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1 # Mass spectrometry-based untargeted metabolomics approach for
2 # differentiation of beef of different geographic origins

3

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26

27 ## Abstract

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

39

40

41 **Keywords:**

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

43

44

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

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

91 The geographical origin of beef is most commonly determined using a genomics approach,
92 stable isotope ratio analysis and multi-elemental analysis. For example, 24 cattle breeds from
93 seven member states of the European Union (France, Denmark, Italy, the Netherlands,
94 Switzerland, Spain and the UK) were assigned correctly to their geographical origin with a
95 success rate of 90% by using single nucleotide polymorphisms (SNP) (Negrini et al., 2008).
96 Meanwhile, Mannen used mitochondrial DNA to classify Wagyu cattle from Japan (Mannen,
97 2017). Although these studies demonstrated the utility of the genomics approach in determining
98 the geographical origin of beef, no studies have compared DNA profiles among beef from
99 different countries. Therefore, it is unknown whether, for instance, Japanese beef can be
100 accurately differentiated from European beef by using only molecular markers. To this end, in
101 China, researchers have successfully distinguished beef produced from Shandong,
102 Heilongjiang, Yunnan and the Tibet Autonomous Region using carbon isotope ratio, but not
103 the nitrogen isotope ratio (Zhao, Zhang, Guo, Wang & Yang, 2016). Moreover, elemental
104 profiles obtained from elemental analyzer continuous flow isotope ratio MS could distinguish
105 beef from four out of five countries, namely Australia, Brazil, Canada and the US, but
106 misclassification between Brazilian and Swiss beef was observed in the external validation set
107 (Franke, Haldimann, Gremaud, Bosset, Hadorn & Kreuzer, 2008). One major limitation of
108 these approaches is that although the methods may be able to identify the geographic sources
109 of beef, no information is provided on their quality.

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

118 MS- and NMR-based metabolomics are new popular techniques that have been developed and
119 used in the study of food safety, quality and traceability (Capozzi & Bordoni, 2013; Hu & Xu,
120 2013). For example, Jung et al. (Jung, Lee, Kwon, Lee, Ryu & Hwang, 2010) used proton NMR
121 to identify beef samples that originated from Australia, Korea, New Zealand and the US.
122 Carrillo et al. (Carrillo et al., 2016) used integrated metabolomics and transcriptome analysis
123 to characterize the differences between grass- and grain-fed Angus steer. Finally, Kodani et al.
124 (Kodani, Miyakawa, Komatsu & Tanokura, 2017) used NMR data to evaluate the degree of
125 unsaturation in triacylglycerol and fatty acid as well as the ageing duration of Japanese Black
126 cattle.

127 Metabolomics studies can be divided into two general approaches, targeted and untargeted
128 analysis. The targeted approach focuses on identifying and quantifying a number of small
129 subset of known metabolites, whereas the untargeted approach aims at acquiring as many
130 metabolites as possible, annotating metabolites and reviewing changes in quantity (Hu & Xu,
131 2013). Beef is a complex matrix with thousands of metabolites that may be affected by species,
132 flavour, nutrient, production and storage. Therefore, the untargeted approach has the advantage

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

137 In the present work, we developed an untargeted metabolomics approach, including both ultra-
138 high-performance liquid chromatography-Orbitrap-mass spectrometry (UPLC-Orbitrap-MS)
139 and gas chromatography-mass spectrometry (GC-MS) analytical platforms, to evaluate the
140 geographical origin and species of beef as well as to differentiate beef produced from different
141 feeding regimes. The entire metabolomics platform was further validated by analyzing its
142 linearity, accuracy, method precision, limit of quantification (LOQ) and limit of detection
143 (LOD). The overall objective of this study was to provide a more comprehensive metabolite
144 map for the identification of potential biomarkers associated with the geographical origin and
145 production conditions of beef. We believe this method will be particularly useful in end-user
146 markets, such as Hong Kong, where information on beef production and storage may not be
147 always fully available or verifiable. Using the developed method, we tested beef purchased in
148 a local Hong Kong market that originated from Australia, the US, and Japan (the top three
149 providers of chilled beef in Hong Kong). The prices of beef from these three countries differ
150 considerably. In 2017, Australia supplied 49.4% of Hong Kong's imported beef, which
151 accounted for 41.4% of total beef sales value, the US supplied 26.6%, which accounted for
152 27.6% of the total sales value, and Japan supplied only 7.3% but accounted for 19.9% of the
153 total sales value (H.K.S.A.R. C&SD, 2018).

