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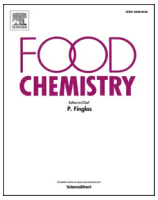

Safe food through better labelling; a robust method for the rapid determination of caprine and bovine milk allergens

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ABSTRACT

Accidental milk cross-contamination is one of the most common causes for costly food recalls. Yet, quantifying trace-levels of allergen is time-consuming and current methods are not adapted for routine analyses making quality control for trace-level allergen content impractical. This perpetuates voluntary "may-contain" statements that are unhelpful for people suffering from food allergies. Here, we developed a rapid LC-MS method enabling milk allergen quantification by comparing all tryptic-peptides of major milk allergens. The bovine-specific αS-2 casein peptide and allergen-epitope NAVPITPTLNR provided excellent performance in sensitivity (LOD 1 mg.kg^{-1}; LOQ 2 mg.kg^{-1}) across various dairy products, good recovery rates in baked croissants (77% with a 10% inter-day RSD) and a linear range of 2–2,000 mg.kg^{-1}. The method can be used for routine determination of trace-contamination with bovine milk allergen and the adulteration of high-value caprine dairy products with lower-value bovine milk products, protecting consumer trust and the growing population suffering from food allergies.

1. Introduction

Food allergy causes a substantial public health burden, with the highest prevalence reported in Australia at 10% and other developed countries varying between 1 and 5%, (Renz et al., 2018; Warren et al., 2020). Almost 90% of all food allergies are caused by only eight food types (i.e., peanut, tree-nut, milk, egg, soy, shellfish, fish and wheat) (Villa et al., 2018). Of these, milk allergy is particularly burdensome. Milk is an ingredient in many products (yoghurt, cheeses, baked goods, and snacks) making avoidance very difficult for the allergic population. Milk allergy reactions can be severe with milk being responsible for 10–19% of all food-allergy induced anaphylactic reactions (Zhang et al., 2022). The Food Standards Australia and New Zealand (FSANZ) and the European Rapid Alert System for Food and Feed (RASFF) databases show undeclared allergens are the number one reason for product recalls, with milk listed as the most common undeclared allergen, responsible for ~30% of all food recalls (FSANZ, 2022)'(Martínez-Pineda & Yagüe-Ruiz, 2022). This is likely due to the common use of milk in mixed and processed foods (FSANZ, 2022). Cross-contamination in allergen-free food can be very costly to industry — $10–30 million US dollars on average per event — due to the effort associated with the recall, the losses in brand trust and supplier relationships (GMA, 2011). In the USA, these costs add-up to $2–3 billion per year (USDA/FDA, n.d.). Accidental cross-contact is one of the most prominent causes for undeclared allergen food recalls by FSANZ and RASFF (FSANZ, 2022; Martínez-Pineda & Yagüe-Ruiz, 2022). Cross-contact is mainly caused by ineffective cleaning of machinery and equipment, premises design and employee training and, is difficult to detect due to the low levels of allergen involved (Martínez-Pineda & Yagüe-Ruiz, 2022). Companies frequently use precautionary allergen labelling statements to label products that are at risk of cross-contamination. However, these statements may be mainly intended to protect manufacturers rather than consumers since they contain no clinically relevant limits (Tsagkaris et al., 2019). A voluntary incidental trace allergen labelling (VITAL) program does provide a quantitative basis for precautionary allergen labelling. The VITAL 3.0 reference dose of milk allergen is 0.2 mg

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protein (Madsen et al., 2020; Voluntary Incidental Trace Allergen Labelling, n.d.). This dose relates to the amount predicted to provoke reactions in 1% of the allergic population. Using an estimated 36% protein in milk powder, this leads to a reference dose of 0.6 mg milk powder (*Voluntary Incidental Trace Allergen Labelling*, n.d.). Considering 100 g of baked goods as an upper limit of what can be expected to be consumed in one portion, this translates to a threshold of approximately 6 $mg.kg^{-1}$. Thus, 99% of the allergic population can be shielded from very restrictive diets if legislation is adopted that restricts precautionary allergen labelling statements and requires allergen free products to maximally contain 6 $mg.kg^{-1}$ milk allergen. A reliable analytical method is required to quantify milk allergen at >6 $mg.kg^{-1}$ total milk to implement such legislation.

There are six major milk allergens, two of which are the major whey components (alpha- and beta-lactalbumin) and four of which are the major casein components (alpha-s1- alpha-s2-, beta- and kappa-casein). Together, these proteins make up about 95% of the total protein content of milk (Croote & Quake, 2016). The lactalbumins cause an allergic reaction in a minority of the milk allergic (5–10%) while the caseins cause an allergic reaction in 50–93% of all milk allergy, depending on the specific casein (Cerecedo et al., 2008; Matsuo et al., 2015). Bovine serum albumin, lactoferrin and immunoglobulins are minor milk allergens that are less present in milk (Conesa et al., 2005; Fiocchi et al., 2010; Linhart et al., 2019; Ueno et al., 1994). There are many different epitopes (both linear and conformational) identified for the major milk allergens, running all along the amino acid chains. The characteristics of these milk allergens and their biological function are summarised in Table S1.

