Developing metabolomics and proteomics tools to identify the provenance of foods and beverages of economic importance in Scotland.

RI-B6-02

Report for Deliverable 1: Survey the use of proteomics in meat authenticity and deliver a report on the advantages and disadvantages of the different MS methods

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Executive summary:

In this report we have surveyed the literature for the use of mass spectrometry based proteomic methods for assessing the provenance of meat, meat fraud and adulteration of meat. Methods could be divided into two broad groups – i. open profiling or non-targeted where as many proteins/peptides are profiled as possible and ii. targeted where a small number of pre-selected proteins/peptides are quantified. Using these methods, a variety of meats have been profiled including chicken, duck, goose, turkey, pork, beef, lamb, rabbit, buffalo, deer, and horse meat. There is no consistent method across the literature and instead there are a number of alternative approaches which have merits and drawbacks for individual analyses. We perform a strength and weakness analysis of the recent literature to identify one method of sample preparation, one workflow for peptide identification and three methods of mass spectrometry (two open-profiling and one targeted) to take forward in subsequent analyses.

1. Introduction:

1.1 Aim: The global aim of this project **is to develop mass spectrometry-based metabolomics and proteomics tools to identify the provenance of foods and beverages of economic importance to Scotland**. In this deliverable we will assess the use of these proteomic approaches to determine the **provenance of meats**, and in particular beef, by demonstrating that proteomics can be used in a high throughput, quantitative manner to prevent substitution with other meats, and determine other factors such as breed of animal and what the animal was fed on, all of relevance to the perceived quality of the meat being sold.

1.2 Meat adulteration: The Threat: The consumer has a right to know what is contained within the food they eat. Allied to this, there is a significant risk in the food supply chain of fraud where a food is replaced by a cheaper or inferior alternative (e.g. the horse meat crisis in 2013 [HM Government PB 14089]). The risks are particularly high for a number of food and drink products produced within Scotland that attract a premium because of their higher quality and consumer desirability (e.g. whisky, beef and honey). As well as safeguarding the provenance of food within Scotland, following Brexit and the need to develop more global food chains, the potential for food fraud has increased and there is a need to develop new high throughput methods that can confirm the provenance of food substances across the food chain.

Identifying the origin of animal species in processed meat products is of great importance for economic, health and religious reasons. The inability to readily identify meat species in processed products gives rise to the potential for species mislabeling, in which one species is substituted intentionally by another, often cheaper one, or unintentionally by cross-contamination when processing different types of meat in the same facilities (2). This may also affect the exposure of consumers to meat allergen risks and cause serious ethical concerns for people who have dietary restrictions due to religious practice.

1.3 Analytical methods for meat adulteration: A variety of methods have been proposed to follow meat adulteration in processed food products including electrophoretic techniques (3), enzyme-linked immunosorbent assay (ELISA) (2), and real-time polymerase chain reaction

(PCR) (4). However, their application to highly processed meat products is limited because of denaturation of proteins and degradation of DNA (5). Cross-reactivity between species for ELISAs has also produced unreliable results (6).

Proteomics is the term used for the comprehensive detection of proteins in a sample (as oppose to methods that focus on a single protein). One of the first developed techniques for meat authentication was electrophoretic based methods which allowed the simultaneous analysis of several proteins or their fragments (peptides) (7) (8). However, these methods have low sensitivity, relatively slow and poor quantitation capability.

In recent years, mass spectrometry based proteomic methods have become more powerful tools in food authentication. Mass spectrometry platforms make possible the high-throughput analysis of thousands of proteins in one experiment. Most Mass Spectrometry approaches for identification and authentication are based on protein pattern profiling (9) or on the identification of specific peptides or proteins to identify the species (10).

1.4 Proteomic workflows for food authentication: Broadly speaking, there are two proteomic approaches used for meat analysis (11). Open profiling or non-targeted workflows profile a wide range of peptides and use a 'fingerprint' derived from the data to identify a particular meat type or characteristic of the meat (e.g. breed of animal, what the animal was fed on, sex, age). This is ideal for discovery workflows. Targeted workflows are designed to detect only a small set of selected and specific peptide markers, and can be much more readily made quantitative.

