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MULTIPLE REACTION MONITORING (MRM) OF PORCINE-SPECIFIC PEPTIDE MARKERS FROM LACTATE DEHYDROGENASE AND SERUM ALBUMIN IN PROCESSED MEAT FOR HALAL AUTHENTICATION

(Pemantauan Tindak Balas Pelbagai (MRM) Penanda Peptida Khusus bagi Khinzir dari *Lactate Dehydrogenase* dan *Serum Albumin* dalam Daging yang Diproses untuk Pengesahan Halal)

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Abstract

Multiple Reaction Monitoring (MRM) of four peptides is presented to detect the presence of pork in meat products using a triple quadrupole system (LC-QQQ-MS). Commercial samples were digested using the tryptic digestion method. MRM acquisition was optimized using Skyline software. Then, the presence of peptides was tested and verified on processed meat products, each with a different type of processing. The result reveals that peptides EVTEFAK (m/z = 450.2873), LVVITAGAR (m/z = 412.2134), FVIER (m/z = 388.7369), and TVLGNFAAFVQK (m/z = 647.8613) were consistently detected in the processed meat and displayed porcine-specific properties. Peptide LVVITAGAR, the most intense peptide, was eluted at 7 min while FVIEIR, as the second highest peak, was eluted at 9.5 min. On the other hand, peptide EVTEFAK was eluted at 4.5 min, and TVLGNFAAFVQK, a peptide with the lowest intensity, appeared between 12.5 to 13 min. Specifically, one peptide is derived from lactate dehydrogenase, and three from serum albumin protein. It demonstrated that porcine-specific peptides could be simultaneously targeted by the MRM method with the help of Skyline software that helps optimize the analytes' specific precursor ion, product ion, and fragmentation behaviour under collision energy. The present result reveals that MRM will enable rapid halal detection of contaminants occurring during the manufacturing or supply chain and ensure food integrity is preserved along the food supply chain.

Keywords: triple quadrupole, halal supply chain, peptide marker, species authentication

Abstrak

Pemantauan tindak balas berganda (MRM) empat peptida untuk mengesan kehadiran daging khinzir dalam produk daging menggunakan sistem caturkutub ganda tiga (LC-QQQ-MS) adalah dilaporkan. Sampel komersil dicerna dengan kaedah penghadaman tryptic. Parameter pemerolehan MRM telah dioptimumkan menggunakan perisian Skyline. Seterusnya, kehadiran peptida telah diuji dan disahkan pada produk daging yang diproses dimana setiap satunya melalui jenis pemprosesan yang berbeza. Keputusan menunjukkan bahawa peptida EVTEFAK (m/z = 450.2873), LVVITAGAR (m/z = 412.2134), FVIER (m/z = 388.7369) dan TVLGNFAAFVQK (m/z = 647.8613) dikesan secara konsisten dan mempunyai sifat khusus daging khinzir. Peptida EVTEFAK, LVVITAGAR, FVIER dan TVLGNFAAFVQK dikesan secara konsisten dalam daging yang diproses dan menunjukkan sifat khusus khinzir. Satu peptida diperoleh khusus daripada laktat dehidrogenase dan tiga daripadanya adalah protein serum albumin. Ia menunjukkan bahawa peptida khusus untuk mengesan khinzir boleh disasarkan secara serentak oleh kaedah MRM dengan syarat pengetahuan tentang ion prekursor khusus analit, ion produk dan tingkah laku pemecahan di bawah tenaga perlanggaran dioptimumkan. Keputusan kajian menunjukkan bahawa MRM akan membolehkan pengesanan cepat bahan halal tercemar yang berlaku semasa proses pembuatan atau rantaian bekalan dan memastikan integriti makanan terpelihara di sepanjang rantaian bekalan makanan.

