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UHPLC/ ESI-Q-TOF-HRMS Analysis for Identification of Collagen Hydrolysates Produced from White Shavings by Locally Isolated Bacterial Strains

Hamed Elsayed [a] *, Mohamed Marzouk [a] , Abdel-Mohsen S. Ismail [b], Mostafa A. El-abd [b], Amira A. H[b], Mohamed I. A[b], Nabil H. El-Sayed [a]

[a] Mohamed Marzouk: https://orcid.org/0000-0002-1344-0617, Mostafa A. El-abd: https://www.orcid.org/0000-0002-4604-7678, Abdel-Mohsen S. Ismail: https://www.orcid.org/0000-0001-6841-8866, Amira A. H, https://www.orcid.org/0000-0002-0339-5103, [a] Nabil H. El-Sayed: https://www.orcid.org/0000-0003-2811-8855, Mohamed I. A: https://www.orcid.org/0000-0002-0077-6621, [a] Hamed Elsayed : https://orcid.org/0000-0002-6463-258X

[a]*Chemistry of Natural and Microbial Products Department, Pharmaceutical Industries Research Institute, National Research Centre, Dokki, Giza, Egypt*

[b]*Chemistry of Tanning Materials and Leather Technology Department, Chemical Industries Research Institute, National Research Centre, Dokki, Cairo 12622, Egypt*

Abstract

Traditional approaches, such as microscopic inspection and PCR, are inadequate for accurately identifying the gelatin types and animal source of leather. The current investigation aimed to establish an Ultra-High-Performance Liquid Chromatography /Electrospray Ionization-Quadrupole Time-of-Flight-High Resolution Mass Spectrometry UHPLC/positive ESI-Q-TOF-HRMS technique for identifying variations in the amino acid sequences of type I collagen hydrolysate. Gelatin, primarily obtained from bovine sources, has found extensive application in various food and pharmaceutical applications. To assure the compliance of food products with halal rules, accurate analytical procedures are very much necessary. A specific marker peptide for bovine gelatins was chosen in this research to establish a system for multiple reaction monitoring using UHPLC /positive ESI-qTOF-HRMS. The current work aimed to optimize the UHPLC/ESI-Q-TOF-HRMS method for the identification of gelatin types and collagen hydrolysates. This was achieved by controlled enzymatic digestion of leather white pickled shaving samples and then matching their LC/ESI-Q-TOF-HRMS output data (R_t, XIC, monoisotopic masses of the molecular and/or selective fragment ions of some peptide markers) with a respective library database. Two bacterial strains, namely BFW (5,7), were isolated from fish wastes and applied on white shavings that were supplied by the Egyptian leather industry. These strains have demonstrated their ability to produce collagenase, which is a potent enzyme that facilitates the controlled hydrolysis of collagen. This enzymatic process gives gelatin of high-quality, which can be utilized as a valuable resource in industrial applications. The present investigation involved identification of a wide-range of collagen marker peptides of bovine collagen hydrolysates using a sequential process of chromatography. The current UHPLC/positive ESI-Q-TOF-HRMS findings indicate that more than 2000 and 4000 peptides were tentatively identified in both investigated collagen hydrolysate samples, i.e. BFW5, BFW7. According to their peptides sequence, the structure of the major peptides was identified from higher to lower MW as GEPGPTGIQGPPGPAGEEGKR, and GPAGPQGPR, respectively, for BFW5 sample. Also, for BFW7 sample, the higher to lower MW of major peptides were identified as GAPGDKGEAGPSGPAGPT and LAGPPGESGR, respectively. The above results showed that the peptides isolated in this study were identified and characterized as collagen marker peptides from bovine collagen hydrolysates. The quantification technique was thus developed using the three most frequently occurring peptides in the digested bovine gelatin, namely GFOGADGVAGPK, GETGPAGROGEVGPOGPOGPAGEK, and GFOGSOGNIGPAGK. When it comes to detecting bovine gelatin, these collagen marker peptides are more unique. When utilized in conjunction with HPLC and mass spectrometry, this technique serves as a precise and very sensitive quantitative approach for the detection of bovine gelatin. Therefore, this approach can be employed to authenticate the halal status of gelatin.

Keywords: UHPLC/ESI-qTOF-HRMS, white pickled shavings, enzymatic hydrolysis, collagen hydrolysate, peptides sequence.

*Corresponding author e-mail: msmarzouk@yahoo.co.uk, hamed_sci@yahoo.com.

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1. Introduction

Liquid chromatography-mass spectrometry (LC/MS) has emerged as a highly prevalent analytical methodology for the qualitative and quantitative assessment of tiny compounds. Over the past decade, its utilization of simple and dependable LC/MS interfaces, such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI), has significantly contributed to the advancement and widespread use of LC/MS methodologies. Indeed, a significant number of laboratories currently employ LC/MS as their primary technique for material analysis. Both ESI and APCI techniques are susceptible to ion suppression effects, with ESI being more susceptible to this phenomenon compared to APCI. Nevertheless, ESI is considered a milder ionization technique as it does not induce the breakdown of conjugates within the ionization source, such as the conversion of glucuronide to the aglycone. A poor separation utilizing APCI has the potential to yield inaccurate quantitative outcomes, despite the deliberate selection of a source that minimizes ion suppression [1, 2]. In recent times, there has been a growing tendency towards achieving better sample throughput, leading to the adoption of faster chromatographic separations and other LC/MS techniques that effectively reduce analytical cycle time. The increased accessibility of modern LC/MS systems have facilitated the widespread adoption of LC/MS techniques, resulting in a scenario where numerous researchers who utilize LC/MS as their principal analytical tool may not possess specialized expertise in the field of LC/MS. Considering the fast development of LC/MS output data and their applications, the utilization has shown significant growth within recent years [3-7]. Currently, the UHPLC/ESI-Q-TOF-HRMS is widely employed as a prominent technique for the determination of the quantity and quality of low-molecular-weight substances, such as gelatin or collagen hydrolysate [2].

The widespread adoption of sophisticated LC/MS systems has led to an increasing acceptance of LC/MS among professionals who may not have had specialized training in this field. The authors posited that an examination of the present condition of the UHPLC/ESI-Q-TOF-HRMS could potentially assist individuals who are unfamiliar with LC/MS in developing efficient LC/MS methodologies that effectively integrate chromatography and mass spectrometry [8, 9]. There has been a growing demand, particularly within the pharmaceutical sector, to reduce the duration of analysis [10]. Upon reviewing the scientific literature, it became evident that the term rapid UHPLC/ESI-Q-TOF-HRMS was employed by several proponents for distinct methodologies. In the context of this article, the researchers have conducted analysis for the examination of collagen hydrolysate [11, 12].