Currently, immunoassays (ELISA) are used for routine food allergen quantification in quality control facilities. These assays may suffer from reduced sensitivity and cross-reactivity in processed foods due to protein modifications and degradation. This leads to the loss or modification of the recognised epitopes, causing variation in method accuracy (Marsh et al., 2020; Parker et al., 2015). Liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM-MS) is a promising technique for allergen monitoring that does not suffer from cross-reactivity issues and has excellent multiplexing potential. Moreover, LC-MRM-MS based allergen quantification may be used as a confirmatory method to validate on-site measurements with portable devices such as smartphone-based detection systems (Ross et al., 2018). Recently, it was shown that allergens can be quantified directly via LC-MRM-MS from rapid antigen tests in under three hours following an innovative extraction protocol (Nelis et al., 2022). The describe pipeline is interesting since it may provide the food industry with a rapid, secure and cost-efficient manner to safeguard allergen free products from cross contamination since it limits the number of LC-MRM-MS analyses required and enables testing at multiple nodes in the food supply chain while assuring visibility of test results for all stakeholders. Nevertheless, the LC-MRM-MS methods reported in the literature do not yet reach the sensitivity, robustness and analyses times required to enable routine analyses for milk allergens. Gomaa et al., reported an LOD of 10 $mg.kg^{-1}$ for α-, β- and *k*-casein (Gomaa & Boye, 2015). Croote et al., reached better sensitivity with an LOD of 5 $mg.kg^{-1}$ for an α-S1-casein peptide (YLGYLEQLLR) and developed an algorithm that enabled matrix dependent interference correction (Croote et al., 2019). However, the method is complex and has a total analyses time of >14 h. Gu et al., reached an LOQ well below 6 $mg.kg^{-1}$ (0.2–0.4 $mg.kg^{-1}$), but the method requires ~18 h and uses Tris-HCl for the protein extraction (Gu et al., 2018), which can cause differences in extraction efficacy (Nelis, Broadbent, Bose, Anderson, & Colgrave, 2022). Matrix-matched calibration and filter aided sample preparation (FASP) has improved LC-MRM-MS based milk allergen determination in processed food (Boo et al., 2019; Xiong et al., 2021). However, FASP protocols are laborious and not fit for routine analyses. Importantly, Boo et al., noted that heat processing altered the relative abundance profile for α-S1 casein peptides YLGYLEQLLR and FFVAPFPEVFGK. Thus, an analyses of milk allergen peptide performance in baked goods may be helpful to determine peptide choice for a quantitative method. Finally, Planque et al., has developed a faster (~4h) protocol for the sensitive (~0.5 $mg.kg^{-1}$) detection of milk and other allergens in a variety of matrices including baked goods (Planque et al., 2017, 2019). Yet, this analysis time does not include the required lengthy concentration step (via evaporation) to reach this sensitivity level.

Various LC-MRM-MS methods have been developed for the quantification of trace-level amounts of milk allergens in processed foods, but none show the required combination of sensitivity, robustness and short analysis time needed for routine adoption. Thus, there is a need for further technological development enabling rapid and robust sample processing for LC-MRM-MS protein quantification in the food allergen section. Indeed, if a rapid and robust LC-MRM-MS method is developed it may be adopted in routine laboratories enabling accurate quantification of cross-contamination (e.g., via processing machinery). Such analyses can provide valuable data to the food industry, enabling food processors to limit the risk of costly, and sometimes life-threatening, contamination and recall events. This is particularly true if the developed method is sensitive enough to determine allergen concentrations below the 6 $mg.kg^{-1}$ threshold. Moreover, only a few peptides from one or two milk allergens are generally used for the method development. This is suboptimal given the potential difference between peptides in robustness towards signal variation caused by food processing. Besides, these peptides are generally conserved across mammalians making the detection of, high-value caprine dairy product adulteration with cheaper bovine milk unfeasible. In this work, a rapid (~2h) protocol was developed for milk allergen quantification. Tryptic peptides of all major milk allergens were compared for robustness towards food processing effects. The peptides were also assessed for their ability to detect adulteration of caprine dairy products via both bioinformatic and empirical methods. Finally, the method's sensitivity, repeatability and recovery were determined to quantify milk powder in baked croissants for the top performing peptides.

2. Materials and methods

2.1. Materials

Instant full cream milk powder, full cream pasteurised cow and goat milk, sour cream, Greek yoghurt, normal and ruby red cheddar, camembert, parmesan, authentic Greek feta made from sheep and goat milk and feta made from cow milk were bought from a local grocery store.

2.2. Matrix preparation

Croissant dough was prepared following an online recipe (Pierre Herme, 2012). Briefly, 6 g dry yeast, 250 g all-purpose flour, 6 g fine sea salt, 37.5 g sugar, 10 g milk powder, 72.5 g water and 100 g unsalted butter was added together until a homogeneous mixture was obtained. This resulted in croissant dough with 20.7% fat and 2.1% milk powder. The dough was risen for one h in a large glass bowl covered with a wet cloth, divided in 100 g batches and rolled out into triangular sheets of approximately 5 mm thickness that were rolled up to croissants and baked for 20 min at 180 °C in an air-heated oven with mechanical convection. Croissants were allowed to cool and weighed. A 3.25% weight loss due to moisture loss during baking was determined on average and corrected for to enable fair comparison between cooked and raw dough.

