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Abstract

Beef is a common staple food in many countries, and there is a growing concern over misinformation of beef products, such as false claims of origin, species and production methods. In this study, we used a mass spectrometry-based metabolomics approach to study the metabolite profiles of beef samples purchased from local retailers in Hong Kong. Using multivariate analysis, beef samples from different a) geographical origins, namely the United States (US), Japan and Australia, and b) feeding regimes could be differentiated. We identified twenty-four metabolites to distinguish beef samples from different countries, ten metabolites to identify Angus beef samples from others and seven metabolites to discriminate Australian beef produced by the organic farming from that produced using other farming modes. Based on results of this study, it is concluded that metabolomics provides an efficient strategy for tracing and authenticating beef products to ensure their quality and to protect consumer rights.

Keywords:

Beef; Foodomics; Geographical origin; Lipidomics; Mass-spectrometry;

1. Introduction

As the third most widely consumed meat (Bijlsma et al., 2006; FAO, 2003), beef constitutes a major dietary component in many parts of the world and is traded internationally. It has a high protein content and is a good source of essential vitamins (e.g. vitamin B) (Wood, 2017) and minerals (e.g. iron) that are frequently deficient in our diet (Ames & Wakimoto, 2002; Pighin et al., 2016). Beef imports in China have grown by nearly 24% in the past 5 years (ResearchAndMarkets, 2018). Beef and veal trade also have been projected to have a steady growth of about 1% per year over the next ten years. (OECD/FAO, 2018). By 2027, it has been projected that the price for beef would increase to USD 4000/t carcass weight equivalent (c.w.e.) (OECD/FAO, 2018).

Despite the steady growing demands, international beef trade has shown substantial volatility in the past few decades. For example, the feeding of meat-and-bone meal contaminated with scrapie to cattle had resulted in a bovine spongiform encephalopathy (BSE) epidemic in cattle in the United Kingdom. In March 1996, there were ten reported cases of the rare but lethal Creutzfeldt Jakob disease in humans after their exposure to beef from BSE–infected cattle, triggering a serious consumer confidence crisis in beef products from the United Kingdom (Anderson et al., 1996). In December 2003, a single dairy cow was found to be infected with BSE in the United States (US), which resulted in 53 countries banning beef imports from the US and an estimated loss of USD 3.2–4.7 billion in 2004 (Coffey, Mintert, Fox, Schroeder & Valentin, 2005). Beef exports for international trade have recovered after the BSE incidents, with notable increases in export volumes from countries such as Australia, Brazil and Argentina. During the absence of US beef in China in the BSE era, Australia gained a large share of the Chinese beef market. However, most Australian beef was grass-fed, and was thus unable to fully capture the market share that the US producers had lost due to meat quality demand (Fields, Therrien, Halstrom, Haggard & Clayton, 2018). An efficient traceability platform or system may greatly reduce the duration, spread and the adverse economic impact of disease outbreak or other food safety incidents.

Beef of different qualities, cuts and geographical origins may have substantially different economic values. Economically motivated food fraud is increasingly subject to public scrutiny. Food products with high commercial values are very often targeted for fraud and adulteration, such as the substitution of an expensive product with a cheaper product, incorrect labelling, and use of illegal additives. For instance, a 2013 report revealed a widespread food fraud of inclusion of horse meat in beef products (e.g. frozen burgers) (O'Mahony, 2013). Although meat from different species can be easily detected using DNA-based techniques, the mixing of meat from different geographical origins is more difficult to detect. Untargeted approaches, such as mass spectrometry (MS)- and nuclear magnetic resonance (NMR)-based metabolomics, have been suggested as a promising strategy for this purpose (Sentandreu & Sentandreu, 2014). Scientific expertise and technologies are constantly being developed to advance the traceability and authentication of food products. The public is increasingly concerned about the origin and authenticity of their food not only for safety reasons but also for economic and quality reasons (Henchion, McCarthy & Resconi, 2017). For instance, the beef industry in the developed countries is facing increased demand for natural meat, driven in part by public concern on the environmental impacts of farming, animal welfare and meat quality. A consumer preference for organic beef has emerged recently because organic beef is considered to be safer as it is

free of antibiotics. Grass-fed beef contains less saturated fat and more omega-3 fatty acid, and is considered to be a healthier choice (Deckelbaum & Torrejon, 2012; Klek, 2016).

The geographical origin of beef is most commonly determined using a genomics approach, stable isotope ratio analysis and multi-elemental analysis. For example, 24 cattle breeds from seven member states of the European Union (France, Denmark, Italy, the Netherlands, Switzerland, Spain and the UK) were assigned correctly to their geographical origin with a success rate of 90% by using single nucleotide polymorphisms (SNP) (Negrini et al., 2008). Meanwhile, Mannen used mitochondrial DNA to classify Wagyu cattle from Japan (Mannen, 2017). Although these studies demonstrated the utility of the genomics approach in determining the geographical origin of beef, no studies have compared DNA profiles among beef from different countries. Therefore, it is unknown whether, for instance, Japanese beef can be accurately differentiated from European beef by using only molecular markers. To this end, in China, researchers have successfully distinguished beef produced from Shandong, Heilongjiang, Yunnan and the Tibet Autonomous Region using carbon isotope ratio, but not the nitrogen isotope ratio (Zhao, Zhang, Guo, Wang & Yang, 2016). Moreover, elemental profiles obtained from elemental analyzer continuous flow isotope ratio MS could distinguish beef from four out of five countries, namely Australia, Brazil, Canada and the US, but misclassification between Brazilian and Swiss beef was observed in the external validation set (Franke, Haldimann, Gremaud, Bosset, Hadorn & Kreuzer, 2008). One major limitation of these approaches is that although the methods may be able to identify the geographic sources of beef, no information is provided on their quality.

Metabolomics focuses on the measurement of metabolites and identifies changes in metabolites as a result of genetic, environmental or dietary factors. (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk & Rodrick, 2009; Cubero-Leon, Peñalver & Maquet, 2014). This approach determines comprehensive metabolite profiles that are not only valuable for identifying the geographical origin of beef, but also important in understanding how these factors associated with the nutritional values, quality, and flavor of beef. Thus, metabolomics provides an efficient technology platform that offers the gathering of essential data for an effective food traceability system.

MS- and NMR-based metabolomics are new popular techniques that have been developed and used in the study of food safety, quality and traceability (Capozzi & Bordoni, 2013; Hu & Xu, 2013). For example, Jung et al. (Jung, Lee, Kwon, Lee, Ryu & Hwang, 2010) used proton NMR to identify beef samples that originated from Australia, Korea, New Zealand and the US. Carrillo et al. (Carrillo et al., 2016) used integrated metabolomics and transcriptome analysis to characterize the differences between grass- and grain-fed Angus steer. Finally, Kodani et al. (Kodani, Miyakawa, Komatsu & Tanokura, 2017) used NMR data to evaluate the degree of unsaturation in triacylglycerol and fatty acid as well as the ageing duration of Japanese Black cattle.

