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**Detection of peptide markers of soy, milk and egg white allergenic proteins
in poultry products by LC-Q-TOF-MS/MS**

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ABSTRACT

Soy, milk and egg white proteins are commonly added to meat products. Proteins of non-meat origin, are applied to improve the water-binding capacity of meat and the texture of the product, as well as for economic reasons as meat substitutes. Detection of low amounts of these proteins is relevant to food safety and human health due to their high allergenicity. In this study, we demonstrate that a nano-LC-Q-TOF-MS/MS method can be used to reliably detect non-meat proteins derived from functional protein additives in various commercial poultry meat products (sausages, frankfurters, pâtés). A large number of heat-stable peptide markers, unique to soy (43 peptides), milk (9 peptides) and egg white (12 peptides), were detected. The source of the peptides were main allergenic proteins, namely soy glycinin and β -conglycinin, milk α -S1-casein and the whey protein β -lactoglobulin, as well as egg white ovotransferrin and lysozyme C. These proteins appeared to be relatively resistant to technological operations used in meat processing including roasting and sterilisation. The mass spectrometry method presented, enables reliable monitoring of the presence of allergenic proteins in severely processed meat products, providing a valuable alternative to PCR and ELISA tests.

Keywords:

Food authenticity, non-meat protein, meat product, nano-LC-Q-TOF-MS/MS

1. Introduction

Modern meat products consist of an increasing number of ingredients and additives. Functional protein additives used in the meat industry, are mainly obtained from sources of vegetable or animal origin. Most vegetable proteins are derived from legumes and cereals, i.e. soy, bean, pea and wheat. Vegetable proteins have a higher water binding capacity and better gelling properties compared to meat proteins, hence, they are commonly added to meat products in order to stabilise the components of the meat emulsion, as well as improve the texture of the final products, through the formation of a strong gel. Sources of non-meat proteins of animal origin, can be milk, blood plasma, connective tissue, mechanically recovered meat and egg white. They mainly act as binders, fillers and stabilisers and have a positive effect on the formation of the texture of meat products due to their good emulsifying properties.

Non-meat proteins are characterised by a relatively high nutritional value and a lower energy content than fat. Thus, they can be used as substitutes for meat and fat, in processed meat products. Their addition reduces the cost of the formulation, while maintaining the high quality and cooking yield. However, less-expensive proteins of non-meat origin, can be fraudulently added to meat products, to substitute more expensive meat components. Furthermore, they may contain hidden allergens that pose a serious threat to the health of allergic consumers. Among soy, milk and egg white proteins, some of the major food allergens, are soy glycinin (Gly m 6) and β -conglycinin (Gly m 5), milk casein α -S1 (Bos d 8) and the whey protein β -lactoglobulin (β -lg; Bos d 5), and egg white ovotransferrin (Gal d 3) and lysozyme C (Gal d 4). Soy, milk and eggs are included in the European Union (EU) list of 14 food allergenic ingredients that require mandatory labelling (Regulation EU No 1169/2011).

Many methods and commercially developed kits are currently available to detect food adulteration, as well as the presence of soy, milk and egg allergens in foods (Monaci, Tregoeat, Hengel, & Anklam, 2006; Pedersen et al., 2008; Lee & Kim, 2010; Azad & Ahmed, 2016; Galan-Malo et al., 2017). Most of these methods are based on immunochemical detection of binding between specific antigens by antibodies. Enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic lateral flow devices, are most commonly used, due to their high sensitivity (ppm range), rapid result and ease of use. However, immunochemical methods have some limitations, particularly when analysing complex and processed food matrices. After thermal processing, proteins are denatured, their conformation is altered, solubility is decreased and some epitopes are destroyed. Consequently, food processing can negatively affect the performance of immunochemical methods, in the detection of processed proteins. The results may also be affected by cross-reactivity, processing method and food matrix (Leduc et al., 1999; Gomaa & Boye, 2015).

DNA-based methods, using polymerase chain reaction (PCR), are very specific and highly sensitive but these methods do not detect proteins and a negative result does not mean the absence of protein. Moreover, DNA is not tissue-specific and, therefore, PCR cannot distinguish, for instance, between egg and chicken meat (Lee & Kim, 2010), as well as milk and beef. Also, proper sample preparation and DNA extraction are crucial issues when using DNA techniques. Moreover, DNA is sensitive to thermal processing and, thus, the results may be affected by the processing conditions and size of the amplified DNA fragment (Şakalar, Abasiyanik, Bektik, & Tayyrov, 2012).

Modern instrumental mass spectrometry (MS)-based methods, developed for authentication purposes, analyse protein and peptide composition, through assigning of protein or species-specific peptide markers. This analytical approach delivers sufficient resolving power and a high degree of specificity and sensitivity. The key advantage of MS-

based methods is detection and identification of many proteins in a single run. Proteins and proteotypic peptides, those unique to a given protein, have been differentiated simultaneously from complex food matrices, when derived from the same species or tissue, even in highly processed products, such as meat pâté or Bolognese sauce (Montowska, Alexander, Tucker, & Barrett, 2015; Prandi et al., 2017). To date, only a few MS-based methods have been reported for the detection of soy proteins in heat-processed meat products. Several soy proteins were identified in heat-treated meat samples spiked with soy protein isolate (SPI), using a quadrupole ion trap instrument (Leitner, Castro-Rubio, Marina, & Lindner, 2006). Houston et al. (2011) compared protein expression profiles of 20 soy varieties of mature seeds and 10 soy allergens, using advanced peptidomic approaches. More recently, liquid chromatography-tandem MS (LC-MS/MS) methods were developed to detect legumes (lupine, pea and soy) in emulsion-type pork sausages, to differentiate cow, buffalo, sheep, and goat milk for milk marker identification in raw materials and cheese products (Bernardi et al., 2015), to detect milk allergens in cow milk and milk products (Ji et al., 2017), residual milk and egg white allergenic proteins in red and white wines (Tolin, Pasini, Simonato, Mainente, & Arrigoni, 2012), as well as soy, egg and milk allergens in cookies (Monaci, Pilolli, De Angelis, Godula, & Visconti, 2014). In our previous study, meat and casein peptides were detected simultaneously, in two commercial smoked and dried sausages, where a portion of meat was replaced with cheese (Montowska et al. 2015).

