MALDI-TOF MS Protein Profiling Combined with Multivariate Analysis for Identification and Quantitation of Meat Adulteration

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Abstract

The problem of adulteration and mislabeling in meat products has raised the public concerns globally. An easy-operation, fast and robust method that applicable to routine inspections is urgently needed. This study showed that the MALDI-TOF MS protein profiling of four meat species (beef, chicken, duck and pork) combining with partial least squares discriminant analysis (PLS-DA) discovered 57 feature peaks for their unambiguous differentiation. Among them, 36 were identified in Uniprot. Based on the linear relation between the intensities of feature peaks, the partial least squares regression was successfully applied to build the prediction models for determining the adulteration ratios of beef meat mixtures containing one of the other three species. Blind tests were applied to evaluate the method and the average prediction accuracy at 94.7% was achieved. Taking duck meat as the adulterant, the detection sensitivity of the method could be down to 5%. Moreover, the method has also been successfully applied to analyze market samples and the results were in agreement with the PCR method, showing the potential of its practical application for qualitative and quantitative analysis of adulterated beef products.

Introduction

Fraud of meat and meat products has become a prevalent issue around the world (Temisak et al. 2021). The adulteration or substitution of higher price meat with lower price meat and mislabeling are common practices (Abbas et al. 2018; Ballin et al. 2009; Black et al. 2016; Temisak et al. 2021), especially in processed meat products. These illegal activities not only cause the economic losses of consumers (Hossain et al. 2020), but also lead to healthy risks such as the exposure of pathogens and allergens (Ballin 2010; Velioglu et al. 2018). To some extent, meat fraud may cause religious problems, for example, pork and pork products consumption is banned according to Kosher and Halal food laws (Alamprese et al. 2013; Hossain et al. 2020; Ortea et al. 2012). Therefore, it is important to develop a simple, sensitive, accurate and high throughput meat authentication method (Ortea et al. 2012; et al. 2020; Wang et al. 2018) to assist governments and enterprises in monitoring the quality and safety of meat products.

Indeed, various analytical approaches have been used to assure the meat authenticity. Polymerase chain reaction (PCR) technique is the most representative DNA-based method widely used for the meat authentication, due to its sensitivity, accuracy and reliability (Alikord et al. 2018; Ren et al. 2017). However, its high sensitivity can also bring the possibility of false positives when the animal origin is contaminated with other meat species during the production (Rahmati et al. 2016; Ren et al. 2017; Kumar et al. 2014). Moreover, the technique has some shortcomings, like high cost, time-consuming and complicated procedures (Ding and Xu 1999; Scott and Knight 2009; Velioglu et al. 2018). Infrared spectroscopy (IRS) and Raman spectroscopy (RS) are commonly used for analyzing characteristic lipids in meat, because of their rapid, simple and noninvasive properties. However, these methods are sensitive to moisture and temperature, thus affecting the detection reproducibility and accuracy (Sørensen et al. 2012). Mass spectrometry (MS), a highly sensitive and specific analytical method, has been widely used in food analysis (Alikord et al. 2018). Moreover, liquid chromatography-mass spectrometry (LC-MS) is one

of the most commonly used MS techniques applied to meat species classification (Gatmaitan et al. 2021). However, the involvement of tedious sample pretreatment (like protein digestion) and slow chromatographic separation process create some limitations for the application of LC-MS in the large-scale analysis (Li et al. 2018; Prandi et al. 2019).

Matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF MS) is regarded as a rapid and reliable MS method (Laakmann et al. 2013; Lagacé-Wiens et al. 2012). Its ultra-high throughput at the analysis rate of 10,000 samples per day (Raad et al. 2016) outperforms most analytical methods, including PCR (Bookout et al. 2006; Taboada et al. 2017), thus being particularly suitable for the large-scale screening of imported food products. In practical applications, it allows the direct analysis of characteristic proteins in complex biological samples, and has been widely utilized in the routine identification of microorganisms (Maier et al. 2006; Sandrin et al. 2012; Santos et al. 2016; Wieser et al. 2011) and discovery of diseases biomarkers (Aslani et al. 2018; Kriegsmann et al. 2014; Rodrigo et al. 2014). It was also used in food identification like detection of milk adulterations (Sassi et al. 2015) and determination of the geographical origin of honey (Wang et al. 2009). In meat identification, it has been applied to the determination of the origin of pork, beef, horse meat, mutton and chicken via the protein-based untargeted analysis (Flaudrops et al. 2015). Moreover, MALDI-TOF MS was combined with the cluster analysis to build a protein fingerprint database of common edible fishes for the analysis of meat adulteration and substitution (Stahl and Schröder 2017). Recently, Shi et al have demonstrated that the specific protein N-glycosylation profiles determined by UPLC and MALDI-TOF MS can be applied to the qualitative and quantitative analysis of meat adulteration (Shi et al. 2019). However, the complicated and tedious sample preparation procedures, including the separation of protein N-glycans, followed by PNGase F enzyme digestion and chemical derivation/labelling, could limit its practical applications. Therefore, the further development of MALDI-TOF MS to enhance its robustness for the qualitative and quantitative analysis of meat adulteration is required.

Herein, a quantitative method using MALDI-TOF MS protein profiling combined with Partial least squares discriminant analysis (PLS-DA) and partial least squares (PLS) model, aiming to determine the adulteration ratio of meat is reported. In this approach, the MALDI-TOF MS method was employed to obtain the protein profiles of four meat species, including chicken, duck, pork, beef, and their two-species binary mixtures containing the expensive meat “beef” adulterated with the other three species, according to Table S1. Principal component analysis (PCA) was used to reveal the differential clustering of the pure meat and the binary mixtures. PLS-DA was applied to select feature peaks to discriminate four meat species, which were then identified through the protein database (Uniprot). Based on the linear relationship of the ion intensities of the feature peaks in the different binary mixtures, PLS model was developed to quantify the adulteration ratios (Aliaño-González et al. 2019; Shi et al. 2019). This integrated approach was successfully validated with the blind tests and applied to the analysis of market samples, showing its potential in performing qualitative identification of meat adulteration and quantitative determination of adulteration ratios.

Materials And Methods

Urea (Aladdin, 99.5%); Thiourea (Aladdin, 99%); Tris (Aladdin, 99%); distilled water (Watsons); Bradford protein assay kit (Biosharp, BL524A); sinapic acid (Aldrich, 98%); trifluoroacetic acid (Alfa Aesar, 99.5%) were used as received.

Four species of meat, including chicken, pork, beef and duck, are common edible meat, were selected in this study. Totally, 103 pure meat samples and 81 mixed-meat samples were collected and the different tissue parts of each species shown in Table S1 were used. Pork, chicken and duck were chosen to mix with beef meat in a binary mixture at the mass percentage of 0.0%, 25.0%, 50.0%, 75.0% and 100.0% for mimicking beef meat adulterated with other species. Moreover, the ternary mixture samples containing 50% beef meat mixed with different percentage of chicken meat (50–0%) and pork meat (0–50%) were used for investigating the ability of the approach in differentiating the ternary mixtures. As the price of duck meat is the lowest among many different meat species, it is likely to partly substitute beef products with duck meat in adulteration. Therefore, duck meat was mixed with beef meat in 3% and 5% to evaluate the detection limit of the approach. All of these meats in fresh were purchased at local markets or supermarkets. Five market samples for testing were purchased in a supermarket and a hotpot restaurant. All the meat samples were stored at -20℃ until use.

For each sample, 5 g of muscle tissues was chopped into paste, fascia and fat were discarded. Then, 0.5 g of meat paste was suspended in 10 mL protein extraction reagent (6M Urea, 1M Thiourea, 50mM Tris, at pH8.2) (Bargen et al. 2014) and homogenized with a homogenizer operated at 8000rpm for 2 min. The homogenate was centrifuged at 12000g for 1 min at 4℃. The supernatant was then filtered with a 0.45μm (polyvinylidene fluoride, PVDF) membrane filter, followed by dialysis in distilled water for 8 hours.