2.3. Protein extraction and defatting

Protein was extracted following Nelis et al., with minor modifications (Nelis et al., 2022). Briefly, 1 mL extraction buffer (2 M urea; 50 mM DDT) was added to 100 mg homogenised sample and the mixture

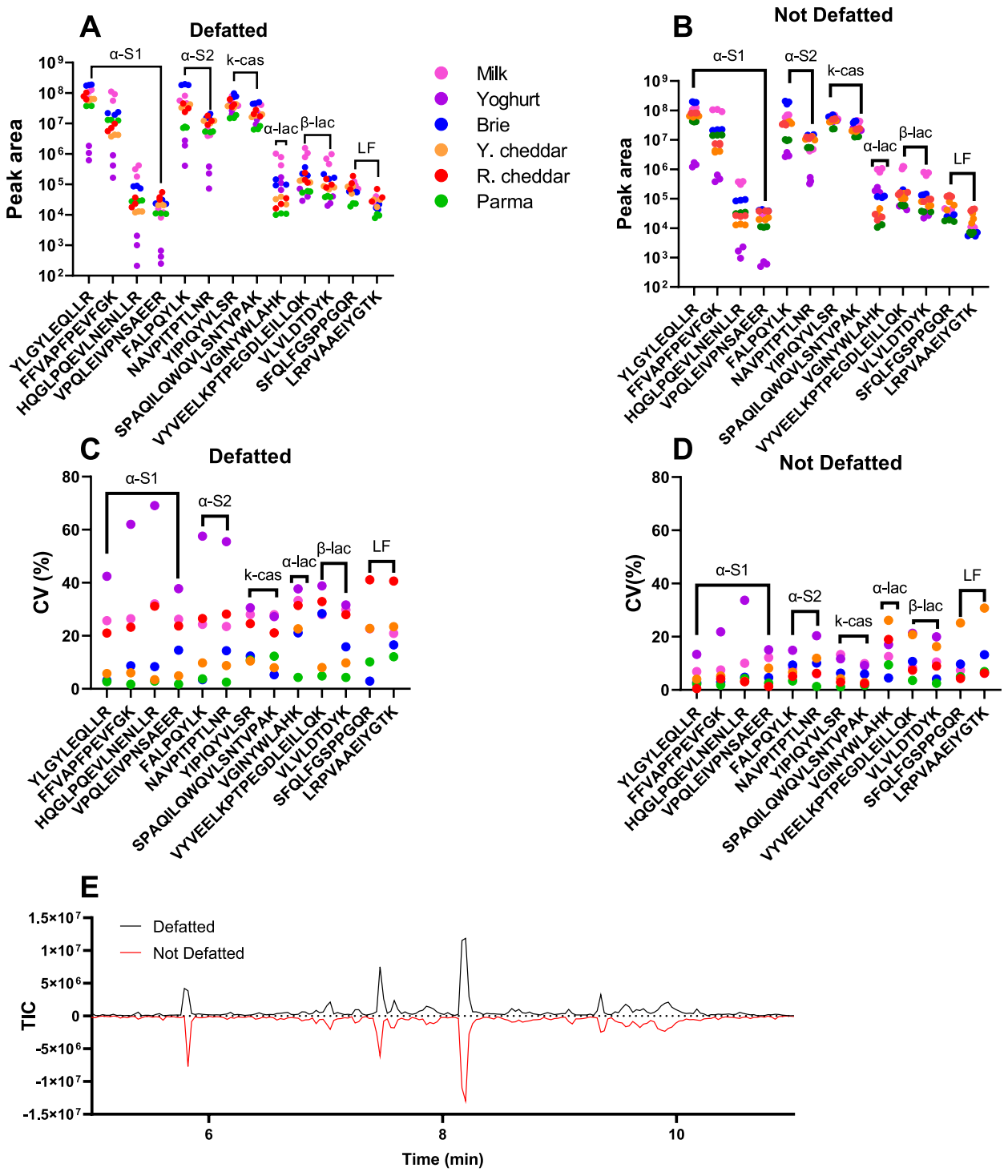

Fig. 1. Method optimisation for candidate milk allergen peptides using LC-MRM-MS. Peak areas (n = 3) for the summed three most intense transitions of peptides for casein, lactalbumin and lactoferrin extracted from various defatted (A) and non-defatted (B) dairy products. (C) and (D) show the CVs calculated over the peak area replicas per dairy type for the defatted (A) and non-defatted samples respectively (B). (E) The summed total ion chromatogram (TIC) of a series of neutral loss and precursor ion scans diagnostic for glycerophospholipids in defatted and non-defatted full cream milk digests.

was thoroughly vortexed followed by sonication (10 min) and 30 min incubation on a shaker plate at room temperature. Samples were then centrifuged at 20,800×*g* for 15 min. All protein extracts were brought to a 2 mg.mL^{-1} total protein concentration in extraction buffer following the protein content estimates indicated on the product packages. Next, 100 μL of these solutions was defatted by adding 400 μL ice-cold (kept at −80 °C) acetone to it and incubated for 1 h at −80 °C. This mixture was centrifuged at 20,800×*g* for 15 min, the supernatant discarded, and the protein pellet allowed to air dry for 20 min. The pellet was reconstituted in 100 μL extraction buffer. Next, proteins were digested by adding 50 μL (~100 μg protein) of this solution to 49 μL protein digestion buffer (100 mM ammonium bicarbonate (ABC), pH 8.0, containing 1 μg sequencing grade trypsin from Promega, USA) and incubating for 1 h at 37 °C on a plate shaker (600 RPM). For comparison, 50 μL of the non-defatted 2 mg.mL^{-1} protein extracts was equally digested by adding 49 μL digestion buffer and incubating for 1 h at 37 °C on a plate shaker (600 RPM).

