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Determination of hidden milk allergens in meat-based foodstuffs by liquid chromatography coupled to electrospray ionization and high-resolution tandem mass spectrometry

M. Bianco[1], C. D. Calvano*[2,3], G. Ventura[1], I. Losito[1,3], and T.R.I. Cataldi*[1,3]

[1]*Dipartimento di Chimica,* [2]*Dipartimento di Farmacia-Scienze del Farmaco,* [3]*Centro interdipartimentale SMART, Università degli Studi di Bari Aldo Moro, via Orabona 4, 70126, Bari, Italy*

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Authors for correspondence, email: cosimadamiana.calvano@uniba.it; tommaso.cataldi@uniba.it

Abstract

The issue of deliberate addition of antigenic proteins to foodstuffs for ameliorating bulk properties or the unintentional cross-contamination poses potentially life-threatening health problems to susceptible subjects. Even the intake of food products declaring the absence of allergens on their labels could lead to severe risks for sensitive consumers due to the presence of the so-called "hidden allergens". Thus, the quantification of low-abundant proteins–as putative allergens has become mandatory. Herein, we present a sensitive and selective analytical method based on reversed-phase liquid chromatography coupled to electrospray ionization and hybrid orbitrap high-resolution mass spectrometry (RPLC-ESI-HRMS) and tandem MS, identifying, and quantifying allergenic milk proteins in complex meat-based foodstuffs from direct measurement of tryptic peptides. Two signature peptides of α-S1-casein and β-lactoglobulin, *i.e.*, FFVAPFPEVFGK (*m/z* 692.868^{2+}) and TPEVDDEALEK (*m/z* 623.295^{2+}), respectively, were chosen to search for hidden allergens in meat-based samples such as cooked meat, sausages, and sterilised pâté. The marker peptides were identified and were exploited for method validation including recovery, matrix effect, precision, linearity, method variation, limit of detection, and limit of quantification. The undeclared occurrence of milk allergens as total milk protein content (TCMP) was verified in commercial meat products; beef and pork pâté were meat-based products which require a major alert because up to 22 μg_{TCMP}/g of matrix *i.e.* more than 10 times the action level was determined.

1 INTRODUCTION

Food allergens are proteins or peptides triggering immune-mediated reactions in susceptible subjects (European Council, 2011). Food allergy is recognized as a serious health problem that currently affects about 3% of the European and 5% of the world population, with a continuously growing incidence (Loh & Tang, 2018; Verhoeckx et al., 2015). The only useful approach for people suffering from food allergies remains the whole avoidance of foods at risk (Arshad, Bateman, Sadeghnejad, Gant, & Matthews, 2007; van Putten et al., 2006); eggs, milk, fish, peanuts, crustaceans, soybeans, wheat, and tree nuts are the most allergenic foods, known as "the big 8" family (Monaci, De Angelis, Montemurro, Pilolli, 2018). Since the number of sensitive consumers suffering from food allergies has increased in recent years, the list of foodstuffs has been extended in Europe and now contains 14 foods, including lupin, shellfish, celery, mustard, sesame, and sulfur dioxide (Verhoeckx et al., 2015). However, in some cases, also the lifelong avoidance of these foods might be not enough because there could be hidden ingredients in food products. Cross-contamination during food processing as a result of inadequate cleaning procedures of machinery, leading to the presence of "hidden allergens", might take place (RÖDER et al., 2008).

With the precise intent of protecting consumer health, the search for allergenic ingredients not reported on labels of food commodities has significantly increased. Many analytical techniques are claimed to be able to detect common allergen proteins, such as direct approaches, *e.g.*, enzyme-linked immunosorbent assays (ELISA) and biosensors (Bremer, Smits, & Haasnoot, 2009; Mohammed, Mullett, Lai, & Yeung, 2001; Trashin, Cucu, Devreese, Adriaens, & De Meulenaer, 2011; Yman, Eriksson, Johansson, & Hellenäs, 2006), and indirect ones, such as polymerase chain reaction (PCR) (Nadal, Pinto, Svobodova, Canela, & O'Sullivan, 2012; Tran et al., 2010) where DNA fragments are targeted as markers of potentially allergenic ingredients. Despite ELISA and PCR are often preferred by the food industry due to the efficiency of routine application, they suffer of either