Metabolomics studies can be divided into two general approaches, targeted and untargeted analysis. The targeted approach focuses on identifying and quantifying a number of small subset of known metabolites, whereas the untargeted approach aims at acquiring as many metabolites as possible, annotating metabolites and reviewing changes in quantity (Hu & Xu, 2013). Beef is a complex matrix with thousands of metabolites that may be affected by species, flavour, nutrient, production and storage. Therefore, the untargeted approach has the advantage

of providing a fuller picture of the relationship among these characteristics. In addition, the sample preparation of the untargeted approach are relatively simple and highly reproducible profiles with broad coverage of metabolites could be obtained. (Castro-Puyana, Pérez-Míguez, Montero & Herrero, 2017).

In the present work, we developed an untargeted metabolomics approach, including both ultra-high-performance liquid chromatography-Orbitrap-mass spectrometry (UPLC-Orbitrap-MS) and gas chromatography-mass spectrometry (GC-MS) analytical platforms, to evaluate the geographical origin and species of beef as well as to differentiate beef produced from different feeding regimes. The entire metabolomics platform was further validated by analyzing its linearity, accuracy, method precision, limit of quantification (LOQ) and limit of detection (LOD). The overall objective of this study was to provide a more comprehensive metabolite map for the identification of potential biomarkers associated with the geographical origin and production conditions of beef. We believe this method will be particularly useful in end-user markets, such as Hong Kong, where information on beef production and storage may not be always fully available or verifiable. Using the developed method, we tested beef purchased in a local Hong Kong market that originated from Australia, the US, and Japan (the top three providers of chilled beef in Hong Kong). The prices of beef from these three countries differ considerably. In 2017, Australia supplied 49.4% of Hong Kong's imported beef, which accounted for 41.4% of total beef sales value, the US supplied 26.6%, which accounted for 27.6% of the total sales value, and Japan supplied only 7.3% but accounted for 19.9% of the total sales value (H.K.S.A.R. C&SD, 2018).

2. Materials and methods

2.1 Reagents

High-performance liquid chromatography (HPLC)-grade acetonitrile, chloroform, isopropanol and methanol were purchased from Duksan Pure Chemicals (Gyeonggi-do, South Korea) while HPLC-grade formic acid was commercially obtained from VWR (Radnor, PA, USA). Double-ionized water was freshly prepared using a Milli-Q water-purification system (Millipore, Bedford, MA, USA). Deuterated cholic acid (2,2,4,4-d4) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

L-alanine, ammonium formate, L-aspartic acid, cis-11-eicosenoic acid (FA 20:1), galactose, glucose, glutamic acid, L-glycine, L-isoleucine, 2-isopropylmalic acid, L-leucine, L-methionine, methoxyamine hydrochloride, ornithine, proline, palmitic acid (FA 16:0), L-serine, stearic acid (FA 18:0), threonine, tyrosine, and L-valine were commercially obtained from Sigma-Aldrich (St. Louis, MO, USA). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and pyridine were purchased from Acros Organics (Morris Plains, NJ, USA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC (16:0/18:1)) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE (16:0/18:1)) were commercially obtained from Avanti Polar Lipids (Alabaster, AL, USA).

2.2 Sample collection

Thirty-nine well-labelled imported beef samples from Australia (n= 20), Japan (n= 5) and the US (n= 14) were purchased from local retailers in Hong Kong (supplementary material, Figure S1). The samples were stored at 4°C during transportation to the laboratory. The samples were separated into different portions and stored in a -80 °C freezer until use. All beef samples were labelled with information such as country of origin, species, feeding regimen, sample weight and price. This information is summarized in the supplementary material (Table S1). The samples from Australia were further classified into organic beef (n=8), Angus beef (n= 4), Wagyu beef (n= 3) or conventional beef (n= 5) according to feeding and breeding.

2.3 Metabolomics analysis

2.3.1 Quality control sample preparation method

For quality assurance and quality control purposes, a small portion of all individual beef samples were pooled and mixed to form a pooled quality control (QC) sample which was stored at -80°C until use. For each analytical batch, a small sample (hereafter, QC samples) was taken from the pooled QC sample and subject to sample preparation in the same manner as all other samples, as described in Section 2.3.2. Before chemical analysis, five repeated injections of the same QC sample were used to verify the working condition of the instruments. In addition, a QC sample was injected to monitor the stability of the instruments after every four sample runs.

QC samples were also used to optimize the sample preparation. Due to the wide chemical diversity of metabolites, the sample extraction procedure employed herein used minimal sample treatment and was expected to be non-selective in order to reveal as much information as possible. Different extraction solvent systems and sample-to-solvent ratios were compared and optimized so as to detect the maximum number of metabolites in beef samples.

2.3.2 Sample preparation

Around 20 g of each beef sample was homogenized, from which a 100 ± 5 mg sample was obtained and stored at -80 °C immediately to inhibit enzyme activities until extraction. A modified Bligh and Dyer's liquid-liquid extraction (LLE) method (Bligh & Dyer, 1959; Dunn et al., 2011) was used to study the lipids and small molecules in beef. Briefly, 800 µL pre-chilled homogenization solvent (50% v/v chloroform: 50% v/v methanol) and beef samples were mixed into a glass test tube, which was then homogenized for 1 minute using an Ultra Turrax T10 homogenizer (IKA, Wilmington, NC, USA). For liquid-liquid extraction, the same volume of double-deionized water was added, and the sample was vortex mixed for 30 s, and then centrifuged at 8000 rpm at 4 °C for 10 min. Next, 200 µL of the organic layer and 200 µL of the aqueous layer were transferred to a glass test tube and a silanized vial, respectively. The aqueous and the organic layer, which contained 100 ppm internal standard (IS) of 2-isopropylmalic acid and 1 ppm IS of cholic acid-d_4, respectively, were then evaporated until dryness under a nitrogen stream. Both dried layers were immediately stored at -80 °C until UPLC-Orbitrap-MS and GC-MS analysis.

For the lipid profile analysis, the dried organic layer was re-dissolved in 150 µL solvent (65% v/v acetonitrile: 30% v/v isopropanol: 5% v/v water, pre-chilled in an ice water bath). After centrifugation at 14000 rpm at 4 °C for 15 min, the supernatant was transferred into an HPLC vial for UPLC-Orbitrap-MS analysis.

For the aqueous profile analysis, two-stage silylation (Dunn et al., 2011) was chosen to derivatize small non-volatile metabolites. The dried aqueous layer was re-dissolved in 75 μL methoxyamine hydrochloride in pyridine (15 mg/mL) under nitrogen protection and subsequently shaken at 30 °C for 1.5 h. After adding 75 μL MSTFA, the mixture was incubated at 70 °C for 1 h, then cooled to room temperature and diluted by adding 150 μL pyridine. This solution was injected into the GC-MS system.

