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

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***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

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

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17 ABSTRACT

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

28 *Keywords:*

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

30 Abbreviations:

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

39

40 **1. Introduction**

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

65 Despite numerous articles regarding meat authenticithy in the last ten years (182 results for "meat
66 authenticity" in Web of Knowledge database, accessed 14/09/2016), only twelve of them took into
67 account processed food products: Motalib Hossain et al. (2016) developed a multiplex polymerase
68 chain reaction–restriction fragment length polymorphism assay to detect beef, buffalo and pork
69 meat also in processed foods such as frankfurters. In this work, detection was possible down to
70 0.1% of adulteration. The same group developed also a short amplicon-length PCR able to detect
71 cat meat in cooked burgers down to 0.01% (Ali et al., 2016). Multiplex PCR, besides fresh meat
72 product, was also applied to some cooked whole muscle meat, detecting several adulteration of beef
73 with chicken meat (Chuah et al., 2016). Cases of substitution of beef with pork meat and of lamb
74 with beef were reported by Premanandh et al. (2013) using a DNA-based approach. Despite the
75 severe processing occurring for gelatine production, a PCR method was able to detect down to 0.1%
76 of pork gelatine in bovine gelatine (Shabani et al., 2015). Duplex PCR was also applied to assess
77 the authenticity of donkey meat (liable of adulteration with horse and mule), with a limit of
78 detection of 1% (Chen et al. 2015). Among game meat, PCR-based methods are available for the
79 detection of roe deer, red deer and (Rak et al., 2014) and for the detection of game birds (Rojas et
80 al., 2009). After the horse meat scandals, a lot of efforts were put in the development of new and
81 sensitive analytical methods for the detection of this meat species. For example, Pegels et al. (2015)
82 developed a TaqMan RT-PCR to detect horse DNA also in processed products, like cured meat,
83 sausages, burgers and pet food. As described by Stefanova et al. (2013), DNA extraction is often a
84 critical step, especially in processed products. In the last years, a promising technique in this field is
85 DNA barcoding, able to detect DNA also in processed fish (Yang et al., 2012). Among methods
86 relying on proteomics, Claydon et al. (2015) identified several horse meat derived peptides that
87 were resistant to food processing and that can be detected in canned corned beef and baby foods.

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

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

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

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

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

133 The development of a quantitative method suitable for commercial products, will allows to detect
134 not only the presence/absence of an undeclared ingredient, but also the relative quantification of a
135 complex matrix with different species mixed together. A quantitative method is indeed a powerful
136 tool also to monitor the entire industrial supply chain, in order to verify the compliance from the
137 raw materials to the finished products. For example, a quantitative method is a helpful tool to
138 discriminate between a simple contamination episode or an intentional food fraud. Moreover, in the
139 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

163 commercial Bolognese sauce. Briefly, the meat was first browned with other ingredients such as
164 celery, carrots and onion, tomato sauce was then added and the mixture was heated to reach about
165 90°C. Glass jars were hot filled and the product was submitted to a final sterilization step at 120° C
166 for few minutes. In the final recipe minced meat reached about the 20% of the total weight.

167 *2.2 Protein extraction*

168 One hundred grams of Bolognese sauce samples were homogenized using an UltraTurrax (IKA T50
169 basic, Staufen, Germany) and then lyophilized at a vacuum level of 0.2 mbar and -50°C (Lio5P,
170 5Pascal, Milan, Italy). 1 g of the lyophilized sample was added to 10 ml of a solution of 50 mmol L^{-}
171 ^{1} TrisHCl (pH 8), 6 mol L^{-1} urea and 1 mol L^{-1} thiourea and homogenized for 5 minutes using an
172 UltraTurrax (IKA T10 basic, Staufen, Germany). The resultant mixture was centrifuged at 8784*g*
173 for 10 min at 4°C and the supernatant was filtered through 0.45 μm syringe driven filters and
174 protein concentration was measured according to the Bradford assay (Kruger, 2002), using bovine
175 serum albumin (BSA) as standard. Samples were desalted at atmospheric pressure using Sep-pak
176 Plus C18 cartridges (particle size 55-105 μm, pore size 125 Å, sorbent weight 360 mg), according
177 to the manufacturer instructions (Waters, Milford, MA, USA) in order to eliminate caothropic
178 agents used during the extraction (urea and thiourea). Briefly, the cartridge was wetted with 5 ml of
179 B solution (water 35%, acetonitrile 65%, plus 0.1% formic acid) and equilibrated with 10 ml of A
180 solution (water 98%, acetonitrile 2%, plus 0.1% formic acid). The sample was flushed through the
181 cartridge, that was subsequently washed with 10 ml of A solution. Finally, adsorbed proteins were
182 eluted with 5 ml of B solution.