Leather production yields 250 kg of finished leather and 120 kg of untanned raw trimmings, tanned blue trimmings, pickled shavings, finished colored shavings, and buffing dust per ton [13-17]. Most of tannin's vast solid waste is collagen, lipids, salts, and chemicals [18-20]. Protein hydrolysate, low-molecular-weight peptides used in cosmetics, health care, and pharmaceuticals may optimize waste advantages [21-26]. Collagen hydrolysis is often accomplished using chemical methods that employ potent acidic and alkaline solutions. Although these technologies are simple, they are both cost-effective and environmentally detrimental. [27-29]. Enzymes are a viable and efficient alternative that also utilize chemical-enzymatic processes, which are environmentally beneficial. [30-33]. In current practice, the enzymatic hydrolysis of collagen has emerged as a prominent method for enhancing the production efficiency of gelatin, mostly employing collagenase. The duration of the treatment is significantly shorter compared to the time needed for alkali or acidic treatment [34]. Industrial gelatin and collagen hydrolysate were optimized from pretreated pickled hide shavings using a microbial collagenase-rich protease combination [10, 35].

Gelatin is a composite of polypeptides acquired from the specific breakdown of collagen, the primary protein found in the skin, hides, white connective tissues, and bones of animals. Gelatin is extensively utilized in the culinary, cosmetic, and pharmaceutical sectors due to its notable benefits, including its ability to decompose naturally, lack of toxicity, compatibility with living organisms, and affordability [36-38]. Gelatin serves as an addition in numerous culinary products, including gummy sweets, canned ham, various luncheon meals, corned beef, chicken rolls, jellied meat, margarine, sour cream, and cottage cheese [39]. One of the most common ingredients in manufactured foods is porcine gelatin. The use of porcine gelatin is strictly prohibited in Islamic nations [40]. However, due to widespread globalization and the food industry, cheaper or banned substances may be employed, leading to misleading labeling, illegal substitution, or mislabeling [41]. Controlling the raw ingredients used in food processing is the best approach for protecting the "Muslim" of the finished food products. An immediate and effective analytical method is required to detect and differentiate the animal origin of gelatin in order to guarantee that food products are halal-compliant and safe to eat. Distinguishing gelatin from different sources using traditional spectroscopic approaches is challenging due to the significant similarities in its structure and characteristics. Gelatins' species origin has been identified using immunochemical methods.

Nevertheless, the immunochemical technique can be influenced by the extent of proline hydroxylation, a crucial factor in defining the antigenic properties of collagen [42]. Polymerase chain reaction (PCR), a technique utilizing DNA, is frequently employed to trace the origin of an animal product or identify potential contamination in other animal tissues due to its exceptional sensitivity and taxonomic specificity [43]. However, gelatin processing leads to the degradation of DNA integrity [44, 45].

Unlike DNA, the protein's amino acid sequence remains remarkably uniform during the gelatin production. The primary amino acid sequence of collagen is a repetitive G–X–Y sequence, with G representing glycine, and X and Y predominantly representing proline and hydroxyproline, respectively [46]. The amino acid sequence, which possesses this particular characteristic, has been employed to determine the animal origin of gelatin by the use of mass spectrometry [47]. This involves detecting certain peptides that serve as markers, following the process of trypsin digestion [48]. Collagen marker peptides, when used in conjunction with sequence search, have the ability to differentiate between bovine and porcine gelatin [49]. Furthermore, Grundy et al.[50, 51] presented an approach utilizing LC-MS/MS to identify the specific species of gelatin present in food and medicinal items. The researchers, led by Sarah [52], discovered that four specific peptides (EVTEFAK, LVVITAGAR, FVIER, and TVLGNFAAFVQK) were consistently identified in cooked hog meat using MRM techniques. Yilmaz et al.[53] introduced an innovative nano UPLC-ESI-qTOF-MSE workflow method with the capability to determine the source of gelatin in certain dairy products such yoghurt, cheese, and ice cream. However, it is imperative to employ a precise and responsive technique that can not only detect but also measure bovine gelatins in order to address issues such as misrepresentation of their origin and composition. Zhu et al.[54] presented Eleven peptide biomarkers which were chosen for pigs, bovine, and donkey after tryptic gelatin peptides were isolated and evaluated with UPLC-tandem high-resolution mass spectrometry. A label-free quantitative approach was developed by optimizing the gelatin pretreatment techniques in three different food matrices. This method relies on the MRM method of the most sensitive biomarker peptides. Sha et al.[55] devised a technique that combines trypsin-catalyzed 18 labeling with high-resolution mass spectrometry to accurately measure the amounts of bovine or porcine gelatin. Guo et al.[12] determined that the sequence AGVMGPOGSR is exclusively present in pigs and a few other animal species that are unsuitable for gelatin production. Therefore, the porcine identify peptide AGVMGPOGSR is highly specific for detecting porcine gelatin.

A commercial leather white pickled shaving samples of animal origin delivered from a production area in Egypt were subjected to conventional collagenase and then trypsin enzymatic digestions. A promising bacterial strain was isolated and identified based on sequence analysis of the 16S rRNA gene, partial sequence. It was from bacterial fish wastes, BFW5 and BFW7 was identified as *Citrobacter freundii,* which acted as the enzyme's producers [35].

The enhancement of the (UHPLC/ESI-qTOF-HRMS) analysis was achieved by the determination of the collagen hydrolysate sequence, hence facilitating the identification of gelatin types and collagen hydrolysates. The UHPLC/ESI-Q-TOF-HRMS was employed to monitor a very intricate mixture comprising several tryptic peptides. This was achieved by analyzing the presence or absence patterns of marker peptides. The main objective of this study is to investigate the rapid UHPLC/ESI-Q-TOF-HRMS analysis of collagen hydrolysate, specifically targeting tiny molecules with a molecular weight of less than about 2,000 within intricate matrices. In this research, we have employed marker peptides identified by UHPLC/positive ESI-Q-TOF-HRMS mode to create a novel analytical approach. This technique enables us to accurately identify and quantify the presence of bovine gelatin. Furthermore, gelatin produced in the laboratory were examined for the specific indicator, bovine peptide. Conclusions of our study definitely showed that this method is highly valuable and efficient for verifying the halal status of commercially available pure gelatin and gelatin-containing processed food items.

2. Experimental

2.1 Materials and methods

Leather wastes (white pickled shavings, WPS) were kindly provided from Elgabass tanneries in ELROBAKI, Cairo. Fish wastes from Egyptian Fish Marketing Company, Cairo. Both BFW5 and BFW7 were obtained from bacterial fish wastes in the current paper discussed from our previous work by Saber et al.,[35].