2.3.3 UPLC-orbitrap-MS data acquisition

For UPLC-Orbitrap-MS analysis, a Thermo Orbitrap Fusion Lumos Tribrid Mass Spectrometry (Thermo Fisher Scientific, Waltham, MA, USA) was connected to a Waters ACQUITY UPLC System (Waters Corp., Milford, USA) via heated electrospray ionization (H-ESI) as the interface. The separation was performed on a Waters ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8μm) with an HSS T3 pre-column (2.1 mm × 5 mm, 1.8μm) at 40 °C. Based on Cajka and Bird's study (Cajka & Fiehn, 2016), a gradient elution of solvent A (60% v/v water: 40% v/v acetonitrile containing 10 mM ammonium formate and 0.1% formic acid) and solvent B (90% v/v isopropanol: 10% v/v acetonitrile containing 10 mM ammonium formate and 0.1% formic acid) was applied with a modified elution program as follows: 0–1.5 min, 30% B; 1.5–8 min, 30–65% B; 8–10 min, 65–70% B; 10–14 min, 70–75% B; 14–17 min, 75–97% B; 17–21 min, 97% B; 21–24 min, 97–30% B; 24–25 min, 30% B. The flow rate was 0.3 mL/min, and the injection volume was 3 μL. The sample chamber temperature was 4 °C. The H-ESI-MS spectra were acquired in both positive and negative ion modes. The H-ESI parameters were as follows: Spray voltage, 3600V for positive ESI and 2300V for negative ESI; sheath gas, 35 arbitrary units; nebulizer auxiliary gas, 20 arbitrary units; sweep gas, 0 arbitrary units. General instrumental parameters were set as follows: ion transfer tube temperature, 350°C; vaporizer temperature, 200°C. For full scan MS, the mass range was set at 100 - 2000 *m/z* with 120,000 mass resolutions. The automatic gain control (AGC) target was set as 2.0×10^5 with a maximum injection time of 100 ms.

2.3.4 UPLC-orbitrap-MS data pretreatment and analysis

The UPLC-orbitrap-MS data obtained in both positive and negative ion modes were pretreated using Progenesis QI (version 2.3; Nonlinear Dynamics) for peak picking and peak alignment. The data matrices were imported into Matlab (MathWorks, Natick, MA, USA) for further processing. Data with a high missing rate (>20% in control) were excluded from subsequent analysis because of unreliable missing value imputation (Wei et al., 2018). Batch correction was then performed by smoothing through QC samples in the injection sequence using cubic spline smoothing to align systemic variations at different injection times (Van der Kloet, Bobeldijk, Verheij & Jellema, 2009). The resulting data were filtered to remove unstable signals with a coefficient of variation (CV%) > 30% across the QC samples.

2.3.5 GC-MS data acquisition

For GC-MS analysis, an Agilent 6890N GC/5975C VL MSD system (Agilent Technologies, Inc., Santa Clara, CA, USA) was connected to an Agilent 7683 Automatic Liquid Sampler. The separation was performed on a HP-5MS column (30 m × 0.25 mm, 0.25 μm; Agilent J&W Scientific, Folsom, CA, USA). The temperature of the inlet was set at 250 °C. In the split-less mode, a 1μL aliquot was injected in helium at a constant flow rate of 1.0 mL/min. The temperature program optimized for GC was as follows: initial oven temperature as 70 °C, held

for 1 min; 4 °C /min to 100 °C; 5 °C /min to 200 °C; 30 °C /min to 250 °C; 250 °C held for 5 min. The MS parameters were as follows: solvent delay, 5 min; ionization energy, 70 eV; temperatures of the ion source and transfer line, 230 °C; full scan mode in *m/z* range 70–550.

2.3.6 GC-MS data pretreatment

The GC-MS raw data were pretreated using the AMDIS software (version 2.70) in batch mode for peak deconvolution and metabolite identification. The data matrices were imported into Matlab (MathWorks, Natick, MA, USA) for further processing. Baseline correction procedure was the same as in Section 2.3.4 and the resulting data were filtered to remove unstable signals with a CV > 20% across the QC samples.

2.3.7 Validation of metabolomics analytical platform

UPLC-orbitrap-MS and GC-MS methods used in this study were validated in the beef extract in terms of linearity, accuracy, precision (both with standards and samples), LOQ and LOD in UPLC-orbitrap-MS and GC-MS, according to the validation guide for untargeted metabolomics (De Paepe et al., 2018; Naz, Vallejo, García & Barbas, 2014; Wiklund et al., 2008).

The linearity of the response for samples was studied by triplicate assay of at least five concentrations, which covered all expected values ranging from 0.2% to 300% of mean values in QC samples. Dilution and re-concentration of the QC samples were carried out by stepwise increase and decrease of the ratio of solvent to the amount of QC sample after centrifugation. Accuracy studies were performed by spiking known amounts of sixteen standards, alanine, aspartic acid, galactose, glutamic acid, glycine, isoleucine, leucine, methionine, proline, serine, valine, fatty acid (FA) 16:0, FA 18:0, FA 20:1, PC(16:0/18:1) and PE(16:0/18:1) into the QC samples before extraction. As for the limits of detection (LOD) and limits of quantification (LOQ), sixteen standard compounds, which resembled the main chemical classes in the beef samples including fatty acid, amino acid, organic acid, glucose, PE and PC, were chosen. LOD and LOQ were measured as the lowest concentrations of each standard with a signal-to-noise (S/N) ratio of 3 and 10, respectively. Instrumental precision was tested by checking for a consistent response to the sixteen selected standards in the mid-range of the calibration curve, evaluated by multiple injection (n=10) of homogeneous standard solution and CV was used to measure the instrumental stability. Inter- and intra-day precision of the method was also evaluated the consistency of analytical platform response for sixteen standards at a specific concentration (midrange of linear curve) and QC samples with ten replications on three different days. Finally, recovery (n=10) was examined by comparing sixteen standard values obtained in spiked samples, within the linearity working range of QC samples. The recoveries were calculated by the formula: recovery (%) = (amount found – original amount) / amount spiked × 100%.

To account for the complexity and heterogeneity of beef tissue, sample homogeneity tests were conducted as follows: Fluctuations in metabolites within the pooled beef and non-pooled beef from the same samples were examined in ten replicates. Preparation of pooled and non-pooled samples followed a previous sampling strategy (Lamichhane et al., 2017) and the CV calculated between one pooled sample and two non-pooled samples from the same beef was used to measure the consistency of the metabolites across the experiments.

2.4 Multivariate analysis

The pretreated UPLC-orbitrap-MS and GC-MS data were combined into a single data set for multivariate analysis. Data were subjected to zero-mean unit-variance scaling to eliminate the influence of high-abundance metabolites for principal component analysis (PCA) and pareto scaling for partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). All the above chemometrics tools were implemented in house (available at https://github.com/DongElkan/pypls.git) by Python (version 3.7) to visualize differences among groups and to find out potential markers. In order to avoid the overfitting of the model during development, leave-one-out cross-validation (Westerhuis et al., 2008) designed for untargeted metabolomics was employed in this study. S-plot and loading plot with jackknife confidence intervals were used to identify reliable potential markers after multivariate analysis. Finally, all potential markers in both aqueous and lipid profiles were identified by external standard, matched with the NIST 11 library, METLIN (http://metlin.scripps.edu) and the Lipidmaps (http://www.lipidmaps.org/), and MS/MS fragments .

2.5 Statistics

Statistically significant differences of the potential markers between groups were analyzed by one-way analysis of variance (ANOVA) with least significance difference test using SPSS PASW Statistics (version 23; IBM).

