Authentication of processed meat products by peptidomic analysis using rapid ambient mass spectrometry

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ABSTRACT

We present the application of a novel ambient LESA-MS method for the authentication of processed meat products. A set of 25 species and protein-specific heat stable peptide markers has been detected in processed samples manufactured from beef, pork, horse, chicken and turkey meat. We demonstrate that several peptides derived from myofibrillar and sarcoplasmic proteins are sufficiently resistant to processing to serve as specific markers of processed products. The LESA-MS technique required minimal sample preparation without fractionation and enabled the unambiguous and simultaneous identification of skeletal muscle proteins and peptides as well as other components of animal origin, including the milk protein such as casein alpha-S1, in whole meat product digests. We have identified, for the first time, six fast type II and five slow/cardiac type I MHC peptide markers in various processed meat products. The study demonstrates that complex mixtures of processed proteins/peptides can be examined effectively using this approach.

Keywords:

Food adulteration, Ambient Mass Spectrometry, Liquid Extraction Surface Analysis Mass Spectrometry, Peptide markers, Fast and slow type MHC isoforms

1. Introduction

Despite the existence of extensive mandatory regulations in most countries, food adulteration is still a global issue which attracts attention at international level and increases public concern regarding food quality. In 2013, the horse meat scandal revealed the weaknesses in the food safety system and contributed to a decrease of confidence in the food industry. Fraudulent practices, i.e. the presence of undeclared horse DNA in food products labeled as containing beef, were confirmed in 4.66% and 0.61% of controlled foods in 2013 and 2014, respectively (European Commission, 2014) as a result of tests in the 28 EU countries. Recent studies have revealed an even higher level of food mislabeling, for example 68% mislabeling was found in sausages, burger patties and meats collected from butcheries and retail outlets in South Africa (Cawthorn, Steinman, & Hoffman, 2013) and in seafood in the USA, a rate of 33% of investigated samples were mislabeled according to U.S. Food and Drug Administration (FDA) guidelines (Kimberly, Walker, Lowell, & Hirshfield, 2013). Similarly, the results of inspections carried out in Poland in 2011 by the Office of Competition and Consumer Protection (UOKiK) and Department of Trade Inspection revealed that 24.7% of the examined batches of luxury processed meat products, i.e. conventional, traditional and organic products sold at high prices, were adulterated/labeled incorrectly (UOKiK, 2012). Continuous monitoring of food quality and safety is now mandatory in the EU and other countries but the increasing sophistication of adulteration means that analytical methods require continuous improvement to ensure effective fraud detection. The rigorous analysis of complex and processed products requires the development of novel analytical methodology which has potential for high-throughput analysis and provides rapid, specific and reliable results.

At present, established methods for meat speciation are based on ELISA and PCR techniques, which are robust when applied to raw or moderately processed samples (Chen & Hsieh, 2000; Ballin, Vogensen, & Karlsson, 2009; Fajardo, González, Rojas, García, & Martín,

2010; Köppel, Eugster, Ruf, & Rentsch, 2012). The reported lower efficiency of these methods in highly processed samples has been linked to processing conditions, thermal denaturation and degradation of the markers compounds monitored (typically DNA or protein epitope) and problems with cross-reactivity between species giving unreliable results (Arslan, Irfan-Ilhak, & Calicioglu, 2006; Şakalar, Abasiyanik, Bektik, & Tayyrov, 2012; Musto, Faraone, Cellini, & Musto, 2014). The difficulty with reliable multiplex detection in a single test and contamination of DNA from other organisms also place severe limitations on analysis of complex samples. However, some proteins are quite resistant to heating (Buckley, Collins, Thomas-Oates, & Wilson, 2009; Montowska & Pospiech, 2012; Buckley, Melton, & Montgomery, 2013) and hence peptidomic analysis techniques have potential advantages when applied to authenticate processed (cooked) food.

Recently, considerable improvement in mass spectrometry (MS) instrumentation has enabled the detection of peptide markers by liquid chromatography-MS techniques (LC-MS) and this has enabled identification of specific proteins from soybean (Leitner, Castro-Rubio, Marina, & Lindner, 2006), fish (Carrera et al., 2011) and meat species (Buckley et al., 2009; Sentandreu, Fraser, Halket, Patel, & Bramley, 2010; Montowska & Pospiech, 2013; von Bargen, Brockmeyer, & Humpf, 2014). In our previous work, we evaluated ambient MS techniques for standard protein identification in mixtures and for the analysis of meat digests to discriminate between five meat species (Montowska, Rao, Alexander, Tucker, & Barrett, 2014a). Subsequently, we detected heat stable peptide markers derived from meat proteins after thermal denaturation using our previously introduced ambient liquid extraction surface analysis mass spectrometry (LESA-MS) methodology (Montowska, Alexander, Tucker, & Barrett, 2014b).

