UNIVERSITÀ DI PARMA
ARCHIVIO DELLA RICERCA

University of Parma Research Repository

Mass spectrometry quantification of beef and pork meat in highly processed food: Application on Bolognese sauce

This is the peer reviewd version of the followng article:

Original
Mass spectrometry quantification of beef and pork meat in highly processed food: Application on Bolognese sauce / Prandi, B., Lambertini, F., Faccini, A., Suman, M., Leporati, A., Tedeschi, T., Sforza, S.. - In: FOOD CONTROL. - ISSN 0956-7135. - 74:(2017), pp. 61-69. [10.1016/j.foodcont.2016.11.032]

Availability:
This version is available at: 11381/2827475 since: 2021-10-15T12:28:23Z

Publisher:
Elsevier Ltd

Published
DOI:10.1016/j.foodcont.2016.11.032

Terms of use:

Anyone can freely access the full text of works made available as "Open Access". Works made available

Publisher copyright

note finali coverpage

(Article begins on next page)

15 October 2026

***Highlights (for review)**

Highlights

- Myofibrillar proteins were extracted from Bolognese sauce and submitted to tryptic digestion
- Marker peptides for beef and pork meat were identified using LC-MS/MS
- Calibration curves were constructed at different beef and pork percentages in the sauce
- The method was accurate in detecting beef and pork amount in blind samples

*Manuscript
Click here to view linked References

Mass spectrometry quantification of beef and pork meat in highly processed food: application on Bolognese sauce

Barbara Prandi[a], Francesca Lambertini[b], Andrea Faccini[c], Michele Suman[b], Andrea Leporati[b], Tullia Tedeschi[a], Stefano Sforza[a*]

[a] Department of Food Science, University of Parma, Parco Area delle Scienze 59/A, 43124, Parma, Italy

[b] Advanced Laboratory Research, Barilla G.R. F.lli SpA, via Mantova 166, 43122, Parma, Italy

[c] Interdepartmental Centre for Measurements G. Casnati, University of Parma, Parco Area delle Scienze 23/A, 43124, Parma, Italy

Corresponding author:

prof. Stefano Sforza

stefano.sforza@unipr.it

Telephone: +39 0521 905406

Fax: +39 0521 905472

ABSTRACT

Food frauds have become a very important issue in the field of food quality and safety. The risk of food adulteration is higher in highly processed food and mainly affects high added value foodstuff. The methods currently available to face this issue, PCR and ELISA, are very sensitive and specific, but they have some limitations. In the present work, tandem mass spectrometry is presented as an emerging approach to detect beef and pork meat in very complex and highly processed food matrices, such as Bolognese sauce, both in qualitative than in quantitative way. The detection is achieved using two different marker peptides, specific for beef and pork meat, both deriving from α2-collagen chain. Then, a calibration curve is set up using real sauces made by different percentages of pork and beef meat in a working range from 0 to 100%. The method here developed allows to quantify beef and pork meat in a complex product such as Bolognese sauce.

Keywords:

Food fraud, meat authentication, marker peptides, mass spectrometry, pork meat, beef meat

Abbreviations:

CE: collision energy; DNA: deoxyribonucleic acid; ELISA: enzyme linked immunosorbent assay; ESI: electrospray ionization; EU: European union; HPLC: high performance liquid chromatography; HRMS: high resolution mass spectrometry; LC: liquid chromatography; LOD: limit of detection; LOQ: limit of quantification; LRMS: low resolution mass spectrometry; LRMSMS: low resolution tandem mass spectrometry; MRM: multiple reaction monitoring; MS: mass spectrometry; MW: molecular weight; PCR: polymerase chain reaction; PDO: protected designation of origin; RP: reverse phase; RT: real time; SIR: single ion recording; Tris: tris(hydroxymethyl)aminomethane; UHPLC: ultrahigh performance liquid chromatography

1. Introduction

Food fraud is a deliberate and intentional substitution, addition, tampering, or misrepresentation of food, food ingredients, or food packaging, labeling, production information, or false or misleading statements made about a product for economic gain that could impact consumer health (Spink and Moyer, 2011). At the moment there is no definition of "food fraud" in EU legislation (http://ec.europa.eu/food/safety/official_controls/food_fraud/index_en.htm). One of the most common types of adulteration is the substitution of an ingredient with a cheaper one, that constitutes, alone, the 95% of the reported cases. The remaining incidents regard the addition of substances able to mask an inferior quality or the undeclared removal of valuable compounds. In the recent years, several scandals regarded meat derived products catalysing public opinion attention (www.foodfraud.org), in particular the partial replacement of beef with horse meat in some ready-to-eat products commercialized by famous brands. However, meat is often exposed to adulteration, the most common being: a false indication about the origin of meats and/or the animal feeding regime (for example in organic/PDO products), the substitution of the specie or the replacement of meat with fat, a missing declaration about a previous meat process (irradiation or thawing) or the possible additive presence (Ballin, 2010). Beside the legal point of view, there are also several concerns regarding quality and safety implications. For example the presence of hidden allergens, the lack of microbiological control, the possible presence of antibiotics, hormones or other food contaminants. Moreover there are also religious and lifestyle issues. Processed products are easier to be adulterated, due to their complexity and to the higher difficulty of detection (Flores-Munguia, Bermudez-Almada and Vazquez-Moreno, 2000). In fact, ground meat cannot be recognized by the only visual inspection, and the mixing with other ingredient in ready to eat foods makes the revelation even more difficult. Given the importance of food frauds, both at economical and safety level, a lot of attention has been focused on the development of analytical methods for adulteration detection (Sentandreu and Sentandreu, 2014).

Despite numerous articles regarding meat authenticithy in the last ten years (182 results for "meat authenticity" in Web of Knowledge database, accessed 14/09/2016), only twelve of them took into account processed food products: Motalib Hossain et al. (2016) developed a multiplex polymerase chain reaction−restriction fragment length polymorphism assay to detect beef, buffalo and pork meat also in processed foods such as frankfurters. In this work, detection was possible down to 0.1% of adulteration. The same group developed also a short amplicon-length PCR able to detect cat meat in cooked burgers down to 0.01% (Ali et al., 2016). Multiplex PCR, besides fresh meat product, was also applied to some cooked whole muscle meat, detecting several adulteration of beef with chicken meat (Chuah et al., 2016). Cases of substitution of beef with pork meat and of lamb with beef were reported by Premanandh et al. (2013) using a DNA-based approach. Despite the severe processing occurring for gelatine production, a PCR method was able to detect down to 0.1% of pork gelatine in bovine gelatine (Shabani et al., 2015). Duplex PCR was also applied to assess the authenticity of donkey meat (liable of adulteration with horse and mule), with a limit of detection of 1% (Chen et al. 2015). Among game meat, PCR-based methods are available for the detection of roe deer, red deer and (Rak et al., 2014) and for the detection of game birds (Rojas et al., 2009). After the horse meat scandals, a lot of efforts were put in the development of new and sensitive analytical methods for the detection of this meat species. For example, Pegels et al. (2015) developed a TaqMan RT-PCR to detect horse DNA also in processed products, like cured meat, sausages, burgers and pet food. As described by Stefanova et al. (2013), DNA extraction is often a critical step, especially in processed products. In the last years, a promising technique in this field is DNA barcoding, able to detect DNA also in processed fish (Yang et al., 2012). Among methods relying on proteomics, Claydon et al. (2015) identified several horse meat derived peptides that were resistant to food processing and that can be detected in canned corned beef and baby foods.

Nowadays, the most diffused analytical methods used by industries for the detection of food frauds (and meat in particular) can be divided essentially into two main groups: DNA-based methods and

protein-based methods. DNA-based methods are constituted mainly by PCR (Natonek-Wisniewska, Krzyscin and Piestrzynska-Kajtoch, 2013), that is sensitive and allows multi-ingredient detection, but, being specie-specific, it cannot distinguish between beef and milk or egg and chicken (and this is a problem for multi-ingredient preparations). Moreover it is an indirect method, and the absence of DNA does not necessarily mean the absence of proteins, since the thermal stability of nucleic acids and proteins is different. Protein-based methods relies essentially on immunoenzymatic assays (ELISA) (Asensio, Gonzalez, Garcia and Martin, 2008), that are highly specific for the target ingredient and sensitive: at the same time, the presence of interfering compounds (e.g. polyphenols, etc.) can negatively affect the analysis and, moreover, denatured proteins could not be detected but still be present. This is particularly true in thermally treated products, where heating induces several modifications in proteins, such as denaturation, lysine reaction with carbonyl groups (Maillard reaction), serine and threonine dehydration, cross linkage due to the formation of isopeptides and lysinoalanine (Gerrard, 2002). All these modification lead to a strong decrease in protein solubility, besides a much harder detectability.

