



Absolute quantification of targeted meat and allergenic protein additive peptide markers in meat products

Magdalena Montowska^{a,*}, Emilia Fornal^b

^a Department of Meat Technology, Poznan University of Life Sciences, Wojska Polskiego 31, Poznan 60-624, Poland

^b Department of Pathophysiology, Medical University of Lublin, Jaczewskiego 8b, Lublin 20-090, Poland

ARTICLE INFO

Keywords:

Food authenticity

Meat

Soy

Milk

Egg

Stable isotope-labelled peptides

ABSTRACT

We present an implementation of the absolute quantification (AQUA) method for monitoring of peptide abundance in complex mixtures of processed proteins. Specific peptide markers from meats (chicken, duck, goose, pork and beef) and common protein allergenic additives (soy, milk and egg white preparations) were chosen and synthesised with stable isotopes (^{13}C and ^{15}N) for use as internal standards. A wide range of food samples, from cooked or raw meat to sterilised pâté, was analysed by a triggered multiple reaction monitoring mode experiment and triple quadrupole mass spectrometry for the direct measure of tryptic peptides representing the amounts of specific proteins. Considerable differences among the abundances of meat and non-meat proteins were observed, and illegal addition and replacement of ingredients were discovered, i.e. undeclared addition of pork and egg white proteins, and illegal substitution of veal, goose and duck meat with cheaper pork.

1. Introduction

The evolution of advanced mass spectrometry (MS)-based methodologies has provided opportunities to quantify specific proteins in complex biological matrices and determine the level of protein expression, by the use of various label-free or labelled strategies; for example, non-labelled techniques based on spectral counting or signal intensity measures for precursor ions, and various stable isotope-labelled methods. Among the isotope-labelling approaches, relative quantification methods are most commonly performed, i.e. isotope-coded affinity tags, isobaric tags for relative and absolute quantification and stable isotope labelling with amino acids in cell culture. In contrast, absolute proteomic quantification using stable heavy isotope peptides (AQUA strategy) has rarely been chosen for meat authentication, due to the need for prior selection and costly synthesis of isotopic peptides (Gerber, Rush, Stemman, Kirschner, & Gygi, 2003; Iammarino, Marino, & Albenzio, 2017; Neilson et al., 2011). Thus, in the analysis of food products, relative label-free quantification strategies are preferred because of their cost-effectiveness, versatility and application for large-scale high-throughput experiments (Neilson et al., 2011; Rodríguez-Suárez & Whetton, 2013; Sandin, Telemann, Malmström, & Levander, 2014). Nonetheless, labelling procedures are undoubtedly more accurate and useful to estimate the extent of food adulteration; for instance, tracking the extent of fraudulent substitution of a portion of a given

component.

Only a few studies have measured an abundance of meat proteins for authentication purposes. The quantity of sarcoplasmic proteins extracted from dry-cured ham at various processing times was investigated based on precursor intensities of extracted ion chromatograms (Gallego, Mora, Concepción Aristoy, & Toldrá, 2016). Pork and beef were quantitated in highly processed food, such as Bolognese sauce, using peak measurements of the area under the curve of two peptides obtained from the $\alpha 2$ collagen chain (Prandi et al., 2017). The last example is the work where the abundance of meat proteins in various meat products was compared using a modified spectral counting technique (the APEX proteomics tool), which includes the peptide MS detection probabilities (Montowska & Fornal, 2017). Other commonly used analytical techniques for meat speciation are immunochemical assays and DNA-based approaches (El Sheikh et al., 2017; Abbas et al., 2018). However, these approaches have some limitations. Enzymatic methods are more vulnerable to thermal treatment and cross-reactivity between closely related species. DNA-based approaches are less affected by processing but cannot discriminate between tissues of the same species, i.e., beef *versus* milk or chicken meat *versus* egg white. Both approaches target one or several species, in contrast to MS-based methods that can identify and quantify a wide range of species and proteins of various origins in a single run.

Regarding MS-based quantification of functional protein additives

* Corresponding author.

E-mail addresses: magdalena.montowska@up.poznan.pl (M. Montowska), emilia.fornal@umlub.pl (E. Fornal).

<https://doi.org/10.1016/j.foodchem.2018.08.131>

Received 14 May 2018; Received in revised form 23 August 2018; Accepted 28 August 2018

Available online 29 August 2018

0308-8146/ © 2018 Elsevier Ltd. All rights reserved.

Table 1

Species and protein additive present in the examined samples.

Sample No.	Sample	Species declared	Additive declared
S.1	Chicken meat	Chicken	–
S.2	Meat mixture	Chicken (1%), turkey (49.5%), pork (49.5%)	–
S.3	Meat mixture	Pork (1%), turkey (49.5%), chicken (49.5%)	–
S.4	Duck meat	Duck	–
S.5	Goose meat	Goose	–
S.6	Homogenised frankfurters	Turkey (70%)	Soy proteins
S.7	Kabanos-type sausage	Chicken, pork	nd*
S.8	Liver sausage	Chicken, pork	Soy, milk proteins
S.9	Smoked and cooked breast	Chicken (80%)	Soy proteins
S.10	Homogenised frankfurters	Chicken (44.7%), turkey (15.4%), pork (10.8%)	Soy proteins
S.11	Homogenised frankfurters	Turkey (30.8%), beef (16.2%), chicken (14.6%), pork (10.8%)	Soy proteins
S.12	Kabanos sausage	Goose	nd
S.13	Pâté (roasted)	Goose (70%), chicken liver, pork	Soy, egg proteins
S.14	Homogenised frankfurters	Chicken (83%), veal (4%)	Milk proteins
S.15	Terrine (sterilised)	Duck	nd
S.16	Pâté (sterilised)	Duck (39%), pork (18%), chicken liver (14%)	Egg proteins

* nd – not declared.

applied in the food industry, similarly, only a few label-free quantification methods have been reported. Vegetable proteins (legumes and cereals, i.e., soy, bean, pea and wheat) or proteins of animal origin (milk, egg, connective tissue or blood plasma) are commonly used in meat processing to form the texture of the final product. These functional protein preparations may act as stabilisers, binders, fillers and sometimes as fat substitutes, to reduce the energy value, but may also be the source of hidden allergens, which are a threat to sensitive consumers (Monaci, De Angelis, Montemurro, & Pilolli, 2018).

MS-based absolute quantification may be performed using isotope-labelled synthetic peptides (known as AQUA strategy), isotope-labelled artificial proteins consisting of concatenated peptides (the quantification concatemer – QconCAT – approach), and using recombinantly expressed and isotopically labelled protein analogues (protein standards for absolute quantification – PSAQ – approach) (Gerber et al., 2003; Beynon, Doherty, Pratt, & Gaskell, 2005; Brun et al., 2007). In the present study, the AQUA method was chosen because synthetic peptides are commercially available and it is more suitable for a small set of proteins (Al Feteisi, Achour, Barber, & Rostami-Hodjegan, 2015).