It is known that the primary structure of some meat proteins is relatively resistant to processing and that certain skeletal muscle proteins are both species- and tissue-specific and

hence there is good potential for the use of specific muscle proteins and peptide markers for meat authentication (Buckley et al., 2009, 2013; Sentandreu & Sentandreu, 2011; Montowska & Pospiech, 2012). We consider that the ease of use and rapid nature of ambient MS has advantages for high-throughput screening of processed food and we wish to explore the potential application of our LESA-MS peptidomic approaches (Montowska et al., 2014a,b) for this purpose. We suggest that the peptidomic analysis can serve as a tool not only for species identification but also for the assessment of the quality of the product. In this study we define 'product quality' as a general term linked with different authenticity issues, such as the detriment of the quality of the product by illegal change of meat to less valuable components of animal origin (e.g. meat of lower class, offal, connective tissue, blood plasma), undeclared plant or milk additives as well as a change in proportion of ingredients. Analysis of myosin isoforms due to their extensive diversity may help to trace some illegal practices in processed meat products.

Unlike highly conserved actin, myosin exhibits extensive variations in vertebrate striated muscles, which is translated into differences in fibre composition and shortening velocity. In adult mammals, pure fibres (slow type I red, and fast type white IIA, IIX, IIB) are expressed by a single myosin heavy chain (MHC) isoform (1, 2A, 2X, and 2B) whereas hybrid fibres may contain several MHC isoforms (1/2A, 2A/1, 2AX, 2XA, 2XB, and 2BX) each encoded by a separate gene (Pette & Staron, 2000). Therefore in this study, besides species identification, we wish to identify heat stable peptides unique to fast and slow type MHC isoforms. Peptidomic analysis may be a viable way to discriminate between the processed meat and non-meat components to examine the quality of the processed meat products.

In this paper, we present the application of our previously established LESA-MS methodology (Montowska et al., 2014a,b) for detection and identification of heat stable beef, pork, horse and poultry peptide markers in various processed meat products. This rapid

peptidomic approach aims to identify heat stable peptides without the need for purification and chromatographic separation. We also describe the application of in-solution tryptic digestion of processed meat samples followed by deposition onto a polymer surface, desorption and direct analysis by LESA-MS for protein/peptide composition of processed meat in order to compare the identified MHC isoforms and select heat stable peptides unique to fast and slow type MHCs.

2. Materials and methods

2.1. Preparation of samples

Meat products (n=18) were purchased at English and Polish supermarkets or manufactured in our own pilot plant. Samples of raw sausages were cooked from chilled in an oven at 190°C for 30 min according to the manufacturers' instructions. In-house processed sausages (3 batches) were prepared in a pilot plant of the Institute of Meat Technology in Poznan (Poland) exclusively from cured pork with the addition of spices and were coarsely minced, smoked and cooked. All samples of about 5 cm length or 5 g were cut from fresh products and kept at -80 °C until further MS analysis. Sample information and details about processing methods and meat composition are given in Table 2.

Washing, digestion and mass spectrometry analysis were performed according to the procedure described previously (Montowska et al., 2014b). Preparation for LESA-MS analysis of samples of processed meat products involved washing procedures followed by digestion. For this purpose, thin sections of sausages (slices of 0.5 g) or 1 g of meat spreads were transferred to glass vials and washed to remove contaminants such as physiological salts, fat, and other soluble low molecular weight compounds. Sample was rinsed twice for 30 s in ethanol/water (70:30) followed by a 15 s wash in ethanol and then by a 30 s wash in methanol/water (90:10). The sample then was rinsed for 2 x 30 s in deionized water, and finally

for 30 s in 100 mM of aqueous ammonium bicarbonate. Washed samples were placed to dry for 30 min in a desiccator.

2.2. In-solution digestion

Dried samples (10 mg) were rehydrated in 100 µL of water and subsequently digested in a solution containing 0.083 µg/µL of trypsin in ammonium bicarbonate at room temperature over a period of 24 h. Digested solution was then centrifuged for 10 min at 13400 rpm, and the supernatant was diluted 10-fold with deionized water. Samples of 1 µL were spotted onto a Permanox slide, 75 x 25 mm (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) and allowed to evaporate in air at room temperature prior to analysis.

