Tryptic Digestion Coupled with Ambient DESI and LESA Mass Spectrometry Enables Identification of Skeletal Muscle Proteins in Mixtures and Distinguishes Between Beef, Pork, Horse, Chicken and Turkey Meat.

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KEYWORDS

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ABSTRACT

The use of ambient desorption electrospray ionization (DESI-MS) mass spectrometry and liquid extraction surface analysis mass spectrometry (LESA-MS) is explored for the first time to analyse skeletal muscle proteins obtained from mixture of standard proteins and raw meat. Single proteins and mixtures of up to five proteins (myoglobin, troponin C, actin, BSA, tropomyosin) were deposited onto a polymer surface, followed by *in-situ* tryptic digestion and comparative analysis using DESI-MS and LESA-MS using tandem electrospray MS. Peptide peaks specific to individual proteins were readily distinguishable with good signal-to-noise ratio in the five-component mixture. LESA-MS gave a more stable analysis and greater sensitivity compared with DESI-MS. Meat tryptic digests were subjected to peptidomics analysis by DESI-MS and LESA-MS. Bovine, horse, pig, chicken and turkey muscle digests were clearly discriminated using multivariate data analysis (MVA) of the peptidomic datasets. The most abundant skeletal muscle proteins were identified and correctly classified according to the species following MS/MS analysis. The study shows, for the first time, that ambient ionization techniques such as DESI-MS and LESA-MS have great potential for species-specific analysis and differentiation of skeletal muscle proteins by direct surface desorption.

INTRODUCTION

Ambient mass spectrometry techniques have several desirable features which make them potentially suitable for the identification of surface proteins, such as minimal requirement for sample preparation and the ability to operate under physiologically relevant analysis conditions. Recent reviews of ambient mass spectrometry[1,2,3,4,5,6] show that many types of biological samples have been tested, such as the surfaces of leaves or fruit[7,1] and tissue sections,[8] but the majority of studies have concerned the analysis of small molecules and lipids[9] or biological tissue imaging.[10,11] In terms of protein analysis, only a few studies have been undertaken to show the potential of DESI-MS for investigation of small[12,13] and large proteins,[14] and tryptic peptides.[15] Most studies have been carried out on intact, model proteins in undigested form, in their native state and aimed at either top-down sequencing[16] or investigation of protein conformations.[17] Since the invention of DESI-MS in 2004[7] only a handful of ambient MS studies of proteins in real biological samples have been reported, such as the analysis of dried blood and serum[1,8] and bacteriophage MS2 capsid protein[13]. Liquid extraction surface analysis mass spectrometry (LESA-MS), a recently introduced chip-based nanoelectrospray technique[18], has been applied successfully to the analysis of hemoglobin variants from dried blood spots[19], glucocorticoids in porcine ear sections[20], pesticides from surfaces of fresh fruits and vegetables[21] and proteins from brain tissue sections subjected to MALDI-TOF analysis[22].

Hence, despite the enormous progress in ambient mass spectrometry (MS) that has taken place in the last decade[5] it still remains a significant challenge to use direct surface MS analysis to identify proteins from tissues and various biological or biomedical surfaces using these technologies. The complexity of biological systems, ion suppression effects and protein-protein interactions[12] all contribute to the lack of significant progress in this area. However, there is

growing interest for further development of ambient ionization techniques for the examination of larger proteins,[12,14] with information available on the analysis of single, standard proteins of small or medium molecular weight up to 66 kDa.[13,17,23,24] The most frequently investigated proteins have been bradykinin, melitin, insulin, ubiquitin, cytochrome C, myoglobin, β-casein, ovalbumin, β-lactoglobulin B and BSA in their native state or after digestion. Proteins have been detected after spotting on solid surfaces, such as glass or various polymers,[1,8,23] or archaeological artefacts[24] as well as directly from liquids.[14,17] Only a few studies have investigated larger proteins up to the size of BSA (66 kDa) and immunoglobulin G (150 kDa) or protein mixtures.[12,14,25,26] In a previous study in our laboratory we implemented DESI in conjunction with imaging multivariate analysis (MVA) to analyse images of protein spots and brain tissue sections,[25] and we successfully applied DESI-MS and LESA-MS to identify protein mixtures adsorbed onto a polymer surface.[26]

Having obtained promising results within a model system consisting of standard proteins, such as insulin, cytochrome C, myoglobin and BSA[25,26] we were interested to evaluate both DESI-MS and LESA-MS for the analysis and identification of mixtures of skeletal muscle proteins and muscle tissue. As far as we are aware, to date no one has reported the use of DESI-MS or LESA-MS to analyse skeletal muscle proteins and meat samples. In the last decade, there have been many studies where mass spectrometry based techniques, most frequently matrix-assisted laser desorption/ionization (MALDI) in combination with electrophoretic and liquid chromatography techniques, have been applied to meat speciation studies, including mapping of full muscle proteomes,[27,28,29,30] comparison of protein profiles between breeds,[31,32,33,34] proteome changes related to postmortem proteolysis and meat aging.[35,36,37,38,39,40]

