



Advances in ultra-high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and processed foodstuffs



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ABSTRACT

Sensitive detection of food allergens is affected by food processing and foodstuff complexity. It is therefore a challenge to detect cross-contamination in food production that could endanger an allergic customer's life. Here we used ultra-high performance liquid chromatography coupled to tandem mass spectrometry for simultaneous detection of traces of milk (casein, whey protein), egg (yolk, white), soybean, and peanut allergens in different complex and/or heat-processed foodstuffs. The method is based on a single protocol (extraction, trypsin digestion, and purification) applicable to the different tested foodstuffs: chocolate, ice cream, tomato sauce, and processed cookies. The determined limits of quantitation, expressed in total milk, egg, peanut, or soy proteins (and not soluble proteins) per kilogram of food, are: 0.5 mg/kg for milk (detection of caseins), 5 mg/kg for milk (detection of whey), 2.5 mg/kg for peanut, 5 mg/kg for soy, 3.4 mg/kg for egg (detection of egg white), and 30.8 mg/kg for egg (detection of egg yolk). The main advantage is the ability of the method to detect four major food allergens simultaneously in processed and complex matrices with very high sensitivity and specificity.

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1. Introduction

Currently, 220 million people, corresponding to 2–3% of adults and at least 8% of children in the whole human population, suffer from food allergies [1,2]. Ingestion of food allergens can induce formation of allergen-specific immunoglobulin E (IgE) antibodies. A reaction to a food allergen can cause symptoms ranging from light (nausea, vomiting, ...) to severe (e.g. anaphylactic shock). To avoid the risk of adverse reactions, allergens must be totally excluded from the diet of allergic people. For these individuals and the people around them, the need to respect a strict diet results in permanent stress [3–5]. In Europe, 14 food allergen ingredients must appear on labels when present: milk, peanut, egg, soybean, fish, crustaceans, cereals containing gluten, tree nuts, celery, lupin, mustard, sesame, molluscs, and sulfur dioxide [6]. Despite the increasing health problem that food allergies represent and because there are no clear legal food allergen thresholds, Voluntary Incidental Trace Allergen Labeling (VITAL) was developed in New Zealand and Australia to

help food producers in the management of cross-contamination during food production. VITAL Reference Doses ED₀₁ (milk, egg, and peanuts) and ED₀₅ (soybean) are the lower confidence interval for the protection of 99% and 95% of allergic people, respectively. On the basis of this referential, even though it does not have a regulatory relevance, food product stated on the label to be allergen free should contain less than 2.5 mg milk proteins, 5 mg peanut proteins, 0.75 mg egg proteins, or 25 mg soybean proteins per kilogram (portion size: 40 g) [7–9]. Despite the endangerment of allergic customers, the numerous and various labeling legislations (directives 2007/68/EC, 2000/13/EC and regulation 2011/1169/EC [10–12]), the undeclared presence of food allergens in food products is still widespread [13,14]. To help food producers declare hidden food allergens, several methods have been developed. Among them, the real-time quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) method is based on the detection and analysis of species-specific DNA sequences (coding or not for allergenic proteins), which are more heat stable than proteins [15–17]. Yet PCR cannot discriminate between DNA from egg (allergenic) and chicken meat (non-allergenic), and thus yields false positives or confounding results [18]. Furthermore, because different food allergens have different DNA-to-protein ratios, conversion of amounts

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of DNA to amounts of protein introduces is imprecise and low DNA content can introduce a lack of sensitivity [19]. The most commonly used method is the Enzyme-Linked Immunosorbent Assay (ELISA) for routine analysis, but the high similarity of some proteins [20] and structural modifications induced by processing [21–23] frequently lead to false positive or false negative results. For example, thermal processing modifies a protein's structure without necessarily abolishing its allergenic potential [24,25].

Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) for the analysis of food allergens is attracting increasing interest [26–31]. Its specificity and sensitivity make it applicable to both processed and raw food allergens, and it can be used to detect several food allergens simultaneously. In developing a specific and sensitive UHPLC–MS/MS method, several challenges must be met. First, the selected marker peptides must be specific to the target food allergen and robust to the thermal process. Second, the test materials must be incurred and not spiked after the process [32,33]. Third, and especially in the case of processed matrices where protein modifications and degradations might occur [34–36], one must use extraction and purification methods that are adequate for obtaining a Limit Of Quantification (LOQ) of approximately 1 mg/kg, consistent with VITAL recommendations [26,27,29,37]. To meet these challenges and to test the specificity of Multiple Reaction Monitoring (MRM) transitions in allergen-free and contaminated matrices, incurred and processed matrices, selected for their complexity or their thermal process, were prepared. The sensitivity of the method was tested by determining, after ascertainment of linearity, a single, common LOQ for each targeted allergen (milk casein, whey, egg yolk, egg white, soybean, and peanut) in two incurred and two processed matrices: chocolate, ice cream, cookie, and tomato sauce.

2. Materials and methods

2.1. Reagents and materials

Urea, ammonium bicarbonate, tris(hydroxymethyl)aminomethane (TRIS), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), iodoacetamide (IAA), and trypsin from bovine pancreas (T8802) were purchased from Sigma-Aldrich (Bornem, Belgium). Acetonitrile (UHPLC–MS/MS grade) and formic acid were from Biosolve (Valkenswaard, the Netherlands). Acetic acid was obtained from Acros Organics (Geel, Belgium) and hydrochloric acid was from Fisher Chemical (Loughborough, UK). Clean-up was performed with sep-pak tC18 solid phase extraction (SPE) columns, (WAT036790, 6 cc, 500 mg) from Waters (Milford, Massachusetts, USA). Lyophilized milk powder (27% protein) and soy flour (36% protein) were from a local store. Eggs obtained from a local store were used to isolate egg yolk (16% protein) and egg white powder (79% protein) were from Barry Farm Wapakoneta, USA. Peanut butter (NIST 2387 22.2% protein) was from the National Institute of Standards and Technology (NIST) (Gaithersburg, Maryland, USA).

2.2. Marker peptide selection

The *open source* software Skyline was used for *in silico* enzymatic digestion of food allergen proteins and to design the MRM. The following parameters were selected: peptide length of 8–25 amino acids, b or y fragmentation, carbamidomethylation modification, precursor ion charge 2 or 3 and product ion charge 1 or 2. After *in silico* digestion, selection of marker peptides in raw food allergens was performed for milk, egg, soy, and peanut prepared individually at 0.1 mg protein/ml. Food proteins were extracted in 200 mM TRIS–HCl; pH 8.2 [32]. Trypsin digestion was carried out (see Section 2.5.2) without any further purification and the

digestion products were analyzed by UHPLC–MS/MS. Afterwards, selection of intensely processed peptides was performed in cookies containing milk, egg, soy, and peanut at 5000 mg/kg each, processed for 18 min at 180 °C. Cookie proteins were extracted in 200 mM TRIS–HCl; pH 8.2 with addition of 5 M urea. Samples were then diluted to 1.0 mg protein/ml in 200 mM ammonium bicarbonate, digested with trypsin without any further purification, and analyzed by UHPLC–MS/MS. Finally, after optimization of protein extraction on the basis of protein contents and analysis of peptides (UHPLC–MS/MS), samples of chocolate, ice cream, cookie, and tomato sauce containing proteins from milk, egg, soy, and peanuts at 200 mg/kg were prepared and analyzed according to the sample preparation protocol described below.

2.3. Preparation of non-contaminated matrices

Four target matrices were selected: chocolate, tomato sauce, cookie, and ice cream. Food-allergen-free chocolate, containing mainly cacao (45%), sugar (35%), and rice powder (20%), and tomato sauce composed mainly of tomatoes (75%) and a mixture of onions, carrots, and celery (15%), were purchased from a local shop and finely ground. Ice cream was prepared by combining coco milk (29.6%), sugar (11.0%), lemon juice (0.4%), and banana (59.0%) and mixing these ingredients with a blender. Cookie dough composed of flour (53.4%), sugar (15.2%), water (14.8%), oil (16.1%), salt (0.3%), ammonium bisulfate (0.1%), and sodium bicarbonate (0.1%) was also prepared.