183 *2.3 Protein digestion*

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

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

195 *2.4 LC-LRMS analysis*

196 The analysis was carried out as previously described (Prandi et al., 2012) and parameters for
197 peptide detection were previously optimized in our laboratory. Digested samples were separated on
198 a RP column (Acquity UPLC BEH 300, C18, 1.7 µm, 2.1×150 mm; Waters Corp., Milford, MA,
199 USA) in an UHPLC/ESI-MS system (Acquity Ultraperformance UPLC with a single quadrupole
200 mass spectrometer, Waters SQD) using a gradient elution. Eluent A was water with 0.1% formic
201 acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid; gradient: 0–7 min
202 100% 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%
203 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
204 ml/min; analysis time 72 min; column temperature 35 °C; sample temperature 18°C; injection
205 volume 10 µl; ionization type was positive ions; capillary voltage 3.2 kV; cone voltage 30 V; source
206 temperature 150 °C; desolvation temperature 300 °C; cone gas flow 100 l/h; desolvation gas flow
207 650 l/h. The samples were analyzed both in Full Scan mode, scanning the range 100–2000 m/z from
208 0 to 58.2 mins, and in SIR mode, monitoring the following m/z: 598.9 (acquisition window 14-24
209 min), 604.9 (15-25 min), 388.2 (22-32 min).

210 *2.5 LC-LRMSMS analysis*

211 The analysis was carried out as previously described (Prandi et al., 2012) and parameters for
212 peptide detection were previously optimized in our laboratory. Digested samples were separated by
213 a RP column (Jupiter Phenomenex 5 µm C18 90 Å 250*2 mm) in an HPLC/ESI-MS/MS (HPLC
214 Waters Alliance 2695 with a triple quadrupole mass spectrometer Waters 4 Micro), using a gradient
215 elution upscaled from the previous described for UHPLC analysis. Eluent A was water with 0.1%
216 formic acid and 0.2% acetonitrile, eluent B was acetonitrile with 0.1% formic acid; gradient: 0-12
217 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%
218 A, 82-90 min 0% A, 90-91 min from 0% A to 100% A, 91-110 min 100% A. HPLC/ESI-MS/MS
219 parameters were: flow 0.2 ml/min; analysis time 110 min; column temperature 35°C; injection
220 volume 40 µl; ionization type positive ions; capillary voltage 3.2 kV; cone voltage 35 V; source
221 temperature 100°C; desolvation temperature 150°C; cone gas flow 100 l/h; desolvation gas flow
222 650 l/h. Samples were analyzed in the MRM mode monitoring the following transitions:
223 598.9→101.1/159.1/455.9 (27-37 min); 604.9→85.7/101/171.1 (30-40 min);
224 388.2→85.9/120.1/173.2 (37-47 min). The run time of 110 min was necessary for a better peaks
225 resolution (given the complexity of the matrix) and to minimize the matrix effect, spreading the
226 eluting compound in a longer run.

227 *2.6 LC-HRMS analyses*

228 Digested samples were separated using RP column (Jupiter Phenomenex C18 4µ, Proteo 90Å
229 150*0.30 mm) and a gradient elution; eluent A was water with 0.1% acetonitrile and 0.1% formic
230 acid and eluent B was acetonitrile with 0.1% formic acid (gradient: 0-4 min from 100% A to 95%
231 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
232 min from 10% A to 95% A, 74-90 min 95% A). HPLC: DIONEX Ultimate3000, Thermo Fisher
233 Scientific (Waltham, Massachusetts, USA); mass spectrometer: LTQ Orbitrap XL, Thermo Fisher
234 Scientific (Waltham, Massachusetts, USA). The analysis parameters were: flow 5 µl/min; analysis
235 time 90 min; column temperature 30°C; sample temperature: 10°C; injection volume 5 µl;

