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IDENTIFICATION OF METABOLITE PROFILE IN HALAL AND NON-HALAL BROILER CHICKENS USING FOURIER-TRANSFORM INFRARED SPECTROSCOPY (FTIR) AND ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY- TIME OF FLIGHT- MASS SPECTROMETRY (UHPLC-TOF-MS)

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ABSTRACT

In Malaysia halal meat is fully defined by Jabatan Kemajuan Islam Malaysia (JAKIM) based on the killing method. Halal meat is usually associated with quality as Muslim sought meat from animal killed using Islamic method. In order to ensure the food are meeting the Halal and thoyyiban aspects, the procedure must be monitored along the supply chains beginning from farm to fork. However, there are lack of studies on effect of slaughtering methods on chickens' metabolite profile. Therefore, metabolomics approach by Fourier-Transform Infrared Spectroscopy (FTIR) And Ultra High Performance Liquid Chromatography- Time of Flight- Mass Spectrometry (UHPLC-TOF-MS) are used in this study to understand the metabolite profile of chickens when subjected to different slaughtering process. The broiler chickens were subjected to Halal (Islamic tradition) and non-Halal slaughtering method (neck poking) where pectoral major muscle tissues from the slaughtered meat were selected for FTIR and UHPLC-TOF-MS analysis. Results from FTIR analysis showed Halal and non-Halal chicken displayed different spectra regardless time of extraction, which was 0 and 24 hours. Spectra obtained from UHPLC-TOF-MS were further analyzed for statistical analysis, which are Principal Component Analysis (PCA) and Partial Least-Squares Discriminant Analysis (PLS-DA) using MetaboAnalyst 4.0. PLS-DA model showed higher intensity of histidine and inosine was recorded in non-Halal chicken while Halal chicken has higher concentration of hypoxanthine. Result from this study indicates that method of slaughter affects the metabolite profile of chicken.

Key words: Broiler chicken, halal, non-halal, slaughtering, metabolite profile, FTIR, UHPLC-TOF-MS, PCA. PLS-DA

INTRODUCTION

Nutritious food encompasses the definition of Halalan thoyyiban according to Islamic jurisprudence. Halal is important in order to meet the quality and wholesomeness of the food produced. It needs a holistic approach in all aspects of ingredients, products, handling methods and halal logistics capabilities. It is interesting to note that the Halal market has accelerated over the past decade, with variety of product range such as food and personal care. In today's world market, it is expected that Muslim population will increase from 1.8 billion in 2014 to 2.2 billion by 2030, a growth of 26.4%. As the numbers illustrated a quarter part of world's population occupied by Muslim, therefore

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it is not a surprise that Halal market is going to be and indeed is currently have a significant and lucrative impact on international business. However, there are quite a number of issues regarding Halal as a slaughtering method. Disputes on various slaughtering methods arise when there is possibility that slaughtered meat did not meet the Halal requirement determined by Islamic jurisprudence.

Research regarding effect of slaughtering methods on chicken meat quality conducted by Hafiz *et al*., 2015; Salwani *et al*., 2015; Wong, 2014; Mohamed and Mohamed, 2012 concluded that slaughtering methods did affect the physical quality of chicken meats in term of pH, color, haem iron content, water holding capacity, lipid oxidation, mineral content and texture. However, there are lack of studies on metabolite profile of broiler chickens when subjected to Halal and non-Halal slaughtering methods. Therefore this study aims to elucidate the effect of Halal and non-Halal slaughtering method on metabolite profiles of broiler chickens.

MATERIALS AND METHOD

Ethics statement

This research was carried out in strict accordance with the recommendations. The full procedure, including consent protocol, was approved by the Animal Ethics Committee of Universiti Sains Islam Malaysia, after specific consideration of the ethical factors relating to the animals involved.

Research methodology

The research was carried out at Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM), Nilai. Two groups of male chickens, (*Gallus gallus domesticus*) (Halal and non-Halal slaughtering method), aged 60 days with approximately the same weight (2 kilogram) were purchased from local farm in Kajang. Each group was represented by 3 chickens. Tissues of pectoral major muscle were snap frozen in liquid nitrogen and were stored at -80°C until extraction.

