Food Chemistry 342 (2021) 128316

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

Hyun Cheol Kim[a], Yoon-Joo Ko[b], Cheorun Jo[a,c,*]

[a] Department of Agricultural Biotechnology, Center for Food and Bioconvergence, and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Republic of Korea
[b] National Center for Inter-University Research Facilities, Seoul National University, Seoul 08826, Republic of Korea
[c] Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang 25354, Republic of Korea

ARTICLE INFO

Keywords:
Chicken breed
2D qNMR
Breast meat extracts
Metabolomics
Multivariate analysis

ABSTRACT

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 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.

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, Napolitano, McAlpine, Chen, & Pauli, 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, Ge, Zhou, Zhang, & Liao, 2019) and to distinguish fresh, abnormal qualities (white striping, wooden breast, and spaghetti meat), and frozen/thawed chicken breast (Soglia, Silva, Lião, Laghi, & Petracci, 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, Martineau, Guitton, Dervilly-Pinel, & Giraudeau, 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, Mappley, La Ragione, Woodward, & Claus, 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

* Corresponding author at: Department of Agricultural Biotechnology, Center for Food and Bioconvergence, and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Republic of Korea.
E-mail address: cheorun@snu.ac.kr (C. Jo).

https://doi.org/10.1016/j.foodchem.2020.128316
Received 25 February 2020; Received in revised form 4 September 2020; Accepted 4 October 2020
Available online 14 October 2020
0308-8146/

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 (Jayasena, Jung, Kim, Kim, et al., 2015; Jung et al., 2013). However, the slow growth rate of KNC has been a crucial limitation for commercialization (Jin, Jayasena, Jo, & Lee, 2017; Kim, Choe, Nam, Jung, & Jo, 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.

2. Materials and methods

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).

2.2. Animal preparation

Four different KNCs [newly developed KNC-A, -C, and -D, and commercial KNC–H (Hanhyup No. 3)] 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. All the rearing process was kept by the protocol of commercial chicken production (Harim Co., Ltd., Iksan, Korea). 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.

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.

2.4. Standard mixture preparation for heteronuclear single quantum coherence (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.

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 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.

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 2 k 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.

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).

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 metabolites of HMDB ID were used for pathway analysis. Before the analysis, samples were log-transformed, and autoscaled. Algorithms for pathway enrichment analysis (global test) and pathway topology analysis (relative betweenness centrality) were used with a chicken (*Gallus gallus*; KEGG) library.

2.9. 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 multiple range 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 triplicates.

3. Results and discussion

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. S1 of Supplementary materials) is linear over the range of 1–5 mM for all free amino acids and 0.2–1 mM for tyrosine (Fig. S2 of Supplementary materials, $R^2 = 0.97$ in proline and $R^2 > 0.99$ in others).

Based on the standard curves, we quantified free amino acids present in chicken breast meat extracts using the HSQC spectrum (Table 1).

Table 1
Metabolite 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	×[1]	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)[2]	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	×	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	×	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	×	193.15 ± 11.37	197.97 ± 5.54
Tryptophan	×	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 ($p < 0.05$).
[1] '×' indicates not found in the 1D ^{1}H spectrum.
[2]1D ^{1}H NMR data of both glutamate and proline were excluded from the calculation.

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 (Markley et al., 2017; Simmler et al., 2014). 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, Kilpeläinen, & Heikkinen, 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. S3 of Supplementary materials). 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, Kamal, & Hashimoto, 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, Warren, & Le Gresley, 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. In spite of this drawback, however, the good linearity of 2D qNMR provide the proportional comparison such as fold changes regardless of metabolites, which make elucidate metabolic differences and/or changes possible. From the present study, both 1D ^{1}H and 2D qNMR methods should be complemented and analyzed together for accurate numerical quantification of metabolites.

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 (Guennec, Giraudeau, & Caldarelli, 2014; Van et al., 2007). 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 2. 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).

