analytical chemistry

Article

pubs.acs.org/ac

Rapid Detection of Peptide Markers for Authentication Purposes in Raw and Cooked Meat Using Ambient Liquid Extraction Surface Analysis Mass Spectrometry

Magdalena Montowska,*[,†,‡] Morgan R. Alexander,[§] Gregory A. Tucker,[#] and David A. Barrett*[,†]

[†]Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

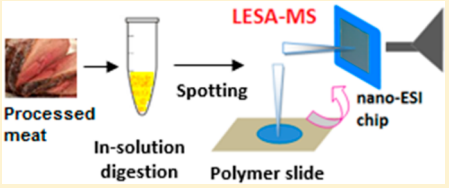
[‡]Institute of Meat Technology, Poznan University of Life Sciences, Wojska Polskiego 31, Poznan 60-624, Poland

[§]Laboratory of Biophysics and Surface Analysis, School of Pharmacy, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

[#]Division of Nutritional Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Leics LE12 5RD, United Kingdom

Supporting Information

ABSTRACT: In this Article, our previously developed ambient LESA-MS methodology is implemented to analyze five types of thermally treated meat species, namely, beef, pork, horse, chicken, and turkey meat, to select and identify heat-stable and species-specific peptide markers. In-solution tryptic digests of cooked meats were deposited onto a polymer surface, followed by LESA-MS analysis and evaluation using multivariate data analysis and tandem electrospray MS. The five types of cooked meat were clearly discriminated using principal component analysis and orthogonal partial least-squares discriminant analysis. 23 heat stable peptide markers unique to species and muscle protein were identified following data-dependent tandem LESA-MS analysis. Surface extraction and direct ambient MS analysis of mixtures of cooked meat species was performed for the first time and enabled detection of 10% (*w*/*w*) of pork, horse, and turkey meat and 5% (*w*/*w*) of chicken meat in beef, using the developed LESA-MS/MS analysis. The study shows, for the first time, that ambient LESA-MS methodology displays specificity sufficient to be implemented effectively for the analysis of processed and complex peptide digests. The proposed approach is much faster and simpler than other measurement tools for meat speciation; it has potential for application in other areas of meat science or food production.



Manufacturers are required to correctly label food products. Appropriate and authentic information is a vital link in ensuring the safety of the food chain, especially in the case of processed food, where it is often difficult to confirm the nature and proportion of constituents. Consumer awareness of food adulteration is continually increasing thanks to the publicity of the cases of fraud detection, such as the recent substitution of horse meat for beef in Europe. In the case of meat products, adulteration most frequently refers to the substitution of meat of high quality by less valuable components. Apart from economic reasons, adulteration of meat products is a problem for moral and religious reasons, as well as the potential danger of serious allergic responses to meat proteins. It is well-established that certain individuals can develop an immune response to proteins, such as serum albumin, actin, myoglobin, and tropomyosin, derived from specific animal species (e.g., bovine, pork, or chicken meat) and that thermal treatment does not reduce the allergenicity of these proteins.[1,2] Therefore, new, robust, rapid, and effective authentication methods require development to ensure the accuracy of food labeling and the security of the food safety system.[3] The ability for a rapid analysis is a crucial issue in the case of nondurable food products.

Many proteomic and genomic methods for species identification have been developed since the mid-1980s. Electrophoretic techniques, enzyme-linked immunosorbent assays (ELISA), mass spectrometry (MS), and polymerase chain reaction (PCR) techniques have been employed.[4,5] However, the application of these established methods to routine analysis of complex and processed products is not always possible and can be ineffective. Electrophoretic techniques are expensive, time-consuming, and only semi-quantitative. Enzymatic methods are sensitive but there are also some severe limitations such as cross-reactivity between species and the susceptibility of the conformational epitopes because of processing effects (e.g., heating). PCR tests are most often used for meat speciation but suffer from time-consuming extraction methods and with a severe limitation as a result of potential contamination of DNA from other organisms. Recently, chemometric analyses using mid-infrared spectroscopy (MID-FTIR) and Fourier transform Raman spectroscopy (FT-Raman) have been suggested as a means to discriminate

Received: July 3, 2014
Accepted: September 26, 2014
Published: September 26, 2014

tissues in minced meat mixtures.[6,7] However, these techniques have not been tested on processed meat products. Chemometric approaches investigate the variance in the chemical composition of the tissue (e.g., content of amino acids, glycogen, fatty acids, and water) and therefore its successful application to complex and processed food matrices is potentially difficult.

Processing conditions, especially high temperature above 100 °C, affect the protein and DNA structure resulting in considerable denaturation of proteins and degradation of DNA. Thus, detection and quantification of meat species in processed samples using real-time PCR is affected by the temperature and size of the amplified DNA fragment.[8] However, the primary structure of proteins is relatively resistant to processing, for example, it is known that proteins survive better than DNA in archeological and heated samples.[9] In our previous work, using traditional proteomic techniques, we noted that in muscle some proteins were only minimally degraded following heating/processing, which enabled us to observe interspecies patterns of specific proteins from various processed meat products.[10,11] Meat proteins thus show robustness to heating and processing and hence have good potential to be used as markers in food authentication. Recently, liquid chromatography–mass spectrometry techniques (LC-MS) have been applied to species identification of fish and meat species on the basis of peptides derived from parvalbumins,[12] bone collagen,[9] and skeletal muscle and sarcoplasmic proteins,[13,14] as well as to identify soybean proteins markers to monitor the addition of soybean protein isolate to meat products.[15] We propose the use of ambient MS for the detection of peptide markers of cooked and processed meat proteins due to its rapid nature and minimal requirement for sample preparation.

Ambient MS techniques have potential for high-throughput screening of food samples because of their ability to operate at atmospheric pressure, and therefore they allow for rapid detection of compounds directly from a biological surface. One of the recently introduced ambient MS techniques is liquid extraction surface analysis mass spectrometry (LESA-MS), a chip-based nanoelectrospray technique, which involves the formation of a liquid/surface microjunction by dispensing an extraction solution on the surface of the sample.[16] To date, only a few proteomic applications of LESA-MS have been reported, for example analysis of hemoglobin variants from dried blood spots[17] and direct analysis of brain tissue sections for protein identification.[18] Previously, in our laboratory, mixtures of standard proteins deposited onto a polymer surface have been investigated using ambient desorption electrospray ionization (DESI-MS) and LESA-MS.[19,20] In the latter paper[20] LESA-MS was shown for the first time to identify skeletal muscle proteins in raw meat digests, and consequently, five meat species were individually discriminated using our methodology.