DNA extraction and PCR amplification of 16s rDNA region

DNA was isolated from the selected isolate according to Sambrook *et al.* [56]. The 16s rDNA was amplified by polymerase chain reaction (PCR), using primers designed to amplify 1500 bp fragment of the 16s rDNA region. The forward and reverse primers were 5'AGAGTTTGATCMTGGCTCAG 3' and 5'TACGGYTACCTTGTTACGACTT 3', respectively. The PCR mixture consisted of 30 p

mole of each primer, 10 ng of chromosomal DNA, 200 µM dNTPs and 2.5 units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. After completion, a fraction of the PCR mixture was examined using agarose-gel electrophoresis according to Ausubleet *et al*. [57] and the remnant was purified using QIA quick PCR purification reagents (Qiagen). DNA sequences were obtained using a 3130 X DNA Sequencer applying BigDye Terminator Cycle Sequencing. The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software [58]. The phylogenetic tree was displayed, using the TREEVIEW program.

DNA Sequencing

Automated DNA sequencing based on the enzymatic chain terminator technique developed by Sanger *et al*. [59] was applied using 3130 X DNA sequences. The sequencing reaction was performed with four different fluorescent labels identifying the ddNTPs, instead of the radioactive labels. These fluorophores were excited with two argon lasers at 488 and 514 nm, respectively when the respective bands passed the lasers during the electrophoresis. The thermal cycling mixture was prepared as follows: 8 µl of Big Dye terminator mixture, 6 µl of the sequencing primer (10 pmol) and 6 µl of the sample (PCR product or plasmid), then the reaction was run in the thermal cycler. The cyclic reaction was carried out for 1 min at 95°C, then 49 cycles of 30 sec at 95°C, 10 sec at 52°C and 4min at 60° C. The products were purified, using a special column according to the instructions of the manufacturer. The elutes were taken and added to high dye formamide with (1:1, v/v), run at 95° C for 5 min for denaturation, shock on ice, then the sample was ready for sequencing in 3130 X DNA sequencer and analysis.

Pipeline analysis: Non targeting shotgun proteomic analysis for the sample

Sample preparation

Protein extraction and denaturation

Same amount of powder was taken equally and 100 µl 8M Urea (500 mMTris pH 8.5) were added on each sample for protein extraction. Samples were homogenized and centrifuged on 10,000 RPM for 30 mins [60].

Protein quantification

Measuring the concentration using bicinchoninic acid assay (BCA assay) was shown in Table 1[61].

Table 1. Measuring concentration using bicinchoninic acid assay (BCA assay) as follows:

Sample	BSA (µl)	Sample (µl)	Sample vehicle (µl)	MilliQ (µl)	BCA working solution (µl)
Blank	0	0	8	12	400
Standard, (1.25 µg/ µl)	8	0	8	4	400
Sample	0	8	0	12	400

Protein digestion

For reduction: 2 µl of 200 mM DTT were added, vortex, and spin down; Incubate for 45 min at RT. For alkylation: 2 µl of 1M IAA were added, and incubate at RT for 45 min in dark; 102 µl of 100 mM Tris pH 8.5.

For trypsinization:6 µl Trypsin containing 1µg procaine enzyme. Incubation overnight at 37°C with shaking at 900 rpm;6µl of 100% formic acid, to acidify the sample to pH 2-3; Spin down for 30 min at RT (see Table 2.) [60, 62].

Table 2. Protein digestion for samples BFW5, BFW7 as follows:

Sample	Conc. (µg/µl)	Total protein needed for digestion (µg)	
		Sample (µl)	Urea buffer (µl), (Mass up to 30 µl)
BFW5	21.32	1.406	28.593
BFW7	19.26	1.557	28.442

Stage tip (MonoSpin Reversed Phase Columns) prod# 5010-21701

For activation:50 µl Methanol on the tip. For initialization:50 µl from "solution B" (0.2% FA+ 80% ACN). For re-equilibration:50 µl from "solution A" (0.2% FA) twice. For sample-trapping: The Eppendorf tube changed and the sample added. For washing: Washed with 15 µl "solution A" twice. For elution: In a collection tube recover 3 times 30 µl "solution B"; Speed-vac, then re-constitute in 20 µl "solution A"; Samples were subjected to peptide quantification.N. B: Centrifuge between each step at 3000 RPM in Stage tip (see Table 3.) [61, 62].

Peptide quantification

Table 3. measure concentration using bicinchoninic acid assay (BCA assay) as follows:

Sample	BSA (µl)	Sample (µl)	Sample vehicle (µl)	MilliQ (µl)	BCA working solution (µl)
Blank	0	0	10	15	25
Sample	0	10	0	15	25
Standard, (1 µg/µl)	10	0	10	5	25

Incubate at 95 °C for 5 min; add 1000 µl prepared BCA; incubate at 600°C for 30 min; then cool down at RT for 20 min; read at A_{562}[60, 63].

Chromatography

LC System

Nano LC system consisting from Eksigent nano LC 400 autosampler attached with EkspertnanoLC425 pump; Injection volume:1µg/ 10 µl; Injection mechanism: Trap and Elute; Needle wash:2 cycles using 10 % isopropanol; Analysis Time:55 min; Trapping parameters: Sample clean up using trapping cartridge CHROMXP C18CL 5um (10x0.5 mm) pumped at flow rate 10 µl/min for 3 min using mobile phase A; Column:3 µm, ChromXP C18CL, 120A, 150 x 0.3mm; Flow rate:10 µl/min; Mobile phase: **A)** MilliQ containing 0.1 % FA**; B)** Acetonitrile containing 0.1 %FA; Gradient profile was given in Table 4 [60].

Table 4. Time program for gradient elution

Time/min	0	38	43	45	48	49	57
A, %	97	70	60	20	20	97	97
B, %	3	30	40	80	80	3	3

MS-Spectrometry

LC-QTOF (SciexTripleTOFTM 5600+); Acquisition mode: Positive; IDA parameters: High resolution TOF MS survey scan followed by product ion scan for the most abundant 40 ions. Cycle time is 1.5 s; TOF mass range: 400 – 1250 m/z; MS2 range (product ion): 170 – 1500 m/z; Ion selection threshold: 150 cps; Total run time:55 min; MS calibration: Sciex tuning solution (P/N 4457953).

Data processing

Analyst TF 1.7.1 is used for data acquisition (Sciex software); Raw MS files, from the TripleTOFTM 5600+ files, were analyzed by Protein pilot (version5.0.1.0,4895), paragon Algorithm (version 5.0.1.0, 4874); Database used is Uniprot*(Bos-Taurus) Bovine* organism (Swiss-prot and TrEMBL database containing 47,386 proteins); The search parameters used in searching were given below (Table 5).

Table 5. Search parameters

Cysteine alkylation	Digestion	Special factors	Search effort	ID focus	FDI analysis	Bias correction
Iodoacetamide	Trypsin	denaturation buffer	Through	Biological modifications	yes	yes

Results and discussion

Molecular identification of selected bacterial isolates

The purpose of this study was to identify the specific isolates obtained from fish waste samples, which were then utilized for the production of enzymes such as collagenase and/or gelatinase. These enzymes were searched for because of their ability to enhance the process of gelatin formation. The bacterial fish waste was denoted by the symbol BFW. Given the varying percentages of gelatin and collagen hydrolysate produced by certain isolates, namely BFW5 and BFW7, it becomes imperative to accurately identify these bacterial strains. The isolates were identified at the molecular level using the process of 16S gene amplification and subsequent sequencing, as depicted in Figure 1.

