Dewi *et al.*
Journal of Analytical Science and Technology (2023) 14:13
https://doi.org/10.1186/s40543-023-00376-3

Journal of Analytical Science and Technology

REVIEW

Open Access

Advances and key considerations of liquid chromatography–mass spectrometry for porcine authentication in halal analysis

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Abstract

The halal food industries are rapidly expanding to fulfill global halal demands. Non-halal substances such as porcine proteins are often added intentionally or unintentionally to products. The development of highly selective and sensitive analytical tools is necessary, and liquid chromatography–mass spectrometry is a powerful tool that can cope with the challenge. The LC–MS method has great potential for halal authentication, because it has high sensitivity and low detection limit and detects several species markers and different tissue origins at once within one species. This article provides an understanding of recent advances in the application of LC–MS for the improvement of porcine authentication. Sample preparation, marker selection, separation and mass spectrometry conditions, quantitative assessment, and data processing for protein identification were all covered in detail to choose the most suitable method for the analytical needs.

Keywords Porcine, Halal authentication, LC–MS

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Introduction

Under Islamic law, "halal" refers to what is permitted and legal, whereas 'haram' refers to the forbidden. Non-halal substances entailing pork and its derivatives are prohibited for Muslim people. Most halal terms are linked to foods, beverages, and consumer goods, such as pharmaceuticals and cosmetics. New technology in the manufacturing industry has resulted in product fraud because goods may contain non-halal materials and undeclared compounds that are dubious to some people (Rohman et al. 2020). Porcine gelatin is a non-halal substance usually found in consumer products because it is commonly added during food, beverage, cosmetic, or pharmaceutical production for its stabilization, emulsification, encapsulation, texture, and thickening properties (Ali et al. 2018; Flaudrops et al. 2015; Hashim and Mat Hashim 2013; Ishaq et al. 2020; Ng et al. 2021; Yilmaz et al. 2013). Among meat products, the most common illegal adulteration is mixing pork meat with other meats, such as beef or horse. Therefore, identifying non-halal substances, such as pork and its derivatives, is essential.

Analytical techniques for authenticating pork and its derivatives include enzyme-linked immunosorbent assays (ELISAs), sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS− PAGE), polymerase chain reactions (PCRs), and mass spectrometry (MS) (Rohman et al. 2020). PCR and ELISA are rapid and popular methods for authenticating raw and processed meat (Mandli et al. 2018; Shabani et al. 2015). Soares et al. developed a PCR method for pork detection in poultry with a 0.1% LOD (Soares et al. 2010), while an ELISA detected the same LOD in Mandli et al.'s report (Mandli et al. 2018). A major limitation of PCR is the critical DNA extraction method because DNA degradation under heat and acidic environments results in lower accuracy of animal source identification (Grundy et al. 2016; Lubis et al. 2016). Moreover, PCR-based methods cannot identify the origin of gelatin tissue (Jannat et al. 2020) and have low sensitivity for DNA detection during food processing (Von Bargen et al. 2014). Likewise, false-positive results and repeatability in ELISA can be challenging (Hsieh and Ofori 2014).

MS has been widely used for characterization of complex samples (Cho et al. 2021; Kim et al. 2019, 2015; Solihat et al. 2022, 2019). Especially current MS instrumentation development using multiple reaction monitoring (MRM) has enabled targeted proteomic and species-specific peptide approaches, especially for complex mixtures (Von Bargen et al. 2014). The liquid chromatography–mass spectrometry (LC–MS) approach has some advantages that outweigh the disadvantages of ELISA, PCR, and SDS-PAGE. In addition, it requires fewer sample preparation procedures and has good reproducibility, sensitivity, and specificity for analyzing meat (Von Bargen et al. 2014, 2013; Jumhawan et al. 2019; Windarsih et al. 2022), low and highly processed foods (Sarah et al. 2016).

Critical factors in LC–MS analysis to ensure no-porcine contamination integrity should be noted, such as sample preparation and instrumentation conditions, because they can considerably impact the sensitivity of downstream analyses. Sample preparation entails peptide extraction from the sample matrix, a process dependent on sample type. For example, in meat-based products, sample preparation before LC–MS analysis includes meat pre-treatment, protein extraction, digestion, and desalting (Von Bargen et al. 2014, 2013; Jumhawan et al. 2017a). Gelatin-based products have simpler sample preparation: gelatin extraction and digestion (Chia et al. 2020; Dal Bello et al. 2021; Jumhawan et al. 2019). Critical parameters in LC–MS instruments include analyte conformation, charge, hydrophobicity, mobile and stationary phases, retention mode, and MS ion suppression (Bhatt and Prasad 2018). Rohman et al. reviewed a few analytical techniques for halal authentication in food and pharmaceutical products, where LC–MS provided better porcine element detection than other physicochemical methods (Rohman et al. 2020). However, their work did not include a comprehensive LC–MS study for porcine component detection.

An overview of porcine protein detection using LC–MS is lacking. Therefore, this review discusses the important concerns and procedures for porcine protein authentication in the complex matrix of foods. It reviewed the principal aspects of LC–MS, followed by sample preparation for LC–MS analysis and critical parameters in the application of LC–MS for porcine identification. The detailed descriptions are including sample pretreatment; reduction, alkylation, and digestion; desalting; biomarker selection; and instrumentation conditions such as separation, MS conditions, and data processing.

LC–MS/MS principles

LC–MS involves two main steps: separating mixtures using LC depending on their chemical and physical properties and identifying the compounds with MS. When employing LC–MS to detect differential protein expression, it is necessary to normalize and align the LC–MS data from several runs to allow for a bias-free assessment of the same biological organisms through repeated testing. This is especially vital for label-free quantification and LC–MS analyte comparisons. The overview of the LC–MS/MS principle can be seen in Fig. 1. An LC–MS experiment produces three types of data: (1) retention