Ambient MS techniques have advantages over these more traditional approaches because of their ability for rapid detection of compounds directly from biological surfaces since they operate in open air at atmospheric pressure. These features give them the potential for high-throughput screening of meat and meat products in their native state or with very little sample preparation. At present the unambiguous identification of meat proteins still requires time-consuming procedures involving protein extraction and chromatographic separation before injection of the sample to the mass spectrometer. Recent achievements in the of species differentiation involve species identification on the basis of bone collagen[41] or skeletal myosin light 3 isoform.[42] In our previous work using traditional approaches to protein analysis we observed that species-specific differences in the protein expression in raw meat were retained after processing in mixtures of minced meat and processed meat products.[43,44] Differences in the expression of myosin light chain (MLC) isoforms[43] and regulatory proteins, metabolic enzymes and blood plasma proteins[44] were observed using two-dimensional electrophoresis (2-DE) coupled with liquid chromatography-mass spectrometry (LC-MS). The above studies encouraged us to believe that proteomic or peptidomic approaches have excellent potential to distinguish both species and tissue differences in raw and processed meat. However, to date the established methodologies for meat speciation, namely 2-DE, enzymatic, near infra-red spectroscopy (NIRS) or polymerase chain reaction (PCR), all have drawbacks which affect their application in routine analysis of processed products; these include high cost of operation, time-consuming analysis, cross-reactivity between closely related species, and limited ability for tissue discrimination in complex processed products. We propose that some of these drawbacks can be overcome by the use of ambient MS due to its flexible, versatile and rapid nature.

In this paper we describe for the first time the development of ambient DESI-MS and LESA-MS methodology to analyse proteins derived from bovine, horse, pig and poultry skeletal muscles. This methodology aims to detect unique peptides for each species of skeletal meat without the application of chromatographic fractionation. Initially, we investigate methodology for single standard proteins and mixtures of up to five proteins (myoglobin, troponin C, actin, BSA, tropomyosin), deposited onto a polymer surface and followed by *in-situ* tryptic digestion and on-surface tryptic digest peptide mass fingerprinting (PMF) analysis by DESI-MS and LESA-MS. Subsequently, DESI-MS and LESA-MS are evaluated for peptidomic analysis of whole meat digests, dried and desorbed directly from a surface using multivariate data analysis and MS/MS to assess the suitability of species specific peptide ions to distinguish between beef, pork, horse, chicken and turkey meat samples.

MATERIALS AND METHODS

Materials. Water, acetonitrile and formic acid were purchased as MS grade from Sigma-Aldrich (Gillingham, UK). Standard proteins, apart from BSA, were from skeletal muscles. Myoglobin (Mb, equine, 17 kDa), actin (bovine, MW 45 kDa), BSA (MW 66 kDa) and tropomyosin (TM, porcine, ~130 kDa) were acquired from Sigma-Aldrich. Troponin C (TnC, rabbit, 18 kDa) was purchased from Alpha Diagnostic Int. (USA). Ammonium carbonate was purchased from BHD Chemicals (Poole, UK), ditiotreitol (DTT) and iodoacetamide (IAA) from Sigma-Aldrich. 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 and kept at -80°C for further proteomic analysis.

On-surface Digestion of Standard Skeletal Muscle Proteins. Proteins were dissolved in acetonitrile:water (50:50) and then single proteins or their mixtures containing up to five proteins were subjected to tryptic digestion. Trypsin was dissolved to a concentration of 0.05 µg/µL with 50 mM ammonium bicarbonate. Sample solutions were spotted onto Permanox™ slides, 75 x 25 mm (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) at 1 µl per spot at 5 mm intervals. After evaporation of the solvent in air, 1 µl of trypsin solution was spotted onto the same area. The digestion proceeded at room temperature (~24 ºC) over a period of 24 h in a humidity chamber made from a Petri dish with dampened paper placed along the rim (Figure 1) to ensure that the trypsin solution did not evaporate.[25] After this period the solution was left to dry at room temperature prior to analysis.

Tissue Preparation. Thin *longissimus* muscle 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*.[45] with modifications as described here. Slice of tissue (0.5 g) was rinsed twice for 30 s in ethanol:water (70:30) followed by 15 s wash in ethanol, and then by 30 s wash in methanol:water (90:10). Finally, tissue was rinsed for 2 x 30 s in deionized water and placed to dry for 30 min in a desiccator.

In-solution Digestion of Meat. Dried muscle tissue (0.5 mg) was 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. The solution was then centrifuged for 10 min at 13,400 rpm and the supernatant was diluted 10 fold with deionized water, and 1 µl of the sample was spotted onto a Permanox™ slide and allowed to evaporate in air at room temperature prior to analysis.

DESI and LESA Mass Spectrometry. All samples were analyzed using both DESI-MS and LESA-MS. The workflow for the experiment is shown in Figure 1. The DESI source was an Omni Spray 2-D® ion source (Prosolia Inc., Indianapolis, IN) and the LESA source was a TriVersa NanoMate® (Advion, Ithaca, NY). Both sources were 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 DESI operating parameters were as follows: DESI spray tip-to-surface distance 0.5 mm, spray tip-to-MS inlet 3 mm, impact angle 50º, Velos MS inlet to surface ~0 mm, spray solvent flow rate of 1.5 µL/min, a cone voltage of 5 kV, 120 psi N_2 nebulising gas, 300°C capillary temperature. The area analysed was 20 x 5 mm at constant velocity profile mode of 1500 µm/s. 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. Dispensed and aspirated solvent extraction volumes were 2.5 and 2.2 µL respectively. Each data set was collected from a single protein spot. The same spray/extraction solvent acetonitrile:water:formic acid (50:50:1) was used in all DESI and LESA experiments. MS data were collected in full scan mode (*m/z* 400-1600), 1 microscan, 100 ms max injection time, AGC mode on. In the case of meat samples spectra were collected in the wider range of *m/z* 400-1600 using DESI and in two narrower ranges of *m/z* 400-1000 and 1000-1600 using LESA due to considerably lower relative abundance of peaks above *m/z* 1000 yielded by LESA-MS. Collision-induced dissociation (CID)