cross-reactivity or lack of detection for thermally degraded/denatured target analytes (Arslan, Ilhak, & Calicioglu, 2006; Musto, Faraone, Cellini, & Musto, 2014; Parker et al., 2015; Platteau et al., 2011; Şakalar, Abasiyanik, Bektik, & Tayyrov, 2012). In the last ten years, mass spectrometry (MS), coupled or not (Cosima D. Calvano, Bianco, Losito, & Cataldi, 2021) with liquid chromatography (LC) has been largely applied to discover proteins and peptides in foodstuffs (Losito, Introna, Monaci, Minella, & Palmisano, 2013; Mattarozzi, Bignardi, Elviri, & Careri, 2012; L. Monaci, Losito, Palmisano, Godula, & Visconti, 2011; Montowska & Fornal, 2019; Pilolli, De Angelis, & Monaci, 2018; M. Planque et al., 2016, 2017). High selectivity, good sensitivity and ruggedness are the main features of MS, along with the possibility to distinguish multiple allergens in a single analysis, thus allowing their quantification in complex food matrices (Cucu, Jacxsens, & De Meulenaer, 2013; Monaci, De Angelis, Montemurro, Pilolli, 2018).

To protect people suffering from food allergies, the European Regulation n° 1169/2011 established that allergens must be signalled on the food products by different sizes, font, or color labels (European Council, 2011). Nevertheless, this regulation did not mention any guideline for allergens deriving from cross-contamination during food production. To indicate the likely, yet unintended, presence of allergenic ingredients in the final products, the food industry adopted a strategy named "precautionary allergen labelling" (PAL) (DunnGalvin et al., 2015). Hence, the expressions "*may contain …*" or "*not suitable for a person with a specified allergy*" are examples of this labelling. However, PAL often does not provide consumers with clear information about the allergenic risk associated with food products and the excessive use of PAL leads the consumers to ignore this label, with consequent serious hazards for allergic subjects (Marchisotto et al., 2017; Pele, Brohée, Anklam, & Hengel, 2007). To avoid this risk and to limit the abuse of PAL on foodstuffs, some national agencies have proposed the Voluntary Incidental Trace Allergen Labeling (VITAL) program (Allen, Remington, et al., 2014; Monaci et al., 2020; Muraro et al., 2014) using an authorized risk

assessment (Allen, Turner, et al., 2014). The VITAL program provides a reference dose (RD) for each allergenic ingredient (*e.g.*, the RD of milk and eggs is 0.2 mg of protein) and an action level (AL) to protect most of the food allergic consumers (Allen, Remington, et al., 2014; Taylor et al., 2014). AL represents the quantity of allergenic protein beyond which it is necessary to declare its presence in the label list considering a reference amount, *i.e.* a typical amount of food ingested. Definitively, the use of the VITAL program with validated RD would increase the importance of the label, reducing the use of PAL to only really risky foods and improving the life quality of food-allergic customers (Taylor et al., 2014).

Low-cost proteins are commonly added to processed meat, such as sliced meats, during the production of meat-based foodstuffs. The reasons are dictated by the need of improving water absorption, gelation, and emulsion of fat droplets and to assure good stability and taste features of cooked products (Gujral, Kaur, Singh, & Sodhi, 2002; Schilling et al., 2004; Toldrá & Nollet, 2016; Zorba, Kurt, & Gençcelep, 2005). Besides economic reasons, extraneous proteins are supplemented to enhance as well organoleptic properties such as flavour, texture and colour (Barbut, 2006; Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017; Rhee, 1992; Ulu, 2004; Yusof & Babji, 1996). The main allergenic additives used are proteins from vegetables as soybean, pea, and lupin (Hoffmann et al., 2017; Leitner, Castro-Rubio, Marina, & Lindner, 2006; Toldrá & Nollet, 2016), or animal ones, such as egg white and milk (Montowska & Fornal, 2018, 2019; Spychaj, Pospiech, Iwańska, & Montowska, 2018; Stella et al., 2020).

Since it is very important to know about hidden proteins, we focused on the detection of bovine milk proteins in meat food products. The main aim was to verify the absence of allergenic proteins, declared or not on the food labels. A method based on reversed-phase liquid chromatography coupled to electrospray ionization and hybrid orbitrap high-resolution mass spectrometry (RPLC-ESI-HRMS) in conjunction with tandem MS was exploited. Once established the specific peptide

markers of α-S1-casein and β-lactoglobulin, representative of milk casein and whey fraction, respectively, protein recovery, matrix effect, precision, linearity, method variation, limit of detection (LOD), and limit of quantification (LOQ) were evaluated. The method allowed us to quantify the undeclared addition of milk proteins in samples of chicken and turkey sausages labelled as milk-free.