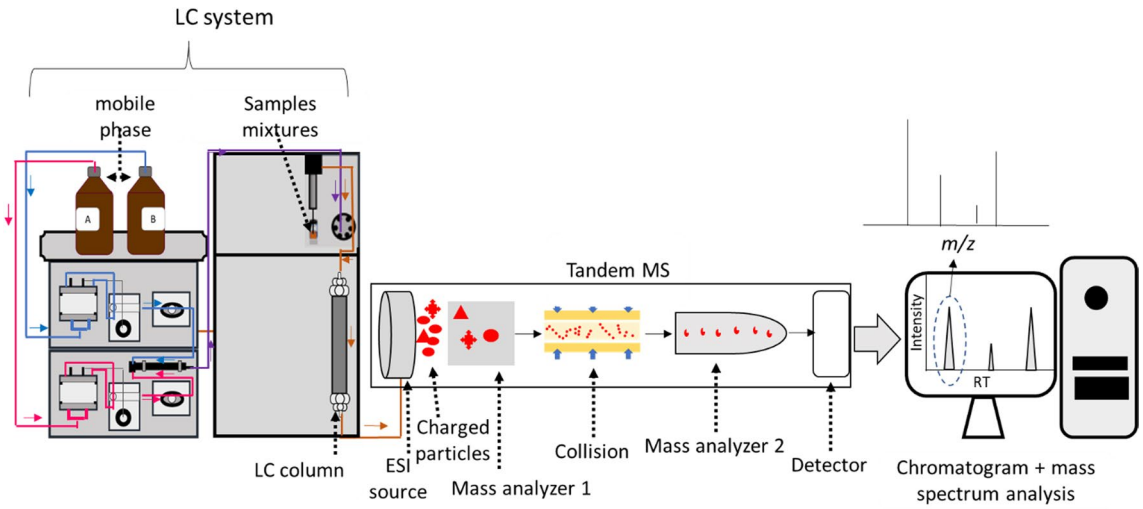


Fig. 1 Liquid chromatography–tandem mass spectrometry (LC–MS/MS) principles

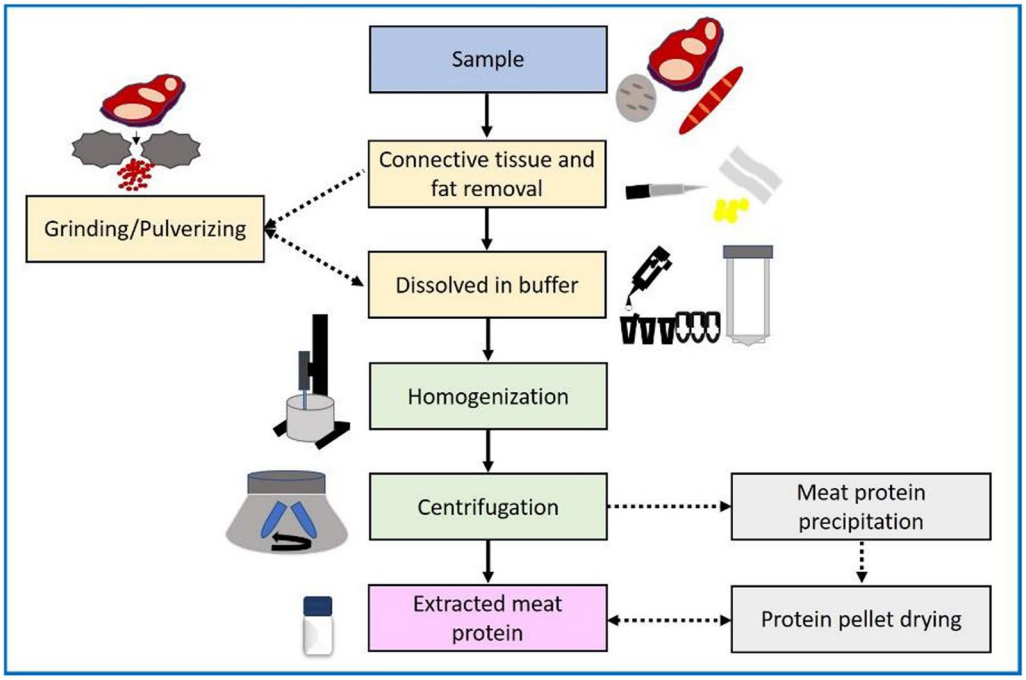
times (RTs), (2) mass-to-charge ratios (m/z), and (3) intensity (Tuli and Ressom 2009).

MS/MS is a two-step process for analyzing a sample that uses one, two, or more mass spectrometers (Fig. 1). MS/MS can provide peptide sequences using the following principles (Rauh 2012; Soares et al. 2012; Tuli and Ressom 2009):

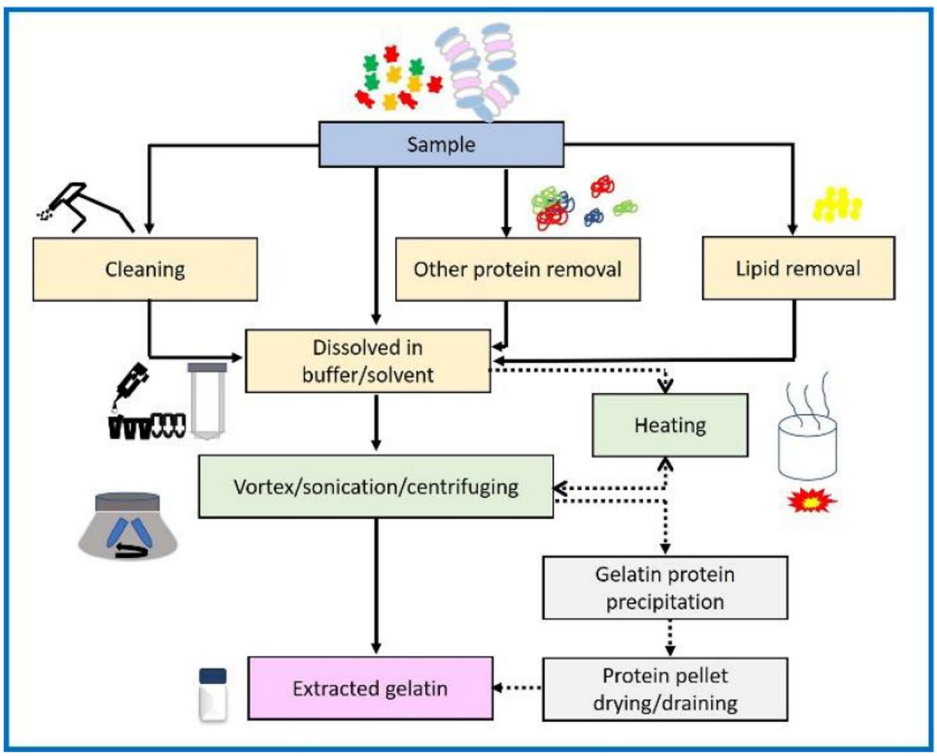
- The mixture of digested peptides is pumped using a high-pressure mobile phase (MP) and passed through an LC column, the stationary phase.
- The separation in the LC column occurs due to a chemical interaction between the sample components, MP, and stationary phase, resulting in various migration rates, known as RT.
- After separation, the effluent is exposed to a mass spectrometer.
- The effluent changes into charged particles after nebulizing, desolvating, and ionizing.
- By introducing electromagnetic fields to these charged particles, they migrate through a succession of mass analyzers (quadrupoles) in a high vacuum.
- MS/MS can be employed for peptide sequencing through a mass analyzer to isolate the precursor ion and collide with inert gas for fragmentation or a second mass analyzer to determine the product ions.
- MS/MS is applied using database search variables, such as parent ion tolerance, peptide charge state, mass calculation methods, and ion-selected types.
- The selected ion types for producing theoretical data may vary according to the type of instrument used for fragmentation.
- The obtained MS/MS spectrum, referring to the distinct peptide sequence, is employed to match peptides with the protein database to identify the sequence.

Sample preparation for LC–MS analysis

Before doing halal analysis, sample preparation is a crucial initial step. Protein is an important component that unlocks various information, such as species identification for halal analysis. According to Lee, selecting a specific protein isolation method depends on the type of sample, whether solid or liquid (Lee 2017). For solid samples, it is homogenized and subsequently lysed. For tissue samples, a mechanical homogenization method can be used. Physical procedures, such as sonication and heat treatment, and chemical approaches, such as detergent treatment to improve protein solubility, are used to lyse cells. Furthermore, chaotropic compounds, such as urea and guanidine hydrochloride, which may break down protein structures and dissolve well in water, can be utilized to improve extraction efficiency. When using liquid samples, we need to consider obtaining dissolved proteins or extracting them from the cells inside the sample. Conventional salting-out and heat denaturation techniques are easy methods for protein isolation. Salting-out decreases protein solubility in water by adding more water-soluble salts, such as ammonium sulfate, whereas heat denaturation can change the conformation of the protein structure and decrease solubility. Isoelectric precipitation can also be utilized by adjusting the pH to the isoelectric point (pI) of the target protein. Isoelectric precipitation can be employed as a fractionation technique because every protein has a distinct pI value. When isoelectric precipitation or salting-out is not possible, polymers or organic solvents (polyethylene glycol, methanol,



(a)



(b)

Fig. 2 General workflow of porcine protein extraction of **a** meat-based food and **b** gelatin-based food prior to the LC–MS analysis

or acetone) can be utilized to increase precipitation (Lee 2017).

The biggest challenge when studying proteins is extracting them from the complex matrix. According to Niu et al., extracting proteins from tissues involves long and complex steps. Several factors can cause incomplete protein extraction. One of the primary reasons for this is the use of ineffective extraction techniques for low-abundance protein-containing samples (Niu et al. 2018).

For further explanation of sample preparation, we divide the discussion into sub-sections of sample pretreatment; reduction, alkylation and digestion for protein digestion; desalting; and biomarker selection.

Sample pretreatment

The type and processing procedure of sample primarily determine the method of pretreatment used. Figure 2a presents the general workflow of porcine protein extraction from meat-based food for LC–MS analysis. The dotted line represents an additional step. The solubility of target proteins in meat-based food products can be reduced by heat treatments, such as boiling, frying, and grilling (Stachniuk et al. 2021). As described in the general workflow shown in Fig. 2a, after connective tissue and fat removal, the process starts with homogenization. Meat-based foods that have been processed or heat-treated are cooled using crushed ice or liquid nitrogen and then ground. The samples are kept under cold conditions before adding the extraction buffer. Alternatively, homogenization and extraction can be combined in a single step, wherein the sample is homogenized by adding an extraction buffer. Homogenization and sample extraction processes use cold extraction buffers in a D500 homogenizer (Sarah et al. 2016) or condition the homogenization process in an ice-water bath environment (Li et al. 2018).

Homogenizing the samples under cold conditions is intended to prevent protein damage due to heat-labile stability (Smith and Xu 2012). The protein in the sample is extracted with an extraction buffer and centrifuged, and the supernatant is collected. The composition of the extraction buffer differed between the studies, usually consists of 7–8 M urea and 2 M thiourea. Some studies added 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate (CHAPS) (Sarah et al. 2016; Wang et al. 2018), whereas another study added 50 mM Tris–HCl (Li et al. 2018). In addition to those already mentioned, dithiothreitol (DTT), carrier ampholytes, and protease inhibitors are added to the extraction buffer mix, after which the supernatant is subjected to cold acetone precipitation and incubated overnight. The mixture is centrifuged, and the pellet is dried and redissolved in an extraction buffer (Sarah et al. 2016).