To minimize the effect of non-biological factors (such as different extraction efficiency and the protein content of different individuals) on the protein profiles, the protein content normalization is applied (Wu and Li 2016). The protein concentration of each dialyzed sample was measured using Bradford method with a microplate reader (Synergy H1 H1MF, Agilent, USA). The sample was diluted with 0.1% trifluoroacetic acid solution to 0.35 mg/ml (protein concentration). Quality control (QC) samples were prepared by mixing 20uL protein solution of each diluted sample for evaluating the stability of this method. The MALDI matrix solution, saturated sinapic acid (SA) solution, was prepared by dissolving SA in 30% acetonitrile solution with 0.1% trifluoroacetic acid. The sample solution (15uL) was mixed with saturated SA solution in 1:1 ratio. Then, 2.5μl of each mixture was applied on the MALDI sample plate (n = 5) and dried at room temperature, which was then analyzed with a MALDI-TOF mass spectrometer. Triplicated analyses were performed to ensure the intraday and interday reproducibility.

The Autoflex Speed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) was employed in this study. All the analyses were conducted in the positive ion mode with a mass range of 3,000 – 22,000 Da in a linear mode. The MS conditions are as follows: laser power: 99.6%; frequency: 500Hz; shot number: 20000 operated at random walk mode; delayed extraction time: 350 ns.

The MALDIquant package (Gibb and Strimmer 2012) of R programming language was used for data preprocessing, including spectra smoothing, baseline correction, alignment, technical replicates averaging and peak detection. Finally, a feature matrix was created and then a normalization method (MS total useful signal, MSTUS) (Wu and Li 2016) built in Microsoft Excel was used to normalize this feature matrix.

Multivariate data analysis was performed with SIMCA-P14.1 software (version 14.1; Umetrics AB, Umeå, Sweden). PCA was applied to reveal the general clustering and grouping trends among four meat species. PLS-DA was used to maximize the separation between samples, and the feature peaks responsible for the separation were found based on the corresponding V + S-plots (VIP $\geq$ 1, |p(corr)| $\geq$ 0.5, |p| $\geq$ 0.05) of PLS-DA (Almeida et al. 2013; Bao et al. 2016; Li et al. 2019; Peerbhay et al. 2013). Then, these feature peaks of four meat species were further confirmed in the MALDI-MS raw spectra based on a sufficient signal-to-noise ratio (S/N > 3) and a good reproducibility, and then identified through Uniprot (the protein database). To build a linear regression prediction model for determining the adulteration ratios in binary mixtures, PLS method is applied, in which, samples among four meat species were separated into training (2/3) and validation set (1/3). (Table S2).

To evaluate the prediction capability of our approach, blind test and market-sample test were adopted. Seven binary mixture samples of beef mixed with chicken, duck or pork in random ratios (sample 1 − 7) were prepared as the blind samples, and five beef products (M1, M2, M3, M4 and M5) purchased from a supermarket and a hotpot restaurant were used as the market samples. The same sample preparation procedures and MS data acquisition conditions mentioned above were adopted for the sample analysis. Moreover, the same multivariate data analysis, including PCA, PLS-DA and the linear regression prediction model based on partial least squares (PLS) method were applied to the data analysis.

Results And Discussion

The representative mass spectra of four species of meat, including chicken, duck, pork and beef, are shown in Fig. 1a. The differential protein profiles shown in the mass range of 3000 to 22000 Da illustrated the different protein composition of the four species. However, the protein profiles of different tissue parts collected from the same species showed similarity (Fig. S1). In the PCA analysis of the processed ion peaks derived from the protein profiles, the score plot (Fig. 1b) revealed a partial differential clustering of four meat species, and the tight clustering of the QC samples illustrated the high stability of the method.