3. Result and Discussion

3.1 Optimization of metabolomics platform

According to literature review, several extraction methods such as single organic solvent extraction, liquid-liquid extraction and solid phase extraction are available for different types of tissue (Jurowski, Kochan, Walczak, Barańska, Piekoszewski & Buszewski, 2017; Teo, Chong, Tan, Basri, Low & Ho, 2015). Liquid-liquid extraction is a common technique used to extract metabolites from tissue, and was applied in this work. For the extraction solvent, we compared three commonly used solvent systems, namely chloroform: methanol: water (1:1:2), chloroform: methanol: water (2:1:0.5) and methyl tert-butyl ether: methanol: water (5:1:1) based on the abundance of ions and the number of metabolites detected. Chloroform: methanol: water (1:1:2) and chloroform: methanol: water (2:1:0.5) provided the maximum number of metabolites with the highest ion abundance. We selected chloroform: methanol: water (1:1:2) on the basis of the green chemistry principles. To optimize the sample quantity, the sample-to-solvent ratios 1:10 and 1:20 were compared. Because the chromatogram acquired using the ratio of 1:10 was saturated under MS measurement, the sample-to-solvent ratio was set at 1:20 (0.1g in 2 mL).

3.2 Reliability of the metabolomics models

The metabolomics analytical methods we developed were evaluated and validated based on linearity, precision and recovery (De Paepe et al., 2018; Naz, Vallejo, García & Barbas, 2014) and the results are shown in Table 1. Good recoveries were obtained for all standards, varying between 93.9% and 104.5%. The CVs of instrumental precision (n=10) ranged from 1.48% to 3.32% for UPLC-Orbitrap-MS and 2.04% to 4.80% for GC-MS. Evaluation of the inter-day (n

= 10) and intra-day (3 days, n = 30) repeatability of the QC samples resulted in CVs from 2.75 to 10.44% and from 2.75 to 17.43%, respectively. In addition, the inter-day and intra-day repeatability of the sixteen selected standards were from 2.39 to 6.58% and from 3.83 to 12.85%, respectively. The LOQs and LODs of sixteen selected standards were in the range of 30.0ppb to 8.0ppm and 3.9ppb to 4.3ppm, respectively. Finally, good linearity ($R^2 > 0.995$) was obtained for the sixteen selected standards after serial dilution and re-concentration of the QC samples.

As for the beef sample homogeneity tests, the CVs calculated from thirty-four metabolites detected via UPLC-Orbitrap-MS and GC-MS are shown in the supplementary material (Figure S4). A high CV value would reflect inconsistency among the samples. In this work, the CV values of the thirty-four metabolites were nearly less than 30%. Thus, the metabolite profile of the beef extracts from the pooled samples did not show any pronounced changes within the same beef samples compared with that of the non-pooled beef samples.

In untargeted metabolomics analytical platform, the use of QC samples for assessing data quality is a common practice. In this study, the instrument precisions of the UPLC-Orbitrap-MS and the GC-MS were monitored by a series of pooled QC samples injected after every four samples throughout the data collection process. There was no significant retention time and *m/z* drift (CV less than 20% on UPLC-Orbitrap-MS and 30% on GC-MS) for internal standards added in the lipid and aqueous profiles of all QC samples (Bijlsma et al., 2006). In addition, the metabolomics profiles obtained from positive and negative electrospray ionization (ESI) modes of UPLC-Orbitrap-MS and GC-MS were plotted using PCA and are shown in the supplementary material (Figure S5a). PCA was used to explore general interrelations among groups. High degree of aggregation in all QC samples for both UPLC-Orbitrap-MS and GC-MS data was observed in the PCA score plots, indicating excellent stability of the two analytical platforms throughout the experiment. The good robustness of the metabolomics model guaranteed the differences among groups resulted from biological variations, such as geographical origin, farming, breeding and feeding regimen.

3.3 Metabolomic profiles of beef

Representative MS chromatograms of beef samples obtained from positive and negative ionization modes of UPLC-Orbitrap-MS and GC-MS are provided in the supplementary material (Figure S2). The GC-MS chromatograms exhibited simpler peak complexity than the UPLC-Orbitrap-MS results. In terms of identification of metabolites, most peaks detected by GC-MS after derivatization were identified by library searching with high matching scores. The derivatized aqueous extracts of beef mainly consisted of amino acids, short chain fatty acids, tricarboxylic acids and simple sugars such as monosaccharides, disaccharides and glucose-phosphate. The selected metabolites in aqueous extracts of beef after multivariance analysis were further confirmed with commercially available standards. In the lipidomic profiles, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were the major classes found in our beef samples. Other lipid metabolites including medium and long chain fatty acids, eicosanoids, sterols and sphingolipids were also detected in lower abundance.

Beef was composed of significant amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which ranged between 78 and 95% of total phospholipid content (Lordan, Tsoupras & Zabetakis, 2017). Our results were consistent with literatures and significant amounts of PC and PE were detected in the collected beef samples. Some of the PC and PE were found to be important in beef produced from different geographical locations (details please refers to the next section). Identification of PC and PE species is a challenging task even with the use of high-resolution MS. One of the complications is the vast number of isomeric molecular species present in the chromatogram, which makes complete structural assignment extremely difficult (Cajka & Fiehn, 2014). In this work, we used detailed mass fragmentation analysis of the mass spectra and MS2 data to confirm the identity of the PC and PE metabolites. In the following, PC(16:0/18:1) and PE(16:0/18:1) were two examples used to illustrate how the fragmentation in the mass spectra leading to confirmation of the identity of the species. The standards of these two were used to further confirm the assignment.

The typical representative mass spectra of standard PC (16:0/18:1) and QC sample and PE (16:0/18:1) standard and QC sample are provided in the supplementary material (Figure. S3a). Due to the presence of ammonium formate in the mobile phase system, PC yielded the predominant adduct ion $[M+HCOO]^-$ and its fragment ion $[M-15]^-$ ($[M+HCOO-CH_3HCOO]^-$) in full scan MS under negative ionization mode (Han, 2016; Hsu, Lodhi, Turk & Semenkovich, 2014). For example, standard PC (16:0/18:1) formed the adduct ion $[M+HCOO]^-$ and fragmented ion $[M-15]^-$ at *m/z* 804.5786 and 744.5560, respectively. In the MS/MS spectrum of the $[M+HCOO]^-$ ion of standard PC (16:0/18:1), the predominant fragment ions were carboxylate ions at *m/z* 255.2333 (sn-1 fatty acid, 16:0) and 281.2492 (sn-2 fatty acid, 18:1). One of the characteristic PC headgroup fragment ions was observed as a very low signal for both the deprotonated demethylated phosphocholine ion at *m/z* 168.0431 and demethylated phosphocholine dehydrated glycerol ester at *m/z* 224.0696. Fatty acid chain related ions, including demethylated lysophosphatidylcholines $[M-FA-15]^-$ at *m/z* 462.2996 and 488.3141, and $[M-15-FA\ ketene]^-$ at *m/z* 480.3100 and 506.3253, were observed under high energy collision-induced dissociation. The MS data showed good agreement between the PC (16:0/18:1) and QC samples.

PE is a class of phospholipids found in cell and biological membranes, and is abundant in beef (Patel & Witt, 2017; Van der Veen, Kennelly, Wan, Vance, Vance & Jacobs, 2017). In contrast to PC, the predominant ion of PE in negative ionization mode was the deprotonated parent ion $[M-H]^-$. Structural information related to fragment ions of PE was deduced from the MS/MS spectra of the standard PE (16:0/18:1) (Han, 2016; Hsu & Turk, 2009), for example, the MS/MS spectrum of deprotonated parent ion [M-H]- of standard PE (16:0/18:1) at *m/z* 716.5236 (Figure S3b). The nature of PE was reflected by a group of PE headgroup fragment ions, a deprotonated PE ion at *m/z* 140.0316 and a deprotonated doubly dehydrated glycerol-phosphocholine at *m/z* 196.0023. The length of fatty acid chains of PE was determined from the carboxylate ions at *m/z* 255.2290 and 281.2270. In contrast to PC, the lyso form of PE, $[M-FA]^-$ and $[M-FA\ ketene]^-$ was absent in the MS/MS spectrum. Thus, the metabolite ion at *m/z* 716.5236 in the QC samples was determined to be PE (16:0/18:1). The MS data showed good agreement between the PE (16:0/18:1) standard and QC samples (Figure S3b). However, there

were still some limitations in deducing the structure of fatty acids in PC and PE, such as location of double bonds on the fatty acid chain (Cajka & Fiehn, 2014).

