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Species specific marker peptides for meat authenticity assessment: a multispecies quantitative approach applied to Bolognese sauce

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Abstract

Food frauds are a critical issue in the field of food safety and quality. Given the high added value, and the complexity of the matrix, processed meat products are among those most susceptible of adulteration. Despite all the efforts made by the official control authorities and by the food industry to counteract these frauds, the undeclared replacement of meat species with cheaper ones is still widespread. The meat species allowed for food consumption are many, and their specific and accurate detection in highly processed food products requires very sensitive and selective analytical methods. In this work, a LC-MS method was developed to identify and quantify eight different meat species (duck, rabbit, chicken, turkey, buffalo, equine, deer and sheep) in a complex food matrix, such as Bolognese sauce. After protein extraction and trypsin digestion, a species-specific peptide marker for each species was chosen for qualification and quantification. The method was validated on real Bolognese sauce samples prepared in an industrial environment, showing a good sensitivity (LOD 0.2-0.8% on whole finished product) and the possibility, using specifically defined calibration lines, to quantify the amount of meat present coming from different species.

Keywords

Food fraud, meat authenticity, peptide markers, LC-MS, Bolognese sauce, species detection

1. Introduction

The progress of globalization, together with the economic opportunity and, in some cases, the low severity and probability of the punishment, has led to a steady increase in food frauds (Pustjens, Weesepoel, & van Ruth, 2016). Thus, a growing attention is being given to food authenticity assessment. Consumers are getting more and more informed about food risks related to frauds, and they ask for valuable tools for food protection and control (Tiozzo, Mari, Ruzza, Crovato, & Ravarotto, 2017). In 2013, the EU Food Fraud Network (FFN) was created in response to the horse meat crisis, and since then meat has always been at the centre of the attention in food frauds cases. From a recent report of FFN, emerged that, of 176 total reported cases of cross-border food frauds, meat products were involved in 47 records (19 for the only poultry and 28 for the remaining meat products) (European Commission, 2016). The types of adulteration that can occur in meat are many and diverse: meat origin, meat substitution, meat processing treatment and non-meat ingredient addition (Ballin, 2010). After the horse meat scandal in 2013, the problem of meat species substitution has obviously gained more and more attention. The substitution of a species with a cheaper one in a meat product is a common fraud in the sector, and it is sometimes difficult to detect, especially in processed products. In a recent survey, the presence of an undeclared species was detected in the 68% of meat products analysed, with prevalence in sausages, burger patties and deli meats (Cawthorn, Steinman, & Hoffman, 2013). The survey was carried out in South Africa, collecting samples from retail outlets and butcheries over a five months period (April 2012 - August 2012), and 95 of the 139 samples contained a meat species which was not declared in the product label. Pork and chicken were the most commonly detected species, but also cases of donkey, goat and water buffalo were detected. Also among game meat, mislabelling cases due to economic gain are reported (18.5% of the samples analysed) (Quinto, Tinoco, & Hellberg, 2016). The substitution of the more expensive game meat with domestic species has also been reported (Amaral et al., 2015), for example the substitution of duck with chicken. These meat species substitutions have important implications on food safety, because the undeclared species is not subjected to any veterinary health check, microbiological and contamination control. In addition to this, some lifestyles exclude the use of particular meat species, for example pork meat is not allowed in Islamic and Jewish dietary rules. In a recent paper undeclared pork meat presence was found in a high percentage (54%) of meat samples, even if declared halal (Amaral, Santos, Oliveira, & Mafra, 2017).

Analytical tools for meat authenticity assessment are continuously evolving in response to the new challenging issues and they cover a wide variety of techniques. In meat species substitution, differences in the genetic materials can be detected by the use of genetic approaches, such as DNA-hybridization and polymerase chain reaction (Rahmati, Julkapli, Yehye, & Basirun, 2016). DNA barcoding is among the most promising genetic techniques for food authentication in processed meat, being able to recognize with 68.3% of success meat species through sequencing of the target gene (cytochrome c oxidase subunit I gene) (Hellberg, Hernandez, & Hernandez, 2017). Another innovative technique is based on the development of chemiluminescent optical fibre genosensors, which has already been applied with success for the detection of pork in minced raw meat mixtures down to 1% (Torelli, Manzano, & Marks, 2017). Despite being a powerful tool for species identification, DNA methods can easily fail in heavily processed food matrices, due to DNA degradation, and also the exact quantitation of the different species detected might be cumbersome and difficult.

Another common technique is based on the recognition of species-specific proteins via immunoassays. A great number of enzyme linked immunosorbent assays (ELISA) have been developed for meat speciation, due to their speed and ease of use (Perestam, Fujisaki, Nava, & Hellberg, 2017). These methods can also be applied to processed products, for example pork can be detected in heat-processed meat products by monoclonal antibody-based ELISA even at low percentages (0.5%). Anyway, also these methods suffer in many cases of false positives and false negatives in heavily processed foodstuff, and their accuracy tends to be very much product-specific.

As mentioned before, food frauds are most likely to occur in processed food products, where minced meat is present (preventing visual recognition). In these thermally treated processed food, several detection problems can arise due to interfering compounds, nucleic acids and proteins degradation (Bauer, Weller, Hammes, & Hertel, 2003; Terry, Harris, & Parkes, 2002). Hydrolysis and denaturation phenomena are particularly harsh in those foodstuffs that also have acid pH (for example lemon juice or vinegar) and/or that undergo to severe heat treatments (for example prolonged cooking or sterilization). Thus, for the most complex food matrices, having different ingredients mixed together or subjected to severe thermal treatments, the need for robust, sensitive and selective methods is still an issue (Sentandreu & Sentandreu, 2014).

A new method for the identification of species-specific molecular markers has been lately gaining momentum, based on the coupling two very performing techniques in the field of food analysis: liquid chromatography (LC) coupled to mass spectrometry (MS), applied to the detection of peptides. Label free relative quantification of meat species-specific proteins is achieved through the detection of marker peptides, generated by enzymatic hydrolysis. The combination of off-gel fractionation and LC-MS detection, allowed the detection of chicken in meat mixtures at very low percentages (0.5% w/w) with high confidence (Sentandreu, Fraser, Halket, Patel, & Bramley, 2010). In more recent researches, even small amount (1% w/w) of beef, pork, chicken, duck and goose in cooked meat could be detected by this methodology (Montowska, 2017; Montowska & Fornal, 2017). With the contribution of off gel fractionation, specific peptides, deriving from myosin light chain 1 and 2, could be identified for buffalo and sheep. 0.5% of buffalo meat could be detected both in raw and cooked mixture (Naveena et al., 2017). LC/MS detection of peptides was demonstrated to be more efficient than PCR and ELISA assays for the determination of the origin in products undergone severe heat treatment or acid/alkali extraction, for example in the case of gelatine, where porcine and bovine collagen peptides could be identified down to 0.4-1% of contamination (Grundy et al., 2016). This method has also been successfully applied to meat mixtures of beef, pork, horse and lamb, exploiting myoglobin derived peptides, with a good sensibility (1%) (Watson, Gunning, Rigby, Philo, & Kemsley, 2015). The good stability of peptides to thermal treatments, rather than intact proteins or nucleic acids, is also demonstrated by the possibility to identify markers for horse and pork also in processed foodstuff even in small amount (0.24%) (von Bargen, Brockmeyer, & Humpf, 2014). Several efforts have been made to make the mass spectrometry analysis much faster and easier, and appreciable results were obtained using the LESA-MS methodology. This technique allowed detecting pork, horse, turkey and chicken meat at amount ranging from 5 to 10% (Montowska, Alexander, Tucker, & Barrett, 2014). Demonstrating the robustness of this approach in heavily processed food matrices, in a previous work we developed a LC-MS method for the identification and quantification of beef and pork meat in a challenging food matrix, Bolognese sauce, where these two types of meat are usually employed as ingredient (Prandi et al., 2017).

In the present paper, with the aim of achieving a fast and robust method for multispecies determination and quantification, we have extended the methodology to other eight species of food interest, covering most of the European meat market. In order to demonstrate its relevance for the food industry in a real environment, its applicability has been demonstrated on real samples prepared at the industrial level.

More specifically, we took into account, beside bovine and pork, poultry and rabbit meat, equine meat (donkey and horse), buffalo, red deer and sheep. Horsemeat has been the focus of attention in 2013, but also turkey was involved in several scandals, such as the substitution of halal lamb with turkey meat at the beginning of 2017 (European Commission, 2017). The method developed and here reported, based on LC/MS determination of peptides, is the first one able to detect and quantify simultaneously these eight species in Bolognese sauce.

Materials and methods

2.1 Samples.

Bolognese sauces were prepared in a pilot plant following the standard industrial procedure. Traditional Italian recipe was opportunely modified to have standard reference matrices of each meat species. Thus, Bolognese sauces were prepared with 19% of pure meat of: duck, rabbit, chicken, turkey, donkey, buffalo, horse and red deer. Other ingredients in the Italian traditional recipe are: tomato pulp 28%, water, tomato concentrate 16.5%, onion, carrots 4%, celery 3%, sunflower oil, corn starch, salt, yeast extract, sugar, laurel 0.06%, sage extract, black pepper. Briefly, chopped onion is suffused with vegetables. Then, tomato sauce, tomato concentrate and freshly minced meat are added, and the sauce is cooked for two hours (approx. temperature 90-100°C). After cooking, Bolognese sauce is poured into the jar at 70°C. Finally, the sterilization is performed into an autoclave: the temperature is brought from 30°C to 121°C, then the jars are cooled again to 30°C (total sterilization time: 90 min).

Besides Bolognese sauce prepared with pure meat species, mixed Bolognese sauces were also prepared, where the total amount of meat (19%) was kept constant. Three different sets of samples were prepared; each consisting of 3 concentration levels (for calibration purpose) and blind samples (for cross validating purpose) as reported in Table 1.

2.2 Reagents and solvents

Deionized water was obtained with Select water purification system (Suez water, Thame, UK). Tris(hydroxymethyl)-aminomethane (99+%), urea (99-101%), hydrochloric acid (≥37%), formic acid (≥95%), DL-dithiothreitol (≥98%), iodoacetamide (crystalline) and trypsin from porcine pancreas (1,000-2,000 BAEE units/mg solid) were purchased by Sigma Aldrich (Saint Louis, MO, USA). Thiourea was purchased by Carlo Erba (Cornaredo, MI, Italy). Acetonitrile (≥99.9%) was purchased by Honeywell-Riedel de Haën (Seelze, Germany). Ammonium bicarbonate (≥99%) was purchased by Fluka Chemie (Buchs, Switzerland).