154

155 **2. Materials and methods**

156 2.1 Reagents

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

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

172 2.2 Sample collection

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

181 2.3 Metabolomics analysis

182 2.3.1 Quality control sample preparation method

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

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

195 2.3.2 Sample preparation

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

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

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

220 2.3.3 UPLC-orbitrap-MS data acquisition

221 For UPLC-Orbitrap-MS analysis, a Thermo Orbitrap Fusion Lumos Tribrid Mass Spectrometry
222 (Thermo Fisher Scientific, Waltham, MA, USA) was connected to a Waters ACQUITY UPLC
223 System (Waters Corp., Milford, USA) via heated electrospray ionization (H-ESI) as the
224 interface. The separation was performed on a Waters ACQUITY UPLC HSS T3 column (2.1
225 mm × 100 mm, 1.8µm) with an HSS T3 pre-column (2.1 mm × 5 mm, 1.8µm) at 40 °C. Based
226 on Cajka and Bird's study (Cajka & Fiehn, 2016), a gradient elution of solvent A (60% v/v
227 water: 40% v/v acetonitrile containing 10 mM ammonium formate and 0.1% formic acid) and
228 solvent B (90% v/v isopropanol: 10% v/v acetonitrile containing 10 mM ammonium formate
229 and 0.1% formic acid) was applied with a modified elution program as follows: 0–1.5 min, 30%
230 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%
231 B; 17–21 min, 97% B; 21–24 min, 97–30% B; 24–25 min, 30% B. The flow rate was 0.3
232 mL/min, and the injection volume was 3 µL. The sample chamber temperature was 4 °C. The
233 H-ESI-MS spectra were acquired in both positive and negative ion modes. The H-ESI
234 parameters were as follows: Spray voltage, 3600V for positive ESI and 2300V for negative
235 ESI; sheath gas, 35 arbitrary units; nebulizer auxiliary gas, 20 arbitrary units; sweep gas, 0
236 arbitrary units. General instrumental parameters were set as follows: ion transfer tube
237 temperature, 350°C; vaporizer temperature, 200°C. For full scan MS, the mass range was set
238 at 100 - 2000 *m/z* with 120,000 mass resolutions. The automatic gain control (AGC) target was
239 set as 2.0×10^5 with a maximum injection time of 100 ms.

240 2.3.4 UPLC-orbitrap-MS data pretreatment and analysis

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

250 2.3.5 GC-MS data acquisition

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

257 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
258 min. The MS parameters were as follows: solvent delay, 5 min; ionization energy, 70 eV;
259 temperatures of the ion source and transfer line, 230 °C; full scan mode in *m/z* range 70–550.

260 2.3.6 GC-MS data pretreatment

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

266 2.3.7 Validation of metabolomics analytical platform

267 UPLC-orbitrap-MS and GC-MS methods used in this study were validated in the beef extract
268 in terms of linearity, accuracy, precision (both with standards and samples), LOQ and LOD in
269 UPLC-orbitrap-MS and GC-MS, according to the validation guide for untargeted
270 metabolomics (De Paepe et al., 2018; Naz, Vallejo, García & Barbas, 2014; Wiklund et al.,
271 2008).

272 The linearity of the response for samples was studied by triplicate assay of at least five
273 concentrations, which covered all expected values ranging from 0.2% to 300% of mean values
274 in QC samples. Dilution and re-concentration of the QC samples were carried out by stepwise
275 increase and decrease of the ratio of solvent to the amount of QC sample after centrifugation.
276 Accuracy studies were performed by spiking known amounts of sixteen standards, alanine,
277 aspartic acid, galactose, glutamic acid, glycine, isoleucine, leucine, methionine, proline, serine,
278 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
279 samples before extraction. As for the limits of detection (LOD) and limits of quantification
280 (LOQ), sixteen standard compounds, which resembled the main chemical classes in the beef
281 samples including fatty acid, amino acid, organic acid, glucose, PE and PC, were chosen. LOD
282 and LOQ were measured as the lowest concentrations of each standard with a signal-to-noise
283 (S/N) ratio of 3 and 10, respectively. Instrumental precision was tested by checking for a
284 consistent response to the sixteen selected standards in the mid-range of the calibration curve,
285 evaluated by multiple injection (n=10) of homogeneous standard solution and CV was used to
286 measure the instrumental stability. Inter- and intra-day precision of the method was also
287 evaluated the consistency of analytical platform response for sixteen standards at a specific
288 concentration (midrange of linear curve) and QC samples with ten replications on three
289 different days. Finally, recovery (n=10) was examined by comparing sixteen standard values
290 obtained in spiked samples, within the linearity working range of QC samples. The recoveries
291 were calculated by the formula: recovery (%) = (amount found – original amount) / amount
292 spiked × 100%.