A number of species-specific proteins and peptides have been identified in open-profiling methods that are capable of detecting different meat products. For example, this includes species-specific peptide markers for raw pork (12), and the simultaneous discrimination of beef, pork, chicken, and duck in raw meat mixed samples (8). Using a targeted approach Watson and co-workers (2015) developed a method for the identification of four species (beef, lamb, pork, and horse) in raw meat and detection of one meat added to another at levels of 1% (w/w) (13). Furthermore, Stachniuk and colleagues (1) recently reported 105 heat-stable peptides detectable in processed meat by liquid chromatography tandem mass spectrometry (LC-MS/MS) (1) which could discriminate chicken, duck, goose, turkey, pork, beef, lamb, rabbit, buffalo, deer, and horse meat. In thermally processed samples, myosin, myoglobin, hemoglobin, L-lactase dehydrogenase A and β-enolase are the main protein sources of heat-stable markers in many studies. The availability of more than one peptide marker for

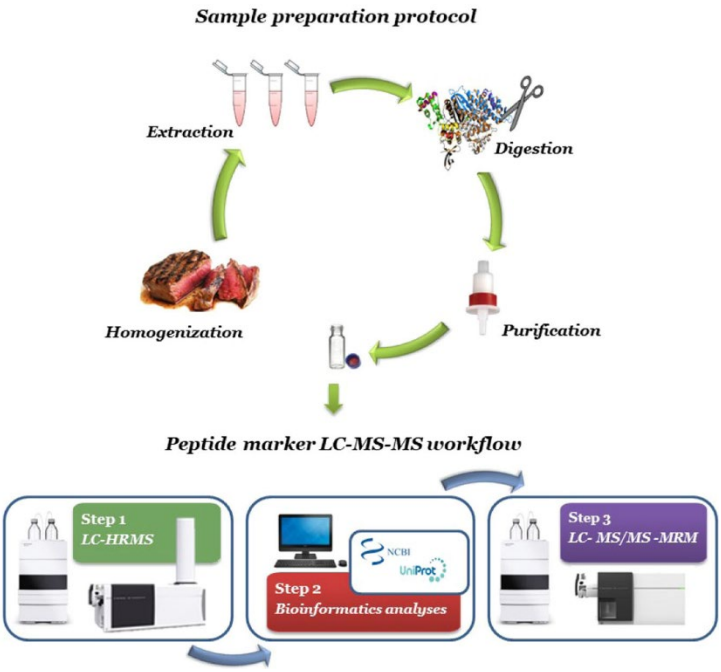

Figure 1. Block diagram for sample preparation and peptide marker liquid chromatography–mass spectrometry (LC-MS/MS) discovery workflow (1).

each species, originating from at least two different target proteins, is highly desirable because different products are characterized by different degrees of protein degradation resulting from food processing (14). The accuracy of LC-MS/MS in detecting food fraud is greatly improved when more than one heat-stable peptide marker is used for each species. Open profiling and targeted approaches are not mutually exclusive and (1) described a synergistic approach involving three-step analysis based on: (i) discovery-based experiments with open-profiling approaches; (ii) peptide identification with bioinformatics tools; and (iii) targeted confirmation of discovered peptides **(Fig. 1).**

In this report we have surveyed the literature for proteomic applications for the provenance of meat and its use in the detection of food fraud and adulteration. As part of this process, we have also considered applications to detect processed meats, mixtures of meats and the possibility of discriminating between different breeds of animals and what the animals have been fed on.

2. Methods:

Two independent reviewers (PP and SM) conducted an online database search of Pubmed for all literature studying meat authentication using proteomics in the last ten years. Search terms included one methodology and one application term:

Methodology	*Application*
Proteomics	meat authentication
Protein	meat fraud
Mass spectrometry	meat provenance

Additional eligibility criteria included: English language publications, published in peer-reviewed scientific or medical journals between April 2012 and March 2022. After removing duplicates (including duplicate methods), titles and abstracts of the identified publications were screened by 2 reviewers for eligibility. Publications were rejected on initial screen if the reviewers could determine from the title and abstract that it did not meet the inclusion criteria. Differences were resolved by discussion. One reviewer then identified all primary research papers, rejecting reviews with this being confirmed by the second reviewer.