AQUA is a targeted absolute quantification strategy, based on introducing synthetic stable isotope-labelled peptides into the sample, which can be enriched with ^{13}C , ^{15}N , ^2H or ^{18}O that allows quantification of proteins by measuring the signal intensity derived from endogenous peptides (light peptides), originating from the protein under investigation and their isotope-labelled counterparts (heavy peptides). Synthetic AQUA peptides accurately imitate the MS behaviour of their native light counterparts, in terms of chromatographic elution properties, ionisation efficiency and fragmentation pattern (Kirkpatrick, Gerber, & Gygi, 2005; Kettenbach, Rush, & Gerber, 2011; Mallick & Kuster, 2010). The internal standard incorporated into the sample is easily distinguished due to the 6–10 kDa mass weight increase of the AQUA peptide, depending on which amino acid was labelled. Recently, a proteomic-based method using two isotope-labelled peptides derived from β -lactoglobulin has been developed for quantification of cow milk and goat and sheep milk in raw material of dairy products, milk powder and whey protein powder (Chen et al., 2016).

In this study, the application of the AQUA strategy is presented, to quantify the abundance of meat proteins and non-meat allergenic protein additives, i.e., soy, milk and egg white, in various processed meat products, for authentication purposes. Meat and commercial products selected for examination were made from chicken, turkey, duck and goose meat, with the addition of pork and beef, as well as soy, milk and egg proteins, according to the list of declared ingredients. Quantification of proteins via the AQUA technique is conducted on the peptide level, and thus peptides unique to the examined mammal, poultry and plant species and proteins were assigned for AQUA

synthesis. These peptide markers were detected and identified in our previously reported studies (Montowska & Fornal, 2017, 2018). In this work, we describe the application of the AQUA strategy for the simultaneous absolute quantification of meat and allergenic protein additives in meat products.

2. Materials and methods

2.1. Materials

Acetonitrile (LC-MS grade) was purchased from Merck KGaA (Darmstadt, Germany). Formic acid (MS grade) was obtained from Sigma-Aldrich (Schnelldorf, Germany). Sequence grade, modified trypsin gold, lyophilised, was bought from Promega GmbH (Mannheim, Germany). Reverse-phase (RP) extraction Sep-Pak C18 Plus cartridges, sorbent weight 360 mg/0.7 mL, were obtained from Waters (Milford, MA). All other chemicals were purchased from Sigma-Aldrich in the best available purity grade. Samples of the pectoralis muscle (chicken, turkey, duck, goose) or longissimus muscle (pork), and their mixtures (samples S.1–S.5), as well as poultry products (various sausages, frankfurters and pâtés, samples S.6–S.16), were purchased locally. Some of them contained soy, milk or egg proteins, according to the declared ingredients. Chicken-pork-turkey meat mixtures were prepared by weighting respective amounts of meat, to obtain samples spiked with 1% of the third species. The meat and additives composition of the examined samples is given in Table 1. All samples were collected in triplicate, stored at -80°C and further prepared for peptide analysis following the procedure described previously (Montowska & Fornal, 2017). Peptide markers derived from meat proteins and protein additives were examined in the present study, i.e., chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), goose (*Anser anser*), pig (*Sus scrofa*), cattle (*Bos taurus*), soy (*Glycine max*), cow (*Bos taurus*) milk and egg (*Gallus gallus*) white.

Heavy peptides were synthesised and validated by JPT Peptide Technologies GmbH (Berlin, Germany). Stable isotopes were incorporated into C-terminal lysine and arginine ($^{13}\text{C}_6$, $^{15}\text{N}_2$, $^{13}\text{C}_6$ and $^{15}\text{N}_4$), generating a mass increase of 8 and 10 Da, respectively, for the singly charged ions. The peptides were purified using a C18 RP-high-performance liquid chromatography (HPLC) column, with detection at 220 nm, and their amounts were determined by amino acid analysis. Then, all synthesised stable isotope-labelled peptides were analysed by HPLC-MS, to verify their chromatographic characteristics and spectra pattern of precursor ions.

2.2. Sample preparation and trypsin digestion

Sample preparation and in-solution trypsin digestion were performed as described previously (Montowska & Fornal, 2017). In brief, samples were rinsed with ethanol/water (70:30), ethanol, methanol/water (90:10) and milli-Q water consecutively. Next, they were homogenised in 100 mM aqueous ammonium bicarbonate and vacuum-dried. Pre-treated samples were stored at -80°C until further analyses. Dried samples (10 mg) were rehydrated in 100 μL of 50 mM ammonium bicarbonate. The proteins were reduced by 200 mM dithiothreitol and then alkylated using 200 mM iodoacetamide. The samples were digested in an ammonium bicarbonate solution containing 0.083 $\mu\text{g}/\mu\text{L}$ trypsin at 37°C for 18 h, in a thermoblock. The digests were purified by RP extraction using Sep-Pak C18 Plus cartridges (Waters, Milford, MA). Eluted peptides were dried using a miVac Duo Concentrator (Genevac Ltd., Ipswich, UK). For chromatographic separation, samples were re-suspended in 2% acetonitrile in milli-Q water containing 0.1% formic acid (solvent A) and the heavy peptide mixtures were added to the samples as reference standards before analysis by ultra-high-performance liquid chromatography coupled to triple quadrupole tandem MS (UHPLC-QQQ-MS/MS).

2.3. UHPLC-electrospray ionisation (ESI)-QQQ-MS/MS analysis

MS analyses were performed using Agilent Technologies 1290 Series LC, composed of a binary pump (model G4220A), an autosampler (G4226A) and a column thermostat (G1316C), coupled to Agilent Technologies QQQ 6460 equipped with a JetStream Technology ESI source. Chromatographic separations were carried out on Zorbax Eclipse Plus C18 Rapid Resolution HD column (2.1×150 mm, $1.8 \mu\text{m}$; Agilent Technologies, Santa Clara, CA). Elution was performed using a 35-min gradient of 0.1% v/v formic acid in water (A) and 0.1% v/v formic acid in acetonitrile (B) under the following conditions: 0–1 min, linear gradient 5–10% B; 1–20 min, linear gradient to 35% B; 20–25 min, linear gradient to 40% B; 25–30 min, linear gradient to 93% B; 30–35 min 93% B, and then 7-min column conditioning at 5% B. The mobile phase flow, column temperature and injection volume were 0.4 mL/min, 45°C and 10 μL , respectively. The ion source parameters were set as follows: gas temperature 250°C , gas flow 8 L/min, nebuliser 30 psi, sheath gas heater 250°C , sheath gas flow 11 L/min, capillary 3500 V. Positive ions were acquired in dynamic MRM mode with triggering enabled. The delta EMV, cycle time and fragmentor voltage were set at 300, 1000 ms and 175 V, respectively. The collision energy was in the range of 15–35 V, and the threshold for triggered ions was set at 100–200, depending on the monitored ion. Instrument control, data acquisition and data analysis were performed using Agilent MassHunter

Workstation Software.

2.4. Method optimisation and quantification of the peptide markers

For all the examined samples, triggered MRM experiments were performed to collect and inspect the MS/MS spectra of the light (endogenous) and heavy, i.e., stable isotope-labelled peptides. Ion intensity and retention time were investigated. The resulting, most intense y-ion series were used for monitoring. Collision energy or threshold for triggered ions were adjusted. The aliquots of samples were spiked with 0.1, 1.0, 5.0, 10.0 and 100.0 ng of each stable isotope-labelled peptide, to ensure that the signal ratio of heavy to light peptide was in the range of 0.1–1.0, thereby securing accuracy of the measurement by obtaining linearity of the curve. The mixtures were prepared in the laboratory shortly before use. In total, four to six repeats of triggered MRM were acquired for each sample. The peak area was determined by Agilent MassHunter software. The peak areas of the transitions were summed before the heavy to light ratio of each peptide pair was calculated, based on the areas of respective y-ions. The total amounts of the endogenous peptides were calculated and expressed as nanograms of peptide per 1 mg of total protein extracted.

