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**MRM–MS of marker peptides and their abundance as a tool for authentication of meat species and meat cuts in single-cut meat products**

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**Abstract**

The abundance of protein markers in different types of meat cuts was explored in the context of authentication of raw meat (pork, beef and chicken) and processed meat products. Peptides originating from myoglobin (Mb) and myosin (My) were analyzed using multiple reaction monitoring mass spectrometry (MRM–MS). Analytical protocol was optimized for good repeatability ( $CV < 10\%$ ) and high sensitivity. The MS signal intensity of Mb marker peptides in raw pork depended significantly on the cut type (e.g. ham vs knuckle). Importantly, a similar pattern in the abundance of the marker peptides was found for processed meat products made of different types of pork cut, despite the food processing applied. This suggests the protocol can be used for authentication of raw pork cuts and processed products made of different cuts of pork. More uniform contents of Mb markers were found in raw beef cuts, and for My markers in raw chicken cuts.

**Keywords:**

Meat authentication, Mass spectrometry, Marker peptides, Myoglobin, Pork, Beef

**1. Introduction**

Proteins are one of the most important macronutrients in the human diet. Usually, the type and the amount of protein we consume depend on our worldview (veganism, vegetarianism, etc.), religion (e.g., Islamists and Judaists do not eat pork), diet-related health conditions (food allergies, food intolerances, etc.), or legislation (e.g., horse slaughter and consumption is banned in several states of the USA (Forrest, 2017; Whiting, 2007)). These are some of the reasons why development of efficient methods for protein authentication in food products is required; especially in the light of an increasing scale of food adulteration.

In meat products, a chopped meat can be easily substituted with a meat of another species or from a different than declared part of the animal, usually of a lower price. One of the most infamous meat scandals was discovered in 2013 when horse meat was found in beef burgers available at a number of the UK supermarkets. In some lasagna or spaghetti Bolognese products, horse meat amounted to 100% of the meat content (BBC, 2013). In the wake of the scandal, random tests of meat products were advised by EU authorities, which led to several other food frauds being revealed in Europe. The Czech State Veterinary Administration found horse meat in pork and beef meatballs delivered from

Sweden. The products were withdrawn from 14 European countries (Pollak, 2013). A survey of Turkish meat products published in 2013 (Ulca, Balta, Çağın, & Senyuva, 2013) showed that one of the examined products labeled as containing 5% beef did not contain beef at all, whereas some other products labeled as '100% beef meatballs' contained chicken, and 'turkey meatballs' were predominantly chicken. In turn, in Malmö, three out of ten kebabs advertised as '100% beef' contained up to 18% pork (The Local, 2013). Also in 2013, the Swiss Central Islamic Council found traces of pork in 7 out of 20 halal kebab samples tested in Switzerland (On Islam & News Agencies, 2013). In 2014, a study carried out by the Food Standards Agency showed that 30% of lamb takeaway meals sold in the UK contained other types of meat (beef or chicken) or no lamb at all, with several products made of unidentified meat (Poulter, 2014). In 2017, it was found that in Leicestershire (UK) turkey was offered as halal lamb for wholesale, in butcher shops, and in restaurants (BBC, 2017).

The above examples highlight the magnitude of meat fraud scandals worldwide in recent years, and also emphasize that this is not just an academic but rather a widespread legal problem, which becomes an increasingly important driving force for urgently needed development of new scientific strategies and analytical methodologies that could help in fighting food adulteration. The current analytical methods rely, in general, on detecting DNA or protein that is non-specific to a declared meat species. Detection of foreign DNA requires a polymer chain reaction (PCR) to amplify a segment of DNA to the level detectable by a subsequent analytical technique such as electrophoresis. PCR techniques have become widely used for meat authentication. Traditional, end-point PCR has been used for a qualitative analysis of unprocessed and processed meats (Ballin, Vogensen, & Karlsson, 2009; Calvo, Zaragoza, & Osta, 2001). Real-time PCR can be used as a quantitative technique in meat and fish authentication (Ballin, Vogensen, & Karlsson, 2009; Hird, et al., 2005; Lahiff, Glennon, Lyng, Smith, Shilton, & Maher, 2002). However, for highly processed food products, real-time PCR may not give accurate quantitative results, as severe processing (e.g., canning, autoclaving) can lead to fragmentation of DNA (Hird, et al., 2006). This, in turn, reduces the number of template molecules, thereby altering the quantitative results of analysis. Thus, the applicability of PCR in meat authentication might be limited to food products containing raw or mildly processed meat (e.g., baked or cooked), as DNA fragmentation is usually not observed there (Hird, et al., 2006).