2 MATERIALS AND METHODS

2.1 Chemicals. Water, acetonitrile (ACN), methanol, hexane, formic acid, and ammonium bicarbonate were obtained from Sigma-Aldrich (Milan, Italy). All solvents used were LC-MS grade except for hexane (HPLC grade). Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), DL-dithiothreitol (DTT), iodoacetamide (IAA), α-casein from bovine milk, and β-Lactoglobulin from bovine milk were obtained from Sigma-Aldrich (Milan, Italy). Sequencing grade modified trypsin was from Promega Italia (Milan, Italy) while RapiGest surfactant was obtained from Waters Corporation (Milan, Italy). Skimmed milk powder was purchased from Fonterra (Fonterra, New Zealand) and contained a stated amount of 33 g of protein on 100 g of sample. Solid-phase extraction (SPE) C_{18} tubes were from Supelco (Milan, Italy). Standard solutions for mass spectrometer calibration were purchased from Thermo Scientific (Waltham, Massachusetts, United States). All meat foodstuff samples, *i.e.*, chicken/turkey, chicken and swine sausages, hamburger and pâté of cooked ham, and beef and pork pâté were purchased from local supermarkets.

2.2 Standard solutions, spiked samples, and fortified extracts

Calibration curves were in the range 0.10-2.5 μg by preparing milk powder solutions at five concentration levels (0.10, 0.25, 0.50, 1.0, and 2.5 μg) (Figure S1). To evaluate matrix effect, calibration curves were obtained for samples spiked before extraction. Specifically, the milk powder

148 solution was added to homogenized samples (#2 and #10) at the same concentration of standard
149 solutions (0.10-2.5 μg) (**Figure 1**) to cover a range of 20-500 $\mu g_{ing}/g_{matrix}$. To estimate the recovery
150 of the extraction method, fortified samples (#2 and #10) were obtained by adding the milk powder
151 solution to the sample protein extract at two concentration levels (0.1 and 2.5 μg) (Pilolli et al.,
152 2018).
153
154 **2.3 Protein extraction.** The protocols of extraction, digestion, and purification of the protein
155 fraction were first optimized on standard milk proteins. All samples (spiked and not) were cut into
156 small pieces and then homogenized by a mixer. 0.5 g of each sample was put in a 15 mL centrifuge
157 tube and 9.5 mL of 50 mM Tris-HCl were added. After a vigorous vortex, samples were incubated
158 for 1 h at 55 °C and vigorously shaken every 15 min for 1 min. Then, samples were cooled at room
159 temperature for 15 min and 0.5 mL of methanol were added and incubated for 10 min in an
160 ultrasound bath to facilitate proteins extraction. Afterward, 3.5 mL of hexane was added, and the
161 solutions were shaken to allow fat separation. Samples were centrifuged at 5000 *g* for 20 min and
162 the organic phase was discharged. 100 μL of aqueous solution were collected into a 0.5 mL
163 Eppendorf tube and dried under nitrogen (Pilolli, De Angelis, & Monaci, 2017; Pilolli et al., 2018;
164 Stella et al., 2020).
165
166 **2.4 Protein digestion**. The dried samples were resuspended in 100 μL of Rapigest (0.1 % w/v in
167 50 mM NH_4HCO_3); 10 μL of 50 mM DTT were added followed by incubation at 60 °C for 30 min. After
168 cooling, 10 μL of 150 mM IAA (were added and the samples were kept in the dark at room
169 temperature for 30 min. Subsequently, 5 μL of sequencing grade modified trypsin (0.1 μg/μL) were
170 added and the samples were incubated at 37 °C overnight. The enzymatic digestion was stopped by
171 the addition of formic acid (pH ca. 2) (Cosima Damiana Calvano, De Ceglie, Monopoli, & Zambonin,

2012). Alternatively, the digestion was carried out using a protocol where denaturation was performed by urea instead of RapiGest reagent. Although short digestion times were checked by a microwave-assisted protocol, the use of RapiGest alongside the overnight digestion guaranteed a good efficiency and reproducibility besides higher coverage of standard milk proteins.

2.5 Protein digest purification. Digest samples were dried under nitrogen and resuspended in 100 µL of 0.1 % formic acid. Tryptic digest purification was carried out using a homemade C_{18} SPE tip; specifically, 10 mg of C_{18} stationary phase, weighted from as disassembled commercial SPE tube, and dissolved in 100 µL of ACN were collected into a properly locked 200 µL tip and conditioned twice with 100 µL of 0.1 % formic acid. Then, the sample was loaded, and the tip was washed twice with 100 µL of 0.1 % formic acid. Elution was carried out with 50 µL of ACN/H_2O (70/30 v/v with 0.1% of formic acid); the eluate was dried under nitrogen and then resuspended in 50 µL of a solution having the initial mobile phase composition (H_2O/ACN 95/5 v/v with 0.1% formic acid) (Aresta et al., 2008; M. Planque et al., 2017; M Planque et al., 2019; Stella et al., 2020).