3. Results and discussion

3.1. Selection of AQUA peptides

In the authentication of processed food samples, it is of great interest to develop methodologies for quantitative measurements of main protein components and functional protein preparations, especially when they are food-borne allergens. The first step in this study was to select peptide markers for AQUA synthesis with isotopic labelling. The selection was made from a set of specific peptides that were detected and identified in our previous studies, where peptide markers unique to meat species (Montowska & Fornal, 2017) and soy, milk and egg white proteins (Montowska & Fornal, 2018), were identified in shotgun experiments using liquid extraction surface analysis-MS/MS and LC-Q-time-of-flight-MS/MS methods. All the chosen peptides were stable during product manufacturing, as well as during proteolytic digestion and MS analysis. The selected peptides are also specific to the examined species and proteins. The specificity of the peptide was confirmed using the protein basic alignment research tool (BLAST, USA National Library of Medicine), for species and protein specificity, thereby avoiding overlap with other proteins and species of common bacteria and eukaryotes. In this study, the final peptide selection was based upon results acquired during MRM experiments by the triggered LC-QQQ method. First, all the light peptides were evaluated in all samples,

Table 2
Monitored light- and heavy-labelled peptide pairs.

Species	Protein	Sequence	RT (min)	Light m/z (charge state)	Heavy m/z	Monitored transitions
Chicken	Pyruvate kinase	EPADAMAAGAVEASF	9.2	782.9 (2+)	787.1	y9, y4, y3
Chicken	Myosin-binding protein C	LDVPISGEPAPTWTWK	11.4	855.5 (2+)	859.4	y11, y8, y6
Pig	Myosin-4	TLAFLFAER	13.1	534.3 (2+)	539.3	y7, y6, y5
Pig	β -Enolase	NYPVVSIEDPFDQDDWK	14.3	1034.0 (2+)	1038.4	y12, y15, y8
Cattle	Myosin-2	MEIDDLASNVETISK	10.9	832.9 (2+)	837.0	y9, y8, y10
Cattle	Myosin-1	TLALLFSGPASGEAEGGPK	12.3	901.4 (2+)	905.4	y12, y13, y14
Goose	Haemoglobin β	LLGDILIVLAHFAK	22.2	569.7 (3+)	572.8	y14, y15, y10
Goose	Haemoglobin β	FFSSFGNLSPTAILGNPMVR	16.5	748.1 (3+)	751.7	y6, y7, 11
Duck	α -Enolase	NYPVVSIEDPFDQDDWGAWK	16.3	1191.0 (2+)	1195.4	y12, y11, y7
Duck	Haemoglobin α -a	FMCAVGAVLTAK	10.0	634.3 (2+)	638.5	y10, y7, y8
Soy	Glycinin G1	VLIVPQNFVVAAR	12.8	713.4 (2+)	718.5	y10, y9, y7
Soy	Glycinin G4	ISTLNSLTLPALR	12.9	699.9 (2+)	705.0	y4, y6, y10
Milk/cow	α -s1-casein	YLGYLEQLLR	14.9	634.3 (2+)	639.5	y8, y6, y4
Milk/cow	α -s1-casein	FFVAPFPEVFGK	16.5	692.8 (2+)	697.0	y6, y10, y4
Egg/chicken	Ovotransferrin	FFSASCVPAGATIEQK	8.6	821.4 (2+)	825.4	y8, y9, y5
Egg/chicken	Ovotransferrin	GAIEWEGIESGSVEQAVAK	10.6	980.4 (2+)	984.4	y11, y13, y9
Egg/chicken	lysozyme C	NTDGSTDYGILQINSR	8.7	877.4 (2+)	882.7	y8, y6, y12