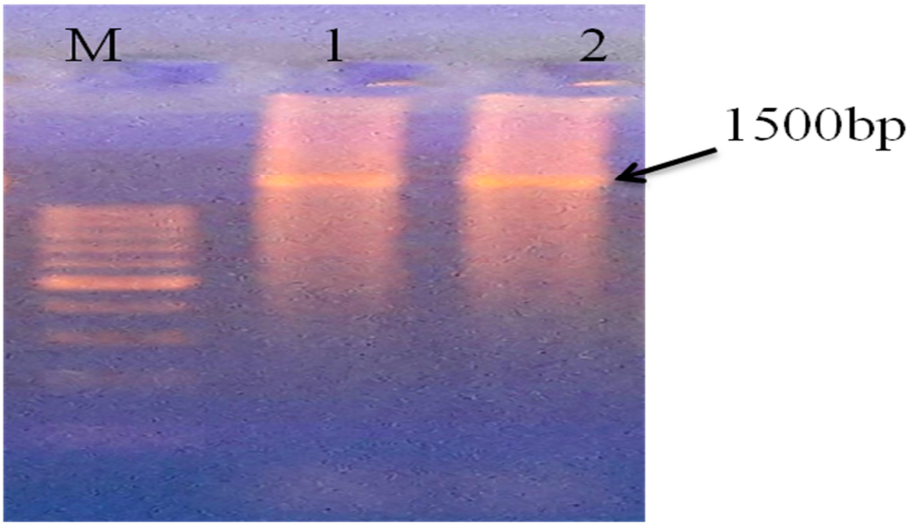


Figure 1. PCR amplification of 16srDNA for bacterial isolates 1500bp lan (1-2) M: 100 bp DNA ladder.

The homology analysis of the sequenced 16S rDNA from a 674 base pair segment of isolate BFW5 was subjected to the blast program. The results of this study matched the previous findings,[10] indicating a significant similarity with a 99% identity to *Citrobacter freundii* strain NBRC 12681 16S ribosomal RNA, partial sequence with accession number PP036901. Also, BFW7 was identified as *Citrobacter freundii* strain NBRC 12681 with a significant similarity with 100 % and max score of 747 with accession number PP177353. The construction of the phylogenetic trees was carried out utilizing the TREEVIEW program, as depicted in Figures 2,3.

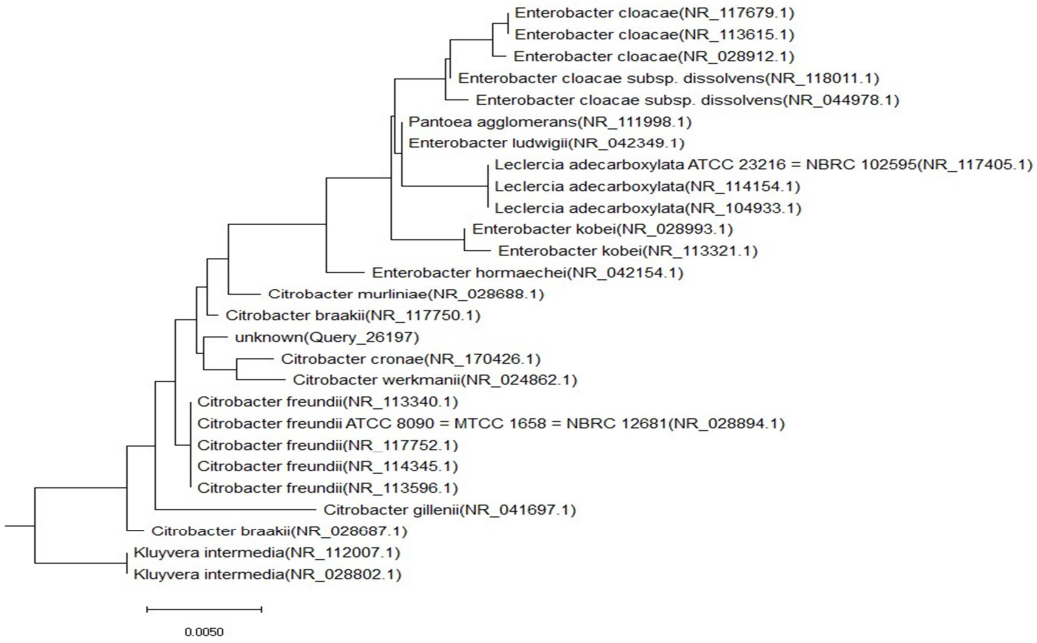


Figure 2. BFW5 isolates phylogenic tree relationships of the bacterial isolate with other 16s rDNA sequences of published strains, showing the relationship between all isolates and other species belong to the genus.

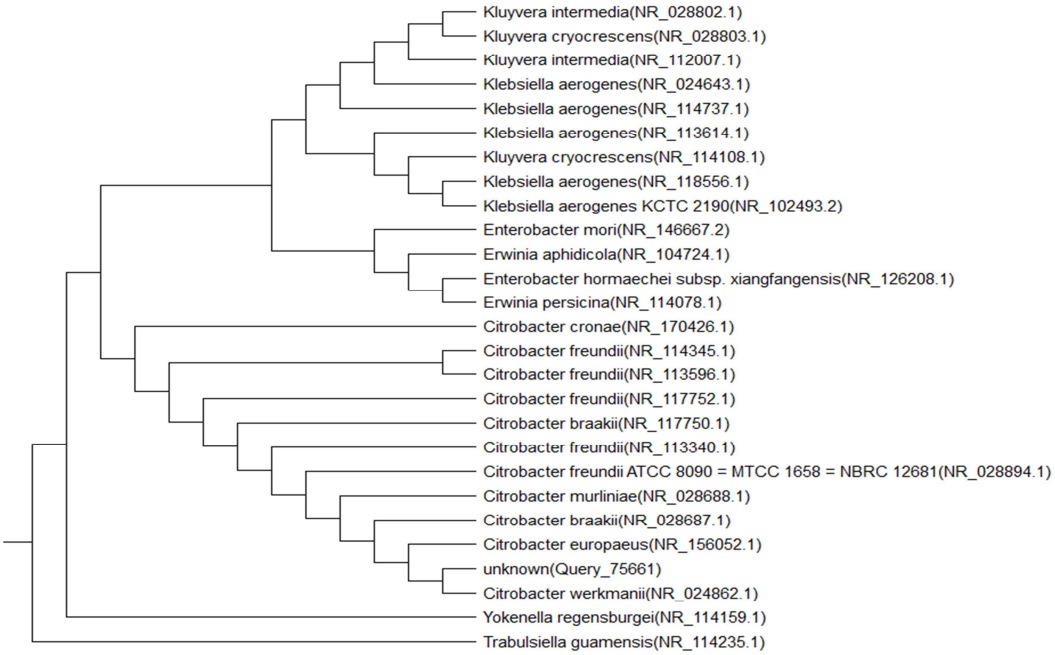


Figure 3. BFW7 isolates phylogenic tree relationships of the bacterial isolate with other 16s rDNA sequences of published strains, showing the relationship between all isolates and other species belong to the genus.