experiments were performed at a normalized collision energy of 35-50%. Data were analyzed using Xcalibur software (Thermo Fisher Scientific). Raw data files were de-isotoped using the Decon software (http://omics.pnl.gov/software/DeconTools.php). Proteins were identified by peptide mass fingerprinting (PMF) against the National Center for Biotechnology Information (NCBInr) database with the assistance of the MASCOT online search engine. The searching parameters were as follows: trypsin enzyme, taxonomy mammals, one missed cleavage, peptide mass tolerance of 0.5 Da, carbamidomethylation of cysteines and variable oxidation of methionines. For MS/MS ions search raw files were converted to MASCOT generic format using MSCONVERT provided by the ProteoWizard project (http://proteowizard.sourceforge.net/tools.shtml). Data-dependent analysis (DDA) mode was used for the analysis of meat samples. The resulting files were searched via MS/MS ions search using MASCOT with following parameters: trypsin enzyme, taxonomy mammals or bone vertebrates for 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+, decoy on. Decoy tool was selected to have the significance threshold adjusted to get a global false discovery rate (FDR) at 1%. 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.

Multivariate Data Analysis. The raw MS data files of beef and horse meat 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 v13.1, Umetrics, MKS Instruments Inc.). The pre-processed beef and horse datasets were initially overviewed using principal component analysis

(PCA-X, unsupervised) to detect outliers in a model and subsequently, using supervised orthogonal partial least squares discriminant analysis (OPLS-DA) to create a model to enhance interpretability.

RESULTS AND DISCUSSION

Ambient MS Detection of Standard Muscle Proteins from On-surface Digests. Single proteins and mixtures containing up to five proteins (Mb, TnC, actin, BSA, TM) were successfully digested on-surface by depositing trypsin solution onto protein spots *in-situ*. An incubation time of 24 h at room temperature was sufficient to ensure that the proteins had undergone digestion. The optimal spray solvent composition for desorption and detection of skeletal muscle peptides (using both DESI and LESA-MS) was found to be acetonitrile:water:formic acid (50:50:1). The addition of low amount of formic acid was found to enhance the efficiency of ionization.

DESI-MS and LESA-MS analyses were applied directly to the dried protein digests on the substrate surface. Typical mass spectra obtained from sampling of five-protein mixture (Mb + TnC + actin + BSA + TM) deposited onto Permanox slides are shown in Figure 2. Examples of other spectra for two-, three-, and four-component mixtures are presented in the Supporting Information (Figures S1-S3). We obtained complex mass spectra similar to electrospray spectra, with mostly singly and doubly charged peptide ions detected, comparable to spectra which have been reported previously for BSA digests.[15,26] As expected, the complexity of the spectra increased with the number of protein components, although many diagnostic peptide peaks were readily distinguishable with good signal-to-noise ratio in the five-component mixture.

Differentiation of at least three diagnostic peaks derived from each protein within the mixture was readily achievable.

The main differences between DESI-MS and LESA-MS analyses were (a) the ion intensities observed in LESA-MS were one to two orders-of-magnitude higher in comparison with DESI-MS analysis of the same samples, (b) a more stable and consistent signal level was observed using LESA-MS, (c) LESA-MS provided more multiply charged peptides across a spectrum resulting in fewer ions above *m/z* 1000, (d) DESI-MS analysis demonstrated more singly charged ions and consequently more ions in the *m/z* 1000-1600 region. The apparently higher efficiency of LESA is likely to be due to a better liquid/surface microjunction process which enables extraction of a higher amount of analyte with reduced losses compared with DESI spray technique. The occurrence of a higher number of multiply charged peptides in LESA-MS indicates differences in the ion formation mechanism. LESA uses a nano-chip system to introduce the analyte to the mass spectrometer, therefore the ionization mechanism is similar to nano ESI-MS. The ionization process in DESI is similar to shock-wave-induced secondary charged droplet formation.[1] Although formation of multiply charged ions excludes the gas-phase ionization mechanism which is typical of small molecules,[1] it is likely that the observed reduction in number of multiply charged ions in DESI is partly affected by this phenomenon as was proposed by Kaur-Atwal *et al*.[15] In spite of possible differences in ionization mechanisms, the identification of proteins and *de novo* peptide sequencing from complex protein mixtures was possible using both techniques. We found that, despite the reduced stability of the signal and less efficient desorption process during DESI analysis, it was still possible to generate a good quality electrospray spectrum suitable for peptide identification and hence retaining the potential of

DESI-MS for surface imaging with its typical lateral resolution of 300-500 μm which is much higher compared with LESA-MS (minimal lateral resolution of 1 mm).

The acquired DESI-MS and LESA-MS spectra of the surface muscle protein digests enabled good sequence coverage and showed efficient digestion. Monoisotopic peaks obtained from DESI-MS and LESA-MS analysis of these proteins were entered manually into MASCOT to search against the NCBInr protein database via PMF. Table 1 presents the search results obtained from the analysis of solutions of single proteins. All of the proteins were correctly identified with significant MASCOT scores of 72-208 (where > 70 was significant; $p<0.05$) and sequence coverage ≥ 45%. Even a large protein such as TM (130 kDa) was readily detected from on-surface digests. Sequence coverage for LESA-MS was higher compared with DESI-MS, apart from actin where the sequence coverage was similar for both methods (49% and 48%). The reasons for the difference in sequence coverage between the proteins are discussed in Supporting Information.