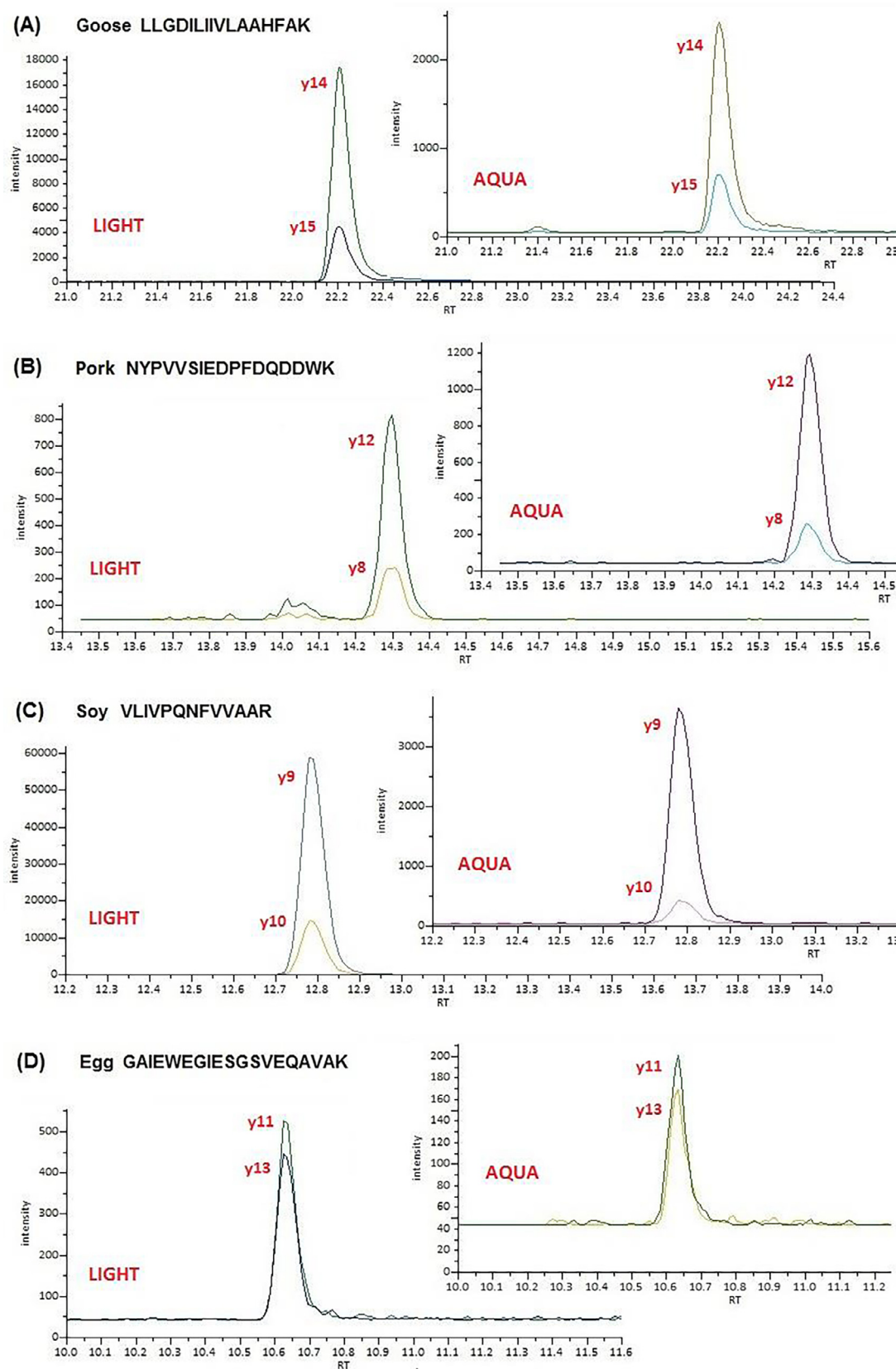


Fig. 1. Chromatograms from two most intense transitions of 1 ng of each heavy AQUA peptide and their light counterparts from roasted pâté (sample S.13). Comparisons of the peptide abundance show that the measured peak area/ion intensity was approximately 10- and 20-fold higher for goose (A) and soy (C) light peptides, respectively, while a similar order of magnitude, compared to the standard, was observed for light pork (B) and egg white peptides (D).

followed by their heavy counterparts in standard solutions and meat samples, to verify their chromatographic behaviour, ionisation and fragmentation pattern. As a result of the evaluation, 17 peptide markers

were selected for chemical synthesis and further used for protein quantification. Table 2 summarises the details of the chosen peptides. Each species and protein additive was represented by at least two

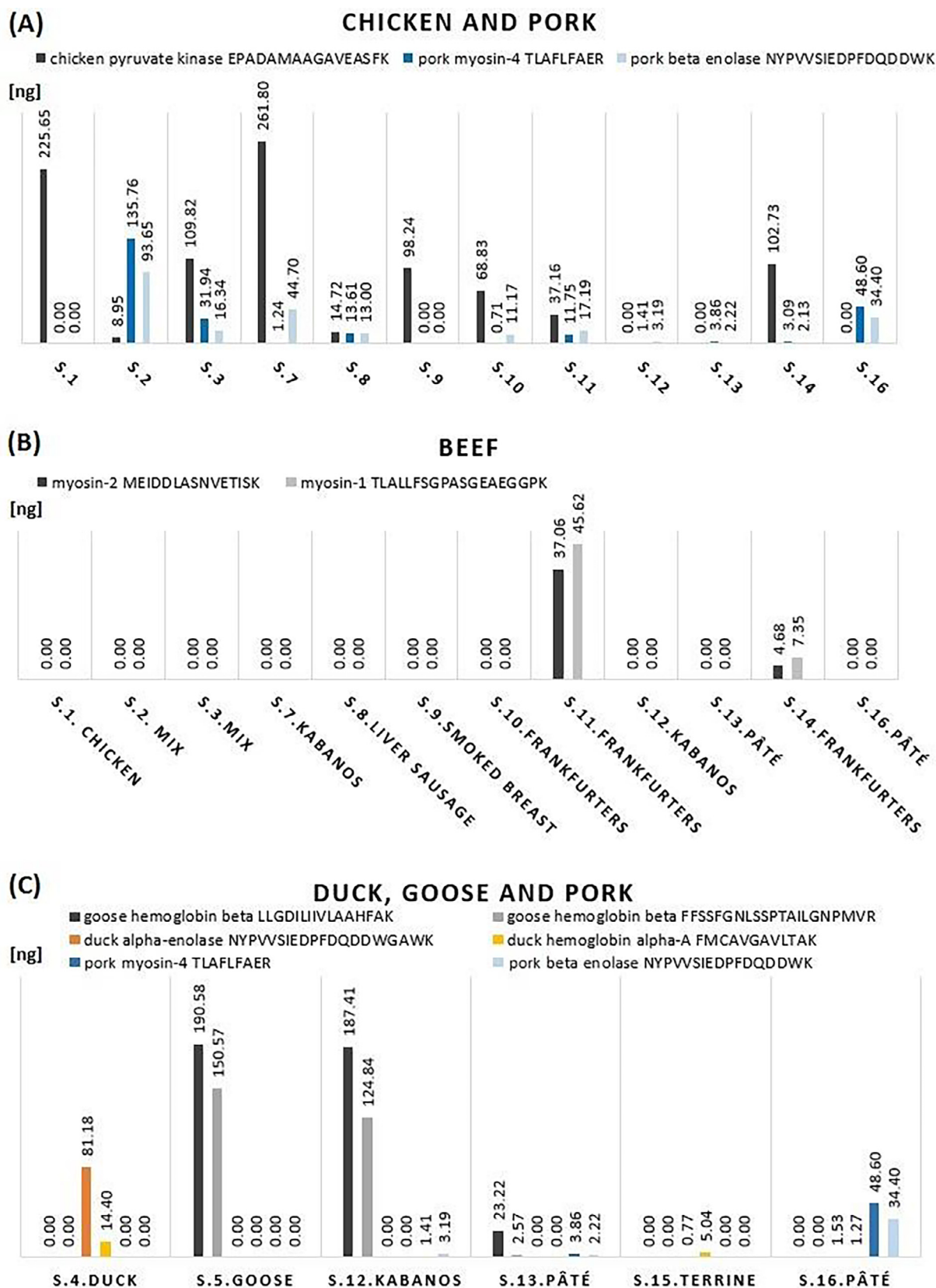


Fig. 2. Comparison of abundances of meat protein peptide markers per sample; (A) chicken and pork proteins; (B) beef; (C) duck, goose and pork proteins. The results are expressed as nanograms of peptide per 1 mg of total protein extracted.

different peptides.

3.2. Quantification of meat proteins

In the present study, commercial meat products of unknown composition were investigated. Single meats (chicken, duck and goose) and three-component mixtures, containing chicken meat and pork at 1% (w/w) were used for comparisons, as the reference samples. For quantification purposes, the samples were spiked with 0.1, 1.0, 5.0, 10.0 and 100.0 ng of each AQUA peptide, and the concentration at which the signal ratio of heavy to light peptide was in the range of 0.1–1.0 was selected for further quantification of meat and protein additives in meat samples, to ensure accuracy of the measurement. The results were expressed as nanograms of peptide per 1 mg of total protein extracted from the sample. Fig. 1 shows the representative ion chromatograms for four light and heavy peptide pairs acquired from roasted pâté (sample S.13), and Fig. 2 presents the quantitative analysis and comparison of abundances of meat protein peptide markers. All results of the absolute quantification of meat and protein additives peptide markers per sample are presented in the Supplementary Material (Table S1).

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.08.131>.