Digestion was stopped in both cases by the addition of 1 μL 100% formic acid (FA). Digests were filtered by centrifugation (5 min; 20,800×*g*) using a 0.45 μM spin-filter (Spin-X, Costar, Saltlake City, USA) and stored at −80 °C until use.

2.4. Liquid chromatography and mass spectrometry analyses

Digested peptides (1 or 5 μL) were sampled with a Shimadzu Nexera UHPLC system equipped with a Kinetex C18 column (length 100 × 2.1 mm; particle size 1.7 μM; pore size 100 Å) for chromatographic separation using a 0.4 mL min^{-1} flowrate. The solvents used were: Solvent A (MQ with 0.1% FA) and solvent B (90% acetonitrile, 0.1% FA). Chromatographic conditions are given in % B and were as follows: Isocratic (5%) for 0.2 min then increased to 45% over 10 min followed by a rapid (0.8 min) increase to 80%, then kept isocratic for 1 min. The %B was then dropped to 5% in 0.1 min and kept isocratic for another 2.9 min. The peptides that eluted were analysed using a 6500 QTRAP (SCIEX) following (Nelis et al., 2022). Briefly, the voltage and temperature applied to the electrospray ionisation source were 5.5 kV and 500 °C. Curtain and GS1 gas flow was 35. GS2 gas flow was 40 (arbitrary units). An 80 eV declustering potential was applied and the collision energy was varied per transition following SCIEX rolling collision energy equations.

2.5. Scheduled multiple reaction monitoring (sMRM)

The peptide sequences for the lactalbumins (alpha- and beta-lactalbumin), the four caseins (alpha-s1- alpha-s2-, beta- and kappa-casein) and lactoferrin were obtained from UniProt and imported into Skyline (MacLean et al., 2010). Peptides (of length 8–25 amino acids) were *in-silico* digested. No missed cleavages or structural modifications were allowed and peptides containing cysteine or methionine were excluded. The top five highest intensity monoisotopic y and b product ions (1 and 2+) were predicted from 2 and 3+ precursors using the Prosit deep-learning algorithm (Gessulat et al., 2019) with a library tolerance of 0.5 *m/z*, a match tolerance of 0.055 *m/z* and the Normalised Collision energy (NCE) set at 33 (NCE was empirically determined to best match the SCIEX Instrumentation). For the initial sMRM method retention times (RTs) were predicted in Skyline using Prosit software and synthetic peptides with an indexed RT (Biognosys) following (Escher et al., 2012). Initially the sMRM detection window was kept large (60 s) and samples (digested full cream milk) were run in triplicate. Accurate RTs were then calculated from these results and used in the final sMRM method which had a 30 s detection window and a 1 s cycle time. MS^2 peak groups were integrated using Skyline. For optimisation experiments the peak areas of the three most intense co-eluting transitions were summed. For the final method this was reduced to the two most intense co-eluting transitions (a qualifying and quantifying ion). An ion ratio (IR) (qualifying ion to quantifying ion) was used in combination with the RT to unambiguously identify peptides. For the IR acceptance the following variance was tolerated: 20% if IR > 0.5, 25% if 0.2 < IR < 0.5, 30% if 0.1 < IR < 0.2. For the LOQ and LOD a signal-to-noise ratio (S/N) > 3 and 10 was used respectively. The quantifying peak was integrated for quantification if the IR, RT and S/N criteria were fulfilled.

2.6. Neutral loss and precursor ion scan

A lipid scan was conducted to determine the efficacy of the defatting protocol. For this we created a method combining neutral loss (NL) scans and precursor ion scans (PIS) that are diagnostic for glycerophospholipids, an abundant class of phospholipids in milk (Contarini & Povolo, 2013). NLs were performed at 141.0, 185.0, 189.0, 115.0 and a PIS at 184.1 *m/z* to scan for phosphatidylethanolamines, phosphatidylserines, phosphatidylglycerols, phosphatidic acid and phosphatidylcholines, respectively, following (Brodesser, 2017; Holčapek et al., 2018). Q1 scans were conducted over the *m/z* 650–850 and collision energy was ramped between 25 and 35 eV while the scan rate was kept at 1000 amu s^{-1}. Source temperature, voltage, gas-flow and chromatography conditions were kept as above.

2.7. Calibration curves, recoveries

For calibration curves, peptides were prepared at concentrations spanning 2,000–0.5 $mg.kg^{-1}$ in both raw and baked croissant dough with a 1 and 5 μL injection. The diluent used was ABC mixed with protein extraction buffer at a 1:1 ratio and all samples were acidified (0.1% FA) prior to analyses. Recoveries were determined for baked croissants using 5, 10, 20, 40 and 80 $mg.kg^{-1}$ milk powder (in triplicate) and interpolating the peak areas on a calibration curve. Recovery experiments were performed four times spread-out over one month (one a week) and results were averaged to determine inter-day repeatability.

