A Dissertation for the Degree of Doctor of Philosophy

Application of Nuclear Magnetic Resonance (NMR) Spectroscopy for Quantitative Analysis and Use in Meat Science

핵자기공명(NMR) 분광학 정량분석의 적용 및 식육과학분야의 이용

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이 논문을 농학박사 학위논문으로 제출함

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Overall Summary

Nuclear magnetic resonance (NMR)-based analyses are originally employed to determine the chemical structures of compounds. In addition, NMR offers both nonbiased and panoramic views, and its quantitative application has increased considerably. However, the accurate analysis of biological samples, which are basically complex mixtures, using NMR spectroscopy is challenging. The biological samples could display an unflattened baseline, overlapping peaks, different T_1 relaxation time, and varied chemical shift, yielding inaccurate quantitative results**.** In addition, multivariate analysis is usually performed to obtain insights into the NMR data. However, in meat science, related analysis is rare. Therefore, in this study, experiments were conducted to 1) optimize the quantitative proton (^{1}H) NMR conditions, 2) confirm the possibility of two-dimensional quantitative NMR analysis, and 3) apply chemometrics using quantitative NMR (qNMR) to meat considering various aspects, including the breed, aging, and storage conditions.

Experiment I.

Optimization of 1D ^{1}H quantitative nuclear magnetic resonance (NMR) conditions for polar metabolites in meat

The objective of this study was to establish an optimized 1D ^{1}H quantitative

nuclear magnetic resonance (qNMR) analytical method for analyzing polar metabolites in meat. Three extraction solutions [0.6 M perchloric acid, 10 mM phosphate buffer, water/methanol (1:1)], three reconstitution buffers [20 mM 3-morpholinopropane-1-sulfonic acid, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, phosphate buffer], and two pulse programs (zg30, noesypr1d) were evaluated. Extraction with 0.6 M perchloric acid and 20 mM phosphate resulted in a stable baseline and no additional overlap for quantifying polar metabolites in chicken breast. In qNMR analysis, zg30 pulse program (without water-suppression) showed smaller relative standard deviation (RSD) and faster running time than noesypr1d (water-suppression). High-performance liquid chromatography was compared with qNMR analyses to validate accuracy. The zg30 pulse showed good accuracy and lower RSD. The optimized qNMR method was able to apply for beef and pork samples. Thus, an optimized 1D ^{1}H qNMR method for meat metabolomics was established.

Experiment II.

Potential of 2D qNMR spectroscopy for distinguishing chicken breeds based on the metabolic differences

Two-dimensional quantitative NMR spectroscopy (2D qNMR) was set up and multivariate analyses were performed on metabolites obtained from breast meat extracts of broilers and four native chicken (KNC) strains. It can accurately identify more metabolites than 1D ^{1}H NMR via separation of peak overlap by dimensional expansion with good linearity, but has a problem of numerical quantification; Complementation of 1D and 2D qNMR is necessary.

Among breeds, KNC-D had superior breast meat quality because of higher amounts of free amino acids, sugars, and bioactive compounds than others. Noticeable differences between KNCs and broilers were observed; KNCs contained higher amounts of inosine 5'-monophosphate, α-glucose, anserine, and lactic acid, and lower amounts of free amino acids and their derivatives. The 2D qNMR combined with multivariate analyses distinguished the breast meat of KNCs from broilers but showed similarities among KNCs. Also, 2D qNMR may provide fast metabolomics information compared to conventional analysis.

Experiment III.

Characteristic metabolic changes of the crust from dry-aged beef using 2D NMR spectroscopy

Two-dimensional quantitative nuclear magnetic resonance (2D qNMR)-based metabolomics was performed to understand characteristic metabolic profiles in different aging regimes (crust from dry-aged beef, inner edible flesh of dry-aged beef, and wet-aged beef striploin) over 4 weeks. Samples were extracted using 0.6 M perchlorate to acquire polar metabolites. Partial least squares-discriminant analysis showed a good cumulative explained variation (R^2 = 0.967) and predictive ability (Q^2 = 0.935). Metabolites of crust and aged beef (dry- and wet-aged beef) were separated in the first week and showed a completely different aspect in the second week via NMR-based multivariable analyses. Moreover, NMR-based multivariable analyses could be used to distinguish the method, degree, and doneness of beef aging. Among them, the crust showed unique metabolic changes that accelerated proteolysis (total free amino acids and biogenic amines) and inosine 5'-monophosphate depletion than dry-aged beef

and generated specific microbial catabolites (3-indoxyl sulfate) and γ-aminobutyric acid (GABA), while asparagine, glutamine, tryptophan, and glucose in the crust were maintained or decreased. Compared to the crust, dry-aged beef showed similar patterns of biogenic amines, as well as bioactive compounds and GABA, without a decrease of free amino acids and glucose. Based on these results, the crust allows the inner dry-aged beef to be aged similarly to wet-aged beef without microbial effects. Thus, 2D qNMR-based metabolomic techniques could provide complementary information about biochemical factors for beef aging.

Experiment IV.

Prediction of chicken freshness and classification of fresh and frozen/thawed meat based on NMR-spectroscopy

We identified key metabolites reflecting microbial spoilage and differentiated fresh meat from frozen/thawed (FT) using 2D qNMR analysis. Fresh and FT chicken breasts were prepared, individually aerobically packaged, and stored for 16 days at 2 °C. Only volatile basic nitrogen (VBN) was significantly changed after 6 log CFU/g of total aerobic bacteria ($p < 0.05$). Extended storage resulted in an increase in organic acids, free amino acids, biogenic amines, and hypoxanthine and a decrease in N,N-dimethylglycine, inosine 5'-monophosphate, and proline. Acetic acid demonstrated the highest correlation with VBN ($r = 0.97$). Fresh and FT breast meat can be differentiated by uniform concentration of carnosine, β-alanine, and histidine levels, consistent changes in nucleotides by storage time, and changes in microbial metabolism patterns that are reflected by some free amino acids. Thus, NMR-based metabolomics can be used to evaluate chicken breast meat freshness and

distinguish between fresh and FT meat.

Keyword: polar metabolites, qNMR, metabolomics, 2D qNMR, chicken, beef, multivariate analysis, classification

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List of Abbreviations

1D ^{1}H NMR		One-dimensional proton nuclear magnetic resonance
^{1}H	:	Proton
2D qNMR	:	Two-dimensional quantitative nuclear magnetic resonance
ANOVA	:	Analysis of variance
AUC		Area under curve
CFU	:	Colony forming unit
COSY	:	Correlation spectroscopy
DDW		Deionized and distilled water
DSS	:	3-(Trimethylsilyl)-1-propanesulfonic acid
GABA		γ-aminobutyric acid
HEPES	:	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HMBC	:	Heteronuclear multiple bond
HPLC	:	High-performance liquid chromatography
HSQC	:	Heteronuclear single quantum coherence
IMP		Inosine 5'-monophosphate
LOD		Limit of detection
LOQ		Limit of quantification
MOPS		3-Morpholinopropanesulfonic acid
NMR	:	Nuclear magnetic resonance
OPLS-DA		Orthogonal partial least square-discriminant analysis

PCA		Principle components analysis
PLS-DA		Partial least square discrimiant analysis
qNMR	:	Quantitative nuclear magnetic resonance
RSD		Relative standard deviation
SVM		Support vector machine
TAB		Total aerobic bacteria
TBARS	:	2-Thiobarbituric reactive substances
TMA		Trimehtylamine
TOCSY	:	Total correlation spectroscopy
TSP	:	3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid
UV/Vis	:	Ultraviolet visible
VBN		Volatile basic nitrogen
VIP		Variable importance in projection

Chapter I.

General introduction

Chemometric analysis is a method of obtaining significant information using multivariate analysis of data obtained by quantitative analysis techniques, such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (Trygg et al., 2007; Markely et al., 2017). Typically, NMR and MS are separately employed to identify the characteristics of a sample; however, when used together, the data quality can be further improved and an increased coverage of target metabolites can be achieved (Seger et al., 2013; Bingol & Brüschweiler, 2017; Bhinderwala et al., 2018). Moreover, relatively high resolution and sensitivity have been achieved with advances in hardware or experimental methods, increasing the quantification accuracy of more target metabolites (Hager, 2002; Kwan et al., 2011). With the improvements in quantitative analysis efficiency, more information can be obtained at once (Simmler et al., 2014). Multivariate analysis can help researchers analyze data better through visualization and clustering analyses for classification, affording meaningful results (Kim et al., 2021b). Multivariate analysis can be divided into supervised and unsupervised analyses (Kim et al., 2021a). An example of unsupervised multivariate analyses is the principal component analysis (PCA), which elucidates the overall differences based only on metabolic information. Conversely, supervised methods, such as the partial least square (PLS)

regression, focus on separation between each group, where variables are arranged based on their contribution (Jayaraman et al., 2014). PCA and PLS-based analyses are the most common approaches in multivariate analysis for distinguishing various classes in a highly complex dataset (Woorley & Powers, 2013). Moreover, many multivariate tools have been developed for data classification, and these tools can be combined with metabolic databases to explain phenomena organically, a subdiscipline known as bioinformatics (Kikuchi & Yamada, 2017).

NMR uses the physical phenomenon of nuclear spin where the energy states of the nuclei are split along the parallel and antiparallel directions in the magnetic field (Kwan et al., 2011). These nuclei, which have a nuclear spin (I) of 1/2, exist in two energy states: ground and excited states, corresponding to +1/2 and −1/2 spins, and the energy gap increases with the magnetic field strength, resulting in a proportionate increase in the sensitivity and resolution of the NMR (Gan, 2000). NMR peaks represent the nuclei of atoms, correlated with the adjacent nuclei of molecules that affect their peak splitting; further, the NMR pattern and peak integration are employed to identify the chemical structures (Pauli et al., 2005). The quantitative properties of NMR have been gradually adopted to quantify metabolites in various biological samples (Simmler et al., 2014). For an accurate interpretation of biological samples, the preparation process should be optimized based on the analytical technique, and standard compounds of the target metabolites are required for qualification and quantification (Le Gresely et al., 2015; Kim et al., 2019). Considering that NMR peaks emerge independent of their chemical groups, peak overlapping

can occur due to various molecules in between (Markley et al., 2017). Prior to application of qNMR analysis, there are some issues that need to be solved. qNMR analysis should be performed using optimum condition because NMR is affected by various chemical condition such as pH, salt concentration of solution, resulting adverse effects on chemical shift and/or relaxation time that could lead incorrect conclusion (Xiao et al., 2009; Kim et al., 2019; Kim et al., 2021). In addition, owing to various chemicals in biological samples, peak overlap could be made it difficult to analyze (Simmler et al., 2014). To overcome this limitation, advanced technique such as two-dimensional NMR analysis have been presented, but they still rare (Marchand et al., 2017).

Meat is obtained from animals, and as living organisms, their metabolites are expressed differently depending on various conditions, such as the breed, age, and storage condition (Kim et al., 2019). To understand and/or elucidate the metabolic changes in the meat, metabolic profiling analysis is used (Jayasena et al., 2015; Choe et al., 2018; Piao et al., 2019). However, a critical limitation of conventional analyses is that the researcher has to predict specific metabolites and apply the appropriate quantification analysis (Jayasena et al., 2015). Thus, there are cases in which important and unexpected changes are missed. NMR-based analyses are good methods for tracing various metabolites simultaneously; moreover, NMR provides unbiased and panoramic views, regardless of the chemical groups of the metabolites (Gallo et al., 2015). Owing to these advantages, NMR spectroscopy has been applied in various fields, including food science (Simmler et al., 2014). However, qNMR-based approaches are very rare in meat science, and thus, the optimization of qNMR

analysis is required.

Therefore, the objective of this study was to optimize the NMR analysis process, from the comprehensive sample preparation to the parameter quantification of various metabolites, following which the optimized NMR spectroscopy analysis is applied to metabolomics with multivariate analysis.

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chemical shift consistency and dilution minimization. *Analyst*, *134*, 916-925.

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Chapter II.

Optimization of 1D ^{1}H qNMR conditions for polar metabolites in meat

2.1. Introduction

The unique flavor of meat is determined by specific metabolites such as amino acids, fatty acids, purines, and sugars, which vary depending on the type of muscle, species, sex, and feeding state (Lin et al., 2007; Jayasena et al., 2013; Piao et al., 2018). Meat flavor is formed during cooking via complex reactions including the Maillard reaction, thiamine degradation, and lipid oxidation, which are depending on the types of metabolites present (Jayasena et al., 2013; Lee et al., 2017). In addition, all post-slaughter conditions such as the packaging method, process temperature, and storage time can affect the metabolite contents and flavor of meat (Zhu et al., 2001; Aliani et al., 2013). To investigate the effects of various conditions on meat flavor, a fast and accurate analysis method for quantifying the metabolites is needed.

In general, each metabolite in meat sample must be quantified using a specific technique. For example, free amino acids, sugars, and nucleotide-degraded compounds are analyzed by chromatography or spectroscopy with specific pre-treatments (Choe et al., 2010; Jung et al., 2013; Jayasena et al.,

2015). Among the different instrumental analytical methods, nuclear magnetic resonance (NMR) spectroscopy provides several advantages compared to conventional methods including easy sample preparation, short running time, and the use of only one reference compound (Simmler et al., 2014). Thus, NMR spectroscopy has become commonly used in the field of quantitative analytical chemistry. Quantitative NMR (qNMR) has been applied to pure substance and complex natural samples in various fields such as pharmaceutical analysis, medical diagnosis, natural products analysis, and food science (Simmler et al., 2014; Galo et al., 2015; Ramakrishnan & Luthria, 2017). In addition, NMR spectroscopy for meat shows excellent reproducibility and accuracy compared to chromatographic analysis, and can quantitatively measure nearly all metabolites simultaneously (Gallo et al., 2015).

Despite these advantages, qualification and quantification conditions must be optimized before NMR analysis of meat metabolites. For example, the chemical characteristics and polarity of metabolites to be tested should be considered to ensure accurate analysis. Because of this, different extraction solutions are used depending on tissue type and metabolite polarity, which can be optimized before analysis (Lin et al., 2017). In addition, the extract must be stored under appropriate condition including pH and salt concentration ($\leq$0.15 M) for accurate quantitative analysis and to prevent adverse effects such as chemical shift or incorrect results in multivariate data analysis (Xiao et al., 2009).

Several previous studies have applied NMR to quantify meat metabolites including free amino acids and fatty acids and evaluated the characteristics of

meat by multivariate data analysis (Graham et al., 2010; Jung et al., 2010; Siciliano et al., 2013). In these studies, an existing quantitative method was applied for different samples without additional verification procedures such as conventional high-performance liquid chromatography (HPLC) quantification. Additionally, they focused only on multivariate data analysis to evaluate the possibility of using NMR (Simmler et al., 2014). Few studies have optimized the analysis conditions for specifically qualifying and quantifying meat metabolites for further related study. Therefore, the objectives of this study were to develop an optimum qNMR method to qualify and quantify polar metabolites in chicken breast based on the extraction solution, reconstitution buffer, and NMR acquisition condition (pulse program). Then, the optimized method was tested for beef and pork.

2.2. Material and methods

2.2.1. Reagents

DSS [3-(Trimethylsilyl)-1-propanesulfonic acid] sodium salt, L-aspartic acid, L-glutamic acid, creatine, deuterium oxide, deuterium oxide including TSP [3-(Trimehtylsilyl)propionic-2,2,3,3,-d_4 acid] sodium salt, HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid], and mono- and di- phosphate sodium salt (anhydrous form) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MOPS (3-Morpholinopropanesulfonic acid) was purchased from Amresco (Solon, OH, USA).

2.2.2. Sample preparation

Chicken breast meat (M. *Pectoralis major*), beef (M. *Semitendinosus*), and pork (M. *Longissimus thoracis*) were purchased from a commercial market (Seoul, Korea). The meats were ground in a laboratory using a meat grinder (MG510, Kenwood Appliances Co., Ltd., Dongguan, Guangdong, China). Five grams of the ground meats were transferred into a 50 mL test tube and stored at -70°C with vacuum packaging. The meats in the frozen state were thawed at 4°C for 24 h before analysis.

2.2.3. Preparation of buffers

To investigate the influence of different buffers on the meat NMR spectra, three extraction solutions [0.6M PCA (perchloric acid), 10 mM phosphate buffer (pH 7.0), and methanol/water (1:1 ratio)] and three reconstitution buffers [20 mM HEPES, MOPS, and phosphate buffer] were prepared, respectively.

All solutions were prepared with deionized distilled water.

2.2.4. Extraction solution

The thawed ground meats (5 g) were extracted with 20 mL of different extraction buffers using a homogenizer (T25 basic, Ika Co., KG, Staufen, Germany) for 2 min at 1,871 ×g. The extraction buffers were 0.6 M PCA, 10 mM phosphate buffer (pH 7.0), and methanol/water (1:1 ratio). The homogenate was centrifuged (Continent 512R, Hanil Co., Ltd., Gyeyang-gu, Incheon, Korea) at 3,500 ×g for 20 min. Each supernatant was transferred into new test tube and titrated to pH 7.0 with 1 M and 6 M sodium hydroxide solution, and then centrifuged again (Continent 512R, Hanil Co., Ltd.) under the same conditions. Each supernatant was filtered (Whatman No. 1, Whatman PLC., Brentford, Middx, UK) and lyophilized (Freezer dryer 18, Labco Corp. Kansas City, MO, USA) prior to NMR analysis.

2.2.5. Reconstitution buffer

The dry mass of the lyophilized extracts was reconstituted using 3 different buffers (20 mM HEPES, MOPS, and phosphate buffer) for comparison. HEPES and MOPS buffers were titrated with sodium hydroxide and phosphate buffer with sodium mono- and di-phosphate to adjust the pH to 7.0. All reconstitution buffers were prepared with D_2O containing 1 mM TSP for quantification of metabolites in the meat sample.

2.2.6. 1D ^{1}H NMR method

2.2.6.1. NMR data processing

1D ^{1}H NMR spectra were acquired by ICON-NMR automation (Bruker Biospin GmbH, Rheinstetten, Baden-Württemberg, Germany). Lock, tune, and shimming were performed automatically. After acquisition, spectra were processed with Topspin 3.5pl7 (Bruker Biospin GmbH). Phase correction and integration of the peak of interest were performed manually.

2.2.6.2. NMR acquisition

A modified standard Bruker pulse program (noesypr1d and zg30) was used for quantification. Each spectrum was recorded in D_2O on a Bruker 600 MHz Cryo-NMR spectrometer (Bruker GmbH). Spectra were obtained at the 1H frequency of 600.130 MHz applying a modified standard noesypr1d (recycle delay of 15 s) or zg30 (recycle delay of 1 s) pulse sequence, with the lock on the deuterium resonance of the solvent. The experimental parameters were as follows: sweep width of 7812.500 Hz, 128 K data points. In noesypr1d and zg30, two prior dummy scans were applied for each spectrum and 64 and 128 scans were recorded, respectively. Spectral processing was carried out using Topspin 3.5p7 (Bruker GmbH). The chemical shifts (δ) were referenced to the TSP resonance or DSS resonance. Baseline correction was performed manually. The major difference between noesypr1d and zg30 is the water suppression function in noesypr1d (Fig. 1)

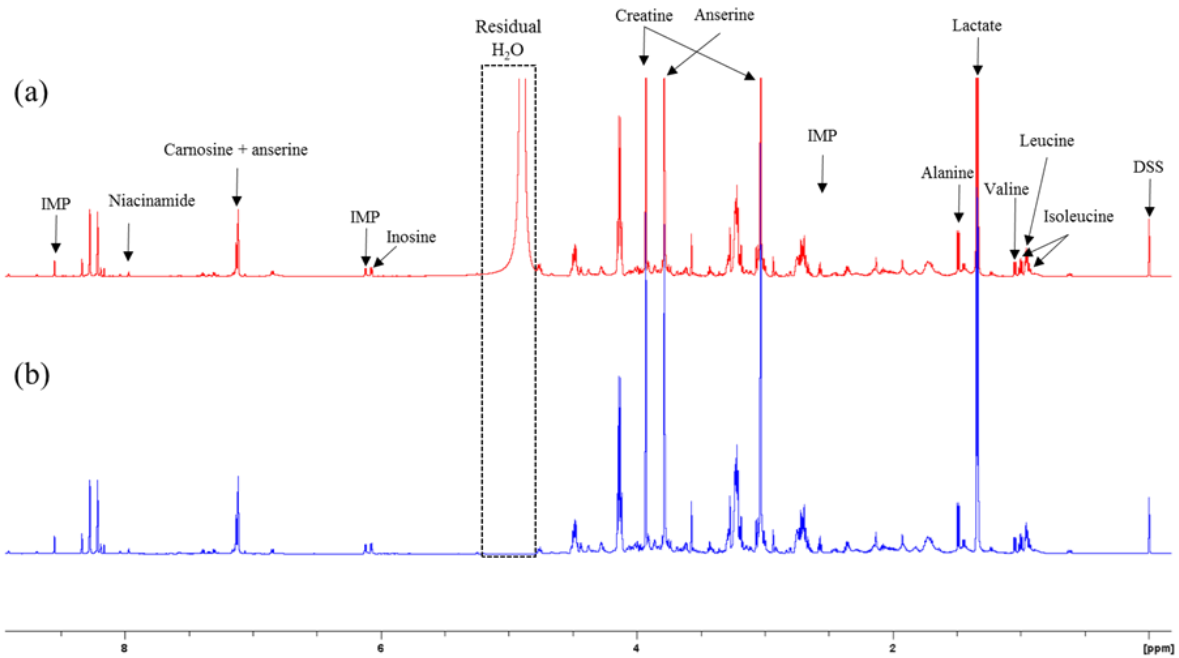

Figure 1. Spectrum of 1D ^{1}H NMR from the chicken breast extracted by 0.6 M perchloric acid and reconstituted by 20 mM phosphate by different pulse programs (a: zg30, b: noesypr1d) using 600 MHz cryo-probe NMR (nuclear magnetic resonance).

2.2.6.3. Identification and quantification of metabolites

Peaks (no or little overlap) of the metabolites were identified based on the chemical shift according to Human Metabolome Database (www.hmdb.ca) and Chenomx NMR suite 7.1 (Chenomx, Inc. Edmonton, AB, Canada). Each metabolite was calculated using 1 mM TSP as an internal standard. The concentration of metabolites was quantified using the following equation.

$$\text{Conc. (metabolite)} = \frac{No.\ of\ proton\ (TSP)}{No.\ of\ proton\ (metabolite)} \times \frac{Peak\ intensity\ of\ metabolite}{peak\ intensity\ of\ TSP} \times Conc.(1\ mM\ TSP)$$

2.2.7. HPLC analysis of amino acids

The reconstituted samples for NMR analysis were diluted 10 times for amino acids quantification (Schwarz et al., 2005). The samples were filtered through a membrane filter (0.2 μm) into a glass vial and injected into an HPLC system (Ultimate 3000, Thermo Fisher Scientific, Inc., Waltham, MA, USA). In the reaction chamber, after injecting 5 μL borate buffer (PN 5061-3339, Agilent Technologies, Santa Clara, CA, USA), each 1 μL sample, o-phthalaldehyde reagent (PN 5061-3335, Agilent), and 9-fluorenylmethyl chloroformate solution (PN5061-3337, Agilent) were reacted and diluted with 32 μL deionized distilled water (DDW). Next, the solution (0.5 μL) was injected to the column with an elution time of 30 min. A VDSpher 100 C18-E column (4.6 × 150 mm, 3.5 μm, VDS Optilab Chromatographie Technik GmbH, Würzburg, Germany) was used with 40 mM sodium phosphate, dibasic (pH 7.8) and DDW/acetonitrile/methanol (10:45:45 v/v %) as the mobile phase with a slightly modified gradient17; the flow rate was 1.5 mL/min. The column

temperature was maintained at 40°C and detection was monitored at wavelengths of 266 and 340 nm. Each amino acid content was calculated from the area of each peak using standard curves obtained using amino acid standards (PN 5061-3330 and 5062-2478, Agilent).

2.2.8. Statistical analysis

Statistical analysis was performed using the procedure of the general linear model. Significance of differences among mean values were determined by Student-Neuman-Keul's test using SAS software with a confidence level of $P<0.05$ (SAS 9.4, SAS Institute Inc., Cary, NC, USA). All the experimental procedures were conducted in triplicate.

2.3. Results and discussion

2.3.1. Extraction solution and reconstitution buffer

For extracting polar metabolites three different solutions (methanol/water, phosphate buffer, and perchloric acid) were selected, which are commonly used, in the present study (Maharjan & Ferenci, 2003; Lin et al., 2007; Dietmair et al., 2010; Römisch-Margl et al., 2012). Among the combinations of extraction solutions and reconstitution buffers used to optimize the buffer conditions, the extracts of chicken breast meat using methanol/water (1:1 ratio) showed an unflattened baseline, regardless of the reconstitution buffers tested. Similarly, Lin et al. (2007) reported that methanol/water (1:1 ratio) extraction showed high-yield and was fast and easy to operate, but exhibited low reproducibility. Phosphate buffer extraction showed results similar to those with methanol/water (1:1 ratio), except that HEPES was used as a reconstitution buffer. Phosphate buffer is mainly used to extract heme pigments such as myoglobin and hemoglobin (Warris, 1979) and these extracts are not used to analyze polar metabolites. In addition, extraction using acidic solution may be advantageous for extracting more amounts of amino acids (Aristoy & Toldra, 1991). In contrast, extraction with perchloric acid using MOPS and 20 mM phosphate buffer as reconstitution buffer showed a flattened baseline (Fig. 2). Wishart (2008) reported that organic buffers (MOPS and HEPES) should be avoided in the complex mixture sample for NMR analysis because overlap of target peaks can occur (Fig. 3). DSS as an internal standard that generates overlap downfield and may disappear, particularly in phosphate buffer extract (Fig. 4). Nowick et al. (2003) reported that DSS can interact with cationic

peptides and convert to different compounds. Thus, DSS was not an appropriate internal standard for the present study and we used TSP to quantify metabolites in chicken meat samples. Based on our results, extraction with perchloric acid and reconstitution in phosphate buffer was the best preparation condition for qNMR to quantify the metabolites in chicken breast.