2.6 RPLC-ESI-MS instrumentation and operating conditions. An LC-MS platform was used, including an Ultimate 3000 UHPLC chromatographic station coupled to a quadrupole-Orbitrap spectrometer (Q-Exactive, Thermo Scientific, Waltham, MA, USA) equipped with a higher collisional-energy dissociation (HCD) cell by a heated electrospray ionization (HESI) source (Thermo Scientific). LC separation was performed at 40 °C using a Phenomenex Aeris WIDEPORE 200 Å C18 column (250 x 2.1 mm, 3.6 µm) equipped with Phenomenex AJO 8783 WIDEPORE C18 (2 x 2.1 mm ID) security guard cartridge. Reverse-phase separation was carried out using H_2O (solvent A) and ACN (solvent B) both containing 0.1% formic acid. The following gradient elution was used during each chromatographic run, with a flow rate of 0.200 mL/min: 0 − 2 min at 5% solvent B; 2 − 20 min linear

196 from 5% to 60% of B; 20 – 22 min linear from 60% to 100% of B; 22−26 min isocratic at 100% of B;
197 26−30 min back to the initial composition, followed by 5 min equilibration time. The ESI and ion
198 optic parameters adopted during acquisitions were the following: sheath gas flow rate, 10 (arbitrary
199 units); auxiliary gas flow rate, 5 (arbitrary units); spray voltage, 3.5 kV in positive polarity; capillary
200 temperature, 200 °C; S-lens radio frequency level, 100 arbitrary units. Positive MS full-scan spectra
201 were acquired in the *m/z* range 150–2500 with 70k of resolution using an automatic gain control
202 (AGC) target of $1x10^6$ and an injection time (IT) of 200 ms. The HCD MS/MS experiments, using an
203 inclusion list containing the marker peptides of each allergenic protein, were carried out, using
204 normalized collision energy (NCE) fixed at 30 with a 17.5k resolution, an isolation window of 2 *m/z*
205 unit, an AGC of $2x10^5$ and IT fill time of 100 ms. The Full-MS/ddMS^2 experiments were performed
206 using NCE fixed at 30 with a 17.5k resolution, AGC of $2x10^5$, IT fill time of 50 ms, isolation window
207 of 4 *m/z*, minimum AGC of $8.00x10^3$, and dynamic exclusion of 10 s. The control of the LC-MS
208 instrumentation and the first processing of data was performed by the Xcalibur software 2.2 SP1.48
209 (Thermo Scientific). Data processing of mass spectra was performed by SigmaPlot 14.5.
210 ProteinProspector (v. 6.2.2) software was used to perform database search of protein or peptides.
211 Proteome Discoverer (version 2.4, Thermo Fisher Scientific) was used to process Full-MS/ddMS^2
212 data.
213

214 # 3 RESULTS AND DISCUSSION

215 ## 3.1 Biomarker selection criteria

216 As already mentioned, to improve texture, color, flavor, and other organoleptic features, extraneous
217 proteins are commonly added to meat-based foodstuffs (Barbut, 2006)(Yusof & Babji, 1996). Yet,
218 milk proteins could accidentally occur also as “hidden allergens” due to cross-contamination during
219 the manufacturing processes. Using a reversed-phase liquid chromatography method coupled to