Another approach to meat authentication is based on protein analysis. Immunological methods can be applied. However, commercial kits for immunoassays (e.g. MBM, MELISA-Tek, FeedCheck) often do



not meet the acceptance criteria of the Food and Drug Administration in terms of selectivity, sensitivity, ruggedness, and specificity (Ballin, Vogensen, & Karlsson, 2009). Other methods, such as HPLC (Ashoor, Monte, & Stiles, 1988), UPLC (Di Giuseppe, Giarretta, Lippert, Severino, & Di Maro, 2015; Giarretta, Di Giuseppe, Lippert, Parente, & Di Maro, 2013), Raman spectroscopy (Boyacı, Temiz, Uysal, Velioglu, Yadegari, & Rishkan, 2014; Sowoidnich & Kronfeldt, 2012), visible and infrared spectroscopy (Al-Jowder, Defernez, Kemsley, & Wilson, 1999; Cozzolino & Murray, 2004), and FTIR (Rohman, Erwanto, & Man, 2011), can be used in order to detect the presence of proteins or peptides in meat samples.

Myoglobin (Mb) and myosin (My) are often targeted as protein markers in authentication of meat species. For example, Mb was successfully applied for detection of pork in raw beef burgers (Giarretta, Di Giuseppe, Lippert, Parente, & Di Maro, 2013) or horse meat added to raw beef (Di Giuseppe, Giarretta, Lippert, Severino, & Di Maro, 2015). Montowska et al. (Montowska, Alexander, Tucker, & Barrett, 2014) focused on detecting peptide markers that originated from several proteins (Mb, My, troponin C) in identifying pork, horse, turkey and chicken in cooked meat. The same researchers also applied liquid extraction surface analysis with mass spectrometry for detecting beef, pork, horse and poultry peptide markers in commercially available and laboratory-made meat products (Montowska, Alexander, Tucker, & Barrett, 2015). Watson et al. (Watson, Gunning, Rigby, Philo, & Kemsley, 2015) have proposed an application of HPLC–MS/MS in multiple reaction monitoring (MRM) mode for verification of raw meat samples authenticity, based on detection of Mb peptide markers. The MRM mode is a feature of tandem mass spectrometry and provides an exceptionally high selectivity and sensitivity of detection. It gives a possibility of detecting as little as 1%-addition of undeclared meat in raw meat samples (Watson, Gunning, Rigby, Philo, & Kemsley, 2015). The MRM–MS approach was successfully applied for detection of horse and pork in halal beef (von Bargaen, Dojahn, Waidelich, Humpf, & Brockmeyer, 2013), as well as pork or horse meat in highly processed beef matrix (von Bargaen, Brockmeyer, & Humpf, 2014), which suggests a potentially higher applicability of MRM–MS in detecting marker peptides for meat authentication in highly processed food products compared with the use of PCR, due to the limitations of the latter explained above.

The Mb content can vary significantly between different types of muscle (Beecher, Cassens, Hoekstra, & Briskey, 1965), suggesting that Mb peptide markers can serve not only as indicators of meat species but potentially also for authentication of the type of a raw meat cut or even processed meat products

made of specific types of cuts. We, therefore, hypothesize that the physiological differences in the contents of specific proteins (e.g., Mb) between different types of muscles can be detected in meat products after food processing has been applied in their manufacturing. If this is to be the case, differences in the abundance of protein markers between specific types of cuts of a given meat species could potentially be used in authentication of meat products made of single types of meat cuts (e.g., processed pork ham vs processed pork knuckle). In order to test the hypothesis, we first optimized the protocol for sample preparation and HPLC–MS/MS analysis in MRM mode, to achieve the high sensitivity and repeatability required for reliable detection of target peptides. We then applied the protocol to analyze a number of different types of pork, beef and chicken raw meat cuts, as well as commercially available processed meat products made of different types of meat cuts.

## 2. Material and methods

### 2.1. Materials

Acetonitrile (ACN, LC–MS grade), urea, trypsin (T0303, type IX-S, 13,000–20,000 BAEE units/mg protein), ammonium hydrogen carbonate and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). Analytical grade potassium chloride, potassium hydroxide, potassium dihydrogen phosphate and formic acid (FA) were obtained from POCH (Gliwice, Poland). Ultrapure water was prepared using an HLP<sub>5</sub> system (Hydrolab, Wislina, Poland).

### 2.2. Meat samples collection and preparation

Raw beef, pork, and chicken meat as well as processed meat products were purchased from local supermarkets in Gdansk, Poland. Samples (5–10 g or ca. 5 cm) were cut from the raw meats and the meat products and stored at –80 °C (Montowska, & Fornal, 2017; Sentandreu, Fraser, Halket, Patel, & Bramley, 2010) in Corning (Amsterdam, Netherlands) plastic centrifugal tubes prior to analysis. The information about the processing type, the type of meat cut, the meat content and the presence of other ingredients in the processed meat products was obtained from product labels (Table 1). The meat contents declared in the products were taken into account during sample analysis and data processing.

