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Metabolite profile based on ^{1}H NMR of broiler chicken breasts affected by wooden breast myodegeneration

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Running title: Metabolite profile of chicken affected by wooden breast

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Abstract

The objective was to characterize the effect of wooden breast (WB) myodegeneration on the metabolite profile of chicken meat by ^{1}H NMR and multivariate data analysis. The results displayed that the metabonome of chicken breast consisted predominantly of 30 metabolites, including amino acids, organic acids, carbohydrates, alkaloids, nucleosides and their derivatives. WB-affected samples showed higher leucine, valine, alanine, glutamate, lysine, lactate, succinate, taurine, glucose, and 5'-IMP levels, but lower histidine, β-alanine, acetate, creatine, creatinine, anserine and nicotinamide adenine dinucleotide levels compared to normal fillets ($p < 0.05$). In conclusion, results indicated that WB-affected fillets possessed a unique biochemical signature. This unique profile could identify candidate biomarkers for diagnostic utilization and provide mechanistic insight into biochemical processes leading to WB myopathy in commercial broiler chickens.

Keywords: chicken breast; wooden breast; metabolite profile; NMR; multivariate data analysis

1 Introduction

Wooden breast (WB) is an emerging myopathy in fast growing broiler chickens (high breast yielding birds), macroscopically considered as hardness and paleness of *pectoralis major* muscle, and is often accompanied with white striping (Sihvo, Lindén, Airas, Immonen, Valaja, & Puolanne, 2017). WB lesion areas are histologically characterized by chronic myodegeneration with interstitial edema and regeneration, and loose connective tissue accumulation (fibrosis) (Sihvo, Immonen, & Puolanne, 2014). White striping shares some histological features with WB, and is characterized by pale striations running parallel to the breast muscle fibers (Kuttappan, Shivaprasad, Shaw, Valentine, Hargis, Clark, et al., 2013). The underlying mechanisms leading to WB are still unclear.

Sandercock, Barker, Mitchell, and Hocking (2009) reported that genetic selection for broiler features could involve changes of cell cations, especially calcium, which seemed to be related to myopathic changes. Soglia, Zeng, Gao, Puolanne, Cavani, Petracci, and Ertbjerg (2018) observed an increase in free calcium in WB muscles at day 7 postmortem. Huang, Ren, Jiang, Xiao, and Lei (2015) found that selenium deficiency was a reason for oxidative damage leading to myodegeneration in birds. Previous studies indicated that white striping was related to heavy birds and a fast growth rate (Bauermeister, Morey, Moran, Singh, Owens, & McKee, 2009; Kuttappan, Brewer, Apple, Waldroup, & Owens, 2012). Because of its healthy nutritional profile, mild flavor and excellent tenderness, chicken breast targets different groups of consumers. However, the WB and white striping myopathies may contribute to the appearance of metabolic and structural abnormalities and have a substantial influence on meat quality (Mudalal, Lorenzi, Soglia, Cavani, & Petracci, 2015; Petracci & Cavani, 2012). A great deal of attention has been paid to the effect of WB on quality

traits, such as texture and processing characteristics (Mudalal et al., 2015; Soglia, Mudalal, Babini, Nunzio, Mazzoni, Sirri, et al., 2015). Chicken meat, which was affected by WB or both WB and white striping myopathies, showed poor texture and reduced water holding ability (Mudalal et al., 2015; Petracci, Mudalal, Bonfiglio, & Cavani, 2013). Especially the superficial layer of WB-affected fillets was harder than that of normal fillets (Soglia, Gao, Mazzoni, Puolanne, Cavani, Petracci, and Ertbjerg, 2017). Nevertheless, no information is currently available on how the WB myopathy is associated with changes in metabolites that can induce the special histological changes of chicken breast.

As a technique for acquiring the metabolite profile, nuclear magnetic resonance (NMR) spectroscopy has made great breakthroughs in the last decade and has been developed as a superior tool for high-throughput and reliable metabolomics analysis in the fields of nutrition and food science (Graham, Kennedy, Chevallier, Gordon, Farmer, Elliott, et al., 2010). Particularly, NMR spectroscopy has been applied to obtain metabolite profiles of meat samples. Castejón, García-Segura, Escudero, Herrera, and Cambero (2015) explored changes in the metabolite profile of beef during storage through ^{1}H NMR spectroscopy. Chen, Ye, Chen, Zhan, and Lou (2017) used ^{1}H NMR-based metabolomics to analyze molecular nutritional properties of vinasse pike eel. Liu, Pan, Ye, and Cao (2013) reported the relationship between age and the quality of duck meat by ^{1}H NMR. These studies highlighted the significance of the metabolite profile in the quality evaluation of meat. In addition, Chung et al. (2005) found that the levels of metabolites in urine, such as creatine, choline-containing compounds, betaine and glycine, increased in patients with juvenile idiopathic inflammatory myopathies. Metabolic myopathies caused by defects in cellular energy metabolism comprise a clinically and etiologically diverse

group of disorders (Berardo, DiMauro, & Hirano, 2010). Sugie, Tsurui, Sugie, and Igarashi (1990) clearly detected a defect in the glycolytic pathway by ^{1}H NMR, suggesting that ^{1}H NMR spectroscopy is useful for the analysis of metabolic myopathies.