Halal slaughtering method

The chickens were slaughtered according to the Islamic traditions where the jugular veins, carotid arteries, trachea and esophagus of the chickens were severed. No stunning was used while performing this method.

Non-halal slaughtering method

A sharp pointed object was used to poke the neck of the chicken to create a small hole for blood drainage and the chickens were immediately drowned in water. Once slaughtered, chickens were quickly processed.

Metabolic extraction

Metabolite extraction was carried out by using the method described by Wu *et al*. (2008) with modifications. Tissue of pectoralis major muscle was homogenized to a fine powder by rapid freezing in liquid nitrogen. The freeze-dried chicken powder (500 mg) was homogenized in 1ml of cold methanol/chloroform/water (2.5:1:1). Supernatants were collected in Eppendorf tubes (named Tube A) after vortex and centrifuged. The remaining pellets were homogenized in 500 µL of methanol/chloroform (1:1). Samples were centrifuged and supernatants were transferred into Tube A. Supernatants in Tube A were added with 250 µL distilled water. Samples were then vortexed, left on ice for 10 min and centrifuged for 10 min at 14000 rpm at room temperature. Aqueous and organic layer was collected in separated 1.5 mL centrifuge tubes (Eppendorf) and stored at -80°C until further analysis. Aqueous layer was used for FTIR and UHPLC-TOF-MS analysis.

Fourier-transform infrared spectroscopy (FTIR) spectral region analysis

The IR data of all the chicken metabolites obtained from FTIR was compared using the IR software Spectragryph version 1.2.8. Nicolet iS50 FTIR Spectrometer was used to obtain full spectrum in the mid-infrared region (650-4000 cm^{-1}). The number of scans was set to 32 with a resolution of 4 cm^{-1}. Measurements were calibrated against the background air. The overall FTIR spectrum corresponds to stretching of functional groups and fingerprint groups that are present in chickens' metabolites.

Ultra high performance liquid chromatography-time of flight- mass spectrometry (UHPLC-TOF-MS) analysis

Untargated metabolomics profile of Halal and non-Halal chicken samples were carried out using UHPLC-TOF-MS (Waters, UK) at Lembaga Koko Malaysia, Nilai. Specifications include column ACQUITY UPLC HSS T3 (100 mm × 2.1 mm × 1.8 µm) also from Waters with temperature of 40°C, injection volume of 1 µL and flowrate of 0.6 mL/min. A linear binary gradient of water (0.1% formic acid) and acetonitrile (mobile phase B) was used as mobile phase A and B respectively. The mobile phase composition was changed during the run as follows: 0 min, 1% B; 0.5 min, 1% B; 16.00 min, 35% B; 18.00 min, 100% B; 20.00 min, 1% B. A Vion IMS QTOF hybrid mass spectrometer from Waters (Manchester, UK) was used to obtain MS data. Analysis was performed in negative ion mode

with the following settings: capillary voltage, 1.50 kV; reference capillary voltage, 3.00 kV; source temperature, 120°C; desolvation gas temperature, 550°C; desolvation gas flow, 800 L/h, and cone gas flow, 50 L/h. Data was acquired in high-definition MS^E ($HDMS^E$) mode in the range m/z 50–1500 at 0.1 s/scan.

Data processing and statistical analysis

MS data was combined, background-subtracted and aligned using Analyst Software (http://sciex.com/). Output data for all samples were binned using MALDIquant R package (Gibb & Strimmer, 2012). Principle Component Analysis (PCA) and Partial Least Square-Discriminant Analysis (PLS-DA) was performed using MetaboAnalyst 4.0. Variable importance for prediction (VIP) scores was also calculated from the PLS-DA.