Study design

A highly sensitive technique utilizing ultra-high performance liquid chromatography (UHPLC) and positive electrospray ionization-quadrupole time-of-flight high-resolution mass spectrometry (ESI-qTOF-HRMS) has been created for multiple reaction monitoring (MRM). Initially, mass spectrometry was employed to identify different types of bovine gelatins after being subjected to trypsin digestion. Subsequently, marker peptides that accurately resemble bovine gelatin were chosen.

UHPLC/positive ESI-qTOF-HRMS analysis of the digested gelatins

Different 16 and 14 proteins were identified in both investigated samples; notably, the six most abundant proteins in each sample were recognized as collagen types, specifically originating from the Bovine species. Accurate characterization of collagen hydrolysates plays a significant role in enhancing the economic potential value add for cosmetics, bone, and skin care goods. The observed decrease in the quantity of proteins detected in both samples can likely be attributed to their inherent characteristics and the prior application of collagenase enzyme. The employed technique facilitated the swift and straightforward identification of collagen types and their hydrolysates (peptides) by comparing them with publicly accessible databases. This enabled the certification of leather, determining whether it originated from a single or multiple animal source. Additionally, it facilitated the identification of the most suitable potential pharmaceutical and industrial applications for the resulting hydrolysates.

The BFW5 sample yielded a total of 16 proteins, which were identified based on the detection of their peptides resulting from enzymatic digestion. These peptides were found to be consistent with the proteins originating from bovine leather. There are six primary classifications of collagen as shown in Table 6 which are as follows:

Table 6. Major six collagen-types proteins identified in BFW5

No.	Name	Peptides (95%)
1	Collagen alpha-1(I) chain OS=Bostaurus OX=9913 GN=COL1A1 PE=1 SV=3	564
2	Collagen alpha-1(III) chain OS=Bostaurus OX=9913 GN=COL3A1 PE=2 SV=1	174
3	Collagen alpha-1(III) chain OS=Bostaurus OX=9913 GN=COL3A1 PE=1 SV=1	169
4	Collagen alpha-2(I) chain OS=Bostaurus OX=9913 GN=COL1A2 PE=1 SV=1	272
5	Collagen alpha-2(I) chain OS=Bostaurus OX=9913 GN=COL1A2 PE=1 SV=2	272
6	Collagen alpha-2(I) chain OS=Bostaurus OX=9913 GN=COL1A2 PE=1 SV=1	269

A comprehensive analysis of the BFW7 sample revealed the presence of a total of 14 proteins that were confirmed to originate from bovine leather. This confirmation was based on their significant representation in the key six proteins outlined in Table 7, as well as their identification through enzymatic hydrolysis and subsequent detection of the resulting peptides.

Table 7. Major six collagen-types proteins identified in BFW7

No.	Name	Peptides (95%)
1	Collagen alpha-1(I) chain OS=Bostaurus OX=9913 GN=COL1A1 PE=1 SV=3	130
2	Collagen alpha-2(I) chain OS=Bostaurus OX=9913 GN=COL1A2 PE=1 SV=2	33
3	Collagen alpha-2(I) chain OS=Bostaurus OX=9913 GN=COL1A2 PE=1 SV=1	33
4	Collagen alpha-2(I) chain OS=Bostaurus OX=9913 GN=COL1A2 PE=1 SV=1	33
5	Collagen alpha-1(III) chain OS=Bostaurus OX=9913 GN=COL3A1 PE=2 SV=1	31
6	Collagen alpha-1(III) chain OS=Bostaurus OX=9913 GN=COL3A1 PE=1 SV=1	27

The tables above present the comprehensive compilation of significant proteins that have been identified in each respective sample. A limited quantity of proteins was detected, potentially attributable to the characteristics of the samples or the utilization of diverse enzymatic pre-treatment methods. The mass chromatograms for each of the two samples (BFW 5, BFW7), namely the base peak (BPC) and total ion (TIC) chromatograms, exhibited a highly intricate composition of collagen hydrolysates (peptides), as depicted in Figures 4 and 5. Practically more than 2000 and 4000 peptides were tentatively identified in both investigated collagen hydrolysate samples (Figure $S_{(1-20)}$ and Table $T_{1,2}$ in the Supporting Information), i.e. BFW 5, BFW7, respectively.

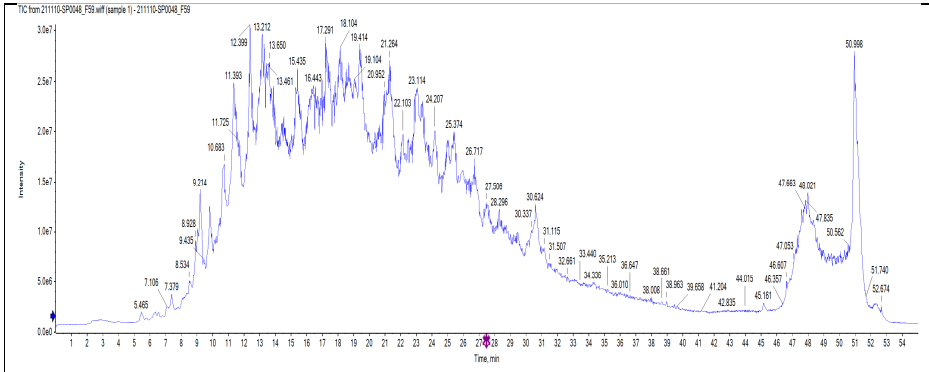
The total ion chromatograms (TIC) of the digested bovine gelatins within a scan spectrum spanning m/z 200–1500 are displayed in Figures 4,5. The elution spectra demonstrate that the peptides in the digested bovine gelatin have a retention time scales which is comparable to the peptides in the other sample digested gelatin. This could be attributed to the significant protein similarity among herbivorous organism mammals. The peptides in the digested samples typically exhibit comparable hydrophobicity/hydrophilicity as a result of the substantial presence of glycine and proline. Visual examination of chromatograms does not allow for accurate differentiation among distinct gelatin. To address this, we extract the ion chromatogram (EIC) from a scan range of m/z 200-1500 for more precise analysis.