220 electrospray ionization and hybrid orbitrap high-resolution mass spectrometry (RPLC-ESI-HRMS), we
221 focused on the development of an analytical protocol for the quantitation of residual declared or
222 not (*i.e.*, milk-free labelled products) milk proteins in meat-based sausages, meat pâté, and
223 hamburger of cooked ham. Two recognized allergic proteins *i.e.*, α-S1-casein and β-lactoglobulin,
224 were chosen as representative, respectively, of milk caseins and whey proteins. Typically, the
225 identification of proteins is carried out by searching for marker peptides deriving from tryptic
226 digestion (Pilolli et al., 2020), thus exhibiting the following features: uniqueness for each protein,
227 stability, absence of chemical modifications, no missed cleavages during enzymatic digestions, more
228 than six amino acids in their sequence, and doubly/triply charged ions (Johnson et al., 2011; Mills et
229 al., 2019). For each allergen protein, a *qualifier* and *quantifier* marker peptides are designated;
230 whilst the first one is employed for unequivocal identification, the second one is chosen for its
231 quantification (Monaci, Pilolli, De Angelis, & Mamone, 2015). In the case of α-S1-casein and β-
232 lactoglobulin, two unique peptides were designated to ensure confidence in the identification of
233 both allergens (Lutter, Parisod, & Weymuth, 2011; L. Monaci et al., 2011; Monaci, Losito, Palmisano,
234 & Visconti, 2011; Parker et al., 2015; Pilolli et al., 2018; M. Planque et al., 2017; Mélanie Planque,
235 Arnould, & Gillard, 2017). Amino acid sequences and mass-to-charge (*m/z*) ratios of marker peptides
236 for α-S1-casein and β-lactoglobulin are reported in **Table 1**. In both cases, the list of selected
237 peptides was refined by removing peptide sequences susceptible to reactions (*e.g.*, post
238 translational modification, oxidation, deamidation, Maillard reaction) during food processing.
239
240 **3.2. Database search and identification of selected peptides**
241 Whilst in plot A of **Figure 2** is shown the extracted ion current (XIC) chromatogram of marker
242 peptides of α-S1-casein at *m/z* 634.355^{2+} and 692.868^{2+}, peaks 1 and 2, respectively, in plot B are
243 displayed peak 3 at *m/z* 623.295^{2+} and peak 4 at *m/z* 533.294^{2+}, referred to designated peptides of

β-lactoglobulin, as obtained upon tryptic digestion of a milk powder solution. Peaks 1 and 2 (plot A) are respectively referred to as qualifier and quantifier marker peptides of α-S1-casein, and peaks 3 and 4 (plot B) are related to quantifier and qualifier peptides of β-lactoglobulin. The amino acid sequences of these peptides were confirmed by database search using Protein Prospector MS-Tag and tandem MS spectra resulting from high-energy collision dissociation (HCD) following RPLC-ESI(+)-FTMS. **Figure 3** illustrates the tandem MS spectra of the doubly charged qualifier and quantifier peptides of α-S1-casein at *m/z* 634.355^{2+} and 692.868^{2+}, plots A and B, respectively. Database search was accomplished by selecting the SwissProt.2017.11.01 database, trypsin as the enzyme with up to two missed cleavages, *Bos taurus* as taxonomy, carbamidomethylation (C) and oxidation (M) respectively as a constant and a probable modification, 2+ as precursor charge, and 10 ppm as tolerance for *m/z* ratios of both precursor and product ions. The database search returned as output the following amino acid sequences, YLGYLEQLLR and FFVAPFPEVFGK with a matched intensity of 100%, based on the recognition of typical peptide product ions like those of *y*, *b* and *a* series, and also of the immonium ion of the amino acid at the *N*-terminus and internal fragments. The complete product ions assignment is listed in **Table S1**.

The same rationale was successfully applied to qualifier and quantifier peptide markers of β-lactoglobulin, at *m/z* 533.294^{2+} and 623.295^{2+}, respectively, as reported in **Figure 4**. The database search of amino acid sequences led to recognize VLVLDTDYK (qualifier peptide) and TPEVDDEALEK (quantifier peptide) with a matched intensity of 94% and 99%, respectively (see **Table S2** for the comprehensive attributions). In all plots of both **Figures 3** and **4**, the detection of the most intense a_2/b_2 pair ions together with *y*-type, *b*-type internal, and immonium ions, resulting from HCD fragmentation, was highlighted since it represented a further confirmation of the amino acid sequence obtained through database search (Michalski, Neuhauser, Cox, & Mann, 2012).

3.3. Method validation

The experimental conditions used to choose and identify the marker peptides of bovine milk proteins were applied to spiked meat samples. It is easily perceivable that there is the need of establishing a series of parameters for all these marker peptides, such as linearity and limits of detection (LOD) and quantification (LOQ), both expressed as $\mu g_{ing}/g_{matrix}$. Specifically, spiked samples were prepared adding standard milk powder in the concentration range of 0.1-2.5 µg (referred to as 20-500 $\mu g_{ing}/g_{matrix}$), and calibration curves were obtained by interpolating peak areas of quantifier peptide versus concentration. LOD and LOQ were calculated as three- and ten-fold, respectively, the intercept standard deviation divided by the slope of the calibration curves (Miller & Miller, 2010). In **Table 2** are summarized the calibration data of the quantifier marker peptides of α-S1-casein and β-lactoglobulin as obtained after spiking samples #2 and #10 listed in **Table 5** (chicken/turkey sausages and pâté of cooked ham, respectively). Sample #2 was chosen for the quantitation of samples from #1 to #8 while sample #10 was used for the quantitation of samples from #9 to #12. The proposed analytical method allowed us to obtain LOD and LOQ values equal to 3.8 and 13 $\mu g_{ing}/g_{matrix}$ for α-S1-casein and 6.3 and 21 $\mu g_{prot}/g_{matrix}$ for β-lactoglobulin, respectively. The determination of each marker peptide permits the quantitation of the individual marker proteins and by applying conversion factors the resultant determination of the total milk protein content (TCMP) in the starting meat product. The conversion from μg_{ing} to μg_{prot} can be obtained considering that the protein content of standard milk powder is equal to 33% w/w. Then, the TCMP can be calculated by the formula $w_{TCMP} = w_j * CF_j$ as very recently reported by Martinez-Esteso et al. (Martinez-Esteso et al., 2020), where w_{TCMP} is the mass fraction of TCMP in the sample, w_j is the mass fraction of the *j*th marker protein in the sample and CF_j is the conversion factor accounting for the contribution of the *j*th marker protein to the total cow's milk protein. CFs are tabulated for milk proteins (Martinez-Esteso et al., 2020). These data suggested the detection and quantification of