RESULTS AND DISCUSSION

Fourier-transform infrared spectroscopy (FTIR)

Halal and non-Halal chicken meat samples were analyzed through FTIR spectrophotometer in the mid infrared range and peaks were obtained ranging from 4000 to 650 cm^{-1}. FTIR analysis was carried out to investigate the effect of extraction time on metabolite profile of chicken meat. The analysis was conducted as a preliminary test to determine whether the metabolite is still retained or are there any changes that occurred when the metabolite is extracted at different time which are 0 hr and 24 hr after slaughtered. Figure 1 presents variations in the peak absorbance analyzed through FTIR for metabolite profile of Halal and non-Halal chicken samples (0 hr and 24 hr). Deviations in the spectral results have been presented in Figure 1.

Metabolite profile of Halal chicken meat (extracted at 0 hr and 24 hr) showed very similar spectra (Figure 1). Similar result was observed in non-Halal chicken meat where similar spectra were recorded either the metabolite extracted at 0 hr or 24 hr (Figure 1). Variations in the absorbance and position of peaks from Halal and non-Halal chicken meat were prescribed by the changes in the band stretching of functional groups. Generally, both Halal and non-Halal chicken meat either extracted at 0 or 24 hr showed strong peaks at the wavelengths of 3293 cm^{-1} and 1637 cm^{-1} and several weak peaks at 2154 cm^{-1} and 1400 cm^{-1}, where these peaks usually correspond to hydroxyl group, amide, alkyne group, and lipids due to O-H stretching, C-N stretching, C≡C stretching and C-H stretching, respectively. It is interesting to highlight that metabolite profile of Halal and non-Halal chicken

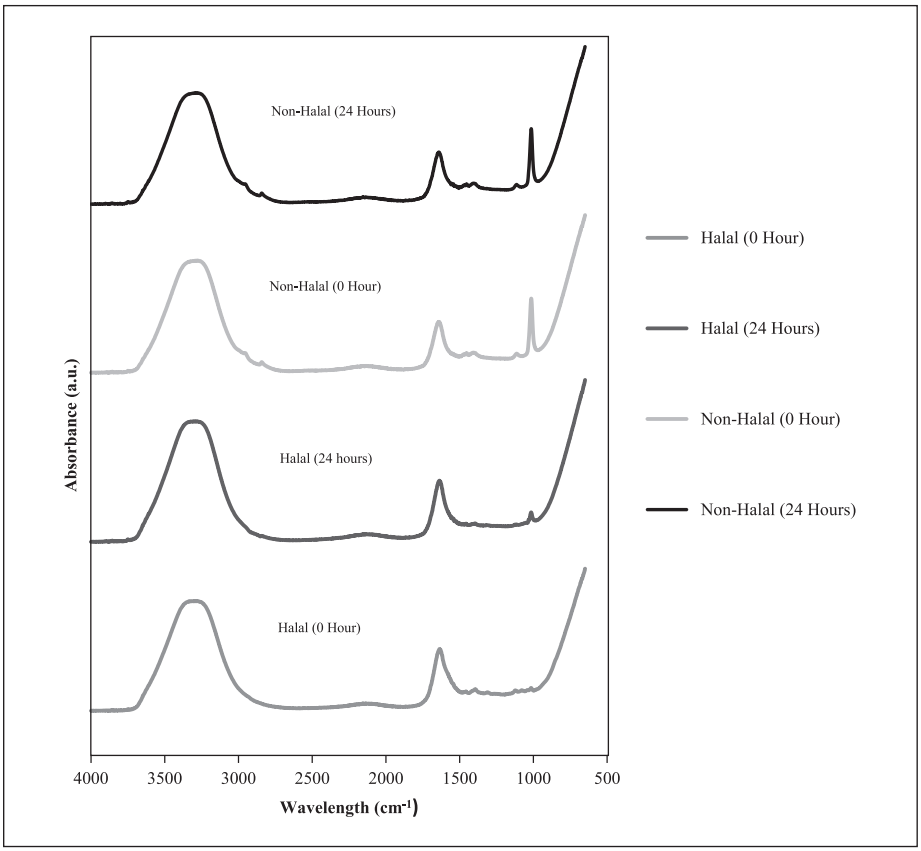

Fig. 1. FTIR spectra of metabolite profile for Halal and non-Halal chickens (0 and 24 hr).