In order to investigate the level of individual variability for each type of raw meat cut, samples were obtained in triplicate on three different occasions and attention was paid to obtain meat cuts of a given meat species from different individual animals. In the case of pork (*Sus scrofa domesticus*), five types

of cuts were investigated, namely: knuckle, shoulder, tenderloin, loin and ham, and for beef (*Bos taurus*), six types of cuts were investigated, i.e. chuck, rib, shank, tenderloin, sirloin and round. In the case of chicken (*Gallus gallus domesticus*), three different types of cuts were investigated, i.e. breast, thigh and drumstick. The meat cuts selected for this study were of the types that are most commonly used in the meat industry, and available to customers as fresh meat or processed meat products. Each sample of a raw meat cut or a processed meat product was analyzed in triplicate, and results presented as means  $\pm$  SD.

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Table 1. Processed single-cut meat products analyzed in the study

ID*	Product	Processing	Composition (wt% meat, or g of meat used /g of product)	Additives
1A	pork knuckle	boiling	knuckle (94%)	salt, dextrose, caramelized sugar
2A	Bavarian knuckle	roasting, steaming	knuckle (104 g/100 g)	salt, wheat fiber, flavorings, pork protein, spices, monosodium glutamate, sodium nitrite
3A	pork loin	roasting, steaming	loin (117 g/100 g)	salt, glucose, extracts of spices, dried garlic, black pepper, green pepper, dried pepper, dried parsley, marjoram
4A	pork tenderloin	smoking, steaming	tenderloin (83%)	water, salt, sugar, triphosphates, pork protein, pea fiber, glucose, sodium ascorbate, aroma, sodium nitrite
1B	pork ham	smoking, steaming	ham (82%)	water, salt, diphosphate, triphosphate, polyphosphate, carrageenan, starch, monosodium glutamate, maltodextrin, sodium isoascorbate, aroma, sodium nitrite
2B	pork ham	smoking, boiling	ham (80%)	water, salt, starch, diphosphate, triphosphate, dextrose, preservative E407a, monosodium glutamate, sodium isoascorbate, maltodextrin, aromas, sodium nitrite
3B	pork loin	smoking, boiling	loin (83%)	water, salt, starch, diphosphate, triphosphate, dextrose, preservative E407a, monosodium glutamate, sodium isoascorbate, maltodextrin, aromas, sodium nitrite
4B	pork tenderloin	smoking, steaming	tenderloin (76%)	water, salt, diphosphate, triphosphate, polyphosphate, carrageenan, starch, monosodium glutamate, maltodextrin, sodium isoascorbate, aroma, sodium nitrite
1C	pork tenderloin	smoking, steaming	pork meat (111 g/100 g)	salt, natural aromas, sugar, natural spices and extracts of spices, sodium nitrite
1D	pork shoulder	boiling	shoulder (83%)	water, salt, glucose, spices, sugar, maltodextrin, hydrolyzed vegetable protein, aromas



1E	pork ham	smoking, steaming	ham (78%)	water, salt, sodium lactate, triphosphate, polyphosphate, glucose, carrageenan, monosodium glutamate, soy protein, sodium isoascorbate, maltodextrin, aromas, sodium nitrite, possible presence of: milk derivatives, gluten, celery, mustard
1F	pork shoulder	roasting	shoulder (140 g/100 g)	salt, spices (containing celery), natural aroma
1G	pork tenderloin	roasting, smoking	tenderloin (135 g/100 g)	salt, sugar, natural spices, sodium nitrite
1H	pork tenderloin	smoking, steaming	tenderloin (80%)	water, salt, dried spices, dried vegetables, stabilizer: E451 (i), soy protein, glucose, pea flour, E407a, native corn starch, pork protein, corn protein hydrolysate, soy protein hydrolysate, antioxidant: E316, E306; yeast extract, sugar, aromas, spice extracts, preservative: E250, possible presence of: gluten, milk, celery

\* Capital letters (A–H) indicate different producers of the meat products analyzed.

On the day of analysis, raw meats and meat products were defrosted at room temperature. Sample of ca. 1 g was taken and placed in a plastic 50-mL centrifuge tube, and the extraction buffer (10 mL, 0.3 M potassium chloride, 0.3 M potassium dihydrogen phosphate, pH 6.5) was added. An actual sample weight was taken into account in data processing. Before taking samples of processed meat products, any material that might have resided on their surface (such as a crust of dried or blackened meat that could have been formed on the outside of the products during the food processing) was removed, and the sampling was done from below such a surface. The samples were homogenized with an Ultra-Turrax homogenizer for 5 min (18 000 rpm). In the next step, the homogenized samples were sonicated for 30 min in an ultrasonic bath and finally centrifuged at room temperature for 40 min at 10733 g. Clear or almost clear supernatants were collected, transferred to plastic, 50-mL centrifuge tubes and vortexed. Subsequently, the extracts were centrifuged at room temperature for 2 min at 1315 g, in order to suppress a foam that could have formed during the previous step. In the case of analyzing processed meat products, 1.5-mL aliquots of extracts were transferred to 12-mL test tubes and mixed with 2.4 mL of 30 mM ammonium hydrogen carbonate, whereas in analyzing raw meat samples, the extracts were first diluted with the extraction buffer (0.3 mL of extract was mixed with 1.2