Having shown the potential of ambient ionization techniques for direct identification of skeletal muscle proteins and discrimination between species, we now wish to evaluate the applicability of LESA-MS in more advanced proteomic applications such as rapid detection and identification of peptide markers derived from thermally treated (cooked) meat species. In this Article, we present the development of LESA-MS methodology for rapid detection of heat stable peptide markers for five meat species, cattle, pig, horse, chicken, and turkey. Raw and cooked meat samples of whole meat digests, dried, and desorbed directly from a polymer surface are evaluated for selection and identification of specific peptides without the need for time-consuming sample preparation and LC-MS analysis. We examine the possibility of identification of markers specific to both animal species and skeletal muscle, those easily detectable after thermal denaturation. Subsequently, mixtures of cooked meat species are analyzed using multivariate data analysis and LESA-MS/MS to assess the sensitivity of the method.

MATERIALS AND METHODS

Materials. Water, acetonitrile, formic acid, and dithiotreitol (DTT) and iodoacetamide (IAA) were purchased as MS grade from Sigma-Aldrich (Gillingham, UK). Ammonium bicarbonate was purchased from BHD Chemicals (Poole, UK). Sequence grade modified trypsin was bought from Promega (Southampton, UK). Meat samples of five species, namely, cattle (*Bos taurus*), horse (*Equus caballus*), pig (*Sus scrofa*), chicken (*Gallus gallus*), and turkey (*Meleagris gallopavo*) were examined in the present study. The samples of *longissimus* muscle or *pectoralis* muscle (poultry) were purchased locally. Samples of about 5 g were cut from the raw and cooked meat and kept at −80 °C until further MS analysis.

Tissue Preparation. Washing, digestion, and mass spectrometry analysis have been performed according to our procedure described previously[20] with minor changes. Meat slices of about 25 mm in thickness were wrapped in aluminum foil and heated in a dry oven at 160 °C for 30 min. Samples of about 5 g were cut and stored at −80 °C. Preparation for LESA analysis of meat samples involved further washing procedures followed by digestion. For this purpose, thin raw and cooked meat sections were transferred to glass vials and washed to remove contaminants, such as physiological salts, fat, and other soluble low molecular weight compounds according to Aerni et al.[21] with modifications as described here. Slice of tissue (0.5 g) 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). Tissue then was rinsed for 2 × 30 s in deionized water, and finally for 30 s in 100 mM ammonium bicarbonate. Washed sliced were placed to dry for 30 min in a desiccator.

Preparation of Meat Mixtures. Meat mixtures were prepared from washed and dried cooked meats. Samples containing two meat species were prepared by weighting respective amounts of meat to obtain beef samples spiked with 10%, 5%, and 1% of other species. A total of 10 mg of mixture prepared from beef and 1, 0.5, and 0.1 mg of horse meat or pork, chicken, turkey meat was weighed in a 1.5 mL Eppendorf tube and was subjected to the process of digestion.

In-Solution Digestion. Dried raw and cooked meat (0.5 mg) were rehydrated in 100 μL of water; 0.25 M DTT was added as a reducing agent and incubated for 1 h at 56 °C and further alkylated by addition of 0.25 M iodoacetamide (IAA) and incubated in the dark for 30 min at room temperature. The excesses of DTT and IAA were removed by filtration using 3 kDa Amicon Ultra-0.5 centrifugal filters (Millipore Merck KGaA, Darmstadt, Germany), followed by washing twice with water. The concentrated sample was digested in a solution containing 0.05 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 and 100-fold with deionized water. Samples of 1 μL were spotted onto a Permanox slide, 75 × 25 mm (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) and

dx.doi.org/10.1021/ac502449w | Anal. Chem. 2014, 86, 10257−10265

allowed to evaporate in air at room temperature prior to analysis. Samples of meat mixtures were not reduced and alkylated since we planned to focus on readily detectable peptides, and to have a simple preparation procedure. Mixtures of 10 mg were directly rehydrated in 100 μL of water and subsequently digested in a solution containing 0.083 μg/μL of trypsin in ammonium bicarbonate.

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. MS data were collected in full scan mode (m/z 400−1000), 1 microscan, 100 ms max injection time, AGC mode on. Data-dependent analysis (DDA) mode as well as standard MS/MS experiments were used for the analysis of all samples. 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). Collision-induced dissociation (CID) experiments were performed at a normalized collision energy of 38%. Data were analyzed using Xcalibur software (Thermo Fisher Scientific) and overviewed using Progenesis MALDI software (Nonlinear Dynamics Limited). 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 following parameters: trypsin enzyme, taxonomy mammals, bone vertebrates for samples containing poultry, one missed cleavage, peptide mass tolerance of 1.2 Da, MS/MS tolerance 0.6 Da, carbamidomethylation of cysteines and variable oxidation of methionines, peptide charge 1+, 2+, and 3+. All samples were analyzed at least in three technical replicates. In order to calculate the charge and exact mass of species-specific ions further MS experiments were carried out using a high resolution Thermo Fisher Exactive Orbitrap mass spectrometer in full scan mode (m/z 400−1000), 1 microscan, 1000 ms max injection time, resolution 100 000 and AGC 500 000.

Multivariate Data Analysis. Data were analyzed as described previously.[20] The raw MS data files of cooked meats and mixtures in-solution digests were imported into SpecAlign (Cartwright Group, PTCL, University of Oxford, UK) for data processing involving normalization and spectral alignment. Processed spectra were imported for multivariate data analysis (SIMCA-P version 13.1, Umetrics, MKS Instruments Inc.). The preprocessed data sets were initially overviewed using principal component analysis (PCA-X, unsupervised) to detect outliers in a model and subsequently analyzed using supervised orthogonal partial least-squares discriminant analysis (OPLS-DA) to create a model to enhance interpretability.

RESULTS AND DISCUSSION

Differentiation between Cooked Meat Species Using Multivariate Data Analysis. LESA-MS parameters, which were optimized previously for raw meat,[20] were tested on cooked meat samples. LESA-MS was applied directly to the dried meat digests on Permanox slides. Average mass spectra of cooked meats with a full m/z range are presented in Figure 1A.