2.3. LESA Mass Spectrometry

The LESA source was a TriVersa NanoMate (Advion, Ithaca, NY) coupled to a Thermo Fisher LTQ Velos ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) operated in positive-ion electrospray ionization mode. The NanoMate platform operated at nanoESI tip voltage of 1.6 kV, with a gas pressure of 0.4 psi and a capillary temperature of 190°C. The same spray/extraction solvent acetonitrile/water/formic acid (50:50:1) was used in all LESA experiments. Total solvent extraction volume was 5 µL, dispensed and aspirated volumes were 3.5 and 3.2 µL, respectively. Each data set was collected from a single protein spot. Data-dependent analysis (DDA) tandem MS/MS data were collected in full scan mode with *m/z* range of 50-2000 divided into four segments (*m/z* 60-600, 550-1050, 1000-1550 and 1500-2000), 1 microscan, 100 ms max injection time, AGC mode on. DDA mode as well as standard MS/MS experiments were used for the analysis of samples. Collision-induced dissociation (CID) experiments were performed at a normalized collision energy of 38%. Data were analyzed using Xcalibur software (Thermo Fisher Scientific). For protein and peptide

identification raw files were converted to MASCOT generic format using MSCONVERT provided by the ProteoWizard project (http://proteowizard.sourceforge.net/tools.shtml). The resulting files were searched via MS/MS ions search using MASCOT against the SwissProt and the National Center for Biotechnology Information (NCBInr) databases with the following parameters: trypsin enzyme, taxonomy bone vertebrates, one missed cleavage, peptide mass tolerance of 1.2 Da, MS/MS tolerance 0.6 Da, no modifications, peptide charge 1+, 2+ and 3+. All samples were analyzed at least in three technical replicates. A decoy search was performed automatically and the matches and MASCOT scores were evaluated at 1% of a false discovery rate (FDR) for identity and homology threshold. Selected peptides in FASTA format were searched against the NCBInr database using the protein BLAST alignment research tool and blastp algorithm for species and protein specificity.

3. Results and discussion

3.1. Myofibrillar proteins as a source of heat stable peptide markers

The workflow for the rapid identification of heat stable peptide markers in processed meat products proposed in this study is presented in Figure 1. The methodology involves three main steps: (a) washing thin slices of samples to remove contaminants which would interfere with the electrospray signal, (b) in-solution tryptic digestion, and (c) data collection using LESA-MS/MS with the acquisition time of 4 min. We focused on the identification of the more abundant proteins using readily detectable peptides which were resistant to processing as well as unique to both species and meat protein. Although, we observed that the sequence coverage of proteins for processed meat products was lower compared to raw and cooked meats (Montowska et al., 2014b) we found this methodology robust and specific and competitive to LC-MS methods, especially when monitoring particular heat stable peptides (see following sections). In our opinion three factors enhanced the efficiency of the analysis: (1) washing all

samples, (2) purification/centrifugation to remove undigested material and reduce the mixture complexity as well as effect of ion suppression, and (3) achieving stable nanoelectrospray during MS data acquisition.

We examined the applicability of the LESA-MS method using various commercial processed meat products purchased from supermarkets as well as in-house processed sausages as reference samples. Data sets were collected with data-dependent tandem LESA-MS for screening of whole products digests and with standard MS/MS experiments using a list of 80 ions which was generated in our previously reported studies of cooked beef, pork, horse, chicken and turkey meat (Montowska et al., 2014b). The dominant MHC isoforms found in processed meat products and their MASCOT output scores are shown in Table 1. In the processed samples we identified the same most abundant skeletal muscle and sarcoplasmic proteins as previously detected for cooked meat, such as MHCs, MLCs, actin, tropomyosin, myoglobin, GAPDH, beta-enolase (Montowska et al., 2014b), but the MASCOT scores and sequence coverages were lower in the case of processed products analysed. In this study turkey MHC was classified to the closely related chicken species since no full sequence of turkey myosin has been published and only short fragments are available in the NCBI database.

A list of peptide markers identified in this study for the 18 different processed meat products is presented in Table 2. Most of the observed peptides were identified as heat stable markers belonging to MHCs, MLCs and myoglobin, and were unique to both species and single muscle protein. Predominantly, MASCOT scores were above the homology or identity threshold and all presented peptides were ranked first in the list of matched peptides of MASCOT results. Figure 2 shows a typical MS/MS spectrum of pork fast type myosin-1 and myosin-4 marker SALAHAVQSSR (563.67^{2+}) obtained from frankfurters (sample 15).

The limit of detection is a critical step towards quantitative analysis using the peptidomic LESA-MS approach. At present, sensitivity rises when chromatographic separation is

involved, for example pork or horse meat can be detected down to 0.24% in processed products using HPLC-MS//MS method coupled with multiple reaction monitoring (Von Bargen, Brockmeyer, & Humpf, 2014). In the aforementioned article, five peptide markers for processed pork and horse meat have been identified. These findings overlap with our studies, since three of the peptides, i.e., TLAFLFAER (pork), SALAHAVQSSR (pork), and LVNDLTGQR (horse) were identified in cooked meats using LESA-MS/MS as described previously (Montowska et al., 2014b). It is likely, that lower sensitivity compared with LC-MS methods due to dynamic range of protein concentration and the lack of fractionation stage, may be enhanced by the use of the LESA instrument with high resolution/accurate mass/MS^n mass spectrometer.