2.3 Protein extraction

One hundred grams of Bolognese sauce were homogenized with a fixed rod homogenizer (Ultra Turrax IKA T50 digital, Staufen im Breisgau, Germany) for 2 min at a speed of 22,000 rpm. The homogenized samples were then lyophilized for 44 hours in a Lio 5P freeze drier (5Pascal, Milan, Italy) at a vacuum level of 0.80 mbar and a temperature of -50°C. One gram of lyophilized Bolognese sauce was extracted with 10 mL of a 0.05 M TrisHCl, 6 M urea and 1 M thiourea (pH 8), using a fixed rod homogenizer (Ultra Turrax IKA T18 digital, Staufen im Breisgau, Germany), for 5 min at 10,000 rpm. Samples were centrifuged at 3,220*g* for 10 min at 4°C (Eppendorf 5810R, Hamburg, Germany) and supernatant was filtered through 1 μm glass fibre syringe filters (Acrodisc, Waters, Milford, MA, USA). Salts and caotropic agents were removed by solid phase extraction (SPE) using Sep-Pak C18 Plus short cartridges (Waters, Milford, MA, USA) according to the manufacturer instructions. The cartridges have 360 mg sorbent per cartridge, with a 55-105 μm particle size. The loaded volume of sample was around 5 mL of protein solution. Desalted protein extracts were lyophilized for 6 hours in a Lio 5P freeze drier (5Pascal, Milan, Italy) at a vacuum level of 0.80 mbar and a temperature of -50°C and stored at -20°C.

2.4 Protein digestion

Freeze dried samples were dissolved in 1 mL of ammonium bicarbonate (0.05 M in water). Disulphide bridges were reduced by adding 5 μL of dithiothreitol (0.2 M in water) and incubating the samples at room temperature for 1 hour. Then, sulfhydryl groups were alkylated by adding 4 µL of iodoacetamide (1 M in water) and incubating in the dark for 1 hour at room temperature. Excess iodoacetamide was neutralized by the addition of 20 µL dithiothreitol (0.2 M in water) and incubation at room temperature for 1 hour. Then, protein content was measured with the Quant-it protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) using the Qubit Fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer instruction. Trypsin digestion was carried out using an enzyme to substrate ratio of 1:20 and incubating overnight at 37°C in an orbital shaker incubator (ES 20, Biosan, Riga, Latvia) with a speed of 100 rpm. Digestion was stopped by the addition of 50 µL of formic acid (10% v/v in water). Samples were stored at -20°C until UHPLC/ESI-MS/MS analysis. Just before injection, samples were centrifuged at 15,093*g* for 10 min at 4°C and the supernatant was saved for the analysis.

2.5 UHPLC/ESI-MS/MS analysis

Peptides generated by enzymatic cleavage were analysed using reverse phase ultra-high performance liquid chromatography (UHPLC) coupled to electrospray ionization tandem mass spectrometry (ESI-MS/MS).

Aeris PEPTIDE 1.7µm XB-C18 column (100 Å, 150 × 2.1 mm; Phenomenex, Torrance, CA, USA) was used for the chromatographic analysis, equipped with a Security Guard ULTRA Cartridge (C18-Peptide, ID 2.1 mm; Phenomenex, Torrance, CA, USA). Chromatographic separation was run in a Dionex Ultimate 3000 UHPLC (Sunnyvale, CA, USA). Flow was set at 0.2 mL/min, column temperature at 35°C and sample temperature at 18°C; eluent A was water with 0.1% (v/v) of formic acid and 0.2% (v/v) of acetonitrile, eluent B was acetonitrile with 0.1% (v/v) formic acid and 0.2% of water. A gradient elution was performed, according to the following parameters: 0–7 min 100% A, 7–50 min from 100% A to 50% A, 50–52.6 min 50% A, 52.6–53 min from 50% A to 0% A, 53–58.2 min 0% A, 58.2–59 min from 0% A to 100% A, 59–72 min 100% A (total analysis time 72 min). Injection volume was 2 µL for 100% pure species and 10 µL for calibration curves and blind samples.

Detection was achieved using a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Waltham, MA, USA) with the following parameters: solvent delay 0-7 min, acquisition 7-58.2 min, ionization type positive ions; spray voltage 3,500 V, vaporizer temperature 250°C; sheath gas pressure 22 (arbitrary units); capillary temperature 250°C. For the Q1MS Scan mode, the acquisition range was set at 100-1,500 m/z. For the Product Scan mode, different collision energies were applied (20, 25, 30, 35 and 40 V) to fragment the selected ions, and the acquisition range for fragments detection was 100-1,500 m/z. For the Selected Reaction Monitoring (SRM) method, the monitored transitions are reported in Table 2. The first reported fragment was used as quantifier, the second one as qualifier. UHPLC/ESI-MS data were elaborated using Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA). The calibration curves were constructed using both the “TIC” trace (sum of the two monitored transitions), and the trace of the first transition (the most intense), giving fully comparable results.

2.6 µHPLC-LTQ-OrbiTRAP analysis

For protein and peptide identification through tandem mass spectrometry analysis, samples were analyzed with µHPLC (Dionex Ultimate 3000, Sunnyvale, CA, USA) coupled to Orbitrap LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). Software employed for the analysis was Xcalibur 2.0.7 (Thermo Scientific, Waltham, MA, USA). Eluent A was water + 0.2% formic acid, eluent B was acetonitrile with 0.2% formic acid. Sample loading conditions were: enrichment cartridge: µ-Precolumn Cartridge, Acclaim PepMap100 C18 5 µm, 100 Å, 300 µm × 5 mm, loading flow: 30 µL/min, 50% eluent A and 50% eluent B. Sample elution condition were: column: Phenomenex Jupiter 4 µm Proteo 90Å 150 mm × 0.3 mm, column temperature: 35°C, gradient: 0-4 min 10% B, 4-60 min linear from 10% B to 50% B, 60-62 min from 50 to 95% B, 62-72 min 95% B (column washing), 72-73 min from 95% B to 10% B, 73-82 min 10% B (column equilibration). HRMS acquisition was performed through 5 subsequent events: event 1: full scan acquisition from 250 to 2000 m/z in high resolution mode (resolution at 400 m/z = 30,000); events from 2 to 5: data dependent scan, at each cycle the four most intense ions (with charge z>1 and with a minimum signal of 500 counts) identified in event 1 are fragmented. The same ion (tolerance 10 ppm and isolation window 2 m/z) can be observed for a maximum of 2 cycles, and then it is automatically inserted in the exclusion list for a maximum time of 20 seconds. Fragmentation is performed in the linear trap of the instrument in CID mode with collision energy of 35.

Proteins were identified with Peaks Studio (Bioinformatics Solutions, Waterloo, ON, Canada). Parameters were: precursor ion tolerance 5 ppm, fragment ion tolerance 0.8 Da, decoy database search: strict 0.01, relaxed 0.05, fixed modifications: cysteine carbamidomethylation, variable modifications: methionine oxidation, hydroxyproline and hydroxylisine. Searches were run both on the specific database of each species (for example *Gallus gallus* or *Equus caballus*) and on the class database (for example Aves or Mammals). Peptides which did not align with any protein present in the database were identified with *de novo* sequencing using both high and low resolution mass spectrometry.

3. Results and discussion

3.1 Marker peptides identification

Bolognese sauces prepared with the meat of several single pure species were first analysed. The proteins were extracted and digested with trypsin (details in the experimental section) and the peptide mixtures were analysed by LC coupled to high resolution mass spectrometry. Samples were analysed in Data Dependent Scan mode using an LTQ-OrbiTRAP instrument. The obtained spectra were analysed with the

Peaks^{TM} software to identify the main proteins and peptides present. The main proteins identified in the Bolognese sauces made with different types of meat are reported in Table S1 (supplementary on line material).

In Bolognese sauce made with poultry, rabbit, goat or sheep, the identified peptides were mostly deriving from collagen, myosin and tropomyosin, demonstrating that stomatic (collagen) and myofibrillar (myosin and tropomyosin) proteins are the most resistant to the thermal treatments applied to Bolognese sauce, generating a consistent amount of peptides. On the opposite, low amount of myofibrillar proteins-derived peptides were found in donkey Bolognese sauce, where collagen (stomatic protein) and haemoglobin (sarcoplasmic protein) gave the highest number of peptides. Haemoglobin derived peptides are found also in buffalo and red deer, together with the muscle counterpart myoglobin and with a good amount of myofibrillar proteins derived peptides (alpha and beta actin). Most of the horse peptides derived instead from myosin (myofibrillar protein). The amount and protein types of the peptides derive from a complex combination of factors: the meat cut used for Bolognese sauce preparation (some parts of the carcasses are richer in collagen), the prevalence of the single protein class in that meat species and the resistance to thermal treatment of the single protein.

To select good candidate marker peptides, only peptides having length longer than six amino acids were chosen, as previously performed by other authors (Watson, Gunning, Rigby, Philo, & Kemsley, 2015). In this way, the peptide length ensures the species-specificity. Then, the candidate peptide ions were cross checked in the chromatogram of the other meat species and only the peptides that were present in one meat types were selected. Moreover, a database search was performed to ensure peptide specificity (Basic Local Alignment Search Tool, BLAST). Among the species specific marker peptides identified, one peptide for each meat species was finally chosen taking into account the following factors: high abundance, good signal to noise ratio at low concentrations, high specificity, no missed cleavages and trypsin specific cleavage sites at both ends. Identified marker peptides are reported in Table 2, together with the MS parameters used for their detection. The SRM chromatograms of the peptide markers for each species are reported in Figure 1. For each chromatogram, all the transitions for all the species were monitored, to ensure the sensitivity of the method (each species having its peptide marker) and the selectivity of the method (only the specific marker peptide being present in each species).

All the identified marker peptides were re-analysed in a triple quadrupole instrument in order to re-confirm their sequence. Some of the marker peptides chosen for red meat samples analysed derived from the sarcoplasmic protein myoglobin. The highest abundance of myoglobin in red meat is probably the basis of the largest amount of myoglobin derived peptides in buffalo and horse. On the other side, marker peptides chosen for chicken and turkey (white meat) derived from fructose 1,6-bisphosphate aldolase, and the myofibrillar proteins troponin T and myosin, respectively. In some cases it was possible to identify a marker peptide very specific for the target species, such in case of rabbit, chicken, turkey and buffalo, whose marker peptides were found to be present only in *O. cuniculus*, *G. gallus*, *M. gallopavo* and *B. bubalis*, respectively. The marker peptide for rabbit (PHSHPALTPEQK) derives from the N-term of the metabolic enzyme fructose bisphosphate aldolase and was previously studied in literature in order to assess chemical modifications to the enzyme fructose 1,6-bisphosphate aldolase (Hopkins, O'Connor, Allen, Costello, & Tolan, 2002), or it has been used as control to evaluate the effect of drift gas polarizability for the separation of tryptic peptides (Ruotolo, McLean, Gillig, & Russell, 2004). So, this work describes for the first time its use for authenticity assessment in food products. The marker peptide for turkey ALGQNPTNAEMNK was already proven to be resistant to heat treatment, as it was detected in cooked beef mixture spiked with 10% turkey meat (Montowska, Alexander, Tucker, & Barrett, 2014). No information was available in literature about the troponin T peptide SDTEEVEHGEAHEAEEVHEEAH and the myoglobin peptide VETDVAGHGQEVL, present only in chicken and buffalo meat (respectively), which have been thus identified here for the first time. The chicken marker peptide shows a post translational modification, an acetylation on the N-term serine residue. This is a common feature in troponin T, where the N-term residue is acetylated and, subsequently, it can be phosphorylated by a protein kinase (Gusev, Dobrovol'skiĭ, & Severin, 1978). For what concerns sheep, the marker peptide AGEVGPPGPPGPAGEK was the most abundant among those that were specie specific for *O. aries*; this peptide derives from collagen α1(I) and it has two hydroxylated prolines (underlined in the sequence). Proline hydroxylation is a typical feature of collagen protein, since it confers the compact structure to collagen helices. This peptide is known in literature because of its use for the identification of sheep in natural leather goods (Izuchi, Takashima, & Hatano, 2016), but it has never been used for food authenticity assessment. There are many species that share a very similar sequence, which only differs for one amino acid: the N-term alanine of the peptide is present, in fact, only in sheep collagen. Unfortunately, no species-specific peptides for goat could be here detected, since all the identified peptides present in goat were also present in sheep. In previous works, it has been possible to discriminate between keratins from sheep and goat exploiting specific keratin-derived peptide markers, with the aim to assess wool and cashmere authenticity (Paolella et al, 2013). However, the amount of keratin in a meat preparation is negligible, thus these peptide markers could not be used here.