Primary research papers were then assessed by a strength and weakness analysis, whereby each method was scored out of 5 for ease of sample preparation, trypsin digestion, mass spectrometry analysis, data analysis (including peptide identification), reproducibility of method, quantification and number of species of meat identified.

3. Results and recommendations:

The literature search identified **98 publications** that were relevant to the provenance of meat and/or meat fraud and proteomics (**Fig 2**). 66 publications were rejected as reviews, book chapters, out of scope or were not available for the reviewers to read. The out-of-scope papers included studies based on ELISA and PCR detection, were metabolomics or lipidomics methods or were based on other food stuffs (e.g. seafood).

Of the remaining manuscripts (32), 22 were rejected as they focused on the detection of a single meat type (e.g. horse meat), focused on the contamination of meat with other sources

of protein (e.g. plant protein), or were incompatible with the mass spectrometry facilities at the Rowett Institute.

The remaining 10 publications were examined by a strength and weakness analysis (**Appendix 1**). This analysis was used to evaluate different key aspects (e.g. steps) of recently published proteomics/peptidomics LC-MS methods used in meat adulteration. This survey demonstrated that there is not a universal LC-MS/MS based method suited for all applications of meat fraud using proteomics. Therefore, we propose that sample preparation, protein digestion and identification of proteins/peptides should be performed following the method described by (15) for overall ease of approach as well as the detailed information these methods provide subsequently. Most importantly, it includes the necessary washing steps of meat before homogenization. The protein digestion uses a simple and most commonly used protocol (100 mM aqueous ammonium bicarbonate).

For LC-MS methods we propose 3 LC-MS methods to take forward for further analysis: Two untargeted peptidomics LC-MS methods (16, 17) and 1 targeted method (18) (**Appendix 2**). For the untargeted peptidomics methods, 2 methods were selected using the 6550 IM-QTOF (Agilent Technologies) (1) and the Orbitrap Q-Exactive (Thermo Fisher) (16) which are both available to researchers at the Rowett Institute. For the targeted LC-MS/MS method, a method that uses the 6490 (Agilent Technologies) that is also available in our facilities was selected (18). Notably, this targeted MRM method is label-free which is advantageous, less complicated, and less expensive compared to an LC-MS/MS method that uses labeled peptides which was also evaluated amongst the 10 papers (19).

