



Analytical Methods

Myoglobin as marker in meat adulteration: A UPLC method for determining the presence of pork meat in raw beef burger

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ABSTRACT

The identification of meat animal species used in raw burgers is very important with respect to economic and religious considerations. Therefore, international supervisory bodies have implemented procedures to control the employed meat species. In this paper we propose myoglobin as a powerful molecular marker to evaluate the presence of non-declared meat addition in raw beef burgers by using ultra-performance liquid chromatography (UPLC) for the separation and identification of edible animal species (beef, chicken, horse, ostrich, pig and water buffalo). Meat samples were pre-treated with sodium nitrite to transform oxymyoglobin and deoxymyoglobin to the more stable metmyoglobin. The developed method was validated, preparing mixtures with different percentages of pork and beef minced meat. The obtained results show that using myoglobin as marker, 5% (25 mg/500 mg) of pork or beef meat can be detected in premixed minced meat samples.

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1. Introduction

Global meat production and consumption continue to rise. In 2013 the total global meat production is expected to be approximately 300 million tonnes (Nierenberg, 2011). Furthermore, demand for meat in the developing world is expected to double by 2020 (FAO, 2008; Pinstrip-Andersen, Pandya-Lorch, & Rosegrant, 1999; Worldwatch, 2011). This expansion of meat demand might be accompanied by commercial fraud; in particular, minced meat (e.g. raw burger), which is a much more heterogeneous product than meat cuts. Consumers want to be protected from falsely labelled food, containing unknown or less desirable meat species (Peter, Brunen-Nieweler, Cammann, & Borchers, 2004).

In this framework, for the sake of public health (Walker, Rhubarb-Berg, McKenzie, Kelling, & Lawrence, 2005), commercial protection (Smulders & Johnson, 1990) and religious customs (absence of pork meat for Jews and Muslims; Sakr, 1971), it is imperative to develop reliable and fast methods for the identification of different animal species in minced meats (Sun, 2008).

At the moment, there are several methods able to recognise pork and beef meat, which are among the most consumed meat types in the world (FAO, 2012). Several authors have proposed different analytical techniques to identify these meats either alone or

to differentiate them when present in mixtures. Immunoassay and enzyme-linked immunosorbent assay (Hsieh, 2006), as well as electrophoresis on polyacrylamide gel, have been suggested to resolve skeletal muscle proteins on the basis of either isoelectric point or molecular weight (Bauer & Hofmann, 1989; Di Luccia et al., 1992, 2007; Käuffer, Beneke, & Bentler, 1990). However, polymerase chain reaction (PCR), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD)-PCR and single nucleotide polymorphism are the most common techniques used for these purposes (Ghovvati, Nassiri, Mirhoseini, Heravi Moussavi, & Javadmanesh, 2009; Koh, Lim, Chua, Chew, & Phang, 1998; Martinez & Malmheden Yman, 1998; Matsunaga et al., 1999; Peter et al., 2004; Saez, Sanz, & Tolda, 2004; Verkaar, Nijman, Boutaga, & Lenstra, 2002).

In particular, SDS-gel electrophoresis on polyacrylamide is a manual technique which does not lend itself easily to full automation for high-throughput analyses (Garfin, 2003), while PCR techniques demand great accuracy in sample preparation, because of the high risk of contamination (Lee & Fairchild, 2006). Furthermore, the electrophoretic techniques frequently need mass spectrometry for protein identification, which in turn is not a routine methodology.

Thus, looking for an alternative method to detect the origin of minced meats, we present an analytical approach based on anionic ultra-performance liquid chromatography [UPLC (Swartz, 2005)], using myoglobin (Mb) as marker. Mbs, found in skeletal and cardiac muscle, are made up of a single polypeptide chain (the globin)