Kata kunci: caturkutub ganda tiga, rantaian bekalan halal, penanda peptida, pengesahan spesies

Introduction

Halal authentication methods have been growing tremendously with the aid of state-of-the-art technology. A DNA-based method such as Polymerase Chain Reaction (PCR) [1] and real-time PCR [2] were consistently reported in the literature, followed by a protein-based approach using enzyme linked immunosorbent assay (ELISA) [3], 2-Dimensional – Polyacrylamide Gel Electrophoresis (2D-PAGE) [4] and until recently, Mass Spectrometry (MS)-based authentication [5] has been proposed as a halal detection method. The analysis of pork adulteration in commercial meat products is challenging due to their composition, complexity, and, very often, inhomogeneity upon processing [6]. Moreover, food matrices that consist of fat, spices, and various salts, including commonly used sodium chloride and sodium nitrite, antioxidants, vegetable additives, or milk proteins, could affect detection sensitivity [7-8]. Therefore, researchers have continuously developed new authentication methods to overcome these challenges.

MS-based analysis has gained increasing consideration in medical, agriculture, animal science, and pharmaceutical fields. It offers excellent sensitivity and selectivity in which the mass-to-charge ratio m/z or structural information of a compound can be obtained in a shorter time. A considerable amount of literature has been published on the MS-based meat speciation technique, which explains the potential of peptides as molecular markers in meat species detection. For instance, [9] developed an Ultra-Performance Liquid Chromatography (UPLC) method that can be used to simultaneously separate myoglobin from water buffalo, bovine, pig, horse, chicken, and ostrich. The biomarker was subsequently used as a fast procedure to detect pork and beef meat in pre-mixed raw beef burgers. In addition, [10] mentioned that quantitative detection of chicken meat using stable isotope peptides was able to detect the presence of chicken in a mixture of meat. Moreover, [11] successfully identified 20 heat-stable peptide biomarkers unique to chicken duck and goose, and a detection method has been successfully developed using nano-LC-QTOF-MS/MS. Meanwhile, species-specific markers for detection in Bolognese sauce [12] and a combination of peptide detection and chemometrics have been demonstrated as well [13].

Targeted MS approaches such as Single Reaction Monitoring (SRM) and Multiple Reaction Monitoring (MRM) have tremendous potential for specific and reproducible qualitative and quantitative measurement of changes in the levels of peptides of interest to biologists and biomedical researchers [14]. The most common application of targeted MS could be observed in medical research, such as the detection of biomarkers for breast cancer [15], neurodegenerative disease [16], and hepatocellular carcinoma or cancer [17]. To this point, there is a current shortage of MRM-based assays in the meat science field, particularly on meat authentication. Previously, [18] developed an MRM method from myoglobin protein for QTRAP triple-quadrupole mass spectrometer to discriminate between beef, lamb, horse, and pork. Meanwhile, research conducted by [19-20] established a peptide-based detection method for the Quadrupole ion Trap Mass Spectrometry (QTRAP-MS) system whereby contaminations of pork with the most sensitive biomarker peptide were detected by MRM3. A previous study [19] reported a peptide-based detection method for a QTRAP-MS system, in which contamination of pork with the most sensitive biomarker peptide was detected down to 0.13% in the beef matrix without pre-enrichment of analytes.

In contrast, the present study was conducted to verify the presence of biomarkers in detecting pork from commercially available meat products using MRM of triple quadrupole (QQQ) MS system. Previously published work on seven peptide biomarkers has successfully been identified using the LC-QTOF platform in heat-treated meat [21]. The present paper is a continuation of previous work whereby the specificity of the peptide was tested on commercial samples. Notably, the MRM platform was pursued as it reduced the time spent screening the total and extracted ion chromatogram. The work of examining the full spectrum in searching the marker peptides is no longer required, and analysis can be conducted in a single run. Moreover, all the listed peptides have been carefully analysed by checking their full MS/MS spectrum scan and species specificity, illustrated in the shotgun proteomics profiling. Once developed and optimized for selectivity and species specificity, the peptide biomarkers were used in the MRM assay to demonstrate proof of concept for detecting adulterated pork meat products in commercial meat products. The development of an MRM assay to verify candidate porcine-specific peptide biomarkers was established in this study. Briefly, it involved the development of MRM transitions and protocol, which was facilitated by the Skyline MRM method development application. Then, verification or refinement of potential biomarkers through MRM into some credible peptides that are, in fact, specific indicators for pork contamination proved that the listed biomarkers are qualified to be used for the species authentication method.

