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RESEARCH

Verification Analyses for the Detection of Bovine and Porcine Species in Foods Containing Animal Gelatin with Q-Exactive ORBITRAP Device

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Abstract

Animal-derived gelatins are widely used in a variety of foods such as biscuits, chewing gum, chocolate, jelly beans, and confectionery, as well as medicines and food supplements. However, high temperatures, chemical treatments, and other methods used in the production process can damage the animal genes in the gelatin. This makes it difficult to extract DNA from gelatin-containing products and prevents the detection of animal species by technological methods such as real-time polymerase chain reaction (RT-PCR). This problem clearly demonstrates the need for new and more advanced technologies for species identification. In such cases where traditional methods are inadequate, more sensitive and reliable technological approaches should be developed. In this study, verification studies were conducted for species detection on 124 animal gelatin–containing products that could not be identified by RT-PCR due to DNA degradation. This was achieved using Quadrupole (Q) Exactive Orbitrap, a new generation molecular technology. As a result of the study, 62 samples containing bovine gelatin were spiked with 1% pork gelatin, and 62 of the samples were found to be positive for porks by Orbitrap. Similarly, 62 of the 62 samples containing pork gelatin spiked with 1% bovine gelatin were detected as bovine positive by Orbitrap. In this context, successful results were obtained in the detection of beef and pork species with LOD 1% level. The developed method was verified in terms of sensitivity, accuracy, and precision. As a result, limits of quantification (LOQ) were ranging from 10.0 µg/L. As a result, the limit of detection (LOD) was found to be 1%. Furthermore, this method was successfully applied to positive and negative controls.

Keywords Gelatin · Beef · Pork · Q-Exactive Orbitrap · Verification · Food analytical method

Introduction

Gelatin is a functional material derived naturally from animal connective tissues of widely used in many fields and sectors such as food, pharmaceuticals, and cosmetics (Deng et al. 2020). Gelatins are animal-derived ingredients used in many commercial food products such as gummy snacks, chewing gum, ice creams, marshmallows, puddings, capsules, candies, biscuits, and chocolates due to their water retaining, thickening, gelling, adhesive, and emulsifying properties (GMIA 2012; Yilmaz et al. 2013; Jannat et al. 2018). Gelatin is produced from the partial denaturation of collagen extracted from the skin, bones, and connective tissues of animals, with a higher proportion from cattle and pigs and a lower proportion from fish and poultry (Liu et al. 2015; Jannat et al. 2018; Michelini et al. 2020).

Global population growth and economic concerns have increased the interest in the production of animal-derived gelatin, which is demanded and produced by consumers (Kazir & Livney 2021; Tümerkan 2021a, b; Santos et al. 2023; Tukiran et al. 2023). The high demand for foods containing animal products brings along a number of illegal practices during the production, distribution, or sale of food. For example, one study found pig DNA in various processed food products, especially those containing gelatin (Demirhan et al. 2012). Similarly, research has detected bovine gelatin in products labeled as containing only porcine gelatin, highlighting the prevalence of species substitution in the gelatin market

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(Grundy et al. 2016). These issues cause concern among a certain segment of the population who want to consume safe and reliable food of known origin due to both consumer deception and their faith in Muslim and Jewish religions (Lubis et al. 2016; Yang et al. 2018). The detection of gelatin is of great importance for food safety and consumer information. It is also a sensitive issue for individuals who do not want to consume certain animal products for religious and ethical reasons.

Due to the different processes applied during the use of gelatin in production, high temperature, or acid applications, most of the gelatin DNA of animal origin is denatured, and therefore, the sensitivity of DNA-based methods in species identification analysis is significantly reduced, resulting in false-positive or false-negative results. (Karni et al. 2013; Sha et al. 2014; Grundy et al. 2016; Kleinnijenhuis et al. 2018). In this context, RT-PCR, one of the methods used in the detection of species and adulteration, is insufficient (Karni et al. 2013; Yang et al. 2018). Another alternative method is enzyme-linked immunosorbent assay (ELISA). Since this method can only detect a limited sequence (i.e., epitope) of proteins, this method can produce false-negative or false-positive results (Wilson 2013; Solier & Langen 2014; Tighe et al. 2015; Kleinnijenhuis et al. 2018). Mass spectrometry has been increasingly applied to food authentication and quality control studies in recent years (Herrero et al. 2012; Ruiz Orduna et al. 2017; Jannat et al. 2018; Yang et al. 2018). The aim of this study is to carry out verification studies to introduce a new analytical method to the field to prevent adulteration by detecting bovine and porcine gelatin species in various product groups using state-of-the-art high-resolution Q-Exactive Orbitrap mass spectrometry.