236 acquisition time 0-75 min; ionization type positive ions; scan range 200-1800 m/z; source voltage
237 3.5 kV; capillary voltage 35 V; source temperature 275°C. Scan Event Details: FTMS + p
238 res=30000 o(250.0-2000.0); ITMS + c Dep MS/MS Most intense ion from; Activation Type: CID;
239 Isolation Width: 2.00; Normalized Coll. Energy: 35.0; Default Charge State: 2; Activation Q: 0.250;
240 Activation Time: 30.000; Dynamic exclusion enabled; Repeat Count: 2; Repeat Duration (s):
241 10.00; Exclusion Duration (s): 30.00. Charge state rejection: enabled; Unassigned charge states:
242 rejected; Charge state 1: rejected; Charge state 2: not rejected; Charge state 3: not rejected; Charge
243 states 4+ : not rejected; ion signal threshold:10000

244 Results and discussion

245

246 *2.1 Marker peptides identification*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

369 *3.2 Beef and pork meat quantification*

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

383 The samples were first analysed using a triple quadrupole instrument, monitoring several MRM
384 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 –

410 40% pork, (D) 40% beef – 60% pork, (E) 20% beef – 80% pork, (F) 0% beef – 100% pork. Despite

411 also percentages below 20% need to be quantified, concentration levels of the calibration curve

412 were designed to be equidistant, so that the calculated slope and intercept are not influenced

413 disproportionally by one data point. LOD and LOQ were estimated on the signal to noise ratio of

414 the weakest (less abundant) marker peptide (pork marker peptide). A good indication on the

415 accuracy of the calculated LOD and LOQ is given by the 2% pork meat blind sample, in which pork

416 meat was detected. A previous example in literature of calibration curves made mixing different

417 amount of meat species (chicken in pork meat) can be found in the work carried out by Sentandreu

418 et al. (2009), in a working range from 0 to 10%, where a good linearity is shown.

419 Samples were extracted and analysed in duplicate, using the SIR mode. Then, the % of beef (pork)

420 is plotted against the value $\frac{\frac{(Area\ beef)}{Area100\%\ beef}}{\frac{Area\ beef}{Area\ 100\%\ beef}+\frac{Area\ pork}{Area\ 100\%\ pork}}$ (or $\frac{\frac{(Area\ pork)}{Area100\%\ pork}}{\frac{Area\ beef}{Area\ 100\%\ beef}+\frac{Area\ pork}{Area\ 100\%\ pork}}$). A good

421 correlation (r^2>0.99) was found using a linear equation (beef: y=0.0102x+0.0041 R^2=0.9933; pork:

422 y=0.0102x-0.0226 R^2=0.9933).

423 The use of synthetic peptides was excluded because of the high costs of the synthesis of isotopically

424 labelled standard, together with the background knowledge needed for the synthesis, that would

425 have probably make the method inaccessible for most of the meat sector stakeholders.

426 To check the accuracy of the method, three blind samples (at unknown meat composition) were

427 analysed, according to current guidelines (Niazi, 2015). Results are reported in Table 4. The method

428 showed a good precision in repeatability conditions both within the same analysis (intraday

429 variability) and between two different analysis carried out with two month of interval (interday

430 variability<15%). The trueness value for acceptance was fixed to be within 20% of the nominal

431 value. The best trueness values were found for sample 8, where the detected values well match with

432 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

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

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

474 **3. Conclusions**

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

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

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

490 4. Declaration of interests

491 The authors declare that they have no conflict of interests.

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496

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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)

633 **Fig. 3.** MS/MS spectra of the marker peptide for beef (upper, IGQpGAVGPAGIR) and for pork

634 meat (lower, TGQpGAVGPAGIR). Peaks are annotated using the conventional peptide

635 fragmentation symbols. Square brackets indicated the fragmentation points.

636 **Fig. 4.** A comparison between SIR detection (A) and MRM detection (B): overlaid traces of the

637 marker peptide for beef in the six points of the calibration curve (from 100% beef to 0% beef).