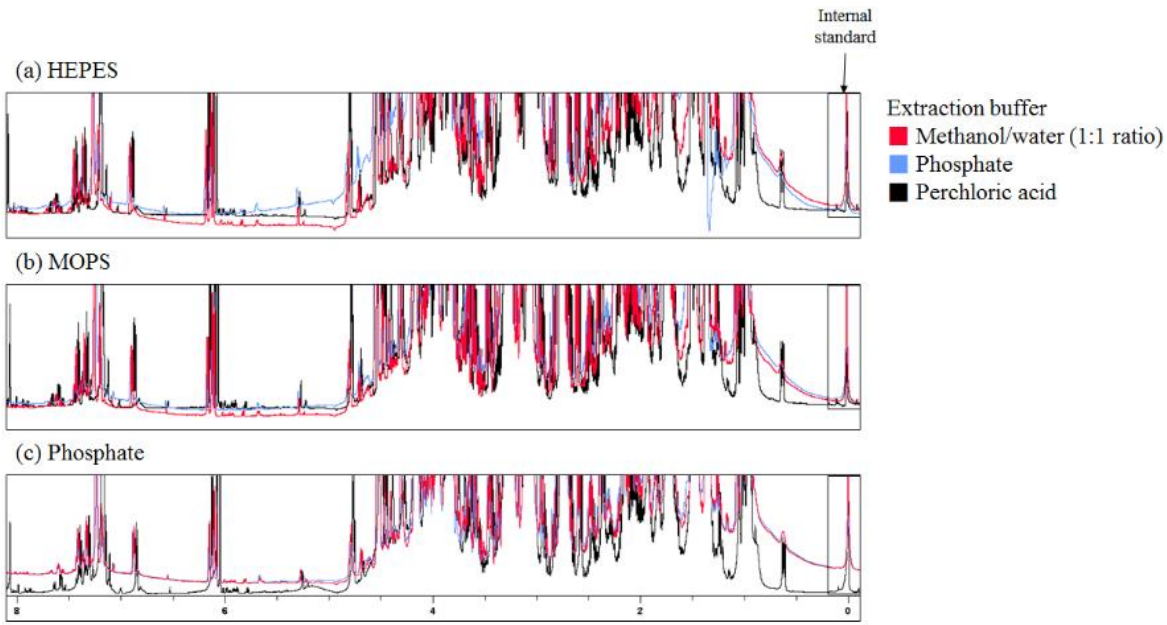

Figure 2. 600 MHz ^{1}H NMR (nuclear magnetic resonance) spectrum of chicken breast extract. All spectra were referenced to the resonance of DSS at 0.00 ppm. The three extraction buffers were methanol/water (ratio 1:1), 10 mM phosphate buffer, and 0.6 M perchloric acid and lyophilized. Dry matter was reconstituted with three buffers with 20 mM of (a) HEPES, (b) MOPS, and (c) phosphate. The residual water peak was removed (noesypr1d pulse)

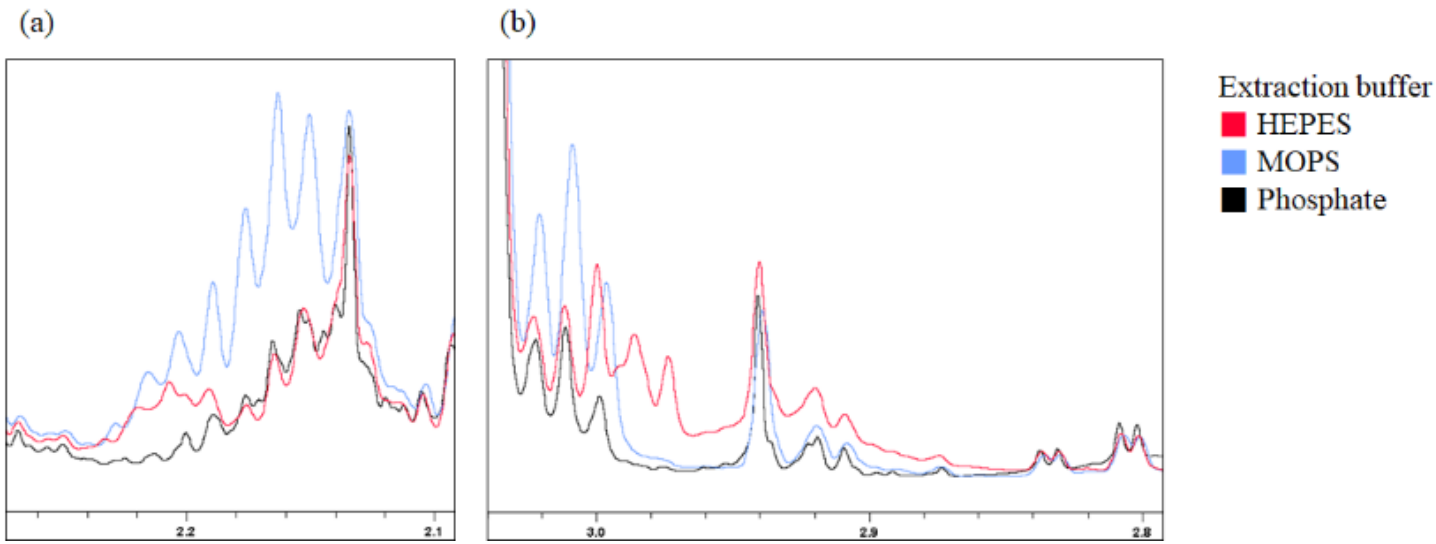

Figure 3. Spectra of 1D ^{1}H NMR (nuclear magnetic resonance) organic buffer overlap region from reconstituted chicken breast extracts (HEPES, MOPS, and phosphate reconstitution buffer) using 600 MHz cryo-probe NMR

2.3.2. Pulse program and spectrum acquisition condition

Two NMR pulse programs (zg30 and noesypr1d) were compared to optimize the method for quantifying polar metabolites in chicken breast. Pulse program noesypr1d, which included a water suppression process, led to an increase in the delay time to obtain the quantitative area of the NMR signal (Bharti & Roy, 2012). Because zg30 involves no water suppression process, NMR spectra were acquired more rapidly (approximately 10 min) than with noesypr1d. For qNMR analysis, whether signal intensity is quantitatively obtained without water suppression process because of different chemical characteristics of each sample should be tested prior to application (Krššák et al., 2004; Bharti & Roy, 2012).

A previous study reported that a delay time with at least 5 T_1 relaxations, the time for an excited state to return to the ground state in NMR analysis, is required to generate a sufficiently intense peak area for quantification (Saito et al., 2004). The delay times of both noesypr1d (over 15 s) and zg30 (1 s) were evaluated before qNMR analysis using a standard mixture (Table 1, 2 and Fig. 3). Water suppression normally provides maximum signal intensity of a sample by removing high water signals from the spectrum (Bharti & Roy, 2012). Both neutral pH and a water suppression process can improve signal/noise ratios and spectra stability (Araníbar et al., 2006). In the present study, qNMR analysis of the metabolites in chicken breast using the zg30 pulse program was less time-consuming and showed no difference in quantification capability.

Table 1. 1D ^{1}H nuclear magnetic resonance signal ratio for quantitative nuclear magnetic resonance (qNMR) according to different pulse program and delay time (D_1) using aspartic acid, malate, and niacinamide using 600 MHz Cryo-probe NMR spectroscopy. As references; 4,4-dimethyl-4-silapentane-1-sulfonic acid.

Pulse program	Delay time (sec)	DSS	Aspartic acid		Malate			Niacinamide		
					Multiplicity[a]					
		(s)	(m)	(m)	(m)	(s)	(dd)	(dd)	(dd)	(s)
					^{1}H (δ)					
		0.00	2.67	2.83	3.87	6.04	7.58	8.24	8.70	8.92
noesypr1d	2	100	107.3	109.4	77.3	78.8	85.2	70.6	66.0	48.3
	5	100	103.7	105.0	86.4	87.1	93.0	79.9	75.6	59.5
	10	100	98.8	99.1	91.4	94.2	97.0	87.7	84.3	71.3
	15	100	100.0	99.7	96.2	98.7	100.0	93.8	91.0	81.2
	20	100	99.7	99.5	97.8	100.0	99.9	95.7	93.5	86.3
zg30	1	100	97.7	99.1	93.7	97.1	96.7	94.0	92.7	88.8
	2	100	101.8	101.9	90.7	90.1	93.8	85.7	83.1	73.4
	4	100	99.0	100.3	90.8	94.5	96.1	89.9	87.6	79.7
	6	100	98.2	99.1	92.4	96.3	97.1	91.7	90.0	83.0
	8	100	98.0	98.4	94.3	96.8	97.4	93.4	91.6	85.6

[a] Represent peak splitting: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; m, multiplet.

Table 2. 1D ^{1}H nuclear magnetic resonance of limit of detection (LOD) and limit of quantification (LOQ) for quantitative nuclear magnetic resonance (qNMR) according to different standard concentration using aspartic acid (δ = 2.83, multiplet) using 600 MHz Cryo-probe NMR spectroscopy. As references; 4,4-dimethyl-4-silapentane-1-sulfonic acid using.

Concentration (mM)	Signal ratio (%)[a]	Shape recognition	Quantification availability
1	100.38	O	O
0.1	100.98	O	O
0.05	101.63	O	O
0.01	161.68	O	X
0.005	-	X	X

[a] Signal ratio (%) = (real signal intensity/theorical signal intensity) × 100

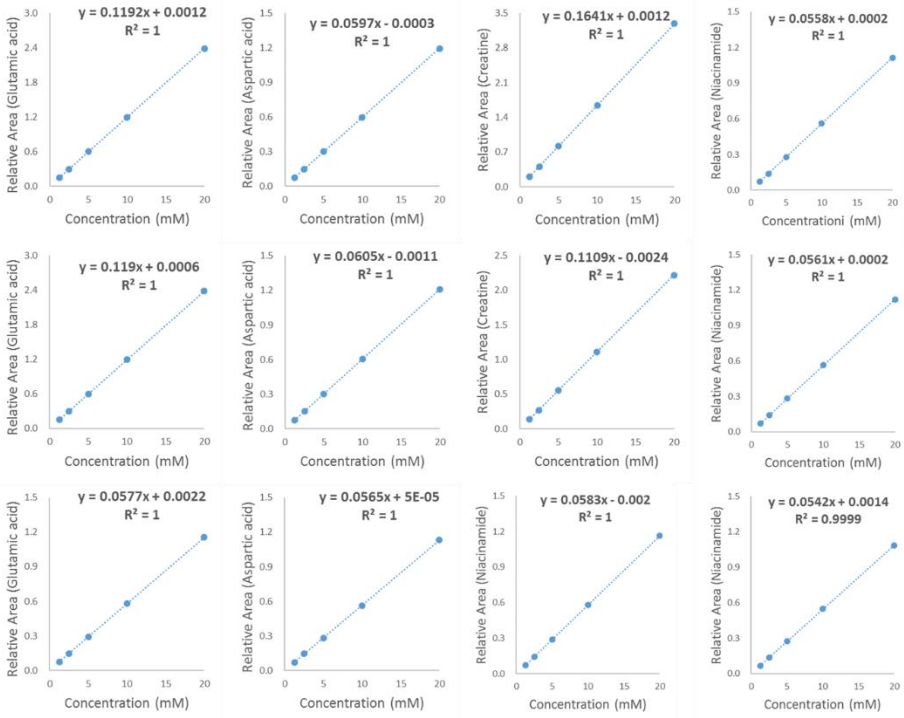

Figure 4. Linearity of standard curve with a 2 mM of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) using L-aspartic acid, L-glutamic acid, creatine and niacinamide. 1D ^{1}H NMR (without water suppression) was used.

Table 3. Comparison of qNMR using 600 MHz ^{1}H NMR and HPLC for determination of concentration of free amino acids (mg/L) of chicken breast meat

Peak no.	^{1}H (δ)	T1 relaxation time (s)
1	1.141	1.28
2	1.289	1.30
3	2.841	1.71
4	3.037	0.74
5	3.381	2.61
6	3.620	1.21
7	3.738	0.95
8	3.949	3.90
9	4.316	1.80
10	5.886	2.59
11	5.922	3.02
12	6.987	1.84
13	8.021	5.13
14	8.163	2.70
15	8.350	1.83

2.3.3. Quantification of metabolites using chicken breast extracts

2.3.3.1. Repeatability of qNMR analysis

Both NMR pulse programs (zg30 and noesypr1d) were performed to quantify metabolites in chicken breast extracts. To verify the accuracy of the developed qNMR method, the results of amino acid contents from HPLC analysis were compared. All analyses showed relative standard deviation (RSD) values less than 5%, regardless of the HPLC and different NMR pulse program, demonstrating good repeatability (Saranadasa, 2000). Especially, leucine and isoleucine showed higher concentration in NMR than HPLC analysis ($P<0.05$). The pulse program of zg30 showed a lower RSD than noesypr1d. Moreover, noesypr1d (water suppression) revealed an incorrect tyrosine concentration compared to the results obtained by HPLC. Tyrosine showed longer T_1 relaxation time (5.13 s) than other target metabolites (Table 3 of the supplementary material). Thus, the delay time (15 s) of noesypr1d was not enough to obtain the signal intensity for quantification of tyrosine (Saito et al., 2004). However, the concentration of tyrosine by NMR analysis using zg30 pulse program was not different from that by HPLC. Mckay (2011) reported that any modified parameter in the analytical process, such as water suppression, may adversely affect the accuracy of NMR analysis. Our results showed that the zg30 pulse program performed equally well to HPLC analysis for free amino acid measurement. Among the tested amino acids, leucine, isoleucine, alanine, tyrosine, and phenylalanine showed better repeatability using NMR with the zg30 pulse program. However, valine, glutamic acid, aspartic acid, and glycine showed better repeatability in HPLC analysis based on the RSD (%).

This wide variation of qNMR for several free amino acids may be because of the slight overlap in the spectra. Overall, we confirmed that qNMR analysis using an internal standard (TSP) is accurate for quantifying free amino acids (Table 4). NMR acquisition using the zg30 pulse program (without water suppression) can analyze metabolites more appropriate than noesypr1d because of the accuracy and its shorter analysis time.

Table 4. Comparison of qNMR using 600 MHz ^{1}H NMR and HPLC for determination of concentration of free amino acids (mg/L) of chicken breast

Metabolite	ppm (δ)	Multiplicity[1]	Chicken breast meat (mg/L)			SEM[2]
			NMR		HPLC	
			zg30	noesypr1d		
Leucine	0.948	*t*, CH	237.77[a]	239.53[a]	231.84[b]	1.599
		RSD[3]	0.25	1.96	0.62	
Isoleucine	0.997	*d*, CH_3	130.88[a]	130.03[a]	126.74[b]	1.205
		RSD	1.24	2.48	1.48	
Valine	0.980	*d*, CH_3	182.85	183.17	181.99	0.773
		RSD	1.06	1.94	0.86	
Alanine	1.460	*d*, CH_3	413.54	417.63	417.87	2.526
		RSD	0.87	0.86	1.18	
Glutamic acid	2.341	*m,* CH_2	440.78	438.92	439.13	3.411
		RSD	1.30	1.63	0.95	
Aspartic acid	2.670	*m,* NH	258.03	268.81	254.56	4.668
		RSD	2.13	4.74	0.84	
Glycine	3.540	*s,* CH_2	230.04	227.50	228.29	2.010
		RSD	1.74	1.10	1.45	
Tyrosine	6.877	*m*, CH	195.09[a]	148.86[b]	197.10[a]	1.568
		RSD	1.00	2.36	1.10	
Phenylalanine	7.320	*d*, CH	124.25	128.83	122.99	1.662
		RSD	2.24	3.01	2.58	

[1] Represent peak splitting: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; m, multiplet.

[2] Standard error of the means (n=9).

[3] RSD: relative standard deviation.

[a,b]Mean with different letters within the same row differ significantly ($P<0.05$).

2.3.3.2. Qualification of ^{1}H NMR spectrum

All metabolites from chicken breast which could be qualified and quantified using the optimized conditions are represented in Fig. 5. Lactate and creatine were notably observed as metabolites in chicken breast meat extracted by perchloric acid. As observed in beef extracts (Graham et al., 2010), nucleotides (IMP, hypoxanthine), dipeptides (creatine, anserine), and other metabolites were confirmed by peak multiplicity and the shape and region of peaks in the present study. However, the chemical shifts of metabolites were slightly varied (Graham et al., 2013; Table. 3). Some metabolites qualified by Graham et al. (2013) were not observed in the present NMR spectra, which may be because of differences in the chemical environment of the samples, such as the metabolite ratio and concentration or ionic strength of the extract, which may lead to different chemical shifts in the NMR spectrum (Govindaraju et al., 2000).

In contrast, 1D ^{1}H qNMR studies have reported chronic overlap issues with different samples including bio-fluid, foods, or natural compounds. In the present study, we also observed peak overlaps in asparagine, serine, glutamine, histidine, threonine, taurine, and tryptophan, which may be a limitation of this method. In this regard, two-dimensional qNMR analysis is recommended to overcome this problem by separating each metabolite peak from the peak cluster (Martineau et al., 2012). Therefore, further development in 2D NMR approaches may help to detect metabolites in meat samples including several amino acids, dipeptides, and nucleotides that cannot be precisely quantified by 1D ^{1}H qNMR.

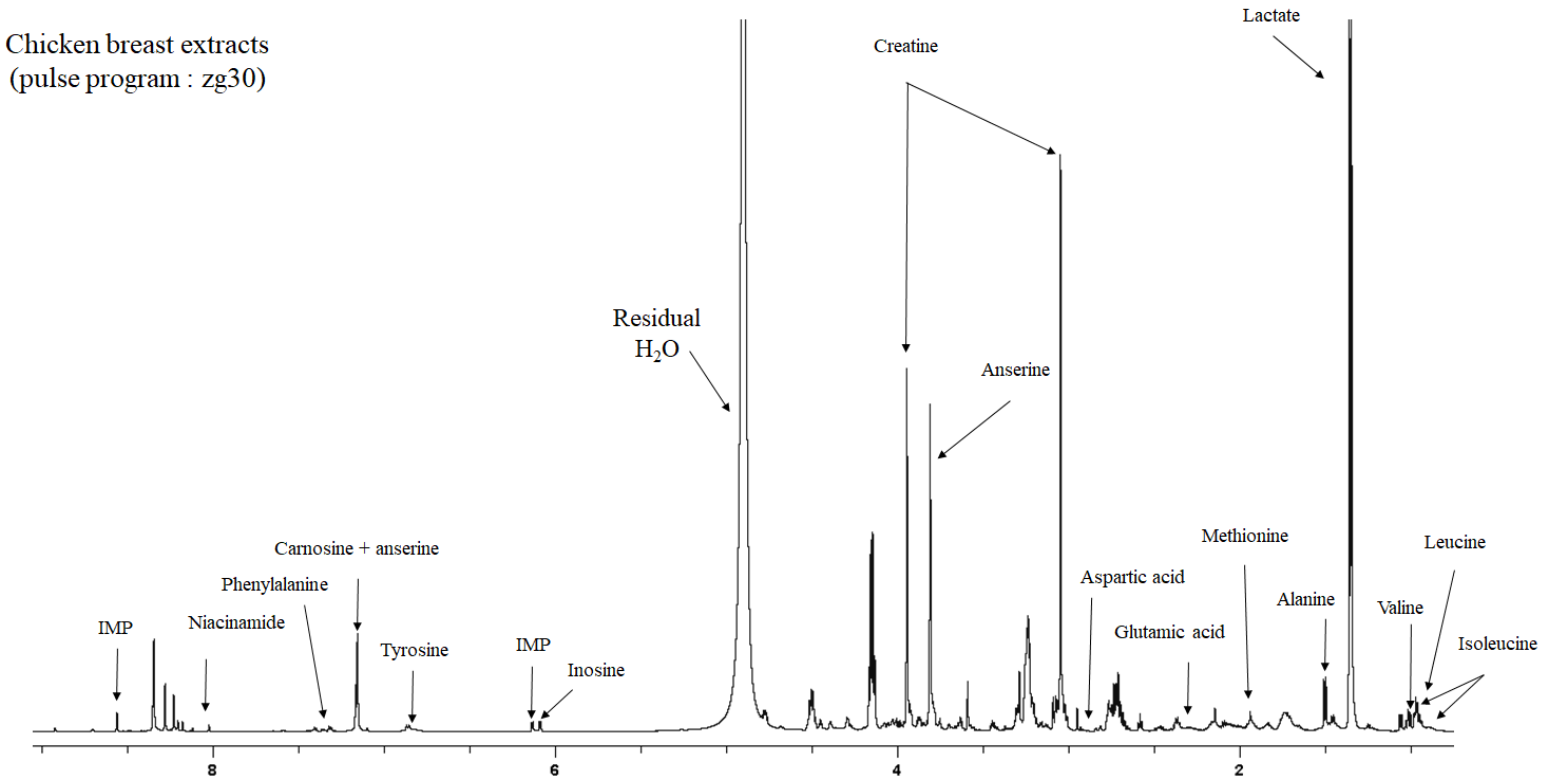

Figure 5. Spectrum of 1D ^{1}H NMR (nuclear magnetic resonance) from chicken breast extracted by 0.6 M perchloric acid (PCA) and reconstituted with 20 mM phosphate (titrated pH 7.0) using 600 MHz cryo-probe NMR

2.3.4. Application of optimized ^{1}H NMR analysis to different animal muscle

The optimized qNMR method using chicken breast was applied for beef and pork. From the method, the concentrations of free amino acids in beef and pork presented relatively lower than chicken breast (Table 5). When we observed spectra, alanine, leucine, isoleucine, glycine, glutamic acid, and phenylalanine were clearly qualified and quantified (Fig. 6). Aspartic acid of both beef and pork presented abnormal shapes of peak with partial overlap when compared with chicken meat but can be quantified. It could be one of sample-specific response among meats. The quantified free amino acid contents of pork and beef were similar to the data from the report of Cornet & Bousset (1999) and Feidt et al. (1996), respectively. The higher amount of free amino acids in chicken than beef and pork could be explained by fast postmortem metabolism rates (Schreurs, 2000).

Table 5. Application of qNMR using 600 MHz ^{1}H NMR with zg30 pulse program (without water suppression) for quantification of free amino acids (mg/L) in beef and pork

	Beef (M. *Semitendinosus*)	RSD[1)]	Pork (M. *Longissimus thoracis*)	RSD
Leucine	71.28 ± 0.77	1.08	85.08 ± 0.85	1.00
Isoleucine	21.40 ± 0.90	4.19	31.95 ± 1.25	3.92
Valine	60.16 ± 1.83	3.04	56.59 ± 0.44	0.77
Alanine	219.64 ± 4.01	1.83	133.79 ± 2.41	1.80
Glutamic acid	122.87 ± 4.40	3.58	131.92 ± 5.28	4.00
Aspartic acid	88.60 ± 3.54	3.99	74.93 ± 2.43	3.25
Glycine	128.22 ± 3.71	2.89	84.81 ± 3.61	4.25
Tyrosine	33.14 ± 1.74	5.23	33.16 ± 1.13	3.40
Phenylalanine	43.18 ± 2.39	5.54	47.83 ±1.99	4.15

[1)] RSD: relative standard deviation.

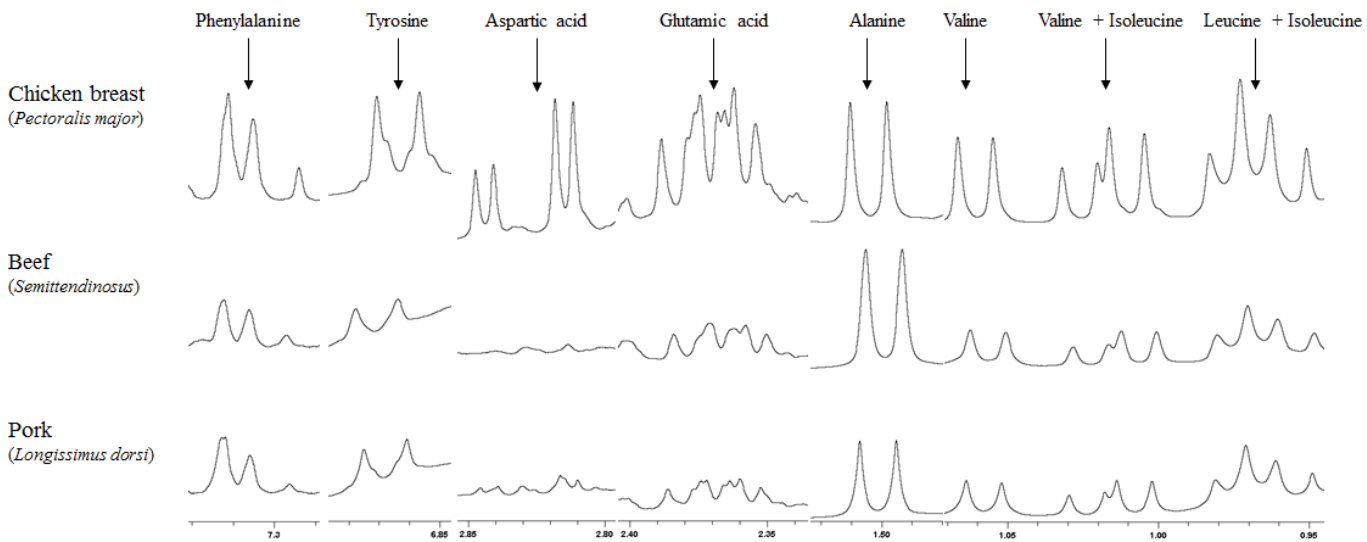

Figure 6. The peak regions of quantified free amino acids of 1D ^{1}H NMR (nuclear magnetic resonance) spectra from chicken breast (M. *Pectoralis major*), beef (M. *Semitendinosus*) and pork (M. *Longissimus thoracis*) using 600 MHz cryo-probe NMR

2.4. Conclusion

To qualify and quantify the polar metabolites in chicken breast, extraction with perchloric acid and reconstitution with phosphate buffer showed the best results for qNMR analysis. In addition, spectra acquisition with zg30 without water suppression can greatly reduce the analysis time without compromising accuracy. Compared to chromatography, 1D ^{1}H qNMR is an alternative analytical method that shows high-sensitivity, short running time, and good accuracy. Moreover, this optimized analysis method can be applied to beef and pork easily. Further studies should investigate a larger number of metabolites in meat for simultaneous quantification via advance techniques such as 2D hetero- and homo-nuclear qNMR analysis.

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Chapter III.

Potential of 2D qNMR spectroscopy for distinguishing chicken breeds based on the metabolic differences

3.1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy has emerged as one of the leading analytical techniques in metabolomics along with mass spectrometry (Markley et al., 2017). NMR-based metabolomics has been widely applied in various fields, such as medical diagnosis, pharmaceutical analysis, herbal products, and food science because it provides a non-targeted and unbiased spectrum regardless of complex chemical characteristics (Simmler et al., 2014). One-dimensional (1D) ^{1}H NMR analysis is rapid, has good reproducibility, and can quantify many metabolites simultaneously (Gallo et al., 2015). Based on these advantages, 1D ^{1}H NMR spectroscopy has been applied to understand metabolome changes of chicken depending on age (Xiao et al., 2019) and to distinguish fresh, abnormal qualities (white striping, wooden breast, and spaghetti meat), and frozen/thawed chicken breast (Soglia et al., 2019). However, despite these advantages, 1D ^{1}H NMR analyses still need to

overcome certain problems, such as low sensitivity and resonance overlapping, which are critical in cases of mixtures, such as herbal and muscle extracts (Simmler et al., 2014).

In a previous study, we optimized 1D ^{1}H quantitative NMR (qNMR) analysis of chicken meat (Kim et al., 2019). We suggested optimal extraction solutions, reconstitution buffers, internal standards, and acquisition parameters for analyzing polar metabolites in meat extracts and confirmed the results via a performance test in comparison to high performance liquid chromatography (HPLC) analysis. However, without a quantification step of the metabolites extracted from the samples, as indicated above, the overlap issue in 1D ^{1}H qNMR could lead to wrong information and erroneous conclusions. For this purpose, two-dimensional quantitative NMR (2D qNMR) approaches can be applied because they provide successful uniform signal intensities depending on metabolite concentrations and quantitative analysis using each targeted standard compound (Giraudeau, 2017; Marchand et al., 2017). In previous study, qualitative analysis of metabolome was performed using 2D NMR for comparison of similarities and differences from different chicken organs (Le Roy et al., 2016). However, quantitative approach using 2D NMR analysis is very rare.