Materials and Methods

Samples

In this study, a total of 124 samples containing pork and bovine gelatin were analyzed. These samples included 62 products with bovine gelatin and 62 products with pork gelatin, such as jelly beans, candies, biscuits, chocolates, chewing gums, food additives, food supplement drug capsules, powdered gelatin, pure pork gelatin, and pure bovine gelatin.

Chemicals and Reagents

These include ammonium bicarbonate (50 mM) (Acros Organics®A0413846, Spain), recombinant trypsin (10 µg) (EMS0006-100 UG®1,003,505,650, Sigma-Aldrich), trifluoroacetic acid (1% TFA) (Carlo Erba® 411,561, UN 2699), water (4 mM Amonyum Format (Analar Normapure®21,254.260) and 1% formic acid (Pancreac®131,030.1612)), methanol (Carlo Erba®414,832, UN 1230), and ultrapure grade water (EMD Millipore Co.).

Preparation of Samples for Analysis

The laboratory working ambient temperature was 20–25 °C during the extraction phase of the animal species determination analysis in gelatin-containing products with the Q-Exactive Focus Orbitrap device. This is important for the quality of the analysis results performed with the Orbitrap device. In addition, all surfaces to be analyzed were cleaned with ethanol before starting the analysis.

Preparation of Solutions to be Used in Analysis

The solutions to be used in the analysis were prepared as follows:

A 50 mM solution of NH_4HCO_3 (3.95 g of ammonium bicarbonate) was prepared by dissolving in 1 L of water (pH ~ 8.5).

Trypsin (10 µg for each sample) = 100 µg trypsin vial was dissolved in 1500 µL with 50 mM NH_4HCO_3. A total of 150 µL of the mixture was added to each sample.

Sample Preparation and Tryptic Digestion

The samples were prepared by weighing 0.5 g of sample into long thin centrifuge tubes, followed by an addition of 5 mL of 50 mM NH4HCO3 solution (Acros Organics®A0413846, Spain). The mixture was sonicated in an ultrasonic water bath (Intersonik® Ultrasonik, Istanbul) at a temperature of 40 °C for 30 min to ensure thorough mixing. After sonication, 200 µL aliquots of the mixture were carefully transferred into 1.5- to 2-mL premium hydrolytic glass and polypropylene vials (brown). These vials were then placed in a reaction block (DLAB® HM100-Pro, China) and heated to 100 °C for 10 min. Following this heating step, the vials were allowed to cool to room temperature for 15 min. Tryptic digestion was performed by adding 150 µL trypsin (EMS0006-100 UG®1,003,505,650, Sigma-Aldrich) (10 µg = 0.01 mg/kg) and incubating at 40 °C (Binder KB 115, Germany) for 24 h. Post-digestion, 1500 µL of 1% TFA solution (Carlo Erba® 411,561, UN 2699) was added, and the mixture was centrifuged at 12,000 G for 10 min (Wisd®WiseSpin CF-10, Korea). Finally, 1 mL of supernatant was collected for Orbitrap analysis.

Q-Exactive Orbitrap Analysis Conditions and Parameters

First Analysis Method (Tryptic Hydrolysate Analyses with UHPLC: Q Exactive Orbitrap)

The separation and detection were performed using UHPLC-Q Orbitrap mass spectrometer with heated electrospray ionization (HESI) (Q Exactive Plus, Thermo Fisher

Scientific, Bremen, Germany). Chromatographic separation was achieved on an AccucoreTMaQ C18 column (100 mm × 2.1 mm, 2.6 µm, Thermo Scientific) maintained at 40 °C. The mobile phases consisted of (A) 0.1% formic acid and 4 mM ammonium formate in water and (B) 0.1% formic acid and 4 mM ammonium formate in methanol. The flow rate was 0.3 mL/min with an injection volume of 10 µL.

The gradient elution was applied as follows: 0.5–0.5 min, 5% B; 0.5–1.0 min, 15% B; 1.0–6.5 min, 40% B; 6.5–7.0 min, 95% B; 7.0–8.5 min, 95% B; 8.5–8.6 min, 5% B; and 8.6–10.0 min, 5% B.

The ionization conditions about HESI parameters were electrospray voltage, 4.0 kV for positive mode; S-lens R, 60 V; the electrospray ionization source temperature, 320 °C; ion transfer capillary temperature; 320 gas flow rate; and 45 arbitrary.

Second Analysis Method (Animal Species Analysis with Q-Exactive Orbitrap)

Animal species separation utilized a 100 mm × 2.1 mm, 2.6 µm AccucoreTM aQ C18 Column coupled to a Thermo Scientific Q-Exactive Orbitrap mass spectrometer. The mobile phases were (A) isopropanol:acetonitrile (85:15, v/v) and (B) acetonitrile:water (65:35, v/v), both containing 0.1% formic acid and 5 mmol/L ammonium formate.