638

639 **8. Tables**

640 **Table 1**

641 Marker peptides present only in Bolognese sauce made with pure beef. The best marker peptide
642 (chosen on the best signal to noise ratio, selectivity, presence of coeluting interferents) is underlined
643 (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

644

645

646

647 **Table 2**

648 Marker peptides present only in Bolognese sauce made with pure pork. The best marker peptide

649 (chosen on the best intensity, selectivity, presence of coeluting interferents) is underlined (fragment

650 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

651

652

653 **Table 3**

654 Common peptides present in both Bolognese sauce. The best peptide (chosen on the best intensity,

655 selectivity, presence of coeluting interferents) is underlined (fragment 1 the most intense was used

656 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
<u>42.63</u>	<u>388.2</u>	<u>2</u>	<u>774.4</u>	<u>120.1</u>	<u>85.9</u>	<u>173.2</u>	<u>30</u>
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

657

658

659 **Table 4**

660 Results of two different analysis done at two months of distance of three blind samples. Results are

661 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

662

663

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%)

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669

Figure 1
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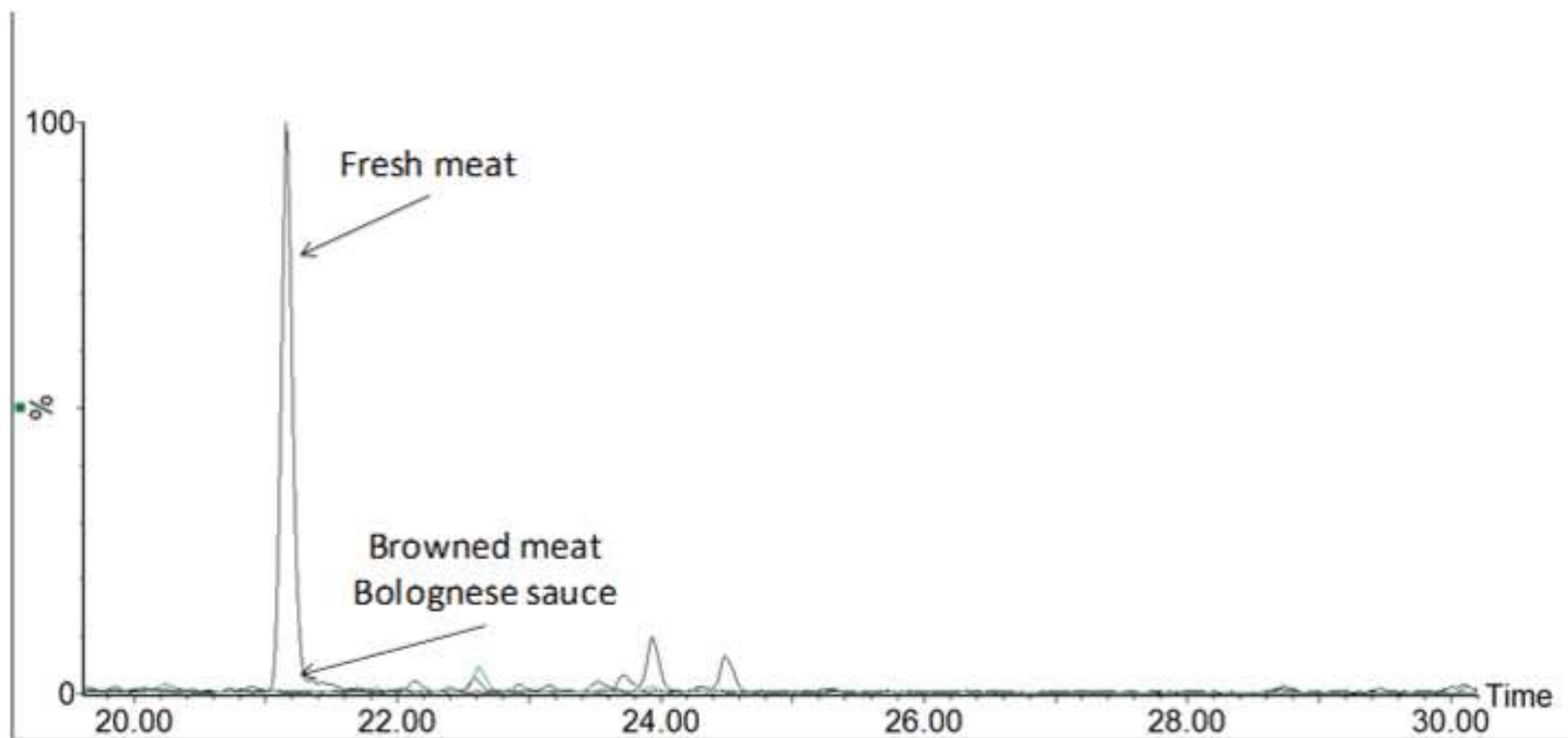