Materials and Methods

Sample preparation for adulterated and commercial samples

One boiled pork and beef meat and seven commercial samples were used in the study (N = 8). Initially, protein extract from boiled pork meat (100°C) was used for peptide screening and selection. Next, seven processed meat samples were purchased from local and international markets (Table 1). Species identity was listed according to the label. All samples were immediately stored in a 50 ml falcon tube at -80°C. Next, the meat and processed meat products were pulverized in liquid nitrogen prior to proceeding with extraction.

Table 1. List of samples used for species authentication using identified peptides

Type of Product	Species Declared (as indicated on the label)	Country of Origin or Manufactured (as indicated on the label)
Pork	pork	Malaysia
Corned beef	beef	Russia
Sausage	pork and chicken	Malaysia
Meatball	pork and chicken	Malaysia
Meatball	beef	Malaysia
Hamburger	pork, chicken and fish	Japan
Smoked Chicken Frankfurter	chicken	Malaysia
Chicken Luncheon	chicken	Malaysia

Extraction of proteins

Sample preparation and in-solution trypsin digestion were performed according to the previously described procedure [21, 22]. Approximately one gram of heat-treated pork and processed meat products were homogenized using handheld homogenizer (Wiggen Hauser D500, USA) in 5 ml of cold extraction buffer containing 7 M Urea, 2 M thiourea, 50 mM DTT, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.4% (v/v) carrier ampholytes (pH 3-10, Bio-Rad, USA) and 50 µL of protease inhibitor cocktail (Calbiochem, USA). Then, the solution was centrifuged at 12,000 g for 10 min at 4°C. The resulting supernatant was subjected to an acetone precipitation procedure in which the total volume of protein was mixed with four volumes of cold acetone (pre-chilled at -20°C overnight). The mixture was then vortexed and incubated at -20ºC overnight.

Subsequently, the samples were centrifuged at 12,000g at 4ºC for 10 min. The resulting pellet was air-dried and resolubilized in 50 µL urea extraction buffer, spun down before the supernatant was collected, and stored at -20ºC. Total extractable protein concentration was determined using a Bradford protein assay kit according to the manufacturer's protocol (Bio-Rad, Hercules, USA). The protein assay was done in triplicates for each sample. The same urea buffer was used as the background reference for the assay. All samples were purchased once and extracted in triplicates, resulting in a total of 24 extracts.

In-solution trypsin digestion

Approximately 100 µg of protein was taken from the protein extracts. Briefly, the protein was incubated in 50 µL of 10 mM DTT/100 mM ammonium bicarbonate for 30 min at 60°C. The mixture was initially allowed to cool prior to proceeding with the alkylation process in 50 µL of 55 mM IAA/100 mM ammonium bicarbonate for 20 min at room temperature (25°C, in a dark place). In-solution tryptic digestion was performed with 1 µg trypsin digestion buffer (Promega trypsin gold, Madison, WI, USA) overnight at 37°C. Tryptic peptides were then dried in a rotational vacuum centrifuge (CHRiST RVC 2-18, Germany) and stored at -20ºC. The desalting procedure using ZipTip C18 (Millipore, USA) was performed according to protocols described by the manufacturer to remove any interfering agents. Next, eluted peptides were re-suspended in milli-Q water containing 0.1% formic acid. Then, chromatographic separation was carried out.

Targeted porcine-specific biomarker in meat products

The initial peptide list for MRM was adopted from the LC-QTOF-MS analysis of cooked pork by Sarah et al. [21]. The list is composed of seven peptides of high-to-moderate abundance in which the targeted peptides were derived from three different proteins: lactate dehydrogenase, creatine kinase, and serum albumin. All the information from the peptides was exported to method optimization tools, namely Skyline Targeted Proteomics Environment Optimization Software, which was downloaded from https://skyline.ms/project/home/software/Skyline/begin.view. Briefly, the full-length protein sequences were imported into Skyline in FASTA format. The peptide filtering condition has been set as follows: maximum length of peptide of 20 and at least eight amino acids. Later, a list of all possible y-series fragment ions, for both z = 2 and z = 3 precursor ion-charge states, spanning a m/z range from 300 to 1,400, was generated. A transition list that detailed the possible collision energy for each precursor ion and product ion was created by the software, and the information was further used to optimize the actual MRM acquisition parameters. During the acquisition, the instrument will be directed to monitor each precursor and product ion transition at various CE voltages during a specific retention time, as suggested by the transition list.