Confirmation of Identity of Diagnostic Tryptic Peptides from On-surface Muscle Protein Digestion. DESI-MS/MS and LESA-MS/MS were successfully performed to confirm the identities of key peptides in the protein digests. Typical diagnostic examples of CID fragmentation obtained for Mb were ions at *m/z* 735.77^{1+} and 1378.92^{1+}, for TnC ions at *m/z* 724.01^{2+}, 913.56^{2+} and 1446.85^{1+}, for actin at *m/z* 998.75^{1+} and 1161.68^{1+}, and for TM at *m/z* 875.63^{1+}, 880.01^{2+}, 1131.66^{1+} and 1243.63^{1+}. Figure 3 shows an average scan MS/MS of the TM ion *m/z* 1131.66^{1+} from standard TM solution using DESI-MS (Fig. 3A) and from the five-protein mixture using LESA-MS/MS (Fig. 3B). The DESI and LESA spectra show the same product ions but of different intensities due to the differing concentrations of TM in the samples.

Examples of MS/MS spectra for TnC peptides (724.01^{2+} and 913.56^{2+}) can be found in Supporting Information (Figures S4 and S5). Figure S6A shows the CID spectrum of the actin peptide (*m/z* 998.75^{1+}) acquired using DESI-MS. In this instance the spectra from all samples, even from mixtures, were matched correctly using MASCOT. The detection of diagnostic peptides from beef and horse meat digests using ambient MS are discussed in Supporting Information (Fig. S6).

Peptidomic Data-dependent Analysis from Meat Samples with LESA-MS/MS. We have shown that ambient MS surface analysis of meat tryptic digests can yield strong and consistent ions from peptides diagnostic of the most abundant proteins in muscle tissue from beef and horse samples (refer to the Supporting Information). The ultimate goal was to check if the differentiation between the five types of meat, namely beef, horse meat, pork, chicken and turkey meat, would be possible by the use of direct surface analysis by desorption/ionization MS techniques. We observed that the average mass spectra were very similar between whole meat digests from the different species (Figures 4A and S8). However, despite the superficial similarities between the average spectral signal of five meat species, there were a number of less abundant ions responsible for the unequivocal discrimination between the five groups (Figure 4B). In-solution tryptic digests after removal of undigested material in order to improve the sequence coverage and protein identification were subjected to direct surface analysis with LESA instrument. LESA allows for setting longer acquisition time compared with DESI, therefore it is more suitable for data-dependent analysis to obtain sequence information automatically via tandem MS and identify a large number of peptides simultaneously. The previous work conducted in our laboratory has shown that LESA gave better identification

MASCOT scores than DESI within samples of standard protein solutions.[26] In this study DDA MS/MS surface analysis of in situ digests was performed for each of the meat species and yielded spectra were submitted for subsequent MS/MS ions MASCOT search. DDA experiment produced a large number of fragmented spectra of which approximately 13% of sequenced peptides were matched above the identity or homology threshold at 1% of a false discovery rate (FDR). As a results, a number of identified proteins ranged from 15 to 29 depending on the sample. There were among them the most abundant proteins present in skeletal muscles, such as myosin, actin, tropomyosin, troponin, myoglobin, glyceraldehyde 3-phosphate-dehydrogenase, fructose-bisphosphate aldolase, glycogen phosphorylase, carbonic anhydrase, beta-enolase. Table 2 presents the MASCOT score for the top five skeletal muscle proteins for each species. Proteins were correctly classified according to the species with only a few exceptions where a closely related species (eg chicken and turkey) was identified because of the lack of species-specific sequences in the NCBI database. That means that LESA-MS was able to detect and identify unique peptides to both skeletal muscle protein and animal species. The results show that it is possible to obtain detailed information from surface digest analysis of a real meat sample using ambient MS.

Differentiation Between Beef, Pork, Horse, Chicken and Turkey Meat Using Multivariate Data Analysis. The same tryptic digests as for DDA MS/MS were examined using general MS experiment, and similarly they were desorbed directly from surface using DESI and LESA following the purification to minimize the sample complexity and ion suppression effect. To provide a more detailed analysis unsupervised multivariate data analysis using PCA was applied to DESI and LESA-MS data sets of whole meat digests and was able to separate the complex

spectra with good spatial distribution and scores of variance. The first two PCA components separating beef from horse meat displayed 62% of the total variance for DESI-MS and 65% for LESA-MS yielded at *m/z* 400-1000 (Figure S9). When analyzing the five types of meat simultaneously, the first two PCA components gave 45% of the total variance for DESI-MS and 56% for LESA-MS. Better grouping was observed for the LESA-MS data sets due to higher reproducibility and sensitivity of the method (Figure S10). No distinct outliers were detected within the created models. DESI models were weaker but satisfactory separation of the groups within the cluster was achieved with OPLS-DA model (Figure 4B).

Supervised multivariate data analysis was performed using OPLS-DA for the same data sets in order to enhance group separation and to identify MS ions which distinguished between species. Grouping the variance only in one predictive component gave correlated coefficients of variations within beef and horse meat clusters at 14% for DESI-MS, and 41 and 36% for LESA-MS of low and high mass range respectively (Figure S9). OPLS-DA between the DESI data sets of the five species gave a good model with R^2=0.97 and Q^2=0.954, where the predictive variation corresponded to 23% (Figure 4B). LESA data sets gave a stronger model with R^2=0.988 and Q^2=0.984, and 44% of the predictive variation (Figure 4C). It is shown in Figures 5 and S9 that excellent separation was obtained within the all data clusters. OPLS-DA comparison of LESA-MS species data collected using nominal (0.7 amu) or accurate mass (<0.005 amu) acquisition (Figure S11) showed that good discrimination of species was obtainable irrespective of the mass resolution/accuracy used.