meat showed different spectra regardless time of extraction (Figure 1), where Non-Halal spectra (0 and 24 hr) showed an increase of absorbance at wavelength 1014 cm^{-1}. This peak usually corresponds to aromatic compound due to C-H bending of aromatic compound. The findings of the current investigation revealed that extraction time did not affect metabolite profiles for each Halal and non-Halal chicken meat. This result also highlighted that Halal and non-Halal chicken meat exhibited different metabolite profiles either at 0 or 24 hr after slaughtered.

Liquid chromatography-time of flight-mass spectrometry analysis

UHPLC-TOF-MS system coupled with an ESI was used to obtain metabolomics data of Halal and non-Halal chicken meat. The data was displayed in UHPLC-TOF-MS spectra (BPI Counts vs Retention time, Figure 2 and 3. Statistical analysis has been performed using MetaboAnalyst 4.0. Principal components analysis (PCA), an unsupervised method was applied to simplify high-dimensional data into a small number of principal components and also to publish strong patterns and trends in

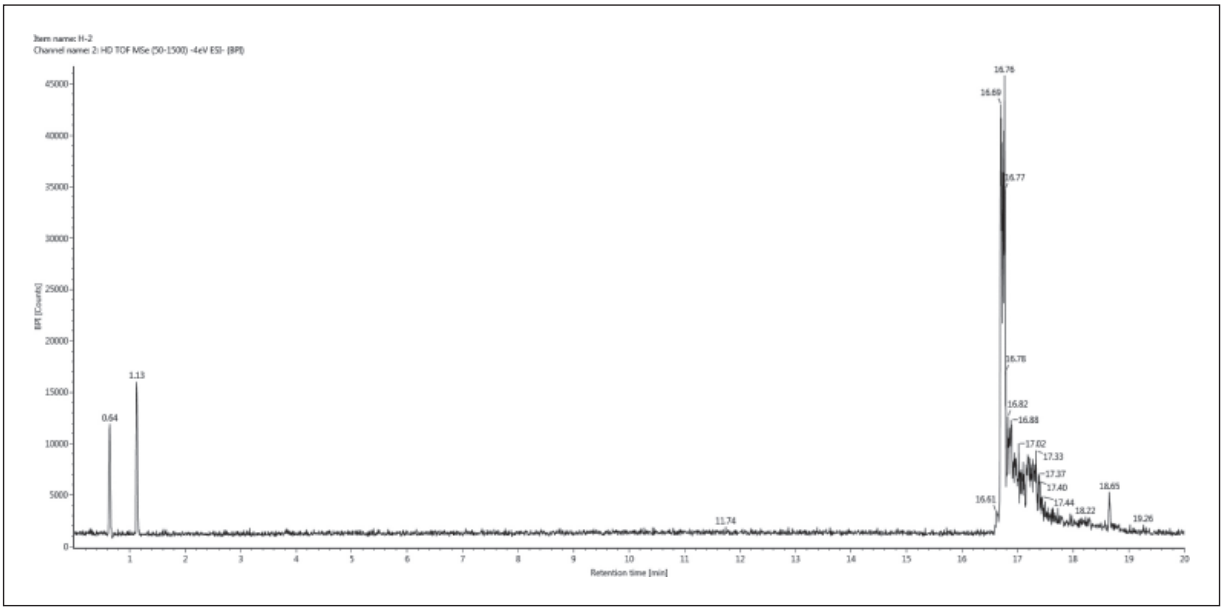

Fig. 2. UHPLC-TOF-MS spectrum of Halal chicken pectoral muscle tissue aqueous extract.