Table 2
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	NA A			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)
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)
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*-dimethylglycine	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)
42	Creatine/Phosphocreatine	Cr/PCr	N-CH_3	3.04	*s*	TOCSY (3.94) HSQC (39.8) HMBC (24.3, 151.1, 168.0)
43	Anserine	Ans	β-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	β-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	β′-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	β′-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-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-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-CH_2	3.45	*t*	COSY (3.3) HSQC (38.1)
55	Carnitine	Crn	γ,γ′-CH_2	3.45		COSY (4.59) HSQC (72.3)
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*-dimethylglycine	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)
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)

(*continued on next page*)

Table 2 (*continued*)

peak	Compound[1]	Abbr.[2]	Group	^{1}H (ppm)	Mult.: J[3] (Hz)	Assignment data
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′-monophosphate	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′-monophosphate	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)
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′-monophosphate	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′-monophosphate	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′-monophosphate	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)
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′-monophosphate	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′-monophosphate	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)
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.

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 (Kim et al., 2019; Simmler et al., 2014).

3.3. Metabolomic differences of broilers and KNC

The PCA scores, VIP scores, and heatmap from four different KNCs and broilers were processed (Fig. 1; R^2 = 0.918, Q^2 = 0.769). 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 (Worley and Powers, 2016). 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, Wishart, & Xia, 2019).

The VIP score is represented by a highly contributing variable (> 1 scores) in the PLS-DA model (Almeida, Fidelis, Barata, & Poppi, 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, Rafii, Wykes, Ball, & Pencharz, 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, Amna, & Hwang, 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.