Thus, in the recent years several mass spectrometry methods were developed to assess meat authenticity (Sentandreu and Sentandreu, 2011). For complex food matrices, mass spectrometry can indeed give the right selectivity, sensitivity and discriminating capacity in order to identify eventual food frauds. It has been demonstrated that horse and pork meat can be detected both in raw and in processed foods using HPLC-MS/MS achieving a detection limit of 0.0024 mass fraction units: in these works MRM^3 experiments were performed on myoglobin tryptic peptides (Von Barghen, Dojahn, Waidelich, Humpf and Brockmeyer, 2013; Von Bargen, Brockmeyer and Humpf, 2014). Four marker peptides for processed pork meat were identified by Sarah et al. (2015), that developed MRM methods for their detection. Montowska et al. (2015) identified, with a fast LESA-MS methodology, 25 heat stable peptides for five meat species (beef, pork, horse, chicken and turkey meat). Claydon et al. (2015) constructed a database of heat stable unique tryptic peptides for nine

meat species: this method was able to detect down to 0.5% cooked and raw horse in a meat mixture. An untargeted approach was instead developed by Ohana et al. (2016) , using shotgun spectral matching: specie identification was possible for 26 different mammalian and bird meats, both in raw and processed foods. However, besides detection and specie identification, a quantification cannot be carried out by the reported methodologies. In another work, raw meat from beef, horse, pork and lamb could be differentiated using myoglobin tryptic peptides reaching a limit of detection of 1%, and in this case the method was demonstrated suitable for raw materials but no food processing was taken into account (Orduna, Husby, Yang, Ghosh, Beaundry, 2015). Beside mammalian differentiation, a mass spectrometry approach was also used for the detection of chicken in meat mixes, and a quantification was achieved using isotopically labelled peptide standards (Sentandreu, Fraser, Halket, Patel and Bramley, 2010).

Ii is our opinion that, at the moment, the main gap in literature concerning meat speciation issue is the lack of proper reference materials, that perfectly resemble the commercial product to be analysed. Most of the published papers (with really few exceptions, Von Bargen et al., 2014) take indeed into account samples made by mixing fresh meat or even cooked meat, but not a real food product, made with different ingredients other than meat that have a strong influence on the detection capability of the method (e.g. dilution effect, matrix effect, interfering compounds). Up to now, no quantitative methods designed on a real food product are available.

The development of a quantitative method suitable for commercial products, will allows to detect not only the presence/absence of an undeclared ingredient, but also the relative quantification of a complex matrix with different species mixed together. A quantitative method is indeed a powerful tool also to monitor the entire industrial supply chain, in order to verify the compliance from the raw materials to the finished products. For example, a quantitative method is a helpful tool to discriminate between a simple contamination episode or an intentional food fraud. Moreover, in the case of multi-ingredient food products such as ground meat mixtures, the presence of beef and pork

140 together is allowed and declared in the label but, since the price of the two commodities is different,
141 a fraudulent shift towards the cheaper specie (even if it is not completely substituted) can be
142 detected with this method.

143 In this work, we focused on the detection and accurate quantification of beef and pork meat in a
144 complex and thermally treated food product (Bolognese sauce), not only to detect the presence of
145 these two species in the products in which they are not declared on the label but also to verify the
146 relative amount of the two species where declared. Beef and pork meat constitute together three
147 fourth of the livestock units in Europe, with beef accounting for 50% and pigs accounting for 25%
148 (European Commission Directorate-General for Agriculture, The meat sector in the European
149 Union). This approach can be subsequently developed to include other meat species, such as horse,
150 chicken and others.

151 For the first time, the samples exploited to set up and validate the method were prepared from a
152 food industry following the same recipe and processing of the commercial Bolognese sauce.

153 A mass spectrometry method was developed using low resolution mass spectrometry, comparing
154 both a single quadrupole instrument (SIR acquisition) and a triple quadrupole mass spectrometer
155 (MRM acquisition). Low resolution mass spectrometer are indeed more accessible to small-medium
156 food companies. Moreover, besides meat specie identification, the method was also developed and
157 validated for a quantification of the amount of beef and pork, so to be used as routine quality
158 control tools in the industry.

159 ## 2. Materials and methods

160 *2.1 Samples*

161 Bolognese sauce samples were prepared at pilot plant scale starting from entire pieces of beef and
162 pork meat that were finely ground and simulating the same industrial thermal processing of

commercial Bolognese sauce. Briefly, the meat was first browned with other ingredients such as celery, carrots and onion, tomato sauce was then added and the mixture was heated to reach about 90°C. Glass jars were hot filled and the product was submitted to a final sterilization step at 120° C for few minutes. In the final recipe minced meat reached about the 20% of the total weight.

2.2 Protein extraction

One hundred grams of Bolognese sauce samples were homogenized using an UltraTurrax (IKA T50 basic, Staufen, Germany) and then lyophilized at a vacuum level of 0.2 mbar and -50°C (Lio5P, 5Pascal, Milan, Italy). 1 g of the lyophilized sample was added to 10 ml of a solution of 50 mmol L^{-1} TrisHCl (pH 8), 6 mol L^{-1} urea and 1 mol L^{-1} thiourea and homogenized for 5 minutes using an UltraTurrax (IKA T10 basic, Staufen, Germany). The resultant mixture was centrifuged at 8784*g* for 10 min at 4°C and the supernatant was filtered through 0.45 µm syringe driven filters and protein concentration was measured according to the Bradford assay (Kruger, 2002), using bovine serum albumin (BSA) as standard. Samples were desalted at atmospheric pressure using Sep-pak Plus C18 cartridges (particle size 55-105 µm, pore size 125 Å, sorbent weight 360 mg), according to the manufacturer instructions (Waters, Milford, MA, USA) in order to eliminate caothropic agents used during the extraction (urea and thiourea). Briefly, the cartridge was wetted with 5 ml of B solution (water 35%, acetonitrile 65%, plus 0.1% formic acid) and equilibrated with 10 ml of A solution (water 98%, acetonitrile 2%, plus 0.1% formic acid). The sample was flushed through the cartridge, that was subsequently washed with 10 ml of A solution. Finally, adsorbed proteins were eluted with 5 ml of B solution.

2.3 Protein digestion

Samples protein extracts were dried with a rotary evaporator. Since the digestion procedure requires a starting volume of 100 µl, this volume was too low to recover the proteins from all the round flask. So, the sample was recovered with 1 ml of deionized water and dried again under nitrogen

flux in a 1.5 ml eppendorf tube. Dry solid residues were reconstituted with 100 µl of NH_4HCO_3 50 mmol L^{-1}. Disulphide bridges were first reduced by the addition of 5 µl of dithiotreitol 200 mmol L^{-1} incubating at room temperature for 1 hour and then alkylated by the addition of 4 µl of iodoacetamide 1mol L^{-1} and incubating in the dark for 1 hour at room temperature. Unreacted iodoacetamide was neutralized by 20 µl of dithiotreitol 200 mmol L^{-1} incubating for 1 hour at room temperature. Then, trypsin was added (enzyme:protein ratio 1:20) and digestion was carried out overnight at 37°C. Digested samples were then centrifuged at 15000*g* for 10 min at 4°C. Supernatant was then directly analysed by LC-MS

2.4 LC-LRMS analysis

The analysis was carried out as previously described (Prandi et al., 2012) and parameters for peptide detection were previously optimized in our laboratory. Digested samples were separated on a RP column (Acquity UPLC BEH 300, C18, 1.7 µm, 2.1×150 mm; Waters Corp., Milford, MA, USA) in an UHPLC/ESI-MS system (Acquity Ultraperformance UPLC with a single quadrupole mass spectrometer, Waters SQD) using a gradient elution. Eluent A was water with 0.1% formic acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid; gradient: 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. Flow was 0.2 ml/min; analysis time 72 min; column temperature 35 °C; sample temperature 18°C; injection volume 10 µl; ionization type was positive ions; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 150 °C; desolvation temperature 300 °C; cone gas flow 100 l/h; desolvation gas flow 650 l/h. The samples were analyzed both in Full Scan mode, scanning the range 100–2000 m/z from 0 to 58.2 mins, and in SIR mode, monitoring the following m/z: 598.9 (acquisition window 14-24 min), 604.9 (15-25 min), 388.2 (22-32 min).

2.5 LC-LRMSMS analysis

The analysis was carried out as previously described (Prandi et al., 2012) and parameters for peptide detection were previously optimized in our laboratory. Digested samples were separated by a RP column (Jupiter Phenomenex 5 µm C18 90 Å 250*2 mm) in an HPLC/ESI-MS/MS (HPLC Waters Alliance 2695 with a triple quadrupole mass spectrometer Waters 4 Micro), using a gradient elution upscaled from the previous described for UHPLC analysis. Eluent A was water with 0.1% formic acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid; gradient: 0-12 min 100% A, 12-77 min from 100% A to 50% A, 77-81 min 50% A, 81-82 min from 50% A to 0% A, 82-90 min 0% A, 90-91 min from 0% A to 100% A, 91-110 min 100% A. HPLC/ESI-MS/MS parameters were: flow 0.2 ml/min; analysis time 110 min; column temperature 35°C; injection volume 40 µl; ionization type positive ions; capillary voltage 3.2 kV; cone voltage 35 V; source temperature 100°C; desolvation temperature 150°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h. Samples were analyzed in the MRM mode monitoring the following transitions: 598.9→101.1/159.1/455.9 (27-37 min); 604.9→85.7/101/171.1 (30-40 min); 388.2→85.9/120.1/173.2 (37-47 min). The run time of 110 min was necessary for a better peaks resolution (given the complexity of the matrix) and to minimize the matrix effect, spreading the eluting compound in a longer run.