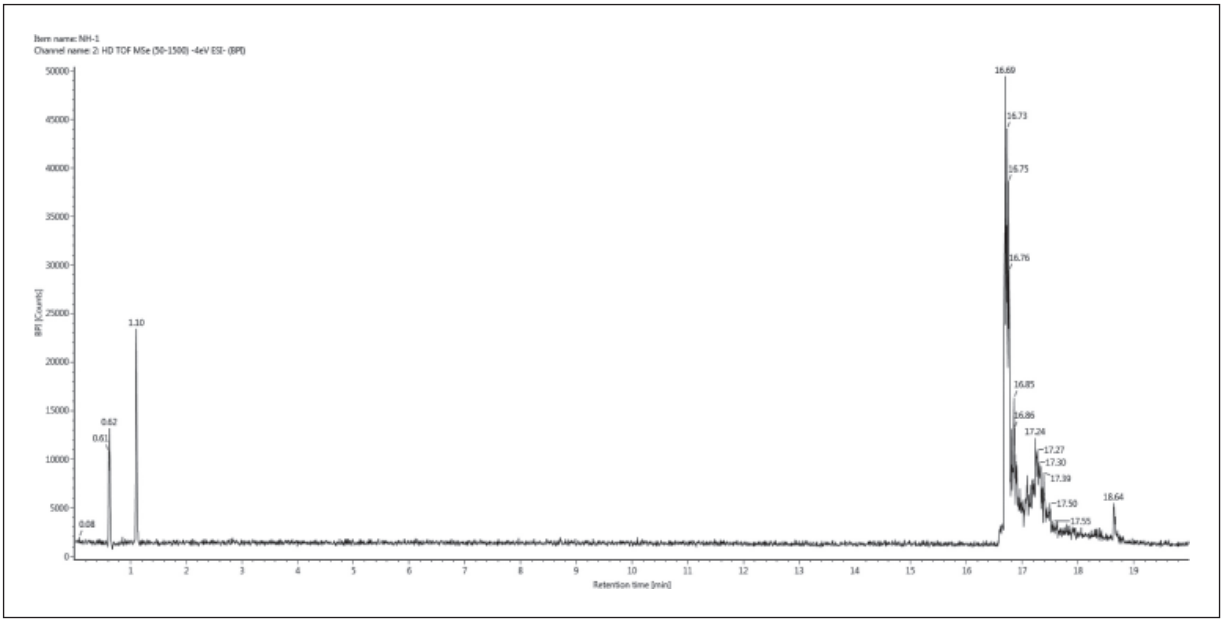

Fig. 3. UHPLC-TOF-MS spectrum of non- Halal chicken pectoral muscle tissue aqueous extract.

data set. The differences between UHPLC-TOF-MS spectral data were explored further by using partial least-squares discriminant analysis (PLS-DA), a supervised method.

Principal component analysis

Principle component analysis (PCA) was applied to dataset in order to reduce its multi-dimensional. PCA method gives advantage in determining natural clusters in data set where the first principal component (PC1) illustrates the largest level of variation followed by the second component (PC2), which is useful in describing the second most important factor of remaining analysis (Sahar & Dufour, 2014). In this analysis, the score plots obtained from PCA are useful in elucidating the differences and similarities between metabolite profiles of Halal and non-Halal chicken. The similarity maps resulted from applying PCA on UHPLC-TOF-MS spectra showed that PC1 and PC2 accounted for 65.8% of the total variance (Figure 4). Besides, the categorization of metabolite profile for Halal and non-Halal chickens presented that PC1 and PC2 predicted 44.6% and 21.2% of total variance respectively (Figure 4). Analyzed samples that showed closeness in the score plot illustrates the similarity between samples with respect to the evaluated principal component score. From the result obtained, it showed that one of the non-Halal biological replicates is similar to Halal chicken. This might be due to variables in the biological samples, thus increasing number of samples is suggested to reduce the error. PCA also highlighted the capability of FTIR as an approach in grouping Halal and non-Halal chickens.

Partial Least-Squares Discriminant Analysis (PLS-DA)

A PLS-DA model was obtained from Metabo Analyst 4.0 and result showed non-Halal chicken had higher intensity of inosine and histidine while Halal chicken showed higher intensity of hypoxanthine (Figure 5).