Fig. 2A demonstrates the changes in the amount of selected chicken and pork peptide markers per sample. In all samples containing chicken meat, a higher abundance of pyruvate kinase EPADAMAAGAVEASFK peptide marker was observed compared with protein C LDVPISGEPA-PTVTWK peptide. The smallest quantities of pyruvate kinase peptide were detected in the chicken-turkey-pork mixture containing 1% (w/w) of chicken meat, and liver sausage (samples S.2 and S.8, with values of 8.95 and 14.72 ng/mg, respectively). For samples that contained a high content of chicken meat, i.e., containing only chicken meat (sample S.1), and high-quality kabanos sausage (sample S.7), the highest amounts of chicken pyruvate kinase were found, with values of 225.65 and 261.8 ng/mg, respectively, whereas, about half of that amount was observed in a mixture containing 50% of chicken meat (sample S.3). Lower amounts of pork myosin-4 TLAFLEAER and pork β -enolase NYPVVSIEDPFDQDDWK is a reliable result considering that pork was not the dominant species in the examined samples. The pork myosin-4 values ranged from 0.71 ng/mg for homogenised frankfurters (sample S.10) to 135.76 ng/mg for chicken–turkey–pork mixture containing 49.5% (w/w) pork (sample S.2). The observed differences in the amounts of individual markers between samples are likely the result of both differences in the composition of raw materials and the different content of individual proteins in tissues.

For products made from chicken and pork but with unknown proportions of these meat components, such as kabanos sausage (sample S.7) and liver sausage (sample S.8), the results showed that sample S.7 contained more chicken meat and much less pork, but equal values for these species were observed for sample S.8, albeit in a significantly smaller amount (Fig. 2A). These results are consistent, given the liver sausage and other pâté-type products contained less meat and more fat and non-meat protein additives, among the samples examined. In goose kabanos-type sausage (sample S.12) and chicken and veal sausage (sample S.14), small amounts of undeclared pork proteins were detected (with values from 1.41 ng/mg to 3.09 ng for the pork myosin peptide marker). Considering that duck and veal are some of the more expensive meats on the European meat market, this can be an example of deliberate adulteration with cheaper meat. In mass production, even a small change in the composition may lead to a large economic profit.

Beef peptide markers were detected in two products, in which the presence of beef or veal was declared (samples S.11 and S.14). Chicken and veal frankfurters (sample S.14) was the product with the lowest declared meat content (4%, w/w veal), which means the real veal protein content of this product was only 0.8%, due to a rough 20% (w/w) protein in the muscle tissue. At that concentration, cattle myosin-1 and myosin-2 peptide marker abundances were quantified as 7.35 and

4.68 ng/mg, respectively (Fig. 2B). These values were lower compared to the signal obtained from the sample S.11 (containing 16.2% beef, and the estimated beef protein content 3.24%). However, regarding the illegal addition of pork detected in this product (Fig. 2A), it is likely that the computed veal peptide markers abundances were correct and the content of veal was below the declared value in the sample S.11. A previously reported label-free MS quantification method that was developed to authenticate processed meat products was able to quantify up to 2% pork in the beef matrix of Bolognese sauce, using the peak area of $\alpha 2$ collagen chain peptide marker as the quantifier (Prandi et al., 2017).

Fig. 2C compares the abundances of duck, goose and pork peptide markers. The highest amounts of goose haemoglobin β LLGDILIVLA-AHFAK and FFSSFGNLSPTAILGNPMVR peptide markers were calculated in the sample made exclusively from goose meat (sample S.5, with values of 190.58 and 150.57 ng/mg, respectively). For goose kabanos (sample S.12), which turned out to be made with an addition of pork, correspondingly slightly lower values were obtained. However, goose pâté (sample S.13), which was supposed to be manufactured from 70% goose meat, contained only 23.22 ng/mg goose haemoglobin β LLGDILIVLA-AHFAK and this is much less than it should be if comparing with a pure meat sample. Instead of meat proteins, high abundances of soy and egg white proteins were observed in this product (see Section 3.3).

In the duck meat, a surprisingly low duck peptide content was observed in pure meat (sample S.4), as well as in two pâté-type products (samples S.15 and S.16). Considering that no other meat and protein additive peptide markers were detected in duck terrine (sample S.15), which is consistent with the label, a relatively low quantity of the duck meat appeared to be the result of poor chromatographic or ionisation behaviour during the MS experiment. Low signals were acquired, especially for light duck α -enolase NYPVVSIEDPFDQDDWGAWK peptide marker. Thus, these two duck peptides turned out to be more suitable for use as qualitative rather than quantitative peptide markers.

Despite the above fault and the noticed cases of adulteration, the association between the abundances of meat proteins of the examined species across the samples was correct. In general, the present results confirm those performed previously using a label-free spectral counting strategy, where, for instance, due to the effect of shared peptides, duck proteins were mistaken for goose (Montowska & Fornal, 2017). Detection and quantification of chicken meat and pork at levels of 1% (w/w) were performed in mixtures of three species. This limit of detection enables discrimination between accidental contamination and intentional adulteration (Claydon, Grundy, Charlton, & Romero, 2015). Turkey meat present in some samples was not quantified within this work because suitable turkey-specific peptide markers were not identified. The main reason was likely the lack of many turkey proteins in the protein sequence databases. The high specificity of the meat authentication approach based on unique peptide markers is chicken meat markers detected in samples of pâtés containing chicken liver (samples S.13 and S.16) when using the highly sensitive UHPLC-QQQ-MS/MS technique. Our selected peptides are only suitable to detect muscle and not offal. Myosin-binding protein C is a fast skeletal muscle isoform that occurs in myofibrils and pyruvate kinase isozymes derived from liver or muscle differ in the primary structure. In our study, the identified sequences come from the muscle isozyme.

3.3. Quantification of protein additives

The presence of functional protein preparations was declared on the label, namely, soy, milk or egg origin, for eight commercially processed products examined in the study. These preparations are commonly used in the meat industry, but manufacturers are not obliged to indicate their quantity or protein composition, more specifically whether they contain caseinates or whey proteins, or yolk or egg white. Seven peptide markers were selected for quantification of these three protein additives (Table 2). The peptides belong to the major food allergens, such as soy

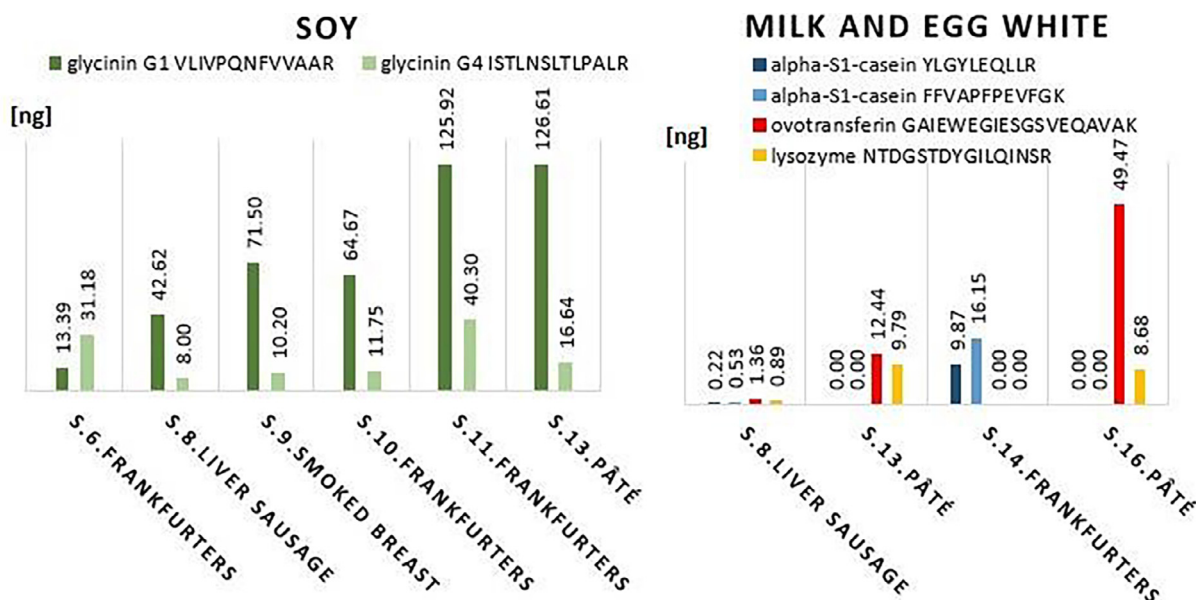


Fig. 3. Absolute quantification data of protein additive peptide markers per sample. The results are expressed as nanograms of peptide per 1 mg of total protein extracted.