2.6 LC-HRMS analyses

Digested samples were separated using RP column (Jupiter Phenomenex C18 4µ, Proteo 90Å 150*0.30 mm) and a gradient elution; eluent A was water with 0.1% acetonitrile and 0.1% formic acid and eluent B was acetonitrile with 0.1% formic acid (gradient: 0-4 min from 100% A to 95% A, 4-60 min from 95% A to 50% A, 60-62 min from 50% A to 10% A, 62-72 min 10% A, 72-74 min from 10% A to 95% A, 74-90 min 95% A). HPLC: DIONEX Ultimate3000, Thermo Fisher Scientific (Waltham, Massachusetts, USA); mass spectrometer: LTQ Orbitrap XL, Thermo Fisher Scientific (Waltham, Massachusetts, USA). The analysis parameters were: flow 5 µl/min; analysis time 90 min; column temperature 30°C; sample temperature: 10°C; injection volume 5 µl;

acquisition time 0-75 min; ionization type positive ions; scan range 200-1800 m/z; source voltage 3.5 kV; capillary voltage 35 V; source temperature 275°C. Scan Event Details: FTMS + p res=30000 o(250.0-2000.0); ITMS + c Dep MS/MS Most intense ion from; Activation Type: CID; Isolation Width: 2.00; Normalized Coll. Energy: 35.0; Default Charge State: 2; Activation Q: 0.250; Activation Time: 30.000; Dynamic exclusion enabled; Repeat Count: 2; Repeat Duration (s): 10.00; Exclusion Duration (s): 30.00. Charge state rejection: enabled; Unassigned charge states: rejected; Charge state 1: rejected; Charge state 2: not rejected; Charge state 3: not rejected; Charge states 4+ : not rejected; ion signal threshold:10000

Results and discussion

2.1 Marker peptides identification

This work focused on one of the most complex and thermally treated meat derived product, i. e. Bolognese sauce . The samples were prepared in a food pilot plant, using exactly the same ingredient (type and amount) and processing conditions of a typical correspondent industrial commercial product.

From the analytical point of view, as far as meat proteins are concerned, Bolognese sauce surely represent a very challenging case in terms of food matrix: finely and highly thermally treated ground meat is indeed mixed with a lot of other ingredients (celery, carrots, onion, tomato sauce, oil, etc.) that can interfere with most of the available analysis (for example immunoenzymatic assays). Considering that meat is around the 20% of the finished product and that is in turn constituted by 20% of proteins, proteins represent only the 4% of the final product. If proteins are used for species identification, and the aim is detecting low percentages of the various species, it is clear that any assay must be able to detect low percentages of specific highly mistreated proteins in a very complex mixture.

In our case, we aimed at the detection of specific peptides after enzymatic hydrolysis, as previously performed on the same food matrix by Von Barghen et al. (2014), and at the development of a quantitative MS method. Thus, as a first step, different methods for protein extractions from Bolognese sauce were tested. Whole Bolognese sauce samples were lyophilized prior to the analysis in order to eliminate water and concentrate the protein fraction. The browning step at which meat is submitted, reaching about 100-130°C, , and the final sterilization step lead to a strong protein degradation. In these conditions, proteins aggregate and have a very low water solubility, so they are difficult to extract, as described previously by Vujadinović et al. in 2014. Several extraction buffer were tried and different protein classes were assessed for suitability by use of LC coupled to single quadrupole instrument:

- Extraction with TrisHCl 50 mmol L^{-1}: sarcoplasmatic proteins were extracted and several marker peptides were identified in raw meat, but just after the browning step of the meat, none of the identified marker was detected. An example is reported in Figure 1, were the peptide LFTGHPETLEK (specific for bovine myoglobin) gives a very intense signal in the fresh meat sample but completely disappears in browned meat and Bolognese sauce.
- Extraction with urea/thiourea 6 mol L^{-1}/1 mol L^{-1}: myofibrillar proteins were extracted and several marker peptides were identified, some of them present also after browning and sterilization steps
- Extraction with phosphate buffer: sarcoplasmatic proteins were extracted and several marker peptides were identified in raw meat, but just after the browning step of the meat, none of the identified marker was detected.

Thus, the best results were achieved by using chaotropic agents (urea and thiourea) at high concentration. The strong thermal treatment requires that the marker peptide(s) to be suitable for meat identification and quantification purposes has to be stable through the entire processing, so that is not degraded by the Maillard reaction, not dehydrated and not involved in cross linking.

Similar observation were seen by Von Barghen et al. (2014), that shifted from a phosphate buffer extraction to an urea/thiourea extraction, since the first one was not able to extract a sufficient amount of protein in thermally treated foods while the second one gave good electrophoretic bands.

Trypsin cleaves at the C-term of arginine and lysine, that are also the two amino acids mostly involved in the Maillard reaction and protein cross linking (Nagaraj et al., 1996). Thus, in a first attempt, chymotrypsin was tried for the proteolytic cleavage in order to generate stable marker peptides. However, chymotrypsin (used following the manufacturer instructions) was observed to be less specific in cleaving than trypsin, and induced a lot of variability both in the type and relative amounts of peptides generated. In fact, chymotrypsin cleavage sites are large hydrophobic residues such as tyrosine, tryptophan and phenylalanine, but it also cleaves after other non polar amino acids such as leucine and methionine. To avoid this variability, trypsin was chosen for all the subsequent analysis.

After having designed a proper protein extraction and digestion protocol, in order to set up the method and to identify the proper marker peptides, fresh meat samples were first analyzed (pure beef and pure pork) allowing to identify many specie specific marker peptides (data not shown). However, most of the peptides were lost just after the browning of the meat and a further reduction (both qualitative and quantitative) was observed in the final Bolognese sauce product. The reduction of identified peptides after meat cooking was also observed by Montowska et al. (2014), that developed a LESA-MS method for the differentiation of beef, pork, horse and lamb down to 10% also in processed meat. This reduction was ascribed to protein aggregation during meat cooking, resulting in a reduced extraction and digestion efficiency. Concerning that, differently as previously reported in the literature (Von Bargen et al., 2014; Sentandreu et al., 2014; Watson, Gunning, Rigby, Philo and Kemsley, 2015), the identification of the marker peptides was performed directly on the Bolognese sauce. For marker peptides identification, a real Bolognese sauce with all the other ingredients (oil and vegetables) made with pure beef and a real Bolognese sauce made with

pure pork were first specially produced in a pilot plant and analysed. Full scan MS chromatograms obtained with a single quadrupole mass spectrometer are reported in Figure 2A and B. Given the high number of ingredient of this product, chromatograms are very complex with hundreds of peptides, and chromatographic peaks not resolved. Matrix effect could not be evaluated due to the unavailability of standard peptides, as well as ion suppression.

However, carefully analyzing manually all the mass spectra of each chromatographic peak, a list of masses can be in any case generated both for beef and pork Bolognese sauce. To facilitate the screening among specie specific marker peptides, only peptides with MW>700 Da were taken into account. Similar parameters were adopted also by Watson et al. (2015), that fixed a minimum peptide length of 6 amino acid residues. Considering an average amino acid weight of 110 Da, this parameter is consistent with 700 Da. In their work, a MRM method for the quantification of beef, horse, pork and lamb (down to 1%) was applied to fresh meat with good results. Then, all the selected peptides were cross checked in the Full Scan chromatogram obtained with the single quadrupole instrument, between the two types of Bolognese sauce. Based on molecular weight and retention time, 14 marker peptides specific for beef were identified, while the marker peptides for pork meat were 13. Only peptides completely absent in the other species and with a good intensity were selected. Moreover, 9 more peptides, common in both types of meat, were also identified.

Then, daughter scan experiments were performed in a triple quadrupole instrument in order to characterize the fragmentation pattern of each peptide. The three most intense fragments were chosen and the optimal collision energy was verified performing different MRM experiments with collision energies (CE) ranging from 10 to 30 eV (Table 1, Table 2, Table 3). Thus the best marker peptides (basing on intensity and specificity) were selected both for beef and pork meat.

Finally, using tandem mass spectrometry the exact amino acid sequence of the two marker peptides was determined: IGQpGAVGPAGIR for beef and TGQpGAVGPAGIR for pork. As it can be observed in Figure 3, the tandem mass spectra of the peptide IGQpGAVGPAGIR is fully consistent

with that reported by Solazzo et al. (2016). This biomarker peptide was used for the determination of the composition of skull coatings from Neolithic artefacts. The peptide TGQpGAVGPAGIR was already identified in the collagen present in porcine and sheep tip antler (Chen et al., 2014), as well as in ancient archaeological skin objects made of sheep, while the peptide IGQpGAVGPAGIR was found in archaeological objects made from bovine skin (Brandt et al., 2014). Consistently, the peptide IGQpGAVGPAGIR was present in bovine leather, while the peptide TGQpGAVGPAGIR was found in pig, sheep, goat and deer leather (Kumazawa et al., 2016).

This two collagen derived peptides were also selected previously as good candidates biomarkers to differentiate between bovine and pork gelatine in complex food samples (dairy products) by Yilmaz et al. (2013). Moreover, they were chosen as marker peptides to detect the presence of bovine and pork proteins in animal feeds (DEFRA, 2009). It is noticeable that these peptides are not among the most abundant in fresh meat, rather they are quite scarce. The thermal treatment undergone by Bolognese sauce (as well as other processed meat derived products) probably enhance the collagen solubilisation, leading to the liberation of a much higher amount of the marker peptides.

The two marker peptides have the same amino acid sequence with the exception of the first N-term amino acid, that is different between beef and pork. This point mutation is indeed very useful for peptide quantification, because the two markers, only having one different amino acid, have thus similar retention time and ionization behaviour . Proline in fourth position is oxidized (hydroxyproline). Making an alignment in the protein database, the specificity of the two marker peptides was verified. The peptide IGQpGAVGPAGIR is specific for *Bos taurus* and *Bos mutus* (a wild yak, genetically related to beef), while the peptide TGQpGAVGPAGIR is specific for *Sus scrofa*, where there are evidence at protein level, and it is a predicted sequence in *Ovis aries* (www.uniprot.org, last accessed 16/09/2016). Both the marker peptides derive from collagen α2-chain. Collagen is a fibrous protein that constitute most of the connective tissue and it is very rich in proline, glycine and hydroxyproline. Since Bolognese sauce is usually made using the front cuts of

beef and pork, these cuts are richer in connective tissue, so it is not surprising that several collagen derived peptides were identified. Meat composition is highly variable among the different cuts used, in terms of collagen percentage, types of protein (myofibrillar and sarcoplasmatic) present, intramuscular fat and so on. Since this natural variability of the starting raw material can induce a certain variability in the method, it is of outmost importance that the calibration curve is made using real food samples, and to standardise as much as possible the raw materials used. The variability of collagen presence in the different tissues can be overcome by a good sampling scheme and sample homogenisation. In the study presented here, trueness was always within 25% of the nominal value, in the worst case situation.