Encouraged by the partial differential clustering of the four meat species, PLS-DA was further applied to select feature peaks for the complete differentiation of each meat species in the binary mixtures, as shown in Table S3. Using the criteria (VIP $\geq$ 1, |p(corr)| $\geq$ 0.5, |p| $\geq$ 0.05), totally 85 important variables with significant contribution to the separation of four meat species were regarded as feature peaks. The quality of the PLS-DA models was assessed by three parameters (R^2, Q^2, RMSECV) supplied by the software. The accumulated explanatory power of the first two principal components on the original

variable (R^2 all greater than 0.98) showed a goodness-of-fit and the accumulation of predictive power (Q^2 all greater than 0.97) showed good model-predictability (Jiang et al. 2017). The root mean square error from cross-validation (RMSECV all smaller than 0.07) indicated that the important features that derived from the PLS-DA models are reliable (Aliaño-González et al. 2019; Li et al. 2015). After checking with MS raw spectra to further confirm the presence of these feature peaks, a total number of 57 characteristic ion peaks were obtained (Table S5). 36 out of the 57 were identified in Uniprot and are summarized in Table 1. In the PCA analysis of the 57 feature peaks derived from the protein profiles, the score plot (Fig. 1c) revealed a complete separation of four meat species at the confidence level of 95% (Fig. S2).

Table 1
36 Feature peaks identified in Uniprot.

MW of Characteristic Ion Peak [Da]	Identified Proteins	MW of Protein in Uniprot [Da]	Accuracy	Species
5531.1	Uncharacterized protein	5,531	99.99%	Beef
6363.9	Uncharacterized protein	6,362	99.97%	Beef
6521.2	Uncharacterized protein	6,524	99.97%	Beef
6675.6	40S ribosomal protein S29	6,677	99.98%	Beef
6947.9	Beta-defensin	6,947	99.99%	Beef
6951.1	Tracheal antimicrobial peptide	6,954	99.96%	Beef
8300.8	Homeodomain-only protein (Odd homeobox protein 1)	8,299	99.98%	Beef
9062.4	Cytochrome c oxidase subunit 7A1, mitochondrial	9,063	99.99%	Beef
9463.5	40S ribosomal protein S27-like	9,463	99.99%	Beef
18914.2	CS domain-containing protein	18,912	99.99%	Beef
18925.4	Uncharacterized protein	18,926	99.99%	Beef
18936.3	Cytochrome P450, family 8, subfamily B, polypeptide 1	18,936	99.99%	Beef
7572.6	Uncharacterized protein	7,575	99.97%	Chicken
9144.8	G_PROTEIN_RECEP_F1_2 domain-containing protein	9,143	99.98%	Chicken
18289.6	Aminoacyl-tRNA hydrolase	18,290	99.99%	Chicken
5548.2	short transmembrane mitochondrial protein 1	5,545	99.99%	Duck
8359.4	ATP synthase subunit e, mitochondrial	8,357	99.97%	Duck
8772.7	serine protease inhibitor Kazal-type 6-like	8,775	99.97%	Duck
9542.8	Molybdopterin synthase sulfur carrier subunit	9,545	99.98%	Duck
15094.6	28S ribosomal protein S12, mitochondrial	15,903	99.99%	Duck
5458.7	KRAB domain-containing protein	5,461	99.96%	Pork

MW of Characteristic Ion Peak [Da]	Identified Proteins	MW of Protein in Uniprot [Da]	Accuracy	Species
6296.0	HMA domain-containing protein	6,295	99.98%	Pork
6322.7	Uncharacterized protein	6,323	99.99%	Pork
6330.2	Uncharacterized protein	6,329	99.98%	Pork
6554.4	Cytochrome b-c1 complex subunit 10	6,553	99.98%	Pork
6627.3	SAFB like transcription modulator	6,624	99.95%	Pork
6857.4	Small integral membrane protein 11A	6,854	99.95%	Pork
7955.7	Uncharacterized protein	7,957	99.98%	Pork
8285.9	Endoplasmic reticulum-golgi intermediate compartment 1	8,287	99.99%	Pork
9034.5	Tachykinin precursor 4	9,038	99.96%	Pork
9312.1	Uncharacterized protein	9,312	99.99%	Pork
9446.0	Chromosome 4 C8orf82 homolog	9,445	99.99%	Pork
16570.1	40S ribosomal protein S15	16,571	99.99%	Pork
18068.0	PhoLip_ATPase_N domain-containing protein;	18,067	99.99%	Pork
18870.0	Ig-like domain-containing protein	18,870	100.00%	Pork
18890.7	MYL2	18,891	99.99%	Pork