Unlike protein extraction from meat, gelatin extraction from matrix products involves simpler steps because gelatin is more soluble (Fig. 2b). If the sample contains lipids, they are removed with hexane. Gelatin is widely used in various food products such as confectionaries, desserts, and dairy products. Different methods are used to extract the gelatin from diverse food systems. A sample is suspended in deionized water and heated until the temperature reaches 50 °C to extract the gelatin from confectionery products, such as jelly, marshmallows, and pastilles. Then, the mixture is centrifuged, and the supernatant is incubated overnight with cold acetone. The mixture is centrifuged again, and the resulting protein pellet is dried and used for further digestion (Jannat et al. 2020). In the case of gelatin extracted from dairy products, picric acid is utilized to form a gelatin-picric acid precipitate (Yilmaz et al. 2013). A mercury (II) nitrate solution is added to the sample to precipitate other proteins, excluding gelatin. Distilled water is then added, and the mixture is left for a few minutes and filtered. The filtrate is added to a saturated picric acid solution. The presence of a yellow precipitate indicates the presence of gelatin. The gelatin precipitate attached to the walls and bottom of the tube is collected, and an ammonium bicarbonate solution is added and sonicated using an ultrasonic homogenizer. Then, the mixture is centrifuged and filtered.

Currently, studies that detect gelatin residues in beverages are limited. Dal Bello et al. conducted a multitarget detection of gelatin and egg white protein residues in wine by supplementing the wine with 50 µg/mL pork skin-based gelatin (Dal Bello et al. 2021). Gelatin was dissolved in hot water before being added to the wine. Before opening the wine bottles, they were shaken manually (60 s) and in a thermostatic bath (30 °C, 15 min) to extract the proteins from the wine. Then, the wines were moved to another container, supplemented with95% FA solution, and vortexed. Cold methanol/chloroform (1:1 v/v, -20 °C) was added, and the sample was vortexed again and agitated for 10 min. The samples were then incubated for 10 min at -20 °C and centrifuged at 7,500 g for 10 min at 4 °C. The supernatant was removed without disrupting the protein-fraction interface. After adding cold methanol to the interface, it was incubated for 10 min at -20 °C, followed by centrifugation for 15 min at 4 °C. The obtained supernatant was removed, and the protein pellet was dried for 15 min, followed by re-suspension in 50 mM warm ammonium bicarbonate buffer (50 °C) to acquire a 20-times concentrated sample. The mixture was vortexed and centrifuged again to remove insoluble deposits. Although applied to extracting gelatin from wine, this procedure is expected to serve as a reference for extracting gelatin from other types of beverages.

Whey is one of the beverage ingredients that must have halal status because whey production is likely to use enzymes derived from porcine. Monaci and coworkers detected elements of whey protein in fruit juices using multiple extraction approaches to determine the best preparation technique based on the maximum recovery of fortified whey protein and the easiness of use (Monaci and van Hengel 2008). The tested extraction methods included ultrafiltration, protein precipitation, and solid-phase extraction (SPE) using an HLB cartridge. Preparing fruit juice samples by ultrafiltration with a cut-off of 10 kDa was initiated using a 0.45 µm cellulose filter. The filtrate was loaded into an ultracentrifuge device, spun for 30 min at 8000 rpm, rinsed with water, and centrifuged again for 30 min. The retentate was collected, dehydrated under a vacuum, and reconstituted. The samples prepared by protein precipitation used water containing 10%, 20%, or 30% trichloroacetic acid (TCA). The centrifuged fruit juices were mixed with the TCA solution and kept on ice for 30 min before recentrifugation. Prior to injection for LC–MS analysis, the supernatant was removed, and the MP was used to dissolve the pellet, which was then filtered through a 0.45 µm cellulose filter. For sample preparation using the Oasis HLB cartridge, fruit juice was initially acidified with FA to reach a final pH of 3. Then, the sample was placed in an SPE cartridge preconditioned with methanol, water, and water containing 0.1% FA. Thick samples were filtered first with a 0.45 µm cellulose filter instead of centrifugation. The column was washed with 0.1% FA in water to remove polar compounds. Elution was carried out using a 95:5 solution of acetonitrile and water with 0.1% FA. The samples from these three different extraction methods were directly injected into LC–MS to determine the recovery value. The extraction method using the Oasis HLB-SPE column resulted in the best recovery. However, we did not include these methods in Table 1 due to the significant differences in sample types and overall sample preparation methods and therefore could not be compared with other methods.

Reduction, alkylation and digestion for protein digestion

After meat protein extraction, the next step is reduction and alkylation. One of the important steps in the bottom-up proteomic analysis is disulfide bond reduction and sulfhydryl group alkylation. When the reduction and alkylation steps are not performed, peptides bound by disulfide bonds are difficult to identify during database searches (Suttapitugsakul et al. 2018). Gelatin samples can directly undergo the digestion step after adding the extraction buffer. Reduction and alkylation are not necessary for gelatin because disulfide bonds are hydrolyzed during the manufacturing process. However, a previous study completed ethanol precipitation prior to digestion (Von Bargen et al. 2013). They achieved this by dissolving and vortexing the gelatin sample with 50 mM ammonium bicarbonate (pH 8), followed by sonication for 30 min. The suspension was transferred to a microcentrifuge tube, and ethanol was added to precipitate the protein. The ethanol was then removed, and the protein pellet was vacuum centrifuged for 20 min at 60 °C to dry it (Yang et al. 2018). Protein purification with ethanol precipitation was performed to increase test sensitivity.

DTT is added for the reduction process, which usually takes 30 min to 1 h at a temperature of 56–60 °C. It is followed by an alkylation step supplemented with iodoacetamide (IAA), and the reaction lasts for 20–30 min in the dark. Protein digestion is performed following reduction and alkylation. Prior to digestion, Wang et al. added a protein solution to a 10 kDa filter unit and removed DTT and IAA by rinsing with ammonium bicarbonate three times (Wang et al. 2018). Solutions of the trypsin enzyme in ammonium bicarbonate (Sarah et al. 2016; Wang et al. 2018) or in 0.1% acetic acid (Li et al. 2018) were used for digestion. Digestion usually occurs overnight or for 12 h at 37 °C (Li et al. 2018; Sarah et al. 2016). Digestion is stopped by adding 0.1% trifluoroacetic acid (TFA) (Li et al. 2018) to a pH < 2 or 0.1% formic acid (FA) (Sarah et al. 2016). Desalting can be performed using ZipTip C18 (Sarah et al. 2016) or HLB cartridges (Waters, USA) (Li et al. 2018).

Gelatin extracted from food products or pure gelatin is dissolved in a 40–50 mM or 1% ammonium bicarbonate solution. Jannat et al. used ammonium bicarbonate containing 9% acetonitrile, then heated the sample at 50 °C for 30 min to denature the gelatin protein (Jannat et al. 2020). The solution was sonicated for homogenization (Yang et al. 2018; Yilmaz et al. 2013). Generally, after gelatin is dissolved, it must be filtered with a 0.22 µm syringe filter before digestion to remove insoluble particles. Digestion is carried out using a trypsin enzyme solution in an ammonium bicarbonate solution (Guo et al. 2018; Jannat et al. 2020; Salamah et al. 2019) or by adding Rapigest to the solution (Yilmaz et al. 2013). Rapigest is a detergent/surfactant that promotes enzymatic protein digestion by facilitating protein unfolding. However, Rapigest relies heavily on sample type (Mosen et al. 2021). Digestion is carried out at 37 °C (Jannat et al. 2020; Salamah et al. 2019; Wang et al. 2018; Yilmaz et al. 2013) to 40 °C (Yang et al. 2018) for 12–24 h (Guo et al. 2018; Jannat et al. 2020; Salamah et al. 2019; Yang et al. 2018; Yilmaz et al. 2013). An FA (Jannat et al. 2020) or TFA (Yang et al. 2018) solution is added to stop digestion. Rapigest is removed by adding TFA and acetonitrile (ACN), then the mixture is kept at 60 °C for 120 min. Then, alcohol dehydrogenase (ADH) tryptic digest

Table 1 Comparison of previously reported sample preparation methods for LC–MS analysis