Korean native chickens (KNC) are indigenous breeds that are known to have a unique flavor and taste, and a chewy texture when compared to commercial broiler chickens (Jin et al., 2018). Among the metabolites present in meat, KNC contains higher amounts of glutamic acid, inosine 5'-monophosphate (inosinic acid; IMP), and arachidonic acid (C20:4) than commercial broilers, which act

as important meat flavor enhancers (Jung et al., 2015). Moreover, KNC meat contains many endogenous bioactive compounds, such as anserine, betaine, carnosine, carnitine, and creatine, which might attract the interest of consumers (Jung et al., 2013; Jayasena et al., 2015b). However, the slow growth rate of KNC has been a crucial limitation for commercialization (Jin et al., 2017; Kim et al., 2018). Recently, several new crossbreds of KNCs have been developed to compensate the limitation and comprehensive metabolomic information of the new crossbreds of KNCs and the major differences from broilers are needed. If a rapid but accurate 2D qNMR analysis is present, it can be applied for other relevant studies as well as future development and characterization of meat from different breeds.

Hence, the major goals of this study were to set up 2D qNMR analytical methods for chicken breast meats followed by a multivariate analysis to distinguish metabolic characteristics of chicken breeds.

3.2. Material and methods

3.2.1. Reagents

L-Alanine, 4-aminobutyric acid (GABA), L-asparagine, L-aspartic acid, glycine, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, taurine, mono- and di- phosphate sodium salt (anhydrous form), deuterium oxide (D_2O), and D_2O with 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.2. Animal preparation

Four different KNCs [newly developed KNC-A, -C, and -D, and commercial KNC–H (Hanhyup 3ho)] were raised under the same conditions for 12 weeks at a pilot-scale farm (Gimje, Korea). The chicks of different KNC breeds were allotted in four pens (25 chicks/pen) within a single house. Food and water were provided *ad libitum* during the entire 12-week experimental period. At 12th week, KNCs were transferred to a slaughterhouse (Iksan, Korea) and held in a lairage overnight. The entire slaughter process proceeded automatically. Chickens were stunned in an electrical water bath, de-feathered, eviscerated, and air-chilled. In the same breed, eight chicken carcasses (2 chickens/pen) of similar size (1,800 ± 50 g) were randomly selected. For comparison, 8 broiler carcasses (Cobb 500f, 30 days old) with the similar weight of KNCs were collected and slaughtered on the same day at the same plant. The selected carcasses were deboned, vacuum-packed, and transferred to the laboratory

(Seoul, Korea) using a cooler with ice. The breast meats were ground using a meat grinder (MG510, Kenwood Appliances Co., Ltd., Dongguan, China) with 3 mm meat screen and homogenized. Then, three samples (approximately 100 g each) were collected, vacuum-packed again, and stored at -70°C until further analyses.

In addition, chicken breast meat was purchased from a local market (Seoul, Korea) for setting up and validation of 2D qNMR analysis prior to breed comparison. This breast meat was also processed as described above and stored under the same conditions.

3.2.3. Extraction of chicken meat

The breast meats in the frozen state were thawed at 4°C for 24 h before analysis. Thawed breast meat (5 g) was homogenized at 1,720×g for 30 s (T25 basic, Ika Co., KG, Staufen, Germany) with 20 mL of 0.6 M perchloric acid. The homogenate was centrifuged (Continent 512R, Hanil Co., Ltd., Incheon, Korea) at 3,086×g for 15 min at 4°C. Each supernatant was transferred to a new test tube and neutralized with potassium hydroxide. Neutralized extracts were centrifuged again under the same conditions. After centrifugation, each supernatant was filtered using a filter paper (Whatman No. 1, Whatman PLC., Middlesex, UK) and lyophilized (Freezer dryer 18, Labco Corp. Kansas City, MO, USA). The lyophilized extracts were stored at -70 °C until NMR analysis.

3.2.4. Standard mixture preparation for HSQC

Standard compounds were prepared in D_2O with 0.5 mM TSP (pH 7.0, 20 mM phosphate buffered saline). Validation was performed using a range of 1 to 5 mM concentration for L-alanine, GABA, L-asparagine, L-aspartic acid, glycine, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-valine, and taurine, and 0.2 - 1 mM for L-tyrosine.

3.2.5. NMR data processing

All acquired spectra were obtained by ICON-NMR automation (Bruker Biospin GmbH, Rheinstetten, Baden-Württemberg, Germany). Lock, tune, and shimming were performed automatically. After acquisition, 2D heteronuclear single quantum coherence spectroscopy (HSQC) spectra were processed with Topspin 3.6pl2 (Bruker Biospin GmbH) for calibration of the frequency of TSP to 0 ppm axis and AMIX (Analysis of MIXtures software v3.9, Bruker Biospin GmbH) for quantification via pattern integration.

3.2.6. Identification and quantification of meat extract metabolites

Assignments of NMR signals were based on standard 2D experiments; correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), HSQC, and heteronuclear multiple-bond correlation spectroscopy (HMBC) were recorded in D_2O at 298 K on a Bruker 850 MHz Cryo-NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Baden-Württemberg, Germany). COSY

and TOCSY experiments were performed with 2 k data points in t_2 domain and 256 increments in t_1, each with 8 and 16 scans, respectively. Spectral widths of 11 ppm were used for TOCSY experiments. HSQC and HMBC experiments were performed with 2k data points in t_2 domain and 512 increments in t_1, each with 8 and 32 scans respectively. Spectral widths were 11 ppm for f_2 dimension and 180 and 240 ppm for f_1 dimension, respectively. The coupling constant values of 145 Hz and 8 Hz were employed to set delay durations for short-range and long-range correlations, respectively. HSQC spectra were also used for quantification.

3.2.7. HPLC analysis of amino acids

The reconstituted samples for NMR analysis were diluted 10 times using deionized distilled water (DDW) for amino acid quantifications (Kim et al., 2019). The samples were filtered through a membrane filter (0.2 μm) into a glass vial and injected into an HPLC system (Ultimate 3000, Thermo Fisher Scientific, Inc., Waltham, MA, USA). In the reaction chamber, after injecting 5 μL borate buffer (PN 5061-3339, Agilent Technologies, Santa Clara, CA, USA), each 1 μL sample, o-phthalaldehyde reagent (PN 5061-3335, Agilent), and 9-fluorenylmethyl chloroformate solution (PN5061-3337, Agilent) were reacted and diluted with 32 μL DDW. Next, the solution (0.5 μL) was injected onto the column with an elution time of 30 min. A VDSpher 100 C18-E column (4.6×150 mm, 3.5 μm, VDS Optilab Chromatographie Technik GmbH, Würzburg, Germany) was used with 40 mM sodium phosphate, dibasic (pH 7.8) and DDW/acetonitrile/methanol (10:45:45 v/v %) as the mobile phase; the flow

rate was 1.5 mL/min. The column temperature was maintained at 40°C and detection was monitored at wavelengths of 266 and 340 nm. Each individual amino acid content was calculated from the areas under each peak using standard curves obtained from amino acid standards (PN 5061-3330 and 5062-2478, Agilent).

3.2.8. Multivariate analysis

The dataset of acquired integral data of each metabolite from HSQC are collected using AMIX (Analysis of MIXtures software v3.9, Bruker Biospin GmbH). The principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), biomarker analysis, and pathway analysis were performed using a web-based metabolomics tool (metaboanalyst.ca) according to Xia and Wishart (2010). Each metabolite of HMDB ID were used for pathway analysis. Before the analysis, samples were log-transformed, and auto-scaled. Algorithms for pathway enrichment analysis (global test) and pathway topology analysis (relative betweenness centrality) were used with a chicken (Gallus gallus; KEGG) library.

3.2.9. Statistical analysis

All experiments were conducted in triplicates. SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Student's *t*-test and Tukey's multiple comparison test were used for statistical analyses. Significant differences among the mean values were established at a significance level of $p<0.05$.

3.3. Results and discussion

3.3.1. Linearity and quantification

Prior to the spectral acquisition of meat extracts, a standard mixture containing free amino acids was prepared for validation of linearity according to their concentrations. This HSQC assay (Fig. 7) is linear over the range of 1 – 5 mM for all free amino acids and 0.2 – 1 mM for tyrosine (R^2 = 0.97 in proline and R^2 > 0.99 in others) (Fig. 8).

Based on the standard curves, we quantified free amino acids present in chicken breast meat extracts using the HSQC spectrum (Table 6). 2D NMR analysis provides a more accurate and larger metabolomic information than a 1D ^{1}H NMR analysis because the resonance overlapping problems are solved via expansion of dimensions from similar chemical structures in the same functional group among metabolites (Simmler, et al., 2014; Markley et al., 2017). HSQC did not show differences in the amounts of alanine, aspartic acid, glutamate, glycine, lysine, methionine, serine, and tryptophan when compared to the conventional HPLC method ($p < 0.05$). However, significant differences were found in the contents of arginine, proline, glutamine, histidine, leucine, phenylalanine, tyrosine, and valine ($p < 0.05$). The major reason for the differences in quantification of amino acids in chicken breast extracts is due to the differences in calibration times of the 90 °pulse (p1) by interactions in mixtures. This difference in p1 time between artificial standard mixtures and chicken meat extracts could generate differences in signal intensities depending on the metabolite's intrinsic optimal p1 (Keifer, 1999; Koskela et al., 2005). Another reason for the differences observed might be due to interferences or

offset caused by adjacent peaks on the HSQC spectra. Despite the expansion in HSQC spectra dimensions, metabolites with similar chemical structures interfered with each other, such as glutamine and histidine in the present study (Fig. 9). Glutamine was slightly interfered by unidentified adjacent peaks. Additionally, histidine was affected by large peak intensities such as creatine (3.95 ppm) and lactate (4.15 ppm). Creatine and lactate dominantly exist in muscle after rigor mortis (Watabe, et al., 1991; Xiao et al., 2019). Peak interferences or offset could be solved via reducing ionic strength using chelating agents, which induces variability of chemical shift (Alves Filho et al., 2016). In addition, the acquisition parameters should be optimized in 2D qNMR for chicken breast (Fardus-Reid et al., 2016; Kim et al., 2019). For accurate numerical quantification of metabolites, these problems need to be cleared and optimized by an optimal p1 pulse of target metabolites, ionic strength, NMR acquisition parameters, the use of suitable concentrations of metabolites to avoid unnecessary peak interference, and to get similar signal intensities between artificial standard curves and chicken breast.

In summary, 1D ^{1}H qNMR generally gives a good precision compared to HPLC and 2D qNMR methods. However, there are limitations in analyzing mixtures due to resonance overlapping. Instead, 2D qNMR strategy also has a drawback of different p1 value between artificial standard mixture and sample extracts and interference of peak in similar chemical structure, even though it can solve the overlapping issues present in 1D ^{1}H qNMR. Therefore, both 1D ^{1}H and 2D qNMR methods should be complemented and analyzed together for accurate numerical quantification of metabolites.

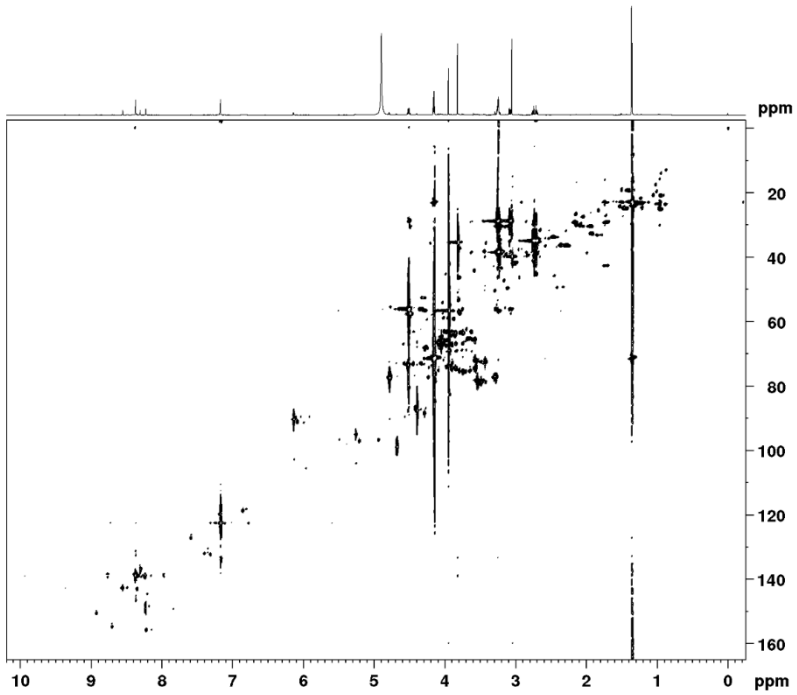


Figure 7. Representative hetero nuclear single quantum coherence (HSQC) NMR spectrum from chicken breast extracts acquired using a 850 MHz cryo-NMR spectrometer.

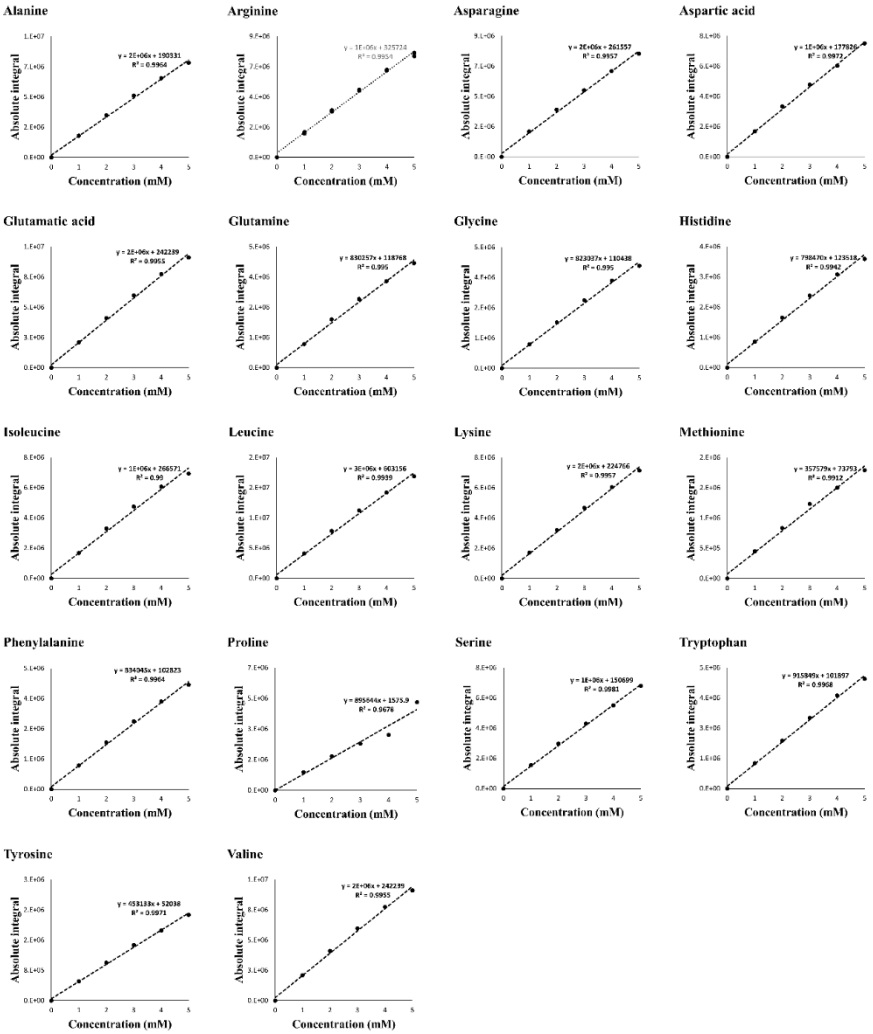


Figure 8. Standard curves of free amino acids for verification of quantification from hetero nuclear single quantum coherence (HSQC) using a 850 MHz Cryo-NMR spectrometer.

Table 6. Metabolites identification and quantification of chicken breast meat from [1]H NMR, 2D HSQC, and HPLC spectra

Compound	[1]H NMR	2D HSQC	HPLC
	(mg/kg)		
Alanine	308.88 ± 5.08	306.58 ± 14.48	304.80 ± 8.24
Arginine	nd	85.36 ± 5.23[b]	215.83 ± 8.03[a]
Asparagine	76.10 ± 2.38[a]	44.82 ± 4.22[b]	77.67 ± 5.08[a]
Aspartic acid	186.05 ± 2.45	170.75 ± 6.11	181.26 ± 12.00
Glutamate	525.65 ± 5.12	363.23 ± 16.43	324.28 ± 9.03
Proline	(Glu + Pro)[1]	158.85 ± 3.37[a]	116.81± 23.91[b]
Glutamine	269.11 ± 1.32[b]	430.98 ± 12.20[a]	269.27 ± 11.57[b]
Glycine	216.57 ± 6.42	222.36 ± 14.49	214.95 ± 4.79
Histidine	nd	126.59 ± 3.76[b]	178.23 ± 3.61[a]
Isoleucine	100.81 ± 2.46[a]	92.40 ± 1.42[b]	82.65 ± 1.45[c]
Leucine	168.49 ± 3.02[a]	153.85 ± 6.01[b]	165.09 ± 1.31[a]
Lysine	nd	68.74 ± 13.14	87.98 ± 6.17
Methionine	73.08 ± 3.72	75.00 ± 0.92	73.00 ± 1.01
Phenylalanine	89.43 ± 0.28[b]	151.52 ± 14.72[a]	87.87 ± 0.86[b]
Serine	nd	193.15 ± 11.37	197.97 ± 5.54
Tryptophan	nd	58.94 ± 9.80	56.27 ± 2.59
Tyrosine	135.04 ± 6.52[a]	112.70 ± 4.94[b]	135.63 ± 1.25[a]
Valine	127.10 ± 2.42[a]	83.32 ± 3.35[b]	125.70 ± 2.37[a]

[a-c] Mean values (n=3) with different letters within the same row differ significantly ($p < 0.05$).
nd, not detected in the 1D [1]H spectrum.
[2] 1D [1]H NMR data of both glutamate and proline were excluded from the calculation.

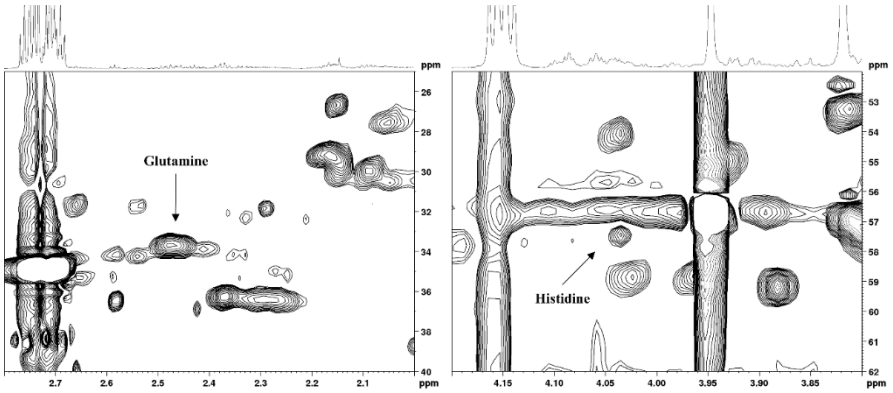


Figure 9. Example of offset and interaction from chicken breast extracts on the hetero nuclear single quantum coherence (HSQC) spectrum using a 850 MHz Cryo-NMR spectrometer

3.3.2. Identification of breast meat extracts by 2D NMR

Two-dimensional NMR analyses (COSY, TOCSY, HSQC, and HMBC) were used to profile metabolites present in various samples (Van et al., 2007; Guennec et al., 2014). For the metabolomic profiling of breast meat extracts, different 2D NMR analyses were carried out. The metabolites which were qualified based on meat quality characteristics via HSQC spectra were detected and listed in Table 7. Based on the HSQC profile, the peak intensities of major polar metabolites (33 in total) present in chicken breast meat which were related to its taste (free amino acids, nucleotides, and sugars) and functionality (dipeptides and vitamin) were quantified. Then, metabolomic comparisons between different chicken breeds were carried out based on the qualification results. The most abundant metabolites from breast meat were lactate > phosphocreatine/creatine > anserine > carnosine, which was similar to the result of a previous study using breast meat of Chinese indigenous chickens (Xiao et al, 2019). However, a verification step of metabolites is absent from the study discussed above. Standard compounds or 2D NMR analyses (COSY, TOCSY, HSQC, and HMBC) are required for verification and reliability because slight differences from different experimental methods could change chemical shifts in a 1D ^{1}H NMR spectra (Simmler et al., 2014; Kim et al., 2019).