To our knowledge, no MS studies have been undertaken, to identify widely heat-stable peptide markers, unique to soy, milk and egg white proteins, in highly processed multi-ingredient meat products. Reliable, qualitative analysis, is suitable to detect the presence or absence of a given protein and, thereby, ensure the accuracy of labelling meat products, which is of utmost importance for consumer safety. The extent of food adulteration, i.e. fraudulent

replacement of a portion of an ingredient, can be further monitored using targeted quantitative MS-based methods.

In this study, we present a peptidomic analysis of meat products, to detect and identify soy-, milk- and egg white-unique heat-stable peptide markers. We examined commercially processed, poultry meat products, with the declared addition of three non-meat protein additives, namely soy protein isolate (SPI), milk and egg white hydrolysates. Tryptic digests of the extracted proteins, were analysed by using nanoflow LC quadrupole time-of-flight MS/MS (nano-LC-Q-TOF-MS/MS). Several proteins specific to the examined additives, were identified as a good source of peptide markers, unique to both non-meat additives and main allergenic proteins present in highly processed 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, lyophilized, was obtained from Promega GmbH (Mannheim, Germany). Reversed-phase Sep-Pak C18 Plus cartridges, sorbent weight 360 mg/0.7 mL, were obtained from Waters (Milford, MA, USA). All other chemicals were purchased from Sigma-Aldrich, in the best available purity grade. Poultry products (various sausages, frankfurters and pâtés; n=12) were purchased locally. For eight of these products, the presence of non-meat proteins (soy, milk or egg origin), was declared on the label. The labels of the remaining four products, did not contain the above-mentioned additives. Table 1 presents the species composition of the eight examined commercial products. Samples of about 5 cm length or 5 g, were cut from fresh products or cooked meat and kept at -80°C , until further

protein analysis. Proteins derived from soy (*Glycine max*), cow milk (*Bos taurus*) and egg white (*Gallus gallus*), were examined in the present study.

2.2. Preparation of samples

Washing and digestion of all samples were performed, according to the procedure described previously (Montowska et al., 2015; Montowska & Fornal, 2017). Thin sections of meat products (0.5 g), were rinsed consecutively in ethanol/water, ethanol, methanol/water, and milli-Q water, to remove physiological salts, fat and other soluble low molecular weight contaminants. Washed samples were homogenised in 0.1 mol/L of aqueous ammonium bicarbonate, using a T25 Ultra-Turrax (IKA Labortechnik, Staufen, Germany) at 9500 rpm for 2×20 s, followed by 13,500 rpm for 30 s and then, vacuum-dried using a CentriVap micro IR (Labconco Corporation, Kansas City, Missouri, USA).

2.3. In-solution trypsin digestion

Dried samples (10 mg) were rehydrated in 100 μ L of 0.05 mol/L ammonium bicarbonate. The proteins were reduced by 0.2 mol/L dithiothreitol (DTT) (56 °C for 1 h) and then alkylated using 0.2 mol/L iodoacetamide (IAA), at room temperature for 30 min in the dark. The remaining IAA was quenched by the addition of 0.2 mol/L DTT and incubation at room temperature for 30 min. The samples were digested in an ammonium bicarbonate solution pH 8.3 containing 0.083 μ g/ μ L trypsin, at 37 °C, overnight (18 h). The digests were purified by reversed-phase extraction, using Sep-Pak C18 Plus cartridges (Waters, Milford, MA, USA). The eluates were dried, using a CentriVap micro IR (Labconco). For chromatographic separation, the samples were resuspended in 2% acetonitrile in milli-Q water, containing 0.1% formic acid (solvent A), before analysis by nano-LC-MS/MS.

2.4. Nano-LC-Q-TOF-MS/MS analysis

Untargeted nano-LC-MS analysis, was performed on an Agilent Technologies (Santa Clara, CA, USA) 1200 series chromatograph, composed of a nano pump, a capillary pump, a thermostat FC/ALS and a microautosampler MicroWPS, coupled to a 6538 UHD Accurate-Mass Q-TOF-LC/MS, equipped with an HPLC-Chip Cube (Agilent Technologies). The LC was performed on an Agilent ProtID-Chip-150 II, 150 mm, 300 Å, C18 chip, with a 40-nL trap column and using an integrated 75 µm nano-LC column (G4240-62006). Instrument control and data acquisition were performed, using Agilent MassHunter Workstation software. The LC parameters were set as follows: 1 µL injection volume, 0.3 µL/min nano pump mobile phase flow, and 5 µL/min capillary pump mobile phase flow. The mobile phase consisted of 0.1% formic acid in 2% acetonitrile/double-distilled water (solvent A) and 0.1% formic acid in 98% acetonitrile/double-distilled water (solvent B). Gradient steps were applied as follows: 0–2 min, 2% B; 2–40 min, to 32% B; 40–45 min, to 37% B; 45–50 min, to 90% B; 50–55 min, 90% B, and a 5 min post-run at 0% B. The ion source gas (nitrogen) temperature was 350 °C and the flow rate was 5 L/min. The capillary voltage was set at 1850 V and the fragmentor set to 200 V. Ions formed in a chip cube nanoelectrospray, were acquired in positive ion scan mode, from 100–1700 m/z range for MS/MS, at a scan rate of 9 scan/s for MS and 5 scan/s for MS/MS. Internal mass calibration was enabled, by using two reference masses at 121.0509 and 922.0098 m/z .

2.5. Protein and peptide marker identification

A UniProtKB/Swiss-Prot database search for proteins and peptides identification was performed, using the Spectrum Mill MS Proteomics Workbench with > 70% score peak intensity and 20 ppm precursor mass tolerance. Raw mass spectra were also converted to Mascot Generic Format (.mgf) and searched using MASCOT software (Matrix Science,

Boston, MA, USA) against the UniProt/Swiss-Prot database, with the following parameters: trypsin enzyme, taxonomy green plants or bone vertebrates, one missed cleavage, 10 ppm peptide mass tolerance, 0.1 Da MS/MS tolerance, carbamidomethylation as fixed modification, methionine oxidation as a variable modification, and 2+, 3+ and 4+ peptide charge states. A decoy search was performed automatically and the matches and MASCOT scores were evaluated at 1% of the false discovery rate (FDR), for identity and homology threshold. Selected peptides, in FASTA format, were searched against the NCBI nr database, using the protein BLAST alignment research tool and BLASTP algorithm (USA National Library of Medicine), for species and protein specificity.