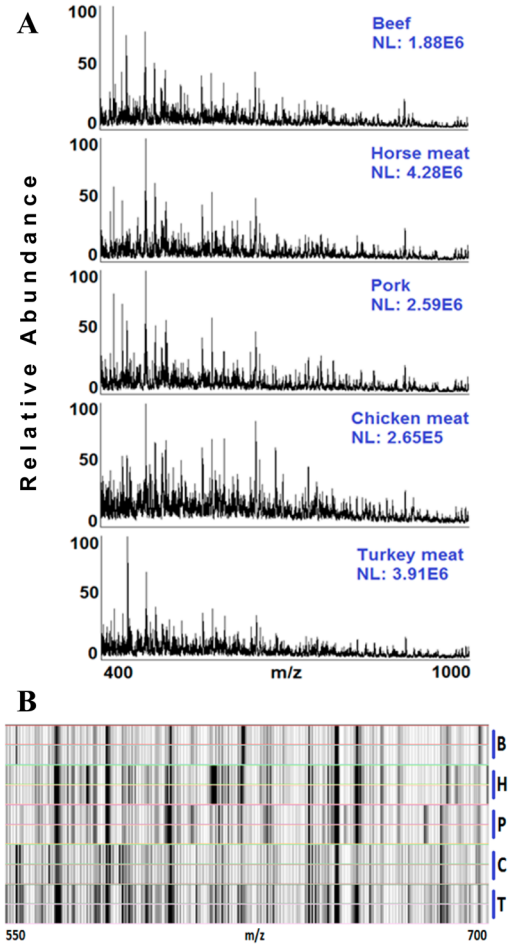


Figure 1. Differentiation between the various types of cooked meat. (A) Average mass spectra of tryptic digests of cooked meats collected using LESA-MS. (B) Fragment of average mass spectrum processed by Progenesis MALDI software showing differences in ions and intensities between five thermally treated meat species. Samples presented in duplicate. Each vertical line represents an individual ion. B, beef; H, horse meat; P, pork; C, chicken meat; T, turkey meat.

Differences in ions and intensities between typical mass spectra within 550−700 m/z range obtained from five cooked meat species are shown in Figure 1B. As reported previously[20] LESA generated complex mass spectra similar to nanoelectrospray spectra (nano ESI-MS) for both raw and cooked meat digests where mostly multiply charged peptide ions were observed. To differentiate between the five types of cooked meat simultaneously, multivariate data analysis was applied to all data sets collected from whole meat digests (Figure 2). Unsupervised PCA was initially performed and was able to discriminate the cooked meat samples with the first four PCA components providing 95% of the total variance (Figure 2A). Subsequently, supervised multivariate data analysis was applied using OPLS-DA for the same data sets and although no further

dx.doi.org/10.1021/ac502449w | Anal. Chem. 2014, 86, 10257−10265

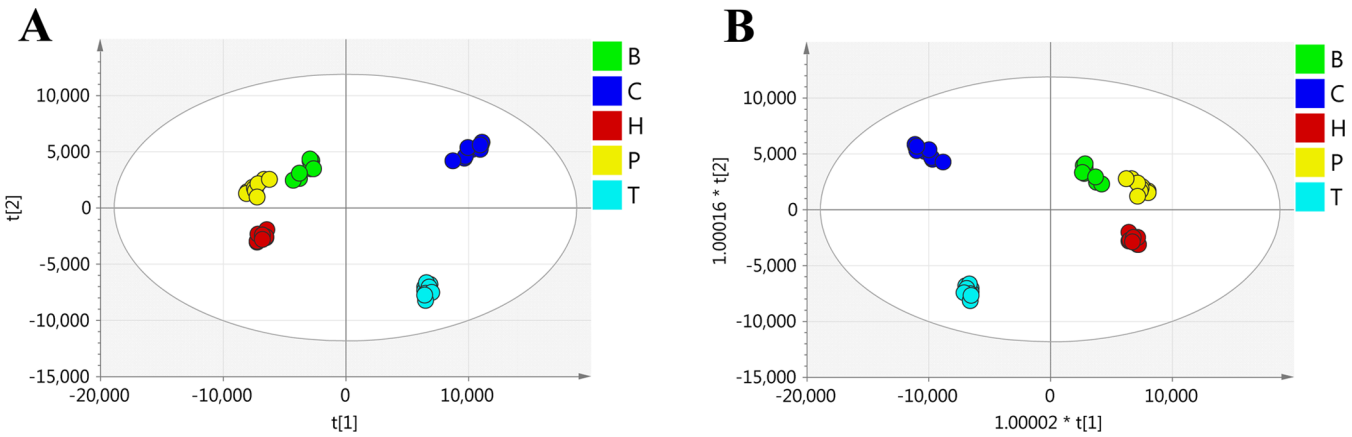


Figure 2. Differentiation between cooked meat species using LESA-MS in the range of m/z 400−1000, n = 50; (A) PCA-X score plots; (B) OPLS-DA score plots. B, beef; H, horse meat; P, pork; C, chicken meat; T, turkey meat.

Table 1. Heat Stable Peptide Markers Unique to the Examined Species and Identified Using the DDA Tandem LESA-MS[a]