In the present study, ^{1}H NMR-based metabonomics was combined with multivariate data analysis to analyze the metabolite profiles of chicken breast within different degrees of WB myodegeneration. The objective of this work was to characterize the biochemical markers of WB myodegeneration through high-throughput metabolomics profile of breast muscle isolated from no, moderate and severe WB samples.

2 Materials and methods

2.1 Sample selection and preparation

Ross 708 broilers from University of Helsinki experimental farm were killed at the age of 38 d by cervical dislocation. The birds were raised in penned groups and fed ad libitum. Ethical permission for the experiment was issued by the Research Animal Resources Committee of the University of Helsinki. The *Pectoralis major* breast muscles were palpated and visual evaluated within 30 min after slaughter and categorized according to the severity of WB abnormality. Those with hardened, out-bulging and pale area in the cranial end of the fillet were identified as WB, while the softer ones with normal appearance were categorized as normal. Consistent with the criteria proposed by Sihvo et al. (2014), breast fillets were then graded into 3 classes consisting of normal (N), moderate (M), or severe (S) based on the severity of the condition. Those with hardness in a local muscle area were categorized as moderate WB and those where a major part of the muscle was affected was categorized as severe. Each group was sampled to consist of 10 muscles. A core

126 samples of 15 g from each breast muscles was sampled, frozen in liquid nitrogen,
127 powdered, mixed, freeze dried and stored at -80°C until further processed for
128 extraction of metabolites. Two g of freeze dried powder from each group was
129 processed in ten replicates for extraction of metabolites and NMR analysis.

130 2.2 Metabolites extraction

131 The extraction of metabolites was according to our previous methods (Yang, Ye,
132 Wang, Sun, Pan, Cao, 2018). Briefly, chicken breast sample was homogenized with
133 methanol/water (2:1, v/v) for 90 s at 20 Hz. After centrifugation at 12,000 g for 10
134 min at 4 °C, the supernatant of the homogenate was acquired. The same extraction
135 processing was repeated once. The two resultant supernatant extracts were combined
136 and condensed in vacuum for removing methanol. After lyophilization, the sample
137 was dissolved by phosphate buffer with 99.9% D_2O, and 0.005% sodium
138 3-trimethylsilyl [2, 2, 3, 3-d_4] propionate. After centrifugation, 550 μL supernatant of
139 each extract was used for NMR analysis in a 5 mm NMR tube.

140 2.3 NMR analysis

141 The NMR analysis and NMR signal assignment were according to our previous
142 methods (Yang et al., 2018). ^{1}H NMR spectra of the chicken breast extracts were
143 obtained at 298 K on a Bruker Avance 600 MHz Spectrometer equipped with an
144 inverse detection probe (Bruker Biospin, Rheinstetten, Germany). For each sample, a
145 standard NOESYGPPR1D pulse sequence was applied to acquire chicken breast
146 metabolite profiles with a weak irradiation during a 2 s recycle delay and a 100 ms
147 mixing time to suppress the water signal. A 90° pulse length was set to 10 μs. A total
148 of 32 scans were collected into 32 k data points with a spectral width of 20 ppm. For
149 NMR signal assignment purposes, a catalogue of two-dimensional NMR spectra were
150 obtained for selected samples and processed following a previously reported method

(Yang et al., 2018), including ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), and ^{1}H-^{13}C heteronuclear multiple bond correlation spectra (HMBC).

2.4 Data analysis

The multivariate data analysis (principal component analysis, PCA), orthogonal projection to latent structure with discriminant analysis (OPLS-DA) and quantitative analysis of chicken breast metabolites were performed according to our previous methods (Zhang, Ye, Sun, Pan, Ou, Dang, et al., 2018). After phase and baseline corrections, the ^{1}H NMR spectra (δ 9.0-0.8) were integrated into regions with a 2.4 Hz width of (0.004 ppm). The regions containing the methanol signals (δ 3.37-3.35) were excluded. Each bucketed region was normalized to the total sum of spectral integral to offset for the whole concentration difference. Afterwards, the normalized NMR datasets were analyzed with multivariate data analysis (SIMCA-P+ software, version 12.0, Umetrics, Sweden). Principal component analysis (PCA) was performed utilizing mean-centered scaling, and the PCA results were displayed as the scores and loading plots; each point in the former showed an individual sample, while the latter showed the magnitude and manners of the NMR signals (thus metabolites) to classification. The orthogonal projection to latent structure with discriminant analysis (OPLS-DA) was sequentially performed using the unit variance-scaled NMR data. All of OPLS-DA models were validated using a cross validation-analysis of variance method (CV-ANOVA) with $p < 0.05$ as significant level (Eriksson, Trygg, & Wold, 2008). The OPLS-DA results were also visualized in the scores and coefficient plot. The loading in the coefficient plot presented the changed metabolites associated with WB myodegeneration, and were acquired by a back-scaled transformation and plotted with a color-coded correlation coefficient with version 7.1 of MATLAB (MathWorks,

Natick, MA, USA). In this study, $|r| > 0.602$ ($r > 0.602$ and $r < -0.602$) was utilized as an absolute cut-off value for the statistical significance ($p < 0.05$).