3.2 Beef and pork meat quantification

Besides meat specie detection, the aim of this work is the development of a method for the quantification of two different species: *Bos Taurus* and *Sus scrofa*. All the quantification experiments were carried out not only with a triple quadrupole instrument, known for its high selectivity and sensitivity, but also with a single quadrupole one, usually not exploited in this kind of analysis due to its quite lower performance capacity. Most of the research the field of meat authenticity is indeed performed using quadrupole-time of flight (qTOF), quadrupole-ion trap or LTQ-Orbitrap instruments. Since the method was meant to be available for food industries, these latter type of instrument was chosen because considered more accessible to small and medium enterprises both in terms of costs and of skilled personnel. A comparison between the two detection modes is reported in figure 4, where the overlaid traces of the marker peptide for beef are reported. As it can be observed, both the methods showed a good signal to noise ratio and a good linearity in terms of gained area for increased beef concentration (from 0% beef, flat chromatogram, to 100% beef, highest peak).

The samples were first analysed using a triple quadrupole instrument, monitoring several MRM transitions (details into the material and methods section). Based on the signal to noise ratio

385 criterion, the limit of detection was estimated corresponding to the 0.003 mass fraction units on
386 whole product, while the limit of quantification is 0.01 mass fraction units on whole product. Limit
387 of detection was calculated as the minimum amount of pork meat able to generate a signal to noise
388 ratio of at least 3, while the for the limit of quantification a signal to noise ratio of 10 was
389 considered, according to previous studies (Shrivastava and Gupta, 2011). The limit of detection was
390 improved compared to that found by Montowska et al. (2014), even if it remained higher than the
391 one reported by von Barghen et al. (2013) and Claydon et al. (2015) that was 0.0024 mass fraction
392 units. LOD was however still acceptable considering the processing undergone by the meat.

393 As previously explained, experiments were performed also using a single quadrupole instrument in
394 the SIR mode (details in the material and method section) and results were fully consistent with
395 those obtained by means of MRM, the only difference, as expected, being slightly higher limits of
396 detection and quantification (LOD=0.01 mass fraction units on whole product and LOQ=0.03 mass
397 fraction units on whole product). LOD and LOQ were calculated on pork marker peptide, that was
398 less abundant than beef marker peptide (worst case). Single ion recording is usually considered less
399 specific than multiple reaction monitoring, especially in the case of complex matrices. In this case,
400 the possible presence of interfering compounds in the SIR analysis was excluded repeating the
401 analysis several times both with SIR and MRM detection, and the comparison and good matching
402 of the results allowed to exclude presence of interfering compounds in the SIR analysis. Thus, the
403 SIR detection was considered suitable for the marker peptides detection and quantification. To our
404 knowledge, it is the first time that single ion recoding was applied to assess meat authenticity.

405 For method calibration six different Bolognese sauce sauces were prepared with a known meat
406 composition, ranging from 100% beef / 0% pork to 0% beef / 100% pork. Protein concentration in
407 the samples was 3.5±0.1 mg/ml (determined by the Bradford assay). Protein concentration was the
408 same for all the calibration points, the only difference was the percentage of beef derived proteins
409 and pork derived proteins: (A) 100% beef – 0% pork, (B) 80% beef – 20% pork, (C) 60% beef –

40% pork, (D) 40% beef – 60% pork, (E) 20% beef – 80% pork, (F) 0% beef – 100% pork. Despite also percentages below 20% need to be quantified, concentration levels of the calibration curve were designed to be equidistant, so that the calculated slope and intercept are not influenced disproportionally by one data point. LOD and LOQ were estimated on the signal to noise ratio of the weakest (less abundant) marker peptide (pork marker peptide). A good indication on the accuracy of the calculated LOD and LOQ is given by the 2% pork meat blind sample, in which pork meat was detected. A previous example in literature of calibration curves made mixing different amount of meat species (chicken in pork meat) can be found in the work carried out by Sentandreu et al. (2009), in a working range from 0 to 10%, where a good linearity is shown.

Samples were extracted and analysed in duplicate, using the SIR mode. Then, the % of beef (pork) is plotted against the value $\frac{\frac{(Area\ beef)}{Area 100\%\ beef}}{\frac{Area\ beef}{Area\ 100\%\ beef}+\frac{Area\ pork}{Area\ 100\%\ pork}}$ (or $\frac{\frac{(Area\ pork)}{Area 100\%\ pork}}{\frac{Area\ beef}{Area\ 100\%\ beef}+\frac{Area\ pork}{Area\ 100\%\ pork}}$). A good correlation ($r^2$>0.99) was found using a linear equation (beef: y=0.0102x+0.0041 R^2=0.9933; pork: y=0.0102x-0.0226 R^2=0.9933).

The use of synthetic peptides was excluded because of the high costs of the synthesis of isotopically labelled standard, together with the background knowledge needed for the synthesis, that would have probably make the method inaccessible for most of the meat sector stakeholders.

To check the accuracy of the method, three blind samples (at unknown meat composition) were analysed, according to current guidelines (Niazi, 2015). Results are reported in Table 4. The method showed a good precision in repeatability conditions both within the same analysis (intraday variability) and between two different analysis carried out with two month of interval (interday variability<15%). The trueness value for acceptance was fixed to be within 20% of the nominal value. The best trueness values were found for sample 8, where the detected values well match with the nominal ones. For sample 7 beef is somehow underestimated. Sample 9 gave good results for

433 what concerns beef amount, while pork is largely overestimated. However in this case we have to
434 consider that 2% is a value below the limit of quantification of the method.

435 To improve this aspect of the method, other calibration curves were constructed by plotting the %
436 of beef with the value $\frac{Area\ beef\ marker}{Area\ meat\ marker}$). In this way, the data were normalized for the meat content
437 of the preparation, moving from a relative quantification in a binary mixture toward an absolute
438 quantification of meat composition. The meat marker is a peptide present in all types of meat.

439 The results obtained with the new quantification formula are reported in Table 5, improving
440 significantly the evaluation of pork content without affecting in a relevant way the parallel beef
441 quantification. Furthermore, with this new approach, the accuracy of the method improved notably,
442 as reported in Table 5, and the method was found able to quantify also very low pork percentages
443 (2%).

444 These results are very important in the field of food authenticity and as a valuable tool to monitor
445 the meat supply chain. Good progresses in the field of meat authentication were achieved in the last
446 years using proteomic-based techniques. A promising methodology for the quantification of beef,
447 pork, horse and lamb in fresh mixtures (exploiting myoglobin derived peptides) was carried out by
448 Watson et al. (2015, reaching the good detection limit of 1% w/w). Chicken meat could also be
449 quantified when mixed to pork meat at 0.5%, even after cooking (Sentandreu et al., 2010) . Peptide
450 biomarkers (among which also the peptide IGQPGAVGPAGIR) were also used to detect the
451 addition of processed animal proteins in feed samples, down to 5% w/w (Marbaix et al., 2016). The
452 work here reported is focused on Bolognese sauce because, given the complexity of the product
453 (both for the high number of ingredients and for the long thermal treatment), it shows a high
454 adulteration risk, as reported recently (2013) by the numerous product recall/withdrawal from the
455 market for the recent horse meat scandal (Report of the investigation by the Food Standards Agency

into incidents of adulteration of comminuted beef products with horse meat and DNA). The same approach is valid for other kind of meat processed foodstuff.

A quantitative approach is also needed to detect food frauds because, if it is true that for mono-varietal or mono-specie products a simple detection of an exogenous component clearly indicates a food fraud, it is also true that nowadays on the market a lot of multi ingredient products are present. Thus, the food fraud can be hidden not in the complete or partial substitution of an ingredient, but also in the manipulation of the declared percentages in mixed product. For examples, beef meat costs about double than pig meat. A lot of meat derived products are made with a mixture of beef and pork (for example meat sauces, burgers, patties, and others) and the buyer of that food product will fix a certain percentage of the two species in the product. Thus, increasing the pork percentage and decreasing the beef (without completely replacing it) would be a very lucrative fraud. To answer this need, some methods are being developed, both in the immune enzymatic assays and in the mass spectrometry field. For example, an efficient methods for the detection of porcine gelatine in edible bird nests was developed by Tukiran et al. in 2015, with a limit of detection below 0.12 ppm. Sarah et al. (2016) identified four marker peptides specific for porcine meat that can be used for meat authentication through MRM. The work developed here on Bolognese sauce, provide a useful tool to detect frauds very difficult to be detected, given the severe processing undergone by this foodstuff.

3. Conclusions

Most of the immunoenzymatic and/or genomic methods currently available for animal species identification are not even able to detect the specie in such a processed product. The recently reported LC/MS methods seem to be very promising for the detection of few amount of meat species also in highly processed food but, to the best of our knowledge, no one faces the topic of the quantification of the species declared.

The present work represents therefore a step in advance in tandem mass spectrometry applications to detect and quantify beef and pork meat in complex and highly processed food matrices. The method, here successfully developed and validated, gave results particularly satisfactory in consideration of the total absence, to date, of analytical strategies able to quantify different meat species in these kind of complex food matrices.