Figure 2

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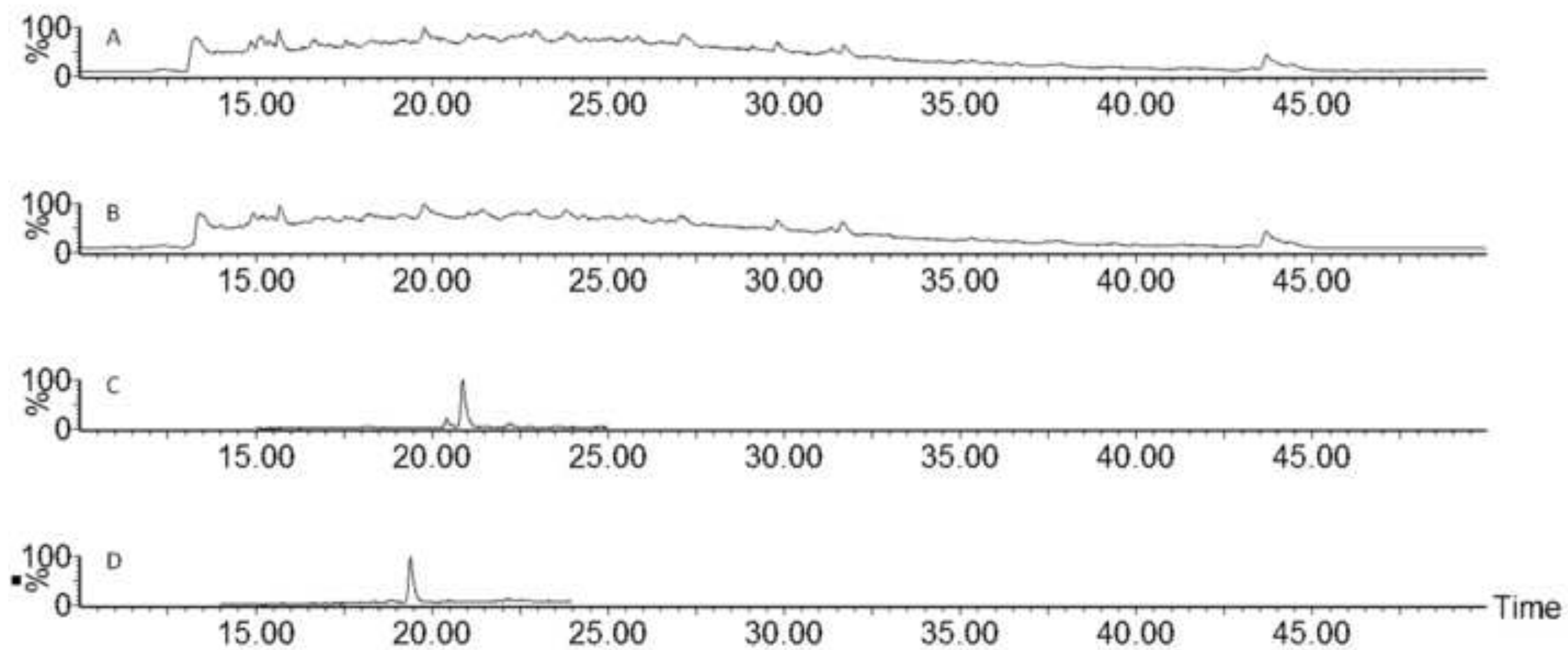