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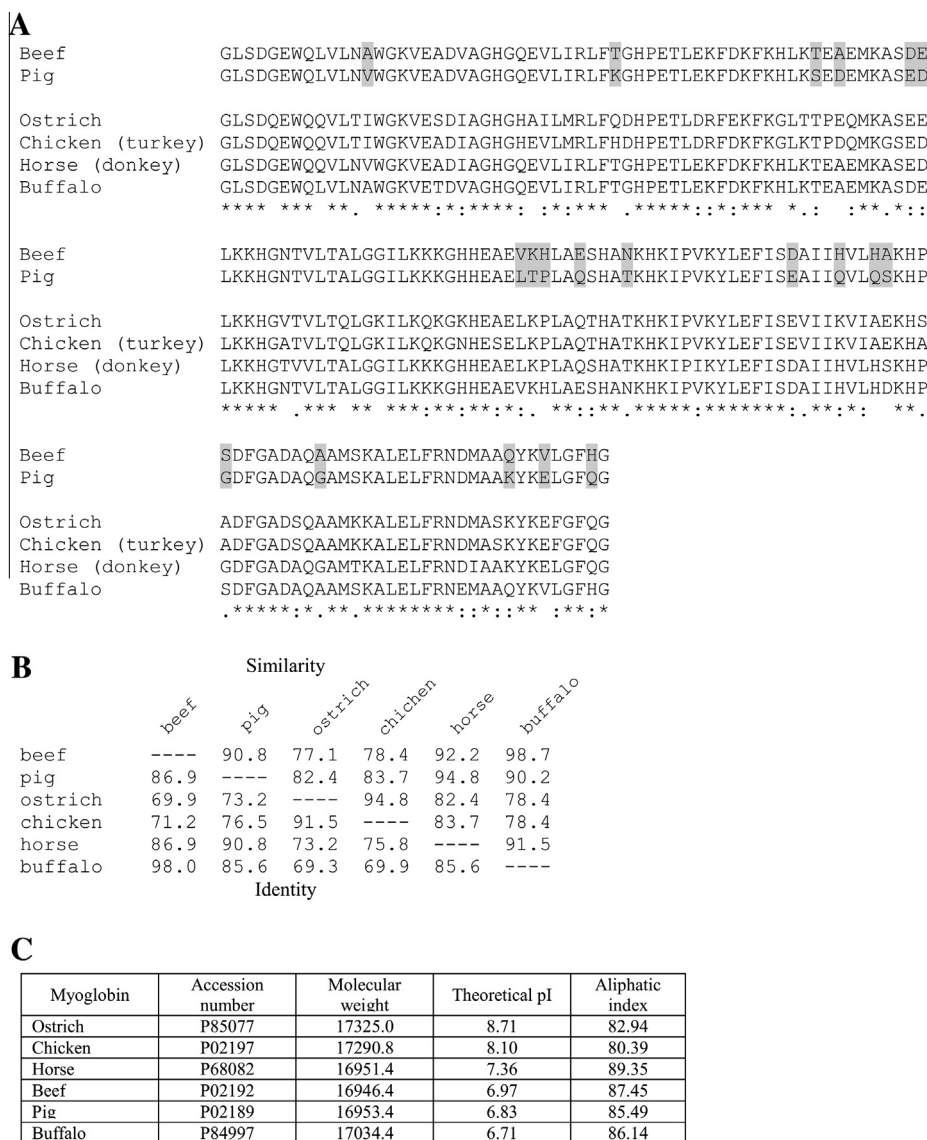


Fig. 1. (A), multiple sequence alignment of myoglobins from beef, pig, ostrich, chicken, horse and water buffalo species. Amino acid substitutions (20) between bovine and pig sequences are reported in light grey. "*" identical, ":" conserved and "." semiconserved residues. (B), identity-similarity matrix of beef, pig, ostrich, chicken, horse and water buffalo myoglobins. (C), accession numbers, molecular weights, isoelectric points (pI) and aliphatic index of the six myoglobins.

and a prosthetic group, an iron protoporphyrin-IX complex with a molecular weight in the range of 14–18 kDa (Ordway & Garry, 2004). Mbs have been widely characterised for their functional role in oxygen storage and cellular diffusion, pO_2 (oxygen partial pressure) buffering, peroxidase activity and their scavenging activity against reactive oxygen species and bioactive NO (Garry & Mammen, 2007). Furthermore, Mb in meat science is extensively studied because it plays a crucial role in meat colouration as the principal haem protein in sarcoplasm (Mancini & Hunt, 2005) and because of its interaction with nitric oxide used to obtain cured meat (Skibsted, 2011).

In this study we developed a UPLC method which can be used to simultaneously separate myoglobins from water buffalo (*Bubalus bubalis* L.), bovine (*Bos taurus* L.), pig (*Sus scrofa domestica* L.), horse (*Equus caballus* L.), chicken (*Gallus gallus domesticus* L.) and ostrich (*Struthio camelus australis* G.). Then, a fast procedure was set up to detect pork and beef meat in pre-mixed raw beef burgers and determine their relative lower detectable amount.