Other MS-based methods have been reported for meat speciation, based on protein detection in the 10-95 kDa range, often involving time-consuming multiple analysis steps prior to MS analysis[27-40,43,44]. For example, a proteomic approach using OFF-GEL isoelectric focusing

followed by SDS-PAGE and subsequent detection by MALDI-TOF and LC-ESI-MS/MS has been applied for the detection of chicken meat in meat mixtures[42] and in mechanically recovered meat[46] with the identification of chicken meat in pork on the basis of two peptides derived from myosin light chain 3. Mammal speciation has also been made based on peptides derived from bone collagen of 32 mammal species using MALDI-TOF/TOF[41] as well as bovine and porcine gelatin using HPLC-ESI-MS/MS.[48] Our approach has the advantage of ambient MS analysis using DESI-MS and LESA-MS to achieve rapid analysis of large skeletal muscle proteins of size up to ~223 kDa, such as tropomyosin and myosin heavy chains, in both meat and complex meat products by direct surface desorption only with minimal sample preparation. The only processing steps applied to the meat samples were washing, digestion and drying of digests on surfaces. One of the reasons for positive peptide detection from meat digests is likely to be the use of a washing step to meat samples to remove potential contaminants, such as physiological salts, fat and other soluble low molecular weight compounds. The entire analysis including the following MS/MS takes ~10 min (excluding sample preparation) and this rapid analysis is important for application to perishable food articles such as meats and meat products. Digestion time (currently 24 hours) could be substantially reduced by incubation of samples with trypsin in 37°C, as well as the application of microwaves or ultrasonication to accelerate protein cleavage, as described previously.[49,50]

CONCLUSIONS

We have performed for the first time analysis of *in-situ* digested mixtures of skeletal muscle proteins, such as troponin C, actin and tropomyosin, using ambient DESI-MS and LESA-MS. The results demonstrate the suitability of surface sampling of protein mixtures by use of

DESI and LESA mass spectrometry for the unambiguous detection and identification of skeletal muscle proteins and peptides. Direct desorption/ionization of whole meat digests without fractionation stage provided sufficient data to discriminate between beef, pork, horse, chicken and turkey meat. A number of ions responsible for the characteristic features of the spectra and significant for unequivocal discrimination between the examined types of meat were observed. After DDA tandem MS skeletal muscle proteins were identified with significant MASCOT score, as well as the proteins were correctly classified according to the examined species.

Novel and rapid surface methods of protein/peptide examination from complex samples, such as meats and processed meats, by direct surface desorption/ionization have many prospective fields of application in meat science and food authentication. In this proof-of-concept work we have demonstrated that both DESI and LESA have the ability to discriminate subtle spectral differences between samples of meat from closely-related species such as beef and horse meat, and chicken and turkey meat. In this study, only the most abundant proteins were identified but implementation of these ambient MS techniques in conjunction with high resolution/accurate mass/MS^n mass spectrometry may open up new possibilities for rapid analysis of less abundant proteins as well as the analysis of proteins in their native state directly from the meat surface.

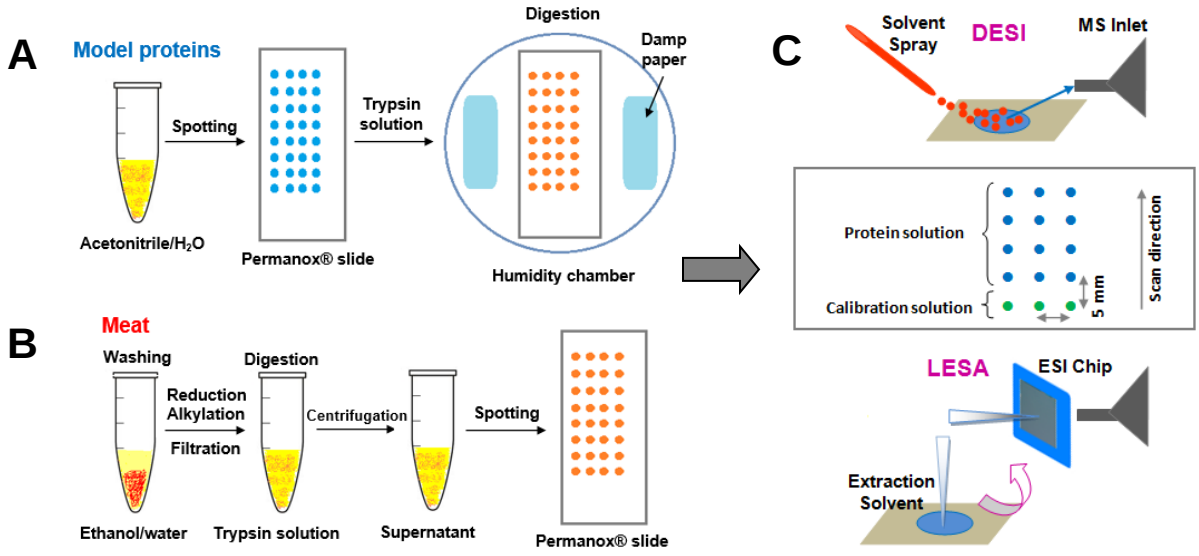

Figure 1. Workflow for DESI and LESA-MS experiments: (A) setup of on-surface digestion of standard skeletal muscle proteins experiments; (B) setup of in-solution digestion of meat experiments; (C) diagram of general DESI and LESA-MS setup. Proteins were spotted at 5 mm intervals. Before analysis rhodamine solution was dropped in first raw for calibration purposes. The DESI area analysed was 20 x 5 mm at constant velocity profile mode. LESA data set was collected from a single protein spot.