The myoglobin peptide HPGDFGADAQGAMTK has been previously used to detect horse in fresh meat (Watson, Gunning, Rigby, Philo, & Kemsley, 2015). Given the genetic proximity of donkey and horse, which belong to the same genus (*Equus*), we found this peptide also in Bolognese sauce prepared with donkey meat. Since the alignment in the Uniprot database gave as the only outputs horse and zebra, this is probably the first time that the myoglobin peptide HPGDFGADAQGAMTK has been found in donkey. Unfortunately, all the peptides identified in the Bolognese sauce prepared with horse meat, were present also in that prepared with donkey meat. Thus, this myoglobin peptide was used as a marker peptide for equine meat (horse and donkey). The amino acid sequence of the marker peptide for duck (*Anas platyrhynchos*) was not present in the protein database, thus it was determined through *de novo* sequencing using a triple quadrupole instrument. The amino acid sequence was determined on the basis of the fragmentation pattern. The amino acid sequence (QELADLAR) has a certain homology with the sequences QENANTLAR and QENANALAR, found in fructose-1,6-bisphosphate aldolase of *A. platyrhynchos* (the same protein from which the marker peptide for rabbit derives). The failure to align the peptide sequences QELADLAR with duck can be explained by the limited number of proteins available in its database. Unfortunately, duck proteome, to date, has not been intensively studied as it happened for other animal species, such as for examples piglet or cattle. However, we found this peptide only in the duck sample, and it was not present in any of the other meat samples under investigation neither in beef or pork meat. Thus, this cross check covered almost all the meat used for human consumption in Western countries. For what concerns red deer, we found that the peptide LNFKPEEEYPDLSK is present in M-type creatine kinase of red deer, and it is present in the protein database of thirteen line ground squirrel, Tasmanian devil and gray short tailed opossum. However, these species are not of food interest and quite of difficult availability, thus it is unlikely that they could be used for meat adulteration. So, the peptide LNFKPEEEYPDLSK was used as a marker for red deer presence.

3.2 Meat species quantification

Once identified, marker peptides for each of the species under study were analysed in order to define the limit of detection and quantification of the method, and also the linearity of the response at very low percentages was studied. More specifically, classical Bolognese sauces were purposely prepared in an industrial pilot plant, following traditional recipe (50% bovine + 50% swine meat), with the addition of 1, 2.5 and 5% (w/w_{meat}) of duck, rabbit, chicken, turkey, donkey, buffalo, horse and red deer.

The aim of this part of the work was to quantify the amount of each meat species (in % w/w) present in Bolognese sauce samples. To do that, the areas of the marker peptides were integrated (sum of both the transitions monitored). Also the area of a common peptide (present in all the meat species) was integrated. The ratio between the area of each marker peptide and the area of the common peptide was made to normalize the data, and to avoid differences due to possible variations in protein extraction efficiency, enzyme activity, and other factors. The peptide used for the normalization of the data was DMIPAQK (deriving from C-term of creatine kinase M-type), used here as a molecular marker for the total meat amount. The obtained ratio was plotted against the amount of each meat species (% w/w). A very good linearity was found for equine, followed by duck and rabbit (Table 4), while lower correlation coefficients were found for turkey and buffalo.

Limits of detection and quantification were determined from the calibration curves, according to the IHC guidelines (2005), using the formulas: $LOD = 3.3 \times \frac{Sy}{S}$ and $LOQ = 10 \times \frac{Sy}{S}$, where Sy is the standard deviation of the response and S is the slope of the calibration curve. The limits of species detection in mixed meat, calculated as percentage on total meat, were in the range 0.8-3.6% (equine-turkey, respectively), while the limits of quantification, always calculated as percentage on total meat were in the range 2.8-12.1%.

Considering that the amount of meat used in Bolognese sauce preparation was 19%, the LOD and the LOQ calculated on the total sauce are 0.15-0.68% and 0.53-2.30% on whole finished product, respectively. Thus, the developed method is able to detect even small contamination of these meat species in the final product and, in the case of massive adulteration, it is able to quantify with a good accuracy the different proportions of meat added. These results were very encouraging also considering the complexity of the tested product (Bolognese sauce), which is composed of many ingredients and submitted to prolonged cooking and a strong sterilization process.

Finally, several blind samples were analysed to check the reliability of the method from very low percentages (such as 0.38% for turkey and buffalo, around the LOD and LOQ values) to 6% (w/w, on whole product) for sheep. The SRM chromatograms of the analysed blind samples are reported in Figure 2.

Very good trueness values (in the range 80-120%) were found for duck, chicken, and sheep 1 (Table 5). It is important to note that, while the amounts of duck and sheep meat added, were higher than LOQ, chicken meat was added in a percentage slightly lower than the LOQ. However, the developed method was able to detect it with a good accuracy. Considering the total amount of meat present in the Bolognese sauce (19% w/w), in most cases the amount of each added species was very low: from 0.38% (w/w, on whole product) for turkey and buffalo to 6% (w/w, on whole product) for sheep. Turkey and buffalo (the two species tested in lower amount) were correctly detected in the samples, even if with less satisfactory accuracy: turkey was overestimated in the blind sample (5.8% instead of 2%, trueness 290%), while buffalo was underestimated (1% instead of 2%, trueness 50%). However, it has to be taken into account that, since the absolute values are low (low % of meat species added), the error percentage becomes high. For higher values (higher % of meat species added), the trueness improves. Intermediate accuracy values were obtained for the other species. The developed method has good specificity and sensitivity for the meat species under investigation. Its application to quantify the amount of meat using a calibration curve in processed food can be improved for some species; however, considering that no analytical methods are available at the moment for this purpose, the results here shown represent a step forward in the field of food authentication, also considering the complexity of the product, and the strong thermal treatment occurred.

Once established the threshold of the method for each species, several samples at unknown meat composition were subjected to the new developed analysis for fraudulent meat species checking. The SRM chromatograms of the blind samples analysed are reported in Figure 3.

As shown in Table 6, the proposed UHPLC/ESI-MS/MS method was always able to detect the meat addition, thus no false negatives were present, demonstrating a high sensitivity of the method. Moreover, the developed method has showed a high specificity, because when a certain meat species was not present, samples always resulted below the LOD, thus no false positives were reported. This is particularly valuable because we took into account also closely related species that could cross-react, such as for example turkey and chicken.

3.3 Conclusions

The here developed method allows the simultaneous detection and quantification of 8 different meat species in a 72 minutes run in Bolognese sauce preparations. Selected peptide markers were used to build calibration curves with a good linearity, allowing obtaining the quantification of the meat species present. This comprehensive methodology took into account the most used species in the meat sector, and was applied to real industrial products prepared by a sausage company according to industrial recipes. On the basis of the obtained results, the method showed, on blind samples, a very good specificity (no false positives) and sensitivity (no false negatives). This is of the utmost importance giving the application on a complex food matrix like Bolognese sauce, which is one of the most susceptible to frauds. The quantitative performance of this method will allow to discriminate between an accidental contamination (low amount detected) and a voluntary adulteration (presumably higher detected amount). With the current work, we provide a useful tool both to the control bodies (to assess product authenticity), and to the food industries (to monitor supplies of raw materials). Moreover, the success of the application of this method to a very difficult food matrix demonstrates how the same approach can likely be successfully applied to a broad range of food preparations.

4. Conflict of interest

The authors declare that they have no conflict of interests.

5. Acknowledgements

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6. Captions to figures

Figure 1. SRM chromatograms obtained for each species: A – duck, B – rabbit, C – chicken, D – turkey, E – buffalo, F – horse, G – deer, H – sheep. For each chromatogram, all the transitions for all the species were monitored.

Figure 2. Overlaid SRM chromatograms of the blind samples analysed for sample set 1 (A – duck, rabbit, chicken and turkey) and for sample set 2 and 3 (B – buffalo, equine, deer and sheep).

Figure 3. Overlaid SRM chromatograms of the detected meat species in the unknown samples.

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Table 1. Experimental plan: set 1 comprises poultry and rabbit meat, set 2 contains buffalo, equine and red deer meat, and set 3 contains sheep and goat meat.

Set 1	Set 2	Set 3
Mix beef/pork 96% + 1% duck, 1% rabbit, 1% chicken, 1% turkey	Mix beef/pork 96% + 1% donkey, 1% buffalo, 1% horse, 1% red deer	Mix beef/pork 98% + 1% sheep, 1% goat
Mix beef/pork 90% + 2.5% duck, 2.5% rabbit, 2.5% chicken, 2.5% turkey	Mix beef/pork 90% + 2.5% donkey, 2.5% buffalo, 2.5% horse, 2.5% red deer	Mix beef/pork 94% + 3% sheep, 3% goat
Mix beef/pork 80% + 5% duck, 5% rabbit, 5% chicken, 5% turkey	Mix beef/pork 80% + 5% donkey, 5% buffalo, 5% horse, 5% red deer	Mix beef/pork 90% + 5% sheep, 5% goat
Blind_1	Blind_2	Blind3A + Blind 3B

Table 2. Identified marker peptides for each species are reported with their retention times and SRM parameters.

Peptide	Species	Retention time (min)	Parent ion (m/z)	Product ions (m/z)	Collision Energy
QELADLAR	Duck	24.4	458.1	449.3; 545.0	20
PHSHPALTPEQK	Rabbit	18.8	447.8	234.8; 314.0	30
SDTEEVEHGEAHEAEEVHEEAH	Chicken	24.0	636.3	226.6; 155.8	30
ALGQNPTNAEMNK	Turkey	22.2	694.2	904.5; 185.2	35
VETDVAGHGQEVL	Buffalo	26.0	677.1	1123.5; 200.6	30
HPGDFGADAQGAMTK	Equine	23.1	751.8	234.6; 742.6	35
LNFKPEEEYPDLSK	Deer	27.7	570.1	722.7; 135.5	25
AGEVGPPGPPGPAGEK	Sheep	21.4	724.7	518.3; 1092.6	30
DMIPAQK	Meat	22.3	802.3	443.2; 297.1	30

Table

Table 3. Tandem mass spectrometry identification of the species specific marker peptides chosen for each meat species. Columns from “a” to “z” refer to the types of fragment ions observed in an MS/MS spectrum of the peptides, with the m/z ratio of the detected fragments. Number in brackets indicate the position of the broken bond (count starts from the N-term for a, b, and c fragments; from the C-term for x, y, and z fragments). In the “BLAST” column, the species whose proteins align with 100% identity with the peptide marker are reported.