glycinin (Gly m 6), milk casein α -S1 (Bos d 8), and egg white ovotransferrin (Gal d 3) and lysozyme C (Gal d 4), which are present in high abundances in these three preparations. Fig. 3 illustrates the absolute quantification of the allergenic peptide markers.

In comparison to meat proteins quantified in processed products (Fig. 2), a relatively high soy protein content was obtained, with a general predominance of glycinin G1 over G4 isoform (for sample S.13, with values of 126.61 and 16.64 ng/mg, respectively). As an example, approximately a 20-fold higher signal of glycinin G1 VLIVPQNFFVVAAR light peptide marker was seen, compared to the heavy standard, in the pâté sample (Fig. 1C). Considering milk protein addition, two α -S1-casein peptides, YLGYLEQLLR and FFVAPFPEVFGK, were detected in two commercially processed products but their content varied from low amounts (0.22 and 0.53 ng/mg, respectively) in liver sausage (sample S.8) to much higher values (9.87 and 16.15 ng/mg) in homogenised frankfurters (sample S.14). Among peptides originating from egg white proteins, ovotransferrin and lysozyme marker peptides were quantified within the present study, and similarly to soy and milk additives, the content of ovotransferrin peptide GAIEWEGIESGSVEQAVAK differed significantly depending on the product (12.44 *versus* 49.47 ng/mg in pâté samples S.13 *versus* S.16), whereas the amount of lysozyme remained at a similar level (Fig. 3). These differences could be due to differences in the composition of egg white preparations used by different manufacturers.

The reason for the observed differences between the examined meat products may also be the application of various thermal technological processes during manufacturing (e.g., smoking, cooking, roasting or sterilisation), which are responsible for partial protein degradation. Although all the peptide markers used for quantification were heat-stable and therefore survived industrial processing, part of them could have been affected by the heating conditions. A detrimental effect of heating on bovine whey proteins was reported by Felfoul, Jardin, Gaucheron, Attia, and Ayadi (2017) but also other results were published where lactoglobulins and caseins were resistant to high temperatures applied during processing of cookies, cakes as well as cheese processing (Bernardi et al., 2015; Ji et al., 2017; Monaci, Pilolli, De Angelis, Godula, & Visconti, 2014). Recently, Li et al. (2018) observed that the thermal stability of several meat and plant peptides (e.g., pork and peanut) was not related to the temperature and heating method, because the intensity of some peptides was higher at 100 or 121 °C than at 78 °C. Thus, the impact of heating on the intensity of peptide ions and

how it affects the quantitative analysis of peptides and proteins using MS methods need to be further investigated. Nevertheless, the stable isotope-labelled peptide strategy is a useful and sensitive approach for quantification of various meat and non-meat proteins in meat products, which enabled, for example, a precise quantification of chicken pyruvate kinase and protein C peptides, and pork myosin-4 and β -enolase peptides at 1% (w/w), in the present study. In comparison, the veal/beef myosin-1 and myosin-2 were quantitated at an even lower level of 0.8% (w/w) in homogenised sausages. When using two isotope-labelled β -lactoglobulin peptides for quantification of cow and goat milk in raw material of dairy products, the limit of detection of cow milk was 1% in cow and goat milk mixture (Chen et al., 2016).

A previously introduced label-free LC-MS/MS screening method allowed detection of 6 mg/kg legumes (lupin, pea and soy proteins) in pork emulsion-type sausages (Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017). To date, the main aim of MS-based studies conducted in food matrices was to establish the limit of detection (LOD) for given allergenic proteins. Monaci et al. (2014) obtained LODs of 0.12, 0.3 and 2 μ g/g for milk, egg and soy powder content, respectively, in allergen-spiked cookie samples. For red wines treated with commercial egg white preparations in the process of wine fining, the developed tandem LC-MS method allowed for the detection of 5 g/hL of egg white (Tolin et al., 2012). Using a label-free quantification method with internal standard peptides, made by replacing amino acids in α -lactalbumin, β -lactoglobulin and α -S1-casein marker peptides designed for the MRM experiment, the α -lactalbumin and β -lactoglobulin contents (2.54 and 48.46 μ g/g, respectively) were measured in commercial chocolate chip cookies, and 247.92 μ g/g α -S1-casein was obtained for milk powder (Ji et al., 2017).

In comparison to other MS-based quantification methods, stable isotope-labelled quantification, though more expensive, allows highly accurate, selective and reliable measurements. Using this approach, we were able to confirm illegal addition/replacement of ingredients in the examined commercial meat products (see the Supplementary Material Table S1). For liver sausage (sample S.8), comparable quantities of chicken and pork proteins were observed. However, apart from declared soy and milk proteins, this product was fortified with undeclared egg proteins, including 1.36 ng/mg ovotransferrin and 0.89 ng/mg lysozyme (Fig. 3 and Table S1). Considerably higher soy and egg protein content than goose protein, as well as a higher content of pork and egg proteins compared to duck meat, was detected in both goose and duck

pâtés (samples S.13 and S.16). Additionally, we were able to detect non-declared pork myosin-4 and β -enolase peptides in goose kabanos (sample S.12), but at relatively low concentrations of 1.41 and 3.19 ng/mg, respectively (Fig. 2, Table S1). Also, illegal substitution of veal with pork was discovered in chicken and veal frankfurters (sample S.14). In summary, the stable isotope-labelled quantification approach can be used as a final, confirmatory step of protein quantification for authentication purposes, which may be preceded as needed, by less expensive label-free relative approaches.

4. Conclusions

The application of the absolute quantification (AQUA) method using stable isotope-labelled peptide markers is presented in this article. The advantages of the approach are high sensitivity and accuracy across many samples, which is necessary when the analysis is aimed at fraud detection. The abundance of peptide markers specific to meat proteins (chicken, pork, beef, duck and goose) and non-meat allergenic protein additives (soy, milk and egg white proteins) was quantified in various commercial meat products. Apart from considerable differences in meat and non-meat originated proteins across the examined samples, evident adulteration was detected in commercial samples. Portions of goose, veal and duck were replaced with cheaper pork in poultry sausages and pâtés, the illegal addition of egg white proteins was observed in liver sausage, and considerably higher soy and egg protein contents than goose and duck meat were detected in goose and duck pâtés. Thus, this accurate and sensitive, stable isotope-labelled technique, which is conducted at the peptide level via unique peptides, enables the identification and quantification of selected peptides and proteins that might otherwise be undetected when using relative label-free approaches with shotgun proteomic analysis.