mL of extraction buffer) and then mixed with 2.4 mL of 30 mM ammonium hydrogen carbonate. In the next step, the extracts were heated for 30 min at 90 °C, in order to denature the proteins present in solution. The extracts were then cooled to room temperature, and 105 mg of urea were added to each test tube in order to accelerate the subsequent enzymatic digestion of proteins. Subsequently, 1.5 mL of trypsin solution (1 mg/mL in 30 mM ammonium hydrogen carbonate) were added. The samples were immediately vortexed and incubated for 4 h at 37 °C. Since the extraction and digestion procedure was time-consuming (around 10 h in total for 21 samples), the following steps were performed on the next day. Until then, the digested extracts were stored frozen (-20 °C). After brining the samples back to room temperature, the extracts were cleaned-up and enriched using Strata-X 33 µm SPE cartridges filled with 200 mg/3 mL polymeric reversed-phase material (Phenomenex, Macclesfield, UK). The cartridges were activated with 2 mL of methanol followed by 4 mL of 1% (v/v) aqueous solution of FA. Afterwards, the extracts were loaded into the cartridges. The empty extract vials were additionally washed out with 500 µL of the 1% (v/v) FA, and the washings were loaded into the cartridges to provide a quantitative transfer of the samples. The cartridges were then washed with 4 mL of the 1% (v/v) FA. Finally, the peptides were eluted with 4 mL of the ACN:water mixture (1:1 v/v, containing 0.1% v/v FA) into 12-mL glass test tubes containing 10 µL DMSO each. The elution was done in two runs, 2 mL each. The cartridges were left to soak for 10 minutes after the first batch of eluate, in order to provide complete elution of the peptides. Subsequently, the solvents in the purified extracts were completely evaporated under a stream of nitrogen at 45 °C. Prior to the chromatographic analysis, the extracts were reconstituted with 200 µL of the ACN:water mixture (3:97 v/v; containing 0.1% v/v FA), vortexed for 30 s, centrifuged for 3 min at 2470 g and transferred to autosampler vials equipped with 250-µL inserts. The sample preparation procedure has been summarized in the Supplementary Material (Fig. S1).

### 2.3. Instrumentation

Selection of species-specific peptides was based on published studies (Watson, Gunning, Rigby, Philo, & Kemsley, 2015; Sentandreu, Fraser, Halket, Patel, & Bramley, 2010), where they were found to be most valuable as markers over other Mb or My peptides, produced after trypsin digestion of the proteins, due to very good MS signal quality, high discriminating power, etc. The marker peptides have been listed in Table 2.



The HPLC–MS/MS analysis of the marker peptides was performed using an Agilent 1200 LC system (Santa Clara, CA) equipped with a binary pump, an online degasser, an autosampler and a thermostated column compartment equipped with a switching valve. The HPLC system was coupled with a Q-Trap 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Kinetex XB C-18 reversed-phase (RP) column (100 × 2.1 mm, 2.6 μm, Phenomenex) was used for the RP–HPLC separation of the peptides. The mobile phase consisted of water containing 0.1% (v/v) FA (component **A**) and acetonitrile containing 0.1% (v/v) FA (component **B**). The following gradient elution programme was used: a linear increase from 3% to 28.4% **B** over 22 min; next, a linear increase from 28.4% to 90% **B** over 1 min, followed by 90% **B** maintained for 10 min. The last step was a conditioning of the column for 12 min with 3% **B**. The flow rate of mobile phase was 300 μL/min, and the injection volume was 10 μL. The column temperature was maintained at 40 °C throughout the separation process. The ESI source was operated in positive ion mode with the following conditions: curtain gas pressure at 25 psi, source temperature set to 550 °C, ion spray voltage at 5000 V, nebulizer gas and heater gas at 50 psi and 20 psi, respectively. Each peptide (with the exception of peptides specific for chicken meat) was monitored by its four most intense MRM transitions. In the case of pork and beef samples, the intensities of three subsequent MRM transitions were very low (e.g. primary to secondary, tertiary and quaternary transitions for pork were around 100:4:4:4). Therefore, four MRM transitions were observed to confirm the identity of the marker peptides. When analyzing chicken meat, two MRM transitions were sufficiently intense to confirm the identity of marker peptides specific for this kind of meat. The source and MS parameters are shown in Table 2. In order to acquire data and control the instrument, Analyst software Ver. 1.5.2 (AB Sciex, Redwood City, CA) was used.