Furthermore, it could be actually implemented in food industries to check the meat composition of the final product but also to protect itself through the control of the raw materials or intermediate products supplied by other producers. Finally, it involves the use of relatively low cost instruments and, once set up, it does not require a highly specialized technicians, having also the potential flexibility to be extended to other meat species, on the basis of industry needs.

4. Declaration of interests

The authors declare that they have no conflict of interests.

5. Acknowledgements

This work was supported by the European Union, Seventh Framework Program (FP7/2007-2013), under Grant Agreement 613688 (Ensuring the Integrity of the European food chain − FoodIntegrity).

6. References

Ali, M. E., Al Amin, M., Razzak, M. A., Hamid, S. B. A., Rahman, M. M., Rashid, N., Asing. (2016). Short amplicon-length PCR assay targeting mitochondrial cytochrome b gene for the detection of feline meats in burger formulation. *Food Analytical methods*, *9(3)*, 571-581.

Asensio, L., Gonzalez, I., Garcia, T., & Martin, R. (2008). Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control*, *19*, 1–8.

Ballin, N. Z. (2010). Authentication of meat and meat products. *Meat Science*, *86*, 577–587.

Brandt, L., Ø., Schmidt, A. L., Mannering, U., Sarret, M., Kelstrup, C. D., Olsen, J. V., Cappellini, E. (2014). Species Identification of archaeological skin objects from Danish bogs: comparison between mass spectrometry-based peptide sequencing and microscopy-based methods. Plos One, 9(9), e106875.

Chen, J. C., Hsiang, C. Y., Lin, Y. C., Ho, T. Y. (2014). Deer antler extract improves fatigue effect through altering the expression of genes related to muscle strength in skeletal muscle of mice. *Evidence-Based Complementary and Alternative Medicine*, *540580*, 1-10.

Chen, A. L., Wei, C. B., Chen, G., Zhao, Y., Yang, S. M. (2015). Duplex PCR approach for the detection and quantification of donkey, horse and mule in raw and heat-processed meat products. *International Journal of Food Science and Technology*, *50(3)*, 834-839.

Chuah, L. O., He, X. B., Effarizah, M. E., Syahariza, Z. A., Shamila-Syuhada, A. K., Rusul, G. (2016). Mislabelling of beef and poultry products sold in Malaysia. *Food Control*, *62*, 157-164.

Claydon, A. J., Grundy, H. H., Charlton, A. J., Romero, M. R. Identification of novel peptides for horse meat speciation in highly processed foodstuffs. *Food Additives and contaminants – Part A*, *32(10)*, 1718-1729.

519 DEFRA - Department for Environment Food and Rural Affairs (2009). Detection of the presence of
520 species specific processed animal proteins in animal feed. Research Project Final Report.

521 Flores-Munguia, M. E., Bermudez-Almada, M. C., & Vazquez-Moreno, L. (2000). A research note:
522 detection of adulteration in processed traditional meat products. *Journal of Muscle Foods*, *11*, 319–
523 325.

524 FSA (2013). Report of the investigation by the Food Standards Agency into incidents of
525 adulteration of comminuted beef products with horse meat and DNA.
526 http://www.food.gov.uk/sites/default/files/multimedia/pdfs/board/board-papers-2013/fsa-130704-
527 fsa-investigation-report.pdf

528 Gerrard, J. A. (2002). Protein–protein crosslinking in food: methods, consequences, applications.
529 *Trends in Food Science and Technology*, *13*, 391–399.

530 http://www.foodfraud.org/search/site?search_api_views_fulltext=meat (Accessed 14/06/2016).

531 Kruger, N. J. (2002). The Bradford method for protein quantitation. In: *The protein protocols*
532 *handbook* (pp. 15-21). Totowa: Humana Press Inc.

533 Hossain, M. A. M., Ali, M. E., Abd Hamid, S. B., Asing, Mustafa, S., Desa, M. N. M., Zaidul, I. S.
534 M. (2016). double gene targeting multiplex polymerase chain reaction− restriction fragment length
535 polymorphism assay discriminates beef, buffalo, and pork substitution in frankfurter products.
536 *Journal of Agricultural and Food Chemistry*, *64*, 6343-6354.

537 Kumazawa, Y., Taga, Y., Iwai, K., Koyama, Y. a rapid and simple LC-MS method using collagen
538 marker peptides for identification of the animal source of leather. *Journal of Agricultural and Food*
539 *Chemistry*, *65*, 6051-6057.

540 Izuchi, Y., Takashima, T., Hatano, N. (2016). Rapid and accurate identication of animal species in
541 natural leather goods by liquid chromatography/mass spectrometry. *Mass Spectrometry*, *5*, A0046.

542 Marbaix, H., Budinger, D., Dieu, M., Fumiere, O., Gillard, N., Delahaut, P., Mauro, S., Raes, M.
543 (2016). Identification of proteins and peptide biomarkers for detecting banned processed animal
544 proteins (PAPs) in meat and bone meal by mass spectrometry. *Journal of Agricultural and Food*
545 *Chemistry*, *64*, 2405-2414.

546 Montowska, M., Alexander, M. R., Tucker, G. A., Barrett, D. A. (2014). Rapid detection of peptide
547 markers for authentication purposes in raw and cooked meat using ambient liquid extraction surface
548 analysis mass spectrometry. *Analytical Chemistry, 86,* 10257-10265.

549 Montowska, M., Alexander, M. R., Tucker, G. A., Barrett, D. A. (2015). Authentication of
550 processed meat products by peptidomic analysis using rapid ambient mass spectrometry. *Food*
551 *Chemistry, 187,* 297-304.

552 Nagaraj, R. H., Shipanova, I. M., Faust, F. M. (1996). Protein cross-linking by the Maillard
553 reaction. *Journal of Biological Chemistry*, *271(32),* 19338-19345.

554 Natonek-Wisniewska, M., Krzyscin, P., & Piestrzynska-Kajtoch, A. (2013). The species
555 identification of bovine, porcine, ovine and chicken components in animal meals, feeds and their
556 ingredients, based on COX I analysis and ribosomal DNA sequences. *Food Control, 34*, 69–78.

557 Niazi, S. K. (2015). *Handbook of bioequivalence testing*. (2nd ed.). Boca Raton: CRC press, Taylor
558 & Francis Group, (pp.213-686).

559 Ohana, D., Dalebout, H., Marissen, R. J., Wulff, T., Bergquist, J., Deelder, A. M., Palmblad, M.
560 (2016). Identification of meat products by shotgun spectral matching. *Food Chemistry, 203,* 28-34.

561 Orduna, A. R., Husby, E., Yang, C. T., Ghosh, D., & Beaundry, F. (2015). Assessment of meat
562 authenticity using bioinformatics, targeted peptide biomarkers and high-resolution mass
563 spectrometry. *Food Additives and Contaminants - part A, 32(10),* 1709–1717.

564 Pegels, N., Garcia, T., Martin, R., Gonzalez, I. (2015). Market analysis of food and feed products
565 for detection of horse DNA by a TaqMan real-time PCR. *Food Analytical Methods*, *8(2)*, 489-498.

566 Prandi, B., Faccini, A., Tedeschi, T., Cammerata, A., Sgrulletta, D., D'Egidio, M. G., Galaverna,
567 G., & Sforza, S. (2014). Qualitative and quantitative determination of peptides related to celiac
568 disease in mixtures derived from different methods of simulated gastrointestinal digestion of wheat
569 products. *Analytical and Bioanalytical Chemistry*, *406(19)*, 4765-4775.

570 Premanandh, J., Sabbagh, A., Maruthamuthu, M. (2013). Misdescription of packaged foods: a case
571 study from the United Arab Emirates. *Food Additives and Contaminants - Part A*, *30(12)*, 2022-
572 2026.

573 Rak, L., Knapik, K., Bania, J., Sujkowski, J., Gadzinowski, A. (2014). Detection of roe deer, red
574 deer, and hare meat in raw materials and processed products available in Poland. *European Food*
575 *Research and Technology*, *239(2)*, 189-194.

576 Rojas, M., Gonzalez, I., Pavon, M. A., Pegels, N., Lago, A., Hernandez, P. E., Garcia, T., Martin, R.
577 (2009). Novel TaqMan real-time polymerase chain reaction assay for verifying the authenticity of
578 meat and commercial meat products from game birds. *Food Additives and Contaminants - Part A*,
579 *27(6)*, 749-763.

580 Sarah, S. A., Faradalila, W. N., Salwani, M. S., Amin, I., Karsani, S. A., Sazili, A. Q. (2016). LC–
581 QTOF-MS identification of porcine-specific peptide in heat treated pork identifies candidate
582 markers for meat species determination. *Food Chenistry*, *199*, 157-164.

583 Sentandreu, M. A., Fraser, P.D., Halket, J., Patel, R., & Bramley, P.M. (2010). A proteomic-based
584 approach for detection of chicken in meat mixes. *Journal of Proteome Research*, *9*, 3374–3383.

585 Sentandreu, M. A., & Sentandreu, M. (2011). Peptide biomarkers as a way to determine meat
586 authenticity. *Meat Science*, *89*, 280–285.

587 Sentandreu, M. A., & Sentandreu, E. (2014). Authenticity of meat products: Tools against fraud.
588 *Food Research International, 60*, 19–29.

589 Shabani, H., Mehdizadeh, M., Mousavi, S. M., Dezfouli, E. A., Solgi, T., Khodaverdi, M., Rabiei,
590 M., Rastegar, H., Alebouyeh, M. Halal authenticity of gelatin using species-specific PCR. *Food*
591 *Chemistry*, *184*, 203-206.