A solvent gradient consisting of 15–25% A (0–2 min), 25–40% A (2–3 min), 40–75% A (3–15 min), 75–99% A (15–20 min), 99% A (20–25 min), 95–15% (25–26 min), and 15% A (26–30 min) at a flow rate of 0.35 mL/min at 50 ℃ in gradient elution. The injection volume was 10 µL for each analysis. The resolution was set to 17,500. The spray voltage was set to 4000 V. The flow rate of sheath gas and auxiliary gas was 45 arb and 10 arb, respectively. The temperature of the auxiliary heater was 320 ℃. The scanning range of full MS was *m/z* 400 ~ 2000. The number of the highest ions monitored in each acquisition cycle was 10. The collision energies were 45 eV.

The Q-Exactive Orbitrap instrument is an instrument for the arrival of masses versus time and matching by column (protein molecule mass/weight) principle and analyzes them by verifying them with TraceFinder (4.1 General Quan) software and Thermo Proteomics "Proteome Discoverer 2" software. Advancements in targeted protein qualitative technology allowed for the qualitative detection and analysis of signature peptides generated by trypsin using the Q-Exactive Orbitrap system. After the analysis of the samples, the chromatograms obtained were processed and evaluated in the Proteome discoverer 2.2 program. This program has a fasta file for both pork gelatin and bovine gelatin. The software gives results by comparing the spectra obtained with the library.

Method Validation and Sample Analysis

In this study, a total of 124 samples containing pork and bovine gelatin were analyzed. These samples included 62 products with bovine gelatin and 62 products with pork gelatin, such as jelly beans, candies, biscuits, chocolates, chewing gums, food additives, food supplement drug capsules, powdered gelatin, pure pork gelatin, and pure bovine gelatin. The method validation was performed using three different sample groups with a comprehensive analysis design. Verification studies were conducted at concentrations of 10%, 5%, 2.5%, and 1%, with the limit of detection (LOD) set at 1%. For the verification analyses, appropriate amounts of gelatin from the two species (pork and bovine) were weighed to create mixtures with defined weight ratios. For instance, a total of 10 mg of mixture 1 was prepared, into which 0.5 mg and 0.1 mg of the other species were added to achieve the desired concentrations. First, reference materials consisting of pure pork gelatin standards (62 samples) and pure bovine gelatin standards (62 samples) were analyzed. Second, mixed-species samples were prepared by weighing appropriate amounts of species to obtain defined ratios, specifically binary mixtures of pork gelatin in bovine gelatin at four different concentrations: 10% (10 mg mixture, $n = 3$), 5% (0.5 mg mixture, $n = 3$), 2.5% (0.25 mg mixture, $n = 3$), and 1% (0.1 mg mixture, $n = 3$). Lower concentrations (0.1%) were also tested but excluded from the validation as they were below the method's detection capability, thus establishing 1% as the method's lowest validated level (LOD). Third, ten commercial gelatin samples were analyzed as real samples, with each sample analyzed in triplicate. Each validation level included both blank (negative control) analyses and positive control samples to ensure method specificity. The blank analyses confirmed the absence of cross-contamination and false positives, while positive controls verified the method's detection capability. The method demonstrated acceptable precision with relative standard deviations (RSDs) of less than 5% for all measurements. The method's detection limit was established at 1% using precursor ion detection. For enhanced sensitivity, signature peptides formed by tryptic digestion were qualitatively detected and analyzed using Q-Exactive Orbitrap.

Statistical Analysis

Peak areas were extracted using Proteome Discoverer 2.2 software (Thermo Fisher Scientific, USA) and normalized relative to the total area. Statistical differences were analyzed at a 99% confidence level ($p < 0.01$) using analysis of variance (ANOVA) in the software to assess significant differences across sample groups. Chromatograms of undetectable pork marker peptides in products containing bovine

gelatin were examined and used to confirm species-specific markers, with detailed molecular weights and retention times listed in the Bovine Species-Specific Marker Peptide List. These chromatographic results were also evaluated for consistency and specificity, using signature peak areas as criteria for species determination.

Results

In verification analyses, samples are prepared by weighing appropriate amounts of meat from two species to obtain mixtures with defined weight ratios. For example, a sample of one species can be spiked.

A total of 10 mg of mixture 1 and 0.5 and 0.1 mg of mixture 1 were added to 10%, 5%, 2.5%, and 1% of other species. That method used precursor ions for qualitative detection so sensivity limited was 1 % could it be detected. With the development of targeted protein qualitative technology, the signature peptides formed by trypsin were qualitatively detected and analyzed by Q-Exactive Orbitrap.