Table 2. MRM transition parameters for detection of marker peptides in meat samples

meat	protein	Uniprot ID	marker peptide symbol and amino acid sequence	parent ion (m/z)	fragments (m/z)	declustering potential (v)	collision energy (v)
pork	myoglobin	P02189	P1 (primary marker), GHPETLEK	455.7	716.4	64.3	21.7
					490.3		
					147.1		
pork	myoglobin	P02189	P2 (confirmatory marker), HPGDFGADAQGAMSK	744.8	619.3	97.7	47.8
					234.1		
					1254.5		
beef	myoglobin	P02192	B1 (primary marker), VLGFHG	315.2	692.3	54.0	14.0
					417.2		
					213.2		
beef	myoglobin	P02192	B2 (confirmatory marker), HPSDFGADAQAAMSK	766.8	530.3	86.0	39.0
					360.2		
					234.1		
chicken	myosin	P02506	Ch1 (primary marker), DQGTFEDFVEGLR	756.8	1298.6	80.0	35.0
					835.4		
chicken	myosin	P02609	Ch2 (confirmatory marker), GADPEDVIMGAFK	675.3	706.4	86.0	39.0
					1395.6		
					964.5		
					880.5		

### 3. Results and discussion

#### 3.1. Optimization of sample preparation

The procedure for meat samples preparation was based on the method developed for raw meat analysis (Watson, Gunning, Rigby, Philo, & Kemsley, 2015). However, a direct application of that method to processed meat products analyzed in this study did not produce sensitive nor reproducible results. Therefore, the procedures for extraction and digestion of proteins as well as the solid-phase extraction of peptides were modified in order to maximize sensitivity and repeatability of the method, which could make it applicable in reliable analyzing of processed meat products. A number of experimental variables were tuned to achieve satisfactory effects. These included parameters of protein extraction, handling of sample extracts, conditions of digestion and solid-phase extraction process. The duration of the mechanical homogenization and the protein extraction seemed to be especially important. For the raw meat samples, 1.5 min of Ultra-Turrax treatment was sufficient for most effective protein extraction. However, we found that for the samples of processed meat products a treatment of 5 min was required. The samples homogenized for 5 min produced an MS signal that was ten times higher than in the samples treated for only 1.5 min (data not shown). Further increase in the homogenization time did not improve the results. However, applying the sonication step improved the repeatability of results. It is well known that thermal treatment of meat significantly decreases protein extractability (Acton, 1972; Klement, Cassens, & Fennema, 1973), due to physical changes in the meat matrix (e.g., increased firmness) and decrease in protein solubility upon heat denaturation. Thus, the homogenization time had to be optimized, as only the prolonged treatment seemed to guarantee sufficient disintegration of the samples, so the maximum quantities of protein could be extracted from the processed meat products studied.

The conditions of centrifugation of the extracts obtained after the homogenization and sonication of samples were found to significantly affect the final results. More than two-fold increase in the intensity of the MS signal was observed for the extracts that were centrifuged for 40 min at 10 733 g, as compared to those that were not centrifuged at all or centrifuged for only 10 min (data not shown). The poorly centrifuged extracts contained fair amounts of insoluble material (most likely collagen particles, etc.). Thus, in the subsequent digestion, substantial amounts of trypsin might have been involved in digesting such debris, instead of the soluble marker proteins (i.e. myoglobin or myosin), which could account for the final level of detection of the peptides derived from these proteins. It was also



observed that the amount of trypsin and the duration of the digestion step contributed significantly to repeatability of results. Only by using substantial quantities of the enzyme (i.e. final concentration of ca. 0.3 mg/mL) and a moderate digestion time (i.e. 4 h), was it possible to achieve a high and repeatable MS signal (i.e. with CV values in the range of 2–10%).

The solid-phase purification of digested extracts was also tuned, in order to maximize sensitivity of analysis. We found it beneficial in terms of avoiding the loss of analytes to use water acidified with FA (1% v/v) instead of acidified methanol for washing the sorbent bed. It was particularly important in analyzing the pork primary peptide marker GHPETLEK, which is poorly retained on the C18 stationary phase. Since the meat samples were larger than those used by Watson et al. (Watson, Gunning, Rigby, Philo, & Kemsley, 2015), SPE cartridges with 200 mg/3 mL of sorbent were used and appropriate volumes of the conditioning, washing and elution solutions applied. The optimized procedure for sample preparation is schematically depicted in the Supplementary Material (Fig. S1).

Representative chromatograms of the pork, beef and chicken peptide markers, obtained after the sample preparation procedure had been optimized, are shown in Fig. 1. The sensitivity and repeatability of the optimized method was estimated in triplicate analyses of raw meats and samples of meat products. Representative comparison of the results obtained before and after the optimization is shown in Fig. 2. In general, significant improvements in both the repeatability and the sensitivity were observed. In particular, the repeatability of results was much better than when using the original procedure (Watson, Gunning, Rigby, Philo, & Kemsley, 2015). The CV values of the results obtained with that method before the optimization were in the range of 35–127%, whereas after the optimization the values dropped below 10%. The sensitivity was enhanced by a factor of 2 to 7 depending on the type of sample (Fig. 2).