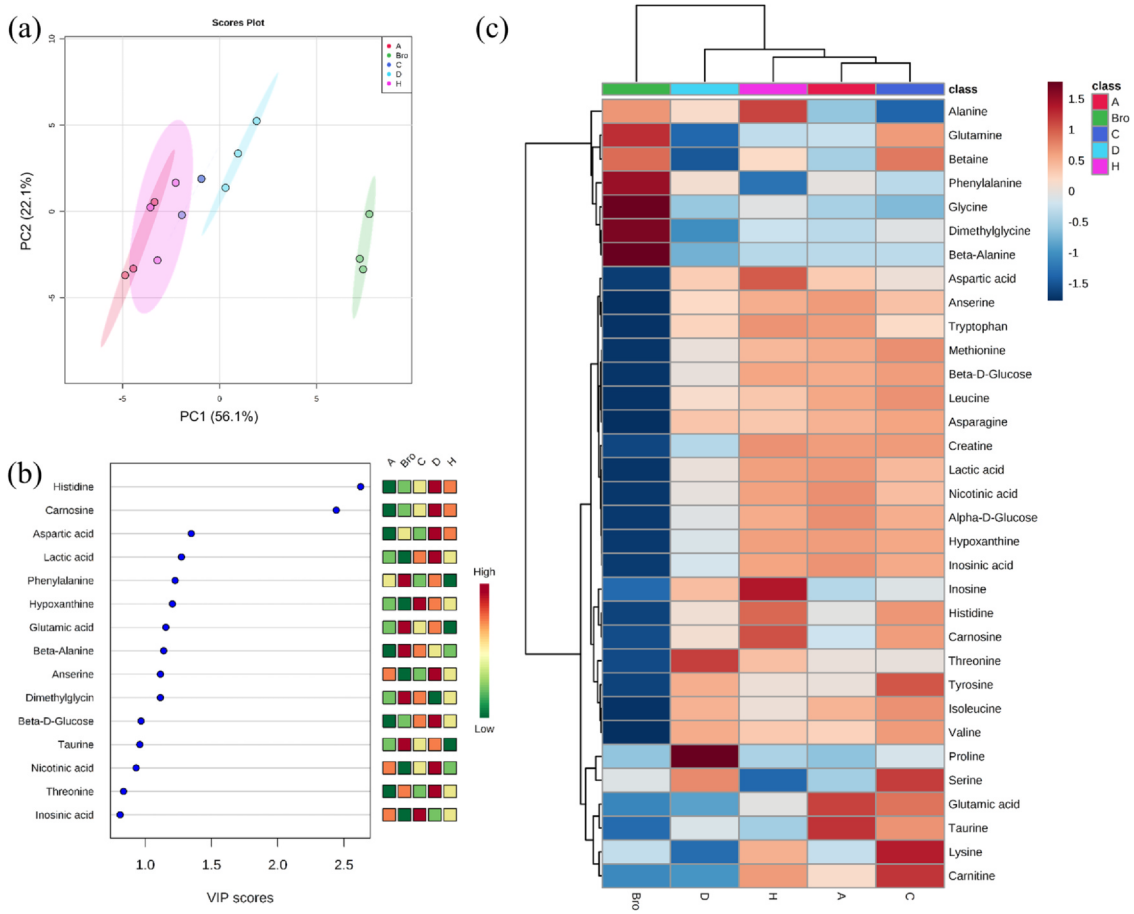


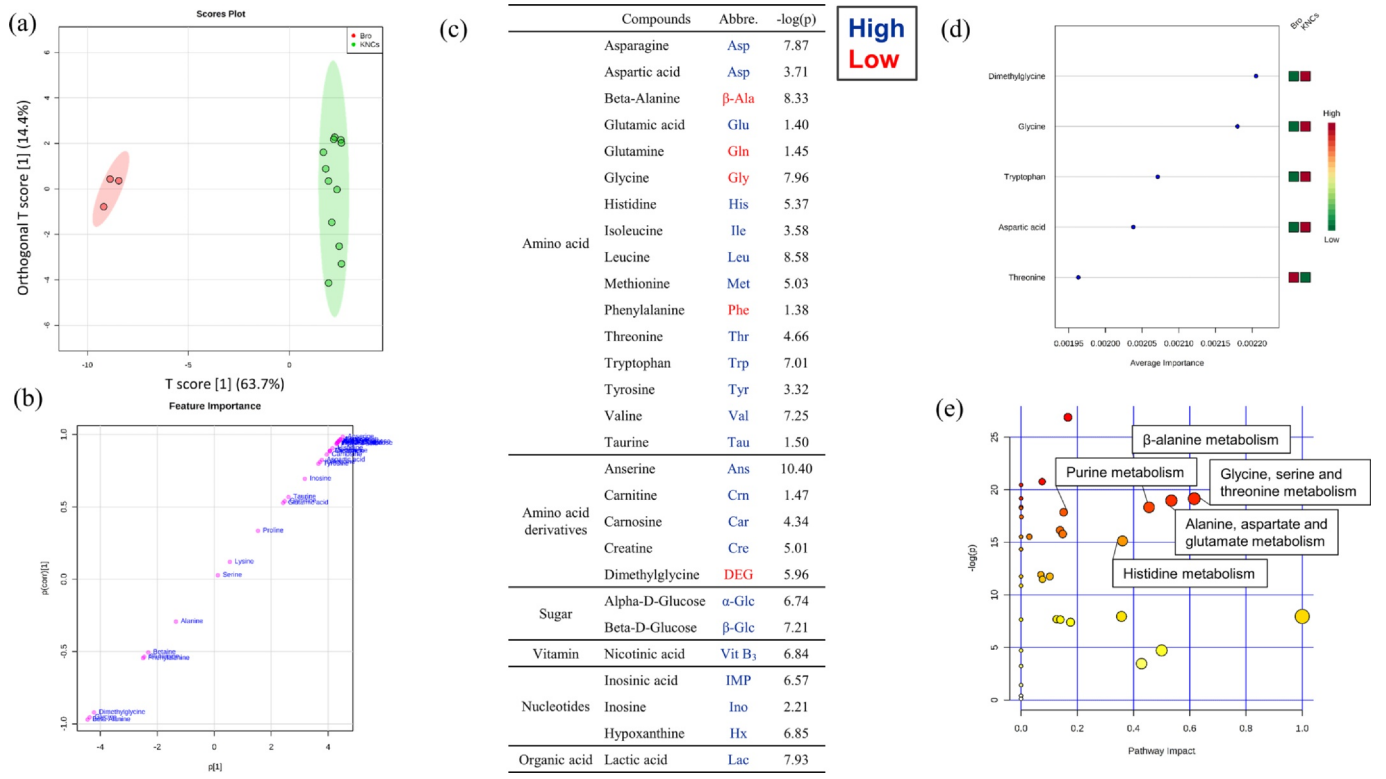
Fig. 1. Principal component analysis (PCA) (a), VIP scores (b), and heatmap (c) from breast meat extracts of different chicken breeds (KNC-A, -C, -D, and -H) by HSQC using a 850 MHz Cryo-NMR spectrometer.