Amino acid metabolism

One of energy source in poultry is amino acid. Amino acid also functions as substrates for proteins biosynthesis and physiological messengers in the biological system (Yamane *et al*., 2009). There are two groups of amino acids, which are ketogenic amino acid and glucogenic amino acids. The categorizations are based on byproducts produced after hydrolization of amino acid. Amino acid that are hydrolyzed to acetyl CoA or acetoacetyl CoA fall into ketogenic amino acid group due to its capability to elevate ketone bodies or fatty acid. Amino acids that degrade to pyruvate, a-ketoglutarate, succinyl CoA, fumarate or oxaloacetate are termed as glucogenic amino acids (Yamane *et al*., 2009). Both hydrolyzation processes leads to energy production in poultry.

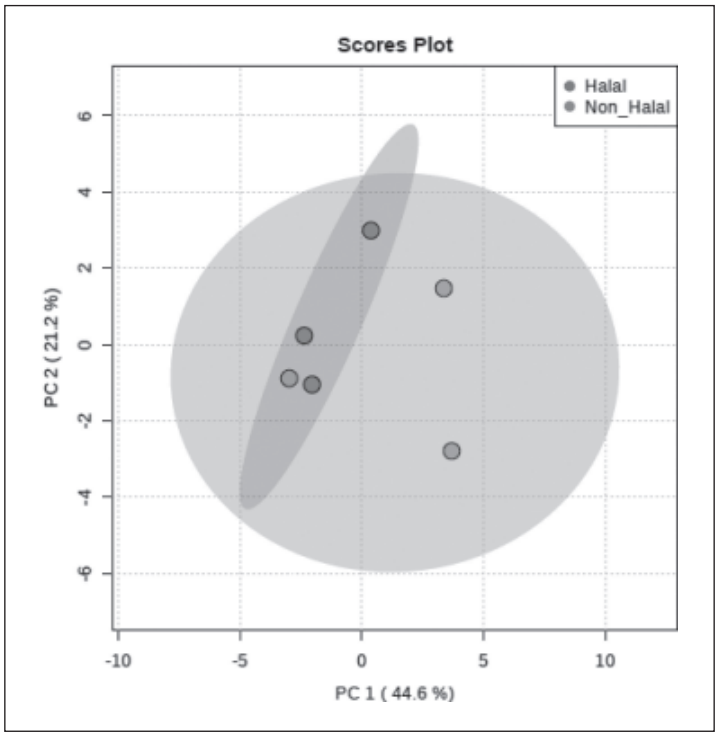

Fig. 4. Scores plot between the selected Principal Components.