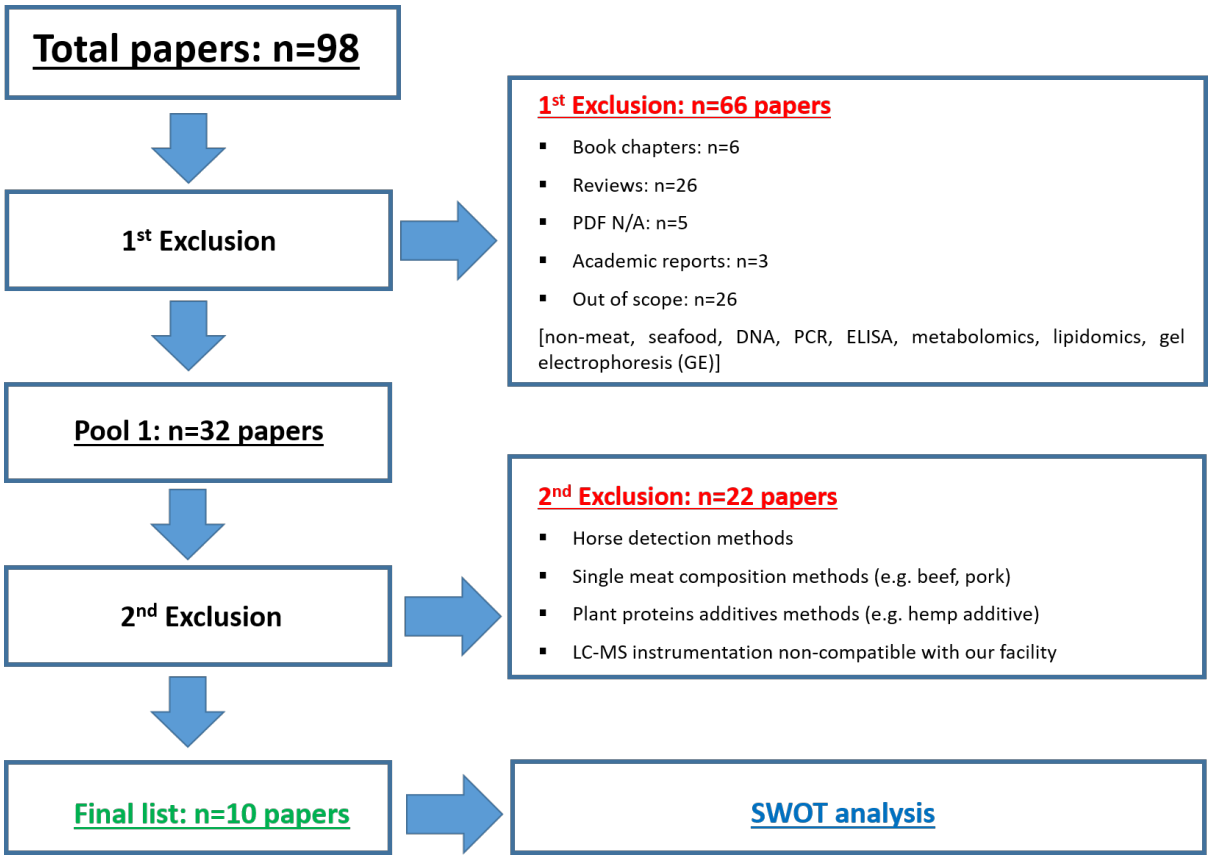

Figure 2. Literature workflow, exclusion criteria, and SWOT-like analysis for 10 selected papers.

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Appendix 1. Strength and Weakness analysis of 10 key representative papers for meat fraud proteomics by LC-MS/MS methods.

Publications	Sample prep	Trypsin Digestion	LC-MS analysis	Data analysis (protein Identification)	Reproducibility of method	Quantification method	Number of meat species identified
PAPER 1 (15) Food Chemistry 237 (2017) 1092–1100	5 Very comprehensively described. CITED IN MASS SPECTROMETRY REVIEWS (2019) as "One of the simplest extraction protocols	4 All steps described, but missing volumes of reagents	2 Untargeted nano-LC-MS analysis peptidomics nanoLC-MS is a complex method 60min analysis	5 A UniProtKB/Swiss-Prot database search for protein and peptide identification was performed using the Spectrum Mill MS Proteomics Workbench (Agilent Technologies).	4 samples were analysed in two technical replicates.	+2 Label-free but complex method; spectral matching technique, termed the absolute protein expression (APEX) proteomics tool.	+5 N=5 species poultry, pork, beef detection of 1% (w/w) of chicken and 1% (w/w) pork in a mixture of the meat of three species, as well as 0.8% (w/w) beef proteins in commercial poultry frankfurters
PAPER 2 (17) Food Chemistry 329 (2020) 127185	+5 Very comprehensively described. Sample (0.3 g) homogenized with 100 mM aqueous ammonium bicarbonate (1 mL) in lab ball homogenizer	+4 All steps described, missing volumes of reagents. trypsin at 37 °C for 18 h	+5 Untargeted peptidomics LC-MS/MS Q-TOF mass spectrometer (6550, Agilent) RRHD Eclipse Plus C18 column (Agilent Technologies) 60 min analysis time	+5 Agilent Mass Hunter Data Acquisition software and MassHunter Bioconfirm software. Spectrum Mill MS Proteomics Workbench. Identified peptides were evaluated for specificity using the BLAST Alignment	+5 RSD were in the range of 0.4–14.9% for three replicates of the same sample (n = 3) and 8.8–35% for six samples of rabbit meat purchased from six sources (n = 6)	+5 Label-free quantification using peak areas.	+5 N=9 species Rabbit, pork, beef, sheep, chicken, turkey, goose, duck, helmeted guinea fowl
PAPER 3 (19) Food Chemistry 274 (2019) 857–864	+5 Samples rinsed with ethanol/water (70:30), ethanol, methanol/ water (90:10) and milli-Q water consecutive Homogenised in 100mM aqueous ammonium bicarbonate and vacuum dried.	+4 All steps described, missing volumes of reagents. Trypsin at 37 °C for 18 h	+5 Targeted using Agilent 6460 QQQ UHPLC- (ESI)-QQQ-MS/MS Zorbax Eclipse Plus C18 Rapid Resolution HD column 42 min analysis time	+5 Data acquisition and data analysis were performed using Agilent MassHunter	0 N/A	+4 stable isotope-labelled peptides (AQUA) triggered MRM	+5 N=5 species Beef, chicken, pork, duck, goose