LC-QQQ-MS analysis

In brief, trypsin-digested peptides were separated by the Agilent 1290 Infinity UHPLC system, while MS analysis was conducted using an Agilent 6490 QQQ mass spectrometer (LC-ESI-QQQ-MS), which was interfaced with a standard-flow ESI (Jet Stream) source. About 5 µl of each sample was injected using the autosampler, with a total of 24 injects altogether. All the samples were maintained at 4°C using a thermostat autosampler. UHPLC separation was performed with AdvanceBio Peptide Map, C18 capillary columns (C18, 2.1 x 100 mm, 2.7 µm particles, cat no. 655750-902) in 95% mobile phase A (100% water, 0.1% formic acid), 5% mobile phase B (90% acetonitrile, 10% water, 0.1% formic acid) at a flow rate of 0.3 ml/min. The total time of analysis was set at 23 min. Initial conditions were maintained for 1 min, and peptide elution occurred at linear gradients of 1-40% mobile phase B for 15 min. This is followed by 5 min at 95% mobile phase B prior to returning to initial conditions in 2 min. Each sample was analyzed in triplicates. Each run was separated by two blank runs to wash and re-equilibrate the column prior to the next run to minimize sample carryover.

The MS conditions were as follows: drying gas temperature, 300°C; drying gas flow, 8 mL/min; nebulizer pressure, 35 psi; corona current, 10 nA; and capillary voltage, 4,000 V. MRM mode was applied for quantitative analysis using precursor/product ion information. All the data acquisition was processed, evaluated, and visualized using MassHunter Workstation (version B.03.01), which was processed using MassHunter Quantitative analysis software (B.04.00) (Agilent Technologies, USA). Ion chromatograms were extracted with a mass tolerance of 10 ppm for MRM data.

Results and Discussion

Optimization of MRM precursor and product ion pairs using Skyline software

Table 2 indicates the final parameter of MRM acquisition suggested by Skyline software in which several parameters such as parent ion, product ion, and collision energy have been assessed to suit the targeted search. Initially, for each parent ion, approximately five to seven product ions were proposed by the software as having a high potential to be detected by LC-ESI-QQQ-MS. Moreover, at least seven collision energies were predicted to suit each product ion, which resulted in a total of 273 possible runs. The information obtained from this analysis is imperative to ensure that the most intense precursor and fragment ions for MRM method development were selected. All the parameters given by Skyline software were assessed to identify the presence of pork. Out of seven peptides with 273 possible runs, only four peptides were consistently detected with a clean and specific signal, whilst chromatograms with considerably high amounts of noise, interfering peaks, and non-specific signals were eliminated. Using Skyline as an optimization tool provides specificity of detection without detecting unnecessary transitions and reduces the time taken by instrument parameters to optimize each peptide [23]. In QQQ MS, three quadrupole (Q) rods were used, in which Q1 is used for precursor ion isolation, Q2 works as a collision cell containing a collision gas (usually argon), and Q3 analyses product ions produced from the precursor ion isolated in Q1. Therefore, only ions with a particular m/z value will be able to escape the quadrupole, while the rest will collide with the quadrupole walls [24, 14].