Sample	Peptide Extraction Procedure			Advantages	Disadvantages	Ref
	Sample Pretreatment	Digestion	Composition of Extraction Buffer			
Heated treated meat (boiling, autoclaving)	[a,b]Homogenizing[c] Acetone precipitation Air-drying pellet	[b,d]Digesting Drying Acidificating Desalting	7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.4% carrier ampholytes, protease inhibitor cocktail	The extraction process is quite simple and easy to do because cold conditions are provided by the ice cold buffer There is additional acetone precipitation so the protein obtained is purer The reducting and alkylating stages are shorter (total 50 min)	Overnight aceton precipitation is quite time consuming, not suitable for routine analysis applications The additional drying process before redissolving and after digesting is time consuming	Sarah et al. (2016)
Heat treated meat and plant (waterbath heating, boiling, high temperature sterilizing, frying, baking)	[a]Pulverizing Cooling in an ice water bath[b] Homogenizing an in ice water bath environment[c]	[d]Digesting Acidificating Desalting	7 M urea, 2 M thiourea, 50 mM Tris–HCl (pH 8)	Does not require additional liquid nitrogen. Hence, it is more cost-efficient. The extraction temperature is more stable due to ice water bath environment, thereby reducing protein degradation The digestion process is quite simple	There are two stages to homogenize the sample, meaning it is less concise Long reducting and alkylating stages (1.5 h)	Li et al. (2018)
Heated treated meat (boiling, grilling)	[a]Grinding in liquid nitrogen[b,c]	[d]Filtrating and washing Digesting[c,b]	8 M urea, 2 M thiourea, 4% CHAPS	Sample homogenization can be smoother, the whole preparation step is very simple and concise	There is a possibility that not all the proteins are perfectly extracted because homogenization with the buffer extraction is not clearly mentioned during extraction Double-stage centrifugation after digestion is less concise	Wang et al. (2018)
Jellies, marshmallows, pastilles, pharmaceutical-grade gelatin capsules	Cutting Heating in deionized water[c] Overnight cold acetone extraction[c] Protein pellet air-drying	(b)Heating Filtering Digesting Stopping digestion	40 mM ammonium bicarbonate containing 9% acetonitrile (pH 8.2)	The additional process with acetone extraction increases the purity of the extracted gelatin	The protein extraction steps are long and time-consuming, not suitable for routine analysis.	Jannat et al. (2020)

Table 1 (continued)

Sample	Peptide Extraction Procedure			Advantages	Disadvantages	Ref
	Sample Pretreatment	Digestion	Composition of Extraction Buffer			
In-house dairy products (yoghurt, cheese, ice cream)	Precipitation of other protein using mercury (II) nitrate solution Filtering Filtrate extraction using picric acid[(c)] Gelatin draining	[(b)]Sonicating[(c)] Filtering[(b,d)] Digesting Detergent removal Adding standard tryptic digest internal calibrant and buffer[(c)]	50 mM ammonium bicarbonate	Proteins other than gelatin can be eliminated, increasing the purity of extracted gelatin Suitable for application in dairy products	The sample preparation stages are very complex and complicated, not suitable for routine analysis The cost is more expensive because there is the addition of reagents such as the use of detergents, reduction and alkylation are not really needed	Yilmaz et al. (2013)
Gelatin used in preparing charcuterie meats, fruit snacks (Jello), Solaray empty gelatin capsules	[(b)]Sonicating Ethanol precipitation Vacuum centrifuge drying	[(b)]Sonicating Heating Digesting Stopping digestion[(c)]	50 mM ammonium bicarbonate (pH 8)	Ethanol precipitation can increase assay sensitivity, the gelatin obtained is purer	The sample preparation stages are long, not suitable for routine analysis	Yang et al. (2018)
Commercial powdered gelatin	[(b)]Sonicating	Digesting Filtering	Phosphate-buffered saline (PBS)	Rapid, simple procedure, cost-effective	Limited/no research as to whether it can be applied to more complex matrices The method is relatively new and needs to be validated for other complex matrix samples	Cai et al. (2021)
Pure gelatin standard	[(b)]Filtering	Digesting	1% ammonium bicarbonate (pH 8)	Easy and simple steps, suitable for routine analysis	Not clearly mentioned whether it is suitable for gelatin extracted from complex matrices	Guo et al. (2018), Salamah et al. (2019)
Gelatin-containing beverages	Shaking in thermostatic bath Adding formic acid and cold methanol/chloroform Cooling in agitation Cool incubating[(c)] Cold methanol precipitation[(c)] Protein pellet drying[(b,c)]	[(d)]Digesting	50 mM ammonium bicarbonate	Multiple stages of protein precipitation — the protein obtained can be purer Shorter time for reduction and alkylation	Sample preparation stages are long and not suitable for routine analysis Reduction and alkylation are not quite necessary, using more enzymes for digestion	Dal Bello et al. (2021)

The following common steps are represented by letter symbols in order to concise the writing: a: chilling sample; b: adding buffer; c: centrifuging, and d: reducting and alkylating. The symbols given at the beginning or end of the steps describe the order of procedure

internal calibrant and ammonium bicarbonate buffer are added. Acid hydrolysis or tryptic cleavage by separating the hydrophilic head and hydrophobic tail can remove Rapigest (Mosen et al. 2021). The mixture is centrifuged, and the aliquot is ready for analysis (Yilmaz et al. 2013).

The gelatin digestion process in proteomic analysis for gelatin detection is quite time-consuming and a barrier to routine applications in gelatin authentication. High-intensity ultrasounds are used to reduce overnight digestion to several minutes. Cai et al. prepared a gelatin sample by dissolving the gelatin sample in phosphate-buffered saline (PBS), followed by sonication for 2 min (Cai et al. 2021). The gelatin solution was treated with trypsin and digested using an ultrasonic machine at 500 W for 10 min. Then, a 0.22-μm filtration membrane was used to filter the digested gelatin sample. The study showed that the 5–45 min digestion time was not significantly different. Hence, the 5-min digestion time was confirmed as the optimal condition for digestion using an ultrasound. This method is still relatively new and needs to be validated to detect gelatin in food products.

The previously reported sample preparation methods for LC–MS analysis were summarized and are compared in Table 1.

Desalting

Due to the complexity of the biological matrix, especially the meat matrix, desalting is one of the critical steps in LC–MS peptide determination. Desalting after or before trypsin digestion can reduce the complexity of the matrix for peptide ionization. This can be achieved by SPE or liquid–liquid extraction (Von Bargen et al. 2014, 2013; Bhatt and Prasad 2018; Sarah et al. 2016). SPE is a common method for desalting because it can also remove ionic detergents and enrich peptides. SOLAμ^{TM} HRP SPE spin plate and ZIPTIP® C18 pipette tip effectiveness, in terms of robustness, reproducibility, and performance, were evaluated. SPE had equal analytical performance in peptide purification before LC–MS analysis, yet SOLAμ^{TM} was more user-friendly for routine laboratory work (Schmelter et al. 2018). Before passing through the cartridge, the digested samples were dissolved in water. After subjecting the sample to cartridges, the peptides were eluted with ACN/water (Von Bargen et al. 2014).

Biomarker selection

Marker peptides are separated using liquid chromatography with a specific column, MP, and elution conditions then detected using an MS detector. Biomarkers are chosen based on a set of criteria and must be unique to a single species. Selecting marker peptides to differentiate between animal species is important because surrogate peptides in a complex mixture with the same m/z cannot be distinguished. According to Zhang et al., proline hydroxylation is a vital element for peptide identification (Zhang et al. 2009). Since the mass shift caused by proline hydroxylation and Ser-Ala mass differences might be mistaken, the sequence should be carefully checked such as in GPPGSAGSPGK and GPPGSAGAPGK because this sequence each appears in bovine and porcine. Prior to analysis, peptide digestion was needed to decrease the molecular weight. The digested bovine peptide was similar to the porcine peptide because of the high homology between the α1(1) and α2(1) chains. One repetition count was employed, and the exclusion period unit was reduced to half a minute in a dynamic exclusion mode to obtain as many MS spectra as feasible during HPLC–MS analysis at m/z 400–2000. Five thousand MS spectra generated from each sample were verified according to the marker sequence. Since specific bovine marker peptides have MS spectra identical to those of porcine marker peptides, their sequences needed to be verified. In addition, LC–MS can confirm proline hydroxylation on peptides because it enhances stability, mechanical properties, and antigenicity. For instance, Fig. 3a shows a peak in digested bovine serum (m/z 924). The MS spectrum (Fig. 3c) confirmed that the peak was from the sequence of GP*P*GPSGISGPP*GPPGP*AGK with four hydroxylation prolines (marked with an asterisk). Meanwhile, a unique peak corresponding to digested porcine was found at m/z 930 (Fig. 3b) with the MS spectrum in Fig. 3d. This peak corresponded to the GP*P*GPSGISGPP*GP*PG PAGK marker sequence at location α2(I). Notably, some fragment peaks from m/z 924 and m/z 930 were observed, such as the y19 ion at m/z 844.7 as a double-charge form and m/z 639.3–1574.4 as y7 to y18 ions (Fig. 3c–d). The difference in mass between bovine and porcine samples was consistent with the different threonine and isoleucine masses. These findings show that HPLC–MS is a viable method to detect flag peptides in digested gelatin samples and distinguish between porcine and bovine gelatin.

