed which components were different and which components separated groups under each condition. From ANOVA assay, a univariate analysis method, the concentrations of 4 amino acids (alanine, asparagine, isoleucine, and valine), 3 types of sugars (fructose, glucose, and xylose), 1 type of organic acid (lactate) and choline were found in samples showing browning. It was confirmed that there was a significant increase. Nutrients significantly decreased among 19 species could not be found. In addition, using multivariate statistical analysis methods (PCA, PLS-DA), it was confirmed that the groups before and after browning were clearly separated. This result is very interesting because it is different from our expectation that browning and double nutrient destruction (reduction) will occur.

An increase in the three types of sugars and changes in choline may be associated with the ‘ripening’ of the fruit. It was found that there may be an increase in these nutrients during the aging process. In a study on garlic, it was revealed that browning occurred during the aging process of garlic, the process of making so-called black garlic, and changes in various components. As a result of analyzing the components of free sugar, amino acids, and polyphenols after aging garlic for 200 hours, the contents of fructose and glucose increased by 74% and 78%, respectively, and the total amino acid contents including aspartic acid, alanine, isoleucine, and valine were showed an increase of about 30%. Polyphenols also showed a significant increase.[25] In a research paper investigating the sugar and amino acid content after ripening Daebonggam for 15 and 30 days, total amino acids showed a tendency to decrease with the aging period, but increased fructose and glucose, the sugar components, were confirmed.[26, 27] Considering these results, it can be interpreted that the increase in some components in our experimental results is due to the 'short ripening' process.

In conclusion, we found that some nutritional components of apples may increase after browning.

However, it is not clear whether the apple browning phenomenon directly affects the nutrient increase or decrease. In addition, the possibility that the increase in these nutrients could decrease again after a long time was considered. Although it is not known what the direct cause of this nutrient content and time dependence is, it may be imagined if there is a way to eat more nutrient-rich apples using this property.

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