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, Jung, Alahakoon, et al., 2015) and has been reported to have a similar bioactivity profile to carnosine (Jung et al., 2013).

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, Jung, Kim, Yong, et al., 2015; Jayasena, Jung, Kim, Kim, et al., 2015). 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. 2) 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, Nnanyelugo, James, & Garlick, 1974). This higher free amino acid concentration could be explained by a higher growth rate and productivity of broilers than KNCs (Ali et al., 2019; Kim et al., 2018). 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, Jung, Kim, Kim, et al., 2015).

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, Bodor, Ahmed, & Offermanns, 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, Jung, Nam, & Jo, 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., 2014; Kim et al., 2018), which further explains the different sensory characteristics.



	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

Fig. 2. 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.

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. 2d). To identify the interactive relationship on the metabolomic pathways, KEGG library-based pathway analysis was performed (Fig. 2e). 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 3. Holm *P* allows to solve the problem of Type 1 error by adjusting the criteria (Giacalone, Agata, Cozzucoli, & Alibrandi, 2018).

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. 3, 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

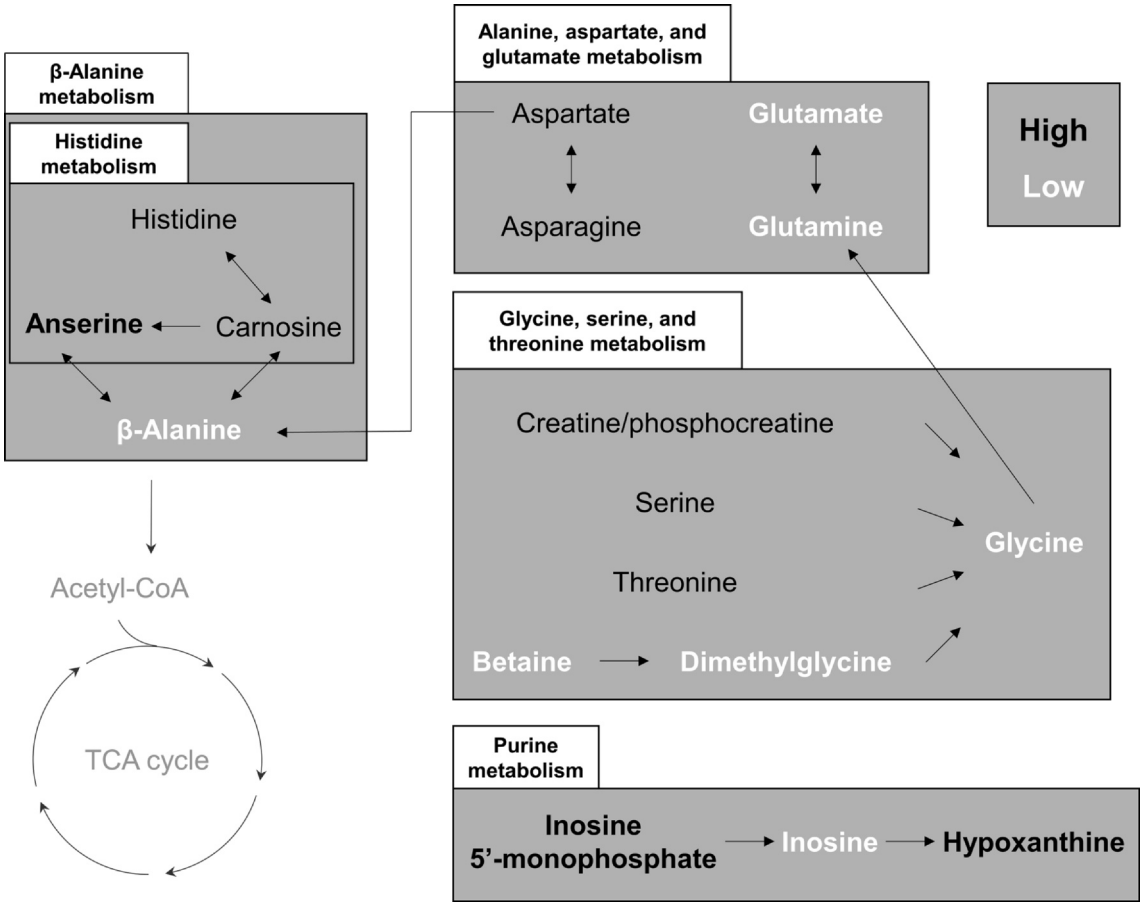


Fig. 3. Schematic illustration of major related pathways of chicken breast meat from native chickens (KNC-A, -C, -D, and H). Metabolites bold colored in black (high) and bold white (low) refer to the metabolites with differences among native chickens in comparison to commercial broilers. Metabolites in grey were not detected.

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, Jung, Alahakoon, et al., 2015; Xiao et al., 2019).

4. Conclusions

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 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.

CRediT authorship contribution statement

Hyun Cheol Kim: Formal analysis, Software, Data curation, Visualization, Writing - original draft. **Yoon-Joo Ko:** Methodology, Validation. **Cheorun Jo:** Conceptualization, Funding acquisition, Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, and Forestry (IPET) through Golden Seed Project, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) and Rural Development Administration (RDA) (213010-05-4-SB410, PJ 012822). Also, this work was supported