Table 7. Assignment of breast meat extracts from chicken using 2D NMR experiments (COSY, TOCSY, HSQC, and HMBC)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
1	Isoleucine	Ile	δ-CH_3	0.95	*t*: 7.44	COSY (1.28, 1.49) TOCSY (2.10) HSQC (14.1) HMBC (27.3, 38.7)
2	Leucine	Leu	δ-CH_3/ δ-CH_3	0.97/ 0.98	*d*/*d*: 6.13/6.17	COSY (1.74) TOCSY (3.76) HSQC (23.8/24.9) HMBC (27.0, 42.6)
3	Valine	Val	γ-CH_3	1.01	*d*: 7.00	COSY (2.30) TOCSY (3.64) HSQC (19.5) HMBC (32.0, 63.1)
4	Isoleucine	Ile	δ-CH_3	1.03	*d*: 7.02	COSY (2.01) TOCSY (1.27;1.47;3.68) HSQC (17.40) HMBC (27.3, 38.7)
5	Valine	Val	γ-CH_3	1.06	*d*: 7.03	COSY (2.30) TOCSY (3.64) HSQC (20.77) HMBC (32.0, 63.1)
6	NAA			1.22	*d*: 6.26	COSY (4.17) TOCSY (2.33, 2.42) HSQC (24.7) HMBC (49.4, 68.4)
7	Isoleucine	Ile	γ-CH_2	1.28	*m*	COSY (0.95, 1.49) TOCSY (2.10) HSQC (27.3) HMBC (14.1, 38.7)
8	Threonine	Thr	γ-CH_3	1.36		COSY (4.29) TOCSY (3.63)
9	Lactic acid	LA	CH_3	1.36	*d*: 6.97	COSY (4.15) HSQC (23.0) HMBC (71.4)
10	Lysine	Lys	γ-CH_2	1.48	*m*	COSY (1.74) TOCSY (3.04) HSQC (25.0)
11	Isoleucine	Ile	γ'-CH_2	1.49	*m*	COSY (0.95, 1.28) TOCSY (2.10) HSQC (27.3) HMBC (14.1, 38.7)
12	Alanine	Ala	β-CH_3	1.51	*d*: 7.26	COSY (3.81) HSQC (19.0) HMBC (53.3, 178.7)
13	Leucine	Leu	β-CH_2	1.71	*m*	COSY (0.97, 0.98, 3.74) HSQC (42.6) HMBC (23.8,24.9)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
14	Lysine	Lys	δ-CH_2	1.72	*m*	COSY (3.01) HSQC (29.1)
15	Leucine	Leu	β'-CH_2	1.75	*m*	COSY (0.97, 0.98, 3.74) HSQC (42.6) HMBC (23.8,24.9)
16	NA B			1.84	*m*	COSY (3.12) HSQC (25.7)
17	Lysine	Lys	β-CH_2	1.94	*m*	COSY (1.48, 3.78) TOCSY (1.72, 3.04) HSQC (32.7) HMBC (29.1, 57.2)
18	NA C			1.95	*s*	HSQC (26.2) HMBC (63.3, 184.3)
19	Isoleucine	Ile	β-CH	2.01	*m*	COSY (1.03) TOCSY (0.95) HSQC (38.7)
20	Glutamate	Glu	β-CH_2	2.09	*m*	COSY (2.16, 2.38) HSQC (29.8) HMBC (36.2, 57.4, 177.4, 184.1)
21	Methionine	Met	S-CH_3	2.15	*s*	HSQC (16.6) HMBC (31.6)
22	Aspartic acid	Asp	β-CH_2	2.15	*m*	COSY (2.22, 2.66) HSQC (32.6)
23	Glutamate	Glu	β'-CH_2	2.16	*m*	COSY (2.09, 2.38) HSQC (29.8) HMBC (36.2, 57.4, 177.4, 184.1)
24	Glutamine	Gln	β-CH_2	2.16	*m*	COSY (2.47, 3.80) HSQC (29.1) HMBC (33.6, 56.9, 177.0, 180.4)
25	Aspartic acid	Asp	β'-CH_2	2.22	*m*	COSY (2.15, 2.66) HSQC (32.6)
26	Valine	Val	β-CH	2.3	*m*	COSY (1.01, 1.06) HSQC (32.0) HMBC (19.5, 176.9)
27	NA A			2.33	*m*	COSY (2.42, 4.17) HSQC (49.4) HMBC (24.7, 68.4, 183.2)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
28	Glutamate	Glu	γ-CH_2	2.38	*m*	COSY (2.09, 2.16) HSQC (36.2) HMBC (29.8, 57.4, 177.4, 184.1)
29	Carnitine	Crn	α-CH_2	2.47		COSY (4.59) TOCSY (3.45) HSQC (45.9)
30	NA A			2.42	*m*	COSY (2.33, 4.17) HSQC (49.4) HMBC (24.7, 68.4, 183.2)
31	Glutamine	Gln	γ-CH_2	2.45	*m*	COSY (2.16) TOCSY (3.80) HSQC (33.6) HMBC (29.1, 56.9, 177.0, 180.4)
32	Glutamine	Gln	γ'-CH_2	2.5	*m*	COSY (2.16) TOCSY (3.80) HSQC (33.6) HMBC (29.1, 56.9, 177.0, 180.4)
33	β-Alanine	β-Ala	α-CH_2	2.58	*t*: 6.63	COSY (3.21) HSQC (36.5) HMBC (39.3, 181.2)
34	Methionine	Met	γ-CH_2	2.66	*t*: 7.69	HSQC (31.6) HMBC (16.6, 32.6, 56.7, 174.4)
35	Aspartic acid	Asp	α-CH	2.66		COSY (2.15, 2.22) HSQC (31.6)
36	Carnosine	Car	NH_2-CH_2-CH_2	2.71	*m*	COSY (3.24) HSQC (34.9) HMBC (38.4, 174.5)
37	Aspartic acid	Asp	β-CH_2	2.75	*dd*	COSY (2.83, 3.93) HSQC (39.3) HMBC (54.9)
38	Anserine	Ans	NH_2-CH_2-CH_2	2.75	*m*	COSY (3.24) HSQC (34.9) HMBC (38.4, 174.5)
39	Aspartic acid	Asp	β'-CH_2	2.83	*dd*	COSY (2.75, 3.93) HSQC (39.3) HMBC (54.9)
40	N,N-dimethylglyc	DMG	N-CH_3	2.96	*s*	HSQC (46.3) HMBC (62.7)
41	Lysine	Lys	ε-CH_2	3.03	*m*	COSY (1.72) TOCSY (1.48, 1.94, 3.78) HSQC (41.8)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
42	Creatine/Phosphocreatine	Cr/PCr	$N\text{-}CH_3$	3.04	*s*	TOCSY (3.94) HSQC (39.8) HMBC (24.3, 151.1, 168.0)
43	Anserine	Ans	$\beta\text{-}CH_2$	3.08	*m*	COSY (3.25, 4.51) HSQC (28.8) HMBC (56.2, 122.7, 133.4, 174.5, 179.6)
44	Carnosine	Car	$\beta\text{-}CH_2$	3.08	*m*	COSY (3.25, 4.50) HSQC (30.6) HMBC (57.41, 119.8, 133.8, 174.5, 179.9)
45	Carnitine	Crn	$N(CH_3)_3$	3.22		HSQC (56.7) HMBC (70.8)
46	Carnosine	Car	$\beta'\text{-}CH_2$	3.23	*m*	COSY (3.25, 4.50) HSQC (30.6) HMBC (57.41, 119.8, 133.8, 174.5, 179.9)
47	Anserine	Ans	$\beta'\text{-}CH_2$	3.25	*m*	COSY (3.25, 4.51) HSQC (28.8) HMBC (56.2, 122.7, 133.4, 174.5, 179.6)
48	Anserine	Ans	$NH_2\text{-}CH_2$	3.25	*m*	COSY (3.08, 4.51) HSQC (38.4) HMBC (35.4, 56.2, 122.7, 133.4, 179.6)
49	β-Glucose	β-Glc	CH-2	3.28	*m*	COSY (4.68) HSQC (77.1) HMBC (78.5, 98.8)
50	Betaine	Bet	$N(CH_3)_3$	3.29	*s*	HSQC (56.2) HMBC (69.03)
51	Taurine	Tau	$S\text{-}CH_2$	3.3	*t*	COSY (3.45) HSQC (50.4)
52	β-Glucose	β-Glc	CH-4	3.43	*m*	COSY (3.49) TOCSY (3.74, 4.68) HSQC (72.5) HMBC (63.5, 78.5)
53	α-Glucose	α-Glc	CH-4	3.44	*m*	COSY (3.49) TOCSY (5.26) HSQC (72.5) HMBC (63.5, 78.5)
54	Taurine	Tau	$N\text{-}CH_2$	3.45	*t*	COSY (3.3) HSQC (38.1)
55	Carnitine	Crn	$\gamma,\gamma'\text{-}CH_2$	3.45		COSY (4.59) HSQC (72.3)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
56	β-Glucose	β-Glc	CH-3	3.49	*m*	TOCSY (4.68) HSQC (78.8)
57	α-Glucose	α-Glc	CH-2	3.56	*m*	COSY (5.26) HSQC (74.2)
58	Glycerol		CH-1	3.57	*m*	COSY (3.66) HSQC (65.5)
59	Glycine	Gly	α-CH	3.59	*s*	HSQC (44.3) HMBC (175.4)
60	Threonine	Thr	α-CH	3.63		COSY (4.29) TOCSY (1.36) HSQC (63.20)
61	Valine	Val	α-CH	3.65	*d*	COSY (2.3) TOCSY (1.00, 1.05) HSQC (63.1)
62	Glycerol		OH-CH_2	3.66	*m*	COSY (3.57) HSQC (65.3)
63	Isoleucine	Ile	α-CH	3.7	*d*	COSY (2.01) TOCSY (1.03) HSQC (62.3)
64	N,N-dimethylglyc	DMG	α-CH_2	3.75	*s*	HSQC (62.7) HMBC (46.3, 173.3)
65	β-Glucose	β-Glc	CH_2-6	3.75		COSY (3.45; 3.90) HSQC (63.6)
66	α-Glucose	α-Glc	CH-3	3.75		TOCSY (5.26) HSQC (75.5)
67	Leucine	Leu	α-CH	3.76	*m*	COSY (1.71) TOCSY (0.97) HSQC (56.2)
68	Lysine	Lys	α-CH	3.78	*m*	COSY (1.94) TOCSY (3.03) HSQC (57.3) HMBC (32.7)
69	Glutamine	Gln	α-CH	3.8	*m*	HSQC (56.9) HMBC (29.1, 33.6, 177.0)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
70	Anserine	Ans	N-CH_3	3.82	*s*	HSQC (35.4) HMBC (133.4, 138.8)
71	α-Glucose	α-Glc	CH-5	3.86	*m*	TOCSY (5.26) HSQC (74.5)
72	α-Glucose	α-Glc	CH_2-6	3.86	*m*	TOCSY (5.26) HSQC (63.5)
73	Inosine	Ino	CH-5 (Rib)	3.87	*m*	COSY (3.93, 4.29) HSQC (64.2)
74	β-Glucose	β-Glc	CH_2-6'	3.91		HSQC (63.5)
75	Inosine	Ino	CH-5' (Rib)	3.93	*m*	COSY (3.87, 4.29) HSQC (64.2)
76	Betaine	Bet	α-CH_2	3.93	*s*	HSQC (69.03) HMBC (56.2, 172.1)
77	Aspartic acid	Asp	α-CH	3.93	*m*	COSY (2.75, 2.83) HSQC (54.9)
78	Inosine 5'-monophosph	IMP	CH_2 (Rib)	4.06/ 4.09	*m*	COSY (4.40) TOCSY (4.53, 4.79, 6.14) HSQC (66.5)
79	Lactic acid	LA	α-CH	4.16	*q*: 6.94	COSY (1.36) HSQC (71.4) HMBC (23.0)
80	Threonine	Thr	β-CH	4.29		COSY (1.36, 3.63) HSQC (68.8)
81	Inosine	Ino	CH-4 (Rib)	4.29		COSY (3.87, 4.46) TOCSY (3.93, 4.77) HSQC (88.5)
82	Inosine 5'-monophosph	IMP	CH-4 (Rib)	4.4	*m*	COSY (4.06, 4.09, 4.53) TOCSY (4.79, 6.14) HSQC (87.6) HMBC (73.4)
83	Inosine	Ino	CH-3 (Rib)	4.46	*dd*	COSY (4.30, 4.77) TOCSY (3.87, 3.93) HSQC (73.2)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
84	Carnosine	Car	CH-COOH	4.49	*m*	COSY (3.08, 3.23) HSQC (57.5)
85	Anserine	Ans	CH-COOH	4.51	*m*	COSY (3.08, 3.25) HSQC (56.3)
86	Inosine 5'-monophosph	IMP	CH-3 (Rib)	4.53	*dd*: 4.72, 4.21	COSY (4.40, 4.79) TOCSY (4.06, 4.09, 6.14) HSQC (73.4) HMBC (66.4, 90.2)
87	Carnitine	Cart	β-CH	4.59		COSY (2.45, 3.45) HSQC (73.2)
88	β-Glucose	β-Glc	CH-1	4.68	*d*: 7.96	COSY (3.28) TOCSY (3.49) HSQC (98.8)
89	Inosine	Ino	CH-2 (Rib)	4.77		COSY (4.46, 6.09) TOCSY (4.29) HSQC (76.9)
90	Inosine 5'-monophosph	IMP	CH-2 (Rib)	4.79	*t*: 5.03	COSY (4.53, 6.14) TOCSY (4.06, 4.09, 4.40) HSQC (77.6) HMBC (87.5, 90.2)
91	α-Glucose	α-Glc	CH-1	5.26	*d*: 3.79	COSY (3.56) TOCSY (3.44, 3.75, 3.86) HSQC (94.9) HMBC (74.2, 75.5)
92	Inosine	Ino	CH-1 (Rib)	6.09	d: 5.64	COSY (4.77) HSQC (91.2)
93	Inosine 5'-monophosph	IMP	CH-1 (Rib)	6.14	d: 5.52	COSY (4.79) HSQC (90.3)
94	Tyramine	Tyrm	CH-3,5	6.81		COSY (7.10) HSQC (118.3)
95	Tyrosine	Tyr	CH-3,5	6.86		COSY (7.16) HSQC (118.6) HMBC (129.4, 157.7)
96	Tyramine	Tyrm	CH-2,6	7.1		COSY (6.81) HSQC (133.42)
97	Tyrosine	Tyr	CH-2,6	7.16		COSY (6.86) HSQC (133.73) HMBC (157.7)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
98	Anserine	Ans	CH-5 (His)	7.16	*s*	COSY (8.36) TOCSY (3.08, 3.25) HSQC (122.8)
99	Carnosine	Car	CH-5 (His)	7.17	*s*	COSY (8.30) TOCSY (3.08, 3.23) HSQC (119.8)
100	Phenylalanine	Phe	CH-2,6	7.32	*d*: 6.97	COSY (7.41) HSQC (132.3)
101	Phenylalanine	Phe	CH-4	7.35	*t*: 7.40	COSY (7.41) HSQC (130.5)
102	Phenylalanine	Phe	CH-3,5	7.41	*t*: 7.60	COSY (7.32, 7.35) HSQC (131.9)
103	Nicotinic acid	NA	CH-5	7.59	*dd*: 8.00, 5.02	COSY (8.23, 8.70) TOCSY (8.94) HSQC (127.1) HMBC (131.9, 154.6)
104	NA D			8.22		COSY (8.84, 9.17)
105	Inosine 5'-monophosph	IMP	CH-8 (purin)	8.23	*s*	HSQC (149.2) HMBC (151.5, 161.3)
106	Nicotinic acid	NA	CH-4	8.23		COSY (7.59) TOCSY (8.86, 8.92) HSQC (139.4) HMBC (151.4, 161.4)
107	Carnosine	Car	CH-2 (His)	8.3	*s*	COSY (7.17) HSQC (137.0)
108	Anserine	Ans	CH-2 (His)	8.36	*s*	COSY (7.16) HSQC (139.0)
109	Inosine 5'-monophosph	IMP	CH-2 (purin)	8.56	*s*	HSQC (142.7) HMBC (90.3, 126.2, 151.5, 161.3, 164.8)
110	Nicotinic acid	NA	CH-6	8.7	*dd*: 1.52, 4.94	COSY (7.59) TOCSY (8.92) HSQC (154.8) HMBC (127.1, 132.0, 139.4, 150.4)
111	NA D			8.84	*dt*: 8.08 1.40	COSY (8.22) TOCSY (9.17) HSQC (148.5) HMBC (142.5, 145.2, 167.9)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
112	Nicotinic acid	NA	CH-2	8.92	*d*: 1.70	TOCSY (7.59, 8.23, 8.70) HSQC (150.4) HMBC (132.0, 139.4, 154.8)
113	NA D			9.17	*d*: 6.04	COSY (8.22) TOCSY (8.84) HSQC (145.2) HMBC (102.9, 142.9, 148.5)
114	NA D			9.36	*s*	HSQC (142.9) HMBC (102.9, 145.2, 148.5, 167.9)

[1] NA; not identified.

[2] Abbreviation of compound.

[3] Represent peak splitting: *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *dd*, doublet of doublet; *dt*, doublet of triplet *m*, multiplet.

3.3.3. Metabolomic differences of broilers and KNC

The PCA scores, VIP scores, and heatmap from four different KNCs and broilers were processed (R^2 = 0.918, Q^2 = 0.769) (Fig. 10). PCA analysis showed a good cumulative explained variation (R^2) and predictive ability (Q^2), which means that the dataset was clearly distinguished by breeds and can eventually predict breeds based on quantified metabolomic information. PCA and PLS-based analyses are the most popular methods in multivariable analysis to differentiate between various classes in a highly complex dataset (Woorley & Powers, 2013). As an unsupervised analysis, PCA could elucidate overall differences based on metabolomic information (Jayaraman et al., 2014). These visualization analyses can be easily discriminated based on the breeds of chicken (KNCs and broilers) and can easily recognize various associated metabolites based on their projections (Chong et al., 2019).

The VIP score is represented by a highly contributing variable (> 1 scores) in the PLS-DA model (Almeida et al., 2013). The intensities of the measured scores were highest to lowest in order of histidine, carnosine, aspartic acid, and lactic acid; the metabolites histidine and carnosine display much higher VIP scores compared to others. These two compounds were important variables when the model was developed in PLS-DA. Histidine is an essential free amino acid that plays an important role of maintaining nitrogen balance in protein synthesis and is related to the synthesis of hemoglobin and carnosine (Kriengsinyos et al., 2002). Furthermore, in a previous study, histidine intake was shown to suppress food intake and fat accumulation in rats (Kasaoka et al., 2004). On the other hand, carnosine (β-alanine-L-histidine) is a dipeptide

synthesized from β-alanine and histidine. Carnosine has been previously reported as a bioactive compound associated with antiglycation, antiaging, antioxidation and neurotransmitter functions, and plays a role in alleviating diseases, such as, Alzheimer's disease, cataracts, diabetes, and ischemia (Jung et al., 2013). Among other metabolites with high VIP scores, aspartic acid is related to umami taste and is important in meat sensory quality (Dashdorj et al., 2015). Lactic acid is a predominant organic acid in postmortem meat and is negatively correlated with physiochemical properties, such as pH, water holding capacity, and tenderness (Xiao et al., 2019). These high VIP-scored metabolites were most abundant in the breast meat of KNC-D, followed by KNC-H and -C. The amounts of these compounds are higher in the breast meat of KNCs than in the broilers. KNC-A had the lowest concentration of these metabolites among all the breeds studied.

The VIP score rankings of the metabolites, phenylalanine, glutamic acid, and β-alanine are significantly higher in the breast meat extract of broilers than in KNCs. Among KNCs, KNC-D had the highest free amino acid content. Moreover, KNC-D had a higher amount of anserine and β-glucose. Anserine (β-alanine-3-methyl-L-histidine) is one of the most abundant bioactive compounds in poultry meat (Jayasena et al., 2014a) and has been reported to have a similar bioactivity profile to carnosine (Jung et al., 2013).

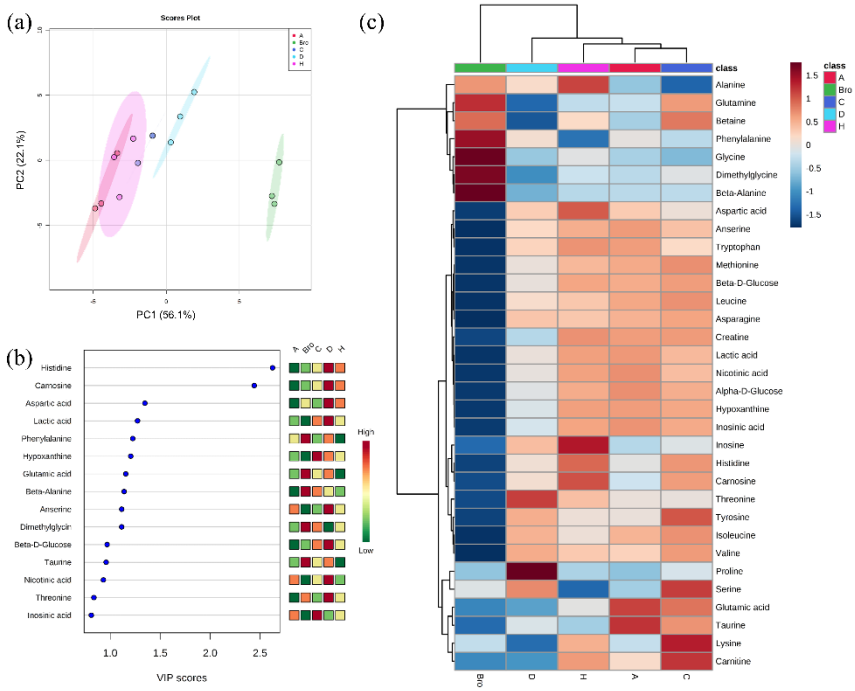


Figure 10. Principal component analysis (PCA) (a), VIP scores (b), and heatmap (c) from breast meat extracts of different chicken breeds (KNC-A, -C, -D, and -H) by HSQC using a 850 MHz cryo-NMR spectrometer

In general, amino acids, sugars, nucleotides, and their derivatives are closely related to meat quality traits, such as sensory, physiochemical, and bioactive properties (Jayasena et al., 2013; Jung et al., 2013; Jayasena et al., 2015a; Jayasena et al., 2015b). In sensory traits, these polar metabolites were related to sweetness (α-glucose and ribose, glycine, alanine, serine, proline, and hydroxyproline), sourness (phenylalanine, tyrosine, alanine, lactic acid), bitterness (hypoxanthine, inosine, histidine, arginine, isoleucine, leucine, lysine, phenylalanine, tyrosine, and valine), and umami (anserine, carnosine, aspartic acid, glutamic acid, and IMP), and sulfurous flavors (methionine) (Dashdorj et al., 2015). Not only the taste of metabolites itself but abundant free amino acids and reducing sugars can act as substrates of Maillard reaction, which is a major reaction responsible for flavor development in muscle foods during cooking (Mottram, 1998). Based on these results, KNC-D may have a superior meat quality when compared to the other KNCs due to its higher concentrations of free amino acids, sugars, and bioactive compounds.

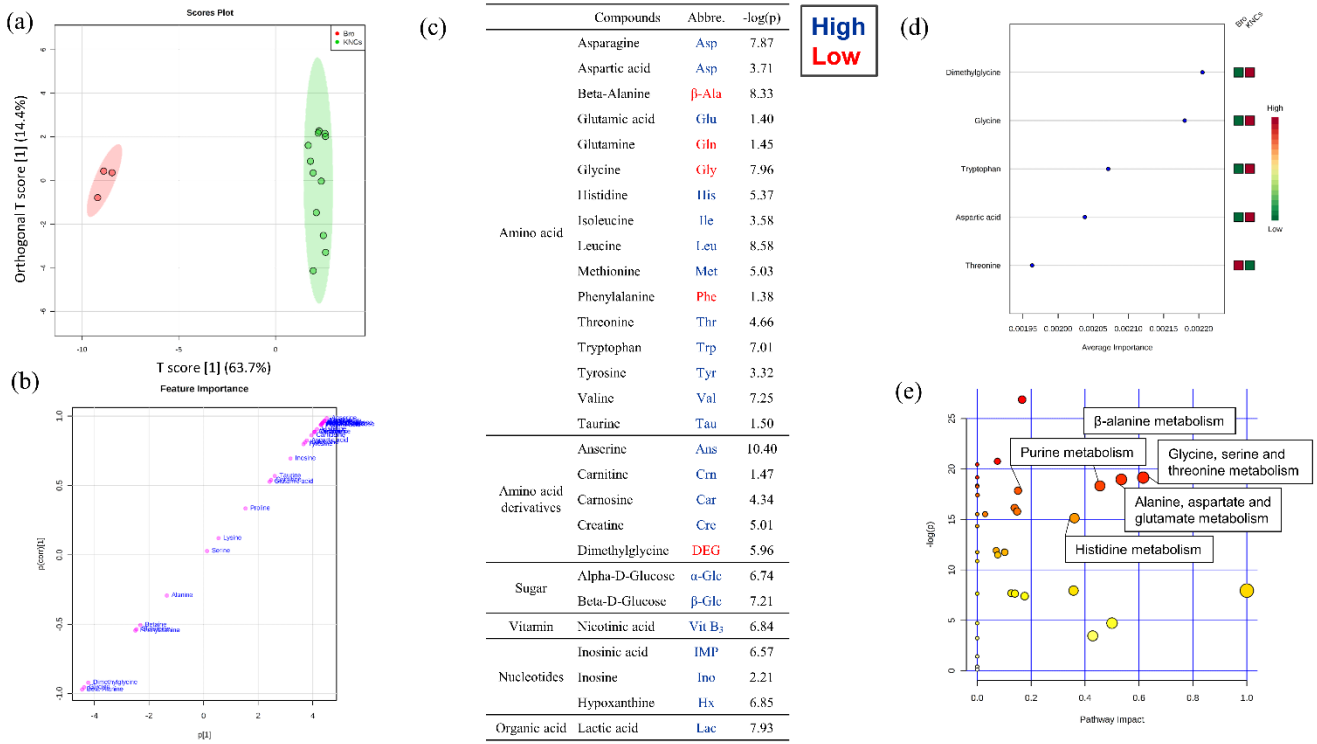
The differences between broilers and KNCs are noticeable on PCA and heatmap analyses. Due to the differences, we can analyze OPLS-DA, t-test, VIP scores, and pathway analysis (Fig. 11) for distinguishing KNCs from broilers. From OPLS-DA, two groups were separated clearly ($R^2X = 0.499$, $R^2Y = 0.910$, and $Q^2 = 0.895$). As noted based on the t-test, KNCs and broilers had distinct differences in the properties of metabolites. Compared to KNCs, broilers had higher concentrations of amino acids and their derivatives. The intracellular free amino acids affect initiation, elongation, and termination of protein synthesis depending on their concentrations (Millward et al., 1974). This higher

free amino acid concentration could be explained by a higher growth rate and productivity of broilers than KNCs (Kim et al. 2018; Ali et al., 2019). On the other hand, KNCs had significantly higher anserine and lower carnitine levels than in broilers. Similar metabolomic trends of KNCs were reported in a previous study in comparison with broilers (Jayasena et al., 2015b).

In addition, breast meat extracts of KNCs had significantly higher concentrations of α-glucose, nicotinic acid, IMP, hypoxanthine, and lactic acid than those of broilers. Carnitine could aid in loss of weight and be advantageous for human health (Jung et al., 2015). Carnosine and anserine have similar vital roles such as suppressing various diseases and improving exercise performance (Jung et al., 2013). Nicotinic acid is a precursor of coenzymes NAD, NADP, and vitamin B complex and have vital roles in reducing total cholesterol levels and improving mortality in coronary heart disease (Gille et al., 2008). IMP is dominant in freshly processed meat and is degraded to inosine and then to hypoxanthine and is closely related to umami taste (Khan et al., 2016). Also, IMP and glutamic acid have synergistic effects on savory taste within a certain ratio (Yamaguchi, 1967). Many previous studies reported that KNCs had a higher nucleotide content than commercial broilers (Choe et al., 2010; Jayasena et al., 2014b; Kim et al., 2018), which further explains the different sensory characteristics.

According to VIP scores and concentration of metabolites, broilers are highly related to free amino acids and their derivatives while KNCs are more related to bioactive compounds, sugars, organic acids, and nucleotides (Fig. 11d). To identify the interactive relationship on the metabolomic pathways, KEGG

library-based pathway analysis was performed (Fig. 11e). Zero points-of-impact score on pathway analysis were excluded because these pathways meant no effect on metabolomic differences. Then, only pathways below 0.05 of Bonferroni-holm methods (*Holm P*) were listed in Table 8. Holm P allows to solve the problem of Type 1 error by adjusting the criteria (Giacalone et al., 2018).



	Compounds	Abbre.	-log(p)
Amino acid	Asparagine	Asp	7.87
	Aspartic acid	Asp	3.71
	Beta-Alanine	β-Ala	8.33
	Glutamic acid	Glu	1.40
	Glutamine	Gln	1.45
	Glycine	Gly	7.96
	Histidine	His	5.37
	Isoleucine	Ile	3.58
	Leucine	Leu	8.58
	Methionine	Met	5.03
	Phenylalanine	Phe	1.38
	Threonine	Thr	4.66
	Tryptophan	Trp	7.01
	Tyrosine	Tyr	3.32
	Valine	Val	7.25
	Taurine	Tau	1.50
Amino acid derivatives	Anserine	Ans	10.40
	Carnitine	Crn	1.47
	Carnosine	Car	4.34
	Creatine	Cre	5.01
	Dimethylglycine	DEG	5.96
Sugar	Alpha-D-Glucose	α-Glc	6.74
	Beta-D-Glucose	β-Glc	7.21
Vitamin	Nicotinic acid	Vit B_3	6.84
Nucleotides	Inosinic acid	IMP	6.57
	Inosine	Ino	2.21
	Hypoxanthine	Hx	6.85
Organic acid	Lactic acid	Lac	7.93

Figure 11. Orthogonal partial least squares-discriminant analysis (OPLS-DA; $R^2X = 0.499$, $R^2Y = 0.910$, and $Q^2 = 0.895$) (a) and variable influence in OPLS-DA (b), list of metabolites with significant differences within native chicken breeds (KNC-A, -C, -D, -and -H) (c), VIP scores (d), and pathway analysis (e) from breast meat extracts of commercial broiler and KNCs by HSQC using a 850 MHz cryo-NMR spectrometer.

Table 3. List of pathway analysis of quantified metabolites by HSQC spectra from chicken breast meat extracts.

Pathway name	Total	Hits	Holm p[1]	log(p)	FDR	Impact
Glutathione metabolism	28	2	<0.001	16.74	0.000	0.102
Purine metabolism	62	4	<0.001	15.42	0.000	0.151
Primary bile acid biosynthesis	46	2	<0.001	14.43	0.000	0.076
Glycine, serine and threonine metabolism	34	6	<0.001	14.31	0.000	0.616
D-Glutamine and D-glutamate metabolism	6	2	<0.001	14.04	0.000	0.500
Glyoxylate and dicarboxylate metabolism	32	4	<0.001	13.99	0.000	0.148
Galactose metabolism	27	1	<0.001	12.29	0.000	0.029
Arginine biosynthesis	13	3	<0.001	12.18	0.000	0.071
beta-Alanine metabolism	21	5	<0.001	10.53	0.000	0.455
Pantothenate and CoA biosynthesis	19	3	<0.001	10.37	0.000	0.075
Alanine, aspartate and glutamate metabolism	28	5	<0.001	10.36	0.000	0.534
Glycolysis / Gluconeogenesis	26	3	<0.001	9.70	0.000	0.001
Taurine and hypotaurine metabolism	8	1	<0.001	9.65	0.000	0.429
Histidine metabolism	16	5	0.003	8.37	0.000	0.361
Aminoacyl-tRNA biosynthesis	48	18	0.010	7.04	0.001	0.167
Cysteine and methionine metabolism	33	2	0.011	6.77	0.002	0.126
Arginine and proline metabolism	38	3	0.018	6.22	0.003	0.176
Phenylalanine, tyrosine, and tryptophan biosynthesis	4	2	0.046	5.15	0.007	1.000
Phenylalanine metabolism	8	2	0.046	5.15	0.007	0.357

[1]Holm adjusted p value.

Based on the VIP scores and pathway analysis, six pathways were selected, including alanine, aspartate and glutamate metabolism, β-alanine metabolism, glycine, serine and threonine metabolism, D-glutamine and D-glutamate metabolism, histidine metabolism, and purine metabolism. Glycine, serine, and threonine metabolism and alanine, aspartate and glutamate metabolism are closely related and connected to glycine levels. As seen in Fig. 12, both glycine, serine, and threonine metabolism and alanine, aspartate, and glutamate metabolism are closely related in broiler breast meats. A series of related metabolites were higher in broilers. When combined with previous results from detected metabolites with high VIP scores, the most noticeable differences between KNCs and broilers were related to free amino acids and their derivatives in β-alanine metabolism and glycine, serine, and threonine metabolism. β-alanine, known to be a precursor of anserine, showed lower amounts in breast meats of KNCs than in broilers, while anserine levels were significantly higher (Jung et al., 2013). With this evidence of metabolomic pathway analysis combined with previously reported higher free amino acid content in broilers, higher growth rate and productivity characteristics of broilers are confirmed. These differences can arise from the breeding levels between broilers and KNCs; KNCs are closer to wild chicken when compared to the broilers (Kim et al., 2018). Among the metabolism pathways, the lowest contributing metabolites are products of purine metabolism, which is related to nucleotide degradation. Among the metabolites related to purine metabolism, IMP is important as it is related to umami taste in meat (Jung et al., 2013). KNCs had significantly higher content of IMP and hypoxanthine compounds

than the broilers. In previous studies, nucleotide levels were found to be proportional to the bird's age, too (Jayasena et al., 2015a; Xiao et al., 2019).