2.8. Software and statistics

For spectral analyses and method development, we used Analyst v1.7.1 and Skyline v21.1.0.146. GraphPad 9.2.0 was used for statistical analyses. Inkscape 0.92.5 and GraphPad 9.2.0 were used for figure preparation and assembly. BlastP (NCBI) was used for peptide alignment using the non-redundant (NR) protein database and the Blossum62 algorithm. Only matches with 100% query cover and 100% match were kept.

3. Results and discussion

3.1. Method optimisation

All tryptic peptides that matched the selection criteria (peptide length 8–25 amino acids, no missed cleavages or structural modifications and no peptides containing cysteine or methionine) were screened for their performance across a range of commercial bovine dairy products (full cream milk, yoghurt, brie, normal and ruby red cheddar, and parmesan cheese). The peptides with at least three co-eluting transitions with an intensity of $>10^3$ for at least one of the assayed products were kept in the final sMRM method. Fig. 1A-D shows the peak areas and the coefficient of variation (CV) obtained for these peptides for both defatted and non-defatted protein extracts. One protein, β -casein, did not produce any useable tryptic digests during method development even though it is quite an abundant milk protein (Table S1) and was excluded from the analysis. Of the other allergens, the α-S1 peptides (YLGYLEQLLR and FFVAPFPEVFGK) and α-S2 casein peptide FALPQYLK featured the most intense peak areas shortly followed by the k-casein peptides SPAQILQWQVLSNTVPAK and YIPIQYVLSR and the α-S1 peptide NAVPITPTLNR. The α- and β-lactalbumins and lactoferrin peptides had similar peak areas and were all 2 to 3 orders of magnitude less intense then the casein peak areas. This is interesting since the lactalbumins are only a factor ~3 less abundant in milk as the caseins, suggesting peptide liberation and or ionisation are the main factors accounting for this difference. The α-S1 and α-S2 casein peptides clustered reasonably well across dairy types except for yoghurt peptides, which were approximately 2 orders of magnitude less intense. The lactalbumin peptides were clearly most abundant in milk, which is unsurprising as these whey fraction proteins are less abundant in cheese (Table S1). Lactoferrin peptides were of similar, albeit quite low, abundance across all dairy types tested. Eliminating the defatting procedure resulted in less variation in signal intensity (Fig. 1A-D) between replicas and between dairy types across the board of tested allergens with average CVs across all peptides for all tested dairy types (9.3 ± 4.7% for non-defatted samples and 21.1 ± 6.5% for the defatted samples). Moreover, the defatting procedure did not result in a high reduction of phospholipids in the full milk sample compared to simple fat/aqueous layer separation by centrifugation (Fig. 1E) while excluding the defatting step saves approximately 2 h in total analysis time. Thus,

Table 1
Suitability of the detected peptides. Position and overlap with allergen epitopes of the *B. taurus* protein sequences are given along with the BlastP results and suitability to detect adulteration of caprine dairy products with bovine milk.

Allergens	Detected peptide	Epitope (Bos taurus)	BlastP	Adulteration Detection
Alpha-S1-Casein	YLGYLEQLLR	Slightly (33–42)	Water buffalo, bison, goat, cattle, sheep, yak, *Jeotgalicoccus coquina, J. aerolatus, J. schoeneichii*	No
	FFVAPFPEVFGK	No (38–49)	Cattle, water buffalo, domestic yak, bison,	Yes
	HQGLPQEVLNENLLR	Mainly (23–37)	Chinese and Indian muntjac (deer); cattle; bison; yak; water buffalo; sheep; goat; *J. coquina, J. aerolatus, J. schoeneichii*	No
	VPQLEIVPNSAEER	Yes (121–134)	Cattle; yak; bison; goat; *J. coquina, J. aerolatus, J. schoeneichii*	No
Alpha-S2-Casein	FALPQYLK	Yes (189–196)	Cattle; yak; bison; *J. coquina, J. aerolatus*	Yes
	NAVPITPTLNR	Yes (130–140)	Cattle; water buffalo; yak; bison; *J. coquina, J. aerolatus*	Yes
Kappa-Casein	YIPIQYVLSR	Mainly (46–55)	Saiga; cattle; yak; goat; sheep; oryx; Deer (various); antelope (various); gazelle (various)	No
	SPAQILQWQVLSNTVPAK	Yes (60–77)	Cattle; yak; antelope (various); bison; *J. coquina*	Yes
Alpha Lactalbumin	VGINYWLAHK	Mainly (118–127)	Cattle; water buffalo; whales; dolphins; goat; sheep; yak; muntjac; oryx; deer (Various)	No
Beta-Lactalbumin	VYVEELKPTPEGDLEILLQK	Mainly (57–76)	Yak; water buffalo; bison; oryx, muntjac; *J. coquina; Staphylococcus aureus*	Yes
	VLVLDTDYK	No (108–116)	Cattle; water buffalo; goat; sheep; deer (various); oryx; bison; *S. aureus; J. coquina; J. schoeneichii*	No
Lactoferrin	SFQLFGSPPGQR	No (304–315)	Cattle; yak; water buffalo; bison; *J. coquina*	Yes
	LRPVAAEIYGTK	No (93–104)	Cattle; yak; water buffalo; bison; *J. coquina*	Yes

the defatting step was omitted. Notably, the κ-casein peptides clustered well across all dairy types and featured large (10^7-10^8) peak areas and relatively low CVs (5–7% for the non-defatted samples across all dairy types) making them excellent candidate peptides for allergen detection across a large variety of dairy products.