Sequence	a	b	c	x	y	z	Protein	Blast
QELADLAR (Duck)		257.5 (2) 370.9 (3) 556.7 (5) 897.6 (8)		384.7 (3)	174.7 (1) 359.0 (3) 474.2 (4) 658.3 (6)	229.1 (2)	*De novo* sequencing	-
PHSHPALTPEQK (Rabbit)	207.0 (2)	235.0 (2) 459.3 (4) 1069.1 (10)			501.2 (4) 602.1 (5) 883.3 (8) 1020.8 (9) 1109.1 (10) 1245.5 (11)	257.6 (2)	Fructose-bisphosphate aldolase	*O. cuniculus*
SDTEEVEHGEAHEAEEVHEEAH (Chicken)		346.0 (3) 604.6 (5) 703.3 (6) 970.6 (9)		510.3 (4)	155.4 (1) 226.4 (2) 357.3 (3) 484.2 (4) 721.3 (6) 1051.7 (9) 1149.5 (20)		Troponin T	*G. gallus*
ALGQNPTNAEMNK (Turkey)	157.0 (2)	185.2 (2) 1127.1 (11) 185.2 (4)	299.2 (6)		391.7 (3) 592.2 (5) 706.5 (6) 807.0 (7) 904.5 (8) 1018.7 (9) 1203.7 (11) 452.4 (8) 573.8 (10)		Myosin essential light chain isoform 1	*M. gallopavo*

VETDVAGHGQEVL (Buffalo)	201.1 (2) 417.2 (4) 516.3 (5) 781.4 (8) 838.4 (9) 1194.6 (12)	229.1 (2) 330.2 (3) 445.2 (4) 866.4 (9) 994.5 (10) 1123.5 (11) 1222.6 (12)	561.3 (5)	708.3 (6)	132.1 (1) 739.4 (7) 810.4 (8) 909.5 (9) 1024.5 (10) 1254.6 (12)	1007.5 (10) 1108.5 (11)	Myoglobin	*B. bubalis*
HPGDFGADAQGAMTK (Horse)	109.7 (1) 526.7 (5)	234.6 (2) 683.2 (7) 1124.9 (12)		406.5 (3)	146.7 (1) 247.6 (2) 379.0 (3) 634.7 (6) 705.8 (7) 892.5 (9) 948.8 (10) 1097.0 (11) 1269.0 (13) 1366.0 (14)		Myoglobin	*E. burchelli* *E. caballus*
LNFKPEEEYPDLSK (Deer)		858.7 (7) 987.3 (8)		372.7 (3)	233.8 (2) 558.9 (5) 722.0 (6) 1206.8 (10)		Creatine kinase	*C. elaphus hippelaphus* *I. tridecemlineatus* *S. harrisii* *M. domestica*
AGEVGPPGPPGPAGEK (Sheep)	229.6 (3) 385.9 (5)	258.2 (3) 357.3 (4) 414.6 (5) 947.1 (11) 1116.7 (13)	146.4 (2) 374.3 (4) 431.8 (5) 658.0 (7) 1134.1 (13)	302.9 (2) 681.3 (7)	147.1 (1) 333.2 (3) 500.7 (5) 558.2 (6) 1035.5 (11) 1092.5 (12)	905.9 (10)	Collagen	*O. aries*

Table 4. Calibration curves and limits of detection and quantification for the different meat species. Table 4 Calibration curves and limits of detection and quantification for the different meat species.

Meat species	Line equation	R^2	LOD (% w/w)	LOQ (% w/w)
Duck	$y = 0.002573x - 0.00138$	R^2=0.962	1.3	4.0
Rabbit	$y = 0.001937x - 0.00046$	R^2=0.931	1.8	5.5
Chicken	$y = 0.000919x - 0.00013$	R^2=0.912	2.1	6.3
Turkey	$y = 0.000367x + 0.000472$	R^2=0.734	4.0	12.1
Buffalo	$y = 0.000285x + 0.000674$	R^2=0.848	2.8	8.6
Equine	$y = 0.001252x + 0.000559$	R^2=0.995	0.9	2.8
Red Deer	$y = 0.000357x - 0.000104$	R^2=0.904	2.2	6.6
Sheep	$y = 0.001188x + 0.00017$	R^2=0.786	3.4	10.4

Table 5. Results obtained for the blind samples, where the different meat species were added.

species	% (w/w) of meat added	% (w/w) of meat calculated
Duck	5.0	4.6±0.2
Rabbit	4.0	5.6±0.0
Chicken	6.0	5.0±0.5
Turkey	2.0	5.8±1.1
Buffalo	2.0	1.0±0.5
Equine	11.0	17.7±3.2
Red Deer	4.0	2.3±0.1
Sheep 1	31.6	30.8±1.4
Sheep 2	15.8	21.0±1.3

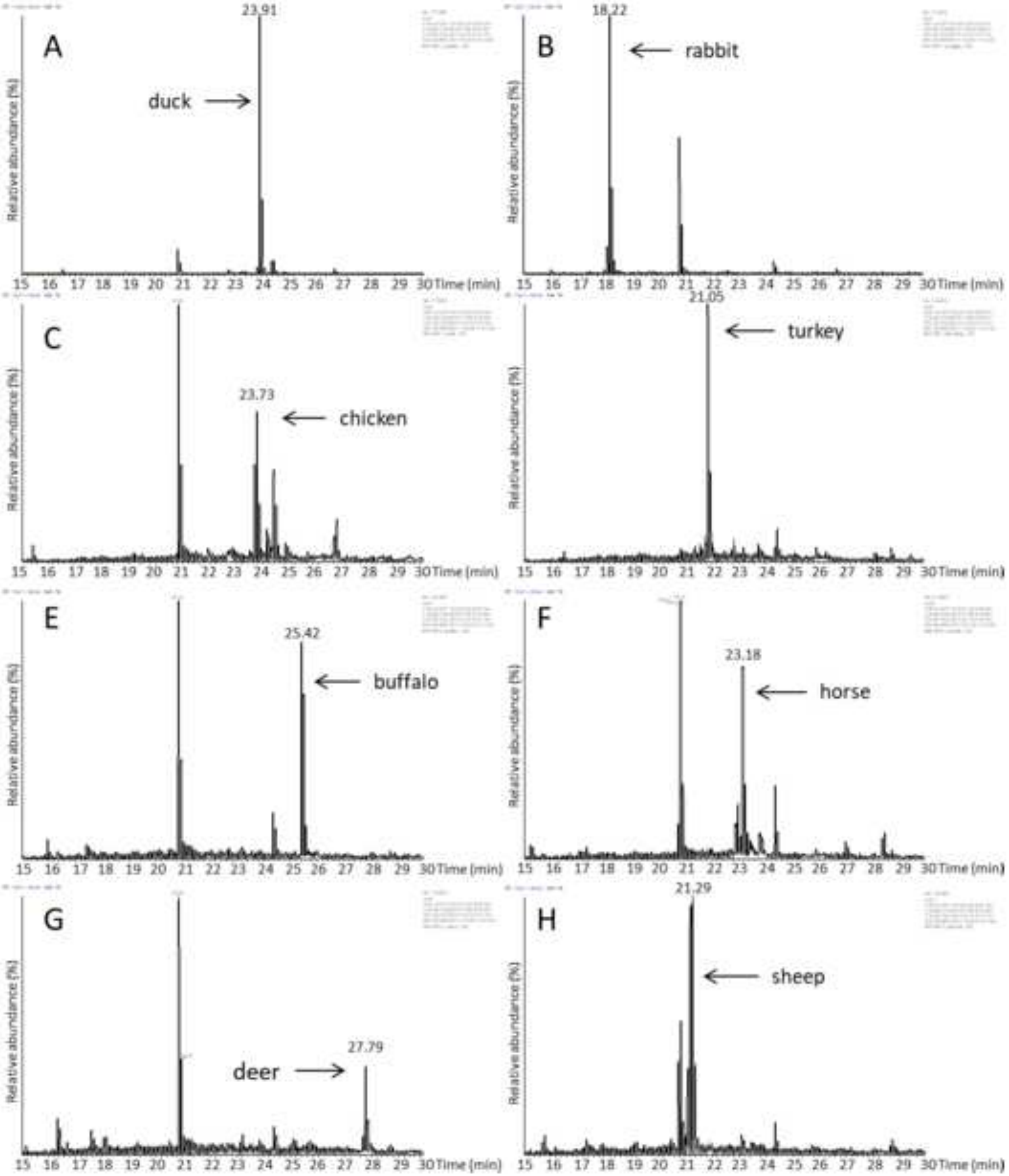
Table

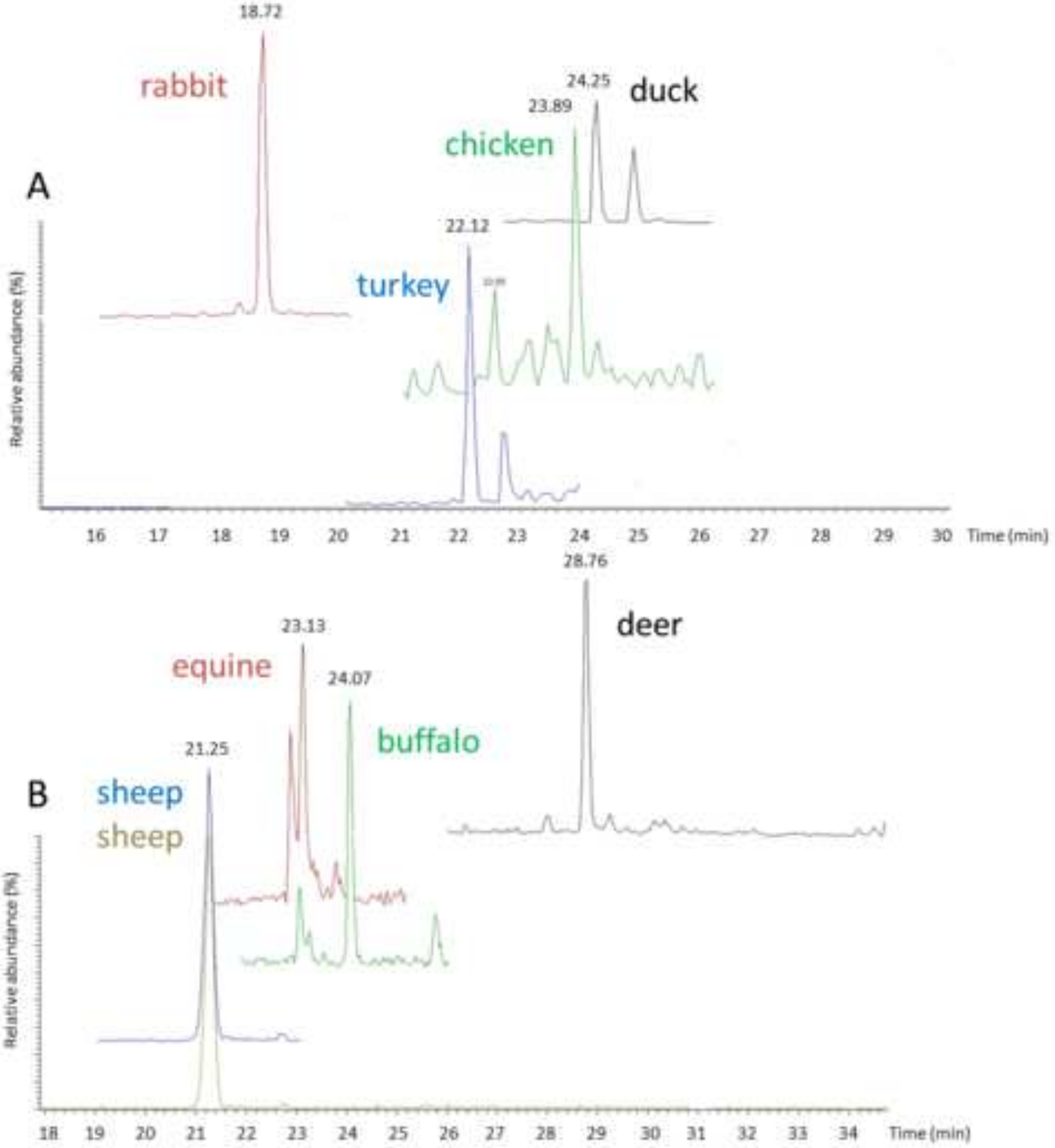
Table 6. Blind samples composition, expressed as % (w/w) of each species on the total amount of meat. Symbol √ means “detected above LOD”.