3.2. Sarcoplasmic proteins as a source of heat stable peptide markers

We also present the detection of heat-stable species-specific markers for sarcoplasmic proteins (glyceraldehyde-3-phosphate dehydrogenase GAPDH, myoglobin, beta-enolase), which can address the needs of species identification (Table 2). Myoglobin peptides were found to be good markers for processed red meats, i.e., beef and horse meat, whereas pork GAPDH peptide WGDAGATYVVESTGVFTTMEK (1125.32^{2+}) is a good marker for the processed products containing pork. This is consistent with previous studies, where enzymatic activity of glycolytic enzymes, for instance GAPDH, was found to be higher in fast-twitch glycolytic muscles (Takekura & Yoshioka, 1987; Okumura et al., 2005). In kabanos sausage (sample 10) and frankfurters (sample 14) milk proteins were detected, and thus two peptides unique to casein alpha-S1 (HQGLPQEVLNENLLR and EPMIGVNQELAYFYPELFR) were identified with significant MASCOT output scores (Table 2). These results are consistent with the product labels since the milk or cheese proteins were included in the list of ingredients. One sample of pork sausages (sample 11) was declared to contain veal at 6%, another two samples

of cocktail sausage (sample 12) and frankfurters (sample 13) were labeled to contain turkey meat at 6% and turkey MRM respectively, however we did not detect cattle and turkey markers using our LESA methodology. We cannot exclude the possibility that the meat content was below the limit of detection of the method. We have already shown in previous work that ambient LESA-MS can detect 10% of cooked cattle, pork, horse, and turkey meat and 5% of chicken meat in a beef matrix (Montowska et al., 2014b). Horse sausage (sample 9) turned out to be made not only from horse meat but also from pork. However there was no list of ingredients on the label, hence we cannot confirm the adulteration of this product.

3.3. Discrimination between fast and slow type MHC isoforms in processed meat products

Having identified markers for protein and species identification in processed meat products our next stage was to discriminate between fast and slow type MHCs to identify heat stable peptides unique to these isoforms. For this purpose, the same data sets as for meat speciation obtained with DDA LESA-MS/MS were analysed individually. Similarly, each potential marker was searched against the NCBInr database with the BLAST tool for isoform specificity. Table 3 shows six fast type II and five slow/cardiac type I MHC unique peptides identified for beef, pork and horse meat using this approach. Examples of type I myosin-7 peptides obtained from cooked ham (sample 1) and fried horse sausage (sample 8) are shown in Figure 3 as fragmented spectra for pig LLSNLFANYAGADTPVEK (962.10^{2+}) and horse MLSNLFANYLGADAPIEK (984.29^{2+}). As far as we are aware, this is the first time that the peptides specific to slow-twitch type 1 myosin-7 isoform were identified in processed meat products.

Frequent detection of slow MHC isoform and peptides unique to slow type isoforms over the fast MHC isoforms implies that processed pork and beef products investigated in this study were manufactured mainly from smaller red or intermediate muscles. In our previous studies,

protein/peptide differentiation between samples of cattle, pig and horse *longissimus dorsi* muscle was performed (Montowska et al., 2014a,b). Since this muscle is composed mostly of fast, white fibres, peptides unique to fast myosin-1(2X) and myosin-4(2B) isoforms were detected in those samples with the highest scores. In two samples of potted beef and corned beef (samples 3 and 4) we were able to detect only slow myosin-7 isoform. The peptide GQNVQQVVYAK, unique to beef myosin-7, was identified in both samples but with the MASCOT scores below the identity and homology threshold (Table 2). This may indicate that: (a) the amount of meat was below the declared content, (b) these products were manufactured from the meat of lower class containing high amount of connective tissue and fat, and/or (c) high amounts of non-meat components, such as collagen preparations, offal and fat were added to the products. Only horse myoglobin was detected in horse sausage (sample 9), therefore the ability/inability to detect markers of specific proteins and tissues may indicate a good/poor quality ingredients in a given product or even an ingredient's substitution.

Because myosin is the most abundant muscle protein and its content corresponds to 40-50% of the total muscle proteins, myosin peptides might be a good indicator of the meat content in the processed products. They also might be used to assess the fibre-type composition of meat components, and thus indirectly to assess the product quality. Although, markers obtained from MHC isoforms, may be a robust tool to indicate the quality of meat ingredients, in our opinion the authentication of processed meat product defined as the quality assessment needs to be based on appropriate and reliable quantitative analysis of several peptide markers of meat and non-meat origin in parallel.