### 3.2. Abundance of marker peptides in raw meat cuts

The optimized method was used for examination of the abundances of peptide markers in raw meats. Different types of cuts of pork (i.e. knuckle, shoulder, loin, tenderloin and ham), beef (i.e. clod, rib, shank, tenderloin, sirloin and round) and chicken (i.e. breast, thigh and drumstick) were analyzed. For each type of meat cut, samples were obtained from three individual animals and analysed separately, in order to assess the level of individual variability. Significant differences in the Mb peptide concentrations were observed between different types of pork cuts (Fig. 3 A,B). Both pork markers

(GHPETLEK and HPGDFGADAQGAMSK) were most concentrated in knuckle and shoulder, and the lowest MS signals were detected for ham and loin. Mb is an oxygen transporting protein, containing heme pigments responsible for the red color of muscles, and the Mb concentration in porcine muscles has been used for classifying them as 'red' (containing >40% red fibers) or 'white' (containing <30% red fibers) muscles (Beecher, Cassens, Hoekstra, & Briskey, 1965). It is known that in pigs the Mb concentration can depend on the metabolic type of a muscle (Lindahl, Lundström, & Tornberg, 2001). Here, we used those physiological differences to identify different cuts of raw meats that are commercially available from food retailers. Importantly, we have shown that, in general, the differences in the Mb markers concentrations between different types of cuts are more significant than the individual variabilities measured for particular types of cuts. This brings about the possibility of using the method for authentication of the types of pork cuts that are offered by meat producers and/or retailers to customers. This is especially important as the price of raw pork can differ considerably depending on the type of cut (EC-MMO, 2017; USDA, 2018), which can create a room for fraud.

We also analyzed Mb markers in different cuts of raw beef (Fig. 3 C,D). In contrast to pork (Fig. 3 A,B), no significant differences in the abundance of peptide markers were observed. In fact, the individual variabilities assessed for particular types of cuts were more significant than differences between different types of cuts. This observation can be supported by the long-known data published by Ginger et al. (Ginger, Wilson, & Schweigert, 1954), which found that the Mb concentrations in beef rib and round did not differ significantly; however, different cuts of beef were not analyzed in that study. This indicates that in beef products the amount of peptide markers can solely depend on the amount of beef, regardless of the type of cut used.

We also examined chicken meat. For this purpose, the peptide markers that originated from two different myosins were analyzed (P02506 and P02609, Table 2). Myosins are more abundant than myoglobins in chicken meat (Nićiforović, Radojčić, & Milosavljević, 1999) and have been recognized before as an adequate source of biomarker peptides for LC-MS/MS (Sentandreu, Fraser, Halket, Patel, & Bramley, 2010), although their abundance in chicken meat has not been reported so far. We did not observe significant differences in the concentrations of the peptides between different types of raw chicken meat cuts (Fig. 3 E,F). As for the analysis of beef, more significant was the individual variability in the MS signal recorded for any particular type of the three types of cuts tested, especially for peptide GADPEDVIMGAFK (Fig. 3F).

### 3.3. Abundance of marker peptides in processed meat products

Having examined the raw meats, we looked at whether similar differences in the abundance of peptide markers can be observed for meat products made of single types of cuts. We focused on pork as it is most commonly used in processed meat products in Europe (Marquer, Rabade, & Forti, 2015). Pork was also the only type of meat in our study that showed different levels of abundance of the Mb marker peptides in different types of raw cuts. Basic characteristics of the composition and the manufacturing processes of all the analyzed meat products have been shown in Table 1. All of them are commercially available in Poland and for export to other European countries.

Despite different combinations of the processing methods used in manufacturing of the meat products (Table 1), the overall abundance of the marker peptides showed a pattern (Fig. 4) similar to that observed for the raw pork cuts (Fig. 3 A,B). Namely, the highest MS signals were observed for (i) processed knuckle and shoulder, and the lowest for (ii) the products made of tenderloin, loin and ham (Fig. 4). The differences were significant between these two groups of products and consistent for both markers analyzed. However, the MS signals were reduced by roughly two orders of magnitude as compared to the raw meats (Fig. 3 A,B).

Considering only non-significant differences in the MS signal for different types of raw beef (Fig. 3 C,D) or chicken (Fig. 3 E,F) cuts, we did not apply the method in analyzing products made of single cuts of these meat species.

## 4. Conclusions

To our knowledge, this is the first time an analytical method for differentiation of raw and processed pork cuts has been described in the scientific literature. We have optimized the MRM-MS method reported by Watson et al. (Watson, Gunning, Rigby, Philo, & Kemsley, 2015) to provide a more reproducible and sensitive procedure for detection of marker peptides, so it could be used in analyzing both raw and processed meats. We have demonstrated that physiological differences in the concentration of certain marker peptides between different muscles can be used for authentication of a type of meat cut. This was only possible for pork as there were no significant differences in the concentration of the Mb peptides between different types of beef cuts or in the concentration of the My peptides between different types of chicken meat cuts.



The high reproducibility of results obtained with the optimized method allows for its application in developing protocols for authentication of commercially available raw pork cuts and meat products made of different types of pork cuts. The ability of differentiating between processed ham, loin and tenderloin vs processed shoulder and knuckle seems to be of great importance as the first group of meat products is, in general, more expensive to customers than the second one (EC-MMO, 2017; USDA, 2018 ). This may create a room for fraud in production and trading of processed pork meat products.