292 milk proteins in very low abundance, including hidden allergens in meat-based foodstuffs (*vide*
293 *infra*). **Figure 5** shows the XIC chromatogram of both quantifier peptides for α-S1-casein and β-
294 lactoglobulin in spiked samples at the lowest concentration level at 20 $\mu g_{ing}/g_{matrix}$. As can be seen,
295 also at the lowermost level, marker peptides are still detectable, suggesting that the protocol may
296 be effective in discovering the cross-contamination occurring during the processing of meat-based
297 products.
298 The evaluation of recovery and matrix effects was assessed by using the pâté of cooked ham and
299 chicken/turkey sausages as matrices, appropriately spiked with milk powder. Specifically, the matrix
300 effect was calculated by computing the ratio between the slopes of the calibration curves obtained
301 for quantifier peptides in spiked samples and milk powder aqueous solutions (Pilolli et al., 2018).
302 The recovery was estimated as the average ratio of peak areas of quantifier peptides obtained for
303 the spiked samples and the extract of the original ones subsequently spiked with milk powder at the
304 same concentration. Two concentration levels were selected (0.1 and 2.5 μg) and two different sets
305 of experiments were carried out, including or not purification by SPE (Pilolli et al., 2018). The
306 resulting data are summarized in **Table 3;** as indicated in the fourth column, the purification step
307 was not critical or detrimental in the recovery of the whole strategy. As far as the matrix effect, its
308 value was not surprising. The co-elution of a meat protein and milk protein marker peptides and
309 their competition for ionization can be expected, thus leading to lower XIC peak areas compared to
310 those obtained for milk powder solutions. Moreover, a lower tryptic digestion yield for milk
311 proteins, when much more abundant meat proteins are also present, might lead to a lower content
312 of marker peptides. Since the matrix effects are rather similar between both #2 and #10 samples,
313 they were fully representative of all the samples for quantitative purposes. These results fully
314 demonstrate that provided a matrix-matched calibration is performed, the proposed method can
315 achieve good sensitive and reliable quantification of milk proteins in meat-based samples.

316 The analytical repeatability and reproducibility including extraction, digestion, and purification steps
317 of milk proteins alongside the stability of milk tryptic marker peptides were also assessed by
318 analyzing three independent extracts of sample #10, preliminarily spiked at a concentration level of
319 200 $\mu g_{ing}/g_{matrix}$ and injecting each sample three times for five working days. The intra-day and inter-
320 day variabilities were evaluated both within and between spiked samples for each milk marker
321 peptide, calculating the RSD values on peak area obtained from XIC chromatograms. The RSD values
322 established for the selected quantifier and quantifier marker peptides of α-S1-casein and β-
323 lactoglobulin are reported in **Table 4**. Note that the intra-day within sample (repeatability) were the
324 lowest obtained, suggesting that the instrumental variability was negligible during a specific day.
325 Conversely, the intra-day between samples reached higher values, especially for α-S1 casein
326 peptides (see **Table 4**), since they account for the overall variability, including extraction, digestion,
327 purification, and analysis. The comparison of variabilities obtained within and between samples in
328 a specific day clearly indicates that the critical stage of the method relies in sample preparation
329 more than analysis. Apparently, the time elapsing between sample preparation and analysis
330 exhibited a limited effect on the variability, since the inter-day values were not much higher than
331 intra-day ones (see rows #1 and #3 in Table 4). Accordingly, RSD values of inter-day between
332 samples and intra-day between samples were comparable. This outcome was confirmed by one-
333 way ANOVA at 95% confidence level performed on all the qualifier and quantifier milk marker
334 peptides, focusing on the day of analysis as the variable factor, thus considering data obtained from
335 all the three samples in a specific day as belonging to the same group. Finally, the short-term and
336 long-term stabilities were assessed on spiked samples after three and six months of storage at 4 °C.
337 Apparently, the content of marker peptides was on average decreased approximately three times
338 upon six months of storage, thus suggesting that these compounds are prone to
339 modification/degradation.