parent ion	M_r(expt)	M_r(calcd)	protein	species	NCBI accession number	start−end	peptide sequence
615.17^{2+}	1228.3254	1227.6459	MLC1/3f	horse	gil545218230	30−41	ALGTNPTNAEIK
694.42^{2+}	1386.8254	1386.6561	MLC1/3f	turkey	gil326922419	79−91	ALGQNPTNAEMNK
756.94^{2+}	1511.8654	1511.6893	MLC1/3f	chicken	gil212330	124−136	DQGTFEDFVEGLR
1001.34^{2+}	2000.7654	1999.9713	MLC2f	cattle	gil115497166	74−91	EASGPINFTVFLNMFGEK
675.52^{2+}	1349.0254	1348.6333	MLC2f	chicken	gil223047	90−102	GADPEDVIMGAFK
806.61^{2+}	1611.2054	1610.7610	MLC2f	chicken	gil223047	115−127	SFLEELLTTQCDR
617.52^{2+}	1233.0254	1232.5455	troponin C	turkey	gil136044	1−11	PSMTDQQAEAR
908.81^{2+}	1815.6054	1814.8952	myoglobin	horse	gil7546624	1−16	GLSDGEWQQVLNVWGK
536.29^{3+}	1605.8482	1605.8475	myoglobin	horse	gil7546624	17−31	VEADIAGHGQEVLIR
803.93^{2+}	1605.8454	1605.8475	myoglobin	horse	gil7546624	17−31	VEADIAGHGQEVLIR
690.10^{2+}	1378.4654	1377.8344	myoglobin	horse	gil7546624	64−77	HGTVVLTALGGILK
766.74^{2+}	1531.4654	1531.6725	myoglobin	cattle	gil27806939	104−119	HPSDFGADAQAAMSK
888.67^{2+}	1775.3254	1774.9101	myosin-1	horse	gil126352470	619−637	TLALLFSGPASADAEAGGK
906.66^{2+}	1811.3054	1810.9101	myosin	chicken	gil13432175	619−637	TLALLFATYGGEAEGGGGK
534.40^{2+}	1066.7854	1066.5811	myosin-4	pork	gil178056718	619−627	TLAFLFAER
838.89^{2+}	1675.7654	1675.8338	myosin	chicken	gil13432175	980−994	NLTEEMAVLDETIAK
573.46^{2+}	1144.9054	1144.5975	myosin-1	cattle	gil41386691	1254−1263	ALEDQLSELK
508.43^{2+}	1015.0054	1014.5458	myosin-1	horse	gil126352470	1272−1280	LVNDLTGQR
563.67^{2+}	1125.3254	1125.5891	myosin-4	pork	gil178056718	1329−1339	SALAHAVQSSR
818.64^{2+}	1635.2654	1634.8008	myosin-1	horse	gil126352470	1596−1609	VVETMQTMLDAEIR
452.40^{2+}	902.7854	902.4457	myosin	chicken	gil13432175	1693−1700	GALEQTER
751.50^{2+}	1501.2454	1500.7784	myosin	chicken	gil13432175	1703−1715	KVAEQELLDATER
687.57^{2+}	1372.1454	1372.6834	myosin	chicken	gil13432175	1704−1715	VAEQELLDATER
631.64^{2+}	1261.2654	1261.5674	myosin	chicken	gil13432175	1824−1834	ELEGEVDSEQK

[a]Peptides belonging to MLCs and MHC are unique to both species and single skeletal muscle protein indicating their potential as meat quality markers.

enhancement of group separation was observed, OPLS-DA gave an equally good model with R^2 = 0.994 and Q^2 = 0.993 and 94.9% of the predictive variation (Figure 2B).

We also evaluated LESA-MS for the direct analysis of thin slices of meat using in situ tryptic digestion. It is a potentially desirable approach for high-throughput analysis because sample preparation would be limited only to the process of digestion, which would radically speed up time of analysis. Although it was possible to achieve similar results compared with the in-solution digestion described earlier, we found that the nano-ESI chip nozzles in TriVersa NanoMate instrument, which are of internal diameter of 5 μm, became blocked during spraying, and a flow of the infused analyte to the mass spectrometer was disrupted after 15−20 s due to the presence of larger particulates on the digested surface. Possibly due to the fibrous structure of muscle tissue we were unable to overcome this problem and hence we found the in situ approach to lack suitable robustness and reliability. Therefore, we decided to evaluate in-solution digest methodology and we found this approach comparatively robust and rapid and thus competitive to LC-MS based methods. We were able using our optimized in-solution digest LESA-MS methodology to identify heat-stable peptide markers and then to investigate the discrimination of mixtures of cooked meat.

Identification of Heat Stable Peptide Markers with Peptidomic Data-Dependent Tandem LESA-MS. Having demonstrated that cooked meats could be discriminated by direct surface analysis of digests using LESA-MS the next stage was to select and identify peptide markers specific to processed skeletal muscle protein and animal species. Tryptic digests of

dx.doi.org/10.1021/ac502449w | Anal. Chem. 2014, 86, 10257−10265

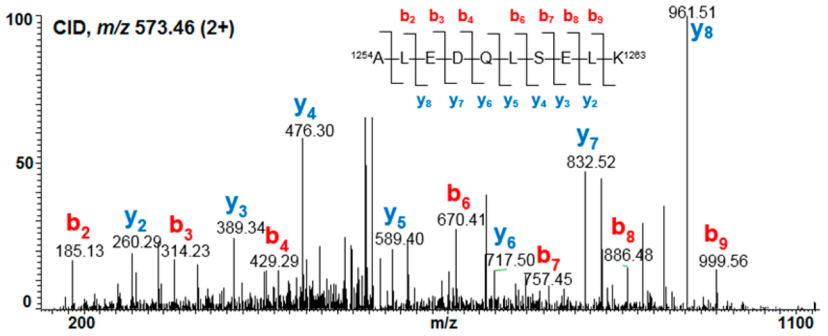


Figure 3. Average scan MS/MS spectrum of the cattle and myosin-1 unique peptide ^{1254}ALEDQLSELK^{1263} obtained from in-solution digestion of cooked beef.