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

299 2.4 Multivariate analysis

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

314 2.5 Statistics

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

318

319 **3. Result and Discussion**

320 3.1 Optimization of metabolomics platform

321 According to literature review, several extraction methods such as single organic solvent
322 extraction, liquid-liquid extraction and solid phase extraction are available for different types
323 of tissue (Jurowski, Kochan, Walczak, Barańska, Piekoszewski & Buszewski, 2017; Teo,
324 Chong, Tan, Basri, Low & Ho, 2015). Liquid-liquid extraction is a common technique used to
325 extract metabolites from tissue, and was applied in this work. For the extraction solvent, we
326 compared three commonly used solvent systems, namely chloroform: methanol: water (1:1:2),
327 chloroform: methanol: water (2:1:0.5) and methyl tert-butyl ether: methanol: water (5:1:1)
328 based on the abundance of ions and the number of metabolites detected. Chloroform: methanol:
329 water (1:1:2) and chloroform: methanol: water (2:1:0.5) provided the maximum number of
330 metabolites with the highest ion abundance. We selected chloroform: methanol: water (1:1:2)
331 on the basis of the green chemistry principles. To optimize the sample quantity, the sample-to-
332 solvent ratios 1:10 and 1:20 were compared. Because the chromatogram acquired using the
333 ratio of 1:10 was saturated under MS measurement, the sample-to-solvent ratio was set at 1:20
334 (0.1g in 2 mL).

335 3.2 Reliability of the metabolomics models

336 The metabolomics analytical methods we developed were evaluated and validated based on
337 linearity, precision and recovery (De Paepe et al., 2018; Naz, Vallejo, García & Barbas, 2014)
338 and the results are shown in Table 1. Good recoveries were obtained for all standards, varying
339 between 93.9% and 104.5%. The CVs of instrumental precision (n=10) ranged from 1.48% to
340 3.32% for UPLC-Orbitrap-MS and 2.04% to 4.80% for GC-MS. Evaluation of the inter-day (n

341 = 10) and intra-day (3 days, n = 30) repeatability of the QC samples resulted in CVs from 2.75
342 to 10.44% and from 2.75 to 17.43%, respectively. In addition, the inter-day and intra-day
343 repeatability of the sixteen selected standards were from 2.39 to 6.58% and from 3.83 to
344 12.85%, respectively. The LOQs and LODs of sixteen selected standards were in the range of
345 30.0ppb to 8.0ppm and 3.9ppb to 4.3ppm, respectively. Finally, good linearity ($R^2 > 0.995$)
346 was obtained for the sixteen selected standards after serial dilution and re-concentration of the
347 QC samples.

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

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

368 3.3 Metabolomic profiles of beef

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

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

394 The typical representative mass spectra of standard PC (16:0/18:1) and QC sample and PE
395 (16:0/18:1) standard and QC sample are provided in the supplementary material (Figure. S3a).
396 Due to the presence of ammonium formate in the mobile phase system, PC yielded the
397 predominant adduct ion $[M+HCOO]^-$ and its fragment ion $[M-15]^-$ ($[M+HCOO-CH_3HCOO]^-$)
398 in full scan MS under negative ionization mode (Han, 2016; Hsu, Lodhi, Turk & Semenkovich,
399 2014). For example, standard PC (16:0/18:1) formed the adduct ion $[M+HCOO]^-$ and
400 fragmented ion $[M-15]^-$ at *m/z* 804.5786 and 744.5560, respectively. In the MS/MS spectrum
401 of the $[M+HCOO]^-$ ion of standard PC (16:0/18:1), the predominant fragment ions were
402 carboxylate ions at *m/z* 255.2333 (sn-1 fatty acid, 16:0) and 281.2492 (sn-2 fatty acid, 18:1).
403 One of the characteristic PC headgroup fragment ions was observed as a very low signal for
404 both the deprotonated demethylated phosphocholine ion at *m/z* 168.0431 and demethylated
405 phosphocholine dehydrated glycerol ester at *m/z* 224.0696. Fatty acid chain related ions,
406 including demethylated lysophosphatidylcholines $[M-FA-15]^-$ at *m/z* 462.2996 and 488.3141,
407 and $[M-15-FA\ ketene]^-$ at *m/z* 480.3100 and 506.3253, were observed under high energy
408 collision-induced dissociation. The MS data showed good agreement between the PC
409 (16:0/18:1) and QC samples.