The mass spectra of beef mixed with chicken at the proportion of 0% (beef only), 25%, 50%, 75% and 100% (chicken only) are shown in Fig. 2a, while mass spectra of the binary mixtures containing beef mixed with other species, like duck and pork at the different proportions, are depicted in Fig. S3. It is worth noting that the intensities of some feature peaks changed gradually with the mixing proportion. For instance, the feature peak at m/z 5442 for beef, m/z 5388 for chicken, m/z 6192 for duck and m/z 6333 for pork, changed in a nearly linear fashion with their mixing proportion, as shown in Fig. 2b. Indeed, the similar trends were also observed in the score plot of the binary mixtures (Fig. 2c) derived from the PCA analysis of the 57 feature peaks. In which, the binary mixtures were located between their corresponding pure species, and shifting toward the pure species as the corresponding proportion increased. Moreover, the ternary mixtures containing 50% beef and different percentage of chicken and pork could be well separated in the PCA score plot (Fig. 2d). In addition, at a fixed percentage (50%) of beef, the positions of the ternary mixtures shifted toward the position of 50% beef-pork binary mixture as the proportion of pork meat increased, while the positions shifted backward to the 50% beef-chicken binary mixture as the

proportion of chicken meat increased. Moreover, in the determination of detection limit, the PCA score plot derived from the 57 feature peaks showed that 5% duck meat mixed in the beef-duck binary sample could be well separated at the confidence level of 95%, but 3% duck meat mixed in the binary mixture could not (Fig. S4). The linear trend provided the basis for building a PLS linear regression prediction model to predict the adulteration ratios in beefs. As shown in Fig. 3, the PLS linear regression prediction model of beef mixed with chicken (a1 and a2), duck (b1 and b2) or pork (c1and c2) at different percentage showed a linear trend with the correlation coefficients of R^2 all greater than 0.99 (for the training data set) and R^2 all greater than 0.99 (for the validation). Moreover, the root mean square error of estimation RMSEE were all smaller than 2.3 (for the training data set) and the root mean square error of prediction RMSEP were all smaller than 2.7 (for the prediction), indicating the good prediction potential of the PLS model (Aliaño-González et al. 2019; Li et al. 2015; Shi et al. 2019).

To verify the accuracy of the integrated approach, i.e., MALDI-TOF MS combined with the multivariate statistical analysis, for the identification of meat species and quantitation of adulteration ratios in the binary mixtures containing beef, seven blind-test samples (sample 1 to 7) were employed, and the results are shown in the PCA score plot (Fig. 4a). Taking the **sample 1** as an example, the location of the data point between the chicken and beef implicated that the beef meat was adulterated with chicken. The adulteration was also confirmed with the presence of feature peaks of chicken and beef in the **sample 1**, as shown in Table 2. The adulteration ratio in the beef-chicken binary mixture determined by the PLS linear regression prediction model was 73.6% (chicken) which was consistent with the true ratio (75.0% chicken) at the accuracy of 98.1%. Other blind test results for the different proportions of chicken, duck and pork in beef samples are shown in Table 2. The prediction accuracies were in the range of 91.6–98.1%, showing the good accuracy and reliability of our developed method.