Using TraceFinder (4.1 General Quan) software on the Q-Exactive Orbitrap instrument, molecular level validation of species-specific genes was performed by comparing masses based on ion chromatography system. In addition, with the Thermo Proteomics (Proteome Discoverer 2) software installed for validation purposes, marker peptides belonging to bovine and porcine species were identified species-wise according to their peptide bond sequences based on ion/mass chromatography. According to the results of the study, 6 PG and 6 BG biomarker peptides (Conf. > 99) were identified for porcine gelatin (PG) and bovine gelatin (BG), respectively. The fragment ion pairing results of these peptides are shown in Tables 1 and 2. With high specificity, high resolution, and low mass bias, HRMS is able to detect small mass differences due to amino acid modifications.

The analysis performed in this study is based on LC–MS/MS (liquid chromatography-mass spectrometry) using the Q-Exactive Orbitrap device. According to the results of the LOD, 1% verification analysis, a total of 124 different matrices, including gelatin from pork platinum extra pure (Carl Roth GmbH® CAS: 9000–70-8), gelatin from bovine skin (Type B® SLCB1685, SIGMA ALDRICH), as well as various products such as powdered gelatin, biscuits, chocolates, confectionery, food additives, and capsule drugs used in food supplements, were analyzed. The Q-Exactive Orbitrap data, including marker peptides and retention times for both bovine and pork species, are presented in Tables 3 and 4.

After the device finishes the analysis, the Proteome Discoverer software is used to search for marker peptides in the "Theoretical masses" (TH + MH) by clicking on the "PSMS" section of the table. The presence of any and/or several or all marker peptides was considered positive (Zhang et al.

Table 1 Pork species-specific marker peptide list for evaluation of study results

List of the biomarker peptides discovery of PG	Precursor ion, *m*/*z* (charge = 1)	Product ion, *m*/*z* (charge = 2)	Retention time (min.)
GPTGPAGVR	811.44208		8.60
GEAGPQGAR	842.41151		2.23
QGPSGPSGER	971.45410		3.59
GETGPSGPAGPTGAR	1311.62877	656.31802	9.22
SAGISVPGPMGPSGPR	1466.74202	733.87465	14.80
GETGPAGPAGPVGPVGAR	1546.79723	773.90225	12.41

m mass/*z* ion

Table 2 Bovine species-specific marker peptide list for evaluation of study results

List of the biomarker peptides discovery of BG	Precursor ion, *m*/*z* (charge = 1)	Product ion, *m*/*z* (charge = 2)	Retention time (min.)
GATGPAGVR	785.42643		7.45
QGPSGASGER	945.43845		1.77
IGQPGAVGPAGIR	1192.67968	596.84348	12.89
GEAGPSGPAGPTGAR	1281.61820	641.31274	9.20
GETGPAGPAGPIGPVGAR	1560.81288	780.91008	13.37
SGDRGETGPAGPAGPIGPVGAR	1975.99443	988.50085	12.79

m mass/*z* ion

2008; Cavus & Ozbay 2017; Yang et al. 2018; Zhu et al. 2023). As a result, chromatograms of pork marker peptides were successfully detected in products containing pork and beef gelatin in the analyses performed using the Q-Exactive Orbitrap device (Figs. 1, 2, 3, and 4).

Discussion

Biomarker peptide screening is critical for ensuring accurate species identification, especially in complex sample matrices where reliable detection methods are essential.

Table 3 Molecular weights of marker peptides detected by Q-exactive orbitrap device from pork gelatin-containing products

No	Sample Type	Content Declaration	Orbitrap														
					m/z (Charger 1) Pig												
			BEEF	PORK	811,44208 GPTGPAGVR	Retention Time (Min)	842,41151 GEAGPQGAR	Retention Time (Min)	971,45410 QGPSGPSGER	Retention Time (Min)	1311,62877 GETGPSGPAGPTGAR	Retention Time (Min)	1466,74202 SAGISVPGPMGPSGPR	Retention Time (Min)	1546,79723 GETGPAGPAGPAGPV GPVGAR	Retention Time (Min)	
1	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
2	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
3	Pure Porcine Gelatin	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
4	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
5	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
6	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
7	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
8	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
9	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
10	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
11	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
12	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
13	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
14	Jelly Bean	Pig Gelatin			+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
15	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
16	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
17	Pure Porcine Gelatin	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
18	Gelatin	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
19	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
20	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
21	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
22	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
23	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
24	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
25	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
26	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
27	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
28	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	
29	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41	

Table 3 (continued)

30	Jelly Bean	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
31	Jelly Bean	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
32	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
33	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
34	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
35	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
36	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
37	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
38	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
39	Jelly Bean	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
40	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
41	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
42	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
43	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
44	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
45	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
46	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
47	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
48	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
49	Jelly Bean	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
50	Biscuit	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
51	Biscuit	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
52	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
53	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
54	Biscuit	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
55	Biscuit	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
56	Jelly Bean	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
57	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
58	Gelatin Powder	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
59	Biscuit	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
60	Biscuit	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
61	Food Additive	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41
62	Food Supplement Pharmaceutical Capsules	Pig Gelatin	-	+	+	8,60	+	2,23	+	3,59	+	9,22	+	14,80	+	12,41

(+) positive amplification; (−) negative amplification

Screening principles and steps for biomarker peptides have been described in previous studies (Zhang et al. 2008; Tan & Lock 2014; Cavus & Ozbay 2017; Yang et al. 2018). The difference is that a database was previously created by downloading all protein sequences of the target species to screen for potential biomarker peptides. This method yielded thousands of spectral matches. This undoubtedly increased the workload of subsequent blast searches. Collecting abundant protein information can provide the sensitivity of low-resolution mass spectrometers while reducing the tedious workload. Furthermore, by obtaining a large amount of protein data, the sensitivity and reliability of the analyses in the verification study are ensured to be high (Zhu et al. 2023).