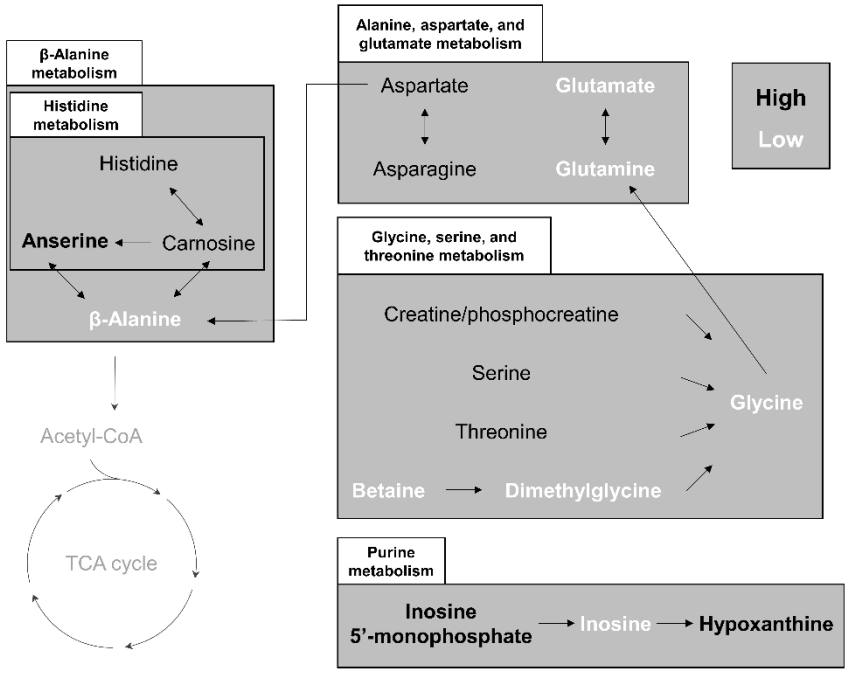


Figure 12. Schematic illustration of major related pathways of chicken breast meat from native chicken breast meat from native chickens (KNC-A, -C, -D, and -H). Metabolites bold colored in black (high) and bold white (low) refer to the metabolites with differences among native chickens in comparison to commercial broilers. Metabolites in grey were not detected

3.4. Conclusion

A combined 1D ^{1}H NMR and 2D HSQC NMR approach for quantification of metabolites present in chicken breast meat extracts was developed and its accuracy was confirmed using different breeds of chicken breast meat extracts. The results obtained showed that KNC-D is superior to other chicken breeds because it contains higher concentrations of free amino acids, sugars, and bioactive compounds. The four different KNC strains analyzed in this study, have a relatively similar metabolomic trend when compared to the broilers. Noticeable differences obtained between KNCs and broilers were higher amounts of IMP, α-glucose, lactate, and anserine, and lower amounts of free amino acids in KNCs meat. From the present study, an integrated peak of metabolites analyzed by a combination of HSQC and multivariate analyses (VIP scores and pathway analysis) may distinguish the differences in breast meat components among chicken breeds. The 2D qNMR when complemented with 1D qNMR can further help in acquiring interactive and accurate information on these differences which could be advantageous when compared to traditional chromatographic analysis.

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Chapter IV.

Characteristic metabolic changes of the crust from dry-aged beef using 2D NMR spectroscopy

4.1. Introduction

Meat aging is a series of biological and physiochemical transitions from muscle to meat after slaughter that causes proteolysis and enhances meat tenderness and palatability (Koohmaraie, 1994; Dashdorj et al., 2015). These changes could be used to improve meat quality under controlled environments, such as temperature, relative humidity, aging period, and the aging method by packaging conditions (Lee et al., 2019a). The major aging methods are divided into dry and wet aging. Meat for wet aging is vacuum-packed, while meat for dry aging is exposed to air without packaging (Smith et al., 2008). Dry-aged meat is known to generate concentrated flavors (beefy and roasted) after aging, while wet-aged meat is known to induce sour and bloody/metallic flavors (Smith et al., 2008; Perry, 2012; Oh et al., 2018).

Dry-aged meat is commonly produced in aerobic conditions, and its surface is dried rapidly. The dried meat surface is called the crust. Significant water loss occurs in both the crust and internal meat during dry aging (Lee et al., 2019a). In general, the crust is trimmed-off and considered inedible because of not only

hardness and dryness but a high concentration of microorganisms, such as mold and yeast (Smith et al., 2008; Ryu et al., 2018; Lee et al., 2019b). Trimming and water losses of dry-aged meat are directly related to economic loss, even if it can create value-added meat after aging (Khan et al., 2016). Lee et al. (2019a). suggested that the microorganisms on the surface of dry-aged beef might be the primary reason for the differences between wet- and dry-aged beef, and the composition of the microorganisms may have a pivotal role in the production of characteristic flavors and textures (Lee et al., 2019b). Therefore, it could be hypothesized that these environmental differences may affect the metabolic changes of the crust and edible portion of dry-aged beef, followed by characteristic sensory properties.

For a quality assessment of beef products, chemometric analyses are required to measure various metabolites, such as free amino acids, peptides, nucleotides, sugars, lipids, and aromatic compounds (Koutsidis et al., 2008). Among various chemometric analyses, nuclear magnetic resonance (NMR) spectroscopy and/or chromatography-based mass spectroscopy have been widely used for elucidating metabolic profiles from various biological samples together with multivariable analyses (Simmler et al., 2014). Between them, NMR can provide intact chemical information without derivatization (Kim et al., 2019). Several NMR studies combined with multivariable analyses have been reported on the metabolic profiles of during beef aging (Castejón et al., 2015). However, to our knowledge, there are no studies reported about metabolic changes in the crust of beef during the aging period.

Therefore, a two-dimensional quantitative NMR (2D qNMR) analysis was

performed to understand characteristic metabolic changes in the crust, dry-aged, and wet-aged beef striploin after aging for 4 weeks.

4.2. Material and methods

4.2.1. Sample preparation and aging process

A total of 60 striploins (*M. Longissimus lumborum*) from both sides of 30 different beef carcasses (21-month-old Holstein steers, quality grade 3) were obtained at 48 h postmortem from a local slaughterhouse and transferred to the meat processing plant (Seoul, Korea). The striploin from the same sides of different carcasses were randomly arranged in each dry- and wet-aged group (Control was randomly selected additionally). Before the aging process, the wet-aged group was vacuum-packaged (HFV-600 L, Hankook Fujee Co., Ltd., Hwaseong, Korea) with a low-density polyethylene/nylon bag (oxygen permeability of 22.5 mL/m^2/24 h atm at 60% relative humidity /25 °C and water vapor permeability of 4.7 g/m^2/24 h at 100% RH/25 °C). Twenty beef samples were randomly selected for both groups and aged for 7, 14, 21, and 28 days under different conditions. Considering the terminal weight loss of dry-aged beef, 1 kg of striploins was aged on a wire rack with the fat surface facing down at 4 °C, 75% RH, and 2.5 m/s airflow velocity. Wet-aged samples (500 g of striploins) was aged at 4 °C after vacuum-packaging. At the sampling stage, the crust of the dry-aged beef was trimmed off approximately 1 cm from the external surface of each dry-aged meat. The crust, dry-, and wet-aged beef samples were vacuum-packaged (HFV-600 L, Hankook Fujee Co., Ltd., Hwaseong, Korea) and stored at −70 °C until the analyses.

4.2.2. Sample extraction

Frozen beef samples were thawed at 4 °C for 24 h before analysis. Thawed

beef samples (5 g) were homogenized at 1720× g for 30 s (T25 basic, Ika Co., KG, Staufen, Germany) with 0.6 M perchloric acid. The homogenate was centrifuged (Continent 512R, Hanil Co., Ltd., Incheon, Korea) at 3086× g for 15 min. The supernatant was transferred to a new test tube and neutralized with potassium hydroxide. Neutralized extracts were centrifuged again under the same conditions. After centrifugation, each supernatant was filtered using filter paper (Whatman No. 1, Whatman PLC., Middlesex, UK) and lyophilized (Freezer dryer 18, Labco Corp., Kansas City, MO, USA). The lyophilized extracts were stored at −70 °C until NMR analysis.

4.2.3. NMR Experiments

One-dimensional (1D) ^{1}H NMR and ^{1}H-^{13}C HSQC were recorded in deuterium oxide (heavy water; D_2O) at 298 K on a Bruker 850 MHz cryo-NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). One-dimensional ^{1}H NMR was performed applying a modified standard zg30 (recycle delay of 1 s) pulse sequence provided by default in Topspin 3.6.2 (Bruker Biospin GmbH, Rheinstetten, Germany), with the lock on the deuterium resonance. The 1D ^{1}H NMR experiment was performed with 64k data points and a sweep width of 17,006.803 Hz with 128 scans. The ^{1}H-^{13}C HSQC experiment was performed as follows: 2k data points in the t_2 domain and 512 increments in the t_1 with 8 scans; spectral widths of 11 ppm for the f_2 dimension and 180 ppm for the f_1 dimension; and coupling constant values of 145 Hz were employed to set delay durations for short-range correlations, respectively. ^{1}H-^{13}C HSQC spectra were also used for qualification. The

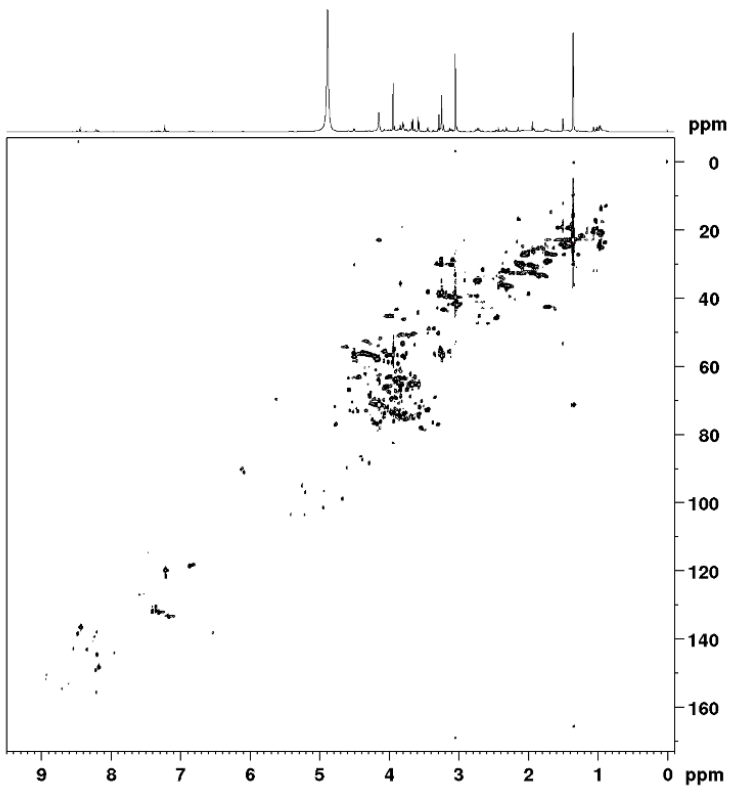
chemical shifts (δ) were referenced to the 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP) resonance. Baseline correction was performed manually.

4.2.4. Multivariate analysis

The dataset [65 (samples) × 46 (metabolites) matrix] of the acquired integral data of each metabolite from ^{1}H-^{13}C HSQC was collected using AMIX (Analysis of MIXtures software v3.9, Bruker Biospin GmbH, Rheinstetten, Germany). The data were analyzed using one-way ANOVA with Tukey's post hoc test where $p < 0.05$ was considered to be significant. PCA biplot, VIP scores of PLS-DA, and heatmap analysis were performed using MetaboAnalyst 4.0 (www.metaboanalyst.ca) according to Xia & Wishart (2010). Prior to the analysis, samples were log-transformed and auto-scaled. Heatmap analysis was evaluated using Euclidean distance and ward cluster algorithm.

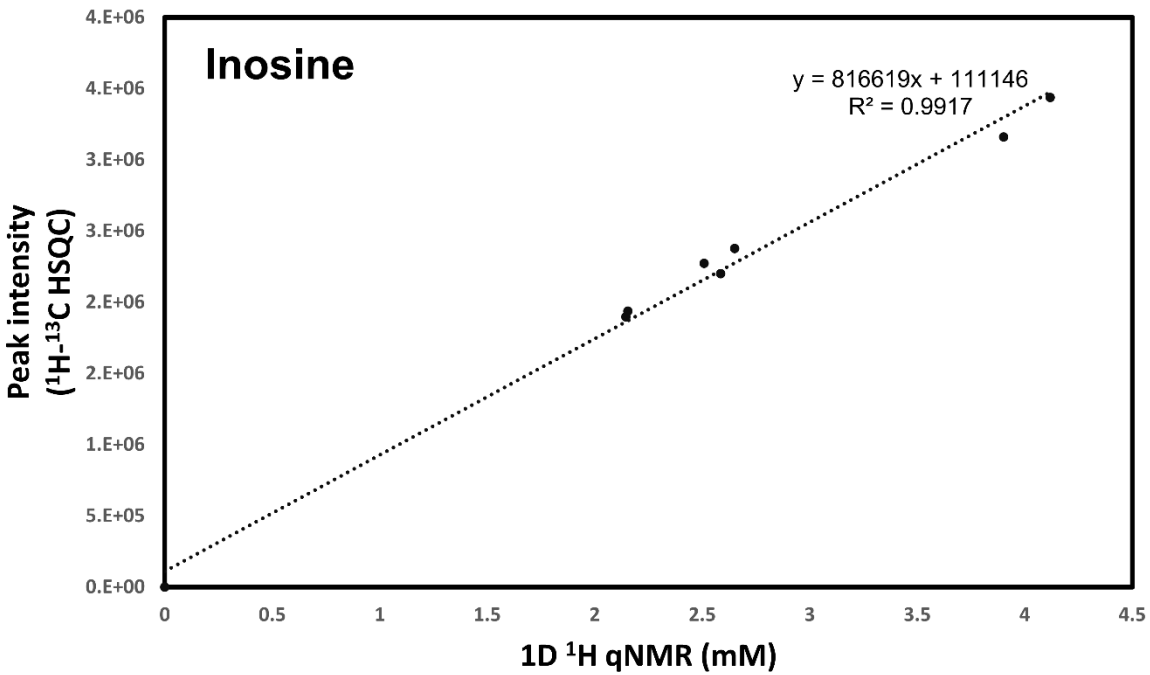
4.2.5. Quantification of Metabolites

Peaks of the metabolites were identified based on the standard compounds and biological magnetic resonance bank (BMRB; bmrb.wisc.edu). Quantification (totally 46 metabolites) was calculated based on the ^{1}H-^{13}C spectra using AMIX (Bruker Biospin GmbH, Rheinstetten, Germany; Fig. 13). Prior to quantification, 1D ^{1}H NMR spectra were acquired and processed according to Kim et al. (2019) to quantify 24 metabolites using an internal standard of 1 mM TSP. Calibration curves were made with the peak intensities from ^{1}H-^{13}C HSQC (Fig. 14)



Compounds	Chemical shift (δ)			
	^{13}C		^{1}H	
	δ_{test}	δ_{mtbd}	δ_{mtbd}	δ_{mtbd}
2,3-Butanediol	21.43	18.62	1.17	1.12
3-Hydroxybutyric acid	25.51	23.75	1.23	1.18
3-Indoxyl sulfate	115.61	113.32	7.50	7.41
Acetate	27.18	25.25	1.97	1.87
Acetone	33.34	31.58	2.24	2.17
Alanine	19.77	18.07	1.52	1.46
Anserine	29.52	27.59	3.12	2.99
Asparagine	38.14	36.44	2.99	2.85
Aspartic acid	40.00	38.46	2.78	2.69
Betaine	56.76	55.36	3.29	3.26
Cadaverine	26.10	24.23	1.50	1.42
Carnitine	46.96	44.25	2.50	2.38
Carnosine	31.58	29.65	3.12	2.99
Citrate	48.17	46.53	2.76	2.68
Creatine	40.59	38.48	3.06	3.00
Ethanol	20.25	18.65	1.21	1.16
Fumarate	139.85	137.22	6.56	6.48
Glutamic acid	58.21	55.22	3.83	3.70
Glutamine	34.63	32.52	2.51	2.40
Glutathione	34.79	33.21	2.62	2.51
Glycine	45.31	43.03	3.61	3.54
Histidine	31.73	29.09	3.27	3.17
Hypoxanthine	149.34	147.05	8.20	8.13
Inosine	91.88	90.30	6.10	6.06
Inosine 5'-monophosphate	88.12	86.72	4.41	4.35
Isoleucine	14.61	12.55	0.99	0.91
Leucine	25.53	22.72	1.00	0.93
Lysine	42.66	40.94	3.06	2.93
Methionine	17.94	15.62	2.16	2.07
Niacinamide	128.26	125.80	7.63	7.55
Phenylalanine	39.97	38.56	3.16	3.09
Proline	69.56	67.82	4.31	4.24
Putrescine	26.10	24.23	1.81	1.76
Pyruvic acid	30.37	28.97	2.38	2.35
Serine	63.89	61.97	4.03	3.94
Taurine	39.07	37.16	3.46	3.40
Threonine	64.06	61.98	3.65	3.57
Trimethylamine	47.96	46.56	2.91	2.88
Tryptophan	54.80	53.10	4.04	3.98
Tyramine	118.95	117.37	6.84	6.77
Tyrosine	119.45	117.71	6.89	6.83
Valine	21.91	19.75	1.09	1.02
α-Glucose	64.36	62.43	3.80	3.75
β-Alanine	37.08	35.49	2.60	2.54
β-Glucose	64.32	62.74	3.93	3.87
γ-Aminobutyric acid	37.08	35.50	2.32	2.25

Figure 13. Representative ^{1}H-^{13}C hetero nuclear single quantum coherence (HSQC) NMR spectrum from 28 day-aged crust of dry aging extracts acquired using a 850 MHz cryo-NMR spectrometer and metabolites list of pattern integration for 2D qNMR analysis



Compounds	R^2
Alanine	0.996
Anserine	0.998
Asparagine	0.999
Aspartic acid	0.997
Carnitine	0.981
Carnosine	1.000
Creatine	0.965
Glutamic acid	0.996
Glutamine	0.986
Glycine	0.968
Hypoxanthine	0.992
Inosine	0.992
Inosine 5'-monophosphate	0.990
Isoleucine	0.990
Leucine	0.994
Lysine	0.996
Methionine	0.996
Phenylalanine	0.996
Proline	0.968
Serine	0.998
Taurine	0.998
Tryptophan	0.997
Tyrosine	0.997
Valine	0.996

Figure 14. Standard curves of quantification from ^{1}H-^{13}C hetero nuclear single quantum coherence (HSQC) based on the 1D ^{1}H quantitative NMR using a 850 MHz cryo-NMR spectrometer

4.3. Results and discussion

4.3.1. Multivariable analyses

Overall metabolic differences were determined using principal components analysis (PCA) based on the peak intensities from ^{1}H-^{13}C hetero single nuclear coherence (HSQC) NMR spectra (Fig. 14). PCA is usually used to discriminate differences of samples composed of multivariable datasets via variance maximization and dimension reduction (Worley & Powers, 2012). As seen in PCA, each meat sample was separated clearly by the aging period, along with PC1 (54.5%). Among all groups, the crust was more strongly correlated with PC1 than the others (wet- and dry-aged beef). However, the crust was not correlated with PC2 (12.5%) during the aging period, while dry- and wet-aged beef correlated with PC2 depending on the aging period. Dry- and wet-aged beef showed a similar appearance in PCA. However, dry-aged beef was better-differentiated by PC1 than wet-aged beef. Wet-aged beef was hard to be distinguished between 21 and 28 days. Along with the PC2, dry- and wet-aged beef exhibited a linear correlation until 21 days, and then the correlation decreased by 28 days of the aging period.

The loading plots of variables, which visualize variables, can be used for understanding metabolic patterns easily (Ergon, 2004). Most of the metabolites were associated with PC1 and were composed of most free amino acids, biogenic amines, and bioactive compounds, except for some free amino acids (asparagine, tryptophan, and glutamine), inosine 5′-monophosphate (IMP), α- and β-glucose, and glutathione (Fig. 15). The proportional increase in free amino acids and their derivatives (biogenic amines and bioactive compounds),

depending on the aging period, was a well-known result of the aged meat (Fu et al., 2017). Biogenic amines such as cadaverine, putrescine, tyramine, and trimethylamine (TMA) commonly increased depending on the aging period, and these compounds are usually considered indicators of freshness in meat samples (Galgano et al., 2009; Shumilina et al., 2016). Moreover, not only IMP and α- and β-glucose are recognized as freshness indicators, but also these metabolites play an important role in meat flavor development during the cooking process (Byun et al., 2003; Lee et al., 2019a). In addition to PCA, partial least squares-discriminant analysis (PLS-DA) was performed to separate between groups of observations (R^2 = 0.967, Q^2 = 0.935). PLS-DA, which is called the supervised method, is similar to PCA, but it can discriminate differences based on each group (Worley & Powers, 2012). In this study, PLS-DA showed a good cumulative explained variation (R^2) and predictive ability (Q^2), which meant that the different aging methods and their aging period were clearly distinguished based on the polar metabolic characteristics.

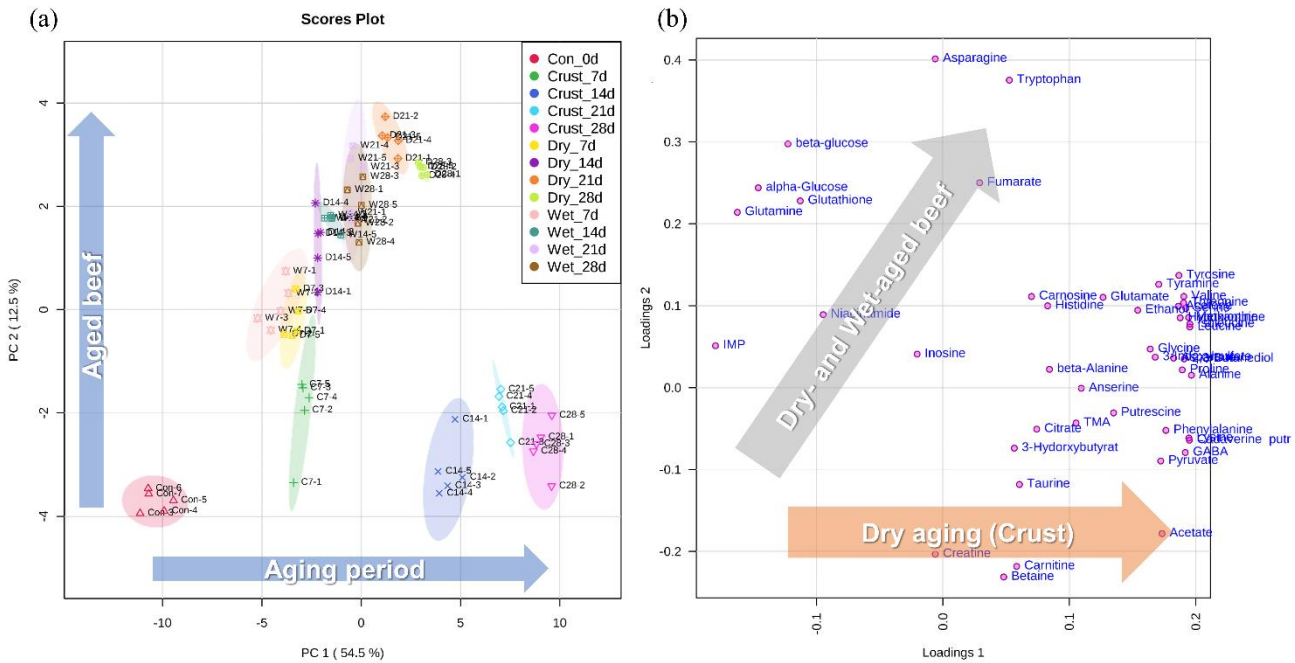


Figure 15. Principal components analysis (PCA) (a) score plots and (b) loading plots from quantified metabolites of beef aged by different method and crust. GABA, γ-aminobutyric acid; TMA, trimethylamine; IMP, inosine 5'-monophosphate.

PLS-DA score plots showed similar appearances compared to PCA (Fig. 16). Since PCA is considered an indicator of the practical reliability of PLS-DA, a similar appearance can explain that the data are clearly distinguished (Worley & Powers, 2012). In PLS-DA, variables can be measured by scores, which are called variable importance in projection (VIP). The overall average of VIP scores is 1, and the metabolites above 1 contribute to the formation of the PLS-DA model (Galindo-Prieto et al., 2014). Based on the VIP scores, twelve metabolites (> 1 score) are presented in Fig. 17. Three metabolites—carnitine, asparagine, and tryptophan—were the most characteristic variables to separate the groups, and these compounds were highly correlated in PC2 of PCA. Asparagine and tryptophan had a positive correlation with PC2, but carnitine had a negative correlation. Along with PCA, the positive correlation was related to the aged beef groups (dry- and wet-aged beef), and the negative correlation was related to the crust. The appearances of the metabolites could be the best way to distinguish between the aging method (dry- and wet-aged beef) and between the edible portion of aged beef and crust. Two other metabolites—fumarate and betaine—were also related to aged beef groups and crust, respectively. Other high VIP score metabolites were related to aged beef (β-glucose, α-glucose, glutamine, and glutathione) and crust (taurine, citrate, and tyramine).