Peptide marker selection

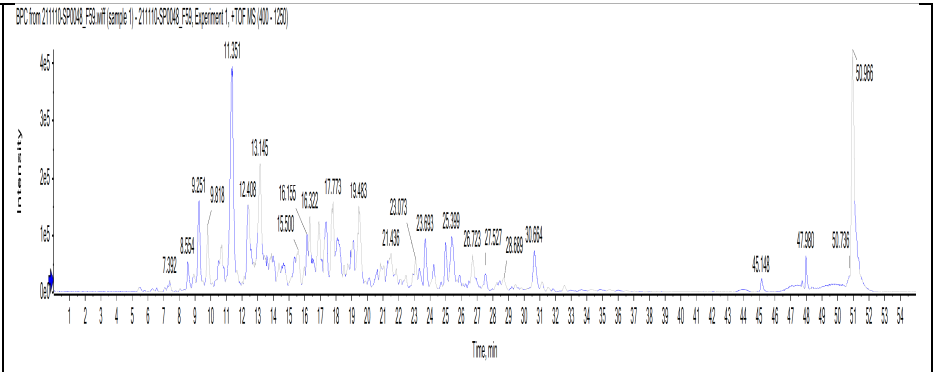
The provided figures display chromatograms, as well as representative extract ion chromatograms (XIC) and matching mass spectrometry (MS) spectra for the collagen marker peptides discovered in each collagen hydrolysate mixture (Figures 6-8) for peptide sequences identified for BFW5 and (Figures 9-11) for BFW7, respectively. The output data in question were acquired using a positive UHPLC/ESI-Q-TOF-HRMS system. The identification process relied on the detection and analysis of $[M+2H]^{2+}$, or $[M+3H]^{3+}$ ions, as well as their respective molecular weights (MW) were illustrated in Table 8. The ions with mass-to-charge ratios of 544.0, 644.0, and 739.0 were detected in the digesting bovine gelatins, as shown in Figures 6-11. The MS/MS spectra of the peptides matched the sequences GFPGADGVAGPK (K representing Lys), GFPGSPGNIGPAGK (P representing Pro), and GETGPAGRPGEVGPPGPPGPAGEK (E representing Glu), respectively. The sequence is situated within the α1 (I) and α2 (I) strands of bovine collagen. The peptide markers exhibited specificity towards bovine gelatins. The ions with mass-to-charge (m/z) values that correspond to the marker peptides were extracted from each UHPLC/ESI-Q-TOF-HRMS run. Table 8 presents a concise overview of their m/z values, charge states, and retention times. The selection of marker peptides for hide was based on three specific criteria. Initially, the peptide may be routinely identified across numerous repeating runs. Additionally, the peptide's optimal lengths should fall within the range of 7 to 15 amino acid residuals, ensuring its suitability for the MRM technique. Furthermore, it is advisable to choose the most prominent signal of the peptide to streamline the procedure.

Table 8. List of selected marker peptides for bovine gelatin identification for the two samples

Chain	Position	Marker peptide	Peptides sequence	(m/z)	MW_{obs}	z^d	Time (Min.)
α1 (I)	316–327	MP1	GFOGADGVAGPK	544.76	1087.52	2	19.4
	733–756	MP2	GETGPAGROGEVGPOGPOGPAGEK	644.31	1287.60	2	17.7
α2 (I)	361–374	MP3	GFOGSOGNIGPAGK	739.33	2215.05	3	15.4

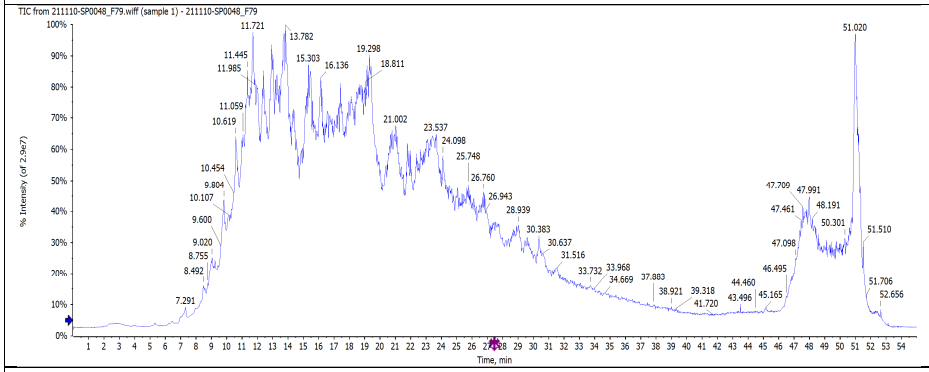


A) TIC: Total Ion chromatogram of BFW5

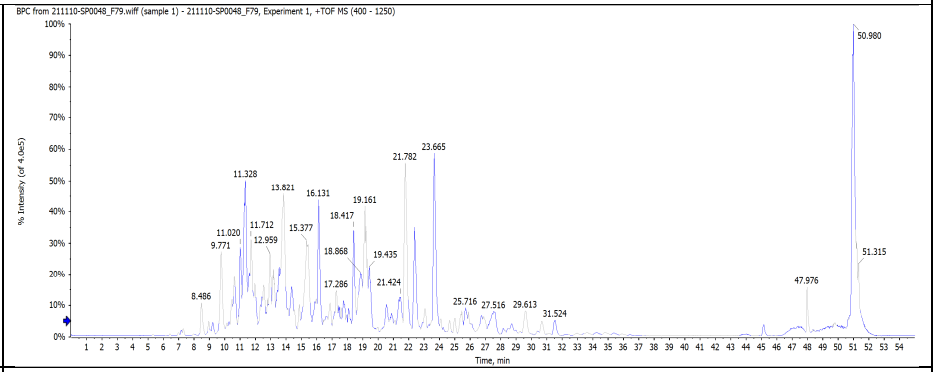


B) BPC: Base Peak chromatogram of BFW5

Figure 4. Represented outputs of UHPLC/Positive ESI-qTOF-HRMS for Sample BFW5



A) TIC: Total Ion chromatogram of BFW**7**



B) BPC: Base Peak chromatogram of BFW7

Figure 5. Represented outputs of UHPLC/Positive ESI-qTOF-HRMS for Sample BFW**7.**

Identifications Collagen Markers for Peptide Sample BFW5

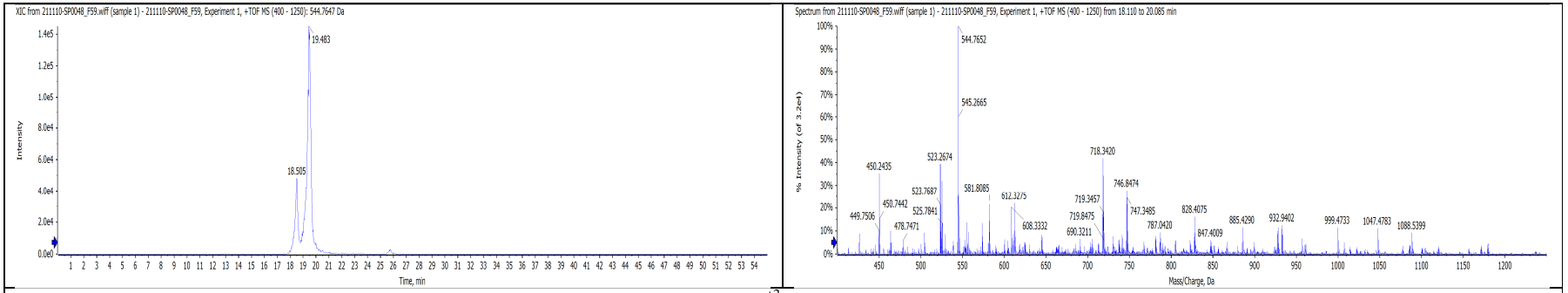


Figure 6. XIC and MS spectra of MP1"GFPGADGVAGPK", $(MS)_{obs}$: 544.7652 Da $[M+2H]^{+2}$, $(MW)_{obs}$: 1087.52979 Da