3.4 Markers selection from the multivariate analysis

As some beef samples from Japan partially overlapped with those from the US under PCA analysis, as shown in the supplementary material (Figure S5), PLS-DA was applied to identify the major difference in metabolic profiles among groups and facilitate the identification of unique metabolites. Three sets of data matrices were subjected to PLS-DA procedure individually or in combination to build the best possible classification model. The results showed that the combination of UPLC-Orbitrap-MS in positive mode and GC-MS resulted in the best separation of the beef samples among Australia, the US and Japan (Figure S5b).

Even though PLS-DA method provided better classification than PCA in our obtained results, it is a supervised learning method and may suffer from overfitting (Westerhuis et al., 2008; Worley & Powers, 2013). In order to minimize overfitting during model development, we searched for potential markers using OPLS-DA with leave-one-out cross-validation among groups, which was followed by an S-plot to identify the metabolites with high covariance and correlation within the model. The jackknife confidence interval was also examined as a complementary tool for identification of metabolites with high correlation with the model and small variability within the group.

Figure 1a presents a cross-validated score plot ($R_2X = 0.37$, $R_2Y = 0.80$, $Q_2Y = 0.62$, p-value = 0.0005) of the discriminating model between Australian beef and US beef using data from UPLC-Orbitrap-MS in positive ionization mode and GC-MS. 90% of the Australia samples were correctly assigned while 100% of US samples were correctly assigned. To further identify metabolites associated with the group's separation, an S-plot was generated and several potential biomarkers were highlighted (Figure 1b). In addition, the loading plot with the jackknife confidence intervals was shown in Figure 1c and metabolites with high statistical reliability were clearly indicated. In general, discriminated metabolites had a high covariance combined with a high correlation, resulting in a small confidence interval. Some unique markers such as PC (15:0/18:1), 9-HODE, myo-inositol and FA 22:4 were screened out by the jackknife confidence intervals and their relative intensities of the beef samples between two countries were depicted in Figure 1d. Since OPLS-DA is designed for classification of two groups only (Brereton & Lloyd, 2014), pairwise comparison of OPLS-DA with leave-one-out cross-validation among groups was performed.

3.5 Discrimination of geographical origins

Cross-validated score plots, S-plots and loading plots with jackknife confidence intervals among beef from Australia, the US and Japan are shown in supplement material (Figure S6). 90% and 92.8% of Australian and US samples were correctly assigned, respectively while 100% of Japanese samples were correctly assigned. Ultimately, twenty-four metabolites with significant differences were identified; their relative concentrations in Australian, Japanese, and US beef samples are shown in Figure 2.

462 The multivariate analysis in our model revealed that metabolites from amino acids played an
463 important role in differentiating beef samples from different countries. Amino acids and their
464 derivatives contributed to 8 out of 24 metabolites that could be used to differentiate the
465 geographical origin of beef. The relative levels of three related amino acids (leucine, isoleucine
466 and valine) in a branched-chain amino acid biosynthesis module showed similar trends among
467 the three countries. In addition, Australian beef had the highest relative level of tyrosine among
468 the three countries. A previous study reported significant differences between the Australian
469 and US beef samples in terms of the absolute content of isoleucine, tyrosine and valine (Jung,
470 Lee, Kwon, Lee, Ryu & Hwang, 2010). This might be a result of the differences in cattle
471 husbandry practices in these countries. In Saleem's metabolomics study on the effect of feeding
472 on cattle (Saleem et al., 2012), rumen fluid was collected and analyzed from dairy cows that
473 were fed four diets with varying ratios of barley grain and silage (i.e., 0, 15, 30, and 45% barley
474 grain in diet dry matter), cows that consumed more barley grain had higher rumen fluid tyrosine,
475 methionine, threonine, leucine, and valine content. In the present study, these amino acids
476 showed a similar trend in the Australian, Japanese, and US beef samples, which might be the
477 result of the feedlot diet formulation. The fact that the length of time cattle spend in the feedlot
478 varied among the three countries was another possible influencing factor (Drouillard, 2018;
479 Gotoh, Nishimura, Kuchida & Mannen, 2018; Greenwood, Gardner & Ferguson, 2018;
480 Koutsidis, Elmore, Oruna-Concha, Campo, Wood & Mottram, 2008).

481 Cyclitols, also called sugar alcohol, are cycloalkanes with at least three hydroxyls, each on a
482 different ring carbon. Cyclitols are widely distributed in the plant kingdom and other living
483 cells and display a broad range of biological activities (Al-Suod, Ligor, Rațiu, Rafińska,
484 Górecki & Buszewski, 2017). In the present study, two cyclitols, scyllo-inositol and myo-
485 inositol, showed similar trends in beef from the three countries. Both scyllo-inositol and myo-
486 inositol were significantly more abundant in US beef than in Australian and Japanese beef.
487 Scyllo-inositol is common in seeds, grains and vegetables with varying degrees of esterification
488 (Al-Suod, Ligor, Rațiu, Rafińska, Górecki & Buszewski, 2017). Therefore, the relative
489 differences in scyllo-inositol content among the Australian, Japanese, and US beef samples
490 were likely to be due to differences in feed. Most Japanese cattle are forced fed a ration to
491 improve their fatty acid composition and flavor (Gotoh, Nishimura, Kuchida & Mannen, 2018).
492 Beef cattle in Australia are primarily grass-fed for most of their life (Ponnampalam, Mann &
493 Sinclair, 2006), so feed requirements focus on the efficient production of pasture, and the
494 conversion of pasture into meat. About one-third of the Australian cattle are eventually
495 'finished' in feedlots where they are fed a largely grain-based diet from 70 to 360 days until
496 they meet the specifications for a particular market (Deblitz, Dhuyvetter & Davies, 2012).

497 In this study, we identified eight PCs, one Lyso PE, three PEs and one free fatty acid
498 metabolites as suitable biomarkers for discriminating the geographical origins of beef samples.
499 It has been reported that PC and PE are the predominant species of glycerophospholipids in
500 beef, contributing 58-65% and 20-30% of total glycerophospholipids, respectively (Lordan,
501 Tsoupras & Zabetakis, 2017). These findings indicated that the metabolomics approach might
502 be useful for assessing the origin of beef samples. Total fatty acid and the fatty acid omega-6/
503 omega-3 ratio in beef samples are often reported and compared from the prospective of

geographical origin, feeding regimen, and nutrition and sensory evaluation. However, as we did not include acid hydrolysis in the extraction protocol, it would be difficult to directly compare our results with those from other works (Horcada, Polvillo, Juárez, Avilés, Martínez & Peña, 2016; Jung, Lee, Kwon, Lee, Ryu & Hwang, 2010; Mezgebo et al., 2017; Scollan et al., 2014). We tried to obtain estimated total fatty acid profiles from our data and the results were shown in section 3.8.