In this study, pork (*Sus scrofa*) and bovine (*Bos taurus*) collagen type I sequences were downloaded from the UniProtKB protein sequence database, and information from a proprietary protein database was used. The discovery of

Table 4 Molecular weights of marker peptides detected by Q-exactive orbitrap device from bovine gelatin-containing products

No	Sample Type	Content Declaration	Beef	Pork	Orbitrap											
					m/z (Charger 1) Cattle											
					785,42643 GATGPAGVR	**Retention Time (Min)**	**945,43845** QGPSGASGER	**Retention Time (Min)**	**1192,67968** IGQPGAVGPAGIR	**Retention Time (Min)**	**1281,61820** GEAGPSGPAGPTGAR	**Retention Time (Min)**	**1560,81288** GETGPAGPAGPIGPVGAR	**Retention Time (Min)**	**1975,99443** SGDRGETGPAGPAGPIGPV GAR	**Retention Time (Min)**
1	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
2	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
3	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
4	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
5	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
6	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
7	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
8	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
9	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
10	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
11	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
12	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
13	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
14	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
15	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
16	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
17	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
18	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
19	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
20	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
21	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
22	Gum	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
23	Confectionery	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
24	Pure Bovine Gelatin	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
25	Gelatine Powder	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
26	Gelatine Powder	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
27	Confectionery	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
28	Food Supplement Pharmaceutical Capsules	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
29	Food Supplement Pharmaceutical Capsules	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
30	Food Supplement Pharmaceutical Capsules	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
31	Food Supplement Pharmaceutical Capsules	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79

Table 4 (continued)

32	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
33	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
34	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
35	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
36	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
37	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
38	Gelatine Powder	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
39	Gelatine Powder	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
40	Food Additive	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
41	Confectionery	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
42	Confectionery	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
43	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
44	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
45	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
46	Food Supplement Pharmaceutical Capsules	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
47	Food Supplement Pharmaceutical Capsules	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
48	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
49	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
50	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
51	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
52	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
53	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
54	Jelly Bean	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
55	Chocolate	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
56	Chocolate	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
57	Chocolate	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
58	Chocolate	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
59	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
60	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
61	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79
62	Biscuit	Beef Gelatin	+	-	+	7,45	+	1,77	+	12,89	+	9,20	+	13,37	+	12,79

(+) positive amplification; **(−)** negative amplification

pork gelatin (PG) and bovine gelatin (BG) biomarker peptides was carried out using UPLC-Q/Exactive MS on our own database. A series of N-terminal fragment ions (b ions) and C-terminal fragment ions (y ions) were generated by high-resolution mass spectrometry (HRMS). The higher the degree of match between the collected fragment ion peaks and the theoretical fragment peaks, the higher the reliability (Conf.) of the peptide.

According to the results of the study, 6 PG biomarker peptides (Conf. > 99) (Table 1) and 6 BG biomarker peptides