For quantitative analysis of chicken breast metabolites, metabolite content was calculated by equating the integrals of non-overlapping NMR signals related to that of internal reference (TSP) with known concentration. The obtained metabolite content data was carried out using the one-way ANOVA procedure (Duncan's Multiple Range Test) with the SAS 8.0 software.

3. Results and discussion

3.1 ^{1}H NMR spectra of chicken breast extract

Figure 1 shows representative ^{1}H NMR spectra of chicken breast muscle extracts from no (A), moderate (B) and severe (C) WB samples. Resonances were identified by a series of 2D NMR spectra (Table 1). The metabonome of chicken breast muscle contained 30 metabolites, including 15 amino acids (leucine, isoleucine, valine, alanine, threonine, glutamate, glutamine, methionine, lysine, β-alanine, tyrosine, glycine, histidine, phenylalanine and taurine), 6 organic acids (lactate, acetate, succinate, creatine, creatinine and taurine), 2 carbohydrates (sucrose and glucose), 2 alkaloids (phosphorylcholine and betaine), and 3 nucleosides and their derivatives (inosine, 5'-IMP and hypoxanthine). These spectra indicated that the contents of metabolites of chicken breast muscles were affected by the WB myopathy. For instance, WB fillets had higher contents of lactate, glucose and 5'-IMP compared to normal fillets.

3.2 Effects of WB myodegeneration on the metabolite profile of chicken breast

In order to acquire an insight of the effect of WB myodegeneration on the metabolite profile of chicken breast muscles, PCA was conducted on the NMR spectral data. PC1 and PC2 explained a total of 65.0 and 23.6% of variables,

respectively. The PCA score plot (Figure 2) shows a clear variation in metabolite profile affected by WB. The no (A), moderate (B), and severe (C) WB samples were thus unambiguously divided into three distinctive groups based on their metabolite profile.

To further investigate the WB-associated change in metabolites, coupled comparative OPLS-DA created from the spectral data of no and moderate WB samples (Figure 3a), no and severe WB samples (Figure 3b), moderate and severe WB samples (Figure 3c) was performed. The OPLS-DA results were displayed as cross-validated score plots (Figure 3, left) and corresponding coefficient plots (Figure 3, right). R^2 and Q^2 values demonstrated reasonable qualities for the 3 OPLS-DA models (Figure 3, left). The results of Duncan's Multiple Range Test were displayed by *p* values. The coefficient plots of OPLS-DA (Figure 3, right) revealed a significant difference of metabolites between no WB, moderate WB and severe WB. The correlation coefficients of metabolites were greater than 0.602, which was regarded to be significant ($p < 0.05$).

Table 2 shows the corresponding correlation coefficients for most metabolites. Compared with normal fillets, severe WB revealed significantly higher levels of leucine, isoleucine, valine, alanine, threonine, glutamate, glutamine, lysine, glycine, lactate, succinate, taurine, glucose, betaine and 5'-IMP, but contained less acetate, creatine, creatinine, β-alanine, histidine, sucrose and nicotinamide adenine dinucleotide (NAD) by the coefficients ($|r| > 0.602$). Moderate WB displayed similar metabolites to severe WB, with the exception of isoleucine, leucine, valine, glutamine, glycine, acetate, taurine and sucrose. In order to acquire more information of the change of metabolites in WB-affected samples, an absolute quantification of metabolites was performed (Table 2). Leucine, valine, alanine, glutamate, lysine,

glycine, lactate, succinate, taurine, glucose, sucrose, phosphorylcholine, 5'-IMP, hypoxanthine and nicotinamide content were at significantly higher levels in moderate WB than in normal ($p < 0.05$), while histidine, β-alanine, phenylalanine, tyrosine, acetate, creatine, creatinine, betaine, inosine, anserine and NAD were at lower concentrations in moderate WB than in normal ($p < 0.05$). Severe WB had similar change for these metabolites compared with moderate WB except for glycine, phenylalanine, tyrosine, sucrose and betaine ($p < 0.05$). These findings were similar to the OPLS-DA results.

WB myodegeneration has significantly influenced the muscle health and meat quality of broiler chickens. Many authors hypothesized that a high growth rate of broiler chickens is associated to the development this myodegeneration (Mudalal et al., 2015; Petracci & Cavani, 2012). Mutryn, Brannick, Fu, Lee, and Abasht (2015) suggested various contributing factors to the myopathy such as an increase of intracellular calcium, localized muscular hypoxia and oxidative stress damages. In this study, we attempted to clarify major metabolic differences between the WB myopathy and healthy chickens, and to further characterize biomarkers of this myopathy. High levels of valine, leucine and isoleucine in WB-affected samples can be candidate biomarkers of extracellular matrix remodeling related to changes in muscle tissue (Abasht, Mutryn, Michalek, & Lee, 2016). The changes in muscle tissue of WB birds can also be related to the appearance of fibrosis (loose connective tissue accumulation) (Sihvo, Immonen, & Puolanne, 2014). The contents of histidine and glutamate were higher than those of other amino acids in the extracts of WB-affected and unaffected chickens, which indicated that histidine and glutamate may be the most important amino acids in the characteristic metabolite profile of chickens. Histidine with anti-oxidant and anti-inflammatory properties is a semi-essential amino