3.2. Bioinformatic analyses of the selected peptides

Fully homologous sequences for the peptides selected in the final sMRM method were retrieved from the NCBI non-redundant protein database using BlastP (Table 1). From the thirteen peptides tested, seven were found to be specific for Bovinae while the remaining six peptides were detected in a range of other mammals. Milk allergen epitopes are conserved across species, and it is common for patients to be allergic to both cow and goat/sheep milk, although this is not always the case (Ah-Leung et al., 2006; del Río et al., 2012). Thus, peptides that accurately quantify milk from multiple species are useful. Yet, such pan-milk allergen peptides cannot detect adulteration of high-value caprine dairy products with common cow milk. The αS-2 peptides FALPQYLK and NAVPITPTLNR, the k-casein peptide SPAQILQWQVLSNTVPAK and the αS-2 peptide FFVAPFPEVFGK may be useful for such determination since they appear to be unique for Bovinae, give good LC-MS intensity and, have been shown to give reasonably consistent results across dairy products (particularly NAVPITPTLNR and SPAQILQWQVLSNTVPAK). The αS-1 peptide YLGYLEQLLR and the k-casein peptide YIPIQYVLSR on the other hand, may be excellent pan-milk allergen peptides since they show good signal intensity and consistent results across dairy products (particularly YIPIQYVLSR). Moreover, the YIPIQYVLSR sequence is mainly covered by a known *B. taurus* allergen epitope (Table 1 and Table S1). The entire FALPQYLK, NAVPITPTLNR and SPAQILQWQVLSNTVPAK sequences are also covered in known *B. taurus* allergen epitopes. As such, these peptides may equally have clinical

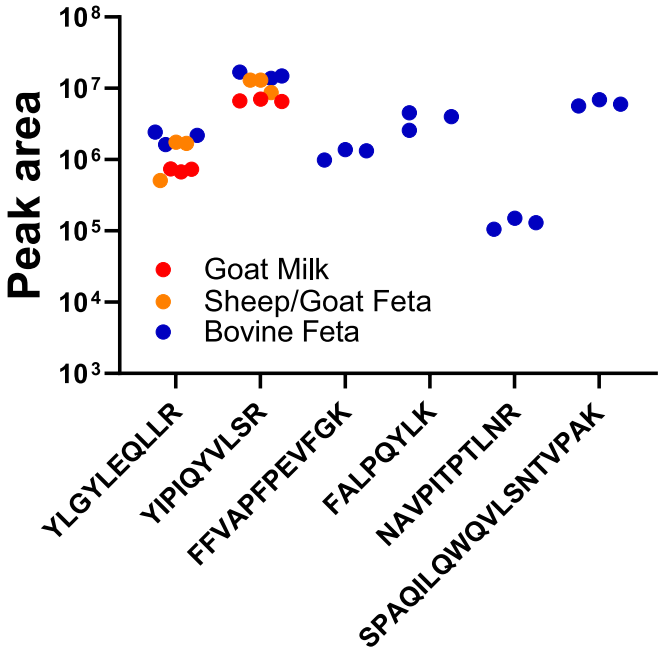

Fig. 2. Peak areas for pan-milk and bovine specific allergen peptides detected in digests of goat milk, feta made from a mixture of sheep and goat milk and feta made from cow milk.