PAPER 4 (18) Food Chemistry 199 (2016) 157–164	+1 Complex sample prep ~1 g of meat was homogenised in 5 mL of ice cold extraction buffer containing 7 M urea, 2 M thiourea, 50 mM DTT, 4% (w/v) (CHAPS), 0.4% (v/v) carrier ampholytes and protease inhibitor followed by centrifugation at 12,000g for 10 min, 4 C.	+5 Very detailed protocol with volumes of reagents cited	+5 Very comprehensive & compatible LC-MS with our lab Both untargeted & targeted LC-MS/MS analysis AdvanceBio Peptide Map, C18 column 42min gradient LC–ESI-Q-TOF; Agilent 6520 Targeted method as a verification step 23 min analysis time	+5 Very comprehensive & compatible with our lab The mass chromatograms were analysed using Agilent MassHunter Qualitative Analysis software The extracted mzML data files of the MS/MS chromatogram were then exported to Spectrum Mill software for protein identification.	+5 Each sample was analysed in triplicate.	+5 Relative quantification Total ion (TIC), base peak (BPC) and extracted ion chromatogram (EIC) were analysed by examining the zoom scan ion of each peptide	+5 N=4 species Pork, beef, chevon and chicken meat
PAPER 5 (20) Food Chemistry Volume 283, 15 June 2019, Pages 489-498	+5 As previously described (Montowska & Fornal, 2017).	+5 trypsin at 37 °C for 18 h.	+3 Both untargeted & targeted LC-MS/MS analysis LC–ESI–QTOF–MS; nanoflow 55min analysis time. Targeted method: Zorbax Eclipse Plus C18 Rapid 35-min analysis tme	+5 Agilent Mass Hunter BioConfirm B.07 software and Agilent SpectrumMill	+5 Each food sample was processed in quadruplicate: The inter-day repeatability of retention time and peptide transition peak area was found not to exceed 1.8% and 5%	+5 Relative quantification And identified peptides unique to duck, goose, chicken, beef and pork.	+5 N=6 species duck, goose, chicken, beef, pork, turkey
PAPER 6 (16) Food Research International 119 (2019) 426–435	+1 Complex sample prep	+1 Complex digestion	+3 nano-LC system; nanoViper C18 trap column connected to C18 analytical column Q-Exactive flow rate 300 nL/min	+4 Raw MS data were analyzed using MaxQuant software version 1.5.3.17 Protein identification was achieved by searching the MS/MS spectra against the UniProt Pig database	+5 The LC-MS/MS experiments were performed in triplicate for each pork sample.	+4 Label-free quantification (LFQ). AUC of the extracted ion chromatogram of peptides (MaxQuant software)	+5 BREED DISCRIMINATION proteomic analysis to discriminate between TP and DLY pork