acid. WB myopathy significantly decreased the content of histidine, suggesting that the WB condition was related to oxidative stress. In agreement, Bao, Boeren, & Ertbjerg (2018) observed decreased histidine content of porcine myofibrils following protein oxidation. In population studies, low level of histidine has been related to chronic kidney disease patients (Watanabe, Suliman, Qureshi, Garcia-Lopez, Bárány, Heimbürger, et al., 2008) and oxidative stress and inflammation in obese women (Niu, Feng, Hou, Li, Kang, Wang, et al., 2012). Yu et al. (2015) found that high level of blood histidine was correlated with risk reduction of developing incident coronary heart disease. Glutamate, the primary excitatory neurotransmitter, is responsible for adjusting a wide range of nervous system functions by glutamate receptors. Glutamate plays an important role in normal central nervous system synaptic function (Choi, Maulucci-Gedde, & Kriegstein, 1987). WB myopathy significantly elevated the content of glutamate. The increased level of glutamate can lead to neurotoxicity in the synaptic cleft (Maragakis & Rothstein, 2004).

Lactate, a mild acid, is the product of glycogenolysis and glycolysis (Chen, Ye, Chen, & Yan, 2016). Mudalal et al. (2015) reported that WB-affected chickens showed higher ultimate pH values compared to unaffected birds. They suggested that these abnormalities may potentially decrease the levels of glycogen in the muscle. As previously reported by Berri et al. (2001 & 2007), postmortem lactate production, which is a main factor affecting ultimate pH, can be limited due to low glycogen content of the muscle. Lactate accumulation, noninvasively evaluated by magnetic resonance spectroscopy, can occur in diseased muscle (Nirkko, Rösler, & Slotboom, 2006). Berardo, DiMauro, and Hirano (2010) reported that lactate was elevated in most mitochondrial disease patients with sporadic isolated myopathies and exercise intolerance. In addition, Pal, Parker, and Costello (2009) found that elevated lactate

in liver was associated with liver damage and disease. Creatine, synthesized in the kidney, liver and pancreas, is taken up by muscle cells involving an active transport mechanism. Creatine is a key constituent of the energy transfer process in several tissues, especially those characterized by the transportation of high-energy phosphate to ADP in muscle cells (Wyss & Kaddurah-Daouk, 2000). Moderate and severe WB contained a significantly higher ratio of creatine:creatinine compared with no WB. Chung et al. (2005) found that urinary creatine:creatinine ratio was significantly higher in patients with juvenile idiopathic inflammatory myopathy than in controls. Furthermore, they reported that the creatine:creatinine ratio was correlated strongly with physician-assessed global disease damage. This ratio may have potential use as a signature of myositis disease damage. Taurine, a sulfur-containing amino acid, was commonly generated in tissues exposed to high levels of oxidants. Inherited or acquired myopathies characterized by metabolic changes, as well as alteration in calcium homeostasis, have been associated with change in muscle taurine content (Camerino, Tricarico, Pierno, Desaphy, Liantonio, Pusch, et al., 2004). Previous studies reported that an increase of taurine was associated with growth enhancement of broilers (Lee, Cheng, Chuang, Shive, Lian, Wei, et al., 2004). Taurine has short- and long-term actions in the control of calcium homeostasis and ion channel function in striated fibers (Camerino et al., 2004). Taurine has marked antioxidant activities and protective effects, and has been shown to be able to elicit neuroprotection and reduce apoptosis (Gharibani et al., 2013). Li et al. (2017) reported that an increased level of taurine in liver tissues could play a role to replenish damaged phospholipid membranes induced by reactive oxygen species, thus representing a self-repair mechanism. The increase of taurine in WB-affected chickens could, therefore, be related to a self-protection mechanism to inhibit cellular damage due to oxidative

stress.

Betaine, a source of methyl groups, is essential as a tissue osmolyte. Betaine and phosphorylcholine are metabolic end products of choline metabolism. Choline-containing compounds are essential constituents of cell membrane metabolism and are synthesized in the liver (Zeisel, Da, Franklin, Alexander, Lamont, Sheard, et al., 2000). Betaine may affect the characteristics of chicken meat and its growth rate. The level of betaine in severe WB was higher than it was in no WB. More betaine can enhance the growth of broiler chickens and delay the impacts of lipid metabolism (Lever & Slow, 2010). Anserine, which has been found in muscle tissues of most vertebrates, has been shown to have antioxidant properties and buffering capacity (Peiretti, Medana, Visentin, Dal Bello, & Meineri, 2012). The decreased level of anserine in WB-affected samples suggests reduced antioxidant protection. The level of 5'-IMP in WB-affected chickens was higher than that in no WB. Annandale, Valberg, and Essén-Gustavsson (2005) found that an increased content of 5'-IMP without depletion of ATP, in individual horse muscle fibers with polysaccharide storage myopathy during submaximal exercise, may lead to the development of rhabdomyolysis and exercise intolerance. Weaver and Kim (2014) reported that an increased level of 5'-IMP in the diet of young pigs could reduce postweaning stress and enhance growth performance. NAD, one of the most important coenzymes in the cell, is important in cell regulation and metabolic reactions in all organisms. Its function as a cofactor is well-established in redox reactions. NAD is generated from tryptophan or aspartic acid (the *de novo* pathway) in organisms. In the salvage pathway, it is produced by recycling degraded NAD products such as nicotinamide. NAD also plays vital roles in longevity, transcriptional regulation, DNA repair, age-associated diseases and calorie-restriction-mediated

life-span extension (Lin & Guarente, 2003). The decreased content of NAD in WB-affected chickens could lead to oxidative stress and impair self-repair.