4. Conclusions

We have examined the applicability of a novel and rapid LESA-MS method to identify peptide markers in different types of processed meat products for authentication purposes. The

entire procedure is radically simplified over other peptidomic methodologies by excluding fractionation steps before and after the protein digestion stage. Sample preparation is therefore limited only to the processes of washing and digestion. By the application of data-dependent LESA-MS/MS for fast screening of whole product digests, we were able to identify a set of 25 heat stable peptide markers derived from myofibrillar and sarcoplasmic proteins. We have also proved that this novel method of peptidomic examination from processed meat products has good specificity to readily identify peptide markers for fast and slow type MHC isoforms. We have demonstrated that there is a set of specific peptides resistant to thermal treatment and easily detectable in various industrially processed meat products which not only serve as markers of meat speciation but also can help to track down other illegal practices linked with the substitution of ingredients.

Since this work was focused on authenticity issues, only peptides from the most abundant proteins were identified. However, the use of LESA interfaced with high resolution mass spectrometry may enhance sensitivity sufficiently to enable analysis of less abundant proteins. This easy to use and versatile ambient methodology has great potential to be implemented in the routine, rapid high-throughput screening of processed products, and in addition displays specificity sufficient to enable examination of other important issues in meat science, e.g., variations in muscle metabolism and meat quality.

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409

Table 1. Dominant skeletal myosin heavy chain (MHC) isoforms identified in tryptic digests of processed meat products

No	Sample	Identified protein	NCBI accession number	Matched peptides[a]	Sequence coverage (%)[b]	MASCOT score[c]
1	Cooked ham	myosin-2 *(Sus scrofa)*	gi|55741490	55	20	388
		myosin-1 *(Sus scrofa)*	gi|157279731	51	19	388
		myosin-7 *(Sus scrofa)*	gi|55741486	31	12	226
2	Beef spread	myosin-2 *(Bos taurus)*	gi|75055812	54	21	429
3	Potted beef	myosin-7 *(Bos taurus)*	gi|41386711	25	14	162
4	Corned beef	myosin-7 *(Bos taurus)*	gi|41386711	54	21	519
5	Beef sausage	myosin-2 *(Bos taurus)*	gi|75055812	66	29	419
		myosin-1 *(Bos taurus)*	gi|41386691	66	28	434
6	Chorizo	myosin-1 *(Sus scrofa)*	gi|157279731	50	20	547
		myosin-7 *(Sus scrofa)*	gi|55741486	24	11	190
7	Hunters sausage	myosin-4 *(Sus scrofa)*	gi|178056718	57	24	392
8	Horse sausage	myosin-2 *(Equus caballus)*	gi|126352598	71	26	842
		myosin-7 *(Equus caballus)*	gi|126352320	48	19	702
9	Horse sausage (smoked)	myosin-7 *(Equus caballus)*	gi|126352320	30	14	155
		myosin-1 *(Sus scrofa)*	gi|157279731	20	9	86
10	Kabanos sausage with cheese	myosin *(Gallus gallus)*	gi|13432175	40	19	376
		myosin-4 *(Sus scrofa)*	gi|178056718	38	19	282
11	Pork sausage	myosin-4 *(Sus scrofa)*	gi|178056718	55	22	797
12	Cocktail sausage	myosin-2 *(Bos taurus)*	gi|75055812	54	22	448
13	Frankfurters poultry	myosin *(Gallus gallus)*	gi|13432175	54	21	520
14	Frankfurters classic	myosin-1 *(Sus scrofa)*	gi|157279731	46	16	425
15	Frankfurters	myosin-2 *(Bos taurus)*	gi|75055812	53	20	325
		myosin-1 *(Sus scrofa)*	gi|157279731	39	15	403
16	Hotdogs	myosin-1 *(Sus scrofa)*	gi|157279731	33	10	164
		myosin *(Gallus gallus)*	gi|13432175	23	11	220
18	In-house sausages	myosin-1 *(Sus scrofa)*	gi|157279731	44	17	274

[a]Number of matched peptides in the database search. [b]Percent of coverage of the entire amino acid sequence. [c]MASCOT score at FDR of 1%.