3. Results and discussion

3.1. Identification of non-meat proteins in processed meat products

Thermal technological processes used in the meat industry, such as smoking, cooking, roasting, grilling and sterilisation, induce protein denaturation, conformational changes and, consequently, protein aggregation and decreased solubility. A lower number of detected peptides, lower protein sequence coverage and an increase in meat protein cross-linkage compared to raw meat, were reported in cooked meats, various sausages and Bolognese sauce (Montowska & Pospiech, 2016; Prandi et al., 2017). Thermal processing that induces protein unfolding or aggregation, may also increase or decrease the allergic reactivity of some proteins, through exposure of hidden epitopes (Thomas et al., 2007). Considering thermal treatment affects MS-based discovery experiments, in this study, the identification of non-meat proteins and heat-stable peptide markers, was carried out on directly, industrially processed poultry products, aimed at detecting these peptides that had survived unmodified, through industrial processing. The industrially processed products, were smoked and cooked, and sample 17 was sterilised (Table 1). Sample preparation was kept very simple. After

washing, to remove the excess of fat and salt, the samples were reduced, alkylated and digested with trypsin, according to our previously described procedure (Montowska et al., 2015; Montowska & Fornal, 2017). The examined products were manufactured exclusively from poultry species (chicken, turkey) or mixed with pork or beef and with the declared addition of soy, milk or egg proteins (Table 1).

Tryptic digests were examined using nano-LC-Q-TOF-MS/MS, with the identification of peptides by MS/MS ions, using the MASCOT or Spectrum Mill searching algorithm. At 1% of an FDR for identity and homology threshold, 80–114 animal proteins, as well as 34–45 vegetable proteins were identified in the digest, depending on the analysed meat product. The acquired spectra of the soy, milk and egg white proteins, enabled both a good sequence coverage and number of matched peptides, which proved the digestion was efficient. The MASCOT output scores for selected non-meat proteins, for each sample, are shown in Table 2. Soy proteins were identified in six samples (for which soy presence was declared on the label), with significant MASCOT scores and sequence coverage in the range of 14–67%. The major soybean storage proteins, i.e. glycinin and β -conglycinin, were identified in all samples and with high scores. Of five major glycinin variants, glycinin G1, G2, G3 and G4, were successfully identified in all examined products, for which soy addition was declared. Previously, the presence of glycinin G2 and glycinin G4, was confirmed in four heat-treated types of meat (chicken, turkey, beef and pork) spiked with SPI, whereas glycinin G3 was detected only in pork and turkey meat (Leitner et al., 2006).

Milk protein preparations, which are widely used in the meat industry, can contain almost exclusively contain casein (caseinates) or whey proteins (whey protein isolates or concentrates), as well as these two groups of proteins. However, manufacturers do not often differentiate between them on the packaging and commonly only general information ‘milk proteins’ is presented in the list of ingredients. Milk proteins were detected in two examined

samples, S.8 and S.15 (Table 2). In these instances, milk proteins were listed among the ingredients on the product label. Three caseins (α -S1-, β - and κ -casein) and β -lg, were identified in homogenised, smoked and cooked frankfurters (sample S.15). Only β -lg was detected in smoked and cooked liver sausage (sample S.8), which implies that this product was likely manufactured exclusively with the addition of whey protein preparation. In other MS-based studies, residues of milk proteins (α -S1- and β -casein), were detected in commercial wines (Tolin et al., 2012) and α -S1-casein in cookies spiked with skimmed milk powder solubilised in water (Lamberti et al., 2016). However, no peptides derived from β -lg were detected in the spiked cookies. Gomaa & Boye (2015) also obtained lower recovery percentages for this protein in processed food matrices, such as bread and boiled pasta, compared with non-processed matrices. In that study, circular dichroism spectroscopy analysis confirmed that β -lg was far less thermostable than casein. In our processed meat samples, however, we acquired good sequence coverage for this protein (44.9 and 38.8% in samples S.8 and S.15, respectively). Considering that either β -lg and α -la were detected in chocolate chip cookies and soda biscuits, using a sensitive LC-multiple reaction monitoring/MS-based method (Ji et al., 2017), confirms that the results may differ, depending on the extraction method implemented, industrial processing and complexity of food matrices.

Chicken egg white is a source of proteins, with a variety of biological and physicochemical properties, useful in the meat industry. In our study, four egg white proteins and major allergens, namely ovalbumin, ovomucoid, ovotransferrin and lysozyme C, were detected in roasted and sterilised pâtés (samples S.14 and S.17, respectively) (Table 2). In both samples, eggs were included in the list of ingredients. Ovalbumin was previously reported as thermolabile (Leduc et al., 1999; Gomaa & Boye, 2015). We acquired good sequence coverage for this protein in processed samples but all the identified peptides appeared to be corresponding peptides, which we found, using the BLASTP tool, to be

present in the sequences obtained from other bird species. Thermal processing could have destroyed specific epitopes and unique fragments of chicken ovalbumin but the other three egg proteins were less affected and specific peptide markers were identified with high confidence scores (see section 3.2). The results demonstrate that the MS-based method can be a suitable tool for detection of egg white proteins in highly processed meat products, particularly, compared with ELISA because masked egg white allergens in sterilised meat products, were not detected by human serum immunoglobulin E (Leduc et al., 1999).

In addition, four “clean” poultry products, i.e. with no declared addition of the functional protein preparations, were analysed in this study (Table 1, samples S.7, S.9, S.13 and S.16). In these instances, negative results were obtained. No single soy, milk and egg protein or proteotypic peptide, was detected, which confirms that the labelling of these four products was correct (data not shown).