410 PE is a class of phospholipids found in cell and biological membranes, and is abundant in beef
411 (Patel & Witt, 2017; Van der Veen, Kennelly, Wan, Vance, Vance & Jacobs, 2017). In contrast
412 to PC, the predominant ion of PE in negative ionization mode was the deprotonated parent ion
413 $[M-H]^-$. Structural information related to fragment ions of PE was deduced from the MS/MS
414 spectra of the standard PE (16:0/18:1) (Han, 2016; Hsu & Turk, 2009), for example, the
415 MS/MS spectrum of deprotonated parent ion [M-H]- of standard PE (16:0/18:1) at *m/z*
416 716.5236 (Figure S3b). The nature of PE was reflected by a group of PE headgroup fragment
417 ions, a deprotonated PE ion at *m/z* 140.0316 and a deprotonated doubly dehydrated glycerol-
418 phosphocholine at *m/z* 196.0023. The length of fatty acid chains of PE was determined from
419 the carboxylate ions at *m/z* 255.2290 and 281.2270. In contrast to PC, the lyso form of PE, [M-
420 FA]$^-$ and $[M-FA\ ketene]^-$ was absent in the MS/MS spectrum. Thus, the metabolite ion at *m/z*
421 716.5236 in the QC samples was determined to be PE (16:0/18:1). The MS data showed good
422 agreement between the PE (16:0/18:1) standard and QC samples (Figure S3b). However, there

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

425 3.4 Markers selection from the multivariate analysis

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

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

441 Figure 1a presents a cross-validated score plot ($R_2X = 0.37$, $R_2Y = 0.80$, $Q_2Y = 0.62$, p-value
442 $= 0.0005$) of the discriminating model between Australian beef and US beef using data from
443 UPLC-Orbitrap-MS in positive ionization mode and GC-MS. 90% of the Australia samples
444 were correctly assigned while 100% of US samples were correctly assigned. To further identify
445 metabolites associated with the group's separation, an S-plot was generated and several
446 potential biomarkers were highlighted (Figure 1b). In addition, the loading plot with the
447 jackknife confidence intervals was shown in Figure 1c and metabolites with high statistical
448 reliability were clearly indicated. In general, discriminated metabolites had a high covariance
449 combined with a high correlation, resulting in a small confidence interval. Some unique
450 markers such as PC (15:0/18:1), 9-HODE, myo-inositol and FA 22:4 were screened out by the
451 jackknife confidence intervals and their relative intensities of the beef samples between two
452 countries were depicted in Figure 1d. Since OPLS-DA is designed for classification of two
453 groups only (Brereton & Lloyd, 2014), pairwise comparison of OPLS-DA with leave-one-out
454 cross-validation among groups was performed.

455 3.5 Discrimination of geographical origins

456 Cross-validated score plots, S-plots and loading plots with jackknife confidence intervals
457 among beef from Australia, the US and Japan are shown in supplement material (Figure S6).
458 90% and 92.8% of Australian and US samples were correctly assigned, respectively while 100%
459 of Japanese samples were correctly assigned. Ultimately, twenty-four metabolites with
460 significant differences were identified; their relative concentrations in Australian, Japanese,
461 and US beef samples are shown in Figure 2.

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

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

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

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

510 One unique metabolite, 9-hydroxy-octadecadienoic acid (9-HODE), was identified in all the
511 beef samples, albeit only existed in low concentration in the Australian samples. 9-HODE has
512 been proposed as an excellent marker for lipid peroxidation in vitro (Spiteller & Spiteller, 1997).
513 Lipid oxidation has a high impact on the overall quality of beef since it adversely affects the
514 colour, texture, nutritional value and safety of the meat. Storage conditions such as repeated
515 freezing-thawing, photooxidation, and a higher degree of fatty acid unsaturation favor meat
516 oxidation (Boselli, Rodriguez-Estrada, Fedrizzi & Caboni, 2009; Chen et al., 2018). Therefore,
517 9-HODE might be a good indicator for the assessment of the quality or freshness of beef.