2. Material and methods

2.1. Chemicals and buffers

Sodium nitrite, glycine and sodium hydroxide were purchased from Sigma-Aldrich Srl (Milan, Italy). PD-10 desalting columns were supplied by GE Healthcare (Milan, Italy). Protein-Pak Hi-Res Q column (4.6 × 100 mm) was purchased from Waters SpA (Vimodrone, Milan, Italy). Bicinchoninic acid (BCA) kit was purchased from Pierce (Rockford, IL).

The following buffers were used: buffer A, 50 mM sodium phosphate (Na/P), pH 7.2, containing EDTA 50 μ M; buffer B, 20 mM glycine-NaOH, pH 9.2; buffer C, 20 mM glycine-NaOH, pH 9.2, containing 0.2 M NaCl.

2.2. Animal meat

Meat samples (myocardium or thigh muscles, and minced meat) of ostrich (*S. camelus australis*), chicken (*G. gallus domesticus*),

horse (*E. caballus*), beef (*B. taurus*), pig (*S. scrofa domesticus*) and water buffalo (*B. bubalis*) were obtained from local hypermarkets and kept frozen at -20°C until use.

2.3. Extraction and purification of myoglobins

Mbs were isolated from ostrich, chicken, horse, beef, pig and water buffalo as previously described (Balestrieri, Colonna, & Irace, 1973) and used with modification (Dosi et al., 2006, 2012). In particular, total proteins were extracted from meat homogenates in 5 mM Na/P, pH 7.2 containing 0.14 M NaCl. Soluble proteins were fractionated by gel filtration and anion exchange chromatography. During the purification procedure, eluted proteins and myoglobin (haem protein) were detected spectrophotometrically at 280 and 409 nm, respectively.

For determination of Mb content from both bovine and pork minced meat, analytical separation methods were used, as previously reported (Dosi et al., 2006). The amount is expressed in g/100 g of minced meat. Samples were analysed in triplicate for each source and mean values presented.

2.4. Electrophoretic and RP-HPLC analyses

Mbs homogeneity was determined by SDS-PAGE with a Mini-Protean II mini-gel apparatus (Bio-Rad; Milan, Italy). 6% (w/v) stacking and 15% (w/v) separation polyacrylamide gels (Laemmli & Favre, 1973) were used and 3 μg of purified ostrich, chicken, horse, beef, pig and water buffalo myoglobins were loaded. Reference proteins (Sigma-Aldrich, Milan, Italy) were: bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), horse myoglobin (17 kDa), and cytochrome C (14 kDa). A further control was carried out by RP-HPLC as previously reported (Di Maro et al., 2009).

2.5. On-line bioinformatics tool for myoglobin amino acid sequence analysis

Mb sequences were aligned using the ClustalW software in the default set-up (<http://www.ch.embnet.org/software/ClustalW.html>). The alignment was then analysed using BOXSHADE software (<http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html>). Molecular weight, theoretical pI and aliphatic index were obtained using the ProtParam tool (<http://web.expasy.org/protparam/>). The obtained data are reported in Fig. 1.

2.6. Separation of metmyoglobin by anion exchange chromatography using the UPLC system

The LC system used for method development and validation consisted of an Acquity UPLC with PDA detector, and a separation module with a Protein-Pak Hi Res Q column [$4.6 \times 100 \text{ mm}$, 5 μm (Waters SpA)], at 40°C operating temperature. Empower software (Waters SpA) was used for data treatment.

The analysis was carried out using a discontinuous gradient (Table 1), made up with buffers B and C. Eluted Mbs were monitored at 409 nm. The injection volume was 25 μL . When necessary, buffer B was used for sample dilution. Separation of all myoglobins was achieved with a total run time of 35 min. The retention times of ostrich, chicken, horse, pig, beef and water buffalo myoglobins were 10.10, 10.55, 14.86, 23.12, 24.29 and 25.74 min, respectively, in good agreement with the decreasing pI values. The resolution (R_s) values were all higher than 2.0 (i.e., 2.3 between ostrich and chicken Mbs, 5.6 between chicken and horse Mbs, 9.7 between horse and pig Mbs, 2.7 between pig and beef Mbs and 7.8 between beef and water buffalo Mbs).

Table 1

UPLC elution program applied for the separation of ostrich, chicken, horse, beef, pig and water buffalo myoglobins.