There are four peptides that gave positive spectra, which were then further analysed to ensure their specificity (Figure 1). Remarkably, two of the peptides, LVVITAGAR and FVIEIR, exhibit product ion with the highest intensity, while the other 2, EVTEFAK and TVLGNFAAFVQK, were present in lower intensity. Peptide LVVITAGAR, the most intense peptide, was eluted at 7 min while FVIEIR, as the second highest peak, was eluted at 9.5 min. On the other hand, peptide EVTEFAK was eluted at 4.5 min, and TVLGNFAAFVQK, a peptide with the lowest intensity, appeared between 12.5 to 13 min. A closer visualization of the extracted MRM chromatogram revealed a good detection of peptide biomarkers despite having the lowest peak. Such chromatogram reflects the relative abundances of distinct proteins in commercial products. The most intense product ions produced for each precursor ion were combined with all detected peptides to develop a final MRM method.

To achieve a true positive identification, all the product ions were confirmed to be eluted at the same point with the corresponding precursor ion, where the elution condition and retention time of all transitions were carefully observed. All the product ions were confirmed to be successfully eluted at the same retention time as the precursor ion with varying collision energy. It was, therefore, necessary to select the optimum pairs of product ion and collision energy that could produce the most intense peak.

Assessment of products ion in processed meat products

The porcine-specific peptide biomarker was evaluated by LC-ESI-QQQ-MS on seven different commercial meat products. Total ion chromatograms of seven products tested (Figure 1) display that the porcine-specific peptides were successfully detected in the tryptic fragmentation of three commercial product samples, which were declared to contain pork as labelled on the packaging. Three samples, Japanese hamburger, pork meatball, and pork sausage, were detected as positive. The result illustrated the overview of the MRM signals detected from all four porcine-specific peptides without any non-specific signal observed in the chromatogram. Meanwhile, the other products, namely, beef ball, chicken luncheon, corned beef, and smoked chicken frankfurter, exhibited a flat spectrum, indicating no traces of pork in the products. The four peaks were consistently absent, which led to the conclusion that they were free from any pork protein component.

Table 2. Parameter of MRM acquisition method for porcine-specific peptides from lactate dehydrogenase and serum albumin tested in processed meat products (Reproduce from [21])

No	Peptide Sequence (Protein)	Accession Number	RT (min)	Precursor Ion (charge state)	Product Ion	Collision Energy	Dwell
-1	LVVITAGAR (Lactate dehydrogenase)	GI:288860136 (*Sus scrofa*)	7	450.2873 (z=2)	786.4932 (y8)	18	20
					687.4048 (y7)	12	20
					588.3364 (y6)	12	20
					475.2523 (y5)	12	20
2	EVTEFAK (Serum albumin)	GI:52353352 (*Sus scrofa*)	4.5	412.2134 (z=2)	595.3286 (y5)	19.8	20
					494.2509 (y4)	10.8	20
					365.2083 (y4)	10.8	20
3	FVIEIR (Serum albumin)	GI:52353352 (*Sus scrofa*)	9.5	388.7369 (z=2)	629.3881 (y5)	10.1	20
					530.3197 (y4)	10.1	20
					417.2356 (y3)	10.1	20
					288.213 (y3)	16	20
4	TVLGNFAAFVQK (Serum albumin)	GI:52353352 (*Sus scrofa*)	12.5	647.8613 (z=2)	592.3553 (y5)	24.1	20
					521.3382 (y4)	30.1	20