Table 2

The presence of feature peaks of different species in blind-test samples (1–7) and market samples (M1, M2, M3, M4 and M5), and determination of adulteration ratios using the PLS linear regression models. (**B**: beef, **C**: chicken, **D**: duck and **P**: pork)

Sample No.	Presence of feature peaks (m/z)	Binary mixtures model	Ture ratio	Predicted ratio	Accuracy
1	**Beef**: 5443, 9463, 18912 **Chicken**: 4939, 6110, 6249, 9143	Chicken-Beef Mixed Model	**B**:25.0% **C**:75.0%	**B**:26.4% **C**:73.6%	98.1%
2	**Beef**: 5443, 5531, 6359, 6677, 6947, 8299, 9463, 18925 **Chicken**: 4939, 6110	Chicken-Beef Mixed Model	**B**:70.0% **C**:30.0%	**B**:68.0% **C**:32.0%	93.3%
3	**Beef**: 5443, 9463, 18926 **Chicken**: 4939, 6110, 6249, 9143, 18290	Chicken-Beef Mixed Model	**B**:20.0% **C**:80.0%	**B**:16.5% **C**:83.5%	95.6%
4	**Beef**: 6362, 9463 **Duck**: 4850, 5342, 5545, 6192, 6656, 6969, 7029, 7160, 7547, 8357, 8775, 15093	Duck-Beef Mixed Model	**B**:10.0% **D**:90.0%	**B**:14.4% **D**:85.6%	95.1%
5	**Beef**: 6358, 6362, 9463, 18926 **Duck**: 4850, 5342, 6192, 6397,	Duck-Beef Mixed Model	**B**:60.0% **D**:40.0%	**B**:57.1% **D**:42.9%	92.8%
6	**Beef**: 5443, 9463, 18926 **Pork**: 5000, 5461, 6295, 6329, 8287, 9445, 16571, 18067, 18891	Pork-Beef Mixed Model	**B**:30.0% **P**:70.0%	**B**:28.1% **P**:71.9%	97.3%
7	**Beef**: 5442, 6358, 18926 **Pork**: 5000, 5461, 6295, 6329, 6553, 6624, 8287, 9445, 16571, 18891	Pork-Beef Mixed Model	**B**:20.0% **P**:80.0%	**B**:26.7% **P**:73.3%	91.6%
M1	**Beef**: 5443, 5531, 6359, 6677, 6947, 8299, 9463, 18926	—	—	**B**:100%	—
M2	**Beef**: 5443, 6359, 6677, 8299, 9463, 18926	—	—	**B**:100%	—
M3	**Beef**: 5443, 6359, 6677, 7327, 9463, 18926	—	—	**B**:100%	—
M4	**Beef**: 5443, 6359, 6677, 6947, 8299, 9063, 9463, 18926	—	—	**B**:100%	—
M5	**Beef**: 9063, 9463, 18926 **Chicken**: 4939,7575	—	—	**B**:77.5% **C**:22.5%	—

To further confirm the practical application of this approach, four fresh beef products (M1, M2, M3 and M4) collected from a hotpot restaurant and one beef ball cooked sample (M5) collected from a supermarket were analyzed. The PCA score plot (Fig. 4b) showed that the fresh beef product is classified into beef without mixing with the other species at 95% confidence level (Fig. S5), which was also confirmed with MS data showing the absence of corresponding characteristic ion peaks of chicken, duck and pork meats. It is worth noting that the data point of the beef ball sample shown in the PCA score plot was away from the cluster of beef samples and approached to the chicken samples. The adulteration of the beef ball sample mixed with chicken meat was confirmed with the presence of their corresponding feature peaks (7 peaks for beef and 2 peaks for chicken, as shown in Table 2). Moreover, the adulteration species was confirmed with the PCR method, as shown in Fig. S6, and the adulteration ratio determined by the PLS linear regression model was 22.5%.