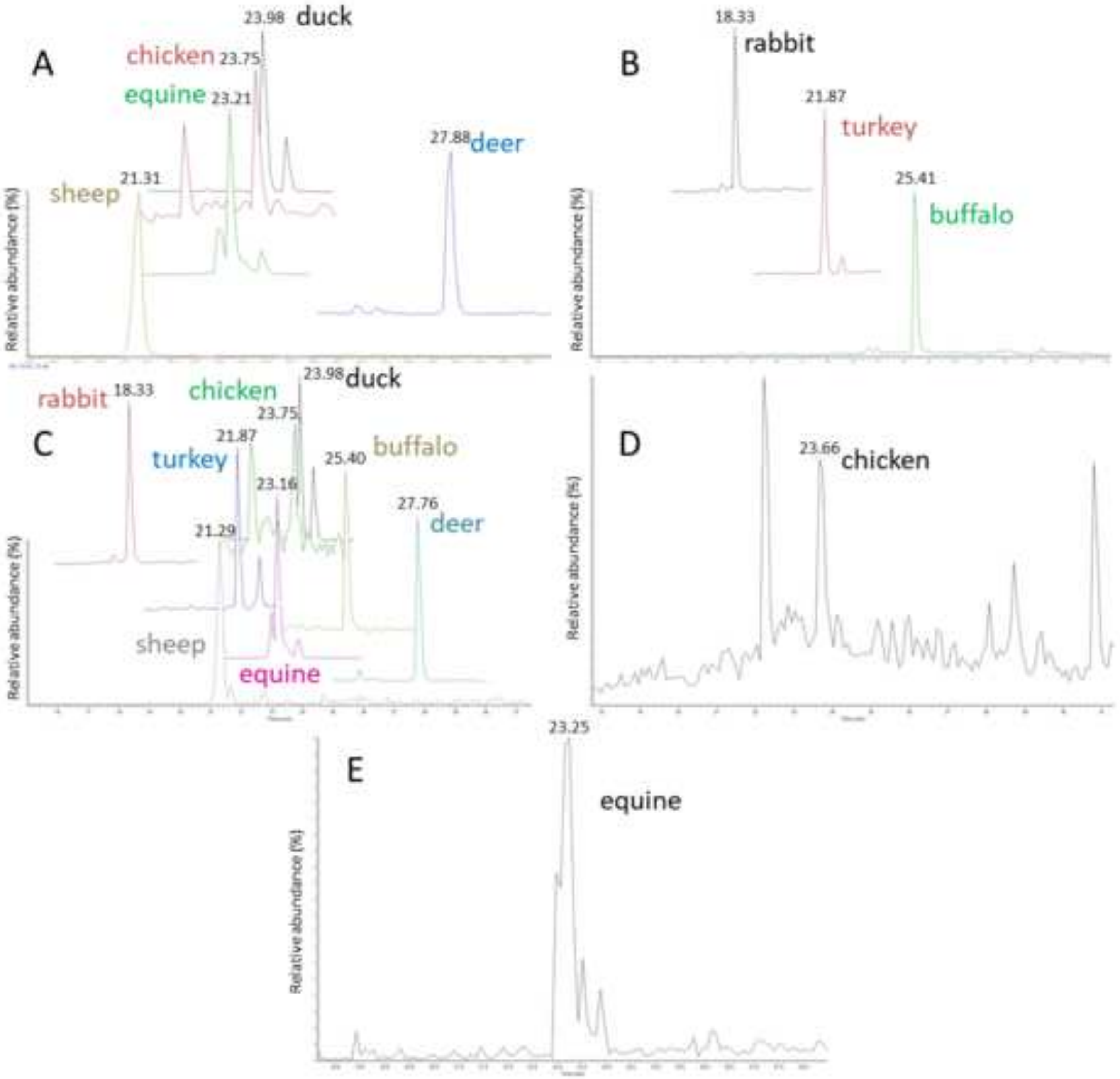
	Recipes (meat % on finished product)					**Results**				
	A	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
Duck	2.00		1.58			√		√		
Rabbit		2.00	1.58				√	√		
Chicken	2.00		1.58	2.00		√		√	√	
Turkey		2.00	1.58				√	√		
Buffalo		2.00	1.58				√	√		
Equine	2.00		1.58		2.00	√		√		√
Deer	2.00		1.58			√		√		
Sheep	2.00		1.58			√		√		

Figure

Click here to download high resolution image




A
18.72
rabbit
24.25
duck
23.89
chicken
22.12
turkey
Relative abundance (%)
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
Time (min)
28.76
deer
23.13
equine
24.07
buffalo
21.25
B
sheep
sheep
Relative abundance (%)
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
Time (min)

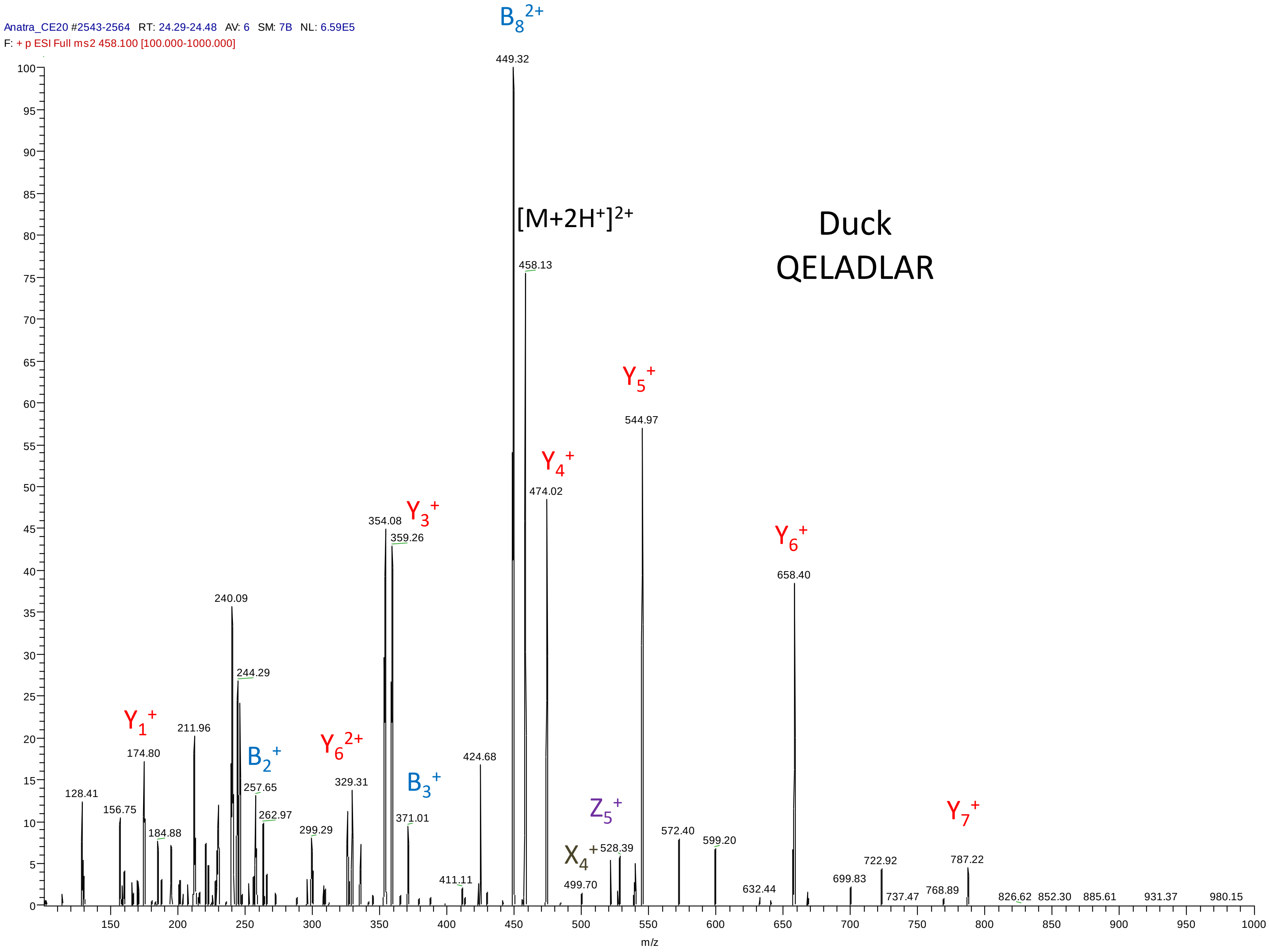

A
23.98 duck
chicken 23.75
equine 23.21
27.88 deer
sheep 21.31
Relative abundance (%)
B
18.33
rabbit
21.87
turkey
25.41
buffalo
Relative abundance (%)
C
rabbit 18.33
chicken
23.98 duck
23.75
21.87
turkey
25.40
buffalo
23.16
21.29
27.76
deer
sheep
equine
Relative abundance (%)
D
23.66 chicken
Relative abundance (%)
E
23.25
equine
Relative abundance (%)