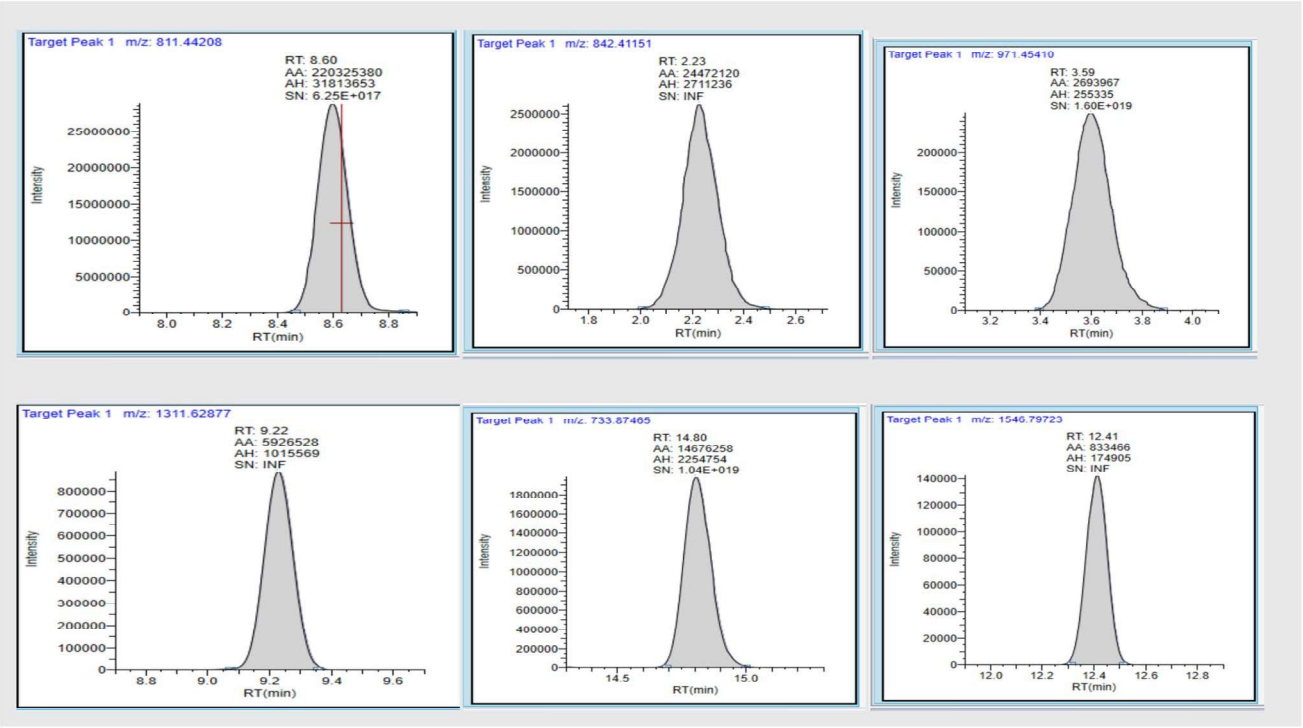

Fig. 1 Chromatograms of pork marker peptides detected in products containing pork gelatin

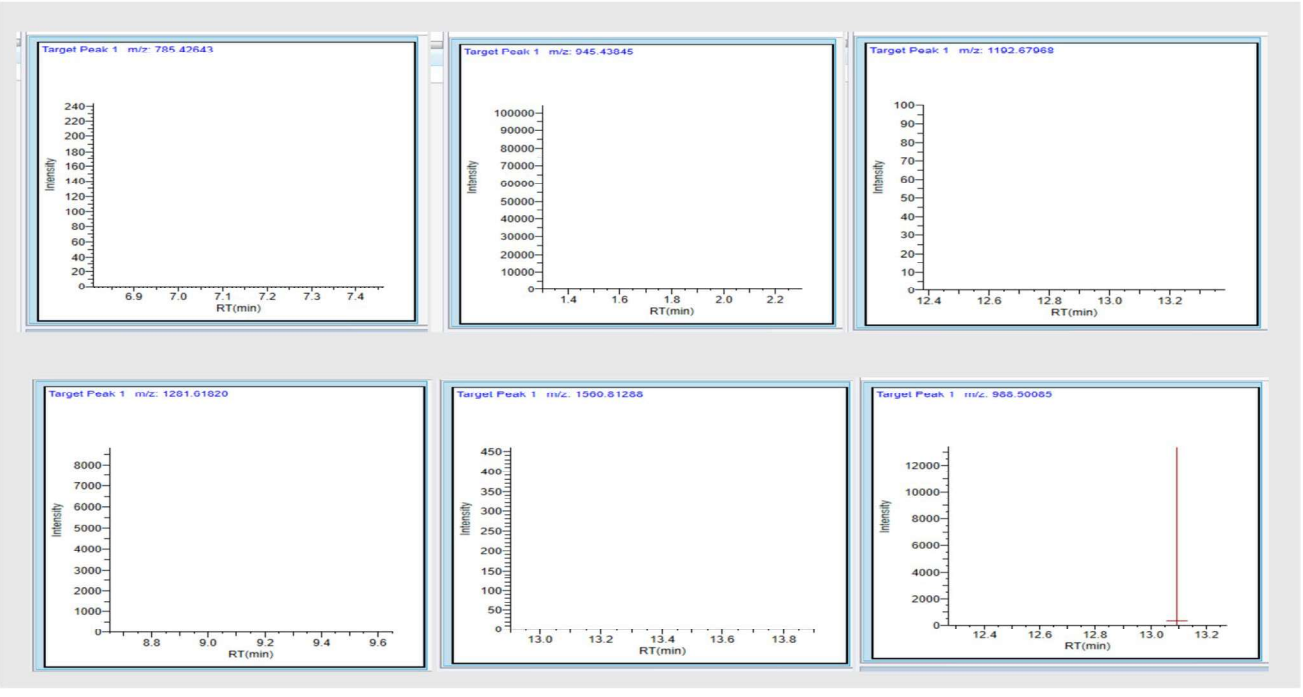

Fig. 2 Chromatograms of undetectable bovine marker peptides in products containing pork gelatin