PAPER 7 (21) Food Chemistry 345 (2021) 128810	+5 Very simple and quick sample prep thermally processed meats (0.3 g) were homogenized with 1 mL of 100 mM ammonium bicarbonate in a laboratory ball homogenizer	+5 Very simple and easy digestion trypsin at 37 ◦C for 18 h.	+5 Agilent 1290 Infinity LC, Agilent RRHD Eclipse Plus analytical column (2.1 × 150 mm, 1.8 µm). flow rate 0.3 mL/min 60min analysis Agilent 6550 Q-TOF mass spectrometer	+5 Agilent Mass Hunter Data Acquisition software (B.09.00) and Qualitative software (B.10.00) The spectra in the raw auto MS/MS data files were extracted using Spectrum Mill MS Proteomics Workbench (Agilent Technologies). The Protein BLAST Alignment Search Tool and blastp.	+3 The limit of detection (LOD) and quantification (LOQ), determined by a signal-to-noise ratio of 3 and 10 method, were 0.8% (w/w) and 2.6% (w/w), respectively.	+5 Relative quantification (EIC)	+3 N=4 species guinea fowl, chicken, turkey and pheasant
PAPER 8 (22) Potravinarstvo Slovak Journal of Food Sciences vol. 14, 2020, p. 149-155	+1 ground in a mortar. Not ideal homogenization for small amounts of samples	+1 Digestion 16 hrs, 37 °C. Volumes cited. Protein content measured by Quant-it protein analysis kit (Thermo Scientific)	+3 Targeted LC-MS/MS ZORBAX Eclipse Plus C18 column 25 min analysis	+3 Skyline program is the best choice in the presence of a previously studied peptide sequence to develop targeted methods. Most often, three transitions were selected.	0 N/A	+3 EIC	+1 N=2 species Beef and pork
PAPER 9 (23) RSC Adv., 2018, 8, 11157	+1 Simple sample extraction BUT lacks homogenization step.	+1 Digestion 15 h at 40 C; volumes cited. Major cons; NO DESALTING STEP	+4 Quick LC-HRMS Total run time of 20 min. Acquity UPLC BEH 300 C18 column Q-Orbitrap MS flow rate 0.3 mL min-1.	+3 Protein identification was performed using Thermo Scientic Proteome Discoverer sofware 2.0 (Thermo Scientific) and MaxQuant sofware	+4 The RSD values (n=5) of 1%, 5% and 50% mixed meat between detected and designated values were 13.8%, 8.5% and 4.6%. Relative LOD of method was 0.5% mixed meat.	+4 EIC 5 Surrogate peptides used for PRM analysis for beef identification 2 peptides heat-stable	+5 N=4 species pork, chicken, sheep and beef
PAPER 10 (24) Food Chemistry 371 (2022) 131075	+1 Simple sample extraction BUT lacks homogenization step.	+1 Digestion 2 h at 37 ◦C, volumes cites. Major cons; NO DESALTING STEP	+4 Thermo Q Exactive Hypersil GOLD C18 column (2.1 mm × 100 mm, 1.9 µm) flow rate 0.2 mL min-1. 25min analysis	+3 Proteome Discoverer software (Version 2.2) (Thermo Fisher Scientific)	0 N/A	+4 EIC	+5 N=7 species pig, beef, sheep, deer, chicken, duck, and turkey

Appendix 2.

Summary of methods that will be used in deliverable 2.

ii) PROPOSED SAMPLE PREPARTION AND PROTEIN IDENTIFICATION METHODS. Taken from (25)

Preparation of samples

Meat slices of approx. 25 mm thickness were wrapped in aluminium foil and heated in a Rational Combi convection oven (Landsberg am Lech, Germany) at 190 °C until reaching a core temperature of 99 °C (38 min was required), to achieve a high degree of protein denaturation. The core temperature was measured with a 6-point core temperature probe from the Rational Combi oven (Landsberg am Lech, Germany). Washing and digestion of all samples were performed, according to a previously described procedure (6). Briefly, thin sections of cooked meats and meat products (0.5 g) were rinsed consecutively in ethanol/ water, ethanol, methanol/water, and milli-Q water, to remove physiological salts, fat and other soluble, low molecular weight contaminants. Washed samples were homogenised in 100 mM of aqueous ammonium bicarbonate using a T25 Ultra-Turrax (IKA Labortechnik, Staufen, Germany) at 9500 rpm for 2 x 20 s, followed by 13,500 rpm for 30 s and then vacuum-dried using a CentriVap micro IR (Labconco Corporation, Kansas City, MO).