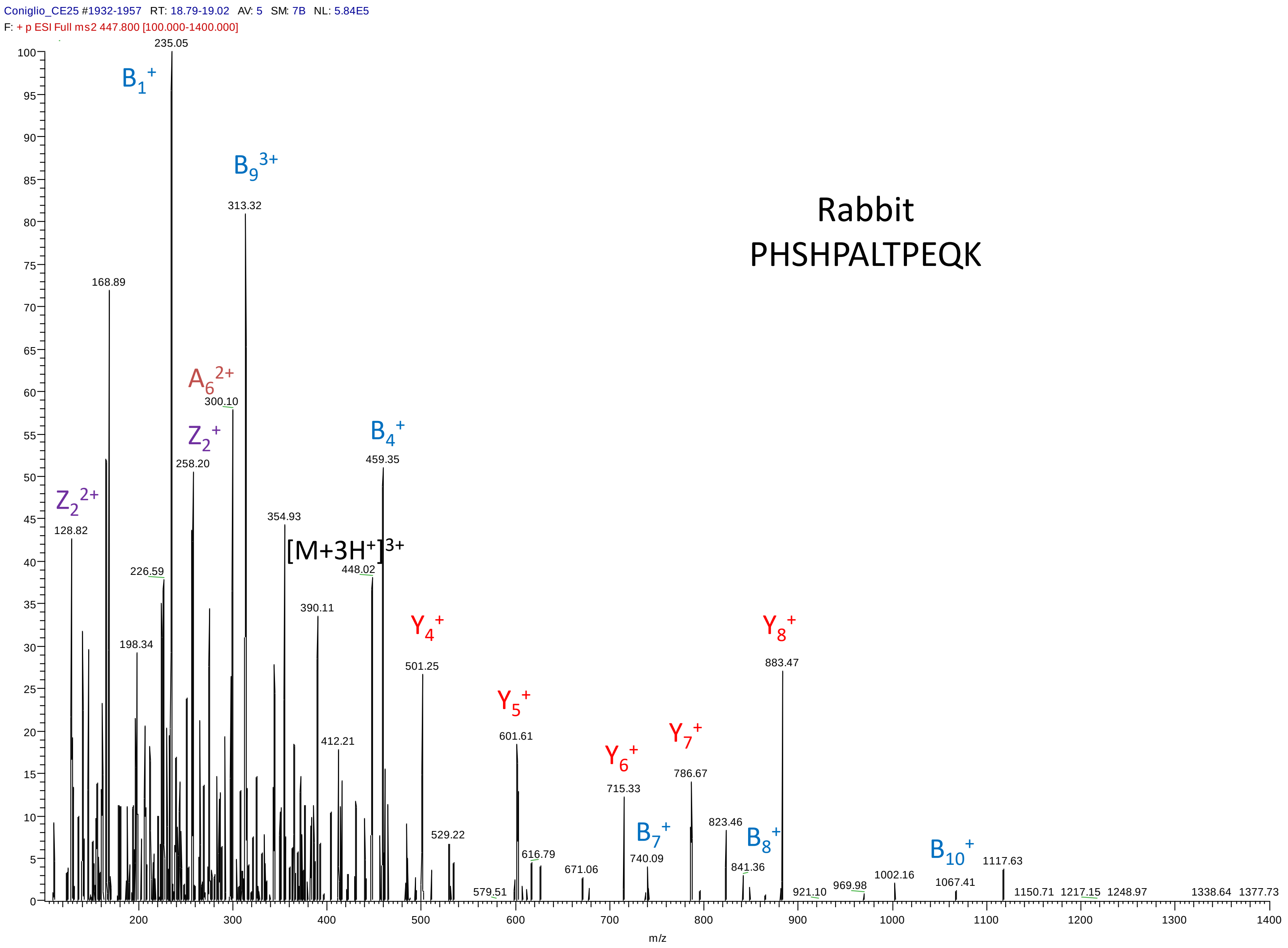
Supplementary Table

Table S1. Main proteins identified through bottom up proteomics in the different Bolognese sauce samples.

Accession	Description	Score	Coverage	MW [kDa]
Duck (Anas platyrhynchos)				
U3IW58	Uncharacterized protein	86	22	128.9
U3J7T0	Uncharacterized protein	81	10	216.0
U3J9U3	Uncharacterized protein	59	15	88.0
R0KZK3	Tropomyosin alpha-1 chain	50	13	23.6
Rabbit (Oryctolagus cuniculus)				
G1SJN7	Myosin-4	220	16	223.1
G1TKS9	Uncharacterized protein	196	13	223.5
L7N1T5	Uncharacterized protein	182	10	223.4
G1T4A5	Collagen alpha-1(I) chain	181	40	109.7
Chicken (Gallus gallus)				
P13538	Myosin heavy chain, skeletal muscle	111	13	223.0
P02457	Collagen alpha-1(I) chain	75	17	137.7
F1NM23	Tropomyosin alpha-1 chain	73	21	31.4
F1P0H9	Collagen alpha-2(I) chain	48	21	128.8
Turkey (Meleagris gallopavo)				
F1P3X1	Uncharacterized protein	113	8	223.3
P02457	Collagen alpha-1(I) chain	101	23	137.7
G1NB83	Uncharacterized protein	64	16	128.0
F1NM23	Tropomyosin alpha-1 chain	64	23	31.4
Donkey (Equus asinus)				
B9VR88	Collagen alpha-1 type I chain	446	50	139.0
B9VR89	Collagen alpha-2 type I chain	350	50	128.7
P01959	Hemoglobin subunit alpha	131	29	15.2
D1MPT0	Hemoglobin beta chain	110	9	16.0
Buffalo (Bubalus bubalis)				
Q9TSN8	Hemoglobin subunit alpha-2	343	56	15.2
P84997	Myoglobin	312	53	17.2
Q0PEU1	Alpha 2 actin	250	35	20.1
A1XSX3	Beta-actin	230	36	41.7
Horse (Equus caballus)				
F6R839	Myosin-2	386	19	223.0
Q8MJV0	Myosin-1	386	19	222.9
Q8MJV1	Myosin-2	360	15	222.7
F6UP52	Uncharacterized protein	332	11	223.1
Red deer (Cervus elaphus)				
C0HJR0	Myoglobin	268	56	17.1
Q4TU70	Alpha hemoglobin chain	259	73	15.2
P01971	Hemoglobin subunit alpha	242	80	15.2
A0S012	Beta-actin	219	36	25.7
Sheep (Ovis aries)				
W5PPG6	Uncharacterized protein	257	12	222.8
W5PT09	Myosin heavy chain 2	240	10	222.4
W5NTT7	Collagen type I alpha 2 chain	223	19	129.0
A0A0H3V7A0	Myosin light chain 1 transcript variant 2	208	56	20.9
Goat (Capra hircus)				
F2X909	Collagen type I alpha 2 (Fragment)	228	84	13.6
F6KVT2	Troponin C type 1 slow	180	32	18.4
F6KVT3	Fast twitch skeletal muscle troponin C2	156	15	18.1
J9V335	Beta-actin (Fragment)	137	26	18.3