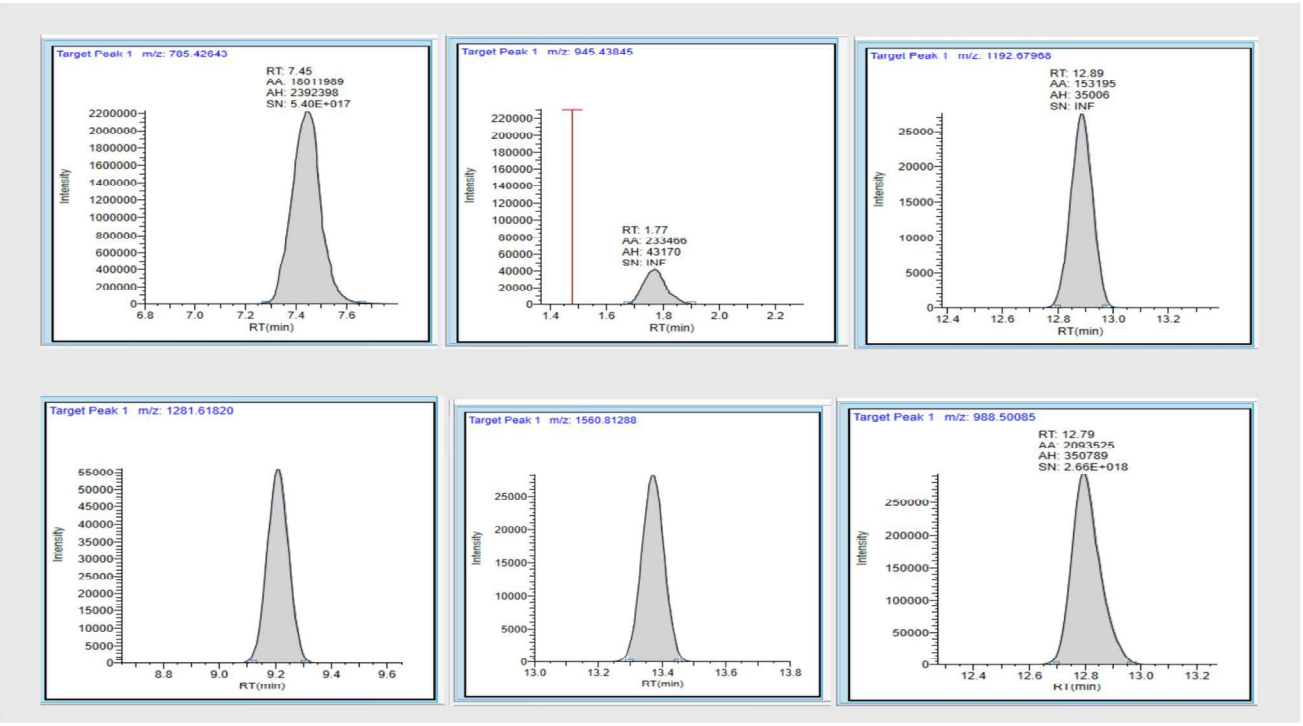

Fig. 3 Chromatograms of the chromatograms of bovine marker peptides detected in products containing bovine gelatin