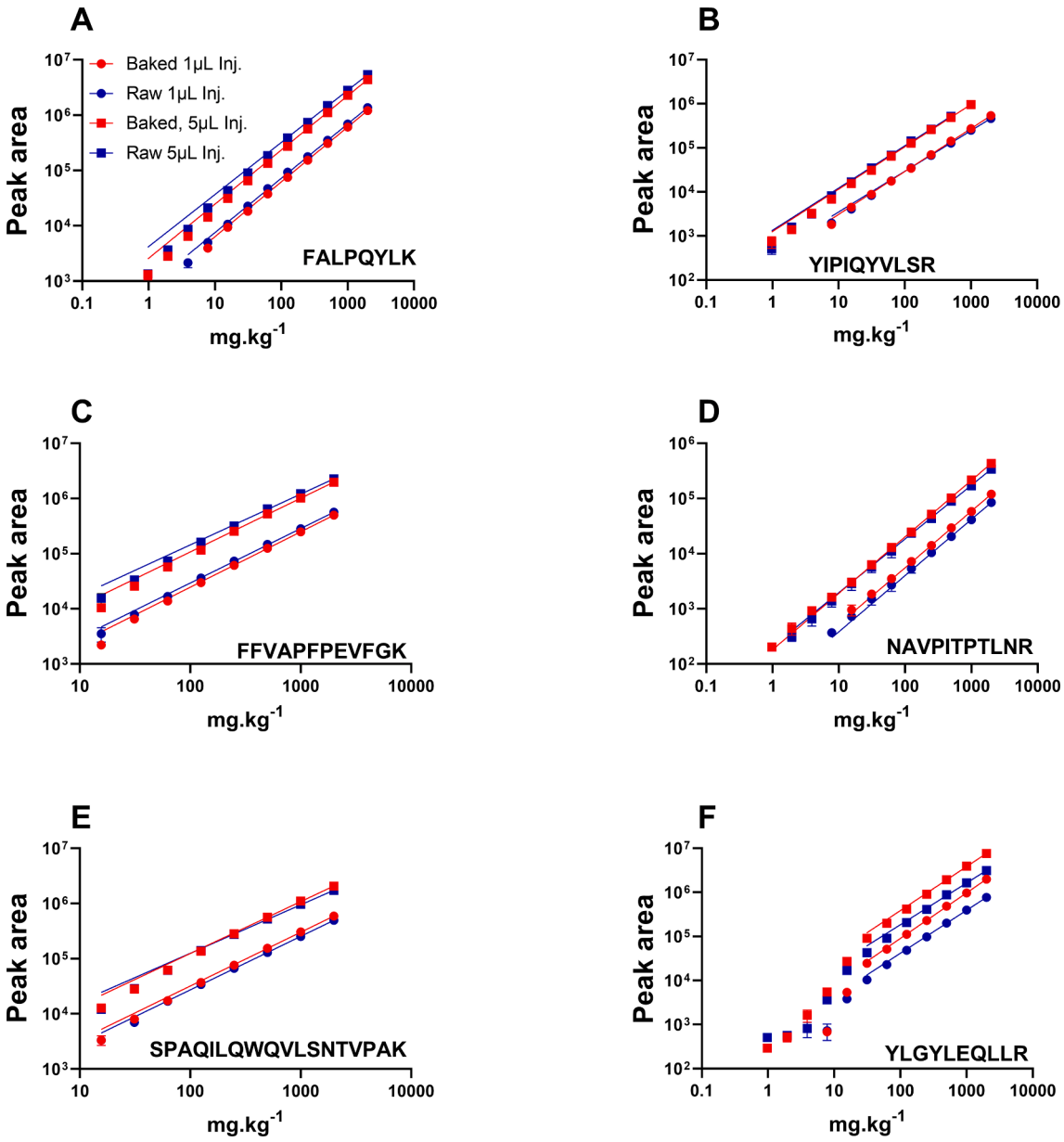

Fig. 3. Calibration curves for the peptides FALPQYLK (A), YIPIQYVLSR (B), FFVAPFPEVFGK (C), NAVPITPTLNR (D), SPAQILQWQVLSNTVPAK (E) and YLGYLEQLLR (F) extracted from baked (red) and raw (blue) croissant extracts using a 1 µL (circles) and a 5 µL (squares) injection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relevance making them attractive choices for allergen quantification.

3.3. Experimental verification of bovine specific milk allergen peptides

The specificity of the identified pan-milk and bovine-specific peptides was tested experimentally using digests from a full cream goat milk, a feta made 100% from sheep and goat milk and a feta made from 100% bovine milk (Fig. 2). The pan-milk peptides (YLGYLEQLLR and YIPIQYVLSR) yielded similar results (in terms of peak area and CVs) for all three products tested. However, the peptides FALPQYLK, NAVPITPTLNR, SPAQILQWQVLSNTVPAK and FFVAPFPEVFGK were absent in the goat milk and goat/sheep feta digests. YIPIQYVLSR gave the best results for the pan-allergen peptides (peak area ~10^7 and a CV of 33% across all dairy types tested versus a peak area of ~10^6 and a CV of 50% for YLGYLEQLLR). Of the bovine-specific peptides, SPAQILQWQVLSNTVPAK gave the highest intensity and lowest CV (9%). NAVPITPTLNR had the lowest signal intensity and a CV of 14%. FFVAPFPEVFGK equally had a CV of 14% while FALPQYLK had the highest CV of these peptides (22%).

3.4. Milk allergen quantification

A quantifying ion (used for peak area calculations), qualifying ion and an ion ratio was established for each peptide to unambiguously identify peptides and determine analytical parameters (linear range, LODs and LOQs) for each peptide (Table S2). Calibration curves were generated for both raw and baked croissant dough using both 1 and 5 µL injections for the peptides YLGYLEQLLR, YIPIQYVLSR FALPQYLK, NAVPITPTLNR, SPAQILQWQVLSNTVPAK and FFVAPFPEVFGK (Fig. 3). The extracted ion chromatograms showing the quantitative and qualitative product ions for these peptides at LOQ level is shown in Fig. S1. For all peptides, except YLGYLEQLLR, the obtained signal for both raw and baked croissant digests overlapped for both the 1 and 5 µL injections, showing that the baking process had little effect on the peptide

Table 2
Analytical parameters of the calibration curves shown in Fig. 3.

Peptide	R^2	LOD (mg. $kg^{-1)}$	LOQ (mg. kg^{-1})	Range (mg. kg^{-1})
NAVPITPTLNR	0.985	1.0	2.0	2–2000
FALPQYLK	0.999	1.0	2.0	2–2000
YIPIQYVLSR	0.996	1.0	2.0	2–1000
FFVAPFPEVFGK	0.991	16.0	31.0	16–2000
YLGYLEQLLR	0.990	1.0	31.0	31–2000
SPAQILQWQVLSNTVPAK	0.998	16.0	31.0	31–2000

Table 3
Recovery rates and inter-day repeatability (RSD) for milk allergen quantification using pan-milk and Bovinae-specific peptides.