Figure 3

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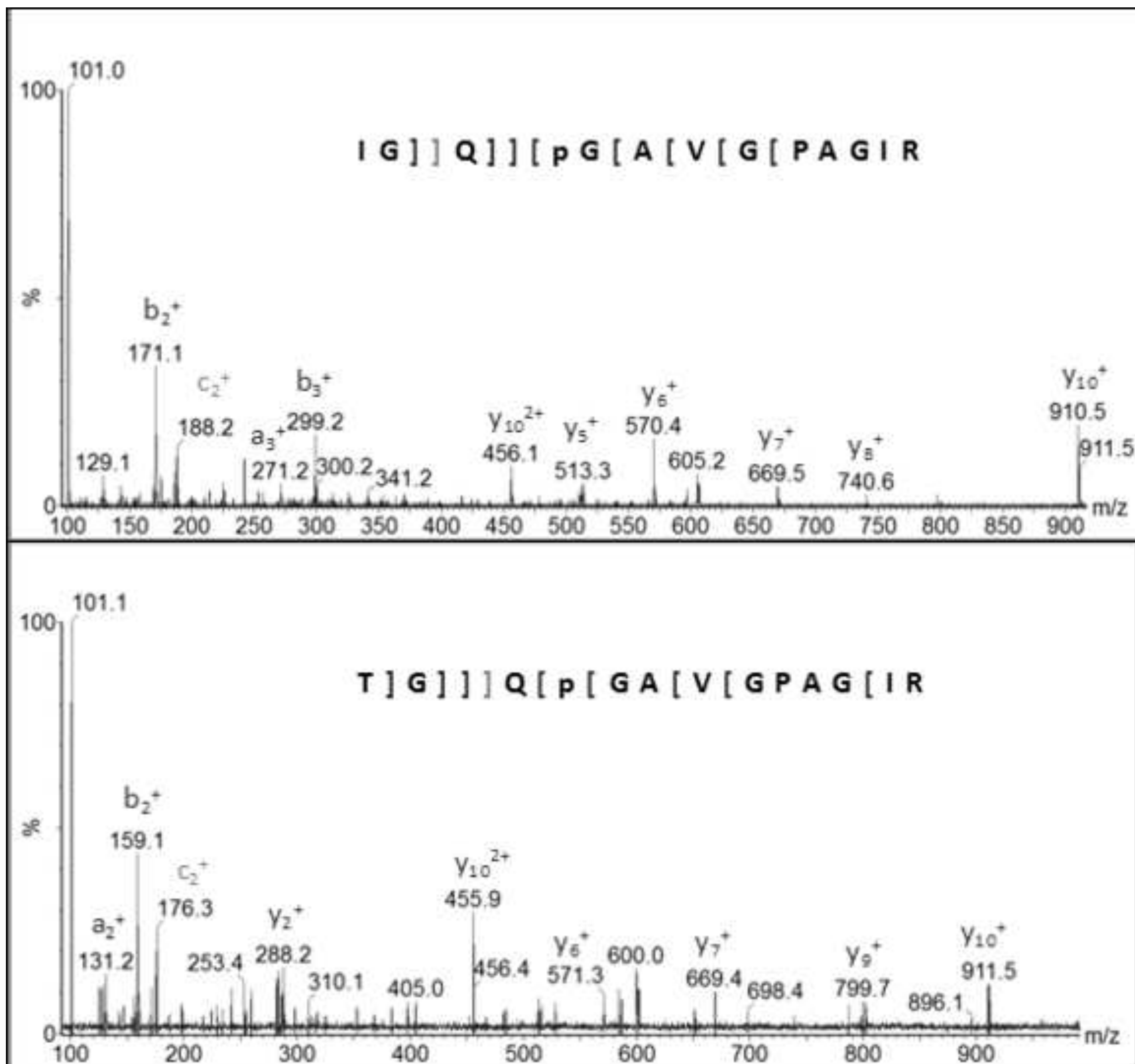