Supplementary Figure



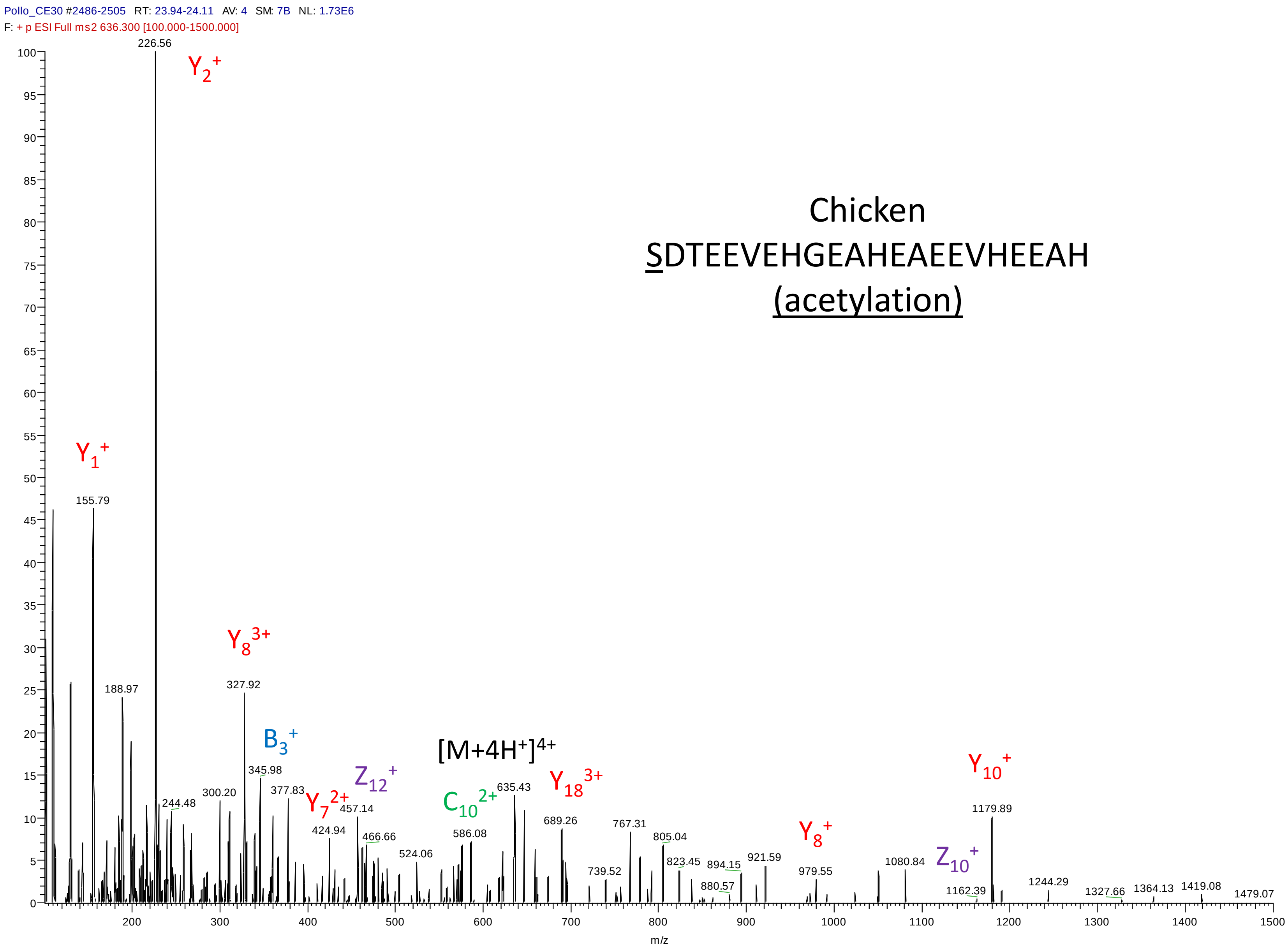


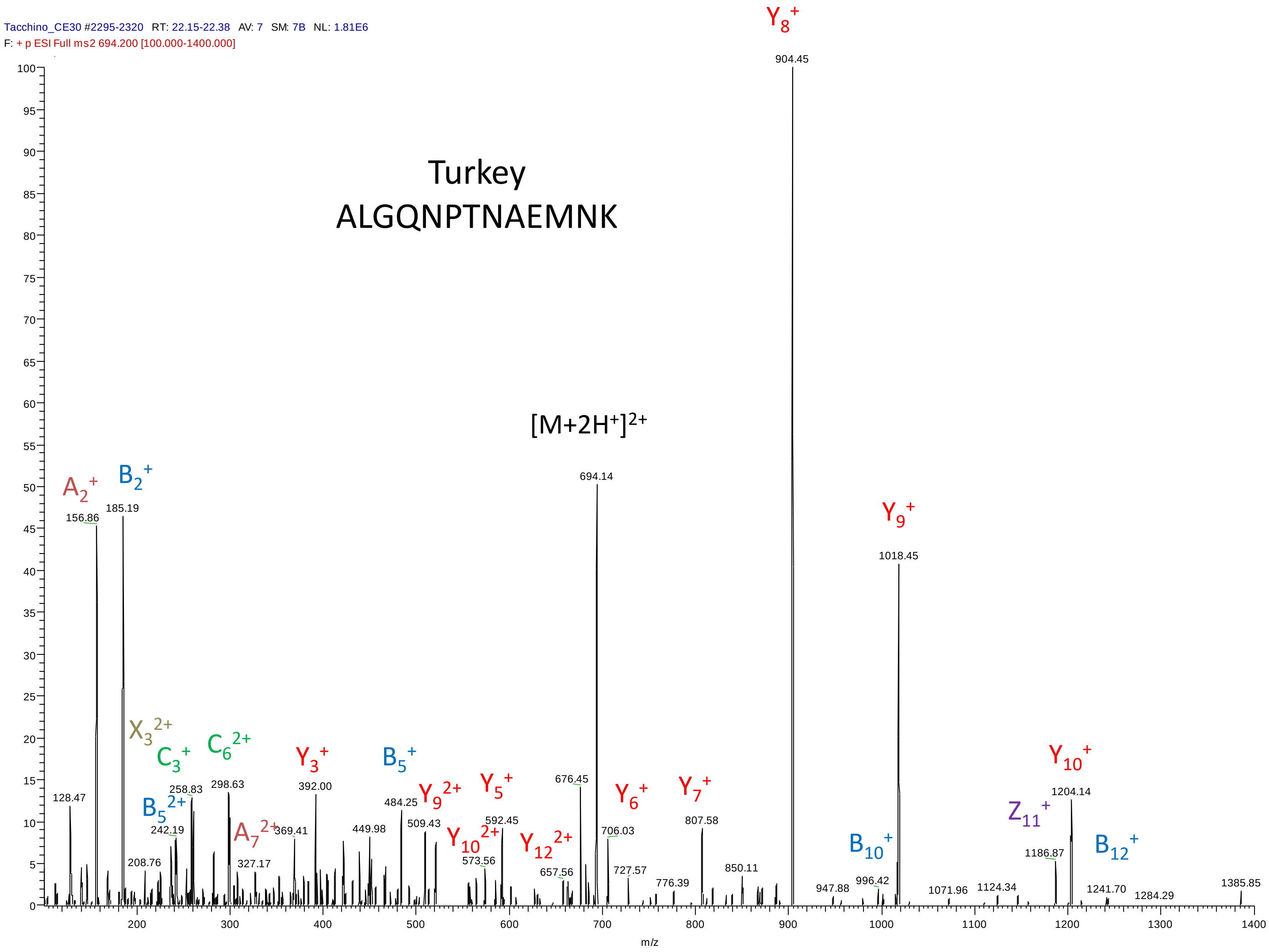
Rabbit
PHSHPALTPEQK

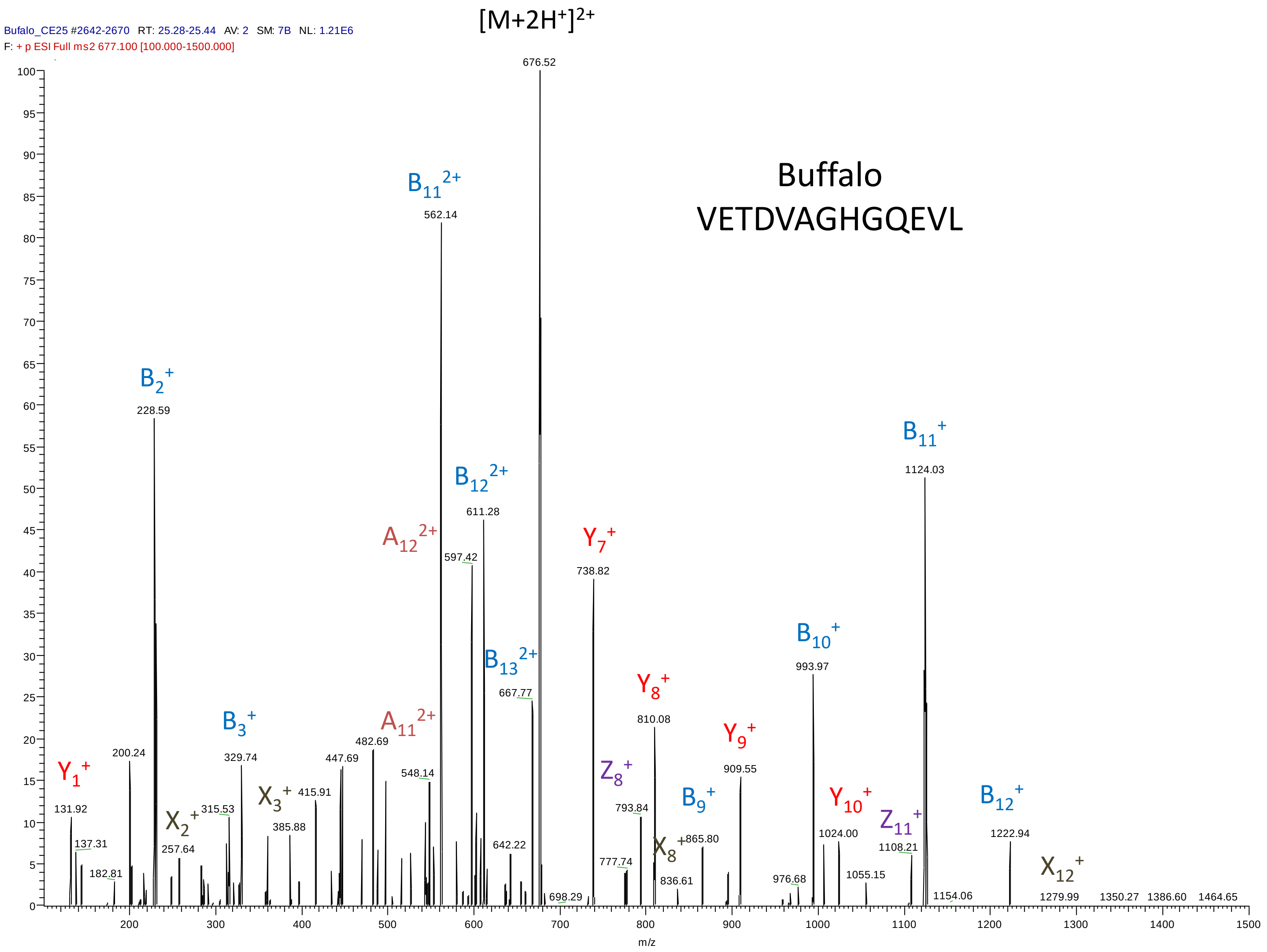
Chicken

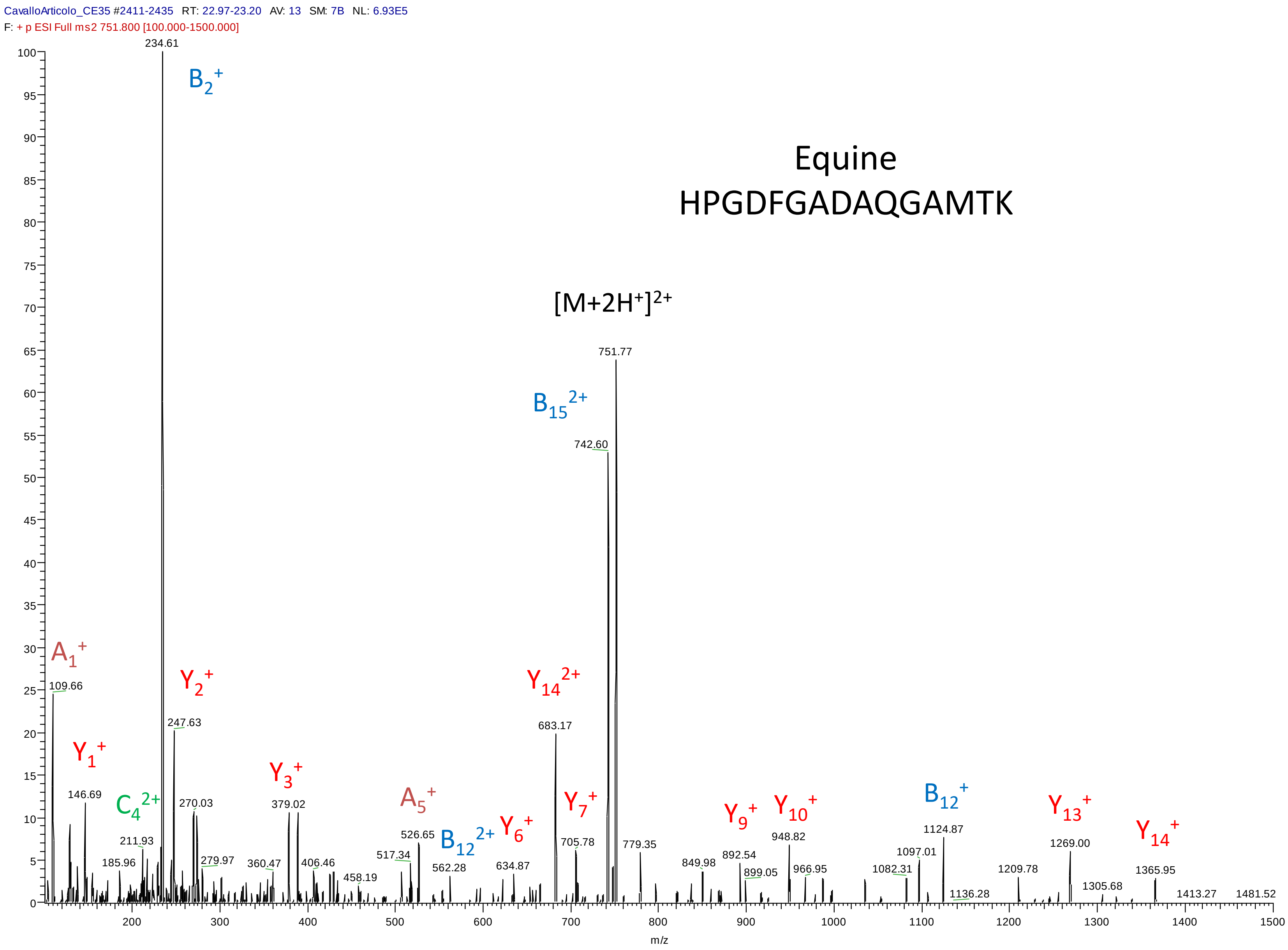
SDTEEVEHGEAHEAEEVHEEAH

(acetylation)