5. Conflict of interest

The authors declare that they have no financial or commercial conflict of interest.

Acknowledgements

This work was supported by the National Science Centre, Poland, based on decision number DEC-2013/11/D/NZ9/02632.

References

- Abbas, O., Zdravcov, M., Baeten, V., Mikuš, T., Lešić, T., Vulić, A., ... Pleadin, J. (2018). Analytical methods used for the authentication of food of animal origin. *Food Chemistry*, 246, 6–17. <https://doi.org/10.1016/j.foodchem.2017.11.007>.
- Al Feteisi, H., Achour, B., Barber, B., & Rostami-Hodjegan, A. (2015). Choice of LC-MS methods for the absolute quantification of drug-metabolizing enzymes and transporters in human tissue: A comparative cost analysis. *The AAPS Journal*, 17(2), 438–446. <https://doi.org/10.1208/s12248-014-9712-6>.
- Bernardi, N., Benetti, G., Haouet, N. M., Sergi, M., Grotta, L., Marchetti, S., ... Martino, G. (2015). A rapid high-performance liquid chromatography-tandem mass spectrometry assay for unambiguous detection of different milk species employed in cheese manufacturing. *Journal of Dairy Science*, 98(12), 8405–8413. <https://doi.org/10.3168/jds.2015-9769>.
- Beynon, R. J., Doherty, M. K., Pratt, J. M., & Gaskell, S. J. (2005). Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. *Nature Methods*, 2, 587–589. <https://doi.org/10.1038/nmeth774>.
- Brun, V., Dupuis, A., Adrait, A., Marcellin, M., Thomas, D., Court, M., ... Garin, J. (2007). Isotope-labeled protein standards: Toward absolute quantitative proteomics. *Molecular & Cellular Proteomics*, 6(12), 2139–2149. <https://doi.org/10.1074/mcp.M700163-MCP200>.
- Chen, Q., Ke, X., Zhang, J. S., Lai, S. Y., Fang, F., Mo, W. M., & Ren, Y. P. (2016). Proteomics method to quantify the percentage of cow, goat, and sheep milks in raw materials for dairy products. *Journal of Dairy Science*, 99(12), 9483–9492. <https://doi.org/10.3168/jds.2015-10739>.
- Claydon, A. J., Grundy, H. H., Charlton, A. J., & Romero, M. R. (2015). Identification of novel peptides for horse meat speciation in highly processed foodstuffs. *Food Additives & Contaminants Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment*, 32, 1718–1729. <https://doi.org/10.1080/19440049.2015.1075256>.
- El Sheikh, A. F., Mokhtar, N. F. K., Amie, C., Lamasudin, D. U., Isa, N. M., & Mustafa, S. (2017). Authentication technologies using DNA-based approaches for meats and halal meats determination. *Food Biotechnology*, 31(4), 281–315. <https://doi.org/10.1080/08905436.2017.1369886>.
- Felfoul, I., Jardin, J., Gaucheron, F., Attia, H., & Ayadi, M. A. (2017). Proteomic profiling of camel and cow milk proteins under heat treatment. *Food Chemistry*, 216, 161–169. <https://doi.org/10.1016/j.foodchem.2016.08.007>.
- Gallego, M., Mora, L., Concepción Aristoy, M., & Toldrá, F. (2016). The use of label-free mass spectrometry for relative quantification of sarcoplasmic proteins during the processing of dry-cured ham. *Food Chemistry*, 196, 437–444. <https://doi.org/10.1016/j.foodchem.2015.09.062>.
- Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., & Gygi, S. P. (2003). Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *PNAS*, 100(12), 6940–6945. <https://doi.org/10.1073/pnas.0832254100>.
- Hoffmann, B., Münch, S., Schwägele, F., Neusüß, C., & Jira, W. (2017). A sensitive HPLC-MS/MS screening method for the simultaneous detection of lupine, pea, and soy proteins in meat products. *Food Control*, 71, 200–209. <https://doi.org/10.1016/j.foodcont.2016.06.021>.
- Iammarino, M., Marino, R., & Albenzio, M. (2017). How meaty? Detection and quantification of adulterants, foreign proteins and food additives in meat products. *International Journal of Food Science and Technology*, 52(4), 851–863. <https://doi.org/10.1111/ijfs.13350>.
- Ji, J., Zhu, P., Pi, F., Sun, C., Sun, J., Jia, M., ... Sun, X. (2017). Development of a liquid chromatography-tandem mass spectrometry method for simultaneous detection of the main milk allergens. *Food Control*, 74, 79–88. <https://doi.org/10.1016/j.foodcont.2016.11.030>.
- Kettenbach, A. N., Rush, J., & Gerber, S. A. (2011). Absolute quantification of protein and post-translational modification abundance with stable isotope-labeled synthetic peptides. *Nature Protocols*, 6(2), 175–186. <https://doi.org/10.1038/nprot.2010.196>.
- Kirkpatrick, D. S., Gerber, S. A., & Gygi, S. P. (2005). The absolute quantification strategy: A general procedure for the quantification of proteins and post-translational modifications. *Methods*, 35, 265–273. <https://doi.org/10.1016/j.ymeth.2004.08.018>.
- Li, Y., Zhang, Y., Li, H., Zhao, W., Guo, W., & Wang, S. (2018). Simultaneous determination of heat stable peptides for eight animal and plant species in meat products using UPLC-MS/MS method. *Food Chemistry*, 245, 125–131. <https://doi.org/10.1016/j.foodchem.2017.09.066>.
- Mallick, P., & Kuster, B. (2010). Proteomics: A pragmatic perspective. *Nature Biotechnology*, 28(7), 695–709. <https://doi.org/10.1038/nbt.1658>.
- Monaci, L., Pilolli, R., De Angelis, E., Godula, M., & Visconti, A. (2014). Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. *Journal of Chromatography A*, 1358, 136–144. <https://doi.org/10.1016/j.chroma.2014.06.092>.
- Monaci, L., De Angelis, E., Montemurro, N., & Pilolli, R. (2018). Comprehensive overview and recent advances in proteomics MS based methods for food allergens analysis. *TrAC Trends in Analytical Chemistry*, 106, 21–36. <https://doi.org/10.1016/j.trac.2018.06.016>.
- Montowska, M., & Fornal, E. (2018). Detection of peptide markers of soy, milk and egg white allergenic proteins in poultry products by LC-Q-TOF-MS/MS. *LWT – Food Science and Technology*, 87, 310–317. <https://doi.org/10.1016/j.lwt.2017.08.091>.
- Montowska, M., & Fornal, E. (2017). Label-free quantification of meat proteins for evaluation of species composition of processed meat products. *Food Chemistry*, 237, 1092–1100. <https://doi.org/10.1016/j.foodchem.2017.06.059>.
- Neilson, K. A., Ali, N. A., Muralidharan, S., Mirzaei, M., Mariani, M., Assadourian, G., ... Haynes, P. A. (2011). Less label, more free: Approaches in label-free quantitative mass spectrometry. *Proteomics*, 11(4), 535–553. <https://doi.org/10.1002/pmic.201000553>.
- Prandi, B., Lambertini, F., Faccini, A., Suman, M., Leporati, A., Tedeschi, T., & Sforza, S. (2017). Mass spectrometry quantification of beef and pork meat in highly processed food: Application on Bolognese sauce. *Food Control*, 74, 61–69. <https://doi.org/10.1016/j.foodcont.2016.11.032>.
- Rodríguez-Suárez, E., & Whetton, A. D. (2013). The application of quantification techniques in proteomics for biomedical research. *Mass Spectrometry Reviews*, 32(1), 1–26. <https://doi.org/10.1002/mas.21347>.
- Sandin, M., Telesman, J., Malmström, J., & Levander, F. (2014). Data processing methods and quality control strategies for label-free LC-MS protein quantification. *Biochimica et Biophysica Acta*, 1844(1), 29–41. <https://doi.org/10.1016/j.bbapap.2013.03.026>.
- Tolin, S., Pasini, G., Curioni, A., Arrigoni, G., Masi, A., Mainente, F., & Simonato, B. (2012). Mass spectrometry detection of egg proteins in red wines treated with egg white. *Food Control*, 23, 87–94. <https://doi.org/10.1016/j.foodcont.2011.06.016>.