Figure 4

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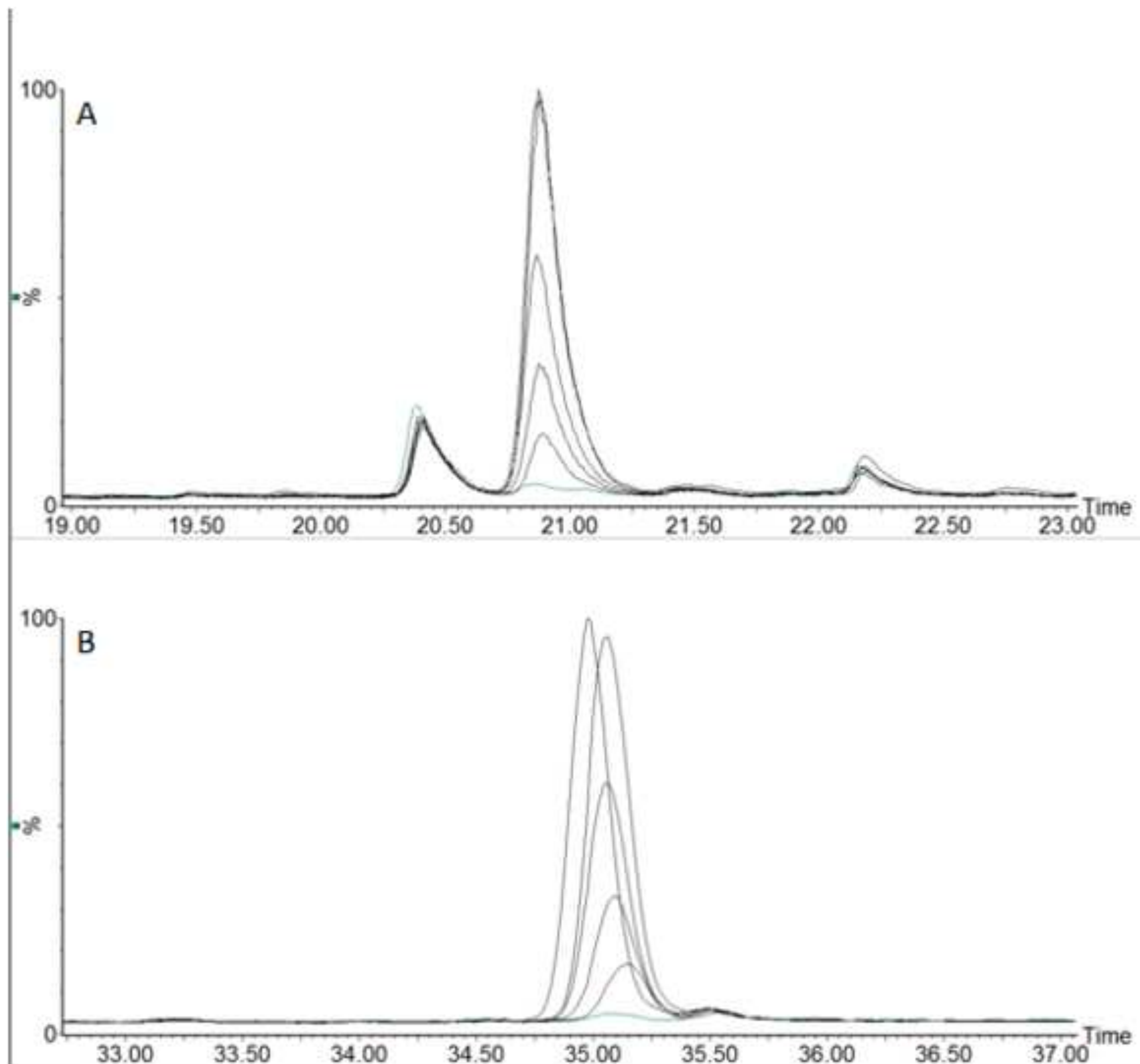

Table 1

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
<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

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
<u>32.84</u>	<u>598.9</u>	<u>2</u>	<u>1195.8</u>	<u>101.1</u>	<u>159.1</u>	<u>455.9</u>	<u>20</u>
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
<u>42.63</u>	<u>388.2</u>	<u>2</u>	<u>774.4</u>	<u>120.1</u>	<u>85.9</u>	<u>173.2</u>	<u>30</u>
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%)