Sarah et al. showed that consistently finding a marker in MRM mode could be a parameter for selecting a potential marker (Sarah et al. 2016). In their report, four consistent marker detections were used to determine the animal origin of gelatin, either bovine or porcine. They found four peptides consistently observed in pork samples—TVLGNFAAFVQK, FVIER, LVVITAGAR, and EVTEFAK—that can be used to distinguish meat species. First, liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF–MS) was used to confirm the species specificity based on m/z, and subsequently, the acquired chromatogram was compared to bioinformatics tools to evaluate peptide characteristics to obtain the potential marker. A list of LC-Q-TOF–MS

analysis-derived species-specific peptides was further confirmed using MRM mode triple quadrupole mass spectrometry (QQQ-MS) to narrow the list to a subset that was consistently found in the meat system.

Recently, Windarsih et al. selected markers based on untargeted metabolomics and proteomic approaches to identify porcine adulteration in fish using LC-Q-TOF–MS (Windarsih et al. 2022). They found potential markers that can be used for pigs with good fitness ($R > 0.95$) and productivity ($Q > 0.5$): FFESFGDLSNADAVMGNPK and HPGDFGADAQGAMSK with LODs of 0.5%. Since these peptide markers were specific to pork, they could be employed as targets to detect pork in contaminated fish.

Kleinnijenhuis et al. described the theoretical justification and validation of LC–MS for authenticating bovine and porcine gelatin markers quantitatively, which is effective for determination of non-porcine contamination (Kleinnijenhuis et al. 2018). Apart from practical factors, including sensitivity and peak shape, seven criteria for theoretical peptide selection were used to determine the optimal quantitative target peptides of gelatins. The rationale for the criteria was as follows:

- The suitable peptide length for MS detection should be between six and 20–25 amino acids because the unique characteristic of short peptides (less than six) is improbable, whereas peptides with a greater length cause signal dropping in MS according to the fragmentation number channels.
- Methionine M should be avoided through oxidization in sample preparation.
- Cysteine C should be modified.
- Asparagine N needs to be deamidated from the prone structure.
- Glutamine Q free.
- Hydroxyproline (Hyp), no other post-translational modification (PTM) site.
- Specific for animal species, including *Bos taurus* and *Sus scrofa*

High-resolution MS, such as Orbitrap MS, can be used to distinguish monoisotopic residues between residues of Hyp and isoleucine, which have differences in monoisotopic masses 113.048 and 113.084. Porcine contains two Hyp residues with porcine GIpGEFGLpGPAGPR (*Sus scrofa*) and bovine GETGPAGPAGPIGPVGAR (*Bos taurus*) target peptide sequences. These markers were confirmed using LC–MS Orbitrap (Fig. 4). Porcine exhibited the following results: m/z 727.40 ([M + 2H]2 +), quantifier ions y7 + m/z 667.35, and qualifier ions m/z 642.30 (Fig. 4). This method was validated per good laboratory practices to ensure scientific quality, data integrity, and traceability (Kleinnijenhuis et al. 2018).

LC–MS parameters for porcine identification

After understanding the basic principles regarding LC–MS and how to prepare samples before LC–MS analysis, the following section is to discuss key instrumentation parameters such as LC separation, MS conditions, and data processing that has been carried out regarding the application of LC–MS for porcine identification.

Von Bargen et al. introduced LC–MS through the MRM and MRM with multistage fragmentation (MRM^3) methods to authenticate horse and pork meat in halal beef (Von Bargen et al. 2013) and highly processed foods (Von Bargen et al. 2014). Before injection into the LC–MS instrument, meat samples were prepared through a sequential process of extraction, digestion, and desalting. The sample was evaporated to remove the solvent and redissolved in ACN-water (3/97; 0.1% FA) to be separated in the HPLC column. Coupled Accela HPLC-LTQ Orbitrap XL (Thermo Scientific, Bremen, Germany) was used to identify peptide markers in the sample. The peptide extract was used as the sample to optimize the MS and MRM parameters. HPLC separation was performed using a Phenomenex Kinetex C18, 100 Å, 2.6 µm, (100 mm × 2.10 mm) column. The XCalibur 2.07 software and Proteome Discoverer 1.1 were used to evaluate the data (Thermo Scientific), where MP A was 0.1% FA in water and MP B was of 0.1% FA in ACN. Only certain peptides were selected for further development of the QTRAP instrument after searching the UniProt Knowledgebase (UniProtKB) database for potential biomarker peptides. The QTRAP 5500 LC–MS instrument coupled with VWR Hitachi HPLC was used to further investigate the specific target by using a Phenomenex Kinetex C18, 100 Å, 2.6 µm, (100 mm × 2.10 mm) column, while 0.1% FA in ACN and 0.1% FA in water were each used as MPs A and B, respectively. The obtained data were interpreted using Analyst software version 1.5.2. Augmenting the column using either Eksigent HALO C18 or Agilent ZORBAX XDB C18 columns enhanced the sensitivity after optimizing the conditions. However, the ZORBAX column created better retention peptide peaks and low backpressure. The results showed that the MRM^3 mode in QTRAP more effectively enhanced the signal-to-noise ratio than the MRM mode in some targeted compounds. For instance, the fragment intensity of m/z 454.6, corresponding to the pork signal, was improved in the MRM^3 signal compared to MRM due to sensitivity. A QTRAP instrument with the MRM^3 mode was used to identify pork contaminants in the beef matrix with biomarker

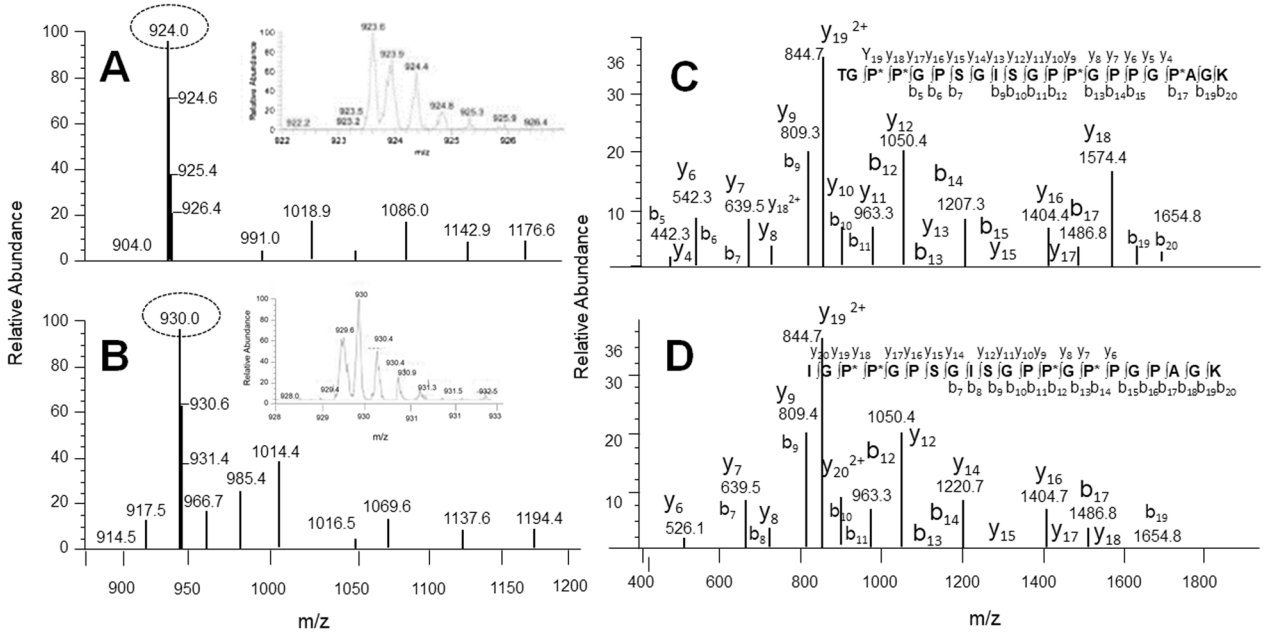


Fig. 3 (left) Mass spectra and inset spectra of **a** digested bovine gelatin (m/z 924) and **b** digested porcine gelatin (m/z 930); (right) MS spectrum of **c** m/z 924.0 conformed to the TGP*P *GP SGISGPP*GPPGP*AGK sequence and **d** MS fragments of m/z 930.0 assigned to the IGP*P*GPSGISGPP*GP*PGPAGK sequence. Note the asterisks (*) represent hydroxylation sites. Zhang et al. (2009) Copyright (2009) with permission from Elsevier

peptides below 0.13%, while it reached 0.55% in the MRM method. However, these data were obtained using unprocessed meat without analyte pre-enrichment (Von Bargen et al. 2013).