518 3.6 Discrimination between Australian organic and non-organic beef

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

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

532 3.7 Discrimination between Angus beef from the US and Australia

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

539 Eight identified lipid metabolites, which belonged to the PC class, were found to be suitable
540 biomarkers for discriminating between US and Australian Angus beef. These findings indicate
541 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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626

627 **Conflict of Interest Statement**

628 The authors have no conflict of interests to declare.

629

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846

847 A list of Table

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

850

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

852

853 A list of Figure:

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

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

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

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

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

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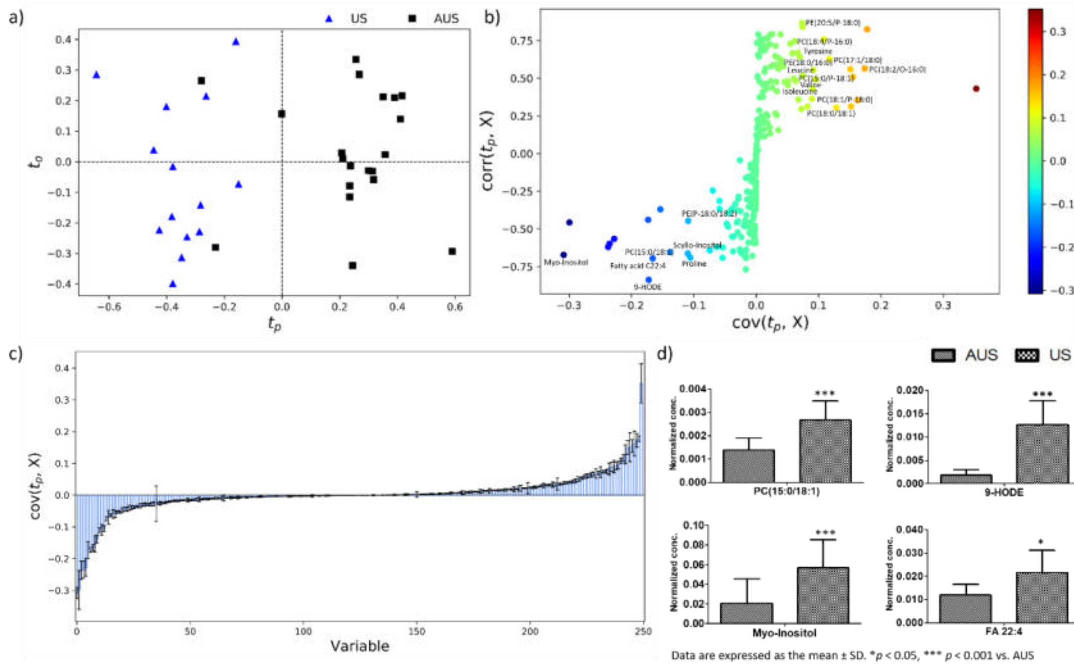