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)	Curve
0	0.4	95	5	6
2	0.4	95	5	6
7	0.4	85	15	6
20	0.4	85	15	6
21	0.4	75	25	6
24	0.4	60	40	6
25	0.4	0	100	6
29	0.4	0	100	6
30	0.4	95	5	6
35	0.4	95	5	6

2.7. Sample preparation for UPLC analysis

In order to transform any oxymyoglobin and deoxymyoglobin form present in minced meat and in standard myoglobins (ostrich, chicken, horse, beef, pig and water buffalo Mbs) to metmyoglobin, meat samples or purified Mbs were subjected to oxidation by using sodium nitrite (Di Iorio, 1981; Fig. 2). These Mb forms show physicochemical properties leading to different chromatographic elution volumes in anionic chromatography (Yamazaki, Yokota, & Shikama, 1964). Briefly, aliquots (0.5 g) of pork and beef minced meat were homogenised in 1.0 mL of buffer A and then centrifuged at 64,000g for 30 min (F1202 rotor, Allegra™ 64R centrifuge; Beckman Coulter, Milan, Italy) (Dosi et al., 2006; Ponce, Linforth, Hall, Guerrero, & Taylor, 1994). Then, the supernatant (~1 mL), containing sarcoplasmic proteins (and principally myoglobin), was incubated with 40 mg (40 mg/mL or 0.579 M) sodium nitrite for 10 min at 25°C . Finally, treated samples were quickly desalted on a PD-10 column (volume 9.1 mL), equilibrated and eluted with buffer B. The same procedure was performed for the standard myoglobins purified from the anionic chromatography. The metmyoglobin content in treated samples was calculated using a spectrophotometric procedure as previously reported (Krzywicki, 1982). All the experiments were performed in triplicate ($n = 3$).

2.8. Methodology validation

Pork and beef minced meats (with labels reporting only the meat species) were purchased in local hypermarkets, manually mixed in a known weight ratio and analysed as reported in Section 2.7.

2.9. Analytical procedures

Protein concentration was determined with the bicinchoninic acid (BCA) kit, following the manufacturer's instructions, using bovine serum albumin (BSA) as standard (Smith et al., 1985).

3. Results and discussion

3.1. Myoglobins purification

Standard Mbs were purified from ostrich, chicken, horse, pig, beef and water buffalo. The purity of Mbs was confirmed by the presence of homogenous peaks eluted from FPLC and RP-HPLC (data not shown), and by SDS-PAGE analysis (Fig. 3). The purified Mbs were used as standards for the development of the rapid UPLC separation.

3.2. Myoglobin sequence analysis

In order to evaluate the physicochemical differences between Mbs, a preliminary identity/similarity analysis was performed.