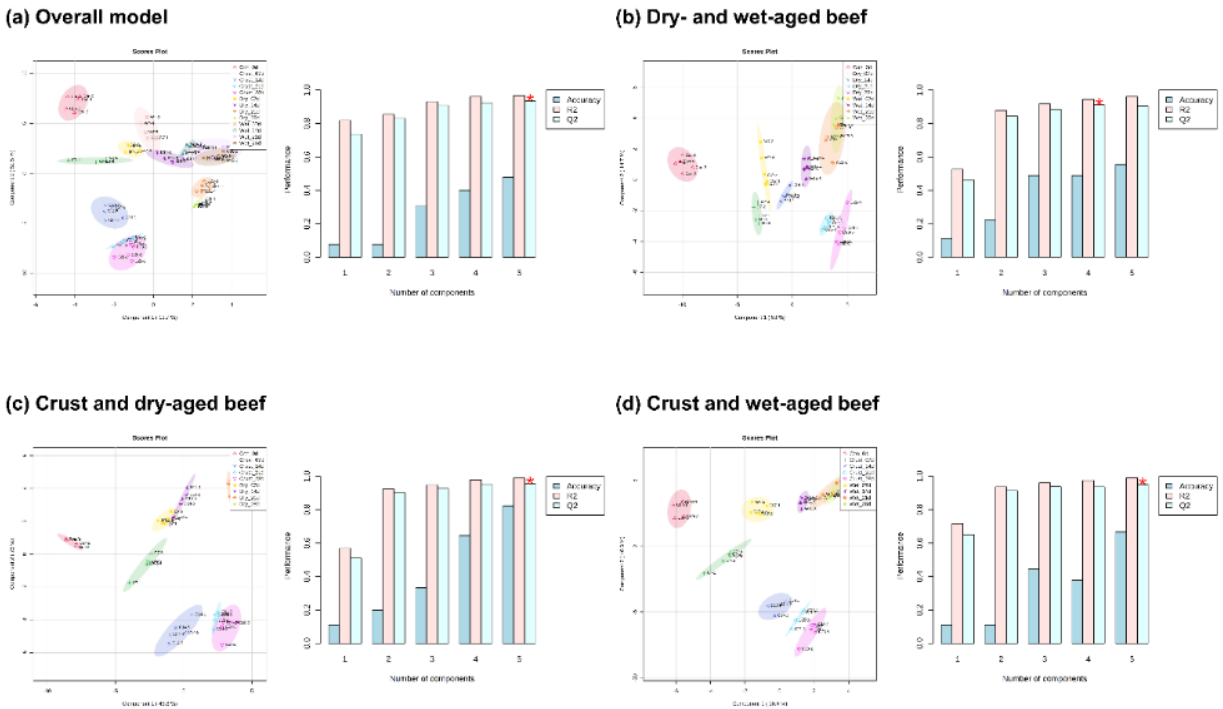


Figure 16. Partial least square-discriminant analysis (PLS-DA) cross validation from quantified metabolites of beef aged by different method and crust. Validation was evaluated (a) overall groups, (b) dry- and wet-aged beef, (c) crust and dry-aged beef, and (d) crust and wet-aged beef

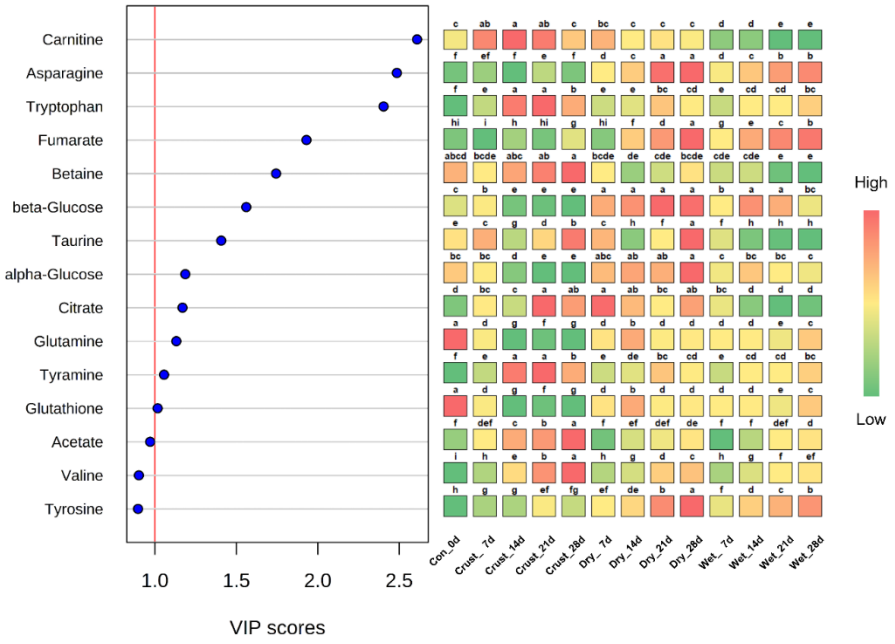


Figure 17. Variable importance in projection (VIP) scores of partial least squares-discriminant analysis (PLS-DA) from the extracts of crust, dry-aged, and wet-aged beef. The colored boxes on the right indicate the relative concentrations of the corresponding metabolites (red, high; yellow, intermediate; green, low). [a-i] Letters different in the same row indicate a significant difference ($p<0.05$)

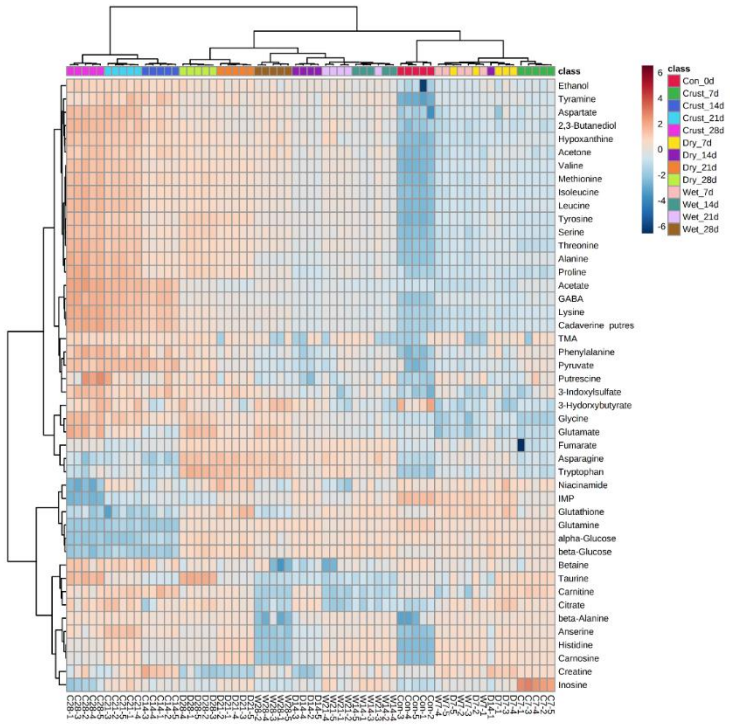


Figure 18. Heatmap analysis based on the quantified metabolites from crust, dry-aged and wet-aged beef using 2D NMR (heteronuclear single quantum coherence, HSQC) on 850 MHz cryo-NMR spectrometer. GABA, γ-aminobutyric acid; TMA, trimethylamine; IMP, inosine 5'-monophosphate

A hierarchical clustering heatmap analysis was performed for the classification between different observations (Fig. 18). This method arranges the groups based on the similarity of the datasets and can be easily recognized using a dendrogram and heatmap (Škuta et al., 2014). As seen in the dendrogram, the greatest differences were shown between the crust and the other groups after the second week. The differences were clearly expressed in PCA. These noticeable differences in the crust could be attributed to water evaporation and microbial effects (Casal et al., 2008; Lee et al., 2019a; Lee et al., 2019b). Among the aged beef, most groups were well clustered by group except for some regions where the first week of dry- and wet-aged beef and the second week of dry-aged beef were mixed and the second and third weeks of wet-aged beef were mixed. Based on the results, the metabolic characteristics were clearly differentiated in the crust and dry-aged beef in the second week. In a previous study, similar results were reported that the unique flavor of dry-aged beef needs to age at least 2 weeks (Lee et al., 2019a).

4.3.2. Metabolic characteristics

4.3.1.1. Proteolysis

Regardless of the treatment, total free amino acids were significantly increased, depending on the aging period (Table 9). The increase in amino acids and biogenic amines is generally proportional to the aging period (Fu et al., 2017). Free amino acids are directly related to meat taste and/or contribute to flavor formation during cooking (Mottram, 1998; Jayasena et al., 2015; Lee et

al., 2019a). Total free amino acids were highest in the crust, then dry-aged, and finally wet-aged beef. In the first week of aging, total free amino acids did not differ significantly between the crust and dry-aged beef, while wet-aged beef was lower than the others ($p < 0.05$). The total free amino acids of the crust began to increase significantly after the second week of aging. The increase in free amino acids in the crust may be due to steady water evaporation during dry aging and accelerated proteolysis by microorganisms (Lee et al., 2019a).

Table 9. Free amino acids contents (mg/100 g) from different aged beef striploin (M. *longissimus lumborum*) using ^{1}H-^{13}C HSQC on 850 MHz cryo-NMR spectrometer

Contents	Aging method	Aging period (day)					SEM[1]
		0	7	14	21	28	
Alanine	Crust	15.95^{e}	27.26^{dx}	37.91^{cx}	47.19^{bx}	52.95^{ax}	0.852
	Dry	15.95^{d}	26.25^{cx}	26.52^{cy}	36.35^{by}	39.93^{ay}	0.264
	Wet	15.95^{d}	23.30^{cy}	27.35^{by}	27.92^{bz}	29.42^{az}	0.312
	SEM[2]		0.359	0.339	0.517	0.980	
Asparagine	Crust	3.79^{b}	4.69^{aby}	3.30^{by}	5.49^{az}	3.95^{bz}	0.363
	Dry	3.79^{d}	7.07^{cx}	8.63^{bx}	13.23^{ax}	13.59^{ax}	0.388
	Wet	3.79^{d}	6.94^{cx}	9.30^{bx}	11.02^{ay}	11.96^{ay}	0.481
	SEM[2]		0.448	0.445	0.518	0.428	
Aspartic acid	Crust	1.39^{d}	4.11^{c}	13.37^{bx}	25.22^{ax}	25.15^{ax}	0.699
	Dry	1.39^{d}	3.77^{c}	5.71^{by}	7.87^{ay}	9.14^{ay}	0.585
	Wet	1.39^{d}	3.54^{c}	7.80^{by}	8.66^{aby}	9.82^{ay}	0.462
	SEM[2]		0.654	0.589	0.630	0.625	
Glutamic acid	Crust	6.74^{e}	27.25^{dx}	67.31^{ax}	62.59^{bx}	56.80^{cx}	0.844
	Dry	6.74^{e}	12.88^{dy}	18.53^{cy}	23.25^{by}	30.44^{az}	0.489
	Wet	6.74^{e}	11.95^{dy}	17.84^{cy}	24.92^{by}	35.83^{ay}	0.855
	SEM[2]		0.369	0.934	1.013	0.865	
Glutamine	Crust	49.09^{a}	34.14^{b}	6.10^{dz}	8.12^{cz}	6.07^{dz}	0.553
	Dry	49.09^{a}	36.12^{c}	42.98^{bx}	34.71^{cx}	35.46^{cy}	0.688
	Wet	49.09^{a}	35.05^{c}	35.21^{cy}	32.16^{dy}	39.22^{bx}	0.781
	SEM[2]		0.828	0.327	0.381	0.567	
Glycine	Crust	25.09^{d}	21.16^{ey}	35.55^{cx}	40.28^{bx}	44.72^{ax}	0.761
	Dry	25.09^{d}	25.83^{dx}	31.83^{cy}	34.15^{by}	39.66^{ay}	0.450
	Wet	25.09^{c}	21.65^{dy}	28.46^{bz}	30.40^{bz}	36.09^{az}	0.702
	SEM[2]		0.832	0.702	0.664	0.700	

Contents	Aging method	Aging period (day)					SEM[1]
		0	7	14	21	28	
Isoleucine	Crust	3.77^{e}	10.08^{dx}	21.79^{cx}	30.62^{bx}	37.25^{ax}	0.616
	Dry	3.77^{e}	10.69^{dx}	13.58^{cy}	20.80^{by}	23.60^{ay}	0.188
	Wet	3.77^{e}	9.07^{dy}	13.93^{cy}	16.78^{bz}	18.32^{az}	0.290
	SEM[2]		0.228	0.396	0.253	0.736	
Leucine	Crust	7.21^{e}	17.79^{dx}	31.11^{cx}	43.72^{bx}	50.38^{ax}	0.821
	Dry	7.21^{e}	17.76^{dx}	20.58^{cz}	30.07^{by}	32.42^{ay}	0.301
	Wet	7.21^{d}	15.83^{cy}	22.63^{by}	25.70^{az}	26.83^{az}	0.519
	SEM[2]		0.257	0.569	0.580	0.989	
Methionine	Crust	4.43^{e}	12.34^{dy}	18.68^{cx}	23.50^{bx}	27.08^{ax}	0.408
	Dry	4.43^{c}	13.77^{bx}	14.56^{bz}	21.61^{ay}	21.67^{ay}	0.344
	Wet	4.43^{d}	12.41^{cy}	16.51^{by}	18.48^{az}	16.75^{bz}	0.283
	SEM[2]		0.285	0.364	0.430	0.416	
Proline	Crust	8.56^{d}	9.89^{dy}	16.97^{cx}	23.66^{bx}	27.18^{ax}	0.627
	Dry	8.56^{d}	10.72^{cx}	11.23^{cz}	17.98^{by}	19.30^{ay}	0.375
	Wet	8.56^{d}	10.58^{cx}	12.89^{by}	14.11^{abz}	14.65^{az}	0.435
	SEM[2]		0.188	0.353	0.491	0.683	
Serine	Crust	6.61^{e}	9.92^{dy}	18.18^{cx}	24.04^{bx}	27.00^{ax}	0.481
	Dry	6.61^{e}	11.73^{dx}	14.58^{cy}	19.93^{by}	22.07^{ay}	0.155
	Wet	6.61^{e}	10.19^{dy}	14.55^{cy}	16.32^{bz}	17.92^{az}	0.322
	SEM[2]		0.358	0.321	0.309	0.511	
Taurine	Crust	45.68^{d}	56.38^{bx}	39.30^{ex}	48.02^{cx}	67.42^{ay}	0.620
	Dry	45.68^{c}	55.03^{bx}	36.44^{ey}	43.50^{dy}	71.26^{ax}	0.692
	Wet	45.68^{a}	41.56^{by}	34.27^{cy}	34.26^{cz}	33.98^{cz}	0.883
	SEM[2]		0.698	0.911	0.689	0.930	
Tryptophan	Crust	3.91^{c}	5.26^{by}	5.29^{by}	6.78^{az}	5.76^{bz}	0.343
	Dry	3.91^{d}	6.86^{cx}	7.71^{cx}	10.92^{bx}	12.39^{ax}	0.303
	Wet	3.91^{e}	6.45^{dx}	8.04^{cx}	9.32^{by}	10.54^{ay}	0.232
	SEM[2]		0.347	0.220	0.274	0.414	

Contents	Aging method	Aging period (day)					SEM[1]
		0	7	14	21	28	
Valine	Crust	3.18^{e}	9.56^{dx}	17.94^{cx}	26.14^{bx}	30.92^{ax}	0.582
	Dry	3.17^{e}	9.88^{dx}	12.51^{cy}	19.48^{by}	20.76^{ay}	0.256
	Wet	3.37^{e}	8.99^{dy}	13.34^{cy}	16.06^{bz}	16.90^{az}	0.263
	SEM[2]		0.109	0.388	0.348	0.702	
Total free amino acids	Crust	200.89^{e}	280.30^{dx}	386.52^{cx}	478.71^{bx}	533.85^{ax}	6.282
	Dry	200.89^{e}	279.48^{dx}	301.81^{cy}	383.80^{by}	446.23^{ay}	2.958
	Wet	200.89^{e}	246.77^{dy}	300.74^{cy}	330.32^{bz}	360.88^{az}	3.759
	SEM[2]		3.884	4.278	4.084	7.102	

[1, 2] Standard error of the mean [1](n=25), [2](n=15).

[a-e] Different letters in the same row indicate a significant difference ($p < 0.05$).

[x-z] Different letters in the same column indicate a significant difference ($p < 0.05$).

4.3.2.2. Bioactive compounds

Anserine in the crust increased up to the third week and decreased during the fourth week, while that in wet-aged beef increased rapidly in the second week and decreased during the aging period ($p < 0.05$; Table 7). Anserine in dry-aged beef tends to increase and decrease significantly every two weeks of aging. Carnosine showed the same pattern as anserine in the crust and dry-aged beef, but in the case of wet-aged beef, it tended to increase significantly during the first week of aging and then decrease as the aging period increased. The changes of anserine and carnosine during the aging period and the tendency of histidine and β-alanine were consistent (Fig. 19). Histidine can be used as a carbon source with glucose during microbial energy metabolism (Stouthamer & Bettenhaussen, 1973). Compared to β-alanine, histidine concentration is closely related to histidyl dipeptides concentration (Mei et al., 1998). The changes in fluctuated histidyl dipeptides could be affected by changing the major strain during the aging period in which initial bacteria increased during the first two weeks and did not change greatly in a later period, while lactic acid bacteria, yeast, and mold increased steadily (Hulankova et al., 2018). Anserine and carnosine are histidyl dipeptides containing β-alanine, which has similar bioactivities, such as a buffering effect and antioxidant activity, to histidyl dipeptides, chelate metal ions, or scavenge free radicals (Chan et al., 1994). Moreover, histidyl peptides are associated with anti-aging, antiglycation, neurotransmitter functions, and the alleviation of diseases, such as Alzheimer's disease, cataracts, diabetes, and ischemia (Jung et al., 2013). Carnosine also contributes to the umami taste in meat (Dashdorj et al., 2015). Regardless of

treatment groups, anserine and carnosine decreased in the fourth week ($p < 0.05$). However, Fu et al. (2017) reported that the bioactivities of wet-aged beef increased proportionally by aging period.

Carnitine is associated with fatty acid-related energy-generating processes, enhancing the utilization of fatty acids, and facilitating the removal of accumulated fatty acids from mitochondria. Thus, it could be advantageous for human health and help with weight loss (Rebouche, 1992). Carnitine in the crust steadily increased up to the third week and then decreased ($p < 0.05$). Carnitine in dry- and wet-aged beef increased up to the first week and remained stable, while that in wet-aged beef decreased during the first week of aging and then remained stable ($p < 0.05$). Carnitine can be synthesized from lysine and methionine (Jung et al., 2015). The decrease in carnitine during aging might contribute to the elevation of TMA by microorganisms, and it could be increased at the terminal stage of dry aging (Falony et al., 2015; Lee et al., 2019a). Creatine increased in the first week in all groups. Creatine increased until the second week and decreased in the third week in the crust ($p < 0.05$). In dry-aged beef, creatine decreased in the second week. On the other hand, wet-aged beef maintained creatine during the aging period. Creatine is related to muscle energy metabolism and provides energy during exercise (Jayasena et al., 2014). Phosphocreatine rapidly converts to creatine postmortem (Harris et al., 1997).

Table 10. Bioactive compounds (mg/100 g) from different aged beef striploin (M. *longissimus lumborum*) using ^{1}H-^{13}C HSQC on 850 MHz cryo-NMR spectrometer

Contents	Aging method	Aging period (day)					SEM[1]
		0	7	14	21	28	
Anserine	Crust	19.93^{c}	90.26^{by}	95.08^{bx}	147.98^{ax}	96.89^{bx}	0.852
	Dry	19.93^{d}	100.21^{ax}	53.12^{cy}	98.30^{ay}	72.07^{by}	0.264
	Wet	19.93^{c}	92.04^{ay}	91.40^{ax}	78.30^{bz}	22.72^{cz}	0.312
	SEM[2]		2.438	3.932	3.193	5.267	
Betaine	Crust	15.36^{ab}	14.78^{b}	16.34^{abx}	17.15^{abx}	17.67^{ax}	0.363
	Dry	15.36^{a}	14.76^{ab}	13.58^{by}	14.19^{aby}	14.98^{abxy}	0.388
	Wet	15.36	14.15	14.18^{y}	13.08^{y}	12.93^{y}	0.481
	SEM[2]		0.302	0.637	0.546	0.873	
Carnitine	Crust	39.61^{c}	44.95^{abx}	46.01^{ax}	45.35^{abx}	42.70^{bx}	0.699
	Dry	39.61^{b}	43.41^{ax}	41.44^{aby}	41.76^{aby}	41.24^{abx}	0.585
	Wet	39.61^{a}	34.97^{by}	34.87^{bz}	32.52^{bz}	32.47^{by}	0.462
	SEM[2]		0.866	0.642	0.451	0.650	
Carnosine	Crust	33.97^{d}	418.83^{bz}	256.24^{cy}	498.79^{ay}	260.74^{cy}	0.844
	Dry	33.97^{e}	471.15^{by}	205.82^{dz}	546.28^{ax}	297.89^{cx}	0.489
	Wet	33.97^{e}	499.34^{ax}	476.09^{bx}	455.32^{cz}	53.02^{dz}	0.855
	SEM[2]		5.908	6.005	5.221	5.655	
Creatine/ Phosphocreatine	Crust	182.13^{c}	215.28^{by}	239.46^{ax}	205.85^{bcx}	192.74^{bc}	0.553
	Dry	182.13^{b}	227.47^{ax}	167.30^{by}	150.83^{by}	157.55^{by}	0.688
	Wet	182.13^{b}	211.49^{ay}	200.98^{abxy}	202.34^{abx}	197.01^{abx}	0.781
	SEM[2]		3.806	12.983	6.496	9.142	

[1, 2] Standard error of the mean [1](n=25), [2](n=15).

[a-e] Different letters in the same row indicate a significant difference ($p < 0.05$).

[x-z] Different letters in the same column indicate a significant difference ($p < 0.05$).

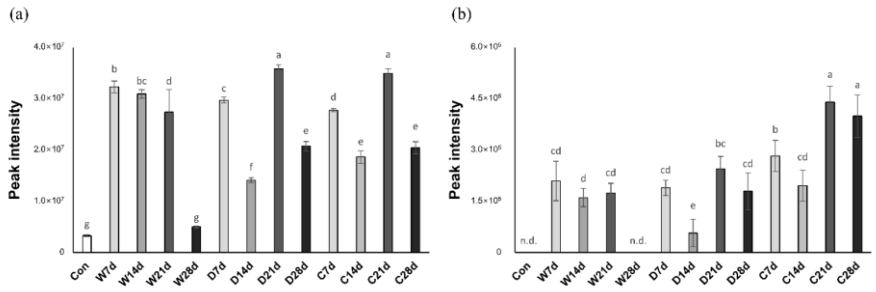


Figure 19. Peak intensities of histidine (a) and β-alanine from different aged beef striploin (M. *longissimus lumborum*) using 2D NMR (heteronuclear single quantum coherence, HSQC) on 850 MHz cryo-NMR spectrometer. [a-g] Different letters in the same row indicate a significant difference ($p < 0.05$). n.d., not detected

4.3.2.3. Nucleotides

The major nucleotides (IMP, inosine, and hypoxanthine) of beef samples are presented in Table 8. Most of ATP in muscles at postmortem is dephosphorylated into AMP and is then converted to IMP, which contributes to an umami taste and imparts flavor to the meat. IMP is converted to inosine and then to hypoxanthine, which contributes to the bitter taste (Dashdorj et al., 2015). Regardless of the aging method, the IMP contents gradually decreased, while the hypoxanthine contents increased as aging length increased ($p < 0.05$). Similarly, IMP contents in the crust rapidly decreased in the first week of aging and were significantly lower than those in the dry- and wet-aged beef during the aging period.

Dry- and wet-aged beef had no differences at the first 2-weeks in the IMP contents. However, the IMP contents of dry-aged beef were higher in the third week and decreased significantly in the fourth week compared to those of wet-aged beef ($p < 0.05$). A similar trend has been observed in previous studies (Kim et al., 2016; Lee et al., 2019a). This decrease in dry-aged beef could be associated with enzymes related to the degradation of IMP (Lee et al., 2019a). IMP was mainly produced at the initial postmortem and degraded during the aging period (Dashdorj et al., 2015). Yamaguchi (1967) reported that a certain ratio of IMP and glutamic acids showed a strong synergistic effect on the umami taste. However, this lower IMP of dry-aged beef compared to wet-aged beef in the fourth week did not cause a difference in the umami taste of dry-aged beef in this study. In the crust, inosine increased in the first week and decreased in the fourth week of aging, and hypoxanthine was proportional during the aging

period regardless of aging methods. As described in PCA, the PC1 scores of IMP, inosine, and hypoxanthine were −0.18, −0.02, and 0.19, while the PC2 scores were 0.05, 0.04, and 0.09, respectively (Fig. 15). Nucleotides are used as freshness indicators, called the k-index (%) and biogenic amines index, that develop during proteolysis (Shumilina et al., 2016). Based on the nucleotides, the crust rapidly decreased in freshness compared to dry- and wet-aged beef.

Table 11. Nucleotide contents (mg/100 g) from different aged beef striploin (M. *longissimus lumborum*) using ^{1}H-^{13}C HSQC on 850 MHz cryo-spectrometer

Contents	Aging method	Aging period (day)					SEM[1]
		0	7	14	21	28	
IMP	Crust	135.21^{a}	57.42^{by}	46.52^{cy}	36.54^{dz}	10.22^{ez}	1.104
	Dry	135.21^{a}	99.78^{bx}	80.49^{cx}	66.71^{dx}	38.29^{ey}	1.291
	Wet	135.21^{a}	102.60^{bx}	75.64^{cx}	56.68^{dy}	53.05^{dx}	1.330
	SEM[2]		1.154	1.612	1.094	0.953	
Inosine	Crust	16.04^{d}	27.48^{ax}	18.58^{cy}	20.48^{b}	14.93^{dy}	0.476
	Dry	16.04^{d}	18.79^{by}	17.59^{cy}	20.17^{a}	17.74^{cx}	0.287
	Wet	16.04^{c}	18.50^{by}	20.24^{ax}	20.03^{a}	16.81^{cx}	0.476
	SEM[2]		0.408	0.423	0.589	0.400	
Hypoxanthine	Crust	12.54^{e}	25.45^{dx}	37.67^{cx}	43.53^{bx}	46.64^{ax}	0.540
	Dry	12.54^{e}	22.72^{dy}	27.64^{cy}	33.51^{by}	37.36^{ay}	0.880
	Wet	12.54^{d}	21.15^{cz}	26.90^{by}	32.18^{ay}	36.04^{ay}	1.415
	SEM[2]		0.502	1.006	0.253	0.765	

[1, 2] Standard error of the mean [1](n=25), [2](n=15).

[a-e] Different letters in the same row indicate a significant difference ($p < 0.05$).

[x-z] Different letters in the same column indicate a significant difference ($p < 0.05$).

4.3.3. Unique metabolic characteristics of the crust during aging

The crust showed a higher concentration of citrate, pyruvate, ethanol, acetone, 2,3-butanediol acetate, and 3-hydroxybutyrate, while both α-glucose and β-glucose were lower than in dry- and wet-aged beef ($p < 0.05$) (Fig. 18). The increased compounds during aging are catabolites of microorganisms through glucose metabolism with glucose as substrates (Sauer et al., 2008). Along with the decrease in glucose, fumarate also decreased, while citrate increased. Therefore, it is shown that microorganisms grown on the outer surface of beef (crust) during aging may play a vital role, affect metabolic changes inside the meat, and significantly influence final meat qualities (Casal et al., 2008; Lee et al., 2019b; Oh et al., 2019). Moreover, it is found that γ-aminobutyric acid (GABA) increased on the crust from the second week of aging and increased on the dry-aged beef at the fourth week of aging compared to wet-aged beef ($p < 0.05$). GABA is usually produced during the fermentation of yeast and fungi (Kumar & Punekar, 1997) and is known to have functional effects, such as lowering blood pressure (Inoue et al., 2003). Previous results demonstrated that the degradation of myofibrillar proteins is not only achieved by endogenous proteolytic enzymes but also by exogenous microbial proteolytic action during the dry aging process (Kumar & Punekar, 1997; Lee et al., 2019a; Lee et al., 2019b).

Despite the beneficial effect, harmful compounds such as biogenic amines and 3-indoxyl sulfate can also be generated in the crust during dry aging (Figure 3). The amounts of glucose, glutamine, asparagine, tryptophan, and fumarate were higher in dry- and wet-aged beef than those in the crust during the second

week of aging. This difference could be explained by the fact that both dry- and wet-aged beef were less influenced by microorganisms as yeast and mold cannot penetrate the crust itself (Lee et al., 2017). On the contrary, Peromingo et al. (2019) suggested fungal secondary metabolites on the surface of dry-cured meat diffused into the meat. Moreover, a previous study reported that mold and yeast growth on the surface of dry-aged beef and their composition (ratio) directly affected meat sensory properties (Lee et al., 2019b).

4.4. Conclusion

Based on this study, the metabolic appearance of crust, dry-, and wet-aged beef were separated from the first week and showed a completely different aspect from the second week, as shown by NMR-based multivariable analyses. Moreover, NMR-based multivariable analyses could be used for distinguishing the aging degree. Among them, the crust exhibited unique metabolic changes that accelerated proteolysis (total free amino acids and biogenic amines) and IMP depletion than in dry-aged beef and generated specific microbial catabolites (3-indoxyl sulfate) and GABA, while asparagine, glutamine, tryptophan, and glucose were maintained or decreased. Compared to the crust, dry-aged beef showed similar patterns of biogenic amines and bioactive compounds and GABA without a decrease in free of amino acids and glucose. Based on these results, the crust allows the inner dry-aged beef to be aged similarly to wet-aged beef. Moreover, the metabolic changes of the crust could affect the metabolites of dry-aged beef. However, understanding how external conditions affect dry-aged beef is necessary to produce high value-added beef products.

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Chapter V.