Our results can contribute to developing robust and reliable analytical methods that are efficient in protecting customers against meat adulteration. The work presented here will serve as a guideline for developing a quantitative method for analyzing more complex meat products that contain different meat species.

### Acknowledgements

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### Supplementary material

Figure S1.

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ACCEPTED MANUSCRIPT



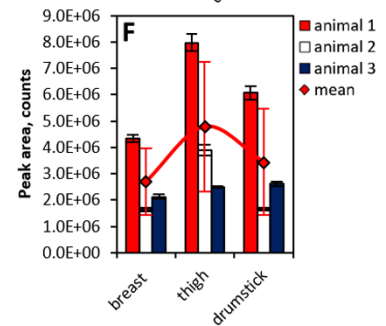
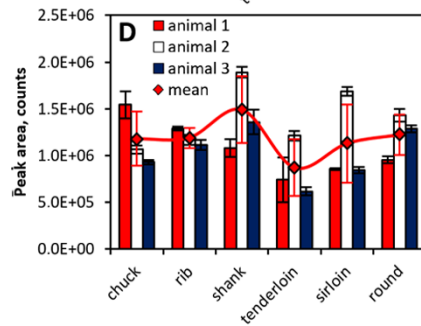
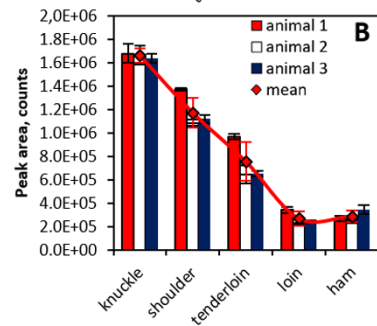
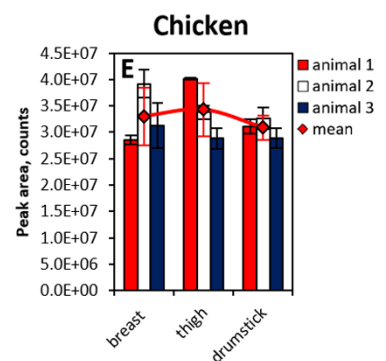
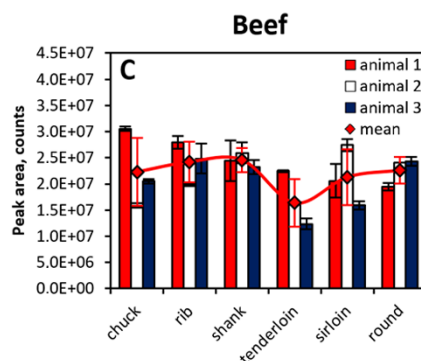
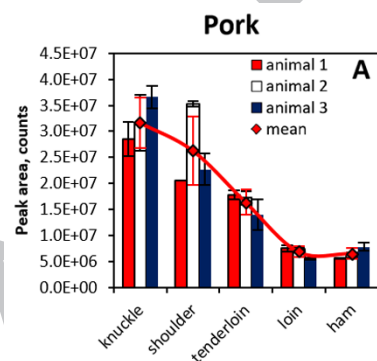
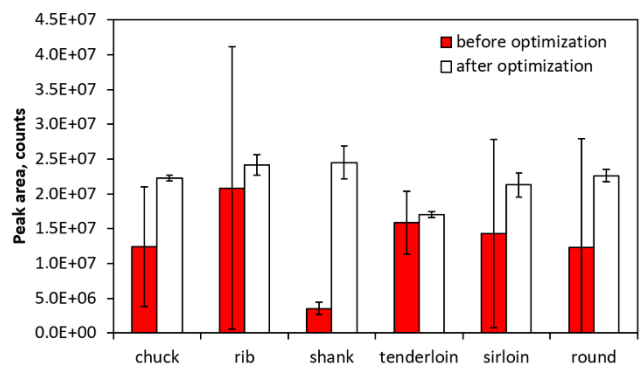
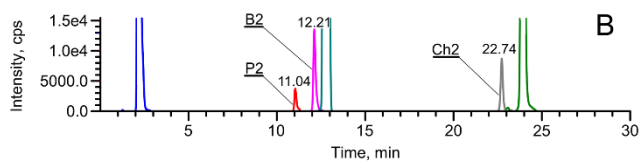
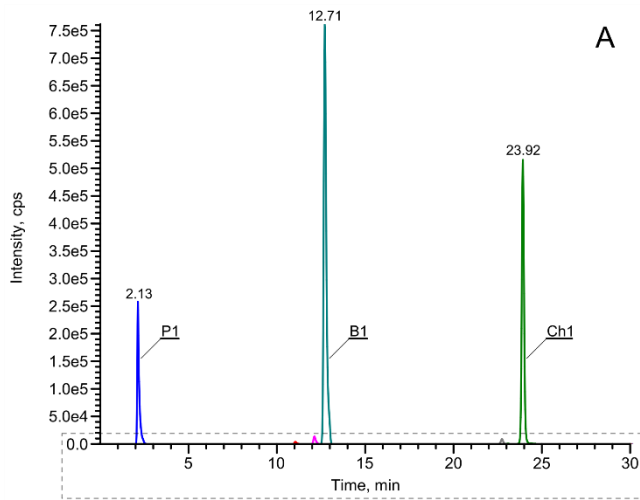
## Figure Captions