Preparation of meat mixtures

Meat mixtures were prepared from washed and dried cooked meats. Samples containing three species (chicken, turkey, pork) were prepared by weighing respective amounts of the meats to obtain samples containing equal amounts of two species (49.5: 49.5), spiked with 1% (w/w) of the third species. A total of 10 mg of the mixture prepared with 1% (w/w) chicken, turkey or pork meat was weighed in a 2-mL Eppendorf tube and trypsin-digested.

In-solution trypsin digestion

Dried samples (10 mg) were rehydrated in 100 µL of 50mM ammonium bicarbonate and 5 µL of beta-lactoglobulin protein (5 mg/mL) were added as an internal standard because it does not occur naturally in meat, except for samples which were manufactured from poultry and milk proteins. In these instances, bovine serum albumin (BSA) was added as a standard protein. The proteins were reduced by 200 mM DTT (56 °C for 1 h) and then alkylated using 200 mM iodoacetamide (IAA) for 30 min in the dark at room temperature. The remaining IAA was quenched by the addition of 200 mM DTT and incubation at room temperature for 30 min. The samples were digested in an ammonium bicarbonate solution containing 0.083 µg/µL, at 37 °C, overnight (18 h). The digests were purified by reversed-phase extraction using Sep-Pak C18 Plus cartridges (Waters, Milford, MA). Eluted peptides were dried using a CentriVap micro IR (Labconco). For chromatographic separation, samples were resuspended in 2% acetonitrile in milli-Q water containing 0.1% formic acid (solvent A) before analysis by nano-LC-MS/MS.

Protein and peptide marker identification

A UniProtKB/Swiss-Prot database search for protein and peptide identification was performed using the **Spectrum Mill MS Proteomics Workbench** with >70% score peak intensity (SPI) and 20 ppm precursor mass tolerance. Raw mass spectra were also converted to Mascot generic format (.mgf) and searched using MASCOT software (Matrix Science, Boston, MA) against the UniProt/ Swiss-Prot database, with the **following parameters:** trypsin enzyme,

taxonomy bone vertebrates, one missed cleavage, 10 ppm peptide mass tolerance, 0.1 kDa MS/MS tolerance, carbamidomethylation as fixed modification, methionine oxidation as a variable modification, and 2+, 3+ and 4+ peptide charge states. A decoy search was performed automatically and the matches and MASCOT scores were evaluated at 1% of the false discovery rate (FDR) for identity and homology threshold. Selected peptides in FASTA format were searched against the NCBInr database using the protein BLAST alignment research tool and blastp algorithm for species and protein specificity.

iii) LC-HRMS method 1. Taken from (26).

Untargeted peptidomics: LC-QTOF-MS/MS analysis (Agilent, 6550)

Peptides were analysed using a high-performance liquid chromatograph **(1290 Infinity, Agilent Technologies,** Santa Clara, CA, USA) connected to an accurate mass **Q-TOF mass spectrometer (6550 iFunnel, Agilent Technologies)** equipped with an ion source (Jet Stream Technology, Agilent Technologies). The peptides were separated using an RRHD Eclipse Plus C18 column (Agilent Technologies; 2.1 × 150 mm, 1.8 µm) and 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as the mobile phase at a flow rate of 0.3 mL/min. The gradient program was as follows: 0–2 min, 3% B; 2–40 min, to 35% B; 40–45 min, to 40% B; 45–50 min, to 90% B; 50–55 min, 90% B; and a 5 min post-run at 3% B. The injection volume was 10 µL and column temperature was maintained at 40 °C. The QTOF/ MS instrument was operated in positive electrospray ionization mode using the following parameters: ion source gas (N_2) temperature 250 °C with a flow rate of 14 L/min; nebulizer pressure 35 psi; sheath gas temperature 250 °C; sheath gas flow 11 L/min; and capillary voltage of 3500 V. The nozzle voltage was set at 1000 V and the fragmentor voltage at 400 V. The mass spectrometer was operated in MS scan mode at 1 scan/s, and in auto MS/MS mode at a scan rate of 9 scans/s for MS and 7 scans/s for MS/MS. Internal mass calibration using two reference ions of m/z 121.0509 and 922.0098 was used. All data were recorded with **Agilent Mass Hunter Data Acquisition software** (B.09.00) and processed **with Agilent Mass Hunter Qualitative analysis software** (B.10.00) and **MassHunter Bioconfirm software** (10.0).