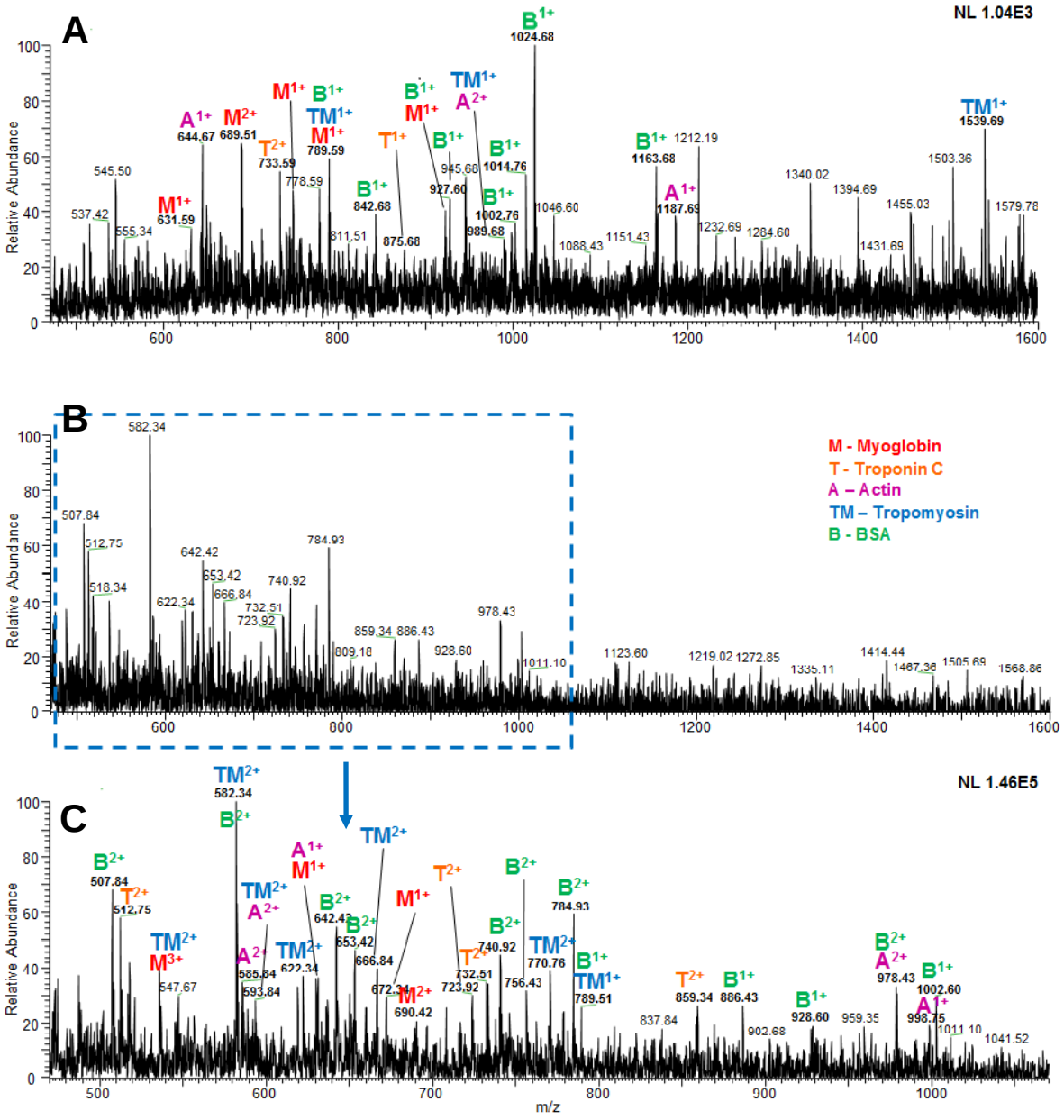

Figure 2. Positive ion mode for tryptic digests; (A) DESI-MS and (B) LESA-MS spectrum of five-component mixture of myoglobin (10 µM), troponin C (55 µM), actin (24 µM), BSA (6.6 µM) and tropomyosin (0.8 µM) in ratio 1:1:1:1:1, mass range *m/z* 400-1600; (C) magnified fragment of LESA-MS spectrum in the mass range of *m/z* 400-1050. Note that some ions were assigned to more than one protein. An example of ion m/z $789^{(1+)}$ is discussed in Supporting Information (Fig. S7, Tables S1 and S2).