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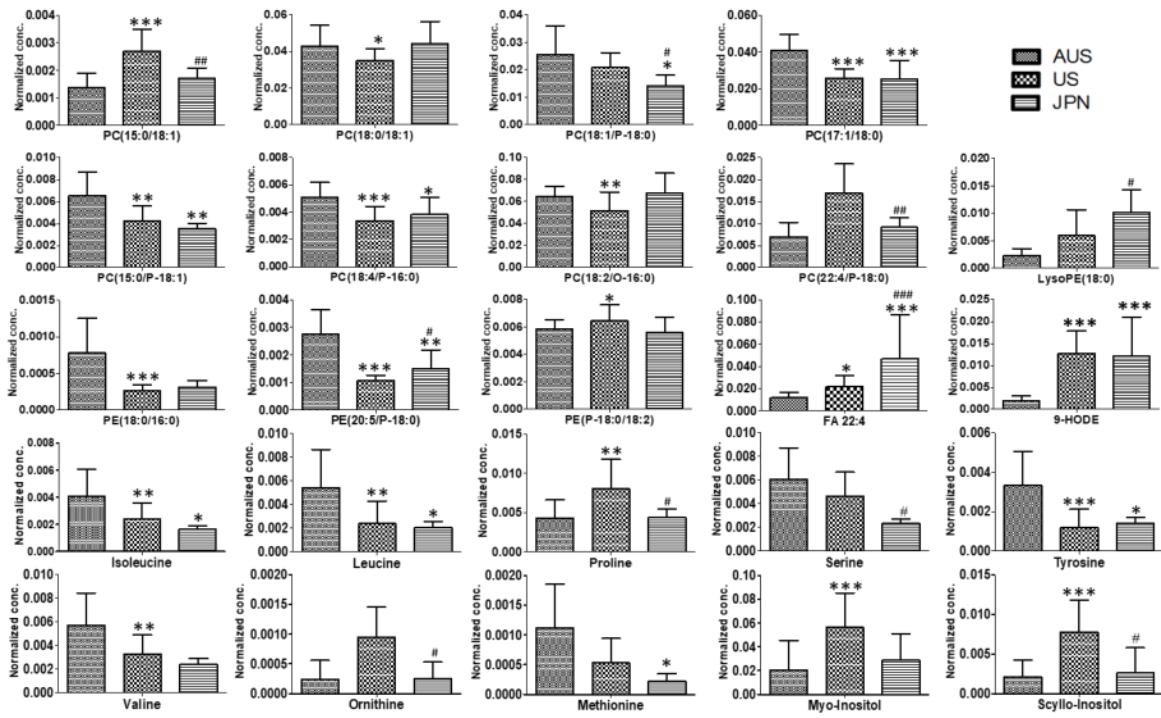

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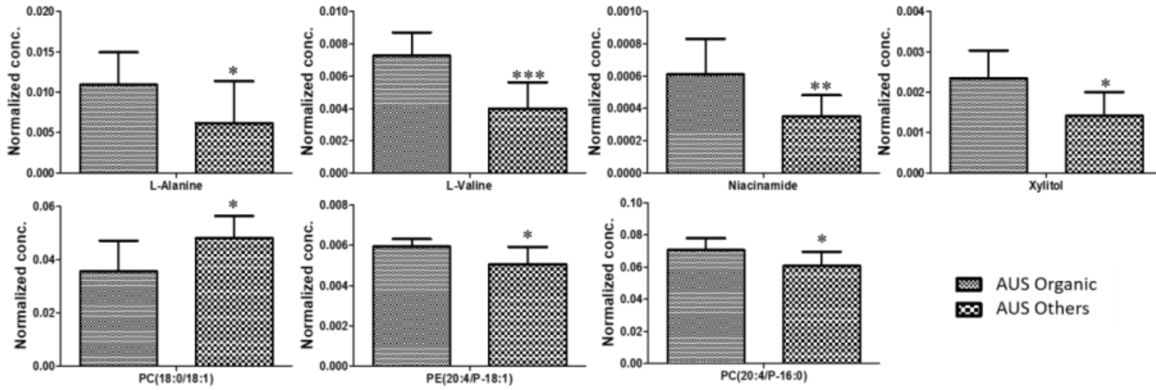

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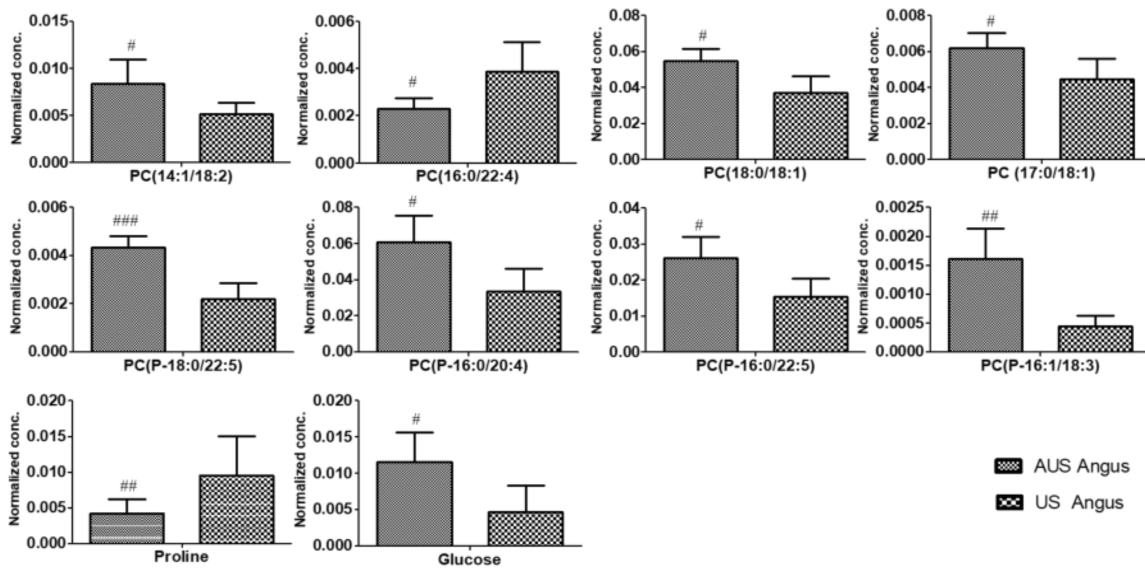