Prediction of freshness of chicken meat and classification of fresh and frozen/thawed meat by 2D qNMR analysis

5.1. Introduction

With increasing incomes, meat and meat-based product consumption has been increasing worldwide because of its superior taste and nutritional value (Godfray et al., 2018). Chicken is a popular source of meat among health-conscious consumers because of its low fat and cholesterol content (Jayasena et al., 2013). Chicken meat has a short shelf life because it is easily spoiled by microorganisms, which result in the deterioration of meat quality through the production of odorous end products such as volatile basic nitrogen (VBN) and lipid peroxides, which deteriorate the sensory quality of meat (Allen et al., 1997; Kim et al., 2004; Lee et al., 2019). Because of its short shelf life, chicken meat is often stored frozen. However, frozen/thawed (FT) chicken has diminished quality, resulting in a lower price than fresh chicken (Jung et al., 2011). Sometimes, FT chicken meat can be illegally mixed with fresh chicken and distributed when the product is in high demand. FT meat is more susceptible to microbial spoilage owing to leakage of water content that contains nutrients for microorganisms (Kim et al., 2013). Microbial contamination level is recognized as an important criterion for evaluating meat freshness and safety (Rukchon et

al., 2014). When microbial contamination occurs, metabolites are consumed differently, depending on the characteristic metabolisms of the microorganisms, resulting in different metabolomes that can be distinguished (Kortstee, 1970; Mason, 1984; Kim et al., 2020a).

Compared with other chemometric analyses, nuclear magnetic resonance (NMR)-based analysis has the advantages of short run times, simple preparation requirements, and good reproducibility (Kim et al., 2019a). Moreover, NMR spectroscopy requires minimal sample preparation (Smolinska et al., 2012). NMR-based analysis is one of the leading types of analysis, along with mass spectroscopy. However, NMR analysis is inherently limited because it involves the measurement of a mixture without an analyte separation process, and thus, the technique has a relatively low sensitivity compared with that of mass spectrometry (Simmler et al., 2014). To overcome this limitation, high-magnetic-field NMR or two-dimensional quantitative NMR (2D qNMR) analysis can be used (Marchand et al., 2017). 2D qNMR-based metabolomics is an advanced method to elucidate the metabolic differences by comparing 2D qNMR results with those of one-dimensional ^{1}H NMR (1D ^{1}H NMR) via expansion dimension, which allows the tracing of higher metabolites simultaneously without erroneous qualification (Kim et al., 2021a). Furthermore, abundant metabolic information can be used to precisely identify key metabolites and differentiate between samples using discriminant analysis (Kim et al., 2021b).

The objective of this study was to identify key metabolites that reflect microbial spoilage during storage and to distinguish fresh meat from FT

chicken breast meat simultaneously based on metabolic information using 2D qNMR analysis.

5.2. Material and methods

5.2.1. Sample preparation

Five packages (1 kg each) of control (fresh) and frozen skinless chicken breast meat (Harim Co. Ltd., Iksan, South Korea) were purchased. The five FT packs were rapidly frozen at −35 °C immediately after slaughter, with the aim of reaching −20 °C in 40 min, and were subsequently stored at −20 °C. Breast meat (5 packages/group) was transferred to the laboratory using a cooler with ice. Transport from the chicken processing plant to the laboratory took 3 h. For simultaneous analysis with fresh breast meat, frozen breast meat was thawed 24 h before the fresh breast meat samples were collected, and both samples were prepared at the same facility. From the same package (8–9 breasts/package), seven breasts were selected, and these were packed aerobically and individually in low-density polyethylene/nylon bags (oxygen permeability of 4.7 g/m^2 for 24 h at 100% RH/25 °C) and marked with the same number. The breast meat was stored for up to 16 days (0, 1, 4, 7, 10, 13, and 16) at 2 °C. After drip loss and color were evaluated daily in quintuplicate, the chicken breast meat was minced using a mini chopper (CH180, Kenwood Appliances Co., Ltd., Dongguan, China). Total aerobic bacteria (TAB) and pH analyses were performed immediately. Samples for 2-thiobarbituric acid (TBA) reactive substances (TBARS), VBN, and NMR analyses were weighed, vacuum-packed, and stored at −70 °C until further analyses.

5.2.2. TAB counts

TAB counts were performed according to the method described by Yong et

al. (2018). Each sample (3 g) was diluted in 27 mL of sterile saline (0.85%) for 2 min using a stomacher (BagMixer® 400 P, Interscience Ind., St. Nom, France). Appropriate dilutions were prepared in sterile saline and spread on plate count agar (Difco Laboratories, Franklin Lakes, NJ, USA). The agar plates were incubated at 37 °C for 48 h, and the microbial counts were calculated. The results are expressed as log numbers of colony-forming units per gram (log CFU/g).

5.2.3. Drip loss

The breast meat was weighed before packaging. Each day during storage, drips were collected to evaluate drip loss. In case of FT breast meat, thawing loss was evaluated using each whole pack instead of drip loss on the first day. Drip loss was represented as a percentage and calculated as follows:

$$\text{Drip loss (\%)} = \frac{original\ sample\ weight - sample\ weight\ after\ storage}{original\ sample\ weight} \times 100$$

5.2.4. Color

Surface color measurements (CIE L*, a*, and b* values representing lightness, redness, and yellowness, respectively) of chicken breast meat samples (skin side of M. *Pectoralis major*) were obtained using a colorimeter (CR-310, Minolta Co., Ltd., Tokyo, Japan). Calibration was performed using a Minolta calibration plate (Minolta Co., Ltd.). Three readings were obtained to minimize error, and the average was used.

5.2.5. pH

Chicken breast meat (1 g) was homogenized with 9 mL of distilled water using a homogenizer (T25 Ultra, Ika Works, Staufen, Germany). The homogenates were centrifuged (Continent 512R, Hanil Co., Ltd., Gimpo, South Korea) at 2,265 ×g for 10 min and filtered (Whatman No. 4, Whatman PLC, Middlesex, UK). The pH value of each filtrate was measured using a pH meter (Seven2Go S2, Mettler-Toledo International Inc., Schwerzenbach, Switzerland). Prior to measuring the pH, the pH meter was calibrated using standard buffers (pH 4.01, 7.00, and 9.21).

5.2.6. TBARS

Chicken breast meat (5 g) was homogenized with 15 mL of distilled and deionized water (DDW) and 50 μL of butylated hydroxyl toluene (in ethanol) for 30 s at 1,513 ×g. The homogenate (2 mL) was transferred to a 15 mL test tube and mixed with 4 mL of TBA and trichloroacetic acid (20 mM TBA in 15% trichloroacetic acid). The test tubes were heated in a water bath at 90 °C for 30 min, cooled, and centrifuged (Continent 512R, Hanil Co., Ltd.) at 2,265 ×g for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (X-ma 3100, Human Co. Ltd., Gwangju, South Korea). The TBARS value was calculated as mg malondialdehyde/kg sample.

5.2.7. VBN

The VBN content was evaluated using the Conway micro-diffusion

technique with slight modifications (Lee et al., 2012). Chicken breast meat (5 g) was homogenized with 20 mL of DDW for 30 s at 1130 ×*g*. The homogenate was filtered using a filter paper (Whatman No.1, Whatman PLC, Middlesex, UK). Further, 1 mL of filtrate and K_2CO_3 were placed at both end sides separately into the outer space of the Conway tool (Sibata Ltd., Saitama, Japan), and 1 mL of 0.01 N H_3BO_3 and 100 μL of Conway reagent (0.066% methyl red:0.0066% bromocresol green, 1:1) were added to the inner space, and the Conway tool was sealed with grease.

$$\text{VBN mg\% (mg/100 g sample)} = 0.14 \times (a - b) \times 5 \times 100$$

where *a* is the titration volume of 0.01N HCl (mL) in the sample and *b* is the titration volume of 0.01 N HCl (mL) in the blank.

5.2.8. Extraction of chicken breast meat for NMR analysis

Chicken breast meat (5 g) was thawed at 4 °C for 24 h before analysis. Thawed chicken breast meat was homogenized at 1,720 ×*g* for 30 s (T25 basic, Ika Co., Kg, Staufen, Germany) with 20 mL of 0.6 M perchloric acid. The homogenate was centrifuged (Continent 512R, Hanil Co., Ltd., Incheon, South Korea) at 3,086 ×*g* for 15 min at 4 °C. Each supernatant was transferred to a new test tube and neutralized with KOH. The neutralized extracts were centrifuged again under the same conditions. After centrifugation, the supernatant was filtered using a filter paper (Whatman No. 1, Whatman PLC) and lyophilized (Freezer dryer 18, Labco Corp., Kansas City, MO, USA). The

lyophilized extracts were stored at −70 °C until NMR analysis.

5.2.9. NMR analysis

All NMR spectra were recorded in D_2O at 298 K using a Bruker 850 MHz Cryo-NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Baden-Württemberg, Germany). 1D ^{1}H NMR was performed by applying standard zg30 with slight modification (recycle delay of 1 s) default in Topspin 3.6.2 (Bruker Biospin GmbH). The 1D ^{1}H NMR experiment was performed using 64 k data points, a sweep width of 17,007.803 Hz, and 128 scans. According to Kim et al. (2021), two-dimensional NMR analyses [correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC)] were performed to quantify and qualify the metabolites. COSY and TOCSY experiments were performed with 2 k data points in the t_2 domain and 256 increments in t_1, each with 8 and 16 scans, respectively. Spectral widths of 11 ppm were used for TOCSY experiments. HSQC and HMBC experiments were performed with 2 k data points in the t_2 domain and 512 increments in t_1, each with 8 and 32 scans, respectively. The spectral widths were 11 ppm for the f_2 dimension and 180 and 240 ppm for the f_1 dimension. Coupling constant values of 145 and 8 Hz were employed to set the delay durations for short-range and long-range correlations, respectively.

5.2.10. Statistical analysis

The acquired integrated HSQC data for each metabolite was processed using

Analysis of MIXtures software v3.9 (Bruker Biospin GmbH). Multivariate analysis was performed using MetaboAnalyst 4.0 (www.metaboanalyst.ca). Prior to multivariate analysis, integrated data were log-transformed and auto-scaled for all multivariate analyses. Interactive principal component analysis (iPCA) and two-way analysis of variance (ANOVA) were performed to distinguish between fresh and FT meat based on treatment and storage time. Additionally, quantified metabolites, physicochemical properties, and TAB were combined for correlation analysis using pattern hunter analysis. Discriminant analysis was completed using receiver operating characteristic (ROC) curve analysis with linear support vector machine (SVM) classification. The feature metabolites were ranked based on the SVM's built-in ranking method. Statistical analysis for selected metabolites in the figure of discriminant analysis was performed using the procedure of the general linear model for the comparison of quantified metabolites from HSQC. Significant differences were determined using the Student–Newman–Keuls multiple range test with the SAS software (SAS 9.4, SAS Institute, Cary, NC, USA) at a confidence level of $p < 0.05$. All experiments were conducted in quintuplicate.

5.3. Results and discussion

5.3.1. Total aerobic bacteria and physicochemical properties

Microbial and physicochemical properties were measured to evaluate the freshness of chicken breast meat (Table 12). The FT breast meat had significantly higher microbial contamination than that in fresh meat ($p < 0.05$). The high microbial contamination levels of the FT breast meat seemed to result from the leakage of exudates, which occurs during the structural destruction of meat during freezing and thawing. The exudate is composed of free amino acids, sugars, and nucleotides, and it can be a good nutrient source for microorganisms (Leygonie et al., 2012; Castejón et al., 2015). The collapse of muscle cell structures during freezing and thawing can result in cavities and meat exudates, which provide optimal conditions for microbial growth (Table 1) (Ngapo et al., 1999; Leygonie et al., 2012). According to previous studies that used the minimal spoilage criterion TAB>6.0, the shelf life of chicken breast meat was less than 10 and 7 days for fresh and FT breast meat, respectively (Rukchon et al., 2014). In fresh breast meat, only VBN demonstrated a significant increase compared with TAB on day 10, whereas increases in a^* value, drip loss, and TBARS value were not significant when the TAB approached the spoilage threshold ($p < 0.05$). In a previous study, the susceptive difference of VBN was used to monitor the freshness of chicken breast meat at a minimal spoilage level using colorimetric array analysis (Lee et al., 2019). However, in FT breast meat, no correlation was observed between drip loss and TAB levels and physicochemical properties at the initial spoilage level (Table 1). A previous study with similar results reported that no direct correlation existed between

TBARS and quality attributes (Kim et al., 2019b). Microorganisms can decrease lipid oxidation in raw and fermented meat products through their own antioxidant activity (Gao et al., 2014), and unsaturated aldehydes, which are indicators of lipid peroxidation development, are eliminated during fermentation (Czerny & Schieberle, 2002). Additionally, various microorganisms have their own antioxidative activities, such as generating non-enzymatic antioxidants or reactive oxygen species scavengers to protect themselves from oxidative damage (Hur et al., 2014).

Of the CIE values, only the a* value significantly increased in a time-dependent manner. The L* value was not different in either treatment group, except on the initial storage day. There was no significant difference in pH between fresh and FT breast meat on different storage days. Likewise, a previous study (Giraud et al., 1991) showed that pH of approximately 6.0, which is similar to that used in the present study, was suitable for microbial growth. According to Rukchon et al. (2014), the initial spoilage level of skinless chicken breast was 6.0 log CFU/g. Based on the results, only VBN had a strong correlation with TAB count, demonstrating that VBN can be used as a freshness indicator of chicken breast meat in both fresh and FT breast meat.

Table 12. Total aerobic bacteria and physicochemical properties on fresh and frozen/thawed (FT) chicken breast meat during storage

		Storage (day)							SEM[1]
		0	1	4	7	10	13	16	
Total aerobic bacteria (log CFU/g)	Con	3.01^{ey}	3.05^{ey}	3.87^{dy}	5.36^{cy}	6.06^{by}	5.98^{by}	6.42^{ay}	0.110
	FT	3.87^{dx}	4.21^{dx}	5.13^{cx}	6.57^{bx}	7.05^{ax}	7.38^{ax}	7.18^{ax}	0.136
	SEM[2]	0.098	0.108	0.094	0.114	0.209	0.095	0.096	
Drip loss (%)	Con	-	0.74^{by}	0.94^{by}	1.29^{by}	1.85^{by}	2.49^{ab}	3.80^{a}	0.482
	FT	1.00^{b3}	2.99^{ax}	2.51^{ax}	2.84^{ax}	3.14^{ax}	3.08^{a}	3.79^{a}	0.448
	SEM[2]		0.408	0.335	0.148	0.382	0.444	0.829	
L* (Lightness)	Con	53.81^{y}	54.25	53.55	54.15	53.46	54.52	55.28	0.777
	FT	57.18^{x}	55.78	56.28	54.45	55.86	55.91	55.32	1.223
	SEM[2]	0.521	1.559	1.028	1.010	0.763	0.734	1.203	
a* (Redness)	Con	4.44^{by}	5.03^{by}	5.66^{aby}	5.65^{aby}	5.87^{aby}	5.79^{aby}	6.69^{ay}	0.350
	FT	6.30^{bx}	6.87^{abx}	7.25^{abx}	8.64^{ax}	8.53^{ax}	8.07^{ax}	7.75^{abx}	0.421
	SEM[2]	0.426	0.309	0.418	0.414	0.415	0.418	0.281	
b* (Yellowness)	Con	13.35^{y}	14.69^{y}	13.43^{y}	13.31^{y}	13.60^{y}	14.05^{y}	13.51^{y}	0.588
	FT	17.58^{x}	17.81^{x}	17.67^{x}	16.29^{x}	18.23^{x}	16.02^{x}	15.77^{x}	0.758
	SEM[2]	0.725	0.759	0.656	0.667	0.788	0.572	0.545	
pH	Con	6.16^{x}	6.17	6.20^{x}	6.08	6.17	6.16	6.07	0.066
	FT	5.95^{y}	6.18	6.01^{y}	6.14	6.17	6.16	6.16	0.062
	SEM[2]	0.048	0.092	0.567	0.039	0.066	0.056	0.076	
TBARS (mg malondehyde/kg)	Con	0.30^{b}	0.30^{b}	0.28^{b}	0.30^{b}	0.32^{b}	0.34^{ab}	0.38^{a}	0.016
	FT	0.29	0.33	0.31	0.30	0.31	0.36	0.31	0.021
	SEM[2]	0.015	0.017	0.018	0.013	0.013	0.027	0.023	
VBN (mg%)	Con	10.10^{c}	10.55^{c}	10.78^{c}	11.34^{cy}	13.59^{b}	17.02^{a}	17.89^{ay}	0.753
	FT	10.48^{d}	11.06^{d}	11.12^{d}	12.75^{dx}	15.75^{c}	18.77^{b}	22.22^{ax}	0.658
	SEM[2]	0.230	0.343	0.448	0.280	0.801	0.917	1.252	

[1] Standard of the mean (n=35), [2](n=10)

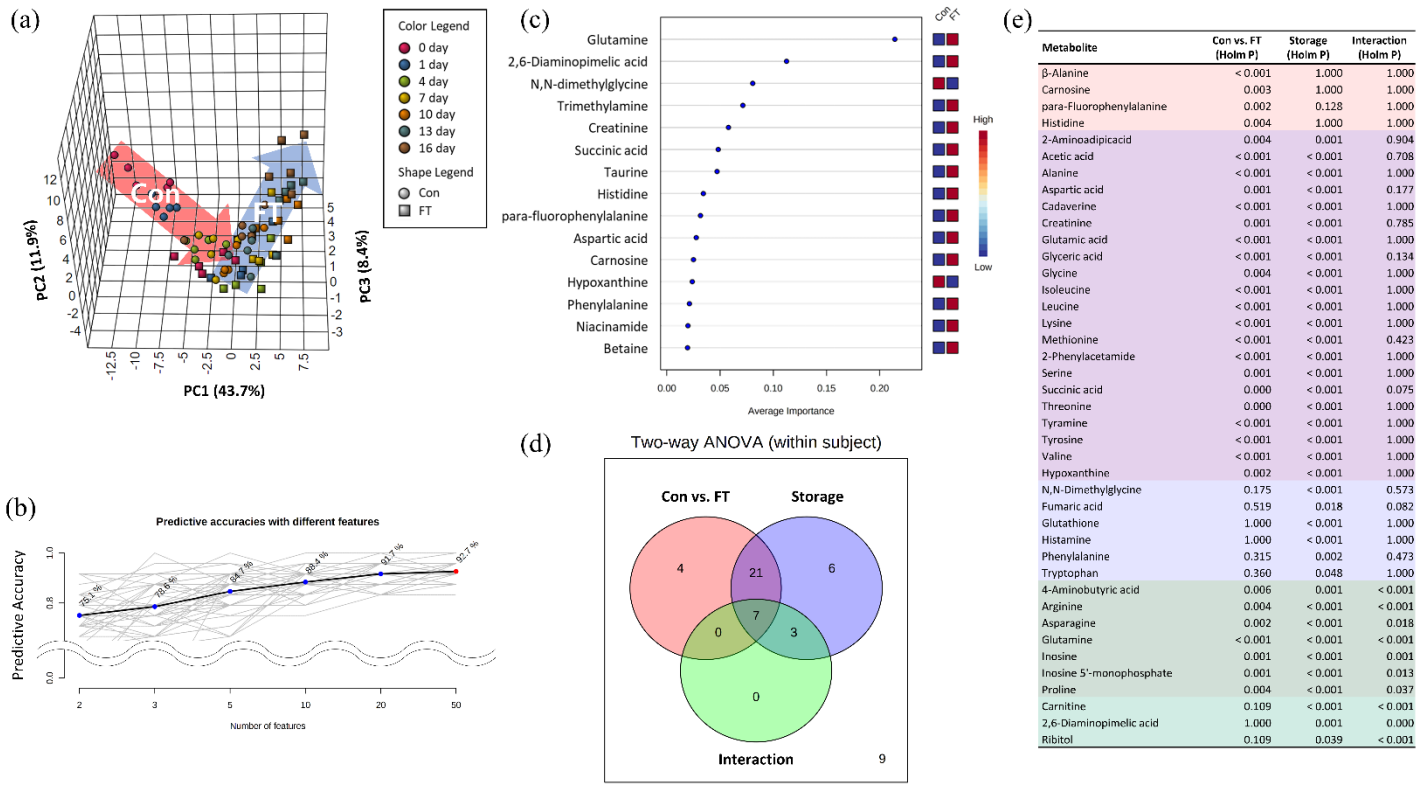
[3] The value is presenting thawing loss (%) at 0th day

5.3.2. Metabolites

For the evaluation of freshness indicators in chicken breast meat, NMR data were acquired and processed by multivariate analysis (Fig. 20). Correlation coefficient analysis was then performed to determine common trends in both fresh and FT breast meat in a time-dependent manner (Fig. 21). The two-way ANOVA results (Fig. 20d and 20e) demonstrated that the quantities of most metabolites were proportional to the storage time. The quantities of organic acid, hypoxanthine, and free amino acids were highly correlated with storage time, whereas those of proline, inosine 5'-monophosphate, and N,N-dimethylglycine (DMG) demonstrated highly negative correlations. These results indicate that metabolic changes can be used as evidence of microbial growth. Succinate and acetate were representative organic acids produced by microorganisms during microbial fermentation (Sauer et al., 2008). Increases in acetic acid and succinic acid can result from glucose fermentation by microorganisms, and this change can be observed in muscle-based foods during storage (Chiou et al., 1998; Wierda et al., 2006; Sauer et al., 2008). According to Bórquez et al. (1994), acetic acid can suppress microbial counts, effectively preserving various organic acids, but the microorganisms steadily grow in a time-dependent manner, even in 5 mg/kg acetic acid.

An increase in hypoxanthine with a decrease in inosine-5'-monophosphate during storage is a typical change that allows prediction of the freshness of chicken meat using *K*-value (%) (Jung et al., 2011). In addition to changes in nucleotides, free amino acids (aspartic acid, valine, isoleucine, lysine, tyrosine, leucine, glutamic acid, methionine, alanine, glycine, and threonine), and

biogenic amines (tyramine and cadaverine), there can be evidence of proteolysis by both meat enzymes and microbial activity during storage (Ngapo et al., 1999; Kim et al., 2020a). Increases in VBN and biogenic amines are normally accompanied by food spoilage and are treated as indicators of freshness in meat (Fraqueza et al., 2008; Lee et al., 2019; Kim et al., 2020a). In addition, DMG and proline can be consumed by microorganisms as carbon or nitrogen sources, and a decrease in DMG and proline is evidence of microbial growth (Kortstee, 1970; Mason, 1984; Moses et al., 2012). As indicated above and in agreement with previous studies, DMG and proline levels decreased during storage ($p < 0.05$). VBN levels were more strongly correlated with metabolites (especially acetic acid, hypoxanthine, lysine, and succinic acid) than that with TAB. This may be a result of stagnating microbial growth in the later storage stage (after minimal spoilage TAB level); TAB did not correlate with VBN and acetic acid at different storage times. Considering the physicochemical properties of chicken breast meat, acetic acid had a strong correlation with VBN (Pearson $r = 0.97$), and could act as an appropriate freshness indicator.



(e)

Metabolite	Con vs. FT (Holm P)	Storage (Holm P)	Interaction (Holm P)
β-Alanine	< 0.001	1.000	1.000
Carnosine	0.003	1.000	1.000
para-Fluorophenylalanine	0.002	0.128	1.000
Histidine	0.004	1.000	1.000
2-Aminoadipicacid	0.004	0.001	0.904
Acetic acid	< 0.001	< 0.001	0.708
Alanine	< 0.001	< 0.001	1.000
Aspartic acid	0.001	< 0.001	0.177
Cadaverine	< 0.001	< 0.001	1.000
Creatinine	0.001	< 0.001	0.785
Glutamic acid	< 0.001	< 0.001	1.000
Glyceric acid	< 0.001	< 0.001	0.134
Glycine	0.004	< 0.001	1.000
Isoleucine	< 0.001	< 0.001	1.000
Leucine	< 0.001	< 0.001	1.000
Lysine	< 0.001	< 0.001	1.000
Methionine	< 0.001	< 0.001	0.423
2-Phenylacetamide	< 0.001	< 0.001	1.000
Serine	0.001	< 0.001	1.000
Succinic acid	0.000	< 0.001	0.075
Threonine	0.000	< 0.001	1.000
Tyramine	< 0.001	< 0.001	1.000
Tyrosine	< 0.001	< 0.001	1.000
Valine	< 0.001	< 0.001	1.000
Hypoxanthine	0.002	< 0.001	1.000
N,N-Dimethylglycine	0.175	< 0.001	0.573
Fumaric acid	0.519	0.018	0.082
Glutathione	1.000	< 0.001	1.000
Histamine	1.000	< 0.001	1.000
Phenylalanine	0.315	0.002	0.473
Tryptophan	0.360	0.048	1.000
4-Aminobutyric acid	0.006	0.001	< 0.001
Arginine	0.004	< 0.001	< 0.001
Asparagine	0.002	< 0.001	0.018
Glutamine	< 0.001	< 0.001	< 0.001
Inosine	0.001	< 0.001	0.001
Inosine 5'-monophosphate	0.001	< 0.001	0.013
Proline	0.004	< 0.001	0.037
Carnitine	0.109	< 0.001	< 0.001
2,6-Diaminopimelic acid	1.000	0.001	0.000
Ribitol	0.109	0.039	< 0.001

Figure 20. Interactive principal component analysis (iPCA) (a), predictive accuracies with different features (b), average importance with 50 features prediction model (c), Venn diagram of two-way ANOVA (d), and list of metabolites which have significance (e).

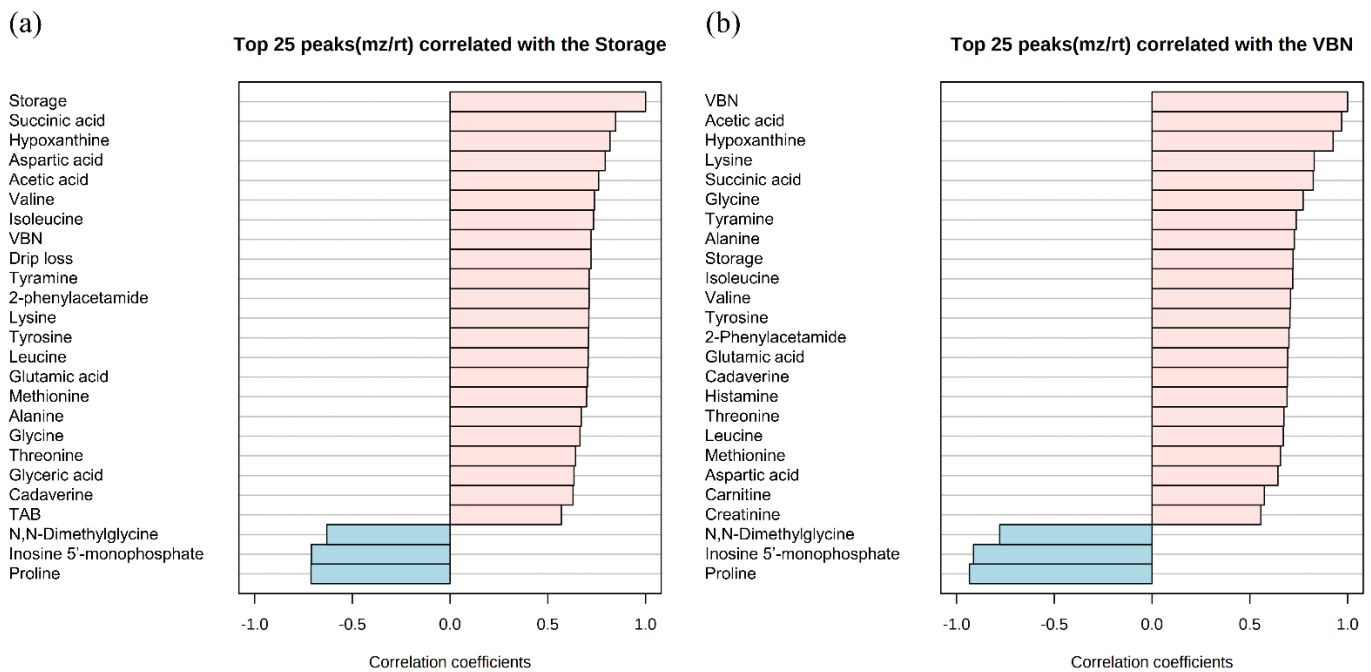


Figure 21. Top 25 metabolites correlated by storage day (a) and volatile basic nitrogen (VBN) (mg%) (b).