In 2014, von Bargen et al. also developed a method for identifying meat in highly processed food with a 2-min protein extraction. Effective sample extraction is one of the most important procedures in focused proteomic analyses of processed samples. Highly processed foods, such as canned meat, meatballs, sausages, and salamis, were extracted in a buffer composed of 50 mM Tris–HCl, 1 M thiourea, and 6 M urea. The supernatant was then digested and desalted. The samples were injected into an HPLC VWR Hitachi coupled with a QTRAP 5500 LC–MS instrument. A Phenomenex Kinetex C18, 100 Å, 2.6 µm, (100 mm × 2.10 mm) column was used with 0.1% FA in ACN and 0.1% FA in water as MPs A and B, respectively. Through MRM and MRM^3 optimized parameters, specific marker peptides were detected in less than 0.24% of pork or horse in the highly processed food matrix. They claimed that without a specific proteomics background, this method is easily usable in normal analytical laboratories (Von Bargen et al. 2014).

Pork adulteration analysis in thermally processed meat is especially difficult since the intricacy and inhomogeneity create low DNA and protein extractability. Currently, proteomic-based analytical techniques, such as LC-Q-TOF–MS, have successfully detected and identified proteins, even after thermal treatment. This technique has been used for the porcine authentication of thermally processed meat. Briefly, the protein extracted from beef was digested with trypsin and desalted using ZipTip C18 before injection into the LC–MS instrument. A C18 (AdvanceBio Peptide) column, with 0.1% FA as MP A and 0.1% FA in 9:1 ACN: water as MP B, was used as the initial condition for LC separation. First, MS between m/z 100–2000 was scanned by a liquid chromatography–electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-Q-TOF–MS) Agilent 6520 system, and the spectra of all unique peptides were studied further. A thorough examination of the LC–MS spectra and a comparison of the species were performed. A peptide must meet the MS auto-validation criteria, be species-specific (over beef, chicken, and chevon), and be consistently detectable following thermal treatment in all repetitions to be considered a possible biomarker. Four peptides can be detected as a pork-biomarker via the MRM method: EVTEFAK (m/z 412.2144, z = 2), LVVITAGAR (m/z 450.2949, z = 2), FVIER (m/z 388.7414, z = 2), and TVLGNFAAFVQK (m/z 647.8641, z = 2) (Stachniuk et al. 2021; Yang et al. 2018). Determining meat origin using MS technology has a strong potential to generate scientifically accurate and reliable outcomes, even at the peptide level. Furthermore, the specificity and

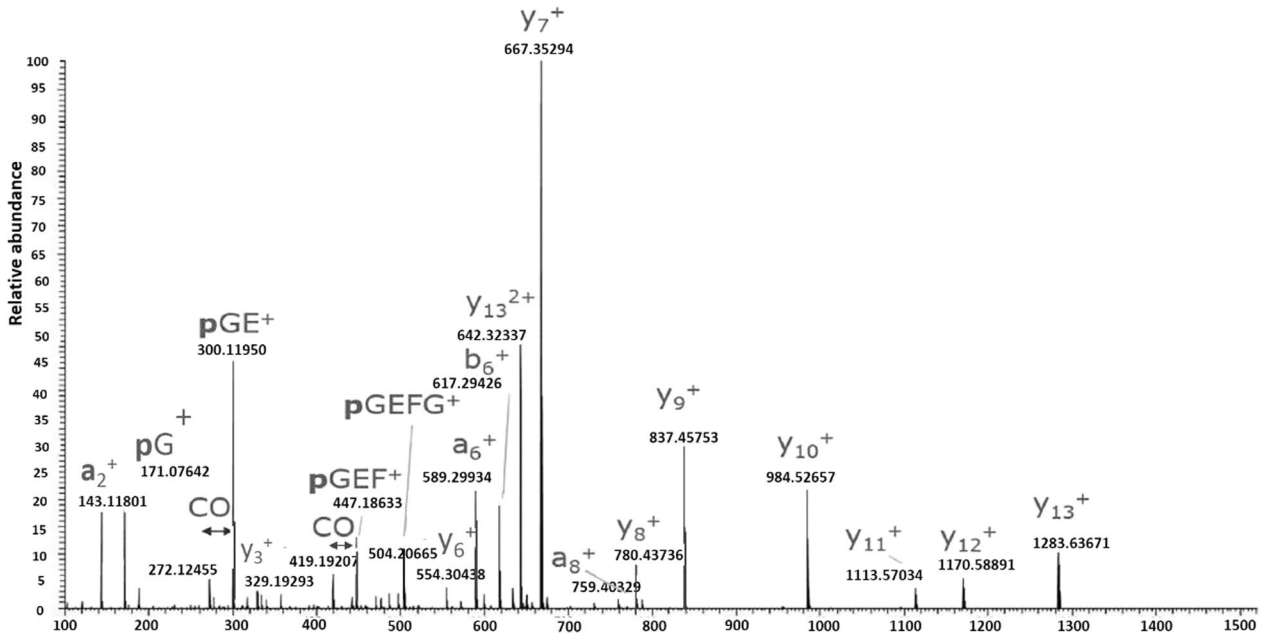


Fig. 4 Internal pG + fragments are represented by the product ions at m/z 171.076, which are extremely characteristic of collagens. Internal **a**, **b**, and **y** ions were the most commonly observed pieces. Kleinnijenhuis et al. (2018) Copyright (2018) with permission from Elsevier

selectivity of proteomics approaches provide a solid platform for halal validation (Sarah et al. 2016).

In Grundy et al. (2016) report, a nanoACQUITY ultra-performance liquid chromatography (UPLC) system coupled with electrospray ionization (ESI)-MS was used to identify sources of gelatin in food products. Since bovine and porcine gelatin is structurally and physicochemically similar, they are difficult to distinguish using standard spectroscopy. Commercialized species determination of gelatin has been limited because of the severe denaturation that occurs during gelatin production. Gelatin was extracted from several sources, such as chicken, porcine, and bovine skin, through in-house preparation. Gelatins were digested in trypsin before being injected into the LC–MS system. These samples were also subjected to ELISA and PCR for comparison. A C18 column with two solvents, 0.1% FA in water and 0.1% FA in ACN, was used for separation in a nanoACQUITY UPLC system. All the samples analyzed produced expected LC–MS results and unexpected PCR and ELISA results. For example, PCR did not detect porcine DNA in a chicken exudate supplemented with 3.5% porcine gelatin, whereas LC–MS identified porcine-specific peptides in 0.4–1% depending on the matrices. Meanwhile, bovine gelatin in chicken exudates resulted in false negatives in ELISA, indicating an invalid result. Although MS provides only qualitative data, it is a reliable analytical technique for determining gelatin sources in food products (Grundy et al. 2016).

Gelatin is quite common in the food industry. Hence, determining the source of gelatin has become important, especially in porcine authentication. Yilmaz et al. described a method to distinguish bovine and porcine gelatin in dairy products (yogurt, cheese, and ice cream) by NanoUPLC-ESI-Q-TOF-MSE (Yilmaz et al. 2013). Figure 5 shows a flowchart of the experiment. Gelatin was extracted from dairy products and used for carbamidomethyl-cysteine modification. Overnight trypsinization was performed to generate the peptides before separation in the LC system using the reverse phase. Peptides were separated on a C18 column with 0.1% FA in water as MP A and 0.1% FA in ACN as MP B. A high-definition mass spectrometer with a NanoLockSpray ion source (SYNAPT HDMS) was used with high collision energy and an alternated low method to quantify and discover the amino acids in the gelatin. The results showed that nano-liquid chromatography could separate specific peptides, which were further analyzed by MS using the database identification algorithm approach. The results showed that, per peptide, there were more fragment ions and more peptides per protein, indicating a higher level of confidence in protein identification. For instance, this method successfully detected porcine peptides in combination with bovine gelatin at a ratio of 1:9 (Yilmaz et al. 2013).