2.4. Preparation of incurred matrices

Incurred matrices were prepared in three independent replicates by adding raw food allergens to non-contaminated matrices to obtain a theoretical allergen protein concentration of 25 mg/kg for milk, 50 mg/kg for soybean, 50 mg/kg for peanut, 100 mg/kg for egg white, and 250 mg/kg for egg yolk. Mixing was carried out with a mechanical blender (robot coupe, Blixer 4 V.V.) for 10 min to ensure homogeneous distribution of food allergens. After incorporation of food allergens, sauce batches were cooked at 95 °C for 45 min, chocolate batches were heated in a water bath at 40 °C for 20 min and frozen at –20 °C before being finely ground, and ice cream was frozen at –20 °C. To obtain incurred matrices with intermediate food allergen concentrations, non-contaminated matrices were mixed with incurred matrices to obtain 0, 0.5, 1.25, 2.5, and 5 mg/kg for milk proteins, 0, 1, 2.5, 5 and 10 mg/kg for soybean and peanut proteins, 0, 3.4, 8.5, 17, 34 mg/kg for egg proteins (egg white), and 0, 12.3, 30.8, 61.6, 123.2 mg/kg for egg proteins (egg yolk). Afterwards, each cookie (40 g dough with a diameter of 7 cm) was baked at 180 °C for 18 min and finely ground.

2.5. Sample preparation protocol

2.5.1. Extraction

Proteins were extracted from 2 g ground matrix in 20 ml of 200 mM TRIS–HCl, pH 9.2, 2 M urea by shaking at 20 °C for 30 min (Agitelec, France) followed by sonication for 15 min at 4 °C to avoid carbamidomethylation with urea. The samples were centrifuged at 4660g for 10 min at 10 °C.

2.5.2. Digestion

A 10-ml aliquot of supernatant was diluted in 10 ml of 200 mM ammonium bicarbonate. After addition of 1 ml of 200 mM DTT to reduce proteins, the samples were incubated at 20 °C for 45 min. Subsequently, alkylation of these proteins was performed by adding 2 ml of 500 mM IAA and incubating for 45 min at 20 °C in the dark. A 16-h trypsin digestion (1 mg/ml in 50 mM acetic acid)

Table 1

Number of proteins, peptides and transitions for milk, egg, soybean and peanut allergens, according five criteria, for the final selection of marker peptides.

		Milk		Egg		Peanuts	Soybeans	Total
		Caseins	Whey proteins	White	Yolk			
1	Proteins	4	3	5	3	43	25	83
Uniprot								
2	Peptides	29	48	63	120	335	345	940
Skyline In-silico digestion	Transitions	648	969	1278	2473	7137	7419	19924
3	Peptides	14	19	41	46	61	44	225
Raw ingredients analysis	Transitions	62	92	197	214	329	179	1073
4	Peptides	11	5	12	11	15	26	80
Processed cookies 5000 ppm	Transitions	39	18	43	38	56	119	313
5	Peptides	4	3	3	5	4	4	23
Incurring and processed matrices	Transitions	12	9	9	15	12	12	69

was performed (protein:trypsin ratio1:20) at 37 °C. The reaction was stopped by addition of 300 µl of 20% formic acid.

2.5.3. SPE purification

Digested proteins were purified on tC18 SPE columns. Cartridge pre-conditioning was performed with 18 ml acetonitrile followed by equilibration with 18 ml of 0.1% formic acid. The digested proteins were centrifuged at 4660g for 5 min at 10 °C and 20 ml supernatant was loaded on the column. Next, 18 ml of 0.1% formic acid was used to flush out impurities. Elution was then performed with 1.8 ml of acetonitrile/0.1% formic acid 30/70 v/v followed by 3.6 ml acetonitrile/0.1% formic acid 80/20: v/v. Before evaporation at 40 °C under a nitrogen flow, 30 µl DMSO was added to avoid dryness. After evaporation, the pellets were dissolved in 200 µl of 0.1% formic acid and centrifuged for 5 min at 11754g. Peptides were then analyzed by UHPLC–MS/MS.