3.4. Identification of milk proteins in meat-based samples

The occurrence of hidden milk allergen proteins was investigated in sausages, meat pâté, and hamburgers of cooked ham. **Table 5** lists all investigated samples, including those possessing the label of milk-free and/or lactose-free. An interesting and important observation of meat-based foodstuffs was that 5 out of 12 samples were labelled as milk-free. Whereas just one sample, among the other 7 ones, stated the addition of milk, the remaining 6 samples did not display explicit hints of its presence. To assess the truthfulness of the label declaration, these samples were subjected to the developed analytical protocol, including protein extraction, digestion, purification, and RLPC-ESI(+)-FTMS analysis as described in the previous sections. Although not labelled as milk-free, samples #1, #6, #7, and #10 did not show peak signals above the LOD of marker peptides of milk allergenic proteins (*vide infra*). Surprising results were observed with the other meat-based foodstuffs, all exhibiting the occurrence of peak signals at *m/z* 634.355^{2+} and 692.868^{2+}, corresponding to qualifier and quantifier peptides of α-S1-casein. This outcome was validated by the correspondence of retention time of marker peptides and HCD tandem MS spectra (*vide infra*). Examples of XIC chromatograms obtained for marker peptides from samples in which the absence of milk allergens was stated (sample #4) and the presence of milk was declared (sample #9) are displayed in plots A and B of **Figure 6**, respectively. As expected for sample #9, an abundant content of milk proteins was proved. The chromatographic plots of samples #1, #2, #3 and #5, #8, #11 are illustrated in Figures S2 and S3 (Supplementary Material), respectively. Even though not labelled as such, sample #1 of chicken and turkey sausages was ascertained as milk-free (see plot A of **Figure S1**).

Besides accurate *m/z* and retention time values, the identity of marker peptides of α-S1-casein in all samples was confirmed by tandem MS spectra. To guarantee the high sensitivity needed for low

abundant species (Kaufmann, 2020), parallel-reaction monitoring with the Orbitrap analyzer, equivalent to multiple reaction monitoring normally employed on triple quadrupole instrumentation for targeted analyses, was adopted. As an example, **Figure 7** shows the tandem MS spectra of ions at *m/z* 634.355^{2+} and *m/z* 692.868^{2+} of sample #11. The former ion was recognised by Protein Prospector software as the qualifier peptide for α-S1-casein, i.e., YLGYLEQLLR, with a matched intensity of 96%. Besides the immonium ion related to tyrosine, representing the *N*-terminus peptide (136.076), fragment ions corresponding to *y*-type product ions, like y_1 (175.119), y_4 (529.344), y_5 (658.386), y_6 (771.468), y_7 (934.530),y_8 (991.3546) and y_9 (1104.634) and the a_2-b_2 pair (249.159-277.155) were recognized in **Figure 7A**. Plot B of the same figure shows the fragmentation spectrum of the quantifier marker peptide FFVAPFPEVFGK, at *m/z* 692.868^{2+}, identified by 100% of correspondence. The immonium ion related to phenylalanine at the *N*-terminus (120.080) and the following product ions: y_2 (204.135), y_3 (351.203), y_4 (450.272), y_6 (676.367), y_7 (823.430), y_8 (920.475), y_9 (991.516) and a_2-b_2 pair (267.149-295.144), were detected. All the product ions of plots A and B of **Figure 7** are summarized in **Table S3**. An additional example of tandem MS of qualifier and quantifier peptides for α-S1-casein referred to sample #4, is given in Figure S4 (Supplementary Material).