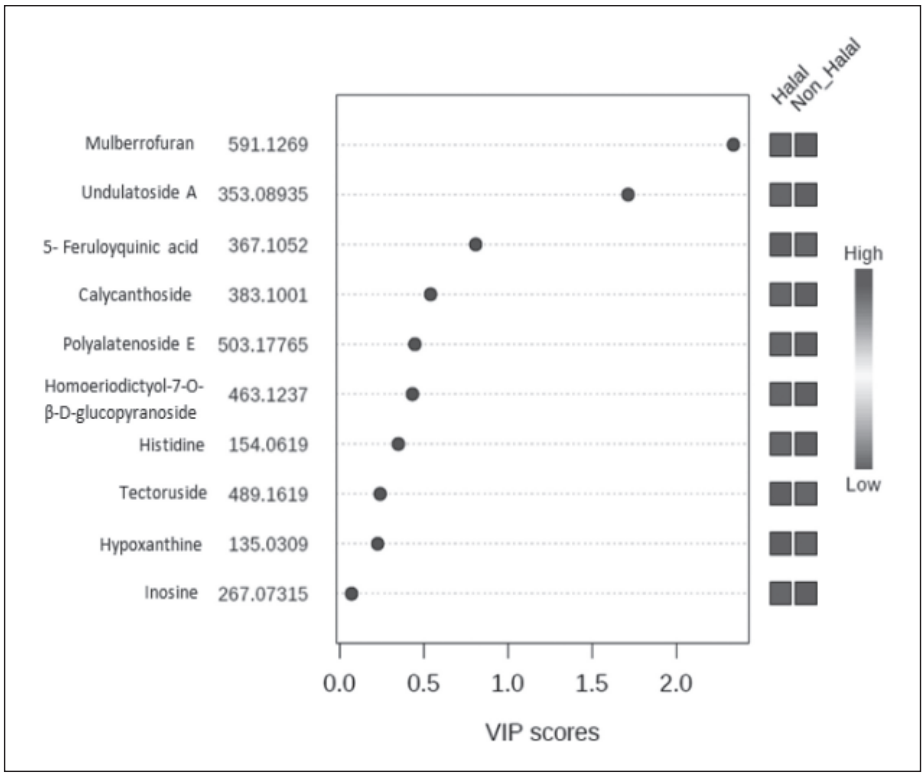

Fig. 5. Important features identified by PLS-DA.

In poultry, histidine is categorized as essential amino acid as well as lysine and arginine since the animal cannot produce these compounds endogenously (Jun *et al*., 2017). These amino acids play their roles in several metabolite pathways. A metabolite role of arginine is exerted through the byproduct production including nitric oxide (NO), L-ornithine (L-Orn), polyamines, proline, glutamate, creatine and agmantine (Morris, 2004). Histidine is degraded to histamine by the function of enzyme histidine decarboxylase and also histidine is degraded to glutamate (Stifel & Herman, 1971).

PLS-DA model showed lower Halal chicken recorded histidine intensity. Due to increased metabolic demands during Halal slaughtering, the chicken likely increases breakdown of amino acids for energy. Therefore, this study suggests that Halal slaughtering process resulted in lower concentration due to an increase catabolization of this amino acids in order to provide energy substrates for the cell as the halal chicken took more time to die while non-Halal chicken is directly drown which it will die faster, thus less energy usage.

Inosine metabolism

It was observed that inosine metabolism of chicken was affected by different slaughtering method. PLSA-DA model (Figure 5) showed higher concentration of inosine in non-Halal chicken as compared to Halal chicken. Lower concentration of hypoxanthine was recorded in non-Halal chicken. Inosine is a metabolite produced from ATP degradation, which can be transformed to hypoxanthine and then released into blood circulation (McConell *et al*., 2005; Bishop, 2010). This study suggests that ATP in the muscle could have been catabolized in Halal chicken to supply energy within the cell as indicated by the higher concentration of hypoxanthine recorded. The increase in energy consumption was observed as shown by lower value of inosine in Halal chicken and the high concentration of hypoxantine as shown by the PLS-DA model (Figure 5).

It is interesting to note the role of inosine and its byproducts in flavor profile quality of poultry. Zhu and Lu (1996), stated that amino acids and inosine monophosphate (IMP) act as the major compounds that enhance and contribute to meat flavor. IMP is said to be an indicator for meat flavor as it plays a major role in this aspect (Suzuki *et al*., 1994; Fuiymura, 1998). Hall (1964) stated that hypoxanthine, inosine, IMP and its corresponding nucleoside and base are compounds that are usually found in meat and these compounds are derived from the degradation of adenosine triphosphate (ATP). Therefore, ATP degradation seems as a desirable metabolic activity due to its capability in contributing to flavor quality of meat.

CONCLUSION

Different metabolite profile of Halal and non-halal chickens were obtained from FTIR and UHPLC-TOF-MS analysis. Result from UHPLC-TOF-MS analysis illustrated the metabolomics activity of amino acid breakdown, inosine hydrolyzation as well as hypoxanthine production in broiler chickens when subjected to different slaughtering methods. Halal slaughter resulted in higher utilization of energy as indicated by the elevated amino acid and inosine breakdown and this give a different metabolite profile from non-Halal chicken. Results from this study revealed that slaughtering method affects the metabolite profiles of chicken. These findings from the analysis also emphasized the effectiveness of FTIR and UHPLC-TOF-MS as a reliable and comprehensive approach for the grouping of Halal and non-Halal chicken.

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