Table 2. Peptide markers for both species and protein identified in processed meat products; all presented peptides were ranked first in the list of matched peptides of MASCOT peptide view results

Sample	Processing method	Declared meat composition	Identified species	Protein	Peptide marker	NCBI accession number	MASCOT ion score[a]	Identity threshold[b]	Homology threshold[c]
1. Cooked ham	sliced, cured, cooked	pork 78%	pig	myosin-1	SALAHAVQSSR	gi|157279731	42	>59	>38
				myosin-2	TLAFLFSGAQTGEAEAGGTK	gi|55741490	41	>60	>57
				myosin-7	LLSNLFANYAGADTPVEK	gi|55741486	86	>64	>49
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	37	>55	>44
2. Beef spread	minced, canned	beef 66%	cattle	myosin-2	TLAFLFSGTPTGDSEASGGTK	gi|75055812	30	>62	>34
				MLC2f	EASGPINFTVFLNMFGEK	gi|115497166	60	>62	>40
				myoglobin	HPSDFGADAQAAMSK	gi|27806939	56	>71	>67
3. Potted beef	minced, canned	beef 67%, beef heart	cattle	myosin-7	GQNVQQVVYAK	gi|41386711	28	>46	>44
				MLC2f	EASGPINFTVFLNMFGEK	gi|115497166	88	>38	>26
4. Corned beef	cured, cooked, corned	beef	cattle	myosin-7	GQNVQQVVYAK	gi|41386711	32	>68	>50
				MLC2f	EASGPINFTVFLNMFGEK	gi|115497166	54	>54	>22
5. Beef sausage	raw, oven cooked from chilled	beef 53%	cattle	myosin-2	TLAFLFSGTPTGDSEASGGTK	gi|75055812	51	>100	>63
				myosin-1	ALEDQLSELK	gi|41386691	38	>40	>39
				MLC2f	EASGPINFTVFLNMFGEK	gi|115497166	87	>54	>28
				myoglobin	HPSDFGADAQAAMSK	gi|27806939	42	>60	>43
6. Chorizo style sausage	raw, oven cooked from chilled	pork 87%	pig	myosin-1	SALAHAVQSSR	gi|157279731	61	>64	>51
				myosin-7	LLSNLFANYAGADTPVEK	gi|55741486	62	>60	>33
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	98	>58	-
7. Hunters sausage	smoked, cooked, roasted	pork 70%, beef 20%	pig	myosin-4	SALAHAVQSSR	gi|178056718	60	>60	>50
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	112	>61	-
			cattle	MLC2f	EASGPINFTVFLNMFGEK	gi|115497166	19	>35	>13
8. Horse sausage	raw, oven cooked from chilled	horse meat	horse	myosin-2	VVETMQTMLDAEIR	gi|126352598	87	>58	-
				myosin-7	MLSNLFANYLGADAPIEK	gi|126352320	83	>58	>40
				myoglobin	GLSDGEWQQVLNVWGK	gi|7546624	72	>56	>36
				myoglobin	VEADIAGHGQEVLIR	gi|7546624	67	>54	-
				myoglobin	HGTVVLTALGGILK	gi|7546624	99	>59	-
9. Horse sausage	smoked, cooked, dried	no data	pig	myosin-1	SALAHAVQSSR	gi|157279731	16	>40	>24
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	63	>35	>17
			horse	myoglobin	GLSDGEWQQVLNVWGK	gi|7546624	59	>38	>22
				myoglobin	VEADIAGHGQEVLIR	gi|7546624	80	>38	-
				myoglobin	HGTVVLTALGGILK	gi|7546624	83	>40	-

10. Kabanos sausage with cheese	smoked, cooked, dried	chicken meat 58%, pork 12%, cheese 7.5%	chicken	myosin	VAEQELLDATER	gi|13432175	81	>44	>43
				MLC1/3f	DQGTFEDFVEGLR	gi|212330	39	>59	>44
				MLC2f	GADPEDVIMGAFK	gi|223047	58	>60	-
			pig	myosin-4	SALAHAVQSSR	gi|178056718	43	>44	>43
			cattle	casein alpha-S1	HQGLPQEVLNENLLR	gi|225632	43	>60	>36
				casein alpha-S1	EPMIGVNQELAYFYPELFR	gi|225632	48	>58	>26
11. Pork sausage	smoked, cooked	pork 92%, veal 6%	pig	myosin-4	SALAHAVQSSR	gi|178056718	64	>44	-
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	88	>45	>27
12. Cocktail sausage	cured, cooked	beef 60%, turkey meat 6%	cattle	myosin-2	TLAFLFSGTPTGDSEASGGTK	gi|75055812	70	>74	>53
				MLC2f	EASGPINFTVFLNMFGEK	gi|115497166	62	>69	>41
				myoglobin	HPSDFGADAQAAMSK	gi|27806939	51	>55	>40
13. Frankfurters poultry	cooked, smoked	chicken & turkey MRM 65%	chicken	myosin	GQTVSQVHNSVGALAK	gi|13432175	60	>56	>46
				myosin	TLALLFATYGGEAEGGGGK	gi|13432175	15	>59	>31
				myosin	VAEQELLDATER	gi|13432175	79	>56	>52
				MLC1/3f	DQGTFEDFVEGLR	gi|212330	63	>58	>54
				MLC2f	GADPEDVIMGAFK	gi|223047	62	>60	-
				beta-enolase	AAIAQAGYTDK	gi|46048765	51	>59	>36
14. Frankfurters classics	smoked	pork 71%, milk proteins	pig	myosin-1	SALAHAVQSSR	gi|157279731	61	>63	-
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	71	>57	-
			cattle	casein alpha-S1	HQGLPQEVLNENLLR	gi|225632	91	>59	>48
				casein alpha-S1	EPMIGVNQELAYFYPELFR	gi|225632	81	>59	>48
15. Frankfurters	cooked, smoked	veal 50%, pork 28%	cattle	MLC2f	EASGPINFTVFLNMFGEK	gi|115497166	73	>37	>18
			pig	myosin-1	SALAHAVQSSR	gi|157279731	31	>45	>34
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	65	>40	>22
16. Hotdogs	cooked, smoked	pork 40%, chicken 18%	pig	myosin-1	SALAHAVQSSR	gi|157279731	47	>62	>41
			chicken	myosin	VAEQELLDATER	gi|13432175	57	>58	>47
				MLC1/3f	DQGTFEDFVEGLR	gi|212347	81	>68	-
				MLC2f	GADPEDVIMGAFK	gi|223047	90	>60	-
17. Roast turkey	sliced, cooked, roasted	turkey meat 84%	turkey	MLC1f	ALGQNPTNAEMNK	gi|326922419	79	>59	>42
				troponin C	PSMTDQQAEAR	gi|136044	42	>63	>41
18. In-house sausages	smoked, cooked	pork	pig	myosin-1	SALAHAVQSSR	gi|157279731	41	>37	-
				GAPDH	WGDAGATYVVESTGVFTTMEK	gi|329744642	63	>54	-
				beta-enolase	NYPVVSIEDPFDQDDWK	gi|113205498	53	>57	>53