3.2. Identification of heat-stable peptide markers

The aim was to identify and select the most intense and reliable peptide markers specific to processed soy, milk and egg white allergenic proteins present in complex meat product matrices. Tandem MS data sets were collected, using a discovery-based experiment, with an accurate mass nano-LC-Q-TOF-MS/MS. The analysis focused on heat-stable peptides, which means the peptides that were stable to heat treatment and survived unmodified through industrial processing. A selection of potential peptide markers was made, based on the results of both the Spectrum Mill and the MASCOT searches. The most intense peptides in FASTA format, with > 70% score peak intensity, and high identity and homology MASCOT scores, were searched for species and protein specificity, in all protein records stored in the NCBI protein sequence database, using the BLAST alignment search tool and BLASTP algorithm. Table 3 presents the selected heat-stable peptide markers unique to soy,

milk and egg white, identified in processed meat products. The peptides belong to the most abundant, as well as allergenic, proteins present in these three additives, such as glycinins G1–G4, β -conglycinin, α -S1-casein, β -lg, ovotransferrin and lysozyme C. Predominantly, the MASCOT score was above the identity and homology threshold and the peptide abundance ranged from $10^3 - 10^7$. For example, the MS/MS spectra specific to glycinin G1, α -S1-casein and ovotransferrin peptides, are presented in Figure 1. Despite the proteins experiencing high temperatures during technological processes, such as roasting and sterilising, it was still possible to generate good quality MS/MS spectra and identify numerous proteins and peptides, for example, over 100 proteins and 30,000 peptide fragments, in the goose and duck pâtés (samples S.14 and S.17).

The two major soy proteins, glycinin isoforms and β -conglycinin share significant homology within soy species but less homology with proteins from other species. For this reason, having performed a BLASTP search, we found that many peptides derived from these proteins, expressed diversity and species-specificity and many of them turned out to be resistant to processing, including sterilisation. Consequently, a large amount, i.e. 43 heat-stable peptide markers, unique exclusively to soy proteins, is presented in this study (Table 3). Among them, was peptide ${}^{236}\text{HFLAQSFNTNEDIAEK}_{251}$ (m/z 621.97³⁺), originating from glycinin G4, which was previously identified in heat-processed meat samples spiked with SPI and was reported as the most reliable marker for the soy protein addition to meat products (Leitner et al., 2006). Recently, the same three soy peptides found in the present study, i.e. ${}^{182}\text{FYLAGNQEQEFLK}_{194}$ (glycinin G1/G2), ${}^{228}\text{EAFGVNMQIVR}_{238}$ (glycinin G2) and ${}^{236}\text{HFLAQSFNTNEDIAEK}_{251}$ (glycinin G4), were selected as suitable marker peptides for soy detection in emulsion-type sausages manufactured with the addition of SPI (Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017). Two other peptides ${}^{391}\text{VFDGELQEGGVLPQNFAVA}_{413}$ (m/z 801.10³⁺) and ${}^{518}\text{AIPSEVLAHSYNLR}_{531}$

(m/z 785.43²⁺), derived from glycinin G2 and G4, respectively, were selected as candidate markers for detection of soy allergenic proteins, in artificially contaminated cookie samples, containing pre-cooked soy flour, egg powder and skimmed milk powder (Monaci et al., 2014).

Felfoul, Jardin, Gaucheron, Attia, & Ayadi (2017) observed that heat treatment (80 °C for 60 min) had a detrimental effect on bovine α -la and β -lg, however, casein fractions remained unchanged under these conditions. Other studies indicate that lactoglobulins are resistant to high temperatures used in daily food processing, for instance, cookies and cakes (Monaci et al., 2014; Ji et al., 2017). Over a 12 of β -lg peptides were identified in the examined cooked and smoked sausages (samples S.8 and S.15), and three of them appeared to be unique to bovine milk (Table 3). One of them, β -lg heat-stable peptide marker $_{165}\text{LSFNPTQLEEQCHI}_{178}$ (m/z 858.41²⁺), has been used for cow milk quantitation in commercial raw materials for dairy products, such as milk and whey powders (Chen et al., 2016). Several peptides unique to bovine casein fractions were identified in poultry sausages, in the present study, which is consistent with recent studies, indicating the casein peptides resistance to cheese and cookie processing (Monaci et al., 2014; Bernardi et al., 2015; Ji et al., 2017). Two α -S1-casein peptides $_{106}\text{YLGYLEQLLR}_{115}$ (m/z 634.36²⁺) and $_{38}\text{FFVAPFPEVFGK}_{49}$ (m/z 692.87²⁺), were detected in both cookie and processed meat matrices. The other two peptides specific to α -S1-casein, namely $_{23}\text{HQGLPQEVLENENLLR}_{37}$ (m/z 587.32³⁺) and $_{148}\text{EPMIGVNQELAYFYPELFR}_{166}$ (m/z 1158.58²⁺), were identified in pork frankfurters and kabanos sausage, manufactured with the addition of milk preparation and cheese, respectively, using ambient liquid extraction surface analysis-MS/MS method (Montowska et al., 2015). Moreover, some of the α -S1-casein and β -casein peptides, were detected in commercial bottled wines (Tolin et al., 2012).

Among peptides originating from ovalbumin, no peptide markers unique exclusively to chicken protein, were observed in roasted and sterilised pâtés (samples S.14 and S.17), however, 12 peptides derived from other egg white proteins, namely ovotransferrin, lysozyme C and ovomucoid, expressed both thermal stability and specificity to chicken species (Table 3). Azarnia, Boye, Mongeon, & Sabik, (2013) detected peptides originating from ovalbumin, in egg samples heated at 99 °C for 15 min and raw pasta samples but they obtained negative results in cooked pasta under the same conditions, using both LC-MS and ELISA methods. Residues of ovalbumin, lysozyme and ovotransferrin, were observed in Italian commercial wines purchased at local supermarkets (Tolin et al., 2012). Ovalbumin peptides were selected as markers for detection of egg allergens in cookies spiked with egg powder (Monaci et al., 2014). Among peptides detected in wines, four ovotransferrin peptides ${}_{174}\text{FFSASCVPGATIEQK}_{188}$ (m/z 821.40²⁺), ${}_{443}\text{TDERPASYFAVAVAR}_{457}$ (m/z 551.62³⁺), ${}_{155}\text{GAIEWEGIESGSVEQAVAK}_{173}$ (m/z 980.49²⁺) and ${}_{689}\text{TCNPSDILQMCSFLEGK}_{705}$ (m/z 1000.45²⁺), as well as three peptides derived from lysozyme C, were also identified in the examined processed meat products. Considering that ELISA and immunoblotting analysis demonstrated that sterilisation affects antigenic epitopes of certain egg white proteins present in meat products, among them ovomucoid, which was reported as heat-resistant (Leduc et al., 1999), MS-based methods are superior in detection of allergenic proteins in severely processed meat products. Our future research direction is to develop a quantitative method based on the selected allergenic marker peptides detected in study, using a labelled approach and MRM-MS, to provide a robust technique that can differentiate between accidental contamination and intentional addition to meat products prepared with non-meat functional preparations containing allergenic proteins.