In general, our findings suggested that WB-affected chickens possessed biological markers of oxidative stress and muscle degradation. Based on these results, we hypothesize that the wooden breast myopathy is related to deficiency of histidine, anserine and NAD, excessive formation of lactate, 5'-IMP and glutamate, and a high ratio of creatine:creatinine.

4. Conclusions

WB myodegeneration in commercial broiler chickens was associated with a unique metabolic signature, which could have diagnostic potential. The deficiency of reduced substances could cause oxidative stress and impair self-repair in WB-affected muscle. Although the exact pathogenesis of the WB disease at present is unknown, our study provides important information related to biochemical markers involved in the etiology of WB myodegeneration.

The work provides the basic information to develop a potentially successful strategy to reduce WB by increasing the level of certain metabolites (histidine, anserine and NAD) or inhibiting accumulation of certain metabolites (lactate, 5'-IMP and glutamate).

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Figures captions:

Figure 1. Representative 600 MHz ^{1}H NMR spectra of broiler chicken breast extracts from no (A), moderate (B) and severe (C) wooden breast (WB) samples. The dotted region was vertically expanded 16 times. Resonance assignments are given in Table 1. Keys: 1. isoleucine, 2. leucine, 3. valine, 4. lactate, 5. threonine, 6. lysine, 7. alanine, 8. acetate, 9. methionine, 10. glutamate, 11. succinate, 12. β-alanine, 13. creatine, 14. creatinine, 15. phosphorylcholine, 16. betaine, 17. taurine, 18. glycine, 19. β-glucose, 20. α-glucose, 21. sucrose, 22. inosine, 23. 5'-IMP, 24. tyrosine, 25. histidine, 26. anserine, 27. phenylalanine, 28. nicotinamide, 29. hypoxanthine, 30. nicotinamide adenine dinucleotide (NAD), 31. glutamine, 32. sucrose and amino acids, 33. residual methanol.

Figure 2. PCA score plot for chicken breast extracts from no (A, stars), moderate (B, circles) and severe (C, diamonds) WB samples. PC1 and PC2 represent 65.0 and 23.6% of the total variance, respectively.

Figure 3. OPLS-DA scores plots (left) and corresponding color-coded correlation coefficient loadings plots (right) generated by comparisons between spectra of meat in extracts from no (A, stars), moderate (B, circles) and severe (C, diamonds) WB samples, respectively. Metabolites keys to the numbers are shown in Figure 1 and Table 1.