592 Shrivastava, A., & Gupta, V. B. (2011). Methods for the determination of limit of detection and
593 limit of quantitation of the analytical methods. *Chronicles of Young Scientists*, *2*, 21-25.

594 Solazzo, C., Courel, B., Connan, J., van Dongen, B. E., Barden, H., Penkman, K., Taylor, S.,
595 Demarchi, B., Adam, P., Schaeffer, P., Nissenbaum, A., Bar-Yosef, O., Buckley, M. (2016).
596 Identification of the earliest collagen- and plant-based coatings from Neolithic artefacts (Nahal
597 Hemar cave, Israel). *Nature Scientific Reports*, *6*, 31053.

598 Spink, J., & Moyer, D. C. (2011). Backgrounder: defining the public health threat of food fraud.
599 Minneapolis, Minn.: National Center for Food Protection and Defense. Available from:
600 http://www.ncfpd.umn.edu/

601 Stefanova, P., Taseva, M., Georgieva, T., Gotcheva, V., Angelov, A. A modified CTAB method for
602 DNA extraction from soybean and meat products. *Biotechnology & Biotechnological Equipment*,
603 *27(3)*, 3803-3810.

604 Tukirana, N. A., Ismail, A., Mustafa S., Hamid, M. (2015). Enzyme immunoassay for the detection
605 of porcine gelatine in edible bird's nests. *Food Additives & Contaminants: Part A, 32(7)*, 1023-
606 1028.

607 Von Barghen, C., Dojahn, J., Waidelich, D., Humpf, H. U., & Brockmeyer, J. (2013). New sensitive
608 high-performance liquid chromatography−tandem mass spectrometry method for the detection of
609 horse and pork in halal beef. *Journal of Agricoltural and Food Chemistry*, *61*, 11986−11994.

610 Von Bargen, C., Brockmeyer, J., & Humpf, H. U. (2014). Meat authentication: a new
611 HPLC−MS/MS based method for the fast and sensitive detection of horse and pork in highly
612 processed food. *Journal of Agricoltural and Food Chemistry, 62*, 9428−9435.

613 Vujadinović, D., Grujić, R.,Tomović, V., Torbica, A. (2014). Effects of temperature and method of
614 heat treatment on myofibrillar proteins of pork. *Chemical Industry & Chemical Engineering*
615 *Quarterly, 20 (3)*, 407−415.

616 Watson, A. D., Gunning, Y., Rigby, N. M., Philo, M., & Kemsley, E.K. (2015). Meat authentication
617 via multiple reaction monitoring mass spectrometry of myoglobin peptides. *Analytical Chemistry*,
618 *87*, 10315−10322.

619 Yang, Y. C., Huang, Y. W., Hsieh, C. H., Huang, Y. R., Chen, C. H. (2012). A unique specification
620 method for processed unicorn filefish products using a DNA barcode marker. *Food Control, 25(1)*,
621 292-302.

622 Yilmaz, M. T., Kesmen, Z., Baykal, B., Sagdic, O., Kulen, O., Kacar, O., Yetim, H., Baykal, A. T.
623 (2013). A novel method to differentiate bovine and porcine gelatins in food products: NanoUPLC-
624 ESI-Q-TOF-MSE based data independent acquisition technique to detect marker peptides in
625 gelatine. *Food Chemistry, 141*, 2450-2458.

626 **7. Captions to figures**

627 **Fig. 1.** Overlaid extract ion chromatograms of the peptide LFTGHPETLEK, from bovine
628 myoglobin, detected in the extraction with TrisHCl buffer of fresh meat, but not in the thermally
629 treated products.

630 **Fig. 2.** Full scan chromatograms of Bolognese sauce made with pure beef (A) and with pure pork
631 (B). SIR chromatograms of beef Bolognese sauce (C, m/z=604.9) and pork Bolognese sauce (D,
632 m/z=598.9)

Fig. 3. MS/MS spectra of the marker peptide for beef (upper, IGQpGAVGPAGIR) and for pork meat (lower, TGQpGAVGPAGIR). Peaks are annotated using the conventional peptide fragmentation symbols. Square brackets indicated the fragmentation points.

Fig. 4. A comparison between SIR detection (A) and MRM detection (B): overlaid traces of the marker peptide for beef in the six points of the calibration curve (from 100% beef to 0% beef).

8. Tables

Table 1

Marker peptides present only in Bolognese sauce made with pure beef. The best marker peptide (chosen on the best signal to noise ratio, selectivity, presence of coeluting interferents) is underlined (fragment 1 the most intense was used as quantifier, fragments 2 and 3 as qualifiers).

Rt (min)	Ion (m/z)	charge	MW (Da)	fragment 1	fragment 2	fragment 3	Best CE
29.5	641.2	2	1280.4	187.2	159	512.8	20
31.35	745.4	1	744.4	85.9	131.1	242.2	30
33.55	548.6	2	1095.2	120.1	128.8	205.3	25
34.03	644.2	2	1286.4	542.3	177	171	20
34.12	747.2	2	1492.4	145.1	167.1	279.3	30
34.36	932.4	1	931.2	839.4	187.4	643.2	20
34.62	636.7	2	1271.3	85.9	110.1	120.2	30
<u>35.18</u>	<u>604.9</u>	<u>2</u>	<u>1207.6</u>	<u>101</u>	<u>85.7</u>	<u>171.1</u>	<u>20</u>
35.55	665.5	2	1329	132.1	87.1	183.1	30
35.88	537.2	2	1072.6	85.9	199.2	227.1	30
37.99	781.3	2	1560.6	155.1	187.1	226.3	30
40.17	707.4	2	1412.8	85.8	169.1	240.3	30
47.09	762.9	2	1523.8	120.1	85.9	175.2	30
48.55	1185.6	2	1184.6	173.2	201.2	119.9	20

Table 2

Marker peptides present only in Bolognese sauce made with pure pork. The best marker peptide (chosen on the best intensity, selectivity, presence of coeluting interferents) is underlined (fragment 1 the most intense was used as quantifier, fragments 2 and 3 as qualifiers).

Rt (min)	Ion (m/z)	charge	MW (Da)	fragment 1	fragmen 2	fragment 3	Best CE
30.85	470.8	2	939.6	86.1	110.1	215.2	30
32.84	598.9	2	1195.8	101.1	159.1	455.9	20
33.94	711.2	2	1420.6	no	no	no	no
34.58	523.2	2	1044.4	85.7	199.2	227.3	20
35.01	795	2	1588	169.2	140.9	846.8	25
35.4	702.4	1	701.4	120	86.1	173.2	25
35.29	830.5	2	1659	169.2	141.2	266.2	30
36.25	774.4	2	1546.8	815.7	261.8	115	20
39.41	388.2	2	774.6	no	no	no	no
40.52	684.3	1	683.2	472.2	340.2	227.1	20
44.67	496.1	2	990.2	no	no	no	no
49.31	586.7	2	1171.4	173.2	201.3	119.9	20
54.14	712.4	1	711.4	302.2	298.3	411.3	25

Table 3

Common peptides present in both Bolognese sauce. The best peptide (chosen on the best intensity, selectivity, presence of coeluting interferents) is underlined (fragment 1 the most intense was used as quantifier, fragments 2 and 3 as qualifiers).

Rt (min)	ion (m/z)	charge	MW (Da)	fragment 1	frgament 2	fragment 3	Best CE
30.84	590.7	2	1179.4	no	no	no	no
33.62	474.2	2	946.4	187.2	175.1	110	20
37.14	532	3	1593	120.2	72	86	30
38.02	666.8	2	1331.6	86.1	143	171.2	25
39.08	872.5	2	871.5	389.2	466.4	484.3	25
40.52	538.7	2	1075.4	199.2	147.2	129	20
42.63	388.2	2	774.4	120.1	85.9	173.2	30
45.39	581.2	2	1160.4	199.2	86.1	120.1	25
46.48	1050.6	1	1049.6	1032.7	no	no	no

Table 4

Results of two different analysis done at two months of distance of three blind samples. Results are reported as the average of two different replicates, together with the standard deviation.

Unknown sample	Real beef %	Real pork %	% beef 1° analysis (trueness)	% beef 2° analysis (trueness)	% pork 1° analysis (trueness)	% pork 2° analysis (trueness)
7	35	65	20.4±5.3	25.3±1.0	79.6±5.3	74.7±1.0
8	75	25	77.2±0.2	76.8±1.0	22.8±0.2	23.2±1.0
9	98	2	92.8±0.7	93.1±1.4	7.2±0.7	6.9±1.4

664 **Table 5**

665 Results of the absolute quantification of beef and pork meat in the three blind samples. Results are
666 reported as the average of two different replicates, together with the standard deviation and the
667 trueness values (in brackets).