One unique metabolite, 9-hydroxy-octadecadienoic acid (9-HODE), was identified in all the beef samples, albeit only existed in low concentration in the Australian samples. 9-HODE has been proposed as an excellent marker for lipid peroxidation in vitro (Spiteller & Spiteller, 1997). Lipid oxidation has a high impact on the overall quality of beef since it adversely affects the colour, texture, nutritional value and safety of the meat. Storage conditions such as repeated freezing-thawing, photooxidation, and a higher degree of fatty acid unsaturation favor meat oxidation (Boselli, Rodriguez-Estrada, Fedrizzi & Caboni, 2009; Chen et al., 2018). Therefore, 9-HODE might be a good indicator for the assessment of the quality or freshness of beef.

3.6 Discrimination between Australian organic and non-organic beef

Cross-validated score plots, S-plots and loading plots with jackknife confidence intervals between Australian organic and non-organic beef are shown in supplement material (Figure S7). 100% of Australian organic beef were correctly assigned while 83.3% of Australian non-organic beef were correctly assigned. Ultimately, seven metabolites that differed significantly among the samples were identified; their relative concentrations among beef from different farming modes are shown in Figure 3.

Three identified lipid metabolites, including two PCs and one PE were suitable as biomarkers for differentiating between Australian beef from organic and from non-organic farming modes. In addition, Australian organic beef had the highest relative levels of L-alanine and L-valine, as well as a high nicotinamide content. Nicotinamide is a component of various biological systems, including vitamin B metabolism, and is a critical part of the structures of NADH and NAD^+. Therefore, a higher nicotinamide content may enhance the functional value of beef (Muroya, Oe, Ojima & Watanabe, 2019).

3.7 Discrimination between Angus beef from the US and Australia

Cross-validated score plots, S-plots and loading plots with jackknife confidence intervals between Angus beef from the US and that from Australia are shown in supplement material (Figure S8). 100% of Angus beef from Australia were correctly assigned while 80% of Angus beef from the US were correctly assigned. Ultimately, ten metabolites that showed significant differences among samples were identified; and their relative concentrations in beef from different countries are shown in Figure 4.

Eight identified lipid metabolites, which belonged to the PC class, were found to be suitable biomarkers for discriminating between US and Australian Angus beef. These findings indicate that the lipidomics approach might be applicable to assess the origin of Angus beef samples.

Regarding the amino acid metabolism, US Angus beef sample had the highest relative levels of proline. Surprisingly, Australian Angus beef had a higher glucose content than US Angus beef. Glucose in meat products may serve as a precursor of volatile compounds such as 3-methylbutanal and 2-methylbutanal (Kosowska, A. Majcher & Fortuna, 2017).

3.8 Comparison of fatty acid contents of the beef samples

Lipid is a major factor determining the quality of beef including its flavor, palatability and nutritive value. Fatty acid profiles, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) content are commonly used to evaluate meat quality in the industry (Pighin et al., 2016). In the literature, the fatty acid profiles were usually determined by hydrolysis of the samples followed by derivatization (e.g. methylation) before the GC-MS analysis. A large number of studies have been reported in the literature relating fatty acid profiles, SFA, MUFA and PUFA contents with the quality of beef (Cifuni, Napolitano, Riviezzi, Braghieri & Girolami, 2004; Elmore, Mottram, Enser & Wood, 1999; Ponnampalam, Mann & Sinclair, 2006). To better understand the characteristics of the beef samples collected in this study with findings from literatures, we tried to estimate the hydrolyzed fatty acid profiles from the UPLC-Orbitrap-MS data collected. Many of the lipids are compounds of fatty acids and more than one lipid species may contain the same fatty acid chain length. For example, PC(16:0/18:0), PC(16:0/18:1) and PE(16:0/18:0) consist of a FA 16:0 that would result in a FA16:0 fragment during MS detection. Therefore, the total peak area in the ion chromatogram extracted at the m/z of FA 16:0 would be an estimate of the content of FA 16:0 in beef. The ion chromatograms at *m/z* values of different fatty acid fragments shown in Table S2 were extracted from the UPLC-Orbitrap-MS data. Based on the contents of individual fatty acid, the SFA, MUFA and PUFA were determined and compared using ANOVA

Japanese beef contained a higher ratio of MUFA/SFA when compared with that from the US and Australia (shown in Figure 5a). The trend of MUFA/SFA ratios obtained in the present study showed good agreement with a previous report about the beef samples from Japan and the US (Smith, 2015). Meanwhile, US beef had the highest omega-6/omega-3 ratio while Australian beef had the lowest omega-6/omega-3 ratio. A high omega 6 to omega 3 ratio may be associated with a higher risk of cardiovascular diseases and other chronic diseases (Simopoulos, 2008). By investigating the potential markers using S-plot and loading plot, FA 18:3 was found as a characteristics marker to differentiate between beef from the US and beef from Australia. A similar trend was observed in the model of Angus beef between the US and Australia (shown in Figure 5b). It was understood that the feeding regime had a major impact on the individual fatty acids of the intramuscular fat in beef. The major feeding diet of the cattle in the US and Australia are grain-fed and grass-fed, respectively (Drouillard, 2018; Ponnampalam, Mann & Sinclair, 2006). A study on the fatty acid compositions between grass silage and barley straw showed significant differences in their summation of omega-6 and omega-3 of the contents. (Horcada et al., 2017)

Figure 5c shows a comparison between Australian beef samples from organic farming and those from other farming modes. Organic beef had a higher ratio of PUFA/SFA and

surprisingly, there were significant differences in one of the characteristic markers, FA 22:6, among the samples. An increase in the proportion of PUFA, particularly n-3 PUFA, would improve the nutritional quality of beef and contribute to a healthy human diet in reducing the risk of human diseases such as CVD (Siri-Tarino, Sun, Hu & Krauss, 2010) . However, the omega-6/omega-3 ratio did not differ significantly between Australian organic and non-organic beef. Taken together, organic beef is a healthier choice than beef produced under other faming modes (Turner et al., 2014).

Obviously, the metabolomics analysis is capable of identifying chemical profiles in far more detail compare to the fatty acid profiles. Beef from different countries and different feeding regimen would result in significantly different lipid and amino acids profiles. Some sugar metabolites are also found to be different among samples. However, metabolomics is still an emerging technique in this area and the relationship of the metabolites identified in this study with the quality, the nutritional values as well as the health implications of beef produced in different ways are yet to be explored.

4. Conclusion

We used MS-based untargeted metabolomics analysis to analyze beef samples collected from local retailers in Hong Kong. Two multi-class analytical platforms were successfully used to assess metabolites with different polarities to differentiate the geographical origin feeding and regimen of the samples. The MS-based approach employed here provided detailed chemical profiles for samples. Potential biomarkers for beef from different countries included amino acids, several sugar metabolites, and a number of PCs and PEs. Thus, the approach adopted in this study offers a method to identify the geographical origin of beef at any point along the supply chain and could be used to develop a verifiable traceability system. However, analysis of more samples is needed to confirm the reliability of the potential biomarkers. With the analysis of more samples and the establishment of a sample database, metabolomics could represent an important technique in a verifiable food traceability system. Our results demonstrated that untargeted metabolomics would be a valuable analytical platform for researchers to obtain comprehensive chemical profiles and to investigate the effects of different farming practices and environmental factors on the quality and nutritional values of beef. More studies are clearly needed to fully understand the implications of the potential biomarkers identified in here on the quality and nutritional values.