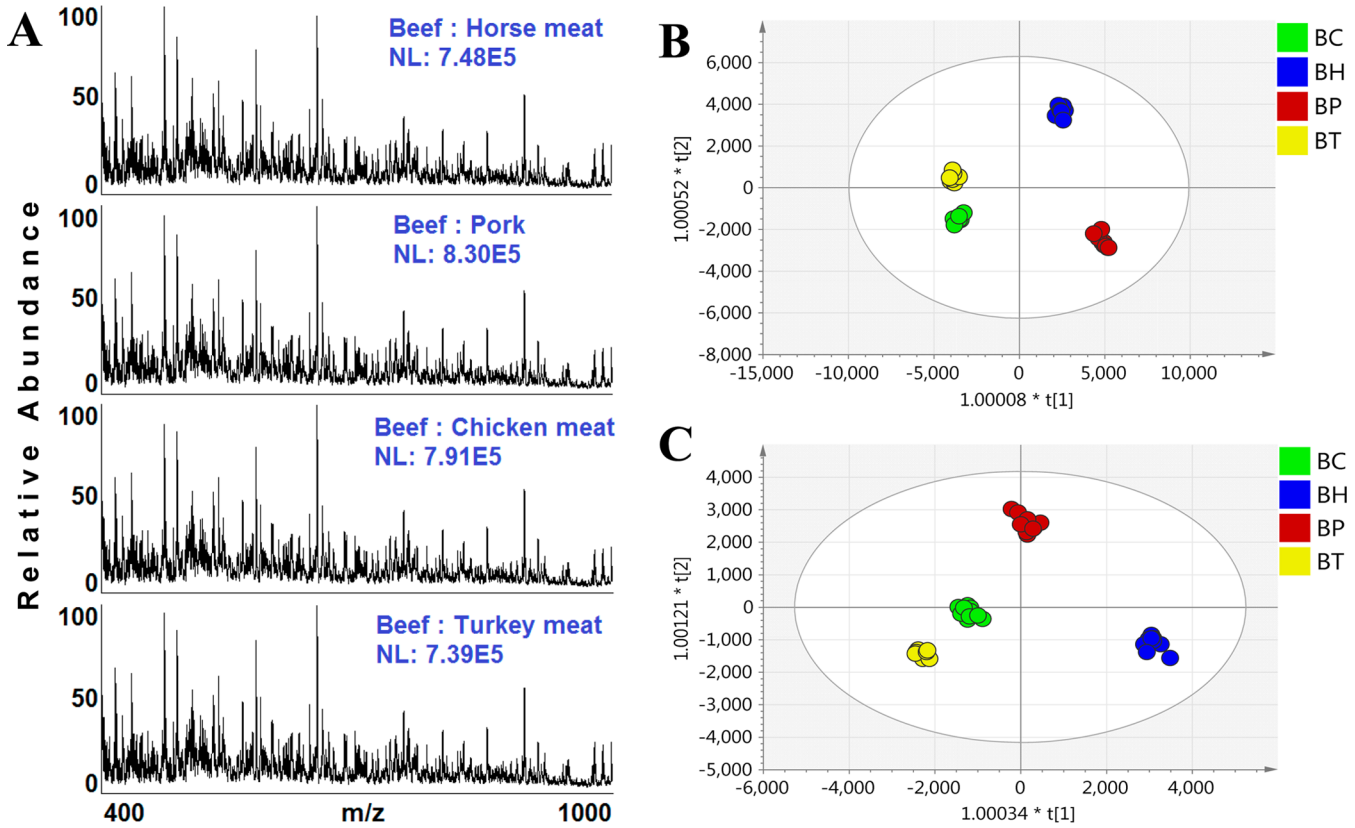


Figure 4. Distinguishing meat species in cooked beef. (A) LESA-MS average mass spectra of tryptic digests of cooked meat mixtures of beef spiked with 1% of horse, pork, chicken and turkey meat. OPLS-DA score plots of data sets collected from beef spiked with 10% (B) and 1% (C) of the second meat species, m/z 400−1000, n = 50. BC, beef/chicken meat; BH, beef/horse meat; BP, beef/pork; BT, beef/turkey meat.

raw and cooked meat were prepared as previously described and examined using LESA coupled to DDA MS/MS with identification of peptides by MS/MS ions using MASCOT searching. As a result of the DDA experiment, at 1% of a false discovery rate (FDR) for identity and homology threshold, it was possible to identify 10 to 15 proteins in the digest depending on the sample. In general, the same skeletal muscle proteins were identified as for raw meat,[20] such as myosin heavy chains (MHC) and myosin light chains (MLC), actin, tropomyosin, myoglobin, but sequence coverage was lower in the case of cooked meat.

The number of identified proteins was lower in cooked meat (15) compared with raw meat (29), with sarcoplasmic proteins mostly responsible for the reduced level of proteins in cooked meat. The MASCOT output scores for MHC and MLC for each species are shown in Table S1 (Supporting Information). In the absence of a species-specific sequence in the NCBI database, proteins were classified to the most closely related species (e.g., turkey and chicken).

Although thermal denaturation does not affect the primary structure of proteins, it causes loss of their solubility and protein aggregation.[22] The lower sequence coverage observed in our complex cooked meat digests seems to be caused by the insolubility of protein aggregates due to the conformational changes of proteins during thermal treatment, resulting in reduced digestion efficiency. Chemical denaturants such as urea, thiourea or guanidine hydrochloride can increase the efficiency of digestion;[22] however, we decided to avoid adding any agents which would suppress the electrospray signal. Our intention also was to keep the procedure as simple as possible so as not to compromise analytical throughput of the method. The aim was to identify easily detectable peptides, which were

dx.doi.org/10.1021/ac502449w | Anal. Chem. 2014, 86, 10257−10265

Table 2. MASCOT Output Scores for Peptide Markers Detected in Two-Component Mixtures of Beef Spiked with 10%, 5%, and 1% of Horse, Pork, Chicken, and Turkey Meat

parent ion	peptide sequence	species	protein	mixture 90:10% B/H	B/P	B/C	B/T	MASCOT score[a]	peptide rank	identity threshold[b]	homology threshold[b]
508.43 $^{2+}$	LVNDLTGQR	horse	myosin-1	√				10	2	>44	>27
818.64 $^{2+}$	VVETMQTMLDAEIR	horse	myosin-1	√				26	1	>41	>25
563.67 $^{2+}$	SALAHAVQSSR	pig	myosin-4		√			30	1	>42	>40
452.40 $^{2+}$	GALEQTER	chicken	myosin			√		5	10	>41	>20
687.57 $^{2+}$	VAEQELLDATER	chicken	myosin			√		7	7	>39	>39
854.13 $^{2+}$	VLNASAIPEGQFMDSK	chicken	myosin			√		5	6	>38	>23
685.54 $^{2+}$	ALGQNPTNAEINK	chicken	MLC1/3f			√		12	4	>38	>31
756.94 $^{2+}$	DQGTFEDFVEGLR	chicken	MLC1/3f			√		20	1	>39	>18
694.42 $^{2+}$	ALGQNPTNAEMNK	turkey	MLC1/3f				√	19	1	>57	>26
				mixture 95:5%							
452.40 $^{2+}$	GALEQTER	chicken	myosin			√		11	2	>41	>25
687.57 $^{2+}$	VAEQELLDATER	chicken	myosin			√		5	7	>39	>24
				mixture 99:1%							
487.46 $^{2+}$	LYDQHLGK	chicken	myosin			√		10	3	>41	>25
576.50 $^{2+}$	NALAHALQSAR	chicken	myosin			√		13	1	>39	>22

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

stable to heat-induced denaturation (termed "heat stable peptides") to directly address the needs of meat authentication in complex processed products. Hence, a selection of heat stable and unique peptides was made based on the results of the MASCOT searches based on spectral data from DDA MS/MS analysis of raw and cooked meat digests.