4. Conclusions

In this study, we have examined the applicability of a nano-LC-Q-TOF-MS/MS method to identify peptide markers for non-meat allergenic proteins derived from functional protein additives in various types of poultry meat products. Application of a non-targeted strategy for screening of whole product protein digests, allowed identifying a set of heat-stable peptide markers originating from major food allergens, namely soy glycinin and β -conglycinin, milk α -S1-casein and the whey protein β -lg, as well as egg white ovotransferrin and lysozyme C. The method displays specificity and sensitivity, sufficient to be implemented to severely processed meat products, for instance, subjected to roasting and sterilisation and, thus, provides a valuable alternative to PCR and ELISA tests.

Conflict of interest

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

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Table 1

Declared meat and non-meat protein composition of commercially processed poultry products.

Sample no.	Product	Meat composition	Non-meat additives
S.6	Frankfurters (homogenised, smoked and cooked)	Turkey	Soy proteins
S.7	'Kabanos' type sausage (smoked, cooked and semidried)	Chicken, pork	Not declared
S.8	Liver sausage (smoked and cooked)	Chicken, pork	Soy proteins, milk proteins
S.9	'Zywiecka' sausage (smoked, cooked and semi-dried)	Turkey	Not declared
S.10	Smoked and cooked breast	Chicken	Soy proteins
S.11	Frankfurters (homogenised, smoked and cooked)	Chicken, turkey, pork	Soy proteins
S.12	Frankfurters (homogenised, smoked and cooked)	Turkey, beef, chicken, pork	Soy proteins
S.13	'Kabanos' sausage (smoked, cooked and semi-dried)	Goose	Not declared
S.14	Pâté (roasted)	Goose, pork	Soy proteins, egg proteins
S.15	Frankfurters (homogenised, smoked and cooked)	Chicken, veal	Milk proteins
S.16	Terrine (sterilised)	Duck	Not declared
S.17	Pâté (sterilised)	Duck, pork	Egg proteins

Table 2

MASCOT output scores for soy, milk and egg white proteins obtained using the nano-LC-QTOF-MS/MS of processed meat products.

Sample no.	Identified protein	Accession no.	Mass (Da)	Matched peptides ^a	Sequence coverage (%) ^b	MASCOT score ^c
S6	Glycinin G1 (<i>Glycine max</i>)	P04776.2	56299	88	61.8	552
	Glycinin G2 (<i>Glycine max</i>)	P04405.2	54927	67	47.8	329
	Glycinin G3 (<i>Glycine max</i>)	P11828.1	54835	21	30.4	86
	Glycinin G4 (<i>Glycine max</i>)	P02858.1	64005	49	37.4	210
	β -Conglycinin, alpha chain (<i>Glycine max</i>)	P13916.2	70535	35	22.0	125
	Histone H4 (<i>Glycine max</i>)	P0CG89.1	11402	6	33.0	31
	Sucrose-binding protein (<i>Glycine max</i>)	Q04672.1	60884	16	14.9	96
S8	Glycinin G1 (<i>Glycine max</i>)	P04776.2	56299	55	56.8	104
	Glycinin G2 (<i>Glycine max</i>)	P04405.2	54927	42	46.2	98
	Glycinin G3 (<i>Glycine max</i>)	P11828.1	54835	7	14.1	32
	Glycinin G4 (<i>Glycine max</i>)	P02858.1	64005	22	26.5	63
	β -Conglycinin, alpha chain (<i>Glycine max</i>)	P13916.2	70535	43	28.1	126
	Histone H4 (<i>Glycine max</i>)	P0CG89.1	11402	17	33.0	62
	Sucrose-binding protein (<i>Glycine max</i>)	Q04672.1	60884	8	14.7	22
	β -Lactoglobulin (<i>Bos taurus</i>)	P02754.3	20269	19	44.9	49
S10	Glycinin G1 (<i>Glycine max</i>)	P04776.2	56299	60	51.3	323
	Glycinin G2 (<i>Glycine max</i>)	P04405.2	54927	35	44.9	168
	Glycinin G3 (<i>Glycine max</i>)	P11828.1	54835	20	31.6	62
	Glycinin G4 (<i>Glycine max</i>)	P02858.1	64005	32	26.7	170
	β -Conglycinin, alpha chain (<i>Glycine max</i>)	P13916.2	70535	28	20.2	87
	Histone H4 (<i>Glycine max</i>)	P0CG89.1	11402	3	23.3	78
	Sucrose-binding protein (<i>Glycine max</i>)	Q04672.1	60884	12	16.0	50
S11	Glycinin G1 (<i>Glycine max</i>)	P04776.2	56299	78	55.6	427
	Glycinin G2 (<i>Glycine max</i>)	P04405.2	54927	51	42.7	195
	Glycinin G3 (<i>Glycine max</i>)	P11828.1	54835	17	16.0	68
	Glycinin G4 (<i>Glycine max</i>)	P02858.1	64005	37	33.6	181
	β -Conglycinin, alpha chain (<i>Glycine max</i>)	P13916.2	70535	34	19.5	108
	Histone H4 (<i>Glycine max</i>)	P0CG89.1	11402	6	21.4	26
	Sucrose-binding protein (<i>Glycine max</i>)	Q04672.1	60884	13	14.9	39
S12	Glycinin G1 (<i>Glycine max</i>)	P04776.2	56299	87	67.9	541
	Glycinin G2 (<i>Glycine max</i>)	P04405.2	54927	57	60.6	370
	Glycinin G3 (<i>Glycine max</i>)	P11828.1	54835	33	51.8	96
	Glycinin G4 (<i>Glycine max</i>)	P02858.1	64005	52	37.5	126
	β -Conglycinin, alpha chain (<i>Glycine max</i>)	P13916.2	70535	45	22.5	137
	Histone H4 (<i>Glycine max</i>)	P0CG89.1	11402	6	21.4	37
	Sucrose-binding protein (<i>Glycine max</i>)	Q04672.1	60884	23	17.0	69
S14	Glycinin G1 (<i>Glycine max</i>)	P04776.2	56299	72	56.2	412
	Glycinin G2 (<i>Glycine max</i>)	P04405.2	54927	54	47.4	245