Another report processed UPLC-MS data using a chemometrics statistical approach, such as principal

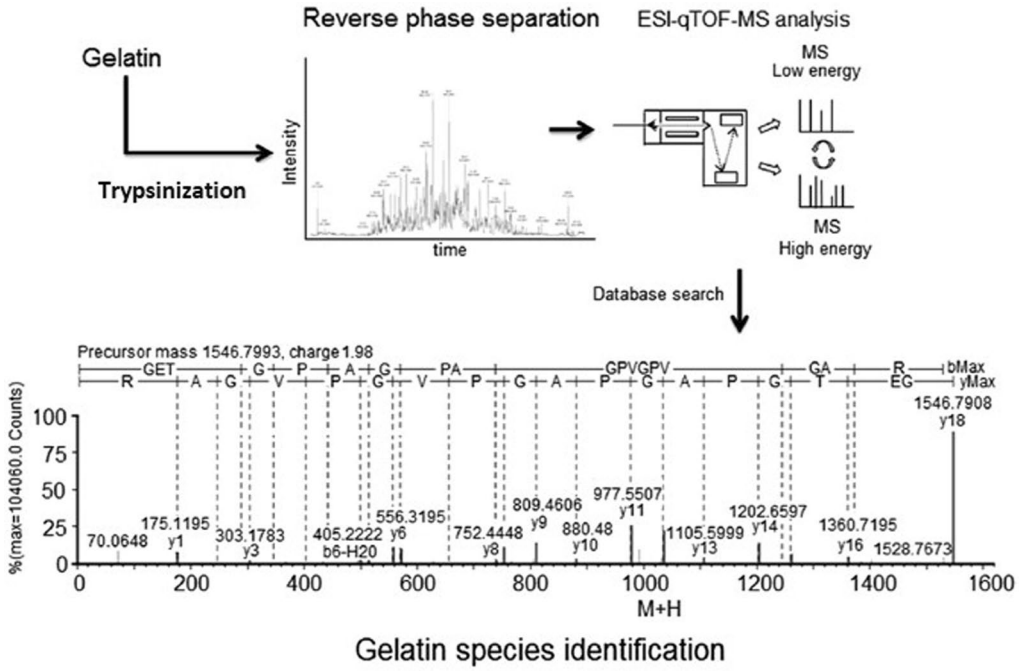


Fig. 5 Workflow for NanoUPLC-ESI-Q-TOF-MSE for gelatin source identification. Yilmaz et al. (2013) Copyright (2013) with permission from Elsevier

component analysis (PCA), and classified bovine and porcine gelatin. RT and m/z from LC–MS were used as variables for principal components (PC) 1 (PC1) and PC2, respectively (Salamah et al. 2019). Another chemometric approach dealing with LC–MS data used partial least squares discriminant analysis to identify the source of gelatin in jelly from bovine skin and bone (Jannat et al. 2020).

Cheng et al. used UPLC-Q-TOF–MS to classify five gelatin sources: deer-horn glue (DH), tortoise shell glue (TS), porcine gelatin (PG), bovine gelatin (BG), and donkey gelatin (DG) (Cheng et al. 2012). The gelatin sample was digested by trypsin before being separated in a UPLC C18 column and infused into a Waters AcquityTM Ultra Performance LC system. Collagen homology in total ion chromatogram (TIC) chromatography revealed indistinguishable tryptic peptides at higher concentrations by visual observation. Therefore, a multivariate statistical tool was required for gelatin profiling. First, the 3D UPLC/MS dataset was converted to an application manager for MassLynxTM, expressed as the exact mass retention time (EMRT). PCA was used to visualize the dataset and check the gelatin classification trends. According to preliminary PCA, 8556 variables were obtained, and the PCA final score plot showed five different types of gelatin inside the Hotelling T2 (0.95) ellipse (Fig. 6a). Figure 6a shows the PCA score plot, where the gelatin types inside the ellipse are DG, PG, BG, DH, and TS, with

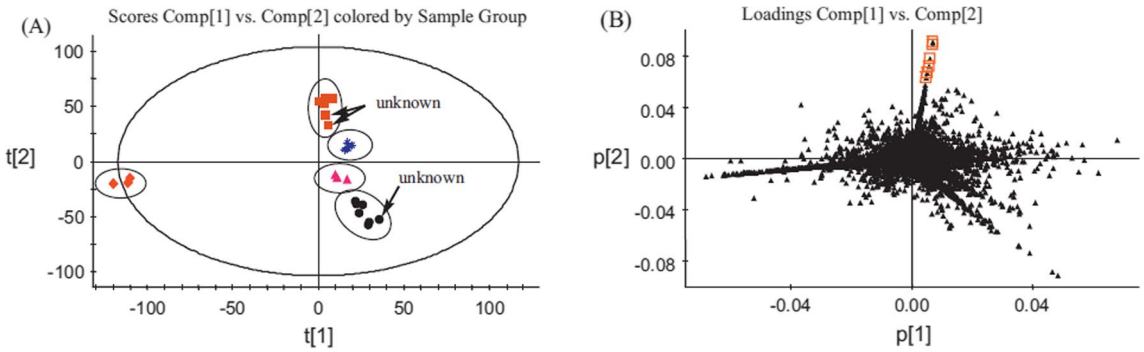


Fig. 6 PCA scoring **a** and loading plots **b** for tortoise shell glue (◆), deer-horn glue (▲), porcine gelatin (✱), bovine gelatin (●), and donkey gelatin (■). (Cheng et al. 2012) Copyright (2012) with permission from Elsevier

Table 2 Summary of the LC–MS parameters to identify porcine in various food types

Food type	Instrument type and data analysis	Optimum condition		m/z and Sequence Marker of Porcine	Ref
		Chromatography	Mass spectrometry		
Meat	Accela HPLC system (Thermo Scientific) coupled with an AB SCIEX TripleTOF 5600 system or LTQ Orbitrap XL (Thermo Scientific) Data analysis: Proteome Discoverer 1.1 and XCalibur 2.07 software	Column: Phenomenex Kinetex C18, 100 Å, 2.6 µm, (100 mm × 2.10 mm) MP A: 0.1% FA in water, MP B: 0.1% FA in ACN with flow rate 0.3 mL/min, gradient setting in min as follows: 0 → MPA 97% MPB 3%, 12 → MPA 97% MPB 3%; 24 → MPA 88% MPB 12%; 182 → MPA 65% MPB 35%; 200 → MPA 40% MPB 60%; 216 → MPA 40% MPB 60%; 217 → MPA 20% MPB 80%; 222 → MPA 20% MPB 80%; 225 → MPA 97% MPB 3%; 240 MPA 97% MPB 3%	ESI MRM mode; Injection volume 25 µL; column oven and autosampler tray temperature 45 °C and 4 °C; source voltage 3.5 kV; capillary 33 V; heater 330 °C; capillary 275 °C; sheath gas flow 35 arb; aux gas flow 12 arb; sweep gas flow 5 arb; HCD energy 40; resolution scan 30,000; tube lens voltage 130 V; isolation width 4	m/z 453.6 YDIINLR, m/z 534.3 TLAFLFAER, m/z 582.8 EFEIGNLQSK	Von Bargen et al. (2013)
Highly processed food: canned meat, meatballs, sausages, salamis	VWR Hitachi HPLC coupled with QTrap 5500 LC–MS AB SCIEX Data analysis: Analyst Software Version 1.5.2	Column: Phenomenex Kinetex C18, 100 Å, 2.6 µm, (100 mm × 2.10 mm) MP A: 0.1% FA in ACN, MP B: 0.1% FA in water, the gradient in min as follows: 0 → MPA 3% MP B 97%; 22 → MPA 28.4% MPB 71.6%; 23 → MPA 100% MPB 0%; 28 → MPA 100% MPB 0%; 29 → MPA 3% MPB 97%; 35 → MPA 3% MPB 97%	ESI MRM mode; Curtain gas 35; source gas (1) 40; source gas (2) 40; spray voltage 5500 V; source temperature 400 °C	m/z 534.4 TLAFLFAER, m/z 453.8 YDIINLR, m/z 376.1 SALAHAVQSSR, m/z 582.8 EFEIGNLQSK, m/z 508.3 LVNDLTGQR	Von Bargen et al. (2014)
Gelatin in chicken fillets	NanoAcquity UPLC system Waters interfaced with maXis LC–MS System (Bruker) Data analysis: compass 1.3 SP1 software	C18 capillary column, nanoAcquity BEH 130 1.7 µm, (75 µm × 250 mm, Waters), flow rate 10 µL/min with MP A: 0.1% FA in water, MP B: 0.1% FA in ACN with the gradient in min as follows: 2 → MPA 95% MP B 5%; 20 → MP A 65% MP B 35%; 2.5 → MP A 5% MP B 95%	(+) ESI scan method at m/z 50–2000; column temperature 60 °C; dry gas temperature 160 °C; dry gas 4 L/min; ion spray voltage 1400 V; acquisition time 0.1 s; threshold 1000 counts	m/z 810.4 GPTGPAGVR, m/z 1545.8 GETGPAGPAGPVGPVGAR, m/z 1547.8, GEPGPAGSVGPAGAVGPR, m/z 1549.8, GPPGESGAAGPAGPIGSR, m/z 2072.0, GSPGADGPAGAPGTPGPQGIAGQR	Grundy et al. (2016)