Supplementary Material

Absolute quantification of targeted meat and allergenic protein additive peptide markers in meat products

Magdalena Montowska ^{a, *}, Emilia Fornal ^b

^a *Department of Meat Technology, Poznan University of Life Sciences, Wojska Polskiego 31, Poznan 60-624, Poland*

^b *Department of Pathophysiology, Medical University of Lublin, Jaczewskiego 8b, Lublin 20-090, Poland*

* Corresponding author.

E-mail address: magdalena.montowska@gmail.com, magdalena.montowska@up.poznan.pl

(M. Montowska).

Table S1. Absolute quantification data of meat and protein additive peptide markers per sample. The results are expressed as nanograms of peptide per 1 mg of total protein extracted. Undeclared proteins are highlighted.

Species	Protein	Sequence	S.1 Chicken	S.2 Mix	S.3 Mix	S.4 Duck	S.5 Goose	S.6 Frankfurt	S.7 Kabanos	S.8 Liver sausage
Chicken	Pyruvate kinase	EPADAMAAGAVEASFK	225.65	8.95	109.82	n.d.	n.d.	n.d.	261.80	14.72
Chicken	Protein C	LDVPISGEPAPTVTWK	29.35	1.57	17.09	n.d.	n.d.	n.d.	43.15	2.59
Pig	Myosin-4	TLAFLFAER	n.d.	135.76	31.94	n.d.	n.d.	n.d.	1.24	13.61
Pig	β -enolase	NYPVVSIEDPFDQDDWK	n.d.	93.65	16.34	n.d.	n.d.	n.d.	44.70	13.00
Cattle	Myosin-2	MEIDDLASNVETISK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cattle	Myosin-1	TLALLFSGPASGEAEGGPK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Goose	Hemoglobin β	LLGDILIIVLAHFAK	n.d.	n.d.	n.d.	n.d.	190.58	n.d.	n.d.	n.d.
Goose	Hemoglobin β	FFSSFGNLSSPTAILGNPMVR	n.d.	n.d.	n.d.	n.d.	150.57	n.d.	n.d.	n.d.
Duck	α -enolase	NYPVVSIEDPFDQDDWGAWK	n.d.	n.d.	n.d.	81.18	n.d.	n.d.	n.d.	n.d.
Duck	Hemoglobin α -A	FMCAVGAVLTAK	n.d.	n.d.	n.d.	14.40	n.d.	n.d.	n.d.	n.d.
Soy	Glycinin G1	VLIVPQNFVVAAR	n.d.	n.d.	n.d.	n.d.	n.d.	13.39	n.d.	42.62
Soy	Glycinin G4	ISTLNSLTLPALR	n.d.	n.d.	n.d.	n.d.	n.d.	31.18	n.d.	8.00
Milk/ cow	α -S1-casein	YLGYLEQLLR	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.22
Milk/ cow	α -S1-casein	FFVAPFPEVFGK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.53
Egg/ chicken	Ovotransferrin	FFSASCVPGATIEQK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.03
Egg/ chicken	Ovotransferrin	GAIEWEGIESGSVEQAVAK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.36
Egg/ chicken	Lysozyme	NTDGSTDYGILQINSR	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.89

n.d. – not detected

Species	Protein	Sequence	S.9 Smoked breast	S.10 Frankfurt	S.11 Frankfurt	S.12 Kabanos	S.13 Pâté	S.14 Frankfurt	S.15 Terrine	S.16 Pâté
Chicken	Pyruvate kinase	EPADAMAAGAVEASFK	98.24	68.83	37.16	n.d.	n.d.	102.73	n.d.	n.d.
Chicken	Protein C	LDVPISGEPAPTVTWK	18.30	16.58	6.10	n.d.	n.d.	20.82	n.d.	n.d.
Pig	Myosin-4	TLAFLFAER	n.d.	0.71	11.75	1.41	3.86	3.09	n.d.	48.60
Pig	β -enolase	NYPVVSIEDPFDQDDWK	n.d.	11.17	17.19	3.19	2.22	2.13	n.d.	34.40
Cattle	Myosin-2	MEIDDLASNVETISK	n.d.	n.d.	37.06	n.d.	n.d.	4.68	n.d.	n.d.
Cattle	Myosin-1	TLALLFSGPASGEAEGGPK	n.d.	n.d.	45.62	n.d.	n.d.	7.35	n.d.	n.d.
Goose	Hemoglobin β	LLGDILIIVLA AHFAK	n.d.	n.d.	n.d.	187.41	23.22	n.d.	n.d.	n.d.
Goose	Hemoglobin β	FFSFGNLSPTAILGNPMVR	n.d.	n.d.	n.d.	124.84	2.57	n.d.	n.d.	n.d.
Duck	α -enolase	NYPVVSIEDPFDQDDWGAWK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.77	1.53
Duck	Hemoglobin α -A	FM-Camc-AVGAVLTAK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.04	1.27
Soy	Glycinin G1	VLIVPQN FVVAAR	71.50	64.67	125.92	n.d.	126.61	n.d.	n.d.	n.d.
Soy	Glycinin G4	ISTLNSLTLPALR	10.20	11.75	40.30	n.d.	16.64	n.d.	n.d.	n.d.
Milk/ cow	α -S1-casein	YLGYLEQLLR	n.d.	n.d.	n.d.	n.d.	n.d.	9.87	n.d.	n.d.
Milk/ cow	α -S1-casein	FFVAPFPEVFGK	n.d.	n.d.	n.d.	n.d.	n.d.	16.15	n.d.	n.d.
Egg/ chicken	Ovotransferrin	FFSAS-Camc-VPGATIEQK	n.d.	n.d.	n.d.	n.d.	6.59	n.d.	n.d.	16.35
Egg/ chicken	Ovotransferrin	GAIEWEGIESGSVEQAVAK	n.d.	n.d.	n.d.	n.d.	12.44	n.d.	n.d.	49.47
Egg/ chicken	Lysozyme	NTDGSTDY GILQINSR	n.d.	n.d.	n.d.	n.d.	9.79	n.d.	n.d.	8.68

n.d. – not detected