Peptide	Recovery (%)	Inter-day RSD (%)	Cal Curve Range (mg. kg^{-1})	Concentrations used for recovery calculations (mg. kg^{-1})
NAVPITPTLNR	77	10	2000–2.0	80–5
FALPQYLK	95	28	2000–30	80–40
YIPIQYVLSR	87	26	1000–30	80–40
FFVAPFPEVFGK	77	19	2000–60	80
YLGYLEQLLR	94	23	1000–60	80
SPAQILQWQVLSNTVPAK	91	9	1000–60	80

abundance. YLGYLEQLLR has been previously used for milk allergen quantification (Croote et al., 2019). However, an algorithm was applied in that work to correct for matrix interference. The present study shows that other peptides may be more suitable for milk allergen quantification due to the improved sensitivity and robustness towards matrix effects. Of the Bovine-specific peptides, NAVPITPTLNR performed exceptionally well with a LOD and LOQ at 1 and 2 $mg.kg^{-1}$ and a linear range from 2 to 2,000 $mg.kg^{-1}$. FALPQYLK and YIPIQYVLSR were the best performing pan-milk allergen peptides with similar analytical parameters — apart from a slightly reduced linear range for YIPIQYVLSR — compared to NAVPITPTLNR (Table 2).

3.5. Recovery

Recovery rates were determined across 5, 10, 20, 40 and 80 $mg.kg^{-1}$ (in triplicate) for baked croissant extracts for all six peptides across four individual experiments, performed across four weeks. Independent calibration curves were generated for each experiment. These experiments informed: (i) the repeatability of calibration curve performance for all the peptides and (ii) the performance of these peptides in terms of recovery efficiency and recovery repeatability. The results are summarised in Table 3. NAVPITPTLNR had the best performance of all peptides compared with excellent repeatability (CV of 10%) and recovery rates (77% on average) across the entire range (5–80 $mg.kg^{-1}$) of milk incursion levels tested at the four different days and is the peptide of choice. FFVAPFPEVFGK, YLGYLEQLLR and SPAQILQWQVLSNTVPAK underperformed in terms of sensitivity, because calibration curves could only be consistently created from 60 $mg.kg^{-1}$ and up due to variation in sensitivity observed for these peptides. FALPQYLK and YIPIQYVLSR performed reasonably well with consistent calibration curves for all days from 30 $mg.kg^{-1}$ and slightly higher recovery rates as NAVPITPTLNR, but also higher inter-day variation (26–28%). The differences in recovery rate and repeatability observed between the peptides may be the result of several factors. For instance, differences in protein hydrophobicity can affect recovery rates. Moreover, different peptides take up different positions in the protein. If a peptide is exposed to the exterior, it may be degraded or modified through food processing (such as baking) more rapidly. Moreover, surface accessibility, hydrophobicity and coil disorder of peptides have been suggested to significantly affect ionisation efficiency as well (Fusaro et al., 2009).

4. Conclusion

A quick and robust LC-MRM-MS protocol for the determination of pan-milk allergen and Bovinae-specific milk allergen peptides was developed. The method enables sensitive quantification (LOQ of 2 $mg.kg^{-1}$) of milk allergens in raw dough and croissants (as an example matrix for baked goods) and across a range of dairy products including milk, yoghurt and cheese while enabling the detection of caprine dairy adulteration with lower-value cow dairy. Although these results are promising, it should be noted that more baked goods should be tested to further evaluate the method performance in other products. The method takes 2 h — a significant improvement compared to previously reported methods, which take ~8–18 h — and does not require lengthy concentration procedures or defatting steps to reach adequate sensitivity. This constitutes a significant step towards the possibility of routine milk allergen quantification via LC-MRM-MS. Nevertheless, time needed for sample transport to a laboratory and instrument cost constitute disadvantages of this method in comparison with portable detection methods. Improvements in quantitation and target identification accuracy in respect to portable methods are, however, equally important. For these reasons it may be particularly attractive to combine LC-MRM-MS confirmatory analyses with on-site rapid and cost-efficient testing. Moreover, the method complies with all the AOAC Standard Method Performance Requirements (analytical range, recovery, reproducibility, and sensitivity) listed for the detection and quantitation of milk allergens (AOAC SMPR 2016.002). If further validated for inter-laboratory performance, this quantitative method has the potential to enable routine milk allergen quantification for quality control purposes. Adoption of such a validated method for milk allergen quantification can accompany legislation that restricts precautionary allergen labelling statements and requires a control system that establishes if milk allergen free products do not contain trace levels of milk allergens above an established threshold, to ensure products are safe for the allergic population. This approach can be implemented for other allergens as well and may substantially reduce the health burden of food allergies.

CRediT authorship contribution statement

Joost L.D. Nelis: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Supervision. **Amanda L. Dawson:** Validation, Formal analysis, Investigation, Writing – review & editing. **Utpal Bose:** Formal analysis, Investigation, Writing – review & editing. **Alisha Anderson:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Michelle L. Colgrave:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **James A. Broadbent:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135885.

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