Table 1 NMR data for metabolites of broiler chicken breast extracts

Key	Metabolites	Moieties	δ^1 H (ppm) and multiplicity[a]	δ^{13} C (ppm)
1	Isoleucine	αCH, βCH, γCH_2, $\gamma' CH_3$, δCH_3	3.67(d), 1.98(m), 1.26(m), 1.45(m), 1.01(d), 0.94(t)	62.4, 38.4, 27.3, 17.7, 13.8
2	Leucine	αCH, βCH_2, γCH, δCH_3, $\delta' CH_3$	3.75(d), 1.72(m), 1.66(m) , 0.98(d), 0.96(d)	62.4, 42.6, 26.9, 25.0, 24.0
3	Valine	αCH, βCH, γCH_3, $\gamma' CH_3$	3.64(d), 2.28(m), 1.05(d), 1.00(d)	63.2, 31.9, 20.9,19.7
4	Lactate	αCH, βCH_3, COOH	4.12(q), 1.34(d)	71.2, 20.3, 185.3
5	Threonine	αCH, βCH, γCH_3	3.58(d), 4.26(m), 1.33(d)	68.7, 63.3, 23.0
6	Lysine	αCH, βCH_2, γCH_2, δCH_2, εCH_2	3.76(t), 1.92(m), 1.44(d), 1.73(m), 3.03(t)	56.9, 32.6, 26.0, 29.3, 41.9
7	Alanine	αCH, βCH_3, COOH	3.81(q), 1.49(d)	53.4, 19.1, 178.2
8	Acetate	CH_3, COOH	1.92(s)	26.2, 184.3
9	Methionine	S-CH_3	2.14(s)	16.9
10	Glutamate	δCO, αCH, βCH_2, γCH_2, COOH	3.77(m), 2.12(m), 2.05(m), 2.36(dt)	184.1, 57.3, 29.8, 36.2, 177.6
11	Succinate	CH_2	2.41(s)	37.0
12	β-Alanine	αCH_2, βCH_2, COOH	2.57(t), 3.18(t)	36.5, 39.3,181.0
13	Creatine	αCH_2, βCH_3, CNH, COOH	3.94 (s), 3.04(s)	56.4, 39.8, 160.0, 177.3
14	Creatinine	CH_2, N-CH_3, C, CO	4.06(s), 3.05(s)	59.1, 33.1, 171.7, 191.4
15	Phosphorylcholine	αCH_2, βCH_2, N-CH_3	4.07(d), 3.54(d), 3.21(s)	70.1, 56.5
16	Betaine	CH_3, CH_2, COO^-	3.28(s), 3.93(s)	56.7, 68.9, 172.2
17	Taurine	CH_2NH_2, CH_2SO_3	3.43(t), 3.27(t)	38.2, 50.6
18	Glycine	αCH_2, COOH	3.58(s)	44.5, 175.4
19	β-Glucose	C_1H, C_2H	4.66(d), 3.26(dd)	98.9, 77.1
20	α-Glucose	C_1H, C_2H	5.24(d), 3.54(dd)	95.0, 74.9
21	Sucrose	G_1H, G_2H, G_3H, G_4H, G_5H, G_6H, F_1H, F_2, F_3H, F_4H, F_5H, F_6H	5.43(d), 3.59(dd), 3.79(t), 3.49(t), 3.83(q), 3.81(q), 3.70(s), 4.23(d), 4.07(t), 3.91(m), 3.83	95.0, 73.9, 75.0, 72.1, 74.9, 62.8, 64.0, 106.5, 79.2, 76.6, 83.9, 65.0
22	Inosine	C_1H, C_2, C_3, C_4H, C_5, $C_1{\cdot}H$, $C_2{\cdot}H$	8.35(s), 8.25(s), 6.10(d), 4.77, 4.45(m)	143.2, 127.2, 161.8, 149.1, 151.5, 91.0, 76.8
23	5'-IMP	C_1H, C_2, C_3, C_4H, C_5	8.57(s), 8.24(s), 6.15(d)	142.7, 126.7, 161.8, 149.1, 152.0, 90.2,
24	Tyrosine	αCH, βCH_2, Ring C_1, Ring $C_{2,6}H$, Ring $C_{3,5}H$, Ring C_4, COOH	3.95(dd), 3.20(dd), 3.06(dd), 7.20(d), 6.90 (d)	59.4, 38.4,129.7, 133.6, 118.8, 157.5, 177.1
25	Histidine	C_1H, C_2H, C_3, C_4H, C_5H, COOH	7.88(d), 7.09(d), 3.15(dd), 3.25(dd), 4.00(dd)	139.0, 119.6, 133.9, 30.8, 57.6, 176.9
26	Anserine	Ring C_2H, Ring C_4H, C_5, Ring NCH_3	8.48(s), 7.19(s), 3.82(s)	136.8, 119.6, 133.5, 35.1
27	Phenylalanine	αCH, βCH_2, Ring C_1, Ring $C_{2,6}H$, Ring $C_{3,5}H$, Ring C_4H, COOH	4.00(dd), 3.13(dd), 3.29(dd), 7.33(q), 7.43(t), 7.38(m)	59.1, 39.1, 138.1, 132.1, 131.8, 130.8, 177.1
28	Nicotinamide	C_2H, C_4H, C_5H, C_6H	8.94(dd), 8.27(dd),7.60(dd), 8.72(dd)	150.1, 139.0, 127.0, 154.2
29	Hypoxanthine	C_2H, C_6H	8.21(s), 8.19(s)	148.2, 160.1
30	Nicotinamide adenine	N_4H, N_5H, $N_1{\cdot}H$, A_2H, A_8H, $A_1{\cdot}H$	8.82(d), 8.18(m), 6.10(d), 8.14(s), 8.43(s), 6.04(d)	148.7, 131.2, 102.6, 155.2, 143.3, 89.3

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	dinucleotide (NAD)			
31	Glutamine	αCH, βCH_2, γCH_2, δCO, COOH	3.77(t), 2.14 (m), 2.46(m)	57.2, 29.2, 33.8, 180.7, 177.2
32	Sucrose and amino acids	αCH resonances	3.46-4.13	
33	Residual methanol	CH_3	3.37(s)	52.0

[a] Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dt, doublet of triples.

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Table 2 Coefficients from OPLS-DA and metabolite contents of broiler chicken breast extracts affected with wooden breast (WB) myopathy.