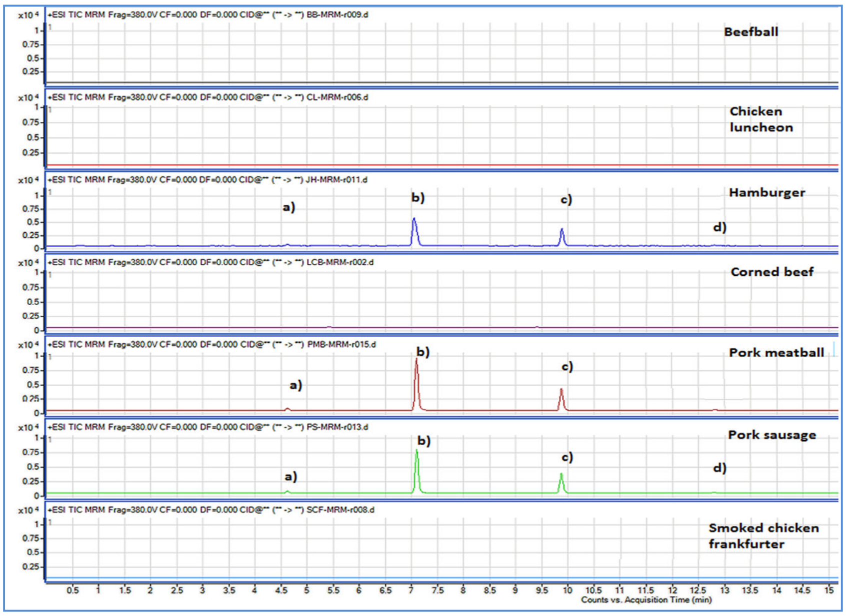

Figure 1. MRM chromatogram of seven different commercial meat products detecting peptides: a) EVTEFAK, b) LVVITAGAR, c) FVIEIR, and d) TVLGNFAAFVQK

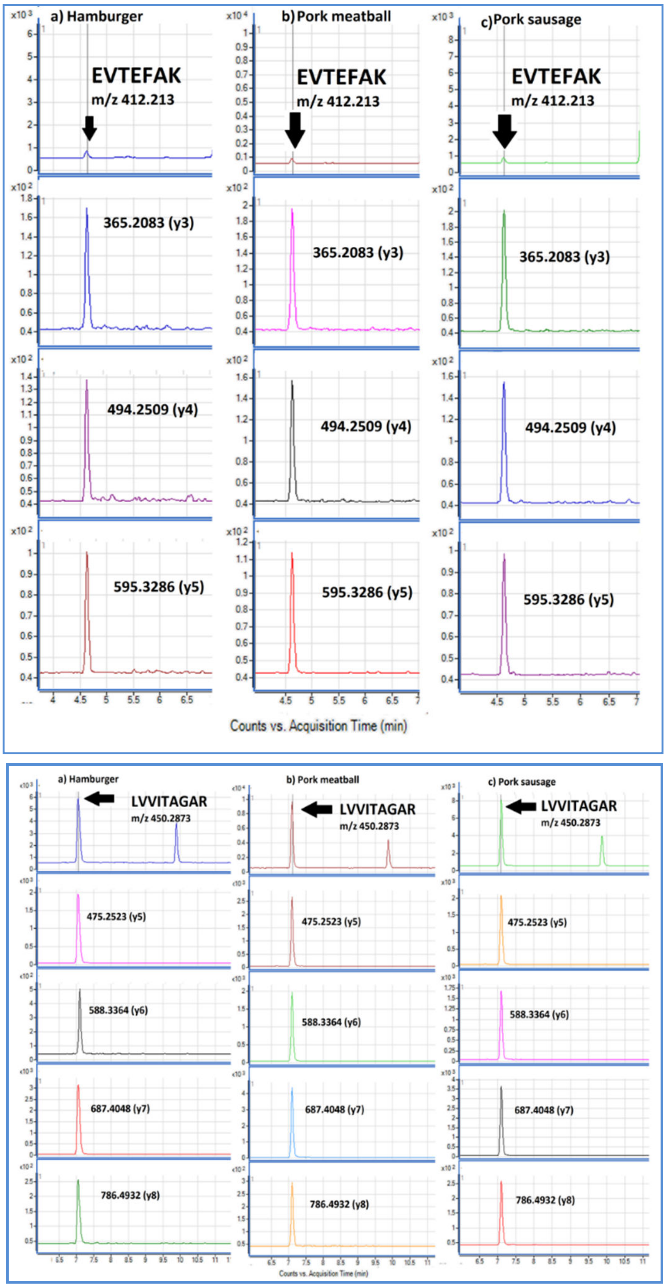

Figure 2**.** MRM chromatogram of peptide EVTEFAK and LVVITAGAR detected in hamburger, pork meatball, and sausage with the specific signal produced by product ion 365.2083, 494.2509 and 595.3286 and 475.2523, 588.3364, 687.4048 and 786.4932, respectively. All product ions were eluted at similar retention