	Glycinin G3 (<i>Glycine max</i>)	P11828.1	54835	25	38.3	81
	Glycinin G4 (<i>Glycine max</i>)	P02858.1	64005	42	34.5	127
	β -Conglycinin, alpha chain (<i>Glycine max</i>)	P13916.2	70535	41	26.0	160
	Histone H4 (<i>Glycine max</i>)	P0CG89.1	11402	12	33.0	45
	Sucrose-binding protein (<i>Glycine max</i>)	Q04672.1	60884	22	22.1	93
	Ovalbumin (<i>Gallus gallus</i>)	P01012.2	43196	92	67.1	655
	Ovomucoid (<i>Gallus gallus</i>)	P01005.1	23660	4	4.0	124
	Ovotransferrin (<i>Gallus gallus</i>)	P02789.2	79551	25	27.7	256
	Lysozyme C (<i>Gallus gallus</i>)	P00698.1	16741	8	32.7	122
S15	α -S1-Casein (<i>Bos taurus</i>)	P02662.2	24570	49	60.7	454
	β -Casein (<i>Bos taurus</i>)	P02666.2	25148	22	40.6	119
	κ -Casein (<i>Bos taurus</i>)	P02668.1	21370	14	14.7	168
	β -Lactoglobulin (<i>Bos taurus</i>)	P02754.3	20269	15	38.8	90
S17	Ovalbumin (<i>Gallus gallus</i>)	P01012.2	43196	61	61.1	431
	Ovomucoid (<i>Gallus gallus</i>)	P01005.1	23660	3	11.0	35
	Ovotransferrin (<i>Gallus gallus</i>)	P02789.2	79551	44	22.3	564
	Lysozyme C (<i>Gallus gallus</i>)	P00698.1	16741	19	40.8	133

^aNumber of matched peptides in the database search. ^bPercentage of coverage of the entire amino acid sequence.

^cMASCOT score at FDR of 1%.

Table 3

MASCOT output scores for heat-stable peptide markers unique to soy, milk and egg white allergenic proteins identified in processed meat products.

Parent ion	Mr(exp)	Pep exp z	Mr(calc)	Sequence (start/end of peptide)	Protein	MASCOT score	Identity threshold*	Homology threshold*	Abundance
Soy peptides									
490.2724	978.5302	2	978.5134	³⁵⁷ LSAEFGSLR ₃₆₅	Glycinin G1	34.25	15	21	6.10E-04
713.4434	1424.8723	2	1424.8504	⁴¹¹ VLIVPQNFVVAAR ₄₂₃	Glycinin G1	54.68	13	13	4.10E-06
725.8381	1449.6617	2	1449.6412	⁴²⁴ SQSDNFEYVSFK ₄₃₅	Glycinin G1	66.65	19	19	1.00E-06
793.9007	1585.7869	2	1585.7776	¹⁸² FYLAGNQEQEFLK ₁₉₄	Glycinin G1/G2	67.22	17	21	5.00E-07
831.4161	2491.2264	3	2491.243	³⁶⁷ NAMFVPHYNLNANSIIYALNGR ₃₈₈	Glycinin G1	49.95	19	19	5.00E-05
889.7626	2666.2659	3	2666.2258	⁹⁸ GIFGMIYPGCPSTFEPPQQPQR ₁₂₀	Glycinin G1	35.63	18	18	9.40E-04
996.5063	2986.4971	3	2986.4607	⁴⁵ IESEGGLIETWNPNNKPFQCAGVALSR ₇₁	Glycinin G1	32.61	15	20	8.70E-04
1093.1945	3276.5615	3	3276.5211	²⁰⁹ HQEEEEENEGGSILSGFTLEFLEHAFSVDK ₂₃₇	Glycinin G1	69.63	16	16	2.70E-07
1141.605	3421.7931	3	3421.7551	⁴³⁶ TNDTPMIGTLAGANSLLNALPEEVIQHT ⁴⁶⁷ FNLK ₄₆₇	Glycinin G1	36.64	17	17	6.40E-04
1215.6198	3643.8375	3	3643.7907	³²³ HNIGQTSSPDIYNPQAGSVTTATSLDFPA ³⁵⁶ LSWLR ₃₅₆	Glycinin G1	63.25	18	18	1.50E-06
632.3395	1262.6645	2	1262.6441	²²⁸ EAFGVNMQIVR ₂₃₈	Glycinin G2	39.12	19	23	5.30E-04
725.8381	1449.6617	2	1449.6412	⁴¹⁴ SQSDNFEYVSFK ₄₂₅	Glycinin G2	66.65	19	19	1.00E-06
937.4715	1872.9284	2	1872.9119	⁴⁶⁶ NNNPFSFLVPPQESQR ₄₈₁	Glycinin G2	26.51	14	21	3.30E-03
966.4767	1930.9388	2	1930.912	²³⁹ NLQGENEEEDSGAIVTVK ₂₅₆	Glycinin G2	25.33	14	20	4.20E-03
1183.5803	2365.1461	2	2365.1073	²⁰⁷ QEEEEENEGSNILSGFAPEFLK ₂₂₇	Glycinin G2	71.61	17	20	1.90E-07
801.1021	2400.2844	3	801.1021	³⁹¹ VFDGELQEGGVLPVQNFVAFAAK ₄₁₃	Glycinin G2	48.68	18	18	5.10E-05
827.097	2478.2691	3	2478.2478	³⁵⁷ NAMFVPHYTLNANSIIYALNGR ₄₇₈	Glycinin G2	57.07	16	22	4.50E-06
862.4689	3445.8466	4	3445.7953	⁴²⁶ TNDRPSIGNLAGANSLLNALPEEVIQHTF ⁴⁵⁷ NLK ₄₅₇	Glycinin G2	52.18	17	17	1.50E-05
915.9861	3659.9154	4	3659.8471	³¹³ QNIGQNSSPDIYNPQAGSITTATSLDFPA ³⁴⁶ LWLLK ₃₄₆	Glycinin G2	62.49	15	15	1.10E-06
725.8381	1449.6617	2	1449.6412	⁴¹⁰ SQSDNFEYVSFK ₄₂₁	Glycinin G3	66.65	19	19	1.00E-06
1045.5026	3133.486	3	3133.4629	²⁰⁹ QEEEEENEGGSILSGFAPEFLEHAFVVDR ₂	Glycinin G3	35.48	17	17	8.60E-04