Motabolite	cofficient[α]			Mean±SD (mg/g)[γ]		
	B/A[β]	C/A	C/B	A	B	C
Isoleucine	—	0.99	0.99	0.04±0.0002b	0.04±0.0003b	0.07±0.001a
Leucine	—	0.99	0.99	0.032±0.0001c	0.034±0.0002b	0.063±0.0004a
Valine	—	0.96	0.99	0.036±0.0002c	0.039±0.0003b	0.066±0.0004a
Threonine	0.96	0.87	—	/	/	/
Alanine	0.72	0.82	0.83	0.36±0.001c	0.40±0.002b	0.53±0.003a
Glutamate	0.99	0.99	—	0.90±0.004c	1.07±0.01b	1.41±0.01a
Glutamine	—	0.92	0.87	/	/	/
Lysine	0.87	0.84	—	0.19±0.001c	0.23±0.002a	0.22±0.002b
β-Alanine	-0.95	-0.96	—	0.37±0.002a	0.25±0.001c	0.27±0.002b
Glycine	—	0.94	0.98	0.16±0.001b	0.18±0.001a	0.09±0.001c
Histidine	-0.95	-0.92	—	7.80±0.03a	6.35±0.06b	6.34±0.05b
Phenylalanine	—	—	—	0.044±0.0002b	0.043±0.0003c	0.064±0.0004a
Tyrosine	—	—	—	0.074±0.0003b	0.070±0.0002c	0.091±0.0005a
Taurine	—	0.98	0.93	1.12±0.004c	1.18±0.01b	2.84±0.02a
Lactate	0.98	0.85	—	6.52±0.02c	11.07±0.07b	11.78±0.06a
Acetate	0.98	-0.93	-0.89	0.08±0.0003a	0.07±0.001b	0.06±0.0004c
Succinate	0.97	0.95	0.87	0.09±0.0002c	0.11±0.001b	0.13±0.001a
Creatine	-0.97	-0.98	—	5.25±0.02a	2.87±0.02b	2.78±0.06b
Creatinine	-0.99	-0.99	—	4.30±0.04a	0.54±0.002b	0.43±0.004c
Glucose	0.94	0.92	0.86	0.21±0.002c	0.28±0.003b	0.49±0.003a
Sucrose	0.79	-0.88	-0.72	21.90±0.07b	23.59±0.14a	21.44±0.11c
Phosphorylcholine	0.78	—	—	0.15±0.001c	0.17±0.001b	0.29±0.003a
Betaine	0.92	0.97	0.92	0.64±0.002b	0.61±0.004c	0.94±0.003a
Inosine	—	—	—	0.26±0.001a	0.21±0.002c	0.25±0.002b
5'-IMP	0.92	0.96	—	0.46±0.001c	0.79±0.003b	1.19±0.01a
Hypoxanthine	—	—	—	0.24±0.01c	0.32±0.01b	0.61±0.02a
Anserine	—	—	—	0.04±0.001a	0.01±0.001b	0.01±0.001b
Nicotinamide	—	—	—	0.05±0.0002c	0.12±0.001b	0.16±0.001a
Nicotinamide adenine dinucleotide (NAD)	-0.89	-0.95	—	0.58±0.003a	0.36±0.003b	0.28±0.004c

[α] The coefficients from OPLS-DA results, positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The coefficient of 0.602 was used as the cutoff value for the significant difference evaluation ($p < 0.05$). — The value of coefficient is lower than 0.602. [β] A, B, C denote broiler chicken breast extracts obtained from no WB, moderate WB and severe WB samples, respectively. [γ] The absolute concentration and standard deviation of mean (SD, mg/g the SMB extracts) were obtained from 10 parallel samples. / The absolute concentration was not determined due to signal overlapping. [a-c] Identical letters in the same row indicate that there was no significant difference in WB-affected chickens ($p > 0.05$).