by the BK21 Plus Program of the Department of Agricultural Biotechnology, Seoul National University, Seoul, Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128316.

References

Ali, M., Lee, S.-Y., Park, J.-Y., Jung, S., Jo, C., & Nam, K.-C. (2019). Comparison of functional compounds and micronutrients of chicken breast meat by breeds. *Food Science of Animal Resources, 39*(4), 632–642.

Almeida, M. R., Fidelis, C. H. V., Barata, L. E. S., & Poppi, R. J. (2013). Classification of Amazonian rosewood essential oil by Raman spectroscopy and PLS-DA with reliability estimation. *Talanta, 117*, 305–311.

Alves Filho, E. G., Almeida, F. D., Cavalcante, R. S., De Brito, E. S., Cullen, P. J., Frias, J. M., ... Rodrigues, S. (2016). ^{1}H NMR spectroscopy and chemometrics evaluation of non-thermal processing of orange juice. *Food Chemistry, 204*, 102–107.

Choe, J.-H., Nam, K.-C., Jung, S., Kim, B.-N., Yun, H.-J., & Jo, C.-R. (2010). Differences in the quality characteristics between commercial korean native chickens and broilers. *Korean Journal for Food Science of Animal Resources, 30*(1), 13–19.

Chong, J., Wishart, D. S., & Xia, J. (2019). Using metaboanalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Current Protocols in Bioinformatics, 68*(1), Article e86.

Dashdorj, D., Amna, T., & Hwang, I. (2015). Influence of specific taste-active components on meat flavor as affected by intrinsic and extrinsic factors: An overview. *European Food Research and Technology, 241*(2), 157–171.

Fardus-Reid, F., Warren, J., & Le Gresley, A. (2016). Validating heteronuclear 2D quantitative NMR. *Analytical Methods, 8*(9), 2013–2019.

Gallo, V., Intini, N., Mastrorilli, P., Latronico, M., Scapicchio, P., Triggiani, M., ... Valerio, M. (2015). Performance assessment in fingerprinting and multi component quantitative NMR analyses. *Analytical Chemistry, 87*(13), 6709–6717.

Giacalone, M., Agata, Z., Cozzucoli, P. C., & Alibrandi, A. (2018). Bonferroni-Holm and permutation tests to compare health data: Methodological and applicative issues. *BMC Medical Research Methodology, 18*(1), https://doi.org/10.1186/s12874-018-0540-8.

Gille, A., Bodor, E. T., Ahmed, K., & Offermanns, S. (2008). Nicotinic acid: Pharmacological effects and mechanisms of action. *Annual Review of Pharmacology and Toxicology, 48*(1), 79–106.