5.3.3. Fresh and FT breast meat

5.3.3.1. Overall metabolic trends

To monitor metabolic changes, iPCA, ROC analysis, and two-way ANOVA were performed (Fig. 20). iPCA results revealed a strong correlation between PC1 (43.7%) and storage time. In addition to PC1, the use of the PC1–PC2 (11.9%) plane explained the difference better by treatment (fresh and FT breast meat) and storage time than the use of PC1 only (Fig. 20a). Fresh breast meat was clearly distinguished from the other clusters until day 1. From day 4, the clusters of fresh breast meat moved in a similar direction to FT breast meat over time but did not overlap. The clusters of FT breast meat moved along PC1 and PC2 over time. In PC3 (8.4%), the fresh breast meat cluster had a negative correlation, whereas the FT breast meat cluster was positively correlated over time. PC3 supports the explanation of overall variation, but it cannot be used independently. Prior to selecting metabolic indicators for distinguishing both groups, the whole dataset (50 metabolites) was used for prediction by groups for screening (Fig 20b). The highest predictive accuracy was the 92.7% for the model made with 50 features. Among them, a total of 41 metabolites that were significantly different were placed on the Venn diagram ($p < 0.05$) (Fig. 20d and 20e). For the selection of indicators, we used a two-way ANOVA. In the Venn diagram, four metabolites were significant only in fresh vs. FT breast meat, and interactions were chosen as candidates for ROC curve analysis because the top 15 metabolites with average importance among the 50 features were not directly related to the results of the two-way ANOVA.

5.3.3.2. Overall metabolic trends

Among the various combinations of candidates, 11 metabolites were selected based on their prediction score, and ROC analysis was performed (Fig. 22). The ROC curves consisting of the selected metabolites had higher predictive accuracy than that of the original model using the whole metabolome, and the accuracy was highest when 10 features were used. The metabolites are listed in order of mean importance from most to least important in Fig. 22d. Quantifying the metabolites is important for evaluating meat quality such as flavor-active and bioactive compounds (Lee et al., 2015). Furthermore, this information can also be used for distinguishing between groups using a machine-learning algorithm (Kim et al., 2021b). The quantity of glutamine rapidly decreased after day 10 in fresh breast meat (0.48-fold) but remained constant throughout the storage period in FT breast meat ($p < 0.05$). In contrast, asparagine gradually increased in fresh breast meat but decreased after day 13 in FT breast meat. Asparagine demonstrated a similar trend in both groups, but tended to be higher in FT breast meat during initial and terminal storage than in fresh breast meat. Proline content decreased gradually in both groups but tended to be higher in fresh breast meat, at 1.28-fold change on day 0 followed by a 3.81-fold change by day 16, than in FT breast meat ($p < 0.05$). Glutamine, arginine, asparagine, and proline can be utilized by microorganisms, and the utilization of free amino acids varies depending on microbial species (Miller & Waelsch, 1952; Abdelal, 1979; Mason, 1984; Vermeulen et al., 2007). However, some free amino acids demonstrated different behavior in fresh meat compared with that in FT meat. It can be inferred that freezing and thawing could change the microbial

composition through the competitive exclusion of microorganisms, resulting in the survival of cold-resistant species and altering the metabolite consumption trend (Mohammed et al., 2021).

Previous studies reported that 2,6-diaminopimelic acid (DAPA) and γ-aminobutyric acid (GABA) are direct evidence of microbial proliferation (Mason, 1984; Kumar & Punekar, 1997). DAPA is a component of cell wall peptidoglycan in various bacteria and is a precursor of lysine (Le Roux et al., 1991). The presence of DAPA can be used to evaluate microbial contamination and the existence of lactobacilli (Schillinger & Lücke, 1987; Wesselinova, 2000). Based on these results, FT breast meat is more vulnerable to microbial spoilage than fresh breast meat because of the exudates during thawing. They contain suitable nutrients for microbial growth, resulting in a high TAB and influencing metabolic changes (an increase in DAPA and GABA and a decrease in proline) in meat. Inosine 5'-monophosphate was gradually decreased and could be used to clearly distinguish between fresh and FT breast meat, whereas inosine could be used to distinguish between fresh and FT breast meat only before day 10. In addition to being freshness indicators, higher carnosine and inosine 5'-monophosphate content can enhance the umami flavor of chicken breast meat (Jayasena et al., 2015). Carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-3-methyl-L-histidine) are histidine dipeptides composed of β-alanine and histidine, which have various bioactivities such as antioxidant activity, a buffering effect, and the potential for metal ion chelation and free radical scavenging. They can also act against various diseases (Kim et al., 2020a).

In the present study, carnosine in FT breast meat appeared to decompose into β-alanine and histidine during freezing and thawing. A similar trend was observed in a previous study, in which the authors suggested that the decrease in carnosine might be the result of degradation by oxidative stress during freezing and thawing (Soglia et al., 2019). However, carnosine is typically transformed by various oxidants (free radicals and lipid peroxidation byproducts) and degraded to various derivatives, instead of being degraded to β-alanine and histidine (Guiotto et al., 2005). The decrease in carnosine content during freezing and thawing has rarely been reported in chicken meat. The concentrations of carnosine, histidine, and β-alanine were relatively consistent throughout the storage period. Contrasting results have also been reported for increasing or consistent trends in carnosine levels during storage, and this difference might result from carnosinase activity levels (Kim et al., 2020b). Based on these results, proteolysis and microbial changes were not relevant to changes in carnosine levels. To understand the decrease in carnosine levels during freezing and thawing, further analysis is needed.

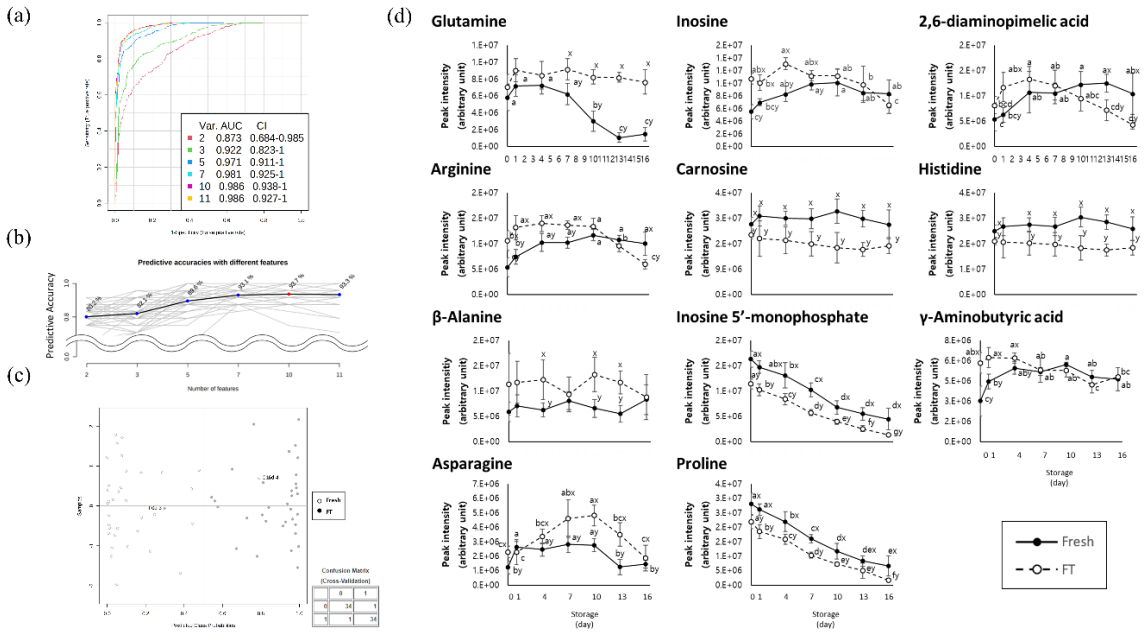


Figure 22. Multivariate area under the curve (AUC) analysis (a), predictive accuracies with different features (b), predicted class probabilities (c), and changes over storage time (d) made with 11 selected metabolites from two-way analysis of variance (ANOVA). [a-f] Different letters in the same group indicate a significant difference ($p < 0.05$). [x-y] Different letters in the same storage day indicate a significant difference ($p < 0.05$).

5.4. Conclusion

A summarized schematic illustration of the present study is represented in Fig. 23. Among all physicochemical properties, only VBN could predict the spoilage of chicken breast meat at the initial TAB level. Generally, proportional increases in organic acids, free amino acids, biogenic amines, and hypoxanthine were observed, while proline and DMG decreased proportionally during storage. Acetic acid was highly correlated with VBN, which could be a simple indicator of freshness in chicken breast meat without VBN analysis for both fresh and FT breast meat. Fresh and FT breast meat can be differentiated by uniform concentration of carnosine, β-alanine, and histidine levels, consistent changes in nucleotides by storage time, and changes in microbial metabolism patterns that are reflected by some free amino acids. Based on these results, 2D NMR-based metabolomics could be useful for evaluating the freshness of chicken breast meat. In addition, it can be used to differentiate fresh from FT breast meat based on different metabolic patterns.

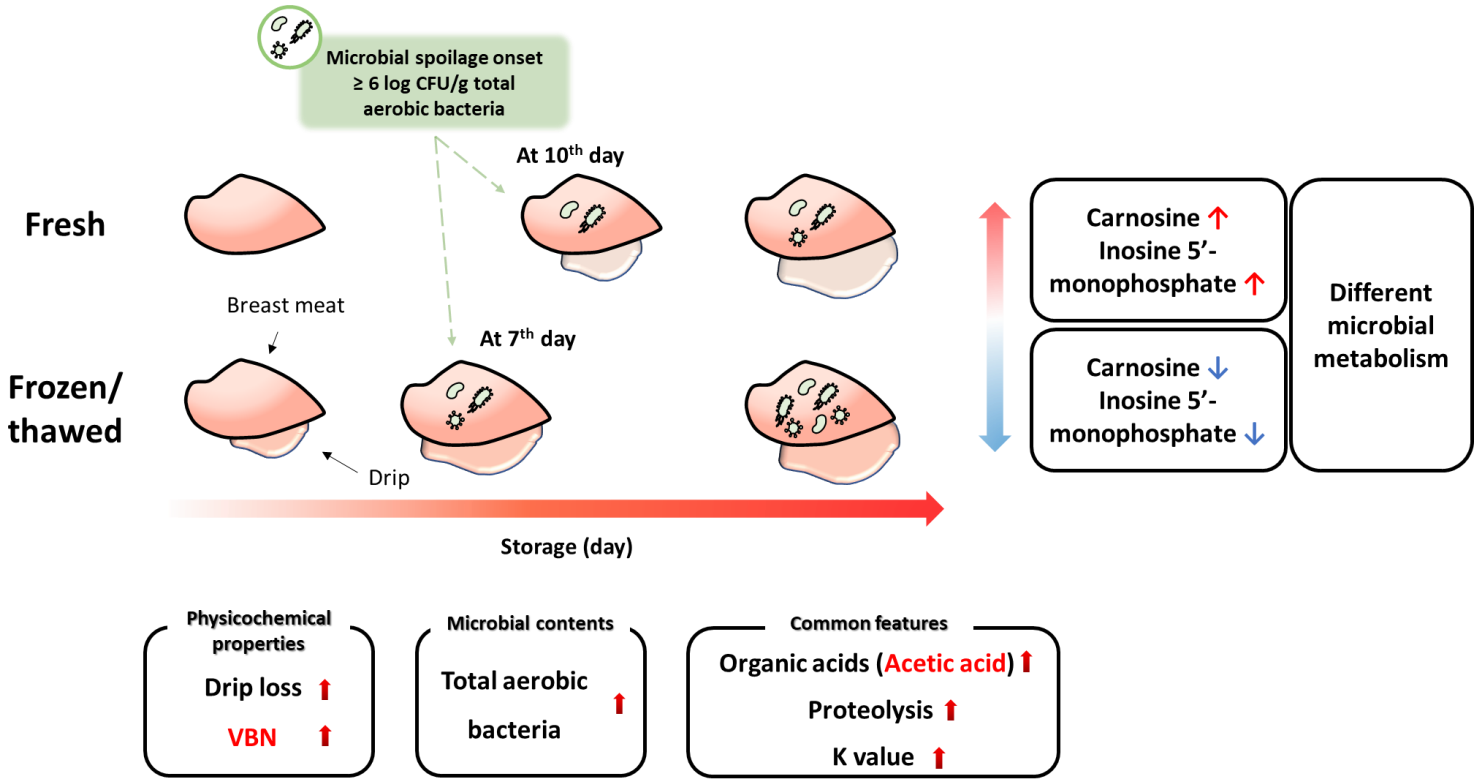


Figure 23. Schematic representation of overall physicochemical and metabolic changes in fresh and frozen/thawed chicken breast meat. Red colored letters represent the most suitable candidates for identifying chicken freshness based on the microbial spoilage level.

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Chapter VI.

Overall conclusion

1D and 2D qNMR analyses for meat were comprehensively optimized from sample preparation to NMR parameters and verified with conventional HPLC. The qNMR-based metabolomics was efficient for elucidation of meat metabolic changes in different chicken breeds, beef aging method, and storage conditions and day by tracing various known and unknown metabolites at once, affording significant results with multivariate analysis. Based on these results, it is believed that qNMR-based analysis will establish itself as an essential analysis method that can unravel the clues of various metabolic changes that we have not been aware in meat science.

Overall summary in Korean

최근 정량분석기기의 발달로 인해 계량화학적 접근방법이 점차 증가하고 있다. 그 중 핵자기공명분광학(NMR spectroscopy)을 기반으로 한 분석방법은 복잡했던 방식을 값싸고 빠르게 처리할 수 있으며, 더욱이 이해가 부족한 현상이나 상황에 빠르게 포착하고, 특정 표적물질에 관계없이 측정하여 정보를 얻을 수 있다. 하지만 식육과학에서 핵자기공명이 이용된 역사는 상대적으로 짧으며, 정량성을 확보하기 위해서는 핵자기공명분광학의 분광학적 특성 중 정량분석에 중요한 완화시간(relaxation time)과 추출물의 pH, 염농도(salt concentration)와 같은 화학적 특성들이 충분히 고려되어야 한다. 따라서, 식육샘플을 이용한 정확한 정량분석을 위해 1) 식육의 극성대사체 추출 방법 및 1차원 수소(^{1}H) 핵자기공명의 정량방법을 확립한 후, 2) 2차원 ^{1}H-^{13}C 이핵 단일 간섭성(HSQC)을 이용한 정성 및 정량 가능성을 확인하고, 3) 이를 바탕으로 우육의 숙성에 따른 변화 및 숙성방법에 따른 차이를 측정할 수 있는지와, 4) 계육 닭가슴살의 신선도와 냉해동되어 유통되는 닭가슴의 차이를 확인할 수 있는지 검증하였다.

실험 I에서는 계육을 시료로 하여 3가지의 추출용매(0.6 M perchloric acid, 20 mM phosphate buffer, methanol/water 1:1)와 3가지의 20 mM의 서로 다른 버퍼를 기반으로 한 재생용매(MOPS, HEPES, phosphate)를 이용하여 핵자기공명분광기에 최적의 조합을 찾고, 조합결과를 기반으로 정량성을 최적화하였다. 총 9가지 조합에서, 과염소산(perchloric acid)을 이용한 추출이 재생용매에 관계없이 베이스

라인이 바르게 나타났으며, 때문에 스펙트럼에 필요 없는 피크를 발생시킬 수 있는 유기용매인 MOPS와 HEPES 대신 인산(phosphate)을 이용한 버퍼 용매 조합이 식육의 극성대사체를 분석하는데 적당하다고 판단되었다. 이를 바탕으로 핵자기공명분광 측정방법을 이용해 용매의 제거 차이를 바탕으로 용매를 제거하지 않는 zg30와 용매를 제거하는 noesypr1d 방법을 이용하여 표준물질을 이용하여 스핀-격자이완(T_1 relaxation) 및 정량성을 확인하고 계육을 이용하여 두 방법 및 액체크로마토그래피(HPLC)와 비교한 결과, 전체적으로 정량성이 동일하게 나타났으나, zg30이 측정시간이 짧으면서도 재현성과 민감도가 좋고, 상대표준편차도 작게 나타났다. 따라서, zg30을 이용한 방법으로 최적화하였으며 돈육 및 우육에도 적용하여 핵자기공명 분광 측정법을 계육 뿐만 아니라 다른 품종에도 적용할 수 있음을 확인하였다.

실험 II에서는 닭가슴살을 추출하여 HSQC를 바탕으로 이차원 핵자기공명 분석 방법의 가능성을 확인하고, 이를 바탕으로 다변량분석을 통해 시료의 차이를 분석하였다. 분석에는 상용 토종닭을 비롯한 신품종 토종닭 3품종 (A, C, D)와 육계(cobb 500f)가 사용되었다. 우선 정량분석의 유효성을 확인하기 위해 일반 육계를 시장에서 구매하여 추출한 뒤 1차원 핵자기공명분광학(1D qNMR), 2차원 핵자기공명분광학(2D qNMR), 그리고 HPLC를 사용하여 유리아미노산을 정량분석하였다. 실험결과, 2D qNMR은 1D qNMR에 비해 훨씬 많은 대사체를 분리하였으나, 부분적으로 정량값이 일치하지 않았다. 이는 표준물질(standard mixture)과의 화학적인 환경이 다른 것으로 판단되나, 각각의 농도에 따른 선형성은 잘 나타나 수적인 정량에는 1D

qNMR과의 혼용이 추천되나, 일반적인 분석은 바로 가능함을 알 수 있었다. 이를 바탕으로 계육에 존재하는 다양한 물질들을 정량한 뒤, 다변량분석을 이용하여 비교하였다. 토종닭 중에서는 신품종 D가 다른 품종에 비해 높은 유리아미노산, 당, 생리활성물질을 나타내었다. 또한 눈에 띄는 차이가 토종닭과 육계에서 나타났는데, 토종닭은 높은 수준의 이노신 일인산(inosine 5’−monophosphate), α−포도당, 안세린, 젖산을 가지나 육계에 비해 유리아미노산과 관련물질들이 낮게 나타났다. 이를 통해 2D qNMR과 다변량분석을 이용하여 품종구별에 사용할 수 있음을 확인하였다.

실험 III에서는 우육 등심(*longissimus lumborum*)을 4주간 건식과 습식숙성하면서 숙성 방법 간 변화와 건식숙성육의 크러스트(crust)에 대한 대사적 변화를 분석하였다. 샘플은 0.6 M의 과염소산을 통해 추출하였고 2D qNMR 분석을 진행하였다. 부분 최소 제곱법(PLS)를 기반으로 하였을 때, 분산(R^2 = 0.967)과 예측능력(Q^2 = 0.935)이 우수하게 나타났다. 크러스트의 경우 가식부위인 건식숙성육, 습식숙성육과 주성분분석에서 숙성 1주부터 다른 양상을 보임을 확인하였으며 2주째에 완전히 분리됨을 확인하였다. 더욱이 핵자기공명분광학을 기반으로 한 다변량분석은 숙성의 방법과 정도에 따른 차이를 잘 나타내는것으로 나타났다. 그 중, 크러스트는 독특한 대사적 변화로 빠른 단백질 분해(proteolysis)와 이노신 일인산(inosine 5’-monophosphate)의 감소가 나타나면서 독특한 미생물 대사체로 인독실 황산염(3-indoxyl sulfate)와 감마 아미노뷰티르산(γ−aminobutyric acid)의 생성이 확인되었고, 아스파라긴, 글루타민, 트립토판, 당의 농도가 유지되거나 감소되는 양상을 보였다. 건식육은 크러스트와 비교해 바이오제닉아민과 생리활성물질, 감마 아미노뷰

티르산의 양상이 비슷하면서도 아미노산이나 당의 감소가 관찰되지 않았다. 이러한 결과를 바탕으로 크러스트는 미생물의 침입을 막아 습식숙성육과 비슷하게 숙성이 되게 하면서도 크러스트 표면에서 일어나는 미생물의 독특한 변화를 통해 건식숙성육 특유의 성질을 나타나게 하는것으로 보인다. 본 실험을 통해 식육의 숙성 중 생화학적 변화를 핵자기공명 분광학으로 쉽게 확인할 수 있음을 알 수 있었다.

실험 IV에서는 닭가슴살(M. *Pectoralis major*)의 저장일차에 따른 대사적 변화와 신선육과 냉/해동육 닭가슴살의 대사적 차이 및 미생물과 물리화학적 변화를 관찰하고 핵자기공명분광학을 통해 신선도와 냉해동육의 분별이 가능한지 확인하였다. 닭가슴살은 함기포장하여 2˚C에 16일간 저장하였고, 이를 분석하였다. 총 호기성 미생물(total aerobic bacteria, TAB) 6 log CFU/g를 닭가슴살 신선도 기준으로 하였을 때 TAB의 기준과 함께 유의적으로 변화하는 것은 휘발성 염기태 질소(volatile basic nitrogen, VBN)가 유일했다. 드립의 양이나 색도, 지질산화정도 또한 시간에 따라 증가는 하나, 총 호기성 미생물의 수치에 따른 민감한 변화를 나타내지 못하였다. 저장기간동안 신선육과 냉/해동육에 관계없이 유기산, 유리아미노산, 생체아민, 그리고 하이포잔틴이 증가하면서 다이메틸글리신과 이노신 일인산, 프롤린은 감소하는 공통적인 경향을 보였다. VBN을 기반으로 상관관계를 보았을 때, 가장 상관관계가 큰 것은 아세트산으로 나타났으며 이는 계육의 드립에서도 같은 양상을 보였다. 50개의 대사체 중, 이원분산분석을 통해 Con과 FT의 차이를 보았는데, 결과를 통해 11개의 대사체를 선정하였고, 이를 기반으로 Con과 FT가 잘 구별됨을 확인하였다. 이러한 차이는 글루타민과, 디아미노피멜산, 아르기닌, 감마 아

미노뷰티르산에서 다른 양상을 보이고 프롤린이 감소하는 양상을 보였다. 또한 특징적인 차이로 FT에서 카르노신의 유의적인 감소가 나타났고 이와 같이 베타알라닌과 히스티딘의 유의적인 차이가 동시에 관찰되었다. 이는 두 그룹 모두 일자에 따른 농도 변화를 나타내지 않아 냉/해동의 결과로만 카르노신의 변화가 일어나는것으로 보인다. 이러한 결과를 바탕으로, 핵자기공명분광법을 이용하여 계육의 신선도를 예측할 수 있고 Con과 FT처럼 다른 상태의 샘플을 구별할 수 있음을 확인하였다.

이상의 연구 결과 핵자기공명분광분석법을 이용하여 식육의 대사물질을 정성 및 정량 분석할 수 있는 최적의 방법을 구축하였다. 이를 기반으로 닭의 품종간 대사체적 차이와 구별, 우육의 숙성 방법 및 부위에 따른 대사체적 차이와 구별, 그리고 저장기간과 저장방법(신선 및 냉동 후 해동)에 따른 대사물질의 변화와 구별에 대해 구명하고 설명할 수 있었다. 이러한 결과를 바탕으로 핵자기공명분광학은 식육을 이용한 연구에서 우리가 지금까지 인지하지 못했던 다양한 생화학적 변화에 대한 실마리를 풀어줄 수 있는 유용한 분석방법으로 사료된다.

감사의 글

어느덧 석·박사 통합과정의 6년이라는 긴 여정을 마무리하게 되었습니다. 연구를 진행하며 많은 어려움이 있었지만, 감사하게도 참 많은 곳에서 도움을 받았고 그 덕분에 만족스러운 결과를 낼 수 있었다고 생각합니다.

먼저 긴 학위과정 간 진심 어린 조언과 지도를 해 주신 저의 지도교수 조철훈 교수님께 감사의 인사를 올립니다. 학부과정때부터 현재까지 제 지도교수님으로 오랜 기간 저를 지켜봐 주시면서 많은 도움과 격려를 아끼지 않으셨고, 또한 저의 잘못을 부단히 바로잡아 주시면서 연구자뿐 아니라 어떻게 인생을 살아가야 하는지 알려주시는 삶의 나침반이 되어 주셨습니다. 업무 와중에도 성심껏 제 논문을 심사해주시고 많은 의견을 주신 김유용 교수님, 남기창 교수님, 장애라 교수님, 김갑돈 교수님께도 깊은 감사의 인사를 올립니다. 교수님들의 세심한 지도를 통해 본 학위논문이 완성될 수 있었습니다. 학위과정 동안 많은 도움을 주신 동물성식품학실의 임동균 박사님, 최주희 박사님, 조은영 선생님, 최은지 선생님을 비롯하여 현정 선배, 해인 선배, 해림, 기호 형, 동진이 형, 다겸, 정연, 소연, 정민, 범진, 상희, 윤지원, 민수, 선진, 지현, 김지원, 예슬, 동헌, 태민, 정아, 민경, 예은, 현준, 성수, 현영, 두연, 조현, 학주에게 깊이 감사합니다. 같이 근무하지는 못했지만 실험실에 자주 들려주시면서 다방면으로 마음 써 주셨던 김현주 박사님께도 감사의 인사를 올립니다. 골든시드프로젝트(GSP)과제를 진행하며 관련해 많은 도움을 주신 강희설 단장님, 김재환 국장님, 안광숙 선생님, 김예송 선생님, 참여

기업 ㈜하림의 방지윤 팀장님, 최병헌 부장님, 김광윤 팀장님, 소성오 농장장님, ㈜한협의 오기석 대표님, 용귀중 부장님, 순천대학교의 지영씨, 문정훈 교수님, 서영씨에게 과제 진행간 많은 도움을 주셔서 감사합니다. 학부생 시절에 화학과 복수전공동안 어떻게 화학을 배워야 하는지에 대해 알려주신 박윤봉 교수님, 학사학위논문을 작성동안 많은 도움을 주신 진동일 교수님, 짧은 실험실 생활에도 많은 가르침을 주셨던 정사무엘 교수님과 생활하는데 많은 도움을 준 육가공학 실험실의 철우, 주리에게도 감사합니다. 또한 지금의 저를 있게 해 주신 많은 교수님들의 격려와 가르침에 감사드립니다.

마지막으로 제가 힘들 때마다 아낌없이 응원해주고 지지해준 가족과 친구들에게 진심을 담아 감사의 인사를 드립니다. 끝없는 지지와 헌신적으로 사랑해주시는 부모님과 누나들에게 감사합니다. 고등학생때부터 늘 응원해주는 석원, 종모, 구용, 진우와 대학 동기인 경민이형, 일식이형, 구영, 인선, 지수, 봉준, 성민, 상열, 남영, 영민, 일은, 혜원, 가희, 여정이와 충남대 다이얼로그 동아리 선후배들, 대학원 다니면서 항상 응원과 격려를 아끼지 않은 동물생명공학과의 광환이형, 동경이형, 혁중이형, 승주, 승훈, 다진솔, 상엽이, 키 큰 민진씨, 바이오모듈레이션학과 소영 모두와 이 영광을 나누고 싶습니다. 앞으로도 부끄럽지 않은 연구자가 되도록 항상 정진하겠습니다. 감사합니다.

2021년 8월

김 현 철 드림