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904 Japanese beef; US, US beef; US Angus, US Angus beef. ** $p < 0.01$, *** $p < 0.001$ vs. AUS,
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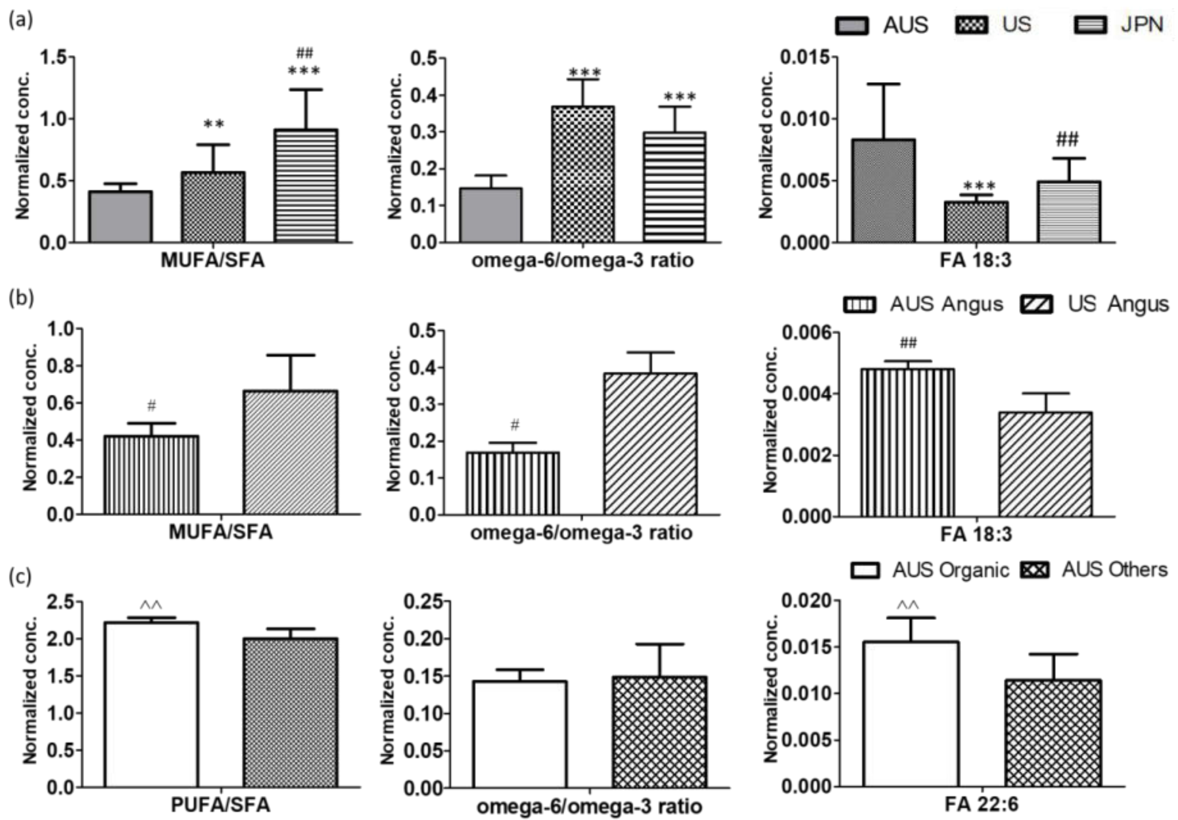

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