Giraudeau, P. (2017). Challenges and perspectives in quantitative NMR. *Magnetic Resonance in Chemistry, 55*(1), 61–69.

Guennec, A. L., Giraudeau, P., & Caldarelli, S. (2014). Evaluation of fast 2D NMR for metabolomics. *Analytical Chemistry, 86*(12), 5946–5954.

Jayaraman, V., Ghosh, S., Sengupta, A., Srivastava, S., Sonawat, H. M., & Narayan, P. K. (2014). Identification of biochemical differences between different forms of male infertility by nuclear magnetic resonance (NMR) spectroscopy. *Journal of Assisted Reproduction and Genetics, 31*(9), 1195–1204.

Jayasena, D. D., Jung, S., Alahakoon, A. U., Nam, K. C., Lee, J. H., & Jo, C. (2015). Bioactive and taste-related compounds in defatted freeze-dried chicken soup made from two different chicken breeds obtained at retail. *The Journal of Poultry Science, 52*(2), 156–165.

Jayasena, D. D., Jung, S., Kim, H. J., Bae, Y. S., Yong, H. I., Lee, J. H., ... Jo, C. (2013). Comparison of quality traits of meat from Korean native chickens and broilers used in two different traditional Korean cuisines. *Asian-Australasian Journal of Animal Science, 26*(7), 1038–1046.

Jayasena, D. D., Jung, S., Kim, S. H., Kim, H. J., Alahakoon, A. U., Lee, J. H., & Jo, C. (2015). Endogenous functional compounds in Korean native chicken meat are dependent on sex, thermal processing and meat cut: Endogenous functional compounds of Korean native chicken meat. *Journal of the Science of Food and Agriculture, 95*(4), 771–775.

Jayasena, D. D., Jung, S., Kim, H. J., Yong, H. I., Nam, K. C., & Jo, C. (2015). Taste-active compound levels in Korean native chicken meat: The effects of bird age and the cooking process. *Poultry Science, 94*(8), 1964–1972.

Jayasena, D. D., Kim, S. H., Lee, H. J., Jung, S., Lee, J. H., Park, H. B., & Jo, C. (2014). Comparison of the amounts of taste-related compounds in raw and cooked meats from broilers and Korean native chickens. *Poultry Science, 93*(12), 3163–3170.

Jin, S., Jayasena, D. D., Jo, C., & Lee, J. H. (2017). The breeding history and commercial development of the Korean native chicken. *World's Poultry Science Journal, 73*(1), 163–174.

Jin, S., Park, H. B., Seo, D., Choi, N. R., Manjula, P., Cahyadi, M., Jung, S., ... Lee, J. H. (2018). Identification of quantitative trait loci for the fatty acid composition in Korean native chicken. *Asian-Australasian Journal of Animal Science, 31*(8), 1134–1140.

Jung, S., Bae, Y. S., Kim, H. J., Jayasena, D. D., Lee, J. H., Park, H. B., ... Jo, C. (2013). Carnosine, anserine, creatine, and inosine 5′-monophosphate contents in breast and thigh meats from 5 lines of Korean native chicken. *Poultry Science, 92*(12), 3275–3282.

Jung, S., Bae, Y. S., Yong, H. I., Lee, H. J., Seo, D. W., Park, H. B., ... Jo, C. (2015). Proximate composition, and L-carnitine and betaine contents in meat from Korean indigenous chicken. *Asian-Australasian Journal of Animal Science, 28*(12), 1760–1766.

Kasaoka, S., Tsuboyama-Kasaoka, N., Kawahara, Y., Inoue, S., Tsuji, M., Ezaki, O., ... Nakajima, S. (2004). Histidine supplementation suppresses food intake and fat accumulation in rats. *Nutrition, 20*(11-12), 991–996.

Keifer, P. A. (1999). 90° pulse width calibrations: How to read a pulse width array. *Concepts in Magenetic Resonance, 11*(3), 165–180.

Khan, M. I., Jung, S., Nam, K. C., & Jo, C. (2016). Postmortem aging of beef with a special reference to the dry aging. *Korean Journal for Food Science of Animal Resources, 36*(2), 159–169.