				36					
867.2142	3464.8278	4	3464.8011	⁴²² TNDRPSIGNLAGANSLNALPEEVIQQTF NLR ₄₅₃	Glycinin G3	47.39	15	15	3.40E-05
1205.6125	3613.8156	3	3613.8053	³⁰⁹ HNIGQTSSPDIFNPQAGSITTATSLDFPAL SWLK ₃₄₂	Glycinin G3	48.20	16	16	3.80E-05
699.9253	1397.836	2	1397.8242	⁴⁰⁸ ISTLNSLTLPALR ₄₂₀	Glycinin G4	67.58	15	15	2.90E-07
763.9503	1525.8861	2	1525.8716	²⁶¹ QIVTVEGGLSVISPK ₂₇₅	Glycinin G4	45.06	17	17	8.00E-05
785.4294	1568.8442	2	785.4294	⁵¹⁸ AIPSEVLAHSYNLR ₅₃₁	Glycinin G4	37.27	15	20	3.20E-04
621.9716	1862.893	3	1862.8799	²³⁶ HFLAQSFNTNEDIAEK ₂₅₁	Glycinin G4	22.01	14	21	8.60E-03
684.003	2048.9872	3	2048.9697	²⁹ LNECQLNNLNALEPDHR ₄₅	Glycinin G4	52.43	16	20	1.30E-05
1058.005	2113.995	2	2113.982	⁵⁴⁰ YEGNWGPLVNPESQQGSPR ₅₅₈	Glycinin G4	25.35	20	20	1.50E-02
718.0328	2151.0765	3	2151.0596	⁴⁶ VESEGGLIQTWNSQHPELK ₆₄	Glycinin G4	30.79	15	20	1.60E-03
919.1136	2754.3191	3	2754.2959	¹⁸⁶ VFYLAGNPDIETPETMQQQQQK ₂₀₈	Glycinin G4	26.49	18	18	8.30E-03
1003.8705	3008.5897	3	3008.5648	⁴⁷⁶ GQLLVVPQNFVVAEQAGEQGFEYIVFK ₅₀₂	Glycinin G4	28.04	18	18	5.60E-03
622.8655	1243.7165	2	1243.7023	³⁷¹ LQESVIVEISK ₃₈₁	β -Conglycinin	20.72	14	23	1.10E-02
464.6344	1390.8813	3	1390.866	³⁰⁶ LITLAIPVKNPGR ₃₁₈	β -Conglycinin	49.34	13	13	1.20E-05
675.9971	2024.9695	3	2024.9399	⁴⁷⁹ EQQQEQQQEEQPLEVR ₄₉₄	β -Conglycinin	68.90	19	19	5.50E-07
1076.532	2151.0495	2	2151.0233	²⁸⁷ VPSGTTYVVPDNNENLR ₃₀₅	β -Conglycinin	33.84	16	20	9.60E-04
820.4018	2458.1834	3	2458.1441	³¹⁹ FESFFLSSTEAQQSYLQGFSR ₃₃₉	β -Conglycinin	43.69	19	19	1.80E-04
901.1406	2700.4	3	2700.3833	⁴⁵³ DLDFLSIVDMNEGALLPHFNSK ₄₅₈	β -Conglycinin	28.43	19	19	6.40E-03
1124.9147	3371.7223	3	3371.6997	⁵³⁷ NFLAGSQDNVISQIPSVQELAFPGSAQ AVEK ₅₆₈	β -Conglycinin	35.40	17	17	8.90E-04
545.3103	1088.606	2	1088.598	¹⁴¹ LLQGIENFR ₁₄₉	Sucrose-binding protein	33.45	15	21	7.80E-04
637.839	1273.6635	2	1273.6514	⁴⁵⁵ DNIVSSLDNVAK ₄₆₆	Sucrose-binding protein	23.24	14	21	6.60E-03
708.3598	1414.705	2	1414.6921	⁵⁰⁵ ESFFFPFELPR ₅₁₅	Sucrose-binding protein	33.89	15	20	6.60E-04
801.0911	2400.2516	3	2400.2325	²⁴⁶ DPESVLSAFSWNVLQAALQTPK ₂₆₇	Sucrose-binding protein	70.11	17	20	2.70E-07
Milk peptides									
587.3236	1758.9471	3	1758.9377	²³ HQGLPQEVNLNENLLR ₃₇	α -S1-Casein	26.73	14	24	3.10E-03
1158.5807	2315.1469	2	2315.1296	¹⁴⁸ EPMIGVNQELAYFYPELFR ₁₆₆	α -S1-Casein	58.85	17	25	3.90E-06

634.3598	1266.705	2	1266.6972	¹⁰⁶ YLGYLEQLLR ₁₁₅	α -S1-Casein	73.32	14	24	6.40E-07
692.8764	1383.7383	2	1383.7227	³⁸ FFVAPFPEVFGK ₄₉	α -S1-Casein	38.35	15	26	2.50E-04
1093.5881	2185.1617	2	2185.1605	¹⁹⁹ DMPIQAFLLYQEPVLGPVR ₂₁₇	β -Casein	49.12	16	24	2.50E-05
990.5547	1979.0948	2	1979.084	⁹⁰ SPAQLQWQVLSNTVPAK ₁₀₇	κ -Casein	64.54	17	23	8.90E-07
771.7631	2312.2674	3	2312.2515	⁵⁷ VYVEELKPTPEGDLEILLQK ₇₆	β -Lactoglobulin	36.95	15	20	3.40E-04
903.1374	2706.3904	3	2706.3687	³¹ VAGTWYSLAMAASDISLLDAQSAPLR ₅₆	β -Lactoglobulin	65.78	17	23	6.80E-07
858.4099	1714.8052	2	1714.7985	¹⁶⁵ LSFNPTQLEEQCHI ₁₇₈	β -Lactoglobulin	26.55	14	34	3.20E-03
Egg white peptides									
529.3032	1056.5919	2	1056.5855	⁶⁸⁰ FYTVISSLK ₆₈₈	Ovotransferrin	27.97	19	27	6.50E-03
512.2919	1533.854	3	1533.8416	¹⁴¹ SAGWNIPIGTLLHR ₁₅₄	Ovotransferrin	45.92	15	23	4.90E-05
821.4087	1640.8028	2	1640.7869	¹⁷⁴ FFSASCVPATIEQK ₁₈₈	Ovotransferrin	30.19	18	26	3.10E-03
551.6215	1651.8427	3	1651.8318	⁴⁴³ TDERPASYFAVAVAR ₄₅₇	Ovotransferrin	36.5	15	27	3.80E-04
980.4899	1958.9652	2	1958.9585	¹⁵⁵ GAIEWEGIESGSVEQAVAK ₁₇₃	Ovotransferrin	45.65	15	25	5.20E-05
1000.4559	1998.8972	2	1998.8849	⁶⁸⁹ TCNPSDILQMCSFLEGK ₇₀₅	Ovotransferrin	50.47	16	22	1.90E-05
512.774	2047.0668	4	2047.052	⁶⁰² ECNLAEVPTHAVVVRPEK ₆₁₉	Ovotransferrin	57.42		13	1.80E-06
523.2758	1044.537	2	1044.5352	¹³⁵ GTDVQAWIR ₁₄₃	Lysozyme C	46.02		13	2.50E-05
714.8263	1427.638	2	1427.643	⁵² FESNFNTQATNR ₆₃	Lysozyme C	39.46		13	1.10E-04
877.4203	1752.8261	2	1752.8278	⁶⁴ NTDGSTDYGILQINSR ₇₉	Lysozyme C	54.05	16	26	8.50E-06
1254.5992	2507.1838	2	2507.1818	⁹² NLCNIPCSALLSSDITASVNCAL ₁₁₄	Lysozyme C	25.27		25	5.40E-02
881.0603	2640.1592	3	2640.1407	¹¹⁴ AFNPVCGTDGVTYDNECLLCAHK ₁₃₆	Ovomucoid	27.83		18	6.40E-03

