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# Protein markers for discrimination of meat species in raw beef, pork and poultry and their mixtures

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ABSTRACT

The purpose of this study was to find discrimination markers for four major meat species such as beef, pork, chicken and duck. Myofibrillar and sarcoplasmic proteins isolated from each meat type were analyzed by one-dimensional gel electrophoresis and some proteins were identified through LC-MS/MS analysis. We confirmed that troponin I (TnI), enolase 3, L-lactate dehydrogenase (LDH) and triose-phosphate isomerase (TPI) could be useful markers for discrimination of mammals from poultry due to their different electrophoretic mobility. Tropomyosin 1 and carbonic anhydrase 3 were observed as muscle fiber type-related proteins and these could also be markers to distinguish mammals from poultry. Species-specific peptides identified by LC-MS/MS spectra allow the identification of each species regardless of the same protein. Therefore, it is easy to discriminate between mammals and poultry by comparing the electrophoretic mobility of TnI, enolase 3, LDH, TPI and CA3, and each species could be identified through LC-MS/MS analysis.



## 1. Introduction

Methods to discriminate meat species from meat products have been commanding researchers' attention because of consumer demands for reliable and safe meat products, as well as higher protection from fraudulent practices such as mislabeling and substitution in the meat industry. Technologies to identify meat species are generally based on protein (Montowska & Pospiech, 2012, 2013; Sarah et al., 2016; Scarpeid, Kvaal, & Hildrum, 1998) and DNA molecules (Arslan, Irfan-Ilhak, & Calicioglu, 2006; Lockely & Bardsley, 2002; Martinez & Yman, 1998; Stamoulis, Stamatis, Sarafidou, & Mamuris, 2010). In recent times, proteomics technologies with mass spectrometry (MS) have been introduced (Montowska & Pospiech, 2012; Sarah et al., 2016) but controversial issues have arisen regarding tissue-specific proteins, proteolysis and denaturation due to storage and processing. DNA-based technologies are considered more stable methods compared to protein-based analyses. However, both of these technologies are not only time-consuming and labor-intensive but are also expensive.

In the meat industry, there is a demand for more rapid and accurate as well as inexpensive technologies to determine the meat species origin. Considering this, a few recent studies have inspired optimism for potentially useful applications in the meat industry: Stamoulis et al. (2010) discovered two mitochondrial DNA molecules to identify poultry meat in food; Sakalar and Abasıyanık (2012) developed a rapid identification method for ruminant and poultry origins using duplex real-time PCR based on SYBR green fluorescens; Montowska and Pospiech (2012, 2013) introduced a proteomic method for meat discrimination in raw and cooked meat as well as meat products; Sarah et al. (2016) discovered porcine-specific peptides using LC and MS; and Rapodi, Pavlidis, Mohareb, Panagou, and Nychas (2015) introduced multispectral image spectroscopy as a new, rapid and non-invasive technique to detect beef and pork in raw meats. Nevertheless, various aspects on the identification of species origin in meat products are still needed in order to develop rapid, accurate, simple and cheap methods that can be easily applied to the meat industry.

In the present study, a new method for the discrimination of meat species using common approaches for protein analysis, such as one-dimensional (1D) gel electrophoresis, MS and immunoblotting, was introduced. In our preliminary experiment, we discovered a different electrophoretic mobility in the same proteins between beef, pork, chicken and duck meat. Therefore, the

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hypothesis of this study is that the same proteins distributed in skeletal muscles of different species have different characteristics such as electrophoretic mobility rate and amino acid sequence. In this study, a few proteins were identified as markers for the discrimination of meat species using 1D gel electrophoresis, and it was confirmed by LC-MS/MS and immunoblotting that these proteins could be candidates for developing a rapid and simple discrimination method.

## 2. Materials and methods

### 2.1. Sample preparation

Loin muscles (*longissimus* m.) from beef and pork and pectoralis major muscles from chicken and duck were purchased from commercial slaughterhouses (10 replicates per meat type) and ground after trimming the connective tissue and visible fat. Each meat type was mixed alone (beef, B; pork, P; chicken, C; duck, D) or together (B + P, BP; B + C, BC; B + D, BD; P + C, PC; P + D, PD; C + D, CD; B + P + C, BPC; B + P + D, BPD; B + C + D, BCD; P + C + D, PCD; B + P + C + D, BPCD) for 5 min using a mixer (KP26 M, KitchenAid®, St. Joseph, MI, USA). The blended samples were prepared to a total weight of 500 g using the same amount of each meat type. Samples (4 g) were taken from all mixtures and homogenized with 30 ml of rigor buffer (75 mM KCl, 10 mM $K_2HPO_4$, 2 mM $MgCl_2$, 2 mM EGTA, pH 7.0, separately). The homogenates were centrifuged at 10,000*g* for 10 min and the supernatant was collected. This process was repeated three times using fresh rigor buffer and the supernatants were used for sarcoplasmic fractions. The remaining pellet was homogenized with 30 ml of fresh rigor buffer and used for myofibrils. The protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin as the standard. The final concentration of samples was adjusted to 2 mg/ml for 1D gel electrophoresis analysis.