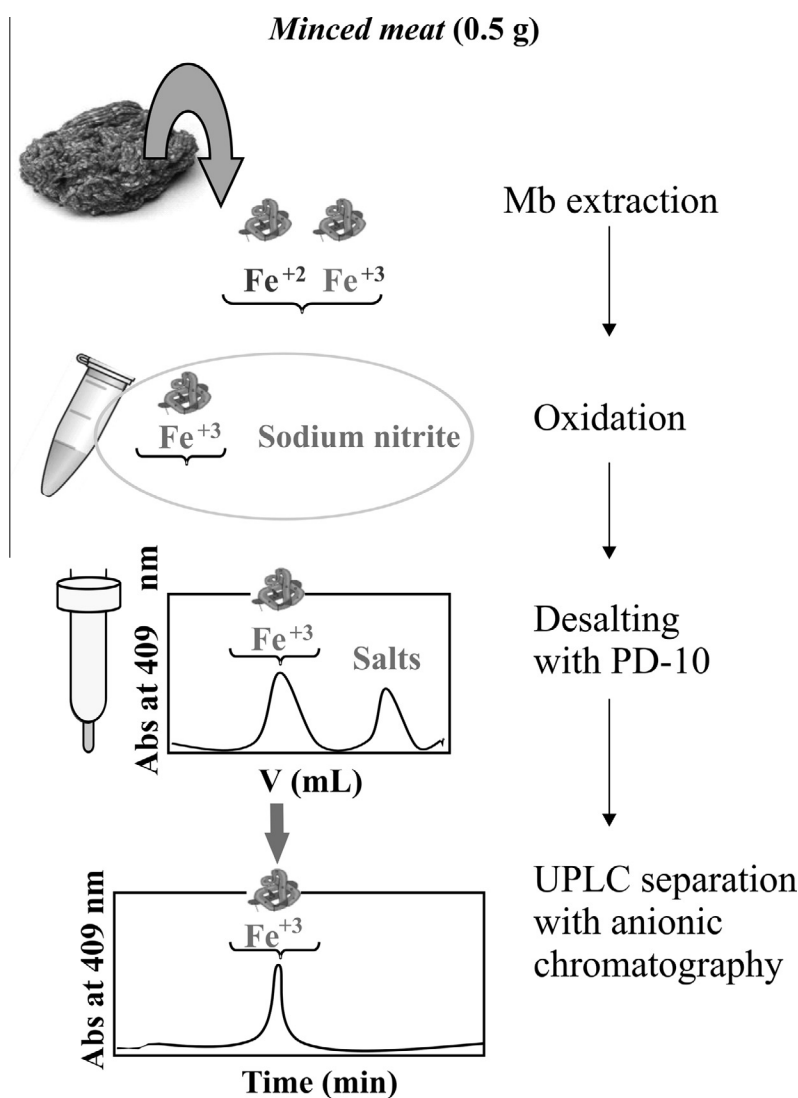


Fig. 2. Schematic workflow developed for the detection of pork meat in beef raw minced meat.

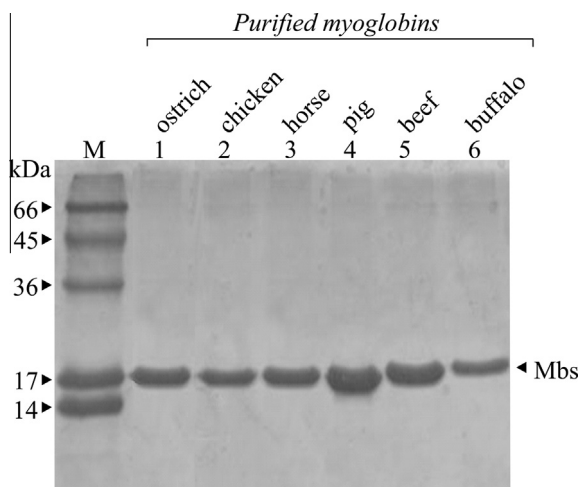


Fig. 3. SDS-PAGE analysis of purified ostrich, chicken, horse, beef, pig and water buffalo Mbs (lanes 1, 2, 3, 4, 5 and 6, respectively; 3 µg). M, protein markers.

The aligned amino acid sequences of ostrich, chicken, horse, beef, pig, and water buffalo Mbs (Fig. 1A) showed high identity/similarity percentages (ranging from 69.3 to 98.0 and 71.1 to 98.7, respec-

tively; Fig. 1B). In particular, 20 substitutions are present between beef and pig Mbs, (86.9% identity, 90.2% similarity). Furthermore, the sequence analysis showed differences (Mw, pI and aliphatic index, Fig. 1C) which could be used to plan a suitable separation method. In particular, distinctive pIs indicated that myoglobins could be separated from one another, based on small differences in their pIs, whereas the aliphatic index, defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine), represents a positive factor that confirms the thermostability of these myoglobins. Previous work showed that turkey (*Meleagris gallopavo*; Joseph, Suman, Li, Claus, Fontaine, & Steinke, 2011) and donkey (*Equus asinus*; Dosi, Carusone, Chambery, Severino, Parente, & Di Maro, 2012) myoglobins were identical to chicken and horse, respectively (data not shown), thus indistinguishable.

3.3. Development and optimisation of the UPLC separation method

Considering the pI values, we decided to use anionic exchange chromatography to separate the Mb samples by UPLC. Preliminarily, an oxidative step was carried out using sodium nitrite.

Setting up of a robust and suitable UPLC method for the separation of ostrich, chicken, horse, beef, pig and water buffalo Mbs