Table 2 (continued)

Food type	Instrument type and data analysis	Optimum condition		m/z and Sequence Marker of Porcine	Ref
		Chromatography	Mass spectrometry		
Gelatin in food (non-specific type)	ACQUITY UPLC H-Class equipped with tandem MS. PCA for data interpretation	Peptide CSH ACQUITY UPLC column (100 mm × 2.1 mm I.D; 1,7 µm). MP A 0.1% trifluoro-acetic acid (TFA) in water and MP B 0.1 TFA in ACN, flow rate 0.2 mL/min with the gradient time in min as follows: 0 → MP A 97% MP B 3%; 3 → MP A 97% MP B 3%; 120 → MP A 65% MP B 35%; 127 → MP A 20% MP B 80%; 130 → MP A 20% MP B 80%; 131 → MP A 97% MP B 3%; 140 → MP A 97% MP B 3%	Sample rate 2 point/s; m/z 350–1250; probe temperature 500 °C; capillary 1.5 V; cone 10 V	m/z 972, sequence undeclared	Salamah et al. (2019)
Dairy products: yoghurt, ice cream, cheese	nanoUPLC-ESI-qTOF-MS^{E} (nano-ACQUITY coupled with SYNAPT HDMS). ProteinLynx Global Server v2.4 software equipped with the IDENTITY^{E} algorithm for protein identification	C18 Trap column, nanoACQUITY UPLC Symmetry (5 µm particle size, 180 µm I.D. × 20 mm length), linear gradient: 5–50% ACN for 90 min with flow rate of 300 nL/min	(+) ESI data independent acquisition mode (MS^{E}); low energy collision 6 V; high energy collision 15–40 V; capillary voltage 3.2 kV; m/z 50–1600	27 marker peptides with an m/z range of 529.2729–2803.3695	Yilmaz et al. (2013)
Food confectionery: gummy, marshmallow, jelly, and candy	Vanquish™ Flex Binary UHPLC system coupled with TSQ Altis™ Triple Quadrupole Mass Spectrometer Thermo Scientific™	C18 LC column Acclaim™ PepMap™ 100 particle size 3 µm (1.0 mm × 150 mm), flow rate 0.1 mL/min; MP A: 0.1% FA in water, MP B: 0.1% FA in ACN with gradient settings in min as follows: 0–2 → A 95% B 5%; 13–15 → A 50% B 50%; 15.1–25 → A 95% B 5%	(+ and -) ESI Vaporizer temp 250 °C; sheath gas 20 arb; aux gas 10 arb; sweep gas 1 arb; ion transfer tube temp 325 °C; cycle time 0.8 s; CID gas 1.6 mTorr; Q1 and Q3 0.7 FWHM; fragmentation source 0 V; chromatography peak width 30 s	m/z 472.7, 406.2, 739.8, 735.7, 774.9, 921.5, 1075, 940.8, 682, 1111, 1095 while sequences are not available	Chia et al. (2020)
Marshmallows, gums, cookies, and chocolates	Shimadzu LCMS-8060 Data analysis: Uniprot database and MRM prediction via SkyLine	Aeris Peptide 1.7 µm XB-C18 100 Å (150 mm × 2.1 mm I.D.) with flowrate 0.3 mL/min; MP A: 0.1% FA in water, MP B: 0.1% FA in ACN with gradient settings in min as follows: 0–2 → 95% A 5% B; 15 → 75% A 25% B; 15.21–16 → 50% A 50% B; 16.01–19 → 95% A 5% B. injection volume 1 µL and temperature 40 °C	(+) ESI MRM mode; block, DL, and interface temperatures 400 °C, 250 °C, and 300 °C, respectively; nebulizing, drying, and heating gas 3 L/min, 10 L/min, and 10 L/min, respectively	m/z 456.2327 GPPGSAGAPGK	Jumhawan et al. (2019, 2017b, 2017a)

unknown sample clustering in the area. Figure 6b shows a loading plot from PCA based on UPLC/MS 8556 variable data, where RT (min)_m/z pairs of 4.65_641.3065, 8.49_925.4326, 8.53_732.8282, 15.59_765.8665, and 16.16 758.8589 were used as marker peptides for BG, PG, DH, DG, and TS, respectively (Cheng et al. 2012).

Chia et al. detected porcine gelatin in food confectionery products (gummies, marshmallows, jellies, and candy) using Vanquish HPLC coupled with a TSQ Altis triple quadrupole mass spectrometer. Gelatin was extracted from the sample and subsequently digested with trypsin before being loaded into an LC–MS instrument. The results were also compared with those of ELISA. This method accurately detected porcine gelatin peptides in diverse sample matrices to a 0.01% contamination level through several confirmation criteria. This method allowed rapid identification with high accuracy by combining a simple sample preparation procedure with a rapid SRM-based LC–MS methodology (Chia et al. 2020).

In another study, Jumhawan et al. reported the source of gelatin in marshmallows, gums, cookies, and chocolates (Jumhawan et al. 2017b). They extracted gelatin from the samples in a similar manner to that that reported previously: extraction and digestion of trypsin before injection into a Shimadzu LCMS-8060 instrument. However, depending on the type of instrument and food, the optimum parameters of chromatography and MS to define porcine in food products may vary. Table 2 summarizes the optimum parameters of LC and MS or MS for each food type and m/z marker and porcine identification. This summary will help researchers reproduce the method for halal authentication.

Future perspective

LC–MS has the potential to be used for routine porcine analysis because it can detect various species simultaneously. The development of various marker peptides will be very interesting because more detailed information can be obtained such as the tissue origin of the porcine protein. In addition, the opportunity to develop a method for quantifying porcine substances with LC–MS is widely open, since it has a smaller LOD of up to 0.01% contamination than any other methods. The efforts to reduce the matrix effect and analysis duration, as well as improve the sensitivity of the results, are still wide open for development. Moreover, LC–MS has proven its potential as a robust, selective, sensitive, and efficient alternative approach for porcine detection, with strong potential for expanding the realm of porcine analysis.

Conclusion

LC–MS method is currently being developed to fulfill the demands of the world's fast-growing halal business and strengthen porcine detection analysis. Extracting porcine materials (such as proteins), the stage of digestion and analysis using LC–MS are all part of the porcine detection technique employing LC–MS. The LC–MS technique is currently utilized for qualitative testing with low detection limits, but quantitative tests can still be developed. The parameters of liquid chromatography and MS include instrument type, data analysis, column type, MP and elution, MS conditions, and selection of the m/z marker, which can differ depending on the type of product being analyzed. Currently, there is no universal method that can be applied to all products because the matrix of each product is unique and requires different handling. However, the references summarized in this review can be considered when choosing which extraction method and LC–MS optimum parameters are most suitable for food product applications. The duration, cost, and simplicity of the process are the main considerations for the method to be applied in routine analysis.

Acknowledgements

The authors wish to thank the Advanced Characterization Laboratories Cibinong—Integrated Laboratory of Bioproduct, National Research and Innovation Agency through E-Layanan Sains, Badan Riset dan Inovasi Nasional for the facilities and scientific and technical support.

Author contributions

KRD, NNS, MI, and SK were involved in the conceptualization and formal analysis; KRD, NNS, MI, and SK contributed to the investigation; KRD and NNS were involved in the data curation; KRD, NNS, MI, SK, TA, HR, and NU assisted in the writing—original draft preparation, review, and editing; NDY, FK, HH, and AH contributed to the supervision; KRD, NDY, and HH contributed to the project administration. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by from Directorate of Research, Technology, and Community Services (DRTPM), the Directorate General of Higher Education, Research, and Technology, The Ministry of Education, Culture, Research, and Technology in the scheme of Penelitian Tesis Magister (PTM) No. 3843/IT3.L1/PT.01.03/P/B/2022; the National Research Foundation of Korea funded by the Korea Government grant No. 2022R1A2B5B02001374 and NRF-2020M3G1A1114560; and the Korea Research Institute of Standards and Science for the establishment of a reliable foundation for the national fine dust measurement system in 2021 (Korea Institute of Standards and Science- 2021—GP2021-0006).

Availability of data and materials

Not applicable.

Declarations

Competing interests

The authors have no competing financial interests to declare.

Received: 2 December 2022 Accepted: 1 February 2023
Published online: 20 February 2023

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