We focused mostly on the identification of the most abundant proteins as potential species and quality markers (MHC, MLC1/3f, MLC 2f, and myoglobin) to take advantage of the specificity of the LESA-MS method and to provide a stable and robust basis for authentication. Identified tryptic peptides in FASTA format were searched against nonredundant protein sequences using the protein BLAST alignment research tool and blastp algorithm for protein and species specificity. Table 1 presents 23 heat stable peptide markers identified with significant MASCOT scores that are unique to species and muscle protein. A list of all 80 peptides identified in this study using the DDA tandem LESA-MS for raw and cooked meat is presented in the Supporting Information (Table S2). Included in these are species-specific peptides derived from sarcoplasmic proteins, such as beta-enolase, glyceraldehyde-3-phosphate dehydrogenase, myoglobin, and peptides from myofibrillar as well the more abundant MHCs and MLCs.

MHCs existing in various isoforms can express diversity even within muscles of the same organism. Therefore, having performed BLAST search for peptide specificity, we found some previously identified MHC peptides that were not absolutely skeletal muscle- or species-specific. Some of them are shared with other cardiac, perinatal or embryonic isoforms of the same species and/or can be found in MHC isoforms of related species (e.g., sheep, goat, duck, or turkey). However, because these peptides are not present in slow isoforms, they were not excluded from our study; the full list of 80 peptides being of interest is presented in the Supporting Information (Table S2). In our opinion, these MHC peptides have the potential to serve as auxiliary markers of product quality since they would help to discriminate between fast and slow muscle isoforms and skeletal and smooth muscles, thereby likely between high and low quality meat components. The term "meat" means the skeletal muscles of mammal and bird species including the diaphragm and the rumen, thus the capabilities of distinguishing between skeletal and smooth muscles in a meat product would give us a quick answer about the quality of applied ingredients. Further, when quantification methodology has been developed, we would get a quick answer on how much meat is in the meat product. However, this hypothesis needs to be verified in the future. Figure 3 shows an average MS/MS scan of the cattle and myosin-1 unique peptide ^{1254}ALEDQLSELK^{1263}. Fragmented peptide specific to skeletal myosin ^{1272}LINDLTTQR^{1280} but not to species (it can be found also in skeletal muscle of sheep, goat and rabbit) is presented in Figure S1 (Supporting Information).

LESA-MS Analysis to Distinguish Mixtures of Cooked Meats: Horse, Pork, Chicken, and Turkey in Beef. To assess the percentage limit of detection (LOD) of the method, we examined two-component mixtures consisting of cooked beef and the second species, pork, horse, chicken and turkey meat at concentration of 10%, 5%, and 1% (*w*/*w*). First, LESA-MS analysis was performed to discriminate between the meat mixtures using MVA. Average mass spectra obtained from in-solution digestion of meat mixtures desorbed from a polymer surface are shown in Figure 4A. We observed excellent differentiation between the mixtures at three concentrations using PCA-X and OPLS-DA multivariate data analysis (Figures 4B and 4C), the first three PCA components displayed 61% of the total variance for data sets collected from beef spiked with 10% of the second meat species. OPLS-DA gave a good model even for mixtures differing in 1% of meat with 27% of the predictive variation and R^2 = 0.986 and Q^2 = 0.925.

Having obtained good initial results within MVA models, all mixtures were analyzed subsequently for species marker peptides using LESA-MS/MS. For the assessment of the LODs, we focused on the most intense heat-stable peptide ions selected previously using DDA. Of the 80 specific ions (presented in Table S2, Supporting Information), which were selected for sequencing with LESA-MS/MS, 15 were detected in our mixtures. Results concerning peptide markers found in samples containing 10% (*w*/*w*) of species, as well as two chicken markers detected in sample containing 5% (*w*/*w*) of chicken meat in the mixture, are shown in Table 2.

dx.doi.org/10.1021/ac502449w | Anal. Chem. 2014, 86, 10257−10265

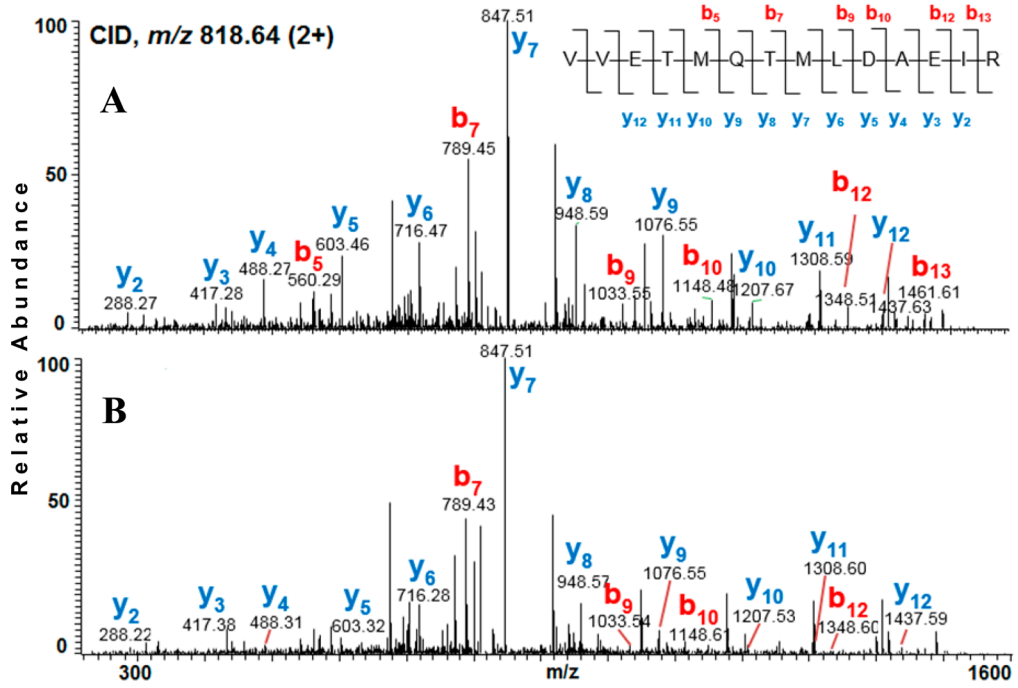


Figure 5. Fragmented spectra of the horse myosin peptide ^{1596}VVETMQTMLDAEIR^{1609} (818.64^{2+}) obtained from in-solution meat digests desorbed from a polymer surface using LESA-MS/MS: (A) cooked horse meat and (B) mixture of cooked beef and horse meat (90:10).