[a]MASCOT score at FDR of 1%. [b,c]Individual ion scores to indicate identity or extensive homology.

Table 3. Peptides unique to fast and slow type MHC isoforms

Species	Protein	Peptide marker
MHC type II fast		
cattle	myosin-1(2X)	ALEDQLSELK
	myosin-2(2A)	MEIDDLASNVETISK
	myosin-2(2A)	TLAFLFSGTPTGDSEASGGTK
horse	myosin-2(2A)	VVETMQTMLDAEIR
pig	myosin-4(2B) myosin-1(2X)	SALAHAVQSSR
	myosin-2(2A)	TLAFLFSGAQTGEAEAGGTK
MHC type I slow/cardiac		
cattle	myosin-7	SAETEKEIALMK
	myosin-7	GQNVQQVVYAK
horse	myosin-7	GTLEDQIIEANPALEAFGNAK
	myosin-7	MLSNLFANYLGADAPIEK
pig	myosin-7	LLSNLFANYAGADTPVEK

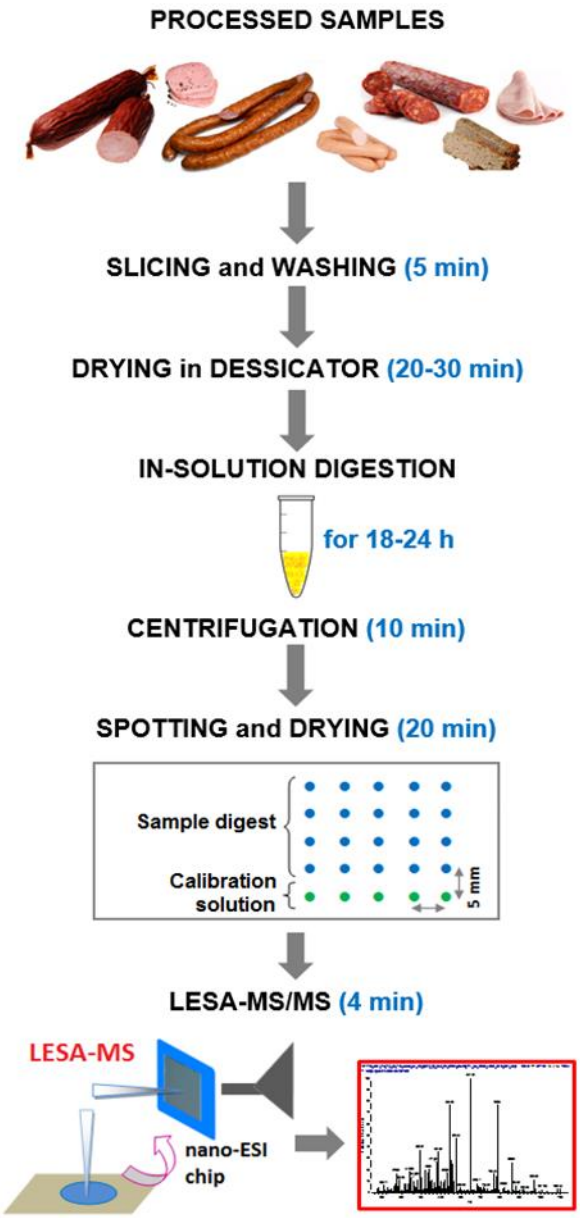

Figure 1. Analytical workflow for fast detection of peptide markers in processed meat products using LESA-MS/MS.