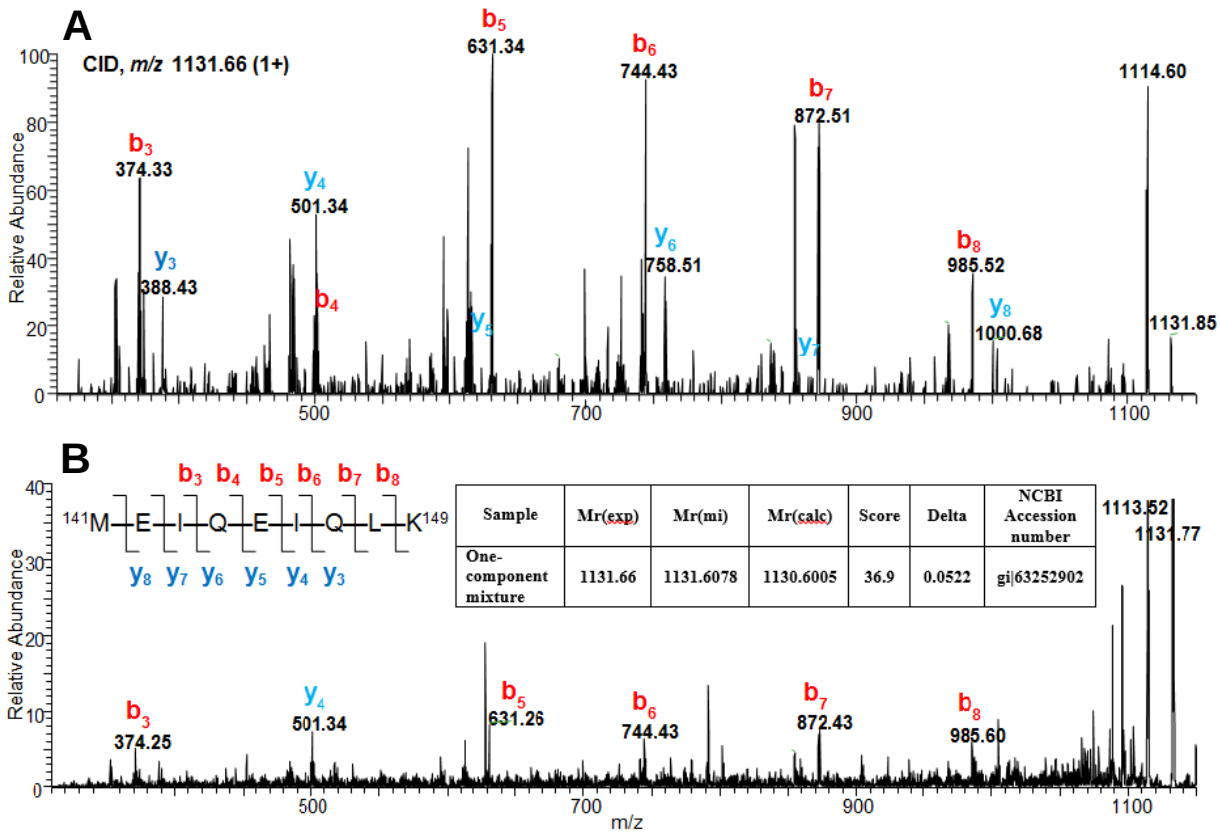

Figure 3. Average scan MS/MS spectra of the tropomyosin peptide ^{141}MEIQEIQLK^{149} (1131.66^{1+}) obtained from on-surface tryptic digest; (A) DESI of porcine skeletal tropomyosin and (B) LESA of five-component mixture of myoglobin (10 µM), troponin C (55 µM), actin (24 µM), BSA (6.6 µM) and tropomyosin (0.8 µM) in ratio 1:1:1:1:1. Inserted table shows MASCOT score. The ion from the single protein solution was correctly matched. The ion from the mixture was not identified after MASCOT search likely due to lower concentration of the protein in the sample.

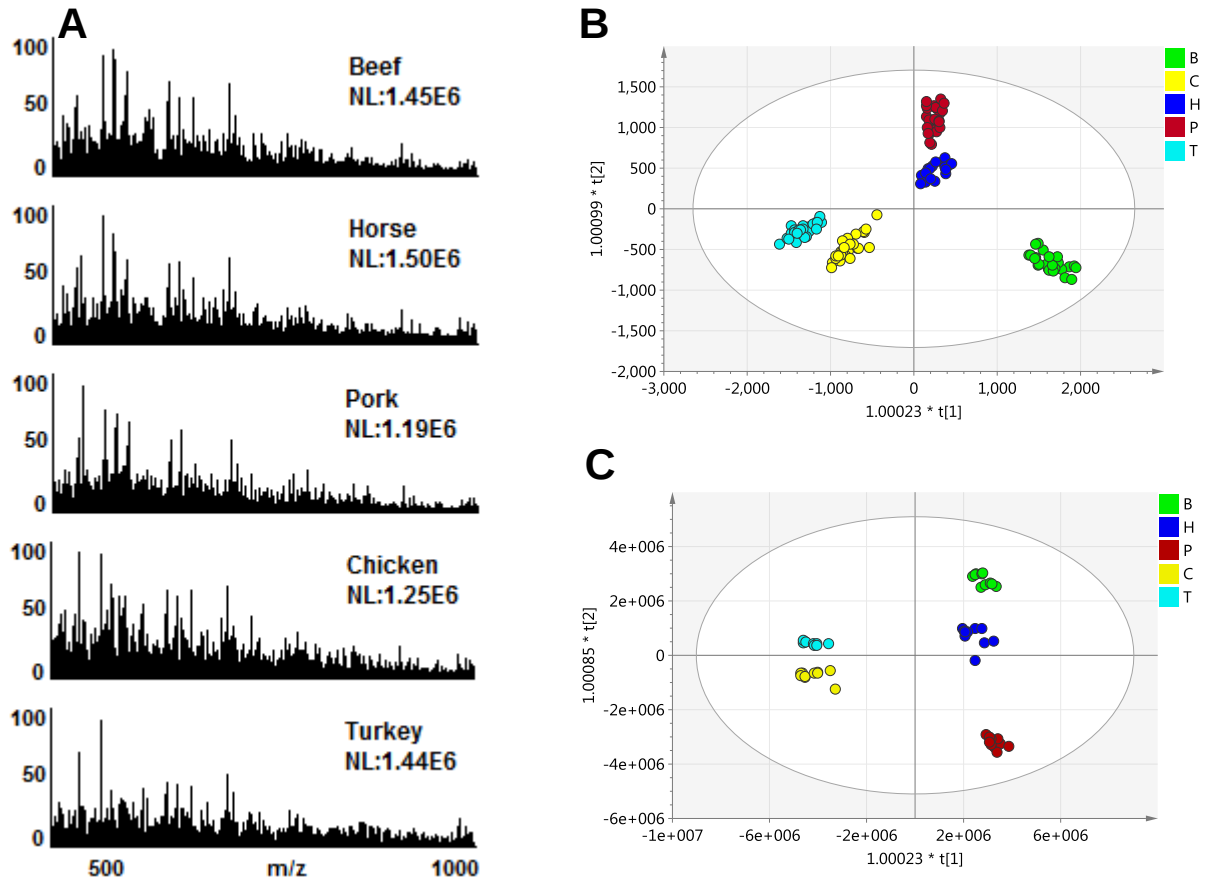

Figure 4. Differentiation between beef, pork, horse, chicken and turkey meat; (A) Average mass spectra of meat digest obtained using LESA-MS; (B) OPLS-DA score plots of data sets collected using DESI-MS in the range of *m/z* 400-1600 (n=125); (B) OPLS-DA score plots of data sets collected using LESA-MS in the rage of *m/z* 400-1000 (n=50). B – beef, H – horse meat, P – pork, C - chicken meat, T - turkey meat.