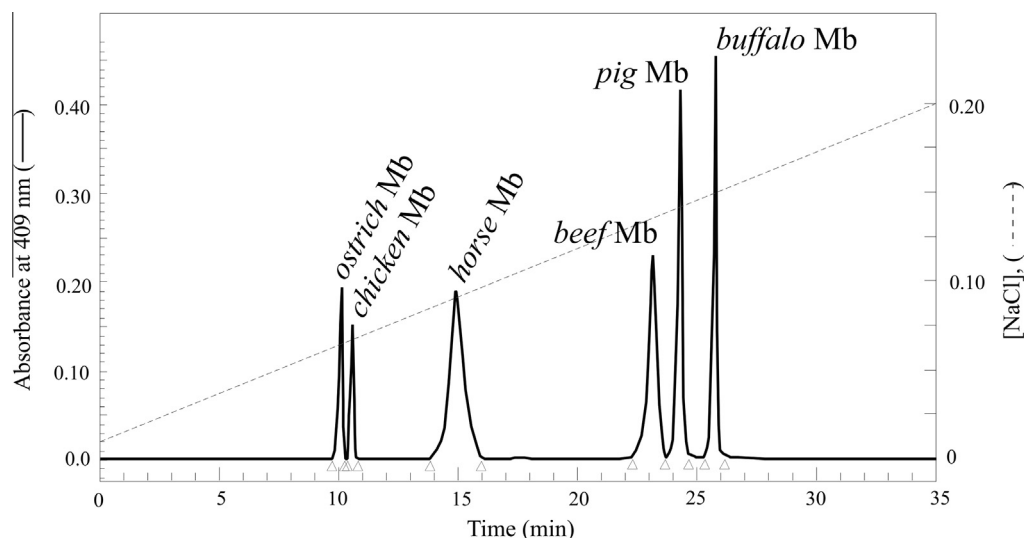


Fig. 4. Elution profiles of ostrich, chicken, horse, pig, beef and water buffalo myoglobins on the UPLC system.

required a number of trials carried out using different mobile phase compositions. Final separation was achieved using the Protein-Pak Hi Res Q column, eluted with a linear gradient made up by buffer A (20 mM glycine-NaOH, pH 9.2) and buffer B (20 mM glycine-NaOH, pH 9.2 containing 0.2 M NaCl), at a flow rate of 0.4 mL^{-1} (Fig. 4). Chromatographic profiles acquired at 280 nm contained only the protein peak corresponding to myoglobin (data not shown). However, Mb elution was monitored at 409 nm, because of the higher extinction coefficient.

Due to the large consumption of pork and beef meats, additional studies were performed on these Mbs, taking into account the abundance of the Mb in pork and beef tissues, i.e. 1.0–3.0 mg/g and 4.0–10.0 mg/g, respectively (Cornforth & Jayasingh, 2004; Keeton & Eddy, 2004). Based on these data, we carried out analyses to quantitate lower limits able to distinguish between pig and beef Mbs in mixtures. Fig. 5A shows the chromatographic profiles obtained using a higher amount of beef Mb (20 μg , corresponding to an average 2.86 mg of beef meat) and 2.0, 1.0 and 0.5 μg of