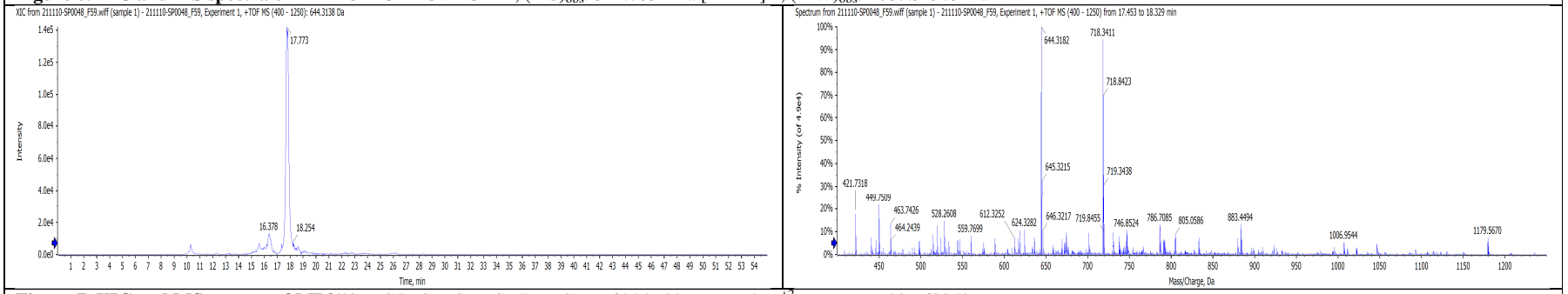


Figure 7. XIC and MS spectra of MP2"GFPGSPGNIGPAGK", $(MS)_{obs}$: 644.3182 Da $[M+2H]^{+2}$, $(MW)_{obs}$:1287.60949 Da

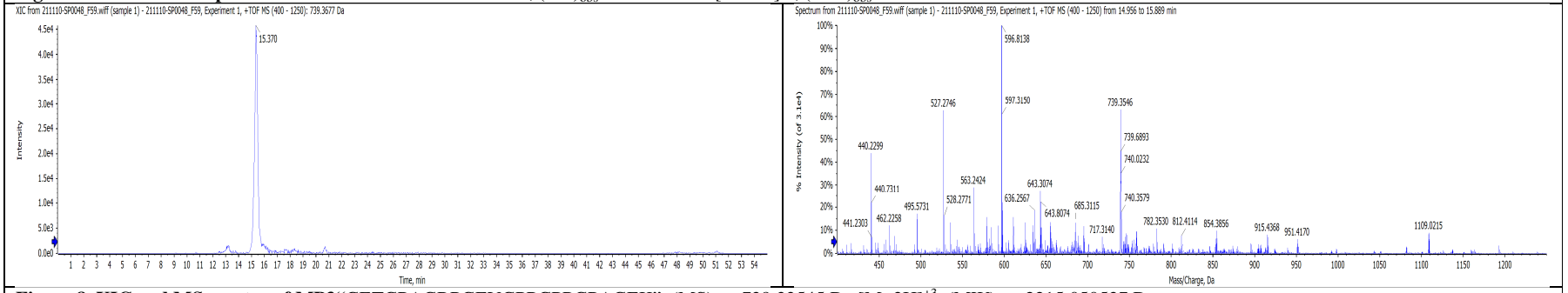


Figure 8. XIC and MS spectra of MP3"GETGPAGRPGEVGPPGPPGPAGEK", $(MS)_{obs}$: 739.33545 Da $[M+3H]^{+3}$, $(MW)_{obs}$: 2215.050537 Da

Identifications Collagen Markers for Peptide Sample BFW7

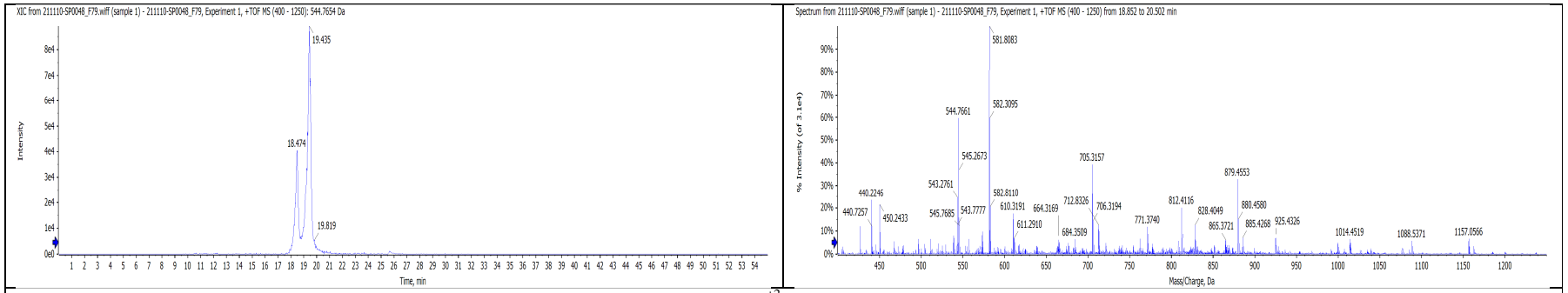


Figure 9. XIC and MS spectra of MP1"GFPGADGVAGPK", $(MS)_{obs}$: 544.7661 Da $[M+2H]^{+2}$, $(MW)_{obs}$: 1087.52979 Da