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Conflict of Interest Statement

The authors have no conflict of interests to declare.

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851 Table 1: Validation data for selected metabolites in QC beef samples with the UPLC-Orbitrap-MS and GC-MS

Type of instruments used for detection	Compounds	Linearity	Correlation coefficient (r^2)	Standard					Sample		Recovery (%, n=10)
				LOD (ppm)	LOQ (ppm)	Instrumental precision (n=10)	Intra-day repeatability (n=10)	Inter-day repeatability (3 days, n=30)	Intra-day repeatability (n=10)	Inter-day repeatability (3 days, n=30)	
UPLC-Orbitrap-MS (+)	PE (16:0/18:1)	0.02 – 2 QC times	0.9983	3.90×10^{-03}	7.80×10^{-02}	1.48%	3.96%	6.10%	7.23%	9.07%	98.72 ± 8.13
	PC (16:0/18:1)	0.002 – 2 QC times	0.9997	7.50×10^{-03}	3.00×10^{-02}	1.57%	6.58%	7.90%	6.82%	8.08%	101.29 ± 9.61
UPLC-Orbitrap-MS (-)	FA 16:0	0.002 – 2 QC times	0.9992	5.57×10^{-02}	1.95×10^{-01}	3.32%	3.54%	5.02%	5.11%	8.77%	96.40 ± 9.45
	FA 18:0	0.002 – 2 QC times	0.9983	2.03×10^{-01}	3.90×10^{-01}	3.08%	3.37%	4.07%	6.20%	8.05%	98.05 ± 6.51
	FA 20:1	0.05 – 2 QC times	0.9974	1.01×10^{-02}	1.01×10^{-01}	1.52%	2.44%	5.60%	7.55%	8.50%	93.92 ± 7.61
	PE (16:0/18:1)	0.01 – 2 QC times	0.9995	2.60×10^{-02}	7.80×10^{-02}	1.53%	2.29%	8.33%	6.57%	9.46%	101.63 ± 9.86
	PC (16:0/18:1)	0.002 – 2 QC times	0.9968	7.80×10^{-02}	1.50×10^{-01}	1.97%	2.95%	4.88%	3.83%	5.67%	102.84 ± 11.10
GC-MS	Alanine	0.1 – 3 QC times	0.9985	1.00	8.00	4.69%	5.50%	10.44%	6.04%	11.10%	100.43 ± 6.03
	Valine	0.1 – 3 QC times	0.9953	1.00	5.00	3.00%	5.05%	8.92%	10.44%	11.58%	99.58 ± 6.94
	Leucine	0.05 – 3 QC times	0.9966	1.00	4.20	2.97%	3.18%	7.55%	3.39%	8.83%	95.98 ± 4.61
	Isoleucine	0.1 – 3 QC times	0.9958	1.00	4.30	3.41%	3.51%	5.91%	4.78%	9.22%	96.83 ± 3.05
	Glycine	0.002 – 3 QC times	0.9951	0.50	2.00	2.42%	4.38%	4.40%	4.46%	16.90%	102.03 ± 4.17
	Serine	0.05 – 3 QC times	0.9972	3.00	5.00	4.76%	4.93%	5.35%	5.52%	16.50%	98.02 ± 6.60
	Methionine	0.2 – 3 QC times	0.9994	4.30	6.25	4.63%	5.83%	10.18%	7.14%	17.43%	97.18 ± 5.52
	Proline	0.005 – 3 QC times	0.9960	4.20	6.50	2.04%	2.39%	4.79%	2.75%	5.09%	104.58 ± 3.30
	Aspartic acid	0.1 – 3 QC times	0.9969	2.00	6.25	3.77%	4.17%	9.23%	4.54%	15.60	102.12 ± 6.62
	Glutamic acid	0.2 – 3 QC times	0.9992	2.00	6.00	4.80%	5.52%	12.85%	6.11%	13.79%	102.74 ± 6.78
	Galactose	0.002 – 3 QC times	0.9991	0.50	1.50	2.47%	2.71%	4.10%	3.15%	13.53%	103.05 ± 6.57

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A list of Figure:

Figure 1: a) Cross-validated score plot of beef samples between Australia and the US, b) S-plot for potential markers selection, c) loading plot with jackknife confidence intervals, d) Bar graphs of the normalized peak area of 4 identified markers, PC(15:0/18:1), 9-HODE, myo-inositol and fatty acid 22:4

Figure 2: Bar graphs of the normalized peak area of differential metabolites for beef samples from different countries: AUS, Australian beef, US, US beef and JPN, Japanese beef. Data are expressed as mean ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs AUS, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs US

Figure 3: Bar graphs of the normalized peak area of differential metabolites for Australian beef samples with different feeding modes. AUS Organic, Australian organic beef; AUS Others, beef from non-organic feeding modes in Australia. Data are expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. AUS Organic

Figure 4: Bar graphs of the normalized peak area of differential metabolites for Angus beef samples obtained from different countries. AUS Angus, Australian Angus beef; US Angus, US Angus beef. Data are expressed as mean ± SD. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. US Angus

Figure 5: Bar graphs of the normalized estimated fatty acid profiles for a) different countries, b) Australian and US Angus beef. and c) feeding regimens in Australia, Data are expressed as the mean ± SD. AUS, Australian beef; AUS Angus, Australian Angus beef; AUS Organic, Australian organic beef; AUS Others, beef from non-organic feeding modes in Australia; JPN, Japanese beef; US, US beef; US Angus, US Angus beef. ** $p < 0.01$, *** $p < 0.001$ vs. AUS, $^{\#\#}p < 0.01$ vs US. a); ^ $p < 0.05$ vs. AUS organic b); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. US Angus c).

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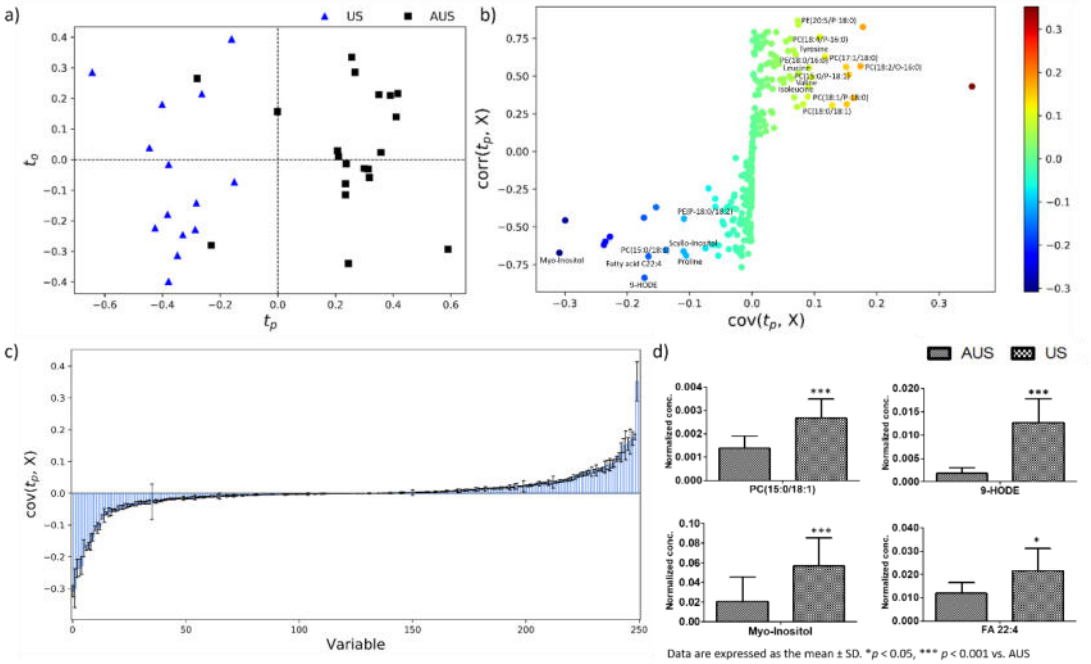