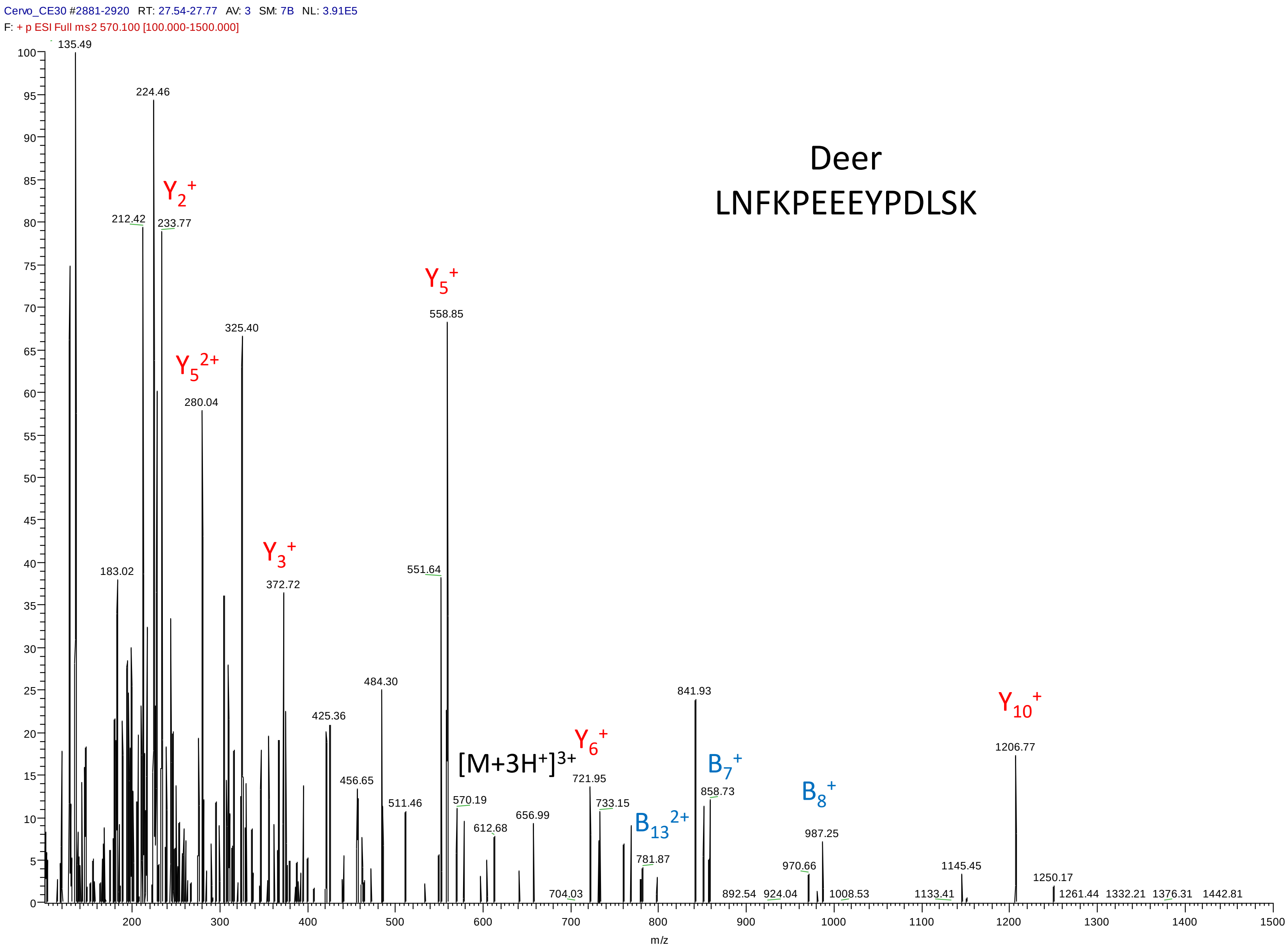



Tacchino_CE30 #2295-2320 RT: 22.15-22.38 AV: 7 SM: 7B NL: 1.81E6
F: + p ESI Full ms2 694.200 [100.000-1400.000]
Turkey
ALGQNPTNAEMNK
[M+2H⁺]²⁺
Y_8^+
Y_9^+
Y_{10}^+
Y_3^+
Y_5^+
Y_6^+
Y_7^+
Y_9^{2+}
Y_{10}^{2+}
Y_{12}^{2+}
A_2^+
A_7^{2+}
B_2^+
B_5^+
B_5^{2+}
B_{10}^+
B_{12}^+
C_3^+
C_6^{2+}
X_3^{2+}
Z_{11}^+
904.45
694.14
1018.45
156.86
185.19
128.47
208.76
242.19
258.83
298.63
327.17
369.41
392.00
449.98
484.25
509.43
573.56
592.45
657.56
676.45
706.03
727.57
776.39
807.58
850.11
947.88
996.42
1071.96
1124.34
1186.87
1204.14
1241.70
1284.29
1385.85
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
m/z


Bufalo_CE25 #2642-2670 RT: 25.28-25.44 AV: 2 SM: 7B NL: 1.21E6
F: + p ESI Full ms2 677.100 [100.000-1500.000]
[M+2H+]2+
676.52
Buffalo
VETDVAGHGQEVL
B11 2+
562.14
B2 +
228.59
B11 +
1124.03
B12 2+
611.28
A12 2+
597.42
Y7 +
738.82
B13 2+
667.77
B10 +
993.97
Y8 +
810.08
B3 +
329.74
A11 2+
482.69
447.69
548.14
200.24
Y9 +
909.55
Y1 +
131.92
137.31
182.81
X2 +
257.64
315.53
X3 +
415.91
385.88
Z8 +
793.84
777.74
B9 +
X8 +
865.80
836.61
642.22
698.29
976.68
Y10 +
1024.00
1055.15
Z11 +
1108.21
B12 +
1222.94
X12 +
1154.06
1279.99
1350.27
1386.60
1464.65
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z



Equine
HPGDFGADAQGAMTK


Cervo_CE30 #2881-2920 RT: 27.54-27.77 AV: 3 SM: 7B NL: 3.91E5
F: + p ESI Full ms2 570.100 [100.000-1500.000]
Deer
LNFKPEEEYPDLSK
135.49
224.46
Y_2^+
212.42
233.77
Y_5^+
558.85
325.40
Y_5^{2+}
280.04
Y_3^+
372.72
183.02
551.64
484.30
841.93
425.36
Y_{10}^+
1206.77
$[M+3H^+]^{3+}$
Y_6^+
721.95
B_7^+
858.73
456.65
511.46
570.19
733.15
B_8^+
656.99
612.68
B_{13}^{2+}
987.25
781.87
970.66
1145.45
1250.17
704.03
892.54
924.04
1008.53
1133.41
1261.44
1332.21
1376.31
1442.81
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
200
300
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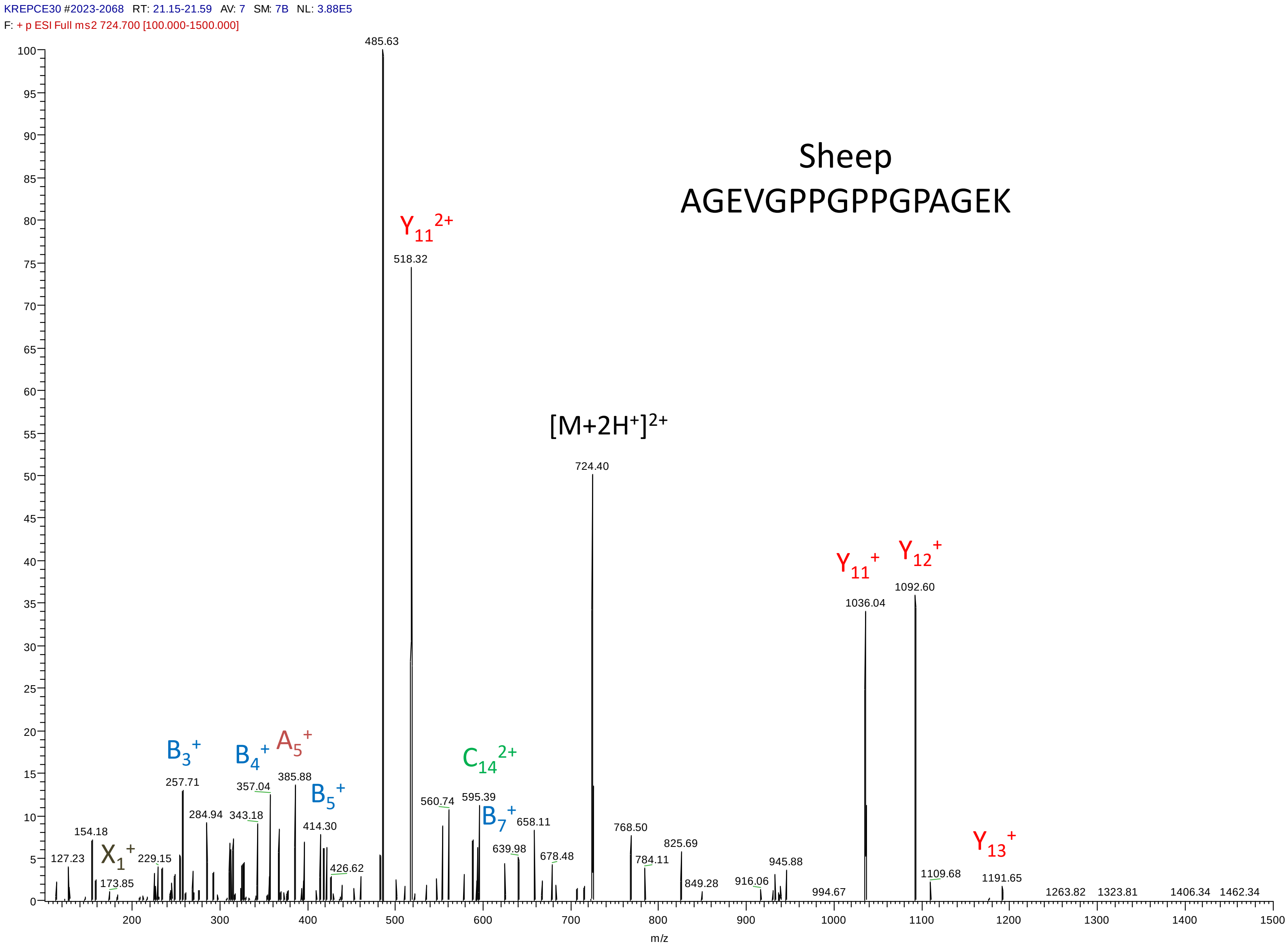
700
800
900
1000
1100
1200
1300
1400
1500
m/z



Sheep

AGEVGPPGPPGPAGEK

HIGHLIGHTS

- Species-specific marker peptides were identified for eight meat species
- A LC-MS based method was developed for the detection of the marker peptides
- Bolognese sauces were prepared with increasing amount of each species
- Using proper calibration curves the method can quantify the meat species present
- A validation was performed analyzing blind Bolognese sauce samples