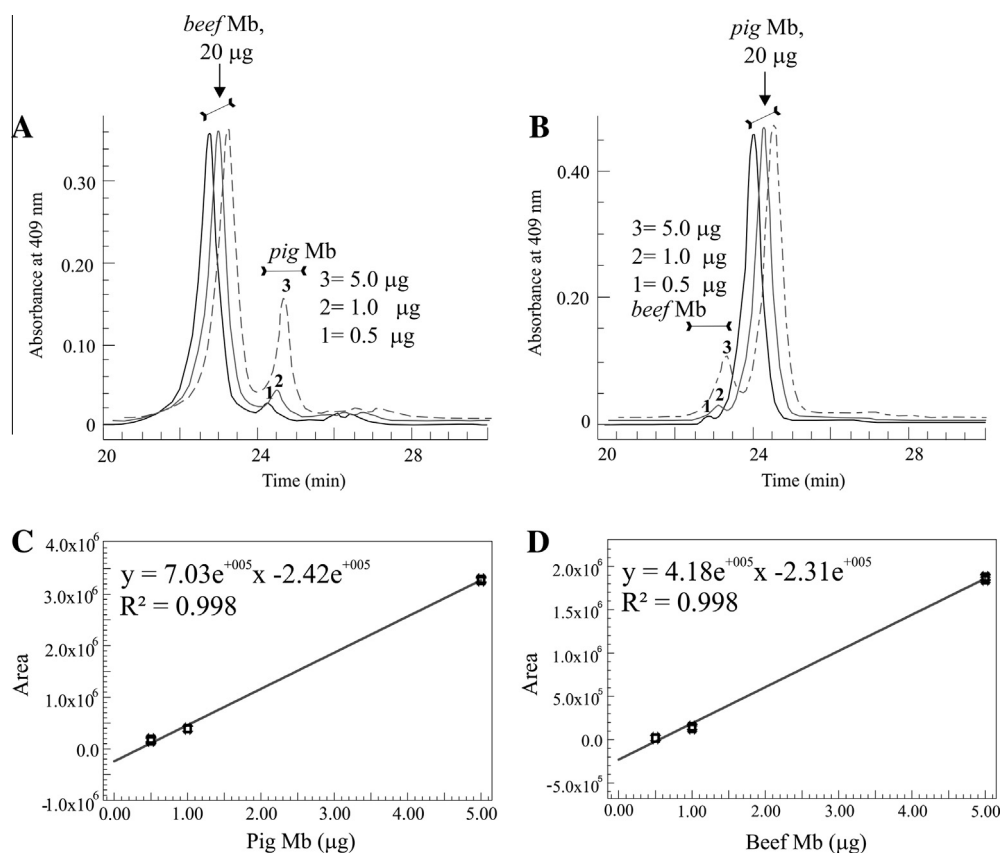


Fig. 5. Chromatographic profiles obtained for the determination of the lower detection limit of standard pig and beef myoglobins (A and B, respectively). In C and D, standard calibration curve and linearity plots for pig (C) and beef (D) myoglobin quantitation, respectively. In the inset, the equation and r^2 values of both calibrations.

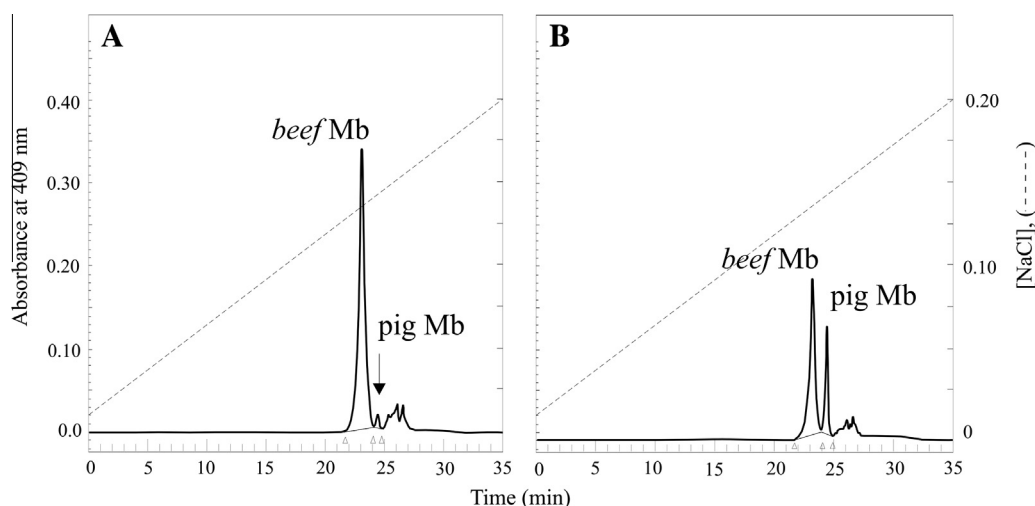


Fig. 6. Typical chromatographic profiles (A and B) of premixed raw burgers with 5% and 50% pork and 95% and 50% beef meat, respectively, after oxidation treatment and UPLC analysis.

pig Mb (corresponding to an average of 1.33, 0.66 and 0.33 mg of pork meat), while Fig. 5B shows the inverse, i.e. higher pig Mb amount (20 μ g: 13.33 pork meat) and 2.0, 1.0 and 0.5 μ g of beef Mb (0.28, 0.14 and 0.07 beef meat). The data show the clear separation between pig and beef Mbs and therefore, the potential use of Mbs as markers to detect the meat origin in raw burgers.

3.4. Calibration curve and linearity

Calibration curves for pig and beef Mbs were obtained by analysing in triplicate three different mixtures of known concentrations (between 0.5 and 5.0 μ g) of standard pig or beef Mbs (Fig. 5C and D, respectively). The obtained curve equation, calculated with the linear regression method, was utilised for the quantitation. The response of the detector was linear in the analysed range for both pig and beef standard Mbs, with an identical correlation coefficient ($r^2 = 0.998$).

3.5. Raw burger analysis

Raw burgers with different percentages of pork and beef minced meats were prepared in our laboratory in triplicate. The relative content of pig and beef Mbs was determined by using the UPLC elution conditions, previously set up. Samples (0.5 g of bulk mixed minced meat) were subjected to the experimental procedure reported in Fig. 2, i.e. treatment with sodium nitrite, desalification onto a PD-10 column and analysis on the UPLC system. In

Fig. 6 A and B are shown typical chromatographic profiles of pre-mixed raw burgers at 5% and 50% pork, and 95% and 50% beef minced meat, respectively. The results obtained for raw beef burgers are summarised in Table 2. The presence of pig and beef Mbs were detected and accurately quantitated in both pre-mixed samples (5–95%). In particular, in the sample with 5% minced pork meat (25 mg/500 mg of beef meat) the presence of pig Mb is clearly detectable, allowing its quantitation (0.297 μ g). Moreover, beef and pork Mb amount in our mixed samples was 1.86 ± 0.08 g/100 g and 0.74 ± 0.04 g/100 g, respectively, with a mean recovery value of 54%. In particular, higher recovery values (about 60%) were obtained when a high amount of beef meat was present in the mixtures. Furthermore, in this set of experiments, the %CV (variation coefficient) was 3% and 9% for pig and beef Mb, respectively.