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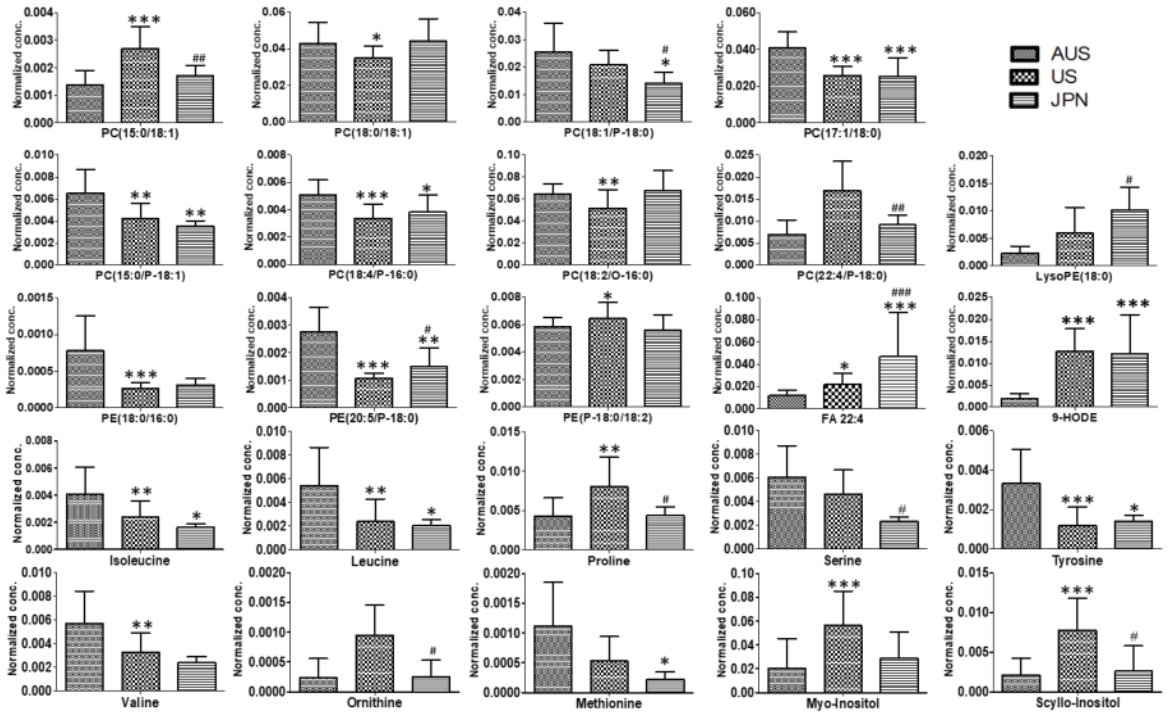

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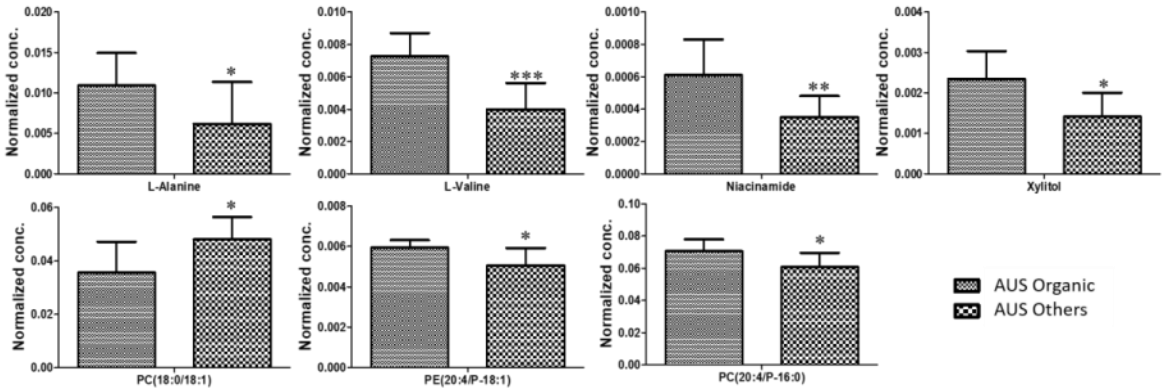

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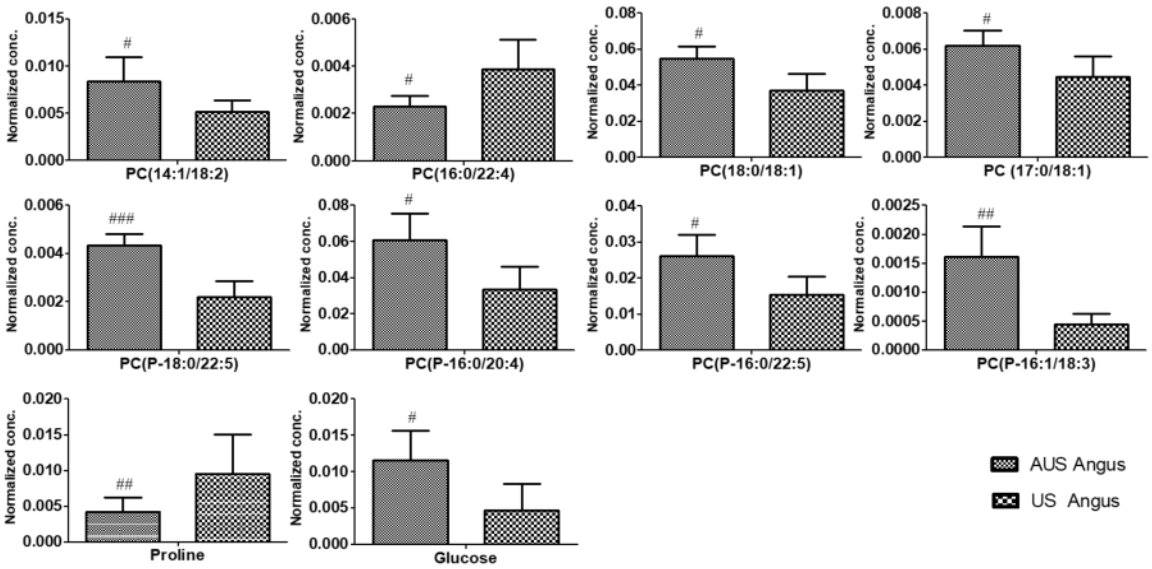

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