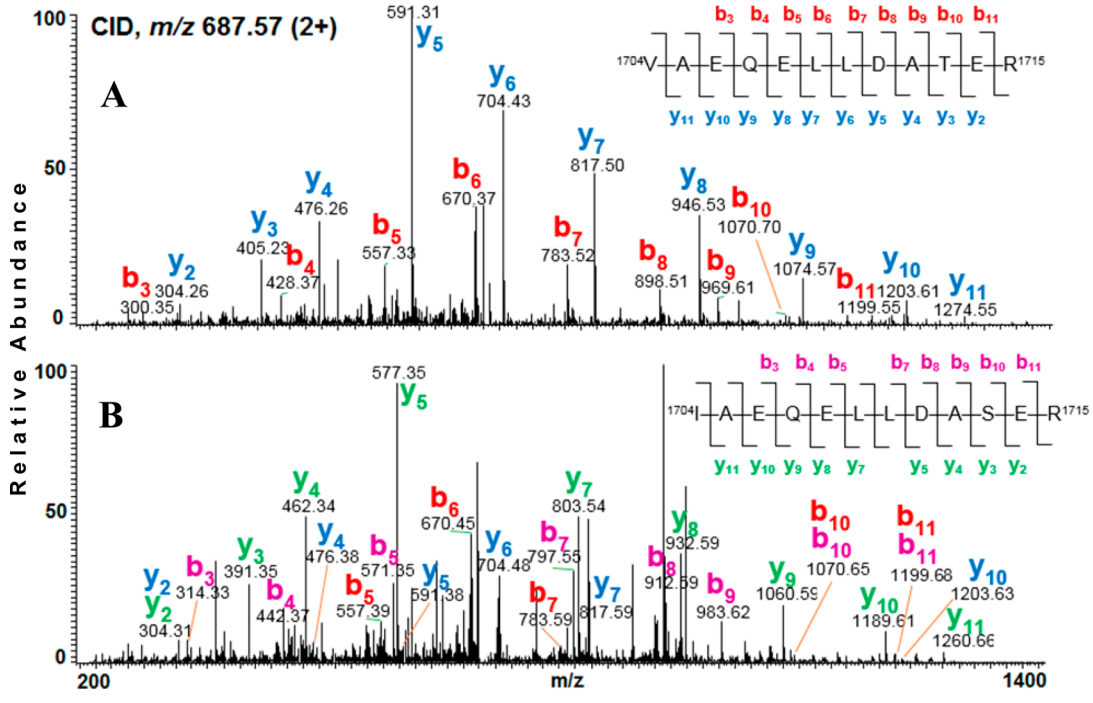


Figure 6. Fragmented spectra of the chicken myosin peptide ^{1704}VAEQELLDATER^{1715} (687.57^{2+}) obtained from in-solution meat digests: (A) cooked chicken meat and (B) mixture of cooked beef and chicken meat (95:5). In the case of mixture, the chicken and beef ion of similar m/z 687.35^{2+}^{1704}IAEQELLDASER^{1715} were sequenced together; beef peptide was ranked first with significant MASCOT score.

Predominantly, MASCOT score was below the identity and homology threshold, however most of the markers were ranked first or second in the list of matched peptides.

To confirm the identities of peptide markers derived from analysis of the meat mixtures, the fragmented spectra were evaluated manually and compared with the spectra of the same parent ions collected from digestion of single meats. Figure 5 shows MS/MS spectra of horse myosin marker ^{1596}VVETMQTMLDAEIR^{1609} (818.64^{2+}) obtained from cooked horse meat (Figure 5A) and from the mixture containing 10% of horse meat in beef (Figure 5B). Spectra of chicken myosin marker ^{1704}VAEQELLDATER^{1715} (687.57^{2+}) from chicken meat and the 5% mixture are presented in Figure 6. Most of the same *b* and *y* product ions were observed within the LESA spectra, however of lower intensities because of low concentration of protein in the mixture. When diagnostic peptide was not ranked

dx.doi.org/10.1021/ac502449w | *Anal. Chem.* 2014, 86, 10257−10265

first in the MASCOT result report, the obtained spectra were more complex, because of the separation and sequencing of several ions having similar m/z (Figure 6B). Another two examples of sequenced peptides unique to pork ^{1329}SALAHAVQSSR^{1339} (563.67^{2+}) and turkey meat ^{79}ALGQNPTNAEMNK^{91} (694.42^{2+}) are illustrated in the Supporting Information (Figures S2 and S3).

Using ambient LESA-MS/MS, we were able to detect 10% of pork, horse, and turkey meat in beef matrix, for chicken meat the limit of detection was 5% (w/w). Two chicken peptides were identified at low concentration of 1% (w/w) (Table 2), but they are not species-specific. The presence of these peptides in cattle myosin embryonic (LYDQKLGK) or cardiac isoform (NALAHALQSAR) and, thus, in small amounts in beef, was probably the reason for their detection in the 99:1 mixture. Interestingly, despite the inability of sequencing of diagnostic peptides from 1% mixture, we obtained good spatial distribution and predictive variation using MVA within the OPLS-DA model (Figure 4C) for all data sets. This proves, that the SIMCA-P algorithm has highly discriminating power enabling differentiation based on the small differences in the ion intensities and spectra composition.

In this Article, we have proof of principle that our rapid and easy to use ambient methodology has the potential to be applied to routine analysis of processed meat products. Time of analysis could be shortened to approximately 1 h by the application of microwaves or ultrasonication[23,24] to reduce the time of digestion process; at present a critical step for the duration of the analysis. This is a big advantage over the more complex and time-consuming protocols based on OFF-GEL[13,25] or liquid chromatography.[9,14] On the other hand, the application of LC separation dramatically increases sensitivity, e.g. low amount of 0.5% chicken meat in pork has been detected using OFF-GEL isoelectric focusing followed by LC-MS/MS.[13] Recently introduced method based on multiple reaction monitoring (MRM) enabled detection of 0.55% raw horse meat in beef or even 0.13% unprocessed pork in beef using MRM^3.[14] However, our method has good specificity, since we were able to detect more unique peptides and among them were some of the same peptide markers which have been reported previously for chicken, pork and horse meat by the use of LC-MS.[13,14] In our approach, the complex peptide mixture was directly analyzed without any pretreatment or fractionation; therefore, the results are altered by sample complexity and dynamic range of protein concentration, mostly due to high abundance of actin and myosin in muscle fibers. Myosin and actin ionize well, thus likely, they suppressed low-abundance peptide species within the mixture, of which the signal was too weak to exceed the noise threshold and to become well charged. For the reasons above, ambient MS analysis lacks sensitivity compared with the aforementioned LC-MS methods. It is highly probable that implementation of this ambient LESA-MS technique in conjunction with high resolution/accurate mass/MS^n mass spectrometry may enhance sensitivity as well as its efficiency in the analysis of less abundant proteins. Our future direction is to implement the LESA-MS methodology to analyze processed products (e.g., sausages, frankfurters, and pâtés), as well as to shorten time of entire analysis to approximately 1 h by the application of microwaves to reduce time of digestion.