Currently, for analytical species identification in raw minced meat, several approaches are used, such as ELISA assays (Hsieh, 2006), PCR amplification (Matsunaga et al., 1999) and species-specific real-time PCR (TaqMan) (Dooley, Paine, Garrett, & Brown, 2004), presenting detection limits of 1% (0.5% for real-time PCR). Instead, the method presented in this paper shows an experimentally determined detection limit of 5%, in the case of pork and beef minced meats. Nevertheless, this method, although with a lower detection limit, is a very robust method, which could be quickly transferred to other laboratories for the determination of non-declared meat addition in beef raw burgers.

Table 2

Amounts of pig and beef myoglobin obtained from the analyses of beef raw burgers containing different percentages of pork meat. Data are means of triplicate analyses ($n = 3$).

Bovine meat (%)	Pork meat (%)	Bovine meat (g)	Pork meat (g)	^a Recovered bovine Mb (μ g)	^a Recovered pork Mb (μ g)
100	0	0.500	0.000	15.0 ± 0.374	^b n.d.
95	5	0.475	0.025	11.1 ± 0.223	0.297 ± 0.010
90	10	0.450	0.050	10.3 ± 0.184	0.663 ± 0.017
80	20	0.400	0.100	9.03 ± 0.271	0.804 ± 0.016
70	30	0.350	0.150	7.94 ± 0.182	1.17 ± 0.022
60	40	0.300	0.200	6.99 ± 0.189	1.72 ± 0.051
50	50	0.250	0.250	3.67 ± 0.66	1.19 ± 0.021
40	60	0.200	0.300	3.51 ± 0.105	2.31 ± 0.053
30	70	0.150	0.350	3.06 ± 0.082	2.82 ± 0.076
20	80	0.100	0.400	1.23 ± 0.033	3.12 ± 0.056
10	90	0.050	0.450	0.912 ± 0.023	3.50 ± 0.105
5	95	0.025	0.475	0.780 ± 0.023	3.71 ± 0.085
0	100	0.000	0.500	^b n.d.	4.02 ± 0.109

^a μ g of bovine and pork myoglobins were calculated by using the areas of standard pig and bovine myoglobins, respectively.

^b n.d., not detected.

Nevertheless, as reported in the literature, myoglobin content of pork and beef tissues is variable considering muscle type and age (Cornforth & Jayasingh, 2004; Keeton & Eddy, 2004). Thus, further studies are needed to verify the lower detection limit of this UPLC-Vis method on mixtures, prepared with different cuts and animal of known age.

4. Conclusion

The development of UPLC methods for fraud detection in foods has recently received much attention because of their speed, resolution, sensitivity and cost effectiveness, important features in quality control procedures. Meat species identification is considered an essential requirement to assure food quality and safety for both consumers and producers. However, to our knowledge, very few chromatographic methods based on HPLC/UPLC have been developed to distinguish meat species in single or mixed samples. The limitations of this approach are the lengthy procedure for sample extraction, the complex chromatographic profiles and, in some methods, the need to derivatise peptides or other biological molecules, to improve detection sensitivity.

Thus, in this paper we developed a new UPLC-Vis method that suggests myoglobin as a molecular marker for simultaneous identification of ostrich, chicken, horse, beef, pig and water buffalo meats. Myoglobin represents a suitable molecular marker for detecting meat species in raw minced products (e.g. raw burgers) for several reasons: (i) it is one of the main constituents of the muscle tissue; (ii) it is possible to purify and obtain Mbs in homogeneous form (metMb); (iii) it is easily detectable because of its high absorbance coefficient at 409 nm. The simplicity of sample extraction/preparation and the proposed UPLC separation conditions show that the developed UPLC-Vis method can be easily automated for high-throughput analyses. In conclusion, we present a simple and versatile UPLC method, using visible detection to supplement PCR techniques and other methods for meat/species differentiation.

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