Table 1. Muscle proteins identified from surface by DESI- and LESA-MS

Experimental protein	NCBI accession number	Identified protein	Matched peptides[a]	Sequence coverage (%)[b]	MASCOT score
DESI-MS					
Myoglobin	gi|118595772	Myoglobin (*Equus caballus*)	19	56	208
Troponin C	gi|223032	Troponin C (*Oryctolagus cuniculus*)	15	61	116
Actin DESI	gi|27819614	Actin, alpha (*Bos taurus*)	26	49	198
Tropomyosin	gi|148222268	Tropomyosin alpha-1 (*Sus scrofa*)	19	45	113
LESA-MS					
Myoglobin	gi|255683511	Myoglobin (*Equus caballus*)	15	76	123
Troponin C	gi|223032	Troponin C (*Oryctolagus cuniculus*)	12	69	56
Actin	gi|27819614	Actin alpha (*Bos taurus*)	21	48	172
Tropomyosin	gi|148222268	Tropomyosin alpha-1 (*Sus scrofa*)	37	83	72

[a]Number of matched peptides in the database search. [b]Percent of coverage of the entire amino acid sequence.

Table 2. MASCOT output scores for the top five skeletal muscle proteins from the examined five types of meat using the DDA tandem LESA-MS analysis of meat digests desorbed from biomedical surface. The homology threshold was adjusted to achieve a specified false discovery rate (FDR) at 1%. As a result, the significance threshold of $p<0.05$ was shifted in beef to $p<0.0303$, in horse to $p<0.0423$, in pork to $p<0.0080$, in chicken to $p<0.0462$ and in turkey to $p<0.0047$ to give the best possible sensitivity at 1% FDR.

Sample	NCBI accession number	Identified protein	Mass (Da)	Matched peptides[a]	Sequence coverage (%)[b]	MASCOT score[c]
Beef	gi|41386691	Myosin-1 (*Bos taurus*)	223764	87	32	877
	gi|27819614	Actin alpha (*Bos taurus*)	42451	22	49	459
	gi|115497166	MLC2f (*Bos taurus*)	19114	7	37	147
	gi|118601750	MLC1/3f (*Bos taurus*)	21033	12	54	111
	gi|27806939	Myoglobin (*Bos taurus*)	17067	7	36	89
Horse meat	gi|126352470	Myosin-1 (*Equus caballus*)	223772	91	33	1145
	gi|114794125	Actin (*Oryctolagus cuniculus*)	41561	22	51	256
	gi|545218230	MLC1/3f (*Equus caballus*)	16022	17	80	162
	gi|157834232	Myoglobin (*Equus caballus*)	16942	11	46	156
	gi|194219044	MLC2f (*Equus caballus*)	19142	10	59	92
Pork	gi|178056718	Myosin-4 (*Sus scrofa*)	224010	92	31	876
	gi|528081968	Actin (*Oryctolagus cuniculus*)	42249	13	39	252
	gi|54607195	MLC2f (*Sus scrofa*)	19080	10	41	124
	gi|117660874	MLC1f (*Sus scrofa*)	21019	10	36	71
	gi|49274641	Troponin C (*Sus scrofa*)	18155	1	10	55
Chicken meat	gi|13432175	Myosin full (*Gallus gallus*)	223976	103	37	1084
	gi|514748971	Actin alpha (*Anas platyrhynchos*)	40730	24	49	561
	gi|223047	MLC2f (*Gallus gallus*)	18739	6	28	292
	gi|212347	MLC1f (*Gallus gallus*)	19525	15	51	111
	gi|211226	Tropomyosin alpha (*Gallus gallus*)	32775	7	19	149
Turkey meat	gi|61657939	Myosin (*Gallus gallus*)	224010	79	29	645
	gi|326920308	Actin (*Meleagris gallopavo*)	42342	10	21	251
	gi|223047	MLC2f (*Gallus gallus*)	18739	6	31	162
	gi|326926446	Tropomyosin alpha (*M. gallopavo*)	32803	13	26	110
	gi|326922419	MLC1f (*Meleagris gallopavo*)	20949	14	64	107

[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%.

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.

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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 authors declare no competing financial interest.

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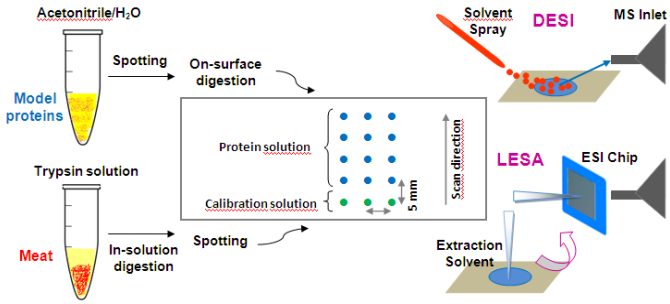
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