CONCLUSIONS

In this work, a set of heat stable peptide markers specific to species and muscle protein has been identified for by use in our previously developed rapid ambient LESA-MS methodology.

The advantage of this approach, over other proposed peptidomic solutions, is the simultaneous analysis of complex peptide digests after only minimal sample preparation. The procedure involves a washing step to remove salts and excess fats which would interfere with the efficiency of ionization, followed by digestion and then direct ionization of dried in-solution digests from a solid surface by LESA-MS. By the application of this approach to peptidomic analysis of thermally treated meat species, we were able to distinguish between cooked beef, pork, horse, chicken, and turkey meat. We have also shown proof of concept that ambient MS can detect meat specific peptides after cooking down to 5% (w/w) of meat mixtures.

We have demonstrated that the proposed method is much faster and simpler than other measurement tools for meat speciation and displays specificity sufficient to be implemented effectively to the analysis of processed and complex meat digests. This peptidomic approach has excellent potential for rapid throughput screening of processed meat products, as well as applications to other fields in meat and food science. We have shown that direct ambient surface MS offers a valuable alternative to the existing methods, such as ELISA tests and more time-consuming PCR tests and LC-MS based methods.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org/.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: magdalena.montowska@gmail.com. Tel.: +48618487251. Fax: +48618487254.

*E-mail: david.barrett@nottingham.ac.uk. Tel.: +44(0) 1159515062. Fax: +44(0)1159515102.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The contents reflect only the authors' views and not the views of the European Commission.

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The postdoctoral fellowship of M.M. was supported by the European Commission under the Marie Curie Intra-European Fellowship Programme (Call: FP7-PEOPLE-2011-IEF).

REFERENCES

(1) Theler, B.; Brockow, K.; Ballmer-Weber, B. K. *Swiss Med. Wkly.* **2009**, *139*, 264−270.

(2) Chruszcz, M.; Mikolajczak, K.; Mank, N.; Majorek, K. A.; Porebski, P. J.; Minor, W. *Biochim. Biophys. Acta* **2013**, *1830*, 5375−5381.

(3) Demarquoy, J. *Meat Sci.* **2013**, *94*, 164.

(4) Ballin, N. Z.; Vogensen, F. K.; Karlsson, A. H. *Meat Sci.* **2009**, *83*, 165−174.

(5) Montowska, M.; Pospiech, E. *Food Rev. Int.* **2010**, *27*, 84−100.
(6) Meza-Márquez, O. G.; Gallardo-Velázquez, T.; Osorio-Revilla, G. *Meat Sci.* **2010**, *86*, 511−519.
(7) Zając, A.; Hanuza, J.; Dymińska, L. *Food Chem.* **2014**, *156*, 333−338.
(8) Şakalar, E.; Abasiyanik, M. F.; Bektik, E.; Tayyrov, A. *J. Food Sci.* **2012**, *77*, N40−44.
(9) Buckley, M.; Collins, M.; Thomas-Oates, J.; Wilson, J. C. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3843−3854.
(10) Montowska, M.; Pospiech, E. *Proteomics* **2012**, *12*, 2879−2889.
(11) Montowska, M.; Pospiech, E. *Food Chem.* **2013**, *136*, 1461−1469.
(12) Carrera, M.; Cañas, B.; López-Ferrer, D.; Piñeiro, C.; Vázquez, J.; Gallardo, J. M. *Anal. Chem.* **2011**, *83*, 5688−5695.
(13) Sentandreu, M. A.; Fraser, P. D.; Halket, J.; Patel, R.; Bramley, P. M. *J. Proteome Res.* **2010**, *9*, 3374−3383.
(14) Von Bargen, C.; Dojahn, J.; Waidelich, D.; Humpf, H.-U.; Brockmeyer, J. *J. Agric. Food Chem.* **2013**, *61*, 11986−11994.
(15) Leitner, A.; Castro-Rubio, F.; Marina, M. L.; Lindner, W. *J. Proteome Res.* **2006**, *5*, 2424−2430.
(16) Kertesz, V.; Van Berkel, G. J. *J. Mass Spectrom.* **2010**, *45*, 252−260.
(17) Edwards, R. L.; Creese, A. J.; Baumert, M.; Griffiths, P.; Bunch, J.; Cooper, H. J. *Anal. Chem.* **2011**, *83*, 2265−2270.
(18) Quanico, J.; Franck, J.; Dauly, C.; Strupat, K.; Dupuy, J.; Day, R.; Salzet, M.; Fournier, I.; Wisztorski, M. *J. Proteomics* **2013**, *79*, 200−218.
(19) Rao, W.; Celiz, A. D.; Scurr, D. J.; Alexander, M. R.; Barrett, D. A. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 1927−1936.
(20) Montowska, M.; Rao, W.; Alexander, M. R.; Tucker, G. A.; Barrett, D. A. *Anal. Chem.* **2014**, *86*, 4479−4487.
(21) Aerni, H.-R.; Cornett, D. S.; Caprioli, R. M. *Anal. Chem.* **2006**, *78*, 827−834.
(22) Park, Z. Y.; Russell, D. H. *Anal. Chem.* **2000**, *72*, 2667−2670.
(23) Ha, N. Y.; Kim, S. H.; Lee, T. G.; Han, S. Y. *Langmuir* **2011**, *27*, 10098−10105.
(24) Santos, H. M.; Kouvonen, P.; Capelo, J.-L.; Corthals, G. L. *Proteomics* **2013**, *13*, 1423−1427.
(25) Surowiec, I.; Koistinen, K. M.; Fraser, P. D.; Bramley, P. M. *Meat Sci.* **2011**, *89*, 233−237.