*Individual ion scores to indicate identity or extensive homology.

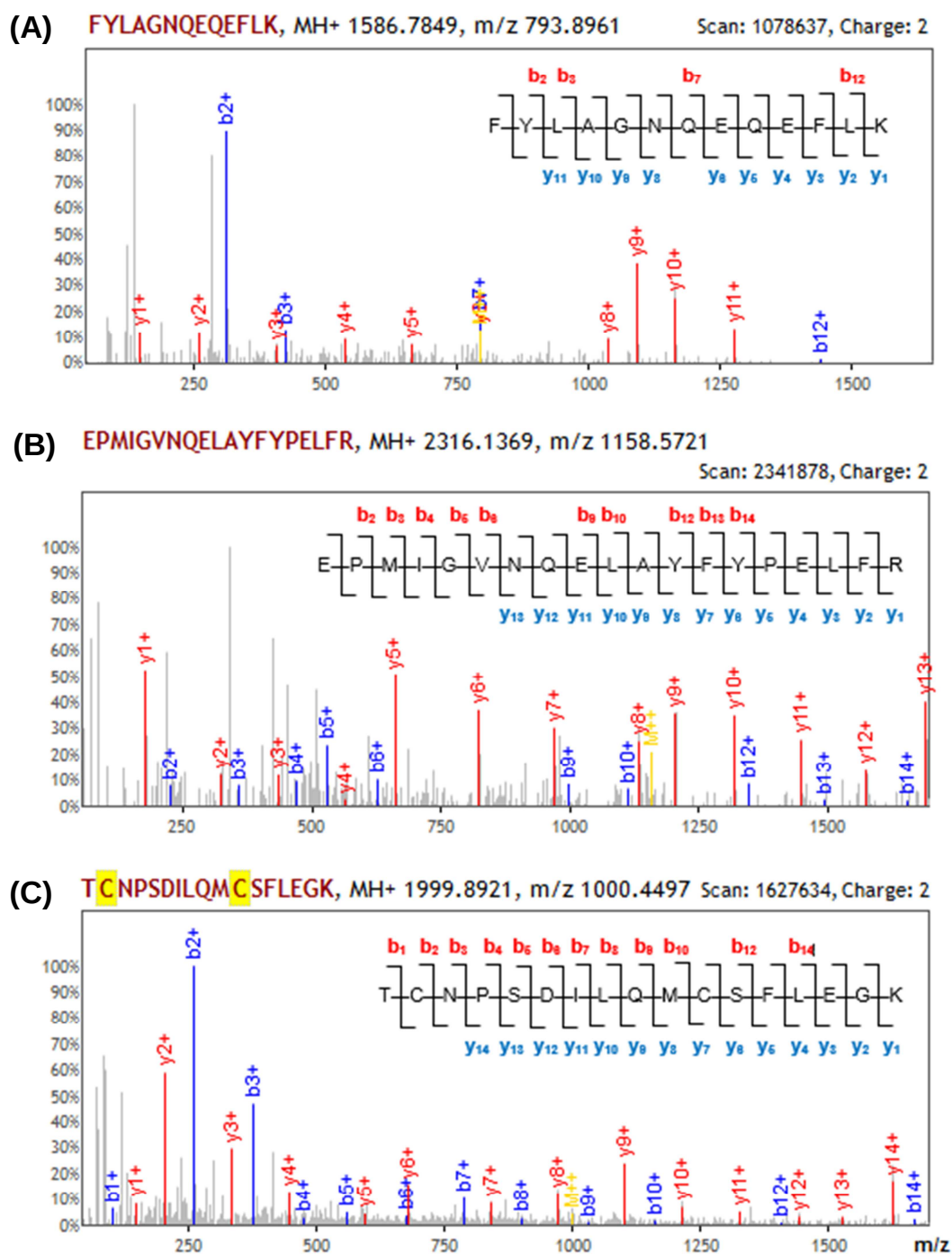


Fig. 1. MS/MS spectra of peptides of non-meat protein additives obtained from in-solution digestion of processed meat products using nano-LC-QTOF-MS/MS; (A) soy glycinin G1 and G2 peptide FYLAGNQEQLK (m/z 793.89²⁺); (B) milk α -S1-casein peptide EPMIGVNQELAYFYPELFR (m/z 1158.57²⁺); (C) egg white ovotransferrin peptide TCNPSDILQMCSFLEGK (m/z 1000.45²⁺).

Highlights:

- Cooked, smoked, roasted and sterilised meat products were examined.
- Protein screening of heat processed products was performed using mass spectrometry.
- The main soy, milk and egg white allergenic proteins were detected.
- Unique peptide markers of soy, milk and egg white were identified.