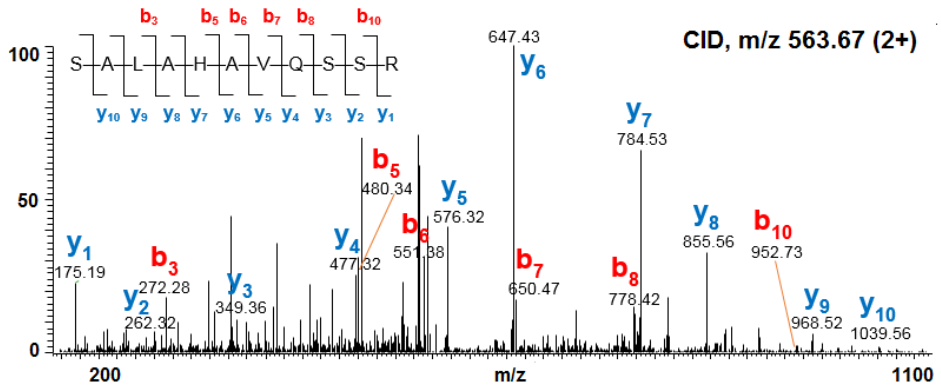

Figure 2. Example of sequenced spectrum of the pork myosin-1 and myosin-4 peptide SALAHAVQSSR (563.67^{2+}), fast type isoforms, obtained from frankfurters made from veal and pork (sample 15) using LESA-MS/MS.

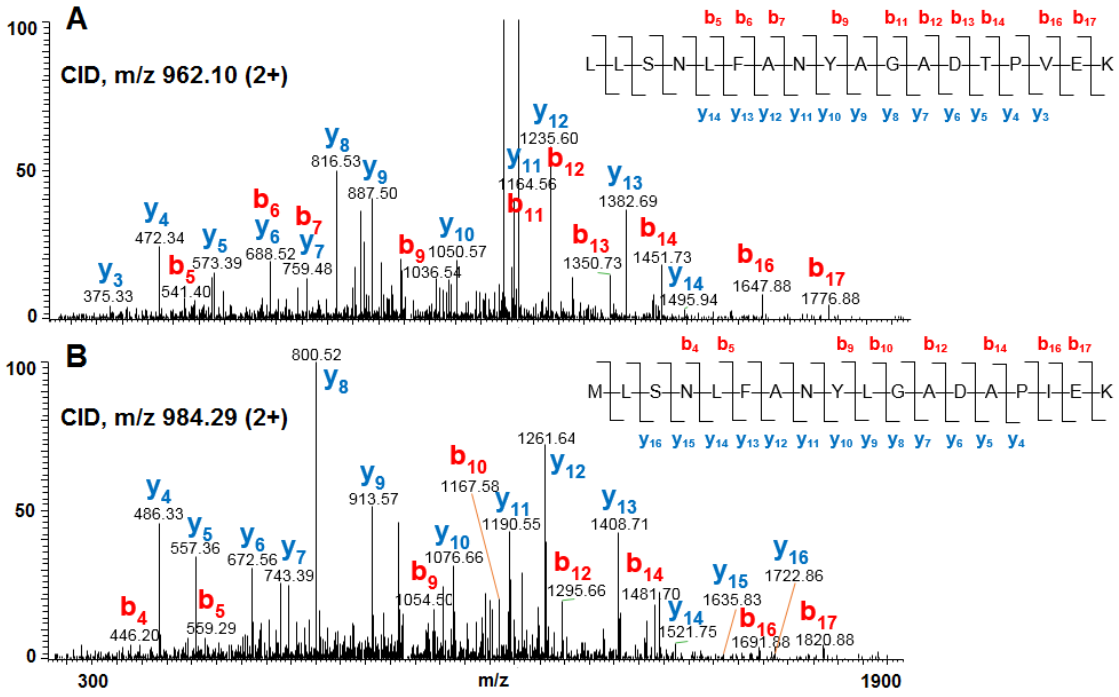

Figure 3. Sequenced LESA-MS/MS spectra of slow type 1 myosin isoform; (A) pig myosin-7 peptide LLSNLFANYAGADTPVEK (962.10^{2+}) obtained from cooked ham (sample 1); (B) horse myosin-7 peptide MLSNLFANYLGADAPIEK (984.29^{2+}) obtained from horse sausage (sample 8) cooked in oven.