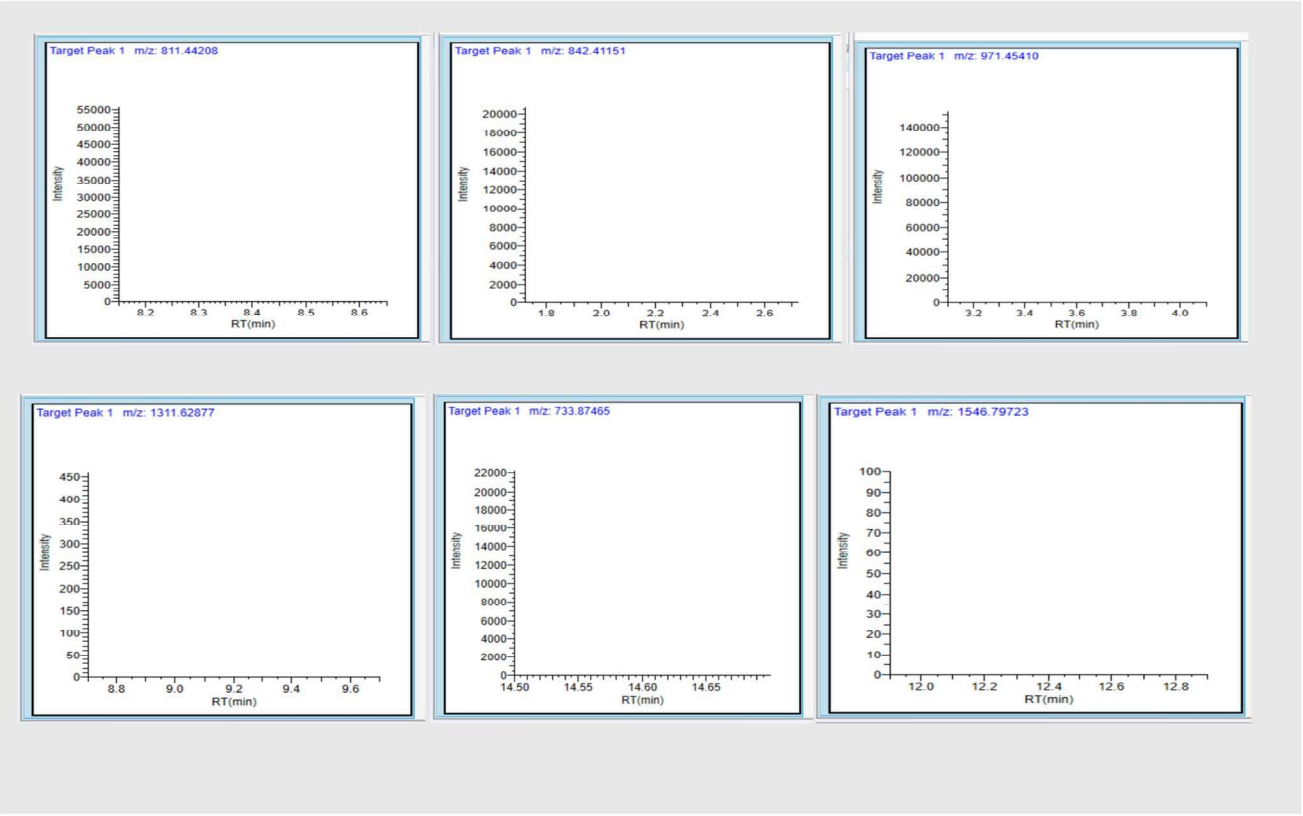

Fig. 4 Chromatograms of undetectable pork marker peptides in products containing beef gelatin

(Conf. > 99) (Table 2) were identified so that we can evaluate our research data. The fragment ion matching results of these 6 biomarker peptides derived from PG and BG are also shown (Tables 1 and 2). With high specificity, high resolution, and low accurate mass deviation, HRMS is able to detect small mass differences due to modification of amino acid residues (Zhang et al. 2008; Lacalle-Bergeron et al. 2021). For this purpose, standards were digested and tested under non-high-resolution MS conditions to confirm that the selected peptides could be stably present in the quantitative instrument (Zhu et al. 2023).

In the Q-Exactive Orbitrap verification studies conducted with 124 different matrices (pure pork gelatin, pure bovine gelatin, powdered gelatin, biscuits, chocolate, confectionery, food additives, capsule drugs used in food supplements) containing 62 bovine and 62 pork gelatin, 100% successful results were obtained at 1% LOD level in all matrices. The results showed that the limits of quantification (LOQ) for the method ranged from 10.0 µg/L, indicating a high sensitivity for the analytes of interest. The limit of detection (LOD) was set at 1%, underlining the ability of the method to detect low levels of target compounds with sensitivity. Furthermore, the method was successfully applied to both positive and negative control samples, demonstrating its robustness, accuracy, and applicability in different sample matrices. This validation underlines the potential utility of the method in a wide range of analytical applications, particularly in the detection and quantification of trace substances.

Conclusions

In animal gelatin–containing products, gelatin is treated with high temperatures and/or various agents during the product process in order to take advantage of the properties of gelatin such as water retention, thickening, gelling, gluing, emulsifying, and carrier. Therefore, since the DNA in animal gelatin–containing products is often more or less damaged, the results obtained as false positive or false negative are not reliable as the sensitivity will be very low with methods that can detect only a small fraction of proteins or DNA recovery. In this context, in order to prevent counterfeiting/adulteration in animal products within the framework of public health and safe food principles and to obtain accurate, reliable, and fast results, the necessity of directing the detection analysis methods with chromatographic methods based on ion and mass spectroscopy at the molecular level in species determination analyses with developing technology has been revealed in this study.

Author Contribution N.G.Y. 1. Made substantial contributions to the conception or design of the work, 2. The acquisition, 3. Analysis, 4. Interpretation of data, 5. The creation of new software used in the work (statistical analsis), 6. Wrote the main manuscript, prepared graphical abstract and tables, 7. Reviewed the manuscript. F.Y. 3. Analysis, 4. Interpretation of data. A.S 3. Analysis, 4. Interpretation of data.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Compliance with Ethics Requirement This study does not contain any studies with human participants or animals performed by any of these authors.

Competing Interests The authors declare no competing interests.

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