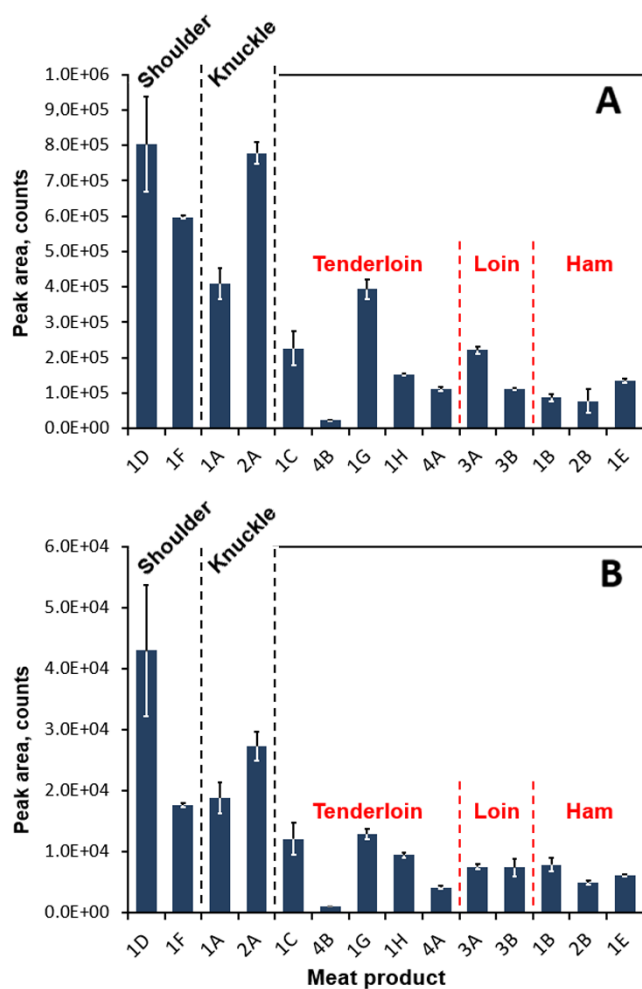
Fig. 1. Representative data obtained in the scheduled MRM mode showing retention times and signal intensities for peptide markers detected in samples of meat products (A). Chromatograms obtained separately for pork, beef and chicken samples have been overlaid. Enlarged view of the area enclosed in the dashed rectangle is shown in B. For clarity, only the most intense MRM transitions of the peptide markers have been shown. Peptide markers: P1, primary pork marker (GHPETLEK); P2, confirmatory pork marker (HPGDFGADAQGAMSK); B1, primary beef marker (VLGFHG), B2, confirmatory beef marker (HPSDFGADAQAAMSK); Ch1, primary chicken marker (DQGTFFEDFVEGLR), Ch2, confirmatory chicken marker (GADPEDVIMGAFK) (for more information see Table 2).

Fig. 2. Representative comparison of repeatability and sensitivity of the results obtained before and after optimization of the method. The primary marker B1 (VLGFHG) was analyzed in raw beef samples. Data are presented as means  $\pm$  SD ( $n = 3$ ).

Fig. 3. Abundance of Mb or My peptide markers in different types of raw (A, B) pork, (C, D) beef and (E, F) chicken meat cuts. Peptide markers: (A) P1, GHPETLEK; (B) P2, HPGDFGADAQGAMSK; (C) B1, VLGFHG, (D) B2, HPSDFGADAQAAMSK, (E) Ch1, DQGTFFEDFVEGLR; (F) Ch2, GADPEDVIMGAFK (for more information see Table 2). An individual bar shows the mean  $\pm$  SD from triplicate analyses of different samples of the same cut obtained from an individual animal. The diamonds represent means  $\pm$  SD for three individual animals the particular types of meat cuts were obtained from (lines are a guide to the eye only).

Fig. 4. Abundance of Mb peptide markers ((A) GHPETLEK, (B) HPGDFGADAQGAMSK) in processed meat products made of different types of single pork cuts. The products characteristics have been given in Table 1. Data are presented as means  $\pm$  SD ( $n = 3$ ).





## Highlights:

- HPLC-MS/MS analysis (MRM mode) was optimized for detection of meat protein markers
- Distribution of myoglobin peptides in pork depends on the type of meat cut
- Myoglobin can be used in authentication of the type of pork cut in meat products
- HPLC-MS/MS (MRM mode) can serve for authentication of raw and processed pork cuts