It is worthwhile mentioning that, except for the hamburger of cooked ham (sample #9), the absence of β-lactoglobulin, representative of whey proteins, was ascertained in all investigated samples. This anomalous outcome may suggest either the cross-contamination or the intended addition of caseinates, rather than whole milk, of samples #2, #3, #4, #5, #8, #11, and #12 (*SAFETY ANALYSIS OF FOODS OF ANIMAL ORIGIN*, n.d.; Yusof & Babji, 1996). To affect the water-retention and to avoid the occurrence of defects (Barbut, 2006; Gujral et al., 2002; Hoffmann et al., 2017; Rhee, 1992; Schilling et al., 2004; Toldrá & Nollet, 2016; Ulu, 2004; Yusof & Babji, 1996; Zorba et al., 2005), sodium/calcium caseinates are used as powder additives of sausages and other meat-based

388 foodstuffs. More, it should be considered that heat treatment is commonly applied to sausages and
389 meat-based products during industrial processing to ensure their microbial safety as well as to
390 extend shelf life. A problem of whey proteins during food treating is their instability to thermal
391 processing, which leads to their denaturation, aggregation, and, under some conditions, gelation
392 (Wijayanti, Bansal, & Deeth, 2014). These heat-induced changes in the physicochemical properties
393 of the β-lactoglobulin could make it less available for extraction and therefore difficult to detect.
394 Cross-contamination due to an inaccurate cleaning of the equipment used during the production
395 processes can occur if, in the same factory, foods containing caseinate among the ingredients are
396 processed (RÖDER et al., 2008). To rule out cross-contamination, we considered meat-based
397 foodstuffs of the same company but related to different factories and/or production batches, such
398 as samples Bb_1, Bb_2, Bc_1, Lm_1, and Lm_2 (see **Table 5**); herein, capital letters indicate the company,
399 lowercase letters indicate the factory, and the subscript number indicates different production lot
400 of the same factory. Unfortunately, the presence of caseins in meat-based products of the same
401 company in different processing plants was confirmed. Since the presence of the same type of cross-
402 contamination in different plants is unlikely, this finding was particularly striking for milk-free
403 labelled samples, suggesting a systematic use of caseinates, although in low amounts. The presence
404 of caseins in a product declared as milk-free is a serious health risk for consumers suffering from
405 milk-related allergies.
406
407 **3.5. Quantitation of milk protein in meat samples**
408 Whether or not meat-based foodstuffs were labelled as milk-free, the quantitation of milk proteins
409 was carried out analysing in triplicate the tryptic digests of protein extracts of all investigated
410 samples (see **Table 5**). Peak areas of the quantifier peptides of α-S1-casein and β-lactoglobulin were
411 calculated from XIC chromatograms after each RPLC-ESI(+)-FTMS analysis and used for quantitation

purposes, while calibration curve parameters of spiked samples were used to determine the concentration of milk proteins in meat foodstuffs as $\mu g_{ing}/g_{matrix}$ and applied to all samples. The content of milk proteins in each sample is reported in **Table 6**. As a comparison in Figure S5 we reported the XIC chromatograms of qualifier and quantifier marker peptides of α-S1-casein at *m/z* 634.355^{2+} and 692.868^{2+} in milk powder standard solution at 0.5 μg_{prot} (A), in sample #2 of chicken/turkey sausage spiked at 100 μg_{ing} (B) and in sample #11 of beef and pork pâté (C) without further addition. Note that in samples #5 and #8, the quantifier peptide of α-S1-casein was detectable, but the relevant peak area was below the limit of quantitation.

Sample #9 is the only meat-based product that is not declared as milk-free; it was then not surprising that both α-S1-casein and β-lactoglobulin exhibited relatively high content of markers peptides and the only sample in which β-lactoglobulin was also detected. Considering the VITAL program guidelines (Monaci et al., 2020), the reference dose for milk is fixed as 0.2 mg of protein. Using a reference 100 g amount of meat product consumed by an adult, it is possible to define the action level equal to 2 μg/g. The concentration values, expressed as $\mu g_{TCMP}/g_{matrix}$ considering an average content of 35% of proteins in milk ingredient, found for samples #2, #3, #4, #11 and #12 are relatively higher than the above-fixed action level, so the presence of milk proteins should be evidenced in the product label to warn and protect sensitive people. Conversely, the milk-free label was reported in 3 out 5 products (see Table 5), thus exposing allergic subjects to serious health risks.

4 CONCLUSIONS

An LC-HRMS method for the identification and quantitation of allergenic milk proteins in complex meat-based foodstuffs, based on protein extraction, tryptic digestion, and peptide analysis, was developed. The work was carried out by using two designed marker peptides of α-S1-casein and β-lactoglobulin. Good recovery, precision, linearity, limit of detection, and limit of quantification

allowed us to quantify undeclared milk proteins, known as “hidden allergens”, on several meat-based samples. These foodstuffs with misleading milk-free labels were investigated and a milk protein content up to 10-fold greater than the action level of allergic ingredients was found. Since even limited exposures of sensitive consumers can provoke significant allergic reactions, the European regulation on allergen indications in food labels needs a revision and a harmonious revision of PAL is mandatory.

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Conflict of interest statement

The authors have declared that no competing interest exists.

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