Conclusions

In summary, a simple, fast and accurate method, namely MALDI-TOF MS protein profiling combined with multivariate statistical analysis, was developed in this study for qualitative and quantitative analysis of beef meat adulterated with chicken, duck and pork. A total of 57 feature peaks (with 36 identified in Uniprot) were discovered for the authentication of the four species. Moreover, PLS was successfully applied to achieve the prediction of different adulteration ratios of the binary mixtures (i.e., beef meat mixed with chicken, duck or pork). The $R^2 > 0.99$, RMSEE < 2.3 (for the data set) and RMSEP < 2.7 (for the prediction) of the three PLS models (i.e., the beef-chicken, beef-duck and beef-pork binary mixtures) indicated the robustness of the models. The average prediction accuracy at 94.7% in the blind tests confirmed the reliability of our developed method. More importantly, the method has been successfully applied to analyze five market samples in real life. One sample containing chicken meat was discovered and confirmed with the PCR method. Using the PLS regression model, the adulteration percentage of chicken in the sample was determined to be 22.5%, showing the practical application of our developed method for beef products screening. This approach is sensitive in detecting the presence of other meat species (duck, chicken, pork meats) in the targeted meat products (beef products), and its detection sensitivity could be down to 5% of adulterant (taking duck meat as the example). The success of this approach is mainly due to its simplicity of incorporating the characteristic feature peaks of targeted meat and adulterants in the simple PCA model, which enables the unambiguous differentiation if the adulterants are present in the products. Therefore, it is anticipated that the approach can be extended to more adulterant meat species when the characteristic feature peaks of other meat species are detected and incorporated into the model.

Declarations

Supplementary Information The online version contains supplementary material available at

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Author Contribution K. Pu designed and performed the experiments and data analysis, and wrote the manuscript; J. Qiu performed the experiments and data analysis, and wrote the manuscript; J. Li performed the experiments; W. Huang assisted the preparation of blind-test samples, and revised the manuscript; X. Lai assisted the data analysis, and revised the manuscript; C. Liu checked the processed data; Y. Lin revised the manuscript; K.-M. Ng contributed to the conceptualization, supervision of the study, and manuscript revision.

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Data Availability Data will be made available on reasonable request.

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

Conflict of Interest Keyuan Pu declares that he has no conflict of interest. Jiamin Qiu declares that she has no conflict of interest. Jiaying Li declares that she has no conflict of interest. Wei Huang declares that he has no conflict of interest. Xiaopin Lai declares that she has no conflict of interest. Cheng Liu declares that he has no conflict of interest. Yan Lin declares that she has no conflict of interest. Kwan-Ming Ng declares that he has no conflict of interest.

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Figures

Figure 1

(**a**) MALDI-TOF MS spectra of four meat species. The m/z values of the major ion peaks are labelled, while others are summarized in Table S4. (**b**) PCA score plot derived from the processed ion peaks of the protein profiles of the four meat species and quality control samples. (**c**) PCA score plot derived from the 57 feature peaks of the protein profiles of the four meat species.

Figure 2

(**a**) MALDI-TOF MS spectra of beef meat mixed with chicken meat in an increasing ratio. Some feature peaks in the range of 4500-7000 Da showing the ratio-dependence are shaded in grey. (**b**) Intensities of selected feature peaks of beef (at 5442 Da), chicken (at 5388 Da), duck (at 6192 Da) and pork (at 6334 Da) increased with mixing proportions, and (**c**) PCA score plot derived from the 57 feature peaks showing the separation of pure beef, chicken, duck and pork meats, two-species binary mixtures and (d) PCA score

plot showing the separation of ternary mixtures containing 50% beef meat mixed with different percentage of chicken and pork ranging from 0% to 50%.

Figure 3

PLS training and validation models for the quantitative determination of mixing ratios of beef meat mixed with chicken (**a1** and **a2**), duck (**b1** and **b2**) and pork (**c1** and **c2**) in the binary mixtures.

Figure 4

PCA score plot derived from the 57 feature peaks showing the locations of (**a**) blind test samples (sample 1 to 7) and (**b**) market samples (sample M1, M2, M3, M4: fresh beef, and M5: cooked beef ball).

Supplementary Files

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