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Figure 1

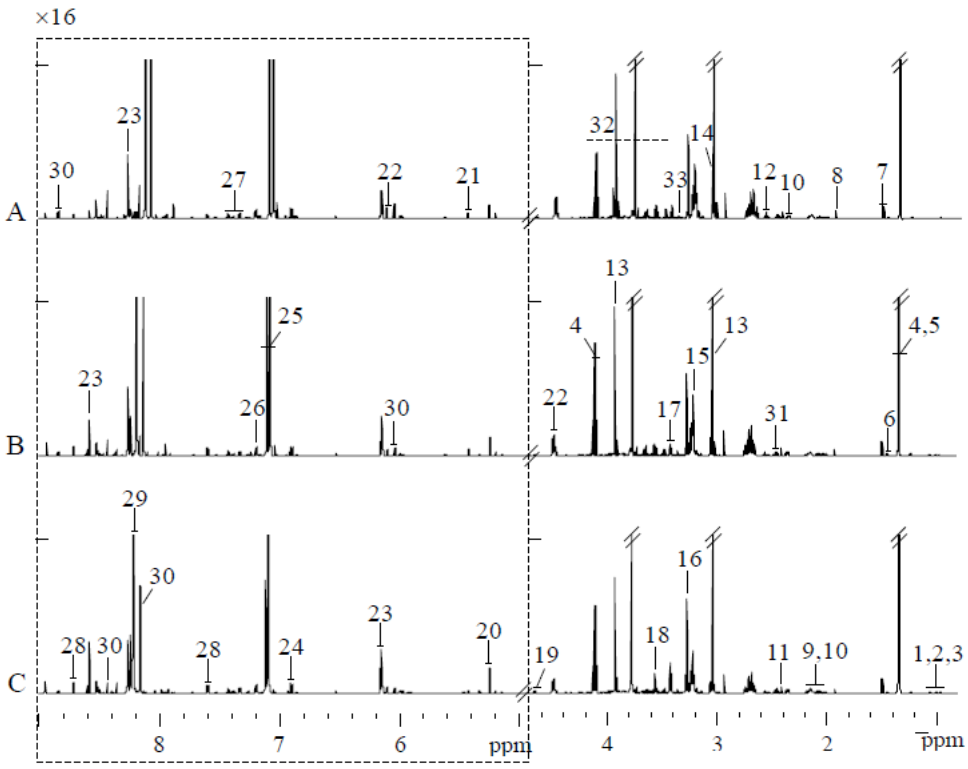

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Figure 2

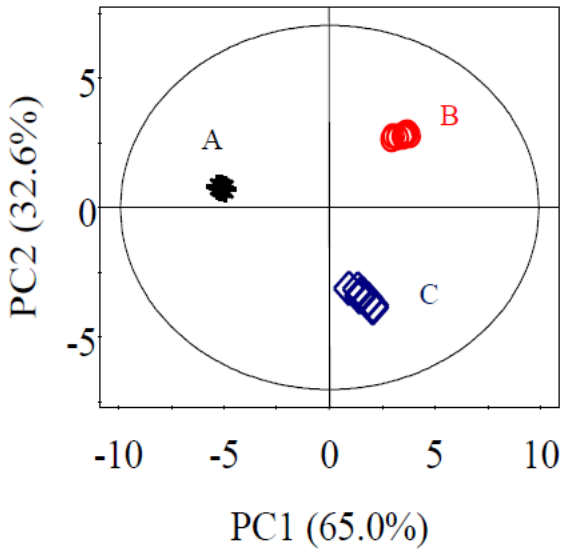

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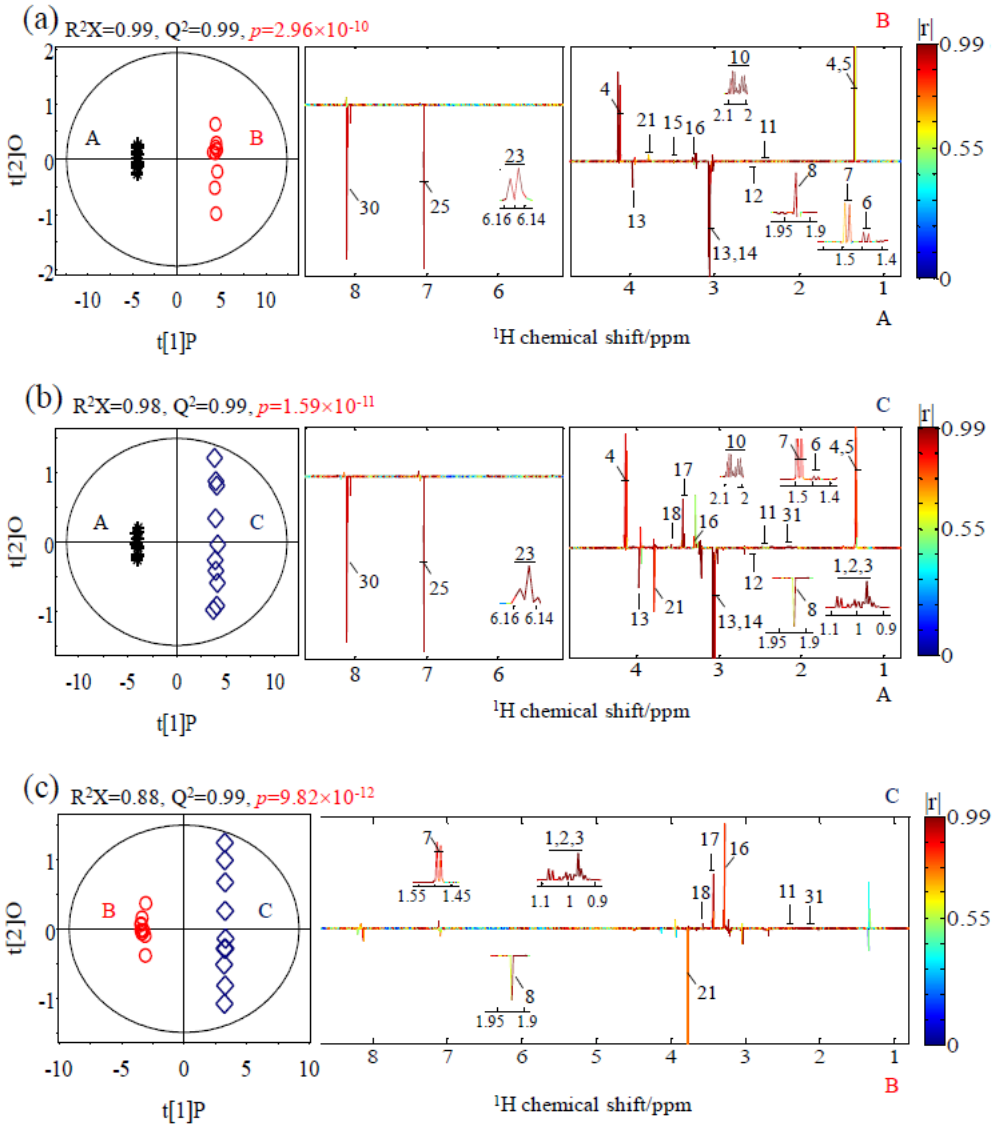
Figure 3


(a) R²X=0.99, Q²=0.99, p=2.96×10⁻¹⁰
(b) R²X=0.98, Q²=0.99, p=1.59×10⁻¹¹
(c) R²X=0.88, Q²=0.99, p=9.82×10⁻¹²
t[1]P
t[2]O
¹H chemical shift/ppm
|r|
0.99
0.55
0

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