iv) LC-HRMS method 2. Taken from (16)

Untargeted peptidomics: LC-MS/MS analysis (Thermo, Q-exactive)

The chromatographic separation was performed using an integrated **nano-LC system** (Easy-nLC, Proxeon Biosystems, Odense, Denmark, now Thermo Fisher Scientific, Waltham, MA, USA). The peptide mixture (5 µL) was loaded onto a nanoViper C18 trap column (Thermo Scientific Acclaim PepMap100, 100 µm×2 cm, 5 µm particle size) connected to the C18 analytical column (Thermo Scientific Easy Column, 75 µm× 10 cm, 3 µm in particle size). Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in 84% acetonitrile. The peptides were eluted using a linear gradient program as follows: 0–55% mobile phase B for 110 min, 55–100% mobile phase B for 5 min, and holding in 100% mobile phase B for 5 min. The flow rate was 300 nL/min, controlled by IntelliFlow technology. Mass spectrometric analysis of peptides was carried out on a **QExactive Orbitrap** mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The mass spectrometer was operated in full-scan high resolution and accurate mass (HRAM) mode. All mass spectra were acquired in positive ion mode. The automatic gain control (AGC) target for MS acquisition was set to 1.0×10^6 with a maximum ion injection time of 50 ms. The dynamic exclusion

duration was set for 60 s. MS survey scans (m/z 300–1800) were acquired with a resolving power of 70,000 at m/z 200. The MS2 Activation Type was higher energy collision induced dissociation (HCD). Resolution for HCD spectra was set to 17,500 at m/z 200. Isolation width for the MS/MS was 2 m/z, and the normalized collision energy was 30 eV. The underfill ratio, which specifies the minimum percentage of the target value likely to be reached at the maximum fill time, was defined as 0.1%. A data-dependent top 10 method was utilized to dynamically choose the most abundant precursor ions from the survey scan for HCD fragmentation. The instrument was operated in an enabled peptide recognition mode. The LC-MS/MS experiments were performed in triplicate for each pork sample.

v) LC-MS/MS method 3. Taken from (27)

Targeted MRM: Multiple reaction monitoring using LC–QQQ-MS

Dried tryptic peptides extracted from pork were reconstituted with 0.1% formic acid prior to separation and analysis by **Agilent 1290 Infinity UHPLC system and Agilent 6490 triple quadrupole (QQQ) mass spectrometer (LC–ESI–QQQ-MS)**, that was interfaced with a standard-flow ESI (Jet Stream) source. The samples were maintained at 4 °C using a thermoregulated autosampler and 5µL of each sample were injected. UHPLC was then performed using an AdvanceBio Peptide Map, C18 column (C18, 2.1x100 mm, 2.7 µm particles) in 95% mobile phase A (0.1% formic acid in water), 5% mobile phase B (0.1% formic acid in 9:1 acetonitrile:water) at a flow rate of 0.3 mL/min. **The total time of analysis was reduced to 23 min.** The initial conditions were maintained for 1 min and elution of peptides was performed with a linear gradient of 5–40% mobile phase B for 15 min. This was followed by 5 min at 95% B before returning to initial conditions in 2 min. Each run was separated by two blank runs to wash and re-equilibrate the column prior to the next run in order of to minimise sample carryover. Each sample was analysed in triplicate.

The MS running conditions were as follows: drying gas temperature, 300 C; drying gas flow, 8 L/min; nebuliser pressure, 35 psi; corona current, 10 nA; and capillary voltage, 4000 V. MRM mode was applied for quantitative analysis using precursor/product ion information. All the data acquisition was processed, evaluated and visualised with **MassHunter Workstation** (version B.03.01) processed using **MassHunter quantitative analysis software (B.04.00) (Agilent Technologies).** Ion chromatograms were extracted with a mass tolerance of 10 ppm for MRM data.