MRM chromatograms from three commercial samples (sausage, meatball, and hamburger) that were positively identified to contain pork were examined in detail. The extracted MRM transitions of porcine specific-peptides marker within 23 min is presented (Figure 2). The first peak, peptide EVTEFAK, was eluted at 4.5 min, while peptide LVVITAGAR, the highest peak detected from lactate dehydrogenase protein, was eluted at 7 min. Peptide FVIEIR, as the second highest peak observed in the chromatogram, was eluted at 9.5 min while peptide TVLGNFAAFVQK, which was detected as the lowest chromatogram peak, was observed at 12.5 min (Table 2).

After examining the spectra, retention time, and abundance of parent ion, the spectra of product ions were further inspected using several parameters, such as the intensity of peptide and the quality of MS/MS spectra. Note that product ions must be eluted within the same retention time as parent ions, indicating the identity of the peptides. This suggests that a highly specified transition that monitors multiple specific ion transitions at high sensitivity from both parent ion and product ion has been performed simultaneously and scanned for a specific mass of interest in Q1 and Q3, respectively [25-26].

The peptide LVVITAGAR was set on finding the specific precursor mass (m/z = 450.2873) on Q1, with the collision energy being optimized to produce a diagnostic charged fragment of that parent ion. Meanwhile, Q3 was set to detect the specific mass of those product ions (786.4932, 687.4048, 588.3364, and 475.2523) produced by their respective parent ions. However, only ions with specified transition were detected in all commercial samples.

The peptide EVTEFAK with precursor ion 412.2134 has been set to detect product ions 595.3286, 494.2509, and 365.2083. Specific signals of precursor and product ions have been observed in three out of four samples, indicating the presence of pork in the products as declared on the label. Similarly, peptide FVIEIR (m/z = 388.7369) produced a good transition signal in both precursor and product ions (629.3881, 530.3197, 417.2356, and 288.213) upon detection in hamburger, pork meatball, and pork sausage. Meanwhile, peptide TVLGNFAAFVQK (m/z = 647.8613) demonstrates signal specificity despite having a slightly low chromatogram with only two product ions, 592.3553 and 521.3382, detected.

All the results above indicate that only analytes with specific precursor/product ion combinations will be detected. In a complex biological sample, there might be other protein components that could produce a 450.2873 m/z precursor ion during ESI. However, there is a low probability that they will also fragment to a 786.4932, 687.4048, 588.3364, and 475.2523 m/z product ion. This technique is termed MRM, and fragmentation is denoted as 450.2873 > 786.4932, 450.2873 > 687.4048.

Targeted MS has become the method of choice for the analysis of complex protein samples. Previous related studies that have documented the importance of targeted MS were mostly from a medical-related background, whereby the technique has been used to quantify and monitor the progress of drug therapy [27] and disease [28], [15]. In relation to the Agilent 6490 QQQ mass spectrometer, an MRM-based assay for the determination of cardiovascular disease and disease classification has been developed using the same instrument [28]. Since the instrument produces high sensitivity and selectivity of peptides, the present study utilizes its capacity to develop a powerful porcine detection method for halal authentication. Research by [29] used LC-ESI-QQQ-MS/MS 6460 from Agilent Technologies, a similar mass detector to this study. They reported positive MRM detection of duck, goose, and chicken peptide markers in various processed types of meat such as duck terrine, semi-dried sausage, and kabanos-type sausages.

As far as the application of MRM for pork detection is concerned, several research articles reported the work on identifying peptide biomarkers for the detection of pork in meat matrices. In a recently published study by [19], a total number of 12 biomarkers were detected. Among the reported peptides, they described peptide YDIINLR from troponin T protein, which was known to provide species-specificity for detecting pork and horses. Moreover, it has been tested against a mixture of raw meat. However, in the present study, no chromatogram of YDIINLR was observed from the cooked meat samples following multiple runs of MRM analysis, which suggested that peptide YDIINLR from troponin T was discovered not to be suitable for detection of thermally processed meat. Meanwhile, MRM of myoglobin peptides was also tested in meat against mixtures of several species of proposed peptide pairs, which is called the Corresponding Protein Corresponding Peptide (CPCP) strategy as a rote to relative quantitation [18].