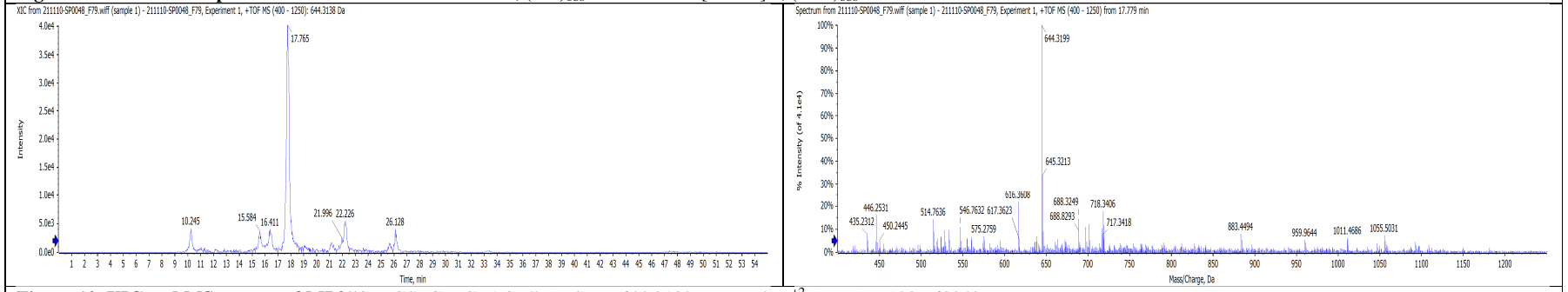


Figure 10. XIC and MS spectra of MP2"GFPGSPGNIGPAGK", $(MS)_{obs}$: 644.3182 Da $[M+2H]^{+2}$, $(MW)_{obs}$:1287.60949 Da

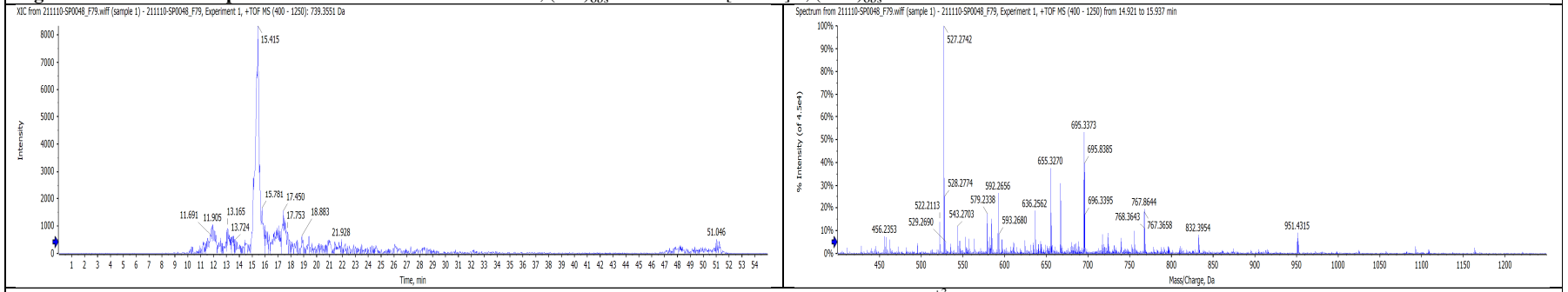


Figure 11. XIC and MS spectra of MP3"GETGPAGRPGEVGPPGPPGPAGEK", $(MS)_{obs}$: 739.33545 Da $[M+3H]^{+3}$, $(MW)_{obs}$: 2215.050537 Da

The approach employed in this study facilitated the fast and simple determination of collagen types and their hydrolysate peptides. This, in turn, allowed for the certification of leather, regardless of its origin from single or multiple animal sources. The identification of collagen types and their corresponding amino acid sequences in the generated peptides was achieved by comparing them with entries in publicly available databases. In both of the samples under investigation, a combined total of 16 and 14 distinct proteins were detected. Among these proteins, six prominent ones were identified in each sample. Notably, these six proteins were determined to be collagen types, originating specifically from the bovine species. Accurate characterization of collagen hydrolysates, specifically peptides, plays a significant role in enhancing the economic potential and value of sectors associated with leather waste, including cosmetics, bone, and skin care goods. The two samples under investigation exhibited a number of proteins, potentially attributable to the inherent characteristics of the samples or their prior treatment with collagenase enzyme. The BPC and TIC chromatograms obtained for both samples exhibited a highly intricate composition of collagen hydrolyzates, specifically peptides. The provided data presented chromatograms of both samples, together with typical extracted ion chromatograms (XIC) and matching mass spectra of the most prominent peptides discovered in each collagen hydrolysate. The output data in question were acquired using a positive UHPLC/ESI-Q-TOF-HRMS system. The method of identification was based on determining the retention time (Rt) information and observing the molecular ions [M+2H]2+ or [M+3H]3+ at a low collision-induced dissociation potential (CID). These ions were then matched with multiple open-library databases for proteins derived from bovine leather collagen. The precise recognition methods, especially amino acid sequencing, rely on a comparison of monoisotopic masses between various charged molecular ions and specific fragments of each peptide. This comparison is done at high selective controlled CID and plays an essential role in the observation of certain fragment/s in the tandem mass spectra (MS2). An important advantage of using the LC/HRESI-MS/MS technology is its ability to accurately identify the type of leather or the original animal source by detecting specific peptides in collagen hydrolysate or related products like as gelatin or glue. Marker peptides, such as those described by Kumazawa et al.,[2], exhibit distinct differences in their molecular weights (MWs) and amino acid sequences among different animal species. The present study identified the three primary marker peptides of bovine leather (typically found in cattle) by analyzing the MS/MS data. These marker peptides, along with other significant peptides, were used to determine the amino acid sequences and molecular weights. The results are presented in Figures 6-11 for both collagen hydrolysate samples. The precise monoisotopic masses of the three markers were identified at m/z 544.7647 $[M+2H]^{+2}$, 644.3138 $[M+2H]^{+2}$, and 739.3677 $[M+3H]^{+3}$. These values were calculated to be 544.77221, 644.81201, and 739.35748, respectively, corresponding to molecular weights of 1087.529785, 1287.60949, and 2215.05054 Da. Their structures were designated as GFPGADGVAGPK, GFPGSPGNIGPAGK, and GETGPAGRPGEVGPPGPPGPAGEK, respectively.

Conclusion

This study containing the identified proteins necessitates the implementation of filtration criteria, which may include a confidentially threshold, tailored to the specific requirements of the researcher. In the preceding part, the utilization of Peak View software version 2.1 was employed for the processing of the output data obtained from the analysis of collagen hydrolysate samples using UHPLC/ESI-Q-TOF-HRMS. The results encompassed the presentation of the Base Peak Chromatogram (BPC), Total Ion Chromatogram (TIC), and Selected Ion Chromatogram (SIC) of the peptides with the highest abundance in the samples. Additionally, the matching Mass Spectra (MS) of these peptides were also included. The hydrolyzed peptides were identified by comparing their molecular weights and amino acid sequences with those in a library database. This database was specifically downloaded to include information on the animal species source (bovine) and the enzymes (collagenase and trypsin) employed for digesting the waste samples. As well, the analytical output MS/MS data was used for the confirmation of the animal source from which the collagen samples were delivered according to the structural identification of the three marker peptides. The marker peptides derived from gelatin can serve as a means to detect the presence of bovine gelatin within a combination. Consequently, the technique can be employed to authenticate the halal status of gelatin.

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Conflicts of interest

There are no conflicts of interest.

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