Kim, H. C., Choe, J., Nam, K. C., Jung, S., & Jo, C. (2018). Productivity and meat quality of the new crossbred Korean native chickens compared with commercial breeds. *Korean Journal of Poultry Science, 45*(2), 125–135.

Kim, H. C., Ko, Y.-J., Kim, M., Choe, J., Yong, H. I., & Jo, C. (2019). Optimization of 1D ^{1}H quantitative NMR (nuclear magnetic resonance) conditions for polar metabolites in meat. *Food Science of Animal Resources, 39*(1), 1–12.

Koskela, H., Kilpeläinen, I., & Heikkinen, S. (2005). Some aspects of quantitative 2D NMR. *Journal of Magnetic Resonance, 174*(2), 237–244.

Kriengsinyos, W., Rafii, M., Wykes, L. J., Ball, R. O., & Pencharz, P. B. (2002). Long-term effects of histidine depletion on whole-body protein metabolism in healthy adults. *The Journal of Nutrition, 132*(11), 3340–3348.

Le Roy, C. I., Mappley, L. J., La Ragione, R. M., Woodward, M. J., & Claus, S. P. (2016). NMR-based metabolic characterization of chicken tissues and biofluids: A model for avian research. *Metabolomics, 12*, 157.

Marchand, J., Martineau, E., Guitton, Y., Dervilly-Pinel, G., & Giraudeau, P. (2017). Multidimensional NMR approaches towards highly resolved, sensitive and high-throughput quantitative metabolomics. *Current Opinion in Biotechnology, 43*, 49–55.

Markley, J. L., Brüschweiler, R., Edison, A. S., Eghbalnia, H. R., Powers, R., Raftery, D., & Wishart, D. S. (2017). The future of NMR-based metabolomics. *Current Opinion in Biotechnology, 43*, 34–40.

Millward, D. J., Nnanyelugo, D. O., James, W. P. T., & Garlick, P. J. (1974). Protein metabolism in skeletal muscle: The effect of feeding and fasting on muscle RNA, free amino acids and plasma insulin concentrations. *British Journal of Nutrition, 32*(01), 127–142.

Mottram, D. S. (1998). Flavour formation in meat and meat products: A review. *Food Chemistry, 62*(4), 415–424.

Simmler, C., Napolitano, J. G., McAlpine, J. B., Chen, S.-N., & Pauli, G. F. (2014). Universal quantitative NMR analysis of complex natural samples. *Current Opinion in Biotechnology, 25*, 51–59.

Soglia, F., Silva, A. K., Lião, L. M., Laghi, L., & Petracci, M. (2019). Effect of broiler breast abnormality and freezing on meat quality and metabolites assessed by ^{1}H-NMR spectroscopy. *Poultry Science, 98*(12), 7139–7150.

Van, Q. N., Issaq, H. J., Jiang, Q., Li, Q., Muschik, G. M., Waybright, T. J., ... Veenstra, T. D. (2007). Comparison of 1D and 2D NMR spectroscopy for metabolic profiling. *Journal of Proteome Research, 7*(2), 630–639.

Watabe, S., Kamal, M., & Hashimoto, K. (1991). Postmortem changes in ATP, creatine phosphate, and lactate in sardine muscle. *Journal of Food Science, 56*(1), 151–153.

Worley, B., & Powers, R. (2016). PCA as a practical indicator of OPLS-DA model reliability. *Current Metabolomics, 4*(2), 97–103.

Xia, J., & Wishart, D. S. (2010). MetPA: A web-based metabolomics tool for pathway analysis and visualization. *Bioinformatics, 26*(18), 2342–2344.

Xiao, Z., Ge, C., Zhou, G., Zhang, W., & Liao, G. (2019). ^{1}H NMR-based metabolic characterization of Chinese Wuding chicken meat. *Food Chemistry, 274*, 574–582.

Yamaguchi, S. (1967). The synergistic taste effect of monosodium glutamate and disodium 5'-inosinate. *Journal of Food Science, 32*(4), 473–478.