Unknown sample	Real beef %	Real pork %	Calculated beef %	Calculated pork %
7	35	65	26.1±0.1 (75%)	67.0±2.5 (103%)
8	75	25	70.4±7.9 (94%)	26.6±7.2 (106%)
9	98	2	80.50±0.03 (82%)	1.7±NA (85%)

668

669

Figure 1

Click here to download high resolution image

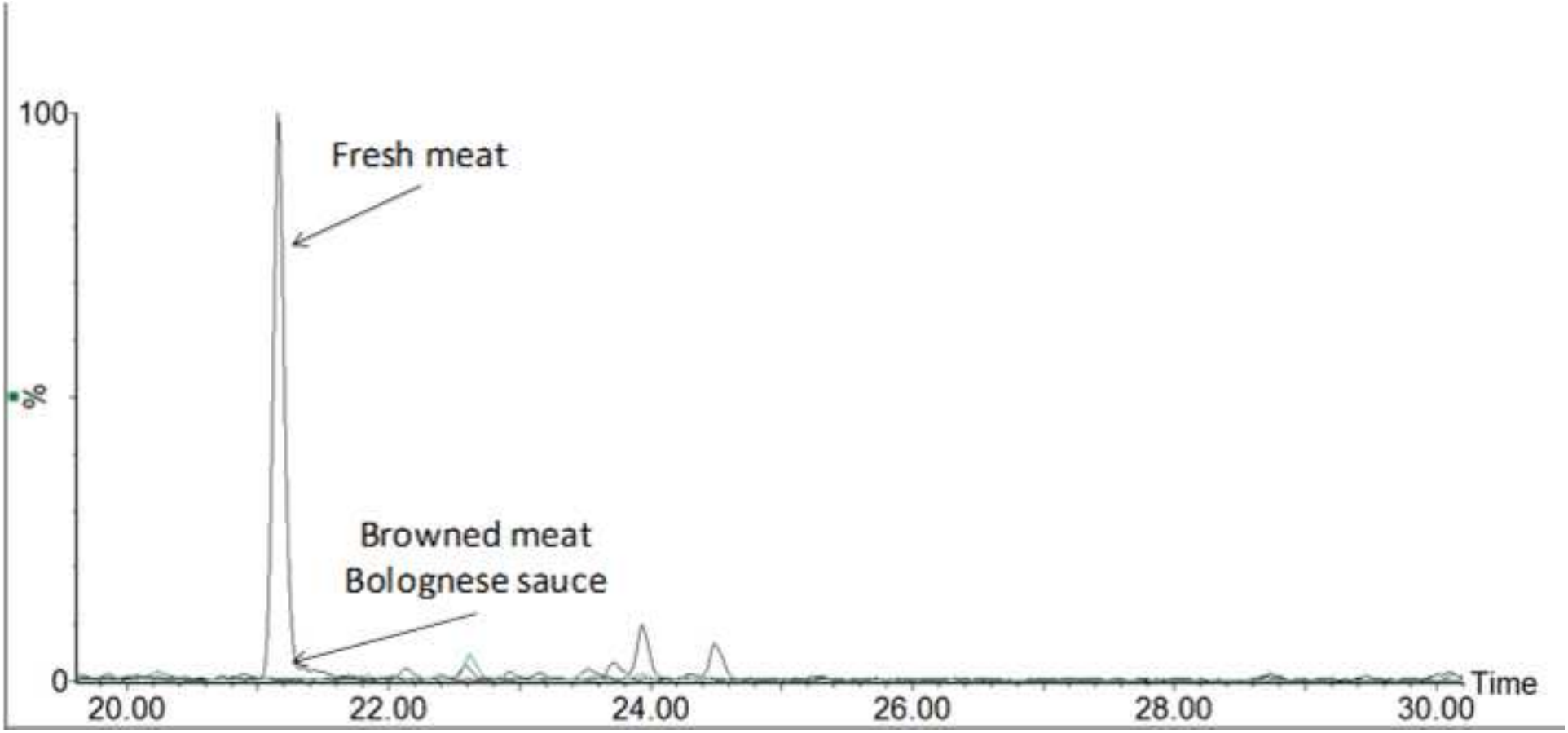

Figure 2

Click here to download high resolution image

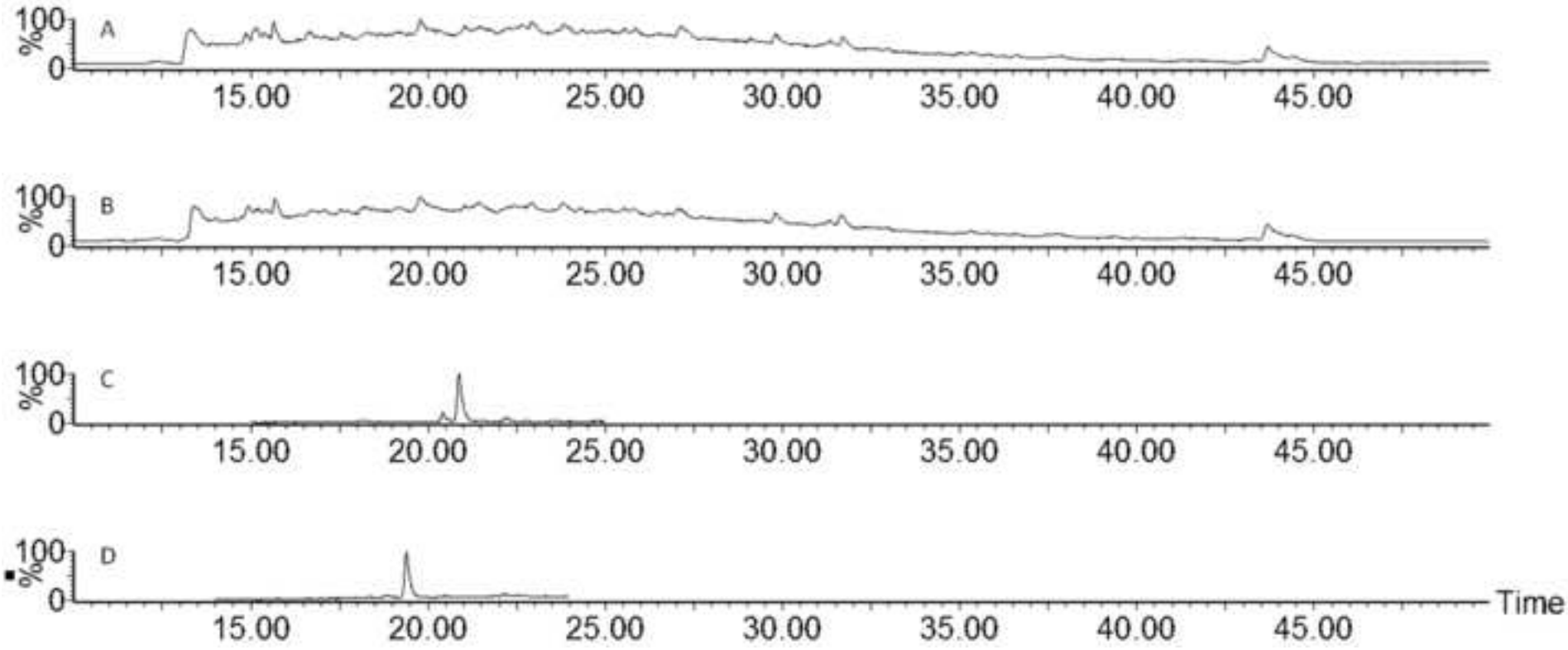

Figure 3

Click here to download high resolution image

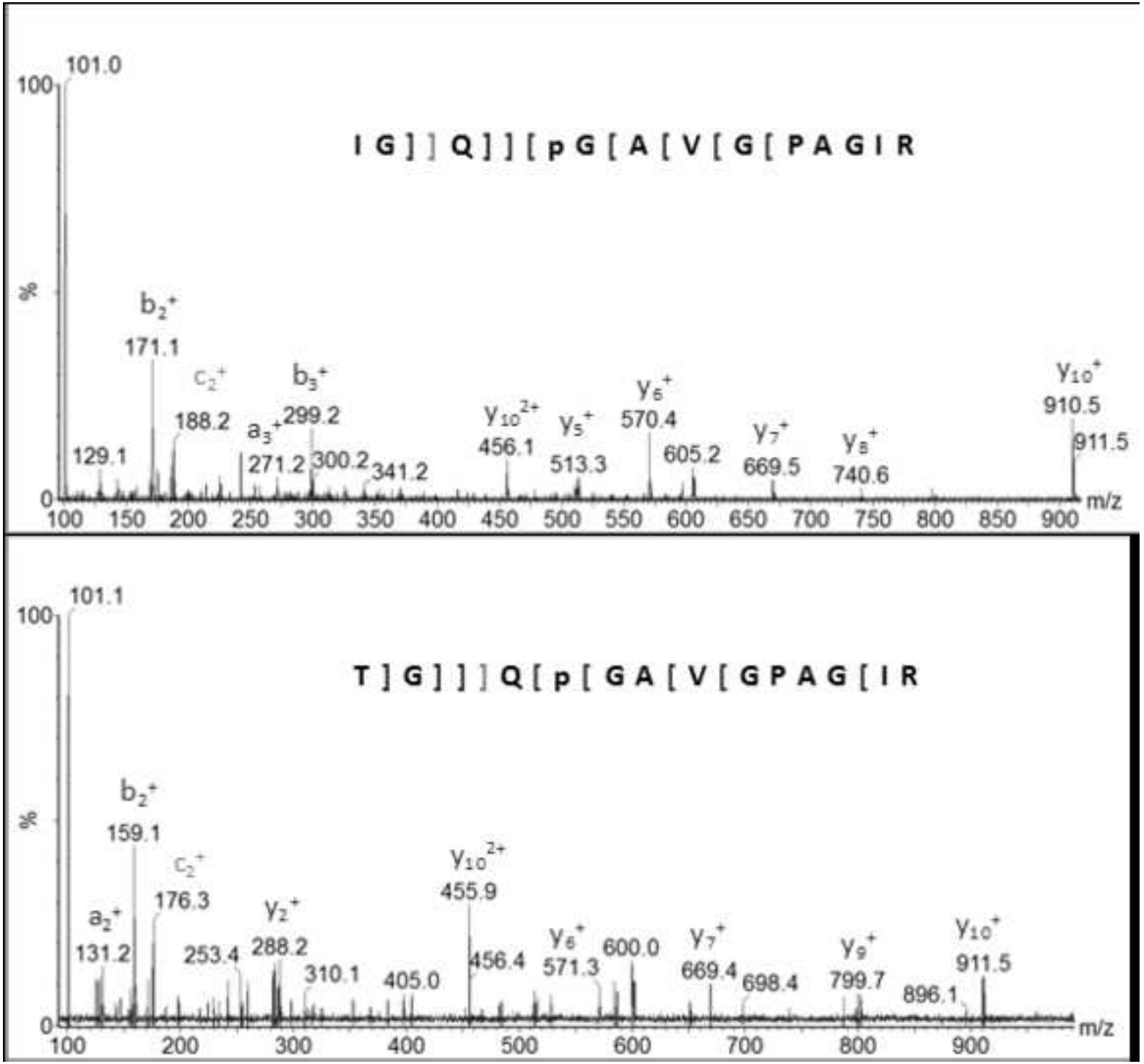

Figure 4

Click here to download high resolution image

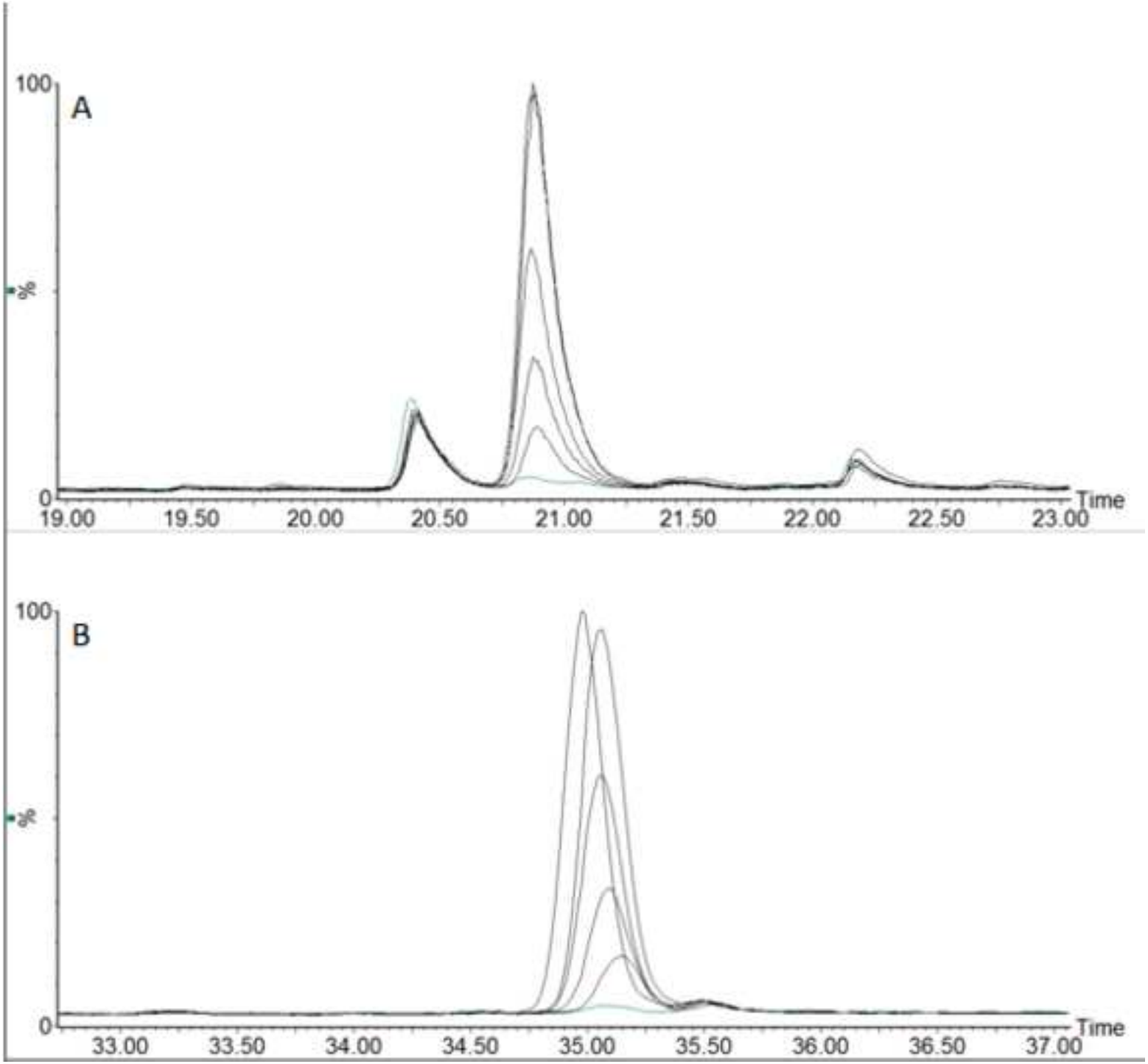

Table 1

Table 1 Marker peptides present only in Bolognese sauce made with pure beef. The best marker peptide (chosen on the best signal to noise ratio, selectivity, presence of coeluting interferents) is underlined (fragment 1 the most intense was used as quantifier, fragments 2 and 3 as qualifiers)

Rt (min)	Ion (m/z)	charge	MW (Da)	fragment 1	fragment 2	fragment 3	Best CE
29.5	641.2	2	1280.4	187.2	159	512.8	20
31.35	745.4	1	744.4	85.9	131.1	242.2	30
33.55	548.6	2	1095.2	120.1	128.8	205.3	25
34.03	644.2	2	1286.4	542.3	177	171	20
34.12	747.2	2	1492.4	145.1	167.1	279.3	30
34.36	932.4	1	931.2	839.4	187.4	643.2	20
34.62	636.7	2	1271.3	85.9	110.1	120.2	30
35.18	604.9	2	1207.6	101	85.7	171.1	20
35.55	665.5	2	1329	132.1	87.1	183.1	30
35.88	537.2	2	1072.6	85.9	199.2	227.1	30
37.99	781.3	2	1560.6	155.1	187.1	226.3	30
40.17	707.4	2	1412.8	85.8	169.1	240.3	30
47.09	762.9	2	1523.8	120.1	85.9	175.2	30
48.55	1185.6	2	1184.6	173.2	201.2	119.9	20

Table 2

Table 2 Marker peptides present only in Bolognese sauce made with pure pork. The best marker peptide (chosen on the best intensity, selectivity, presence of coeluting interferents) is underlined (fragment 1 the most intense was used as quantifier, fragments 2 and 3 as qualifiers)

Rt (min)	Ion (m/z)	charge	MW (Da)	fragment 1	fragmen 2	fragment 3	Best CE
30.85	470.8	2	939.6	86.1	110.1	215.2	30
32.84	598.9	2	1195.8	101.1	159.1	455.9	20
33.94	711.2	2	1420.6	no	no	no	no
34.58	523.2	2	1044.4	85.7	199.2	227.3	20