#### 2.1.1. 1D gel electrophoresis and image analysis

Both sarcoplasmic and myofibrillar proteins were mixed (1:1, v/v) with a sample buffer containing 5% β-mercaptoethanol, 4% SDS, 20% glycerol, 0.2% bromophenol blue and 0.125 M Tris–HCl (pH 6.8) and heated at 100 °C for 3 min. Then, 10 μg of protein was loaded on each lane and 1D gel electrophoresis was performed using 10% SDS-polyacrylamide gel and a 4% stacking gel at a 20 mA constant current per gel. The gel was stained using 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 7% acetic acid. The 1D gels were scanned with a scanner (2100XL, UMAX®, TX, USA) and images were analyzed using an image analysis system (Kodak 1D Image Analysis Software, Eastman Kodak Co., NY, USA).

### 2.2. In-gel digestion

After gel image analysis, 1D gel bands, which displayed different electrophoretic mobility by species, were selected and each band was excised from the stained gel. The bands were destained with 50 μl $NH_4HCO_3$ buffer (50 mM, pH 7.8) containing 30% (v/v) acetonitrile, and dried using a speed vacuum concentrator (SPD1010, Thermo Fisher Scientific Inc., MA, USA). The dried gels were rehydrated using 10 μl of trypsin (Promega, Southampton, UK) solution (2.5 ng/μl) in 50 mM $NH_4HCO_3$ (pH 7.8) at 4 °C for 2 h. The rehydrated gels were incubated at 37 °C for 12 h. Purification of tryptic peptides were performed as described by Choi, Cho, Bae, Zoubaulis, and Paik (2003).

### 2.3. LC-MS/MS and protein identification

LC-MS/MS was performed using a nano-LC and LTQ mass spectrometer (Applied Biosystems, CA, USA) with a capillary column (150 mm × 0.075 mm, Proxeon, Odense M, Denmark) and a Magic C18 stationary phase resin (5 μm, 100 Å pore, Michrom BioResources, CA, USA), as described by Lee et al. (2014). Mobile phase A was 0.1% formic acid in deionized water and mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 5% B to 8% B for 1 min, 8% B to 35% B for 19 min, 85% B for 10 min and 5% B for 10 min. The flow rate was maintained at 300 nl/min. Ion spectra were acquired in a data-dependent acquisition mode using continuous cycles of one full scan from 300 to 1500 *m*/*z* plus three product ion scans from 100 to 1700 *m*/*z*. Precursor *m*/*z* values were selected starting with the most intense ion using a selection quadruple resolution of 4 Da. The MASCOT search engine (Matrix Science, London, UK) was used to identify the MS/MS spectra present in the NCBInr (*Bos taurus*, *Sus scrofa*, *Gallus gallus* and *Anas platyrhynchos*) protein sequence database. Database search criteria were as described: fixed modification, no; variable modification, oxidized at methionine residues; maximum allowed missed cleavage, 1; peptide MS tolerance, 100 ppm; fragment MS tolerance, 0.1 Da; peptides resulting from trypsin digest were considered. Individual ion scores greater than 45 were regarded as significant ($p < 0.05$).

### 2.4. Immunoblotting

Proteins separated by 10% 1D gel were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA) and blocked using Tris-buffered saline with Tween 20 containing 5% skim milk. A battery of seven primary antibodies with specificities for tropomyosin (TM) 2 (sc-134128), TM1 (sc-32516), troponin (Tn) I (sc-8118), TnT (sc-8123), glucose-6 phosphate isomerase (GPI) (sc-30392), L-lactate dehydrogenase (LDH) (sc-27232) and carbonic anhydrase (CA) 3 (sc-99005) and secondary antibodies (anti-rabbit IgG-HRP, sc-2357; anti-goat IgG-HRP, sc-2768) was purchased from Santa Cruz Biotechnology, Inc. (TX, USA). PVDF membranes were visualized using diaminobenzidine in the presence of imidazole.

## 3. Results

#### 3.0.1. 1D gel electrophoresis and image analysis

Myofibrillar and sarcoplasmic proteins from each meat species and their mixtures were separated by 1D gel electrophoresis, as shown in Fig. 1. Many bands showing a different electrophoretic mobility between meat species were observed in both myofibrillar and sarcoplasmic proteins. For myofibrillar proteins (Fig. 1A), myosin and actin were observed as clear bands and their intensities were very strong. Bands B1 and P1 from bovine (B) and porcine (P) muscles, respectively, displayed the same mobility. However, there was no band in the duck (D) lane and a weak band was observed in the chicken (C) lane. The results are supported by the 1D gel image analysis findings (Fig. 2). Band intensity was strong in the B and P lanes (at approximately the 900 pixel position), while the D lane did not show a peak. A small peak was observed in the C lane. In all mixtures except for CD, clear-cut bands and peaks were observed from the 1D gel (Fig. 1A) and image analysis results (Fig. 2). Bands B2, P2, C2 and D2 displayed a similar mobility on the 1D gel. C2 and D2 were more concentrated bands compared to B2 and P2. CD also shows the strongest intensity among