2.5.4. UHPLC–MS/MS

An Acquity system (Milford, Massachusetts, USA) equipped with a C18 Acquity BEH130 Waters column (2.1 × 150 mm) was used to separate the food allergen peptides. Column compartment and thermal autosampler were set at 40 °C and 10 ± 5 °C, respectively. After injection of 20 µl sample, a gradient applied for 24 min (at 0.2 ml/min) allowed separation of the food allergen peptides (solvent: 0.1% formic acid (A) – acetonitrile plus 0.1% formic acid (B)). Elution was carried out as follows: 0–1 min: 86% A; 1–16.5 min: 86% to 60% A, 16.5–16.6 min: 60% to 0% A; 16.6–21 min: 0% A; 21.0–21.1 min: 0% to 86% A, 21.1–24 min: 86% A. A Waters Xevo TQS triple quadrupole system with a positive electrospray and MRM mode were used for detection of food allergen peptides. A 150 l/h cone flow and a 1200 l/h desolvation flow of nitrogen were then applied. The capillary voltage was set at 2.0 kV and the collision gas flow was set at 0.12 ml/min. The source and desolvation temperatures were set respectively at 150 and 500 °C.

3. Results and discussion

3.1. Marker peptide selection

Marker peptides were selected in five steps (Table 1). First, the Uniprot database was searched for target proteins of milk, egg, soy, and peanut. Second, peptides and transitions were generated by Skyline in silico digestion. Third, digested extracts of raw food allergens were analyzed by UHPLC–MS/MS, without prior purification. This allowed elimination of 95% of the 19924 transitions generated by Skyline. Fourth, processed cookies were analyzed to identify a list of peptides resistant to the thermal process and having intense transitions as in the analysis of raw ingredients. Fifth, the target food allergens were incorporated into the chocolate, ice cream, sauce, and cookies and extracted, digested with trypsin, and purified on SPE columns. Marker peptides were determined

by UHPLC–MS/MS in all target matrices and only specific peptides giving high-intensity transitions and detected in all matrices were retained (Table 2). Even through UHPLC–MS/MS method is highly specific and peptide specificity was controlled (blast), the huge number of proteins in complex foodstuffs can lead to unspecific transitions. Moreover, structural protein modifications and interferences in complex foodstuffs can also compromise food allergen detection. For all these reasons, the detection of one peptide per allergen is not enough and it is therefore essential to ensure the specificity and the reliability of the method by keeping the detection of at least 3 or 4 peptides for each allergen. Collision energies were optimized for each peptide transition with a 0.1 mg/ml solution of raw ingredient proteins. The three most intense transitions for each peptide were retained for subsequent analyses.

3.2. Determination of marker peptide specificity

A BLAST analysis of the retained peptide sequences was performed to check for inter-species homology (Uniprot). The egg peptides ISQAVHAAHAEINEAGR and EALQPIHDLADEAISR were found in both hen eggs and *Coturnix japonica* (common farmed quail) eggs. The peptides NIPFAEYPTYK and NIGELGVEK of hen eggs displayed homology to peptides of *Anas platyrhynchos* (mallard duck) eggs. As the production and price of hen eggs totally exclude eggs from other species in industrial preparations, these similarities were not viewed as a drawback of the method. The milk proteins of bovine, ovine, and caprine species tend to show high homology. For instance, casein αS1 of dairy cow milk shares 95.8% similarity to that of *Bubalus bubalis* (buffalo), 99.1% similarity to that of *Bos mutus grunniens* (yak), 88.8% similarity to that of *Ovis aries* (sheep), and 88.3% similarity to that of *Capra hircus* (goat) (Uniprot). However, most people who are allergic to cow milk are also allergic to the milk of these other animals [20]. Therefore, the peptides YLGYLEQLLR and VLVDLDYK, homologous to peptides found in the milk of the above-mentioned species, were retained in the final selection. No homologies relevant to food ingredients were found for the selected soy and peanut peptides.

3.3. Method sensitivity

For each food allergen, a single, common LOQ was determined for all targeted matrices (Fig. 1 A and B). For each peptide, two MRM transitions in allergen-free matrices and in incurred matrices are shown to demonstrate the specificity of the method and to confirm detection of the food allergens at the LOQ. The retention times of peptides were different between matrices. Indeed, matrix effect can affect detection of food allergen peptides and might lead to some shift in the retention time. The introduction of an internal standard will allow to determine relative retention time and to limit the matrix effects. The LOQ was defined as the minimum concentration giving a signal-to-noise ratio (S/N) of 10 for the most intense MRM

Table 2
Multiple Reaction Monitoring (MRM) parameters for the identification of milk, egg, soybean and peanut proteins by UHPLC–MS/MS. The cone voltage was fixed at 35 V.

	Protein	Peptide	Retention time in sauce (min)	Precursor (charge state) (<i>m/z</i>)	Product ion (fragment)	Collision energy (eV)
Soybean	Glycinin P04347	ISTLNSLTLPALR	10.5	699.9 (++)	984.6 (y9 ⁺)	23
					870.5 (y8 ⁺)	25
	Glycinin A3B4	EAFGVNMQIVR	8.1	632.3 (++)	783.5 (y7 ⁺)	25
					859.5 (y7 ⁺)	18
	Glycinin G2 P04405	ELINLATMCR	8.3	610.8 (++)	760.4 (y6 ⁺)	17
					646.4 (y5 ⁺)	22
	2S albumin P19594	LITLAIPVNKPGR	7.9	464.6 (+++)	865.4 (y7 ⁺)	21
					751.4 (y6 ⁺)	21
	Gly m 2S albumin				638.3 (y5 ⁺)	17
					767.5 (y7 ⁺)	15
	Beta-conglycinin P13916				583.4 (y11 ⁺⁺)	9
					476.3 (y9 ⁺⁺)	11
Milk	Casein αS1 P02662	HQGLPQEVLENLLR	8.1	587.3 (+++)	871.5 (y7 ⁺)	17
					758.4 (y6 ⁺)	16
					436.2 (b4 ⁺)	17
	Bos d 8	FFVAPFPEVFGK	13.5	692.9 (++)	991.5 (y9 ⁺)	18
					920.5 (y8 ⁺)	18
					676.4 (y6 ⁺)	28
		YLGYLEQLLR	12.3	634.4 (++)	934.5 (y7 ⁺)	21
					771.5 (y6 ⁺)	20
					658.4 (y5 ⁺)	21
	Casein αS2 P02663	NAVPIPTLNR	5.1	598.3 (++)	911.5 (y8 ⁺)	17
					456.3 (y8 ⁺⁺)	14
					285.2 (b3 ⁺)	12
	β-lactoglobulin P02754	VYVEELKPTPEGDLEILLQK	10.6	771.8 (+++)	912.0 (y16 ⁺⁺)	19
					790.9 (y14 ⁺⁺)	19
					627.9 (y11 ⁺⁺)	20
	Bos d 5	VLVLDTDYK	6.4	533.3 (++)	853.4 (y7 ⁺)	15
					754.4 (y6 ⁺)	14
					641.3 (y5 ⁺)	16
		LSFNPTQLEEQCHIN-terminal peptide	8.9	858.4 (++)	1254.6 (y10 ⁺)	26
					928.4 (y7 ⁺)	27
					627.8 (y10 ⁺⁺)	27
Egg	Ovalbumin P01012	GGLEPINFQTAADQAR	7.5	844.4 (++)	1331.7 (y12 ⁺)	26
					1121.5 (y10 ⁺)	28
					666.3 (y12 ⁺)	25
	Gal d 2	LTEWTSSNVMEER	5.9	791.4 (++)	1052.5 (y9 ⁺)	31
					951.4 (y8 ⁺)	23
					864.4 (y7 ⁺)	23
		ISQAVHAAHAEINEAGR	2.3	887.5 (++)	1138.6 (y11 ⁺)	33
					1067.5 (y10 ⁺)	33
					996.5 (y9 ⁺)	32
	Vitellogenin-2 P02845	EALQPIHDLADEAISR	7.8	593.3 (+++)	761.4 (y7 ⁺)	19
					690.3 (y6 ⁺)	15
					668.8 (y12 ⁺⁺)	15
		NIPFAEYPTYK	7.5	671.8 (++)	1115.5 (y9 ⁺)	15
					508.3 (y4 ⁺)	16
					558.3 (y9 ⁺⁺)	29
		NIGELGVEK	4.0	479.8 (++)	731.4 (y7 ⁺)	12
					674.4 (y6 ⁺)	10
					545.3 (y5 ⁺)	19
	Vitellogenin-1 P87498	YLLDLLPAAASHR	10.4	480.6 (+++)	709.4 (y7 ⁺)	15
					582.3 (y11 ⁺⁺)	10
					355.2 (y7 ⁺⁺)	14
	Apovitellenin P02659	NFLINETAR	6.2	539.3 (++)	816.5 (y7 ⁺)	14
					703.4 (y6 ⁺)	16
					590.3 (y5 ⁺)	16
Peanut	Cupin P43238	NTLEAAFNAEFNEIR	10.7	869.9 (++)	1139.5 (y9 ⁺)	27
					992.5 (y8 ⁺)	26
	Ara h1	RPFYSNAPQEIFIQQGR	7.3	684.4 (+++)	878.4 (y7 ⁺)	26
					748.4 (y6 ⁺)	20
	Cupin Q8LKN1	FNLAGNHEQEFLR	6.2	525.6 (+++)	608.3 (y10 ⁺⁺)	19
					836.4 (b7 ⁺)	17
	Ara h 3/4				692.4 (y5 ⁺)	20
					600.8 (y10 ⁺⁺)	13
		TANELNLLILR	11.2	635.4 (++)	565.3 (y9 ⁺⁺)	14
					983.6 (y8 ⁺)	21
	Cupin Q647H4				854.6 (y7 ⁺)	20
					741.5 (y6 ⁺)	22
	Ahy-1					

transition of the targeted food allergen. The S/N was calculated peak to peak in a range equal to six times the peak width at half height for three independent replicates of the targeted matrix. The results obtained demonstrate the suitability of the method. The sensitivity of detection for the food allergen peptides was determined on processed cookies. The LOQs recorded are: 0.5 mg milk proteins/kg for caseins, 5 mg milk proteins/kg for whey, 3.4 mg egg proteins/kg for caseins, 5 mg milk proteins/kg for whey, 3.4 mg egg proteins/kg for caseins, 30.8 mg egg proteins/kg for egg yolk, 2.5 mg/kg for

peanut proteins, and 5 mg/kg for soybean proteins. On the basis of the LOQ, milk casein and egg white peptides appear better for detecting food allergens than whey and egg yolk proteins, as the sensitivity of detection is ten times higher for these peptides than for whey and egg yolk peptides. In industrial processes, however, whey and egg yolk can easily be separated, respectively, from milk casein and egg white. It is therefore important to have a sensitive

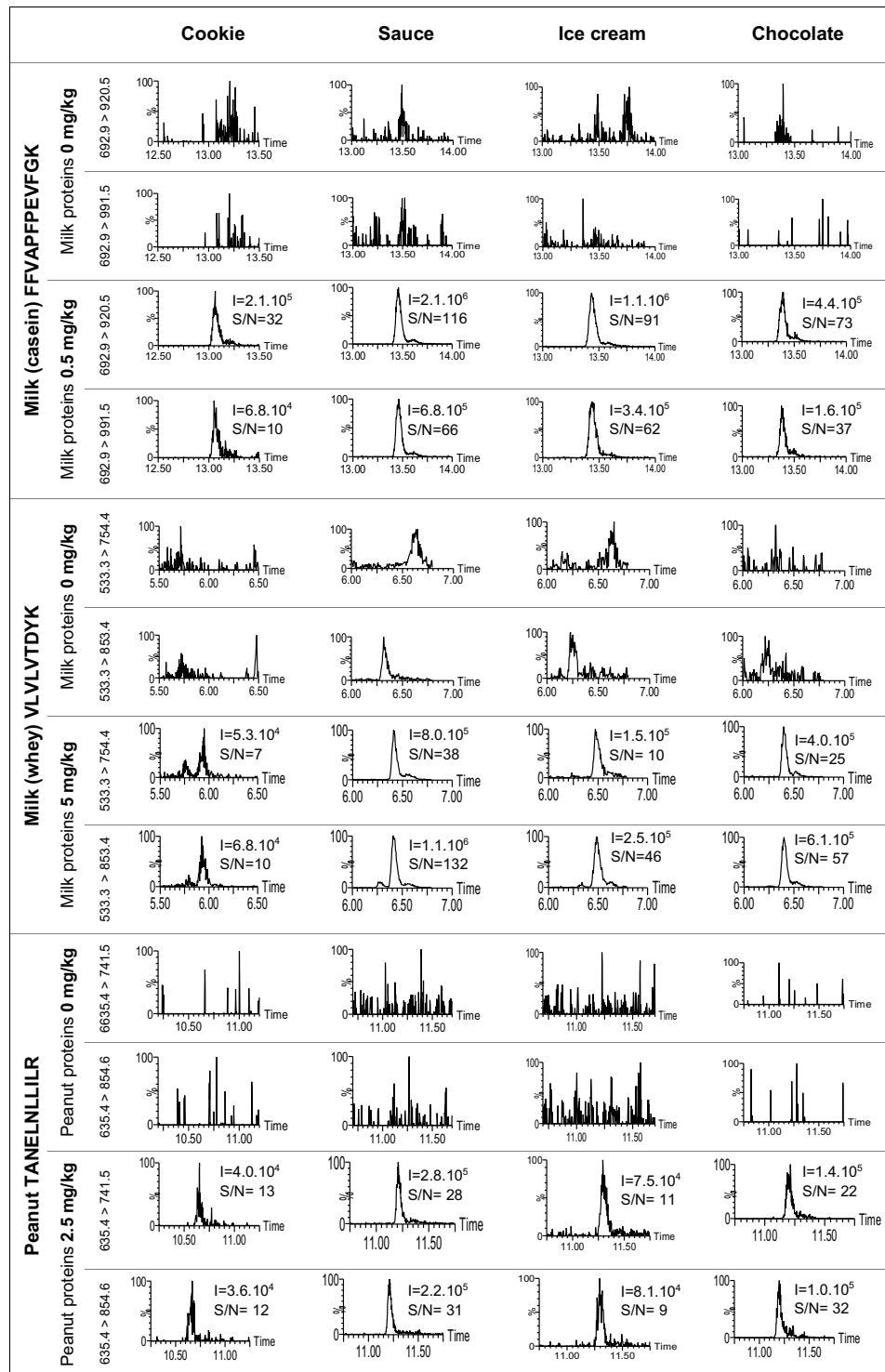


Fig. 1. A and B: Chromatograms of the two higher multiple reaction monitoring MRM transition of 1-A; milk casein peptide FFVAPFPEVFGK, whey milk peptide VLVLTDTYK and peanut peptide TANELNLLILR 1-B; egg white peptide GGLEPINFQTADQAR, egg yolk peptide NFLINETAR and soy peptide EAFGVNMQIVR in chocolate, ice cream, tomato sauce and cookies. Data of non-contaminated matrices (0 mg/kg), incurred or processed matrices at the limit of quantification are presented without any data treatment.

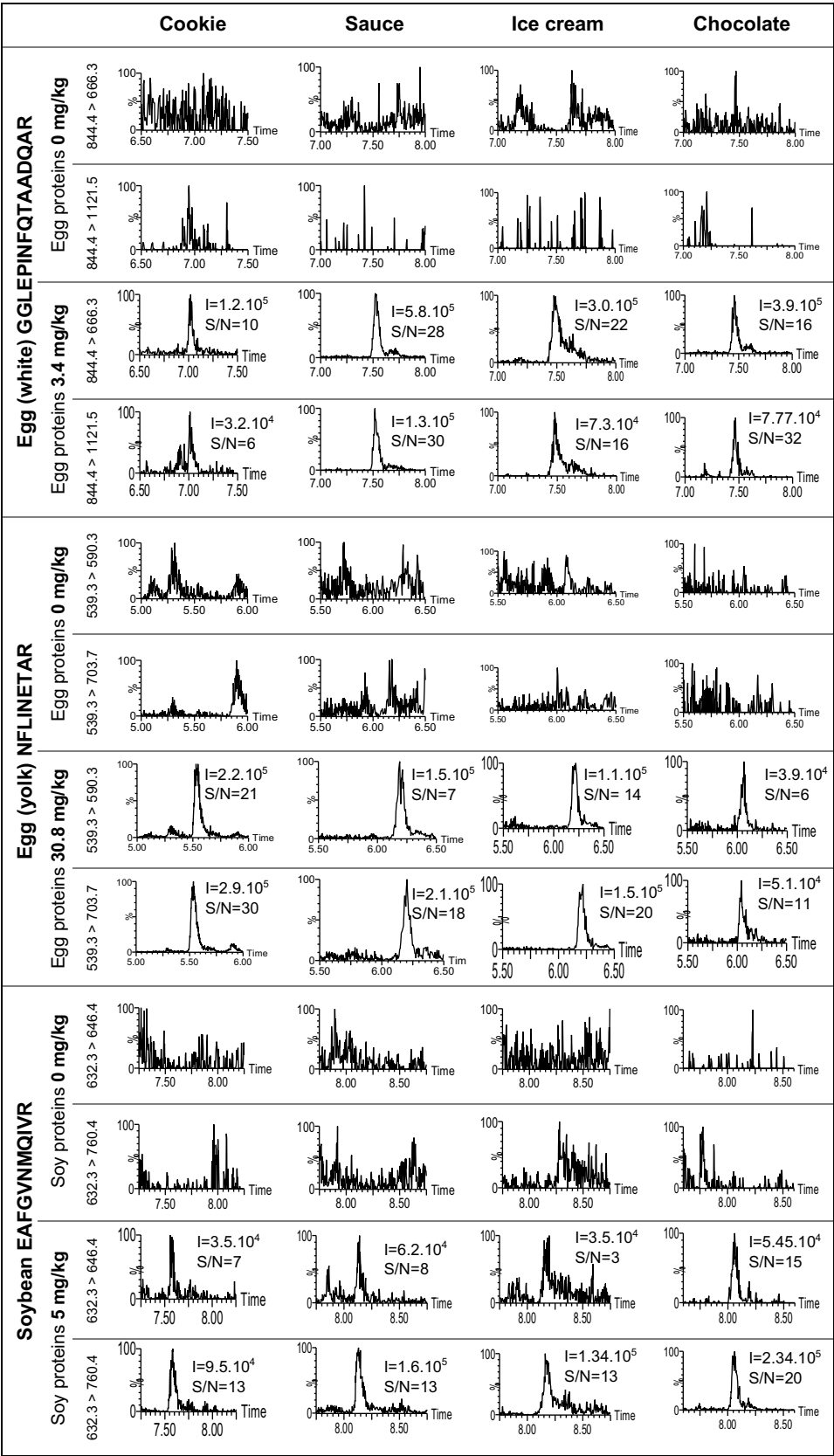


Fig. 1. (Continued)