35.01	795	2	1588	169.2	140.9	846.8	25
35.4	702.4	1	701.4	120	86.1	173.2	25
35.29	830.5	2	1659	169.2	141.2	266.2	30
36.25	774.4	2	1546.8	815.7	261.8	115	20
39.41	388.2	2	774.6	no	no	no	no
40.52	684.3	1	683.2	472.2	340.2	227.1	20
44.67	496.1	2	990.2	no	no	no	no
49.31	586.7	2	1171.4	173.2	201.3	119.9	20
54.14	712.4	1	711.4	302.2	298.3	411.3	25

Table 3

Table 3 Common peptides present in both Bolognese sauce. The best peptide (chosen on the best intensity, selectivity, presence of coeluting interferents) is underlined (fragment 1 the most intense was used as quantifier, fragments 2 and 3 as qualifiers)

Rt (min)	ion (m/z)	charge	MW (Da)	fragment 1	frgament 2	fragment 3	Best CE
30.84	590.7	2	1179.4	no	no	no	no
33.62	474.2	2	946.4	187.2	175.1	110	20
37.14	532	3	1593	120.2	72	86	30
38.02	666.8	2	1331.6	86.1	143	171.2	25
39.08	872.5	2	871.5	389.2	466.4	484.3	25
40.52	538.7	2	1075.4	199.2	147.2	129	20
42.63	388.2	2	774.4	120.1	85.9	173.2	30
45.39	581.2	2	1160.4	199.2	86.1	120.1	25
46.48	1050.6	1	1049.6	1032.7	no	no	no

Table 4

Table 4 Results of two different analysis done at two months of distance of three blind samples. Results are reported as the average of two different replicates, together with the standard deviation and the trueness values (in brackets).

UNKNOWN SAMPLE	REAL BEEF %	REAL PORK %	% BEEF 1° ANALYSIS (trueness)	% BEEF 2° ANALYSIS (trueness)	% PORK 1° ANALYSIS (trueness)	% PORK 2° ANALYSIS (trueness)
7	35	65	20.4±5.3 (58%)	25.3±1.0 (72%)	79.6±5.3 (122%)	74.7±1.0 (115%)
8	75	25	77.2±0.2 (103%)	76.8±1.0 (102%)	22.8±0.2 (91%)	23.2±1.0 (93%)
9	98	2	92.8±0.7 (95%)	93.1±1.4 (95%)	7.2±0.7 (360%)	6.9±1.4 (345%)

Table 5

Table 5 Results of the absolute quantification of beef and pork meat in the three blind samples. Results are reported as the average of two different replicates, together with the standard deviation and the trueness values (in brackets).

UNKNOWN SAMPLE	REAL BEEF %	REAL PORK %	CALCULATED BEEF %	CALCULATED PORK %
7	35	65	26.1±0.1 (75%)	67.0±2.5 (103%)
8	75	25	70.4±7.9 (94%)	26.6±7.2 (106%)
9	98	2	80.50±0.03 (82%)	1.7±NA (85%)