Commercial meat products produced through technological processes possess several challenges for MS analysis due to protein denaturation following the process. Processes like cooking [30], freezing, and thawing methods [31] could influence the protein quantity and extractability of the samples. The porcine-specific peptides obtained [21] were tested on processed products, and it was verified whether the peptide application was suitable for such products or only limited to meat detection. Here, proteins extracted from the meat products with various technological processes and purchased in the local and international supermarkets (Table 1) demonstrated a good match, with three exhibiting positive results. Hence, it indicates that the proposed peptides are suitable for processed products. The present study has successfully validated that a species-specific fragment was identifiable in all complexes of meat products, including sausages, corned beef, or canned meat, by applying MRM.

Previous studies conducted by [19-20] reported that species-specific marker peptides for horse and pork were identified in commercial samples such as frikadeller, meatloaf, and spaghetti sauce, which has been subjected to complex and harsh processing like mixing, cooking, and frying using MRM^3 experiment. Identified marker peptides were demonstrated to resist the thermal processing of different meat products. Furthermore, the sensitive and specific detection of pork or horses in processed food has been demonstrated to be down to 0.24% in a beef matrix system. Contrary to this study, peptides TLAFLFAER and SALAHAVQSSR are from myosin-4 and myosin-1 and -4, respectively, with no marker from serum albumin and lactate dehydrogenase reported [20]. Another research utilized raw or semi-dry ham as study material and employed peptides from Myosin Light Chain (MLC) isoforms, namely KPAAAAAPAPAPAPAPAPAPAPPK and EAFTVIDQNRDGIIDKEDLRDTFAAMGR, which indicated that the fragments of sequence are most likely to be the most resistant to trypsin, proteolytic enzymes as well as to technological processes [32].

Notably, the protein reported in this study agrees with [33], who reported the significance of lactate dehydrogenase as a marker of beef quality between *in vitro*-digested aged and cooked meat. Meanwhile, three peptides, ADFTDVTK, DVFLGSFLYEYSR, and EDPHACYATVFDK, from serum albumin have been demonstrated in MRM experiments as potential peptide biomarkers for the detection of sheep in processed products [34]. Moreover, serum albumin has been reported as the precursor of functional bio-peptide for developing commercial functional foods and nutraceuticals [35] and was also discovered in protein exudates obtained from pig meat injected in different levels of brine [36].

To make this research more meaningful, several recommendations shall be considered for future work. Future work should identify the remaining potential biomarker candidates, which could be present in trace amounts at the low abundance proteins group. Hence, sample fractionation and purification could improve the extraction of biomarker candidates, which are too low for a reliable assay. Moreover, it is recommended that internal standards or labelling of candidate peptides using stable isotopes be introduced. Known labelled standards, such as a stable isotope of the targeted peptide, would need to be introduced into the sample prior to sample injection to ensure a more confident result during routine analysis.

In general, the objectives of the present study were satisfactorily achieved. Notably, this research provided several important findings that may contribute to the advancement of halal authentication procedures. This study also highlighted a better understanding and insight into species specificity provided by precursor and product ion selection. All these findings shall be extended for the detection in other food systems and not just limited to meat and its co-products.

Conclusion

Four peptides (EVTEFAK, LVVITAGAR, FVIEIR, and TVLGNFAAFVQK) were consistently detected in commercial meat products. Apart from determining the specificity of the peptides in heat-treated meat, the MRM method developed through this study was also able to identify the targeted porcine-specific peptides in processed meat products, which suggests the method's selectivity. Moreover, it has been demonstrated that when collision energy is optimized, unique peptides can be detected by targeting their specific precursor ion and product ion through MRM analysis. Indeed, targeted simultaneous analysis of multiple proteins will enable rapid halal detection of large sample sets of contaminated meat products. Thus, a set of porcine-specific markers was proposed rather than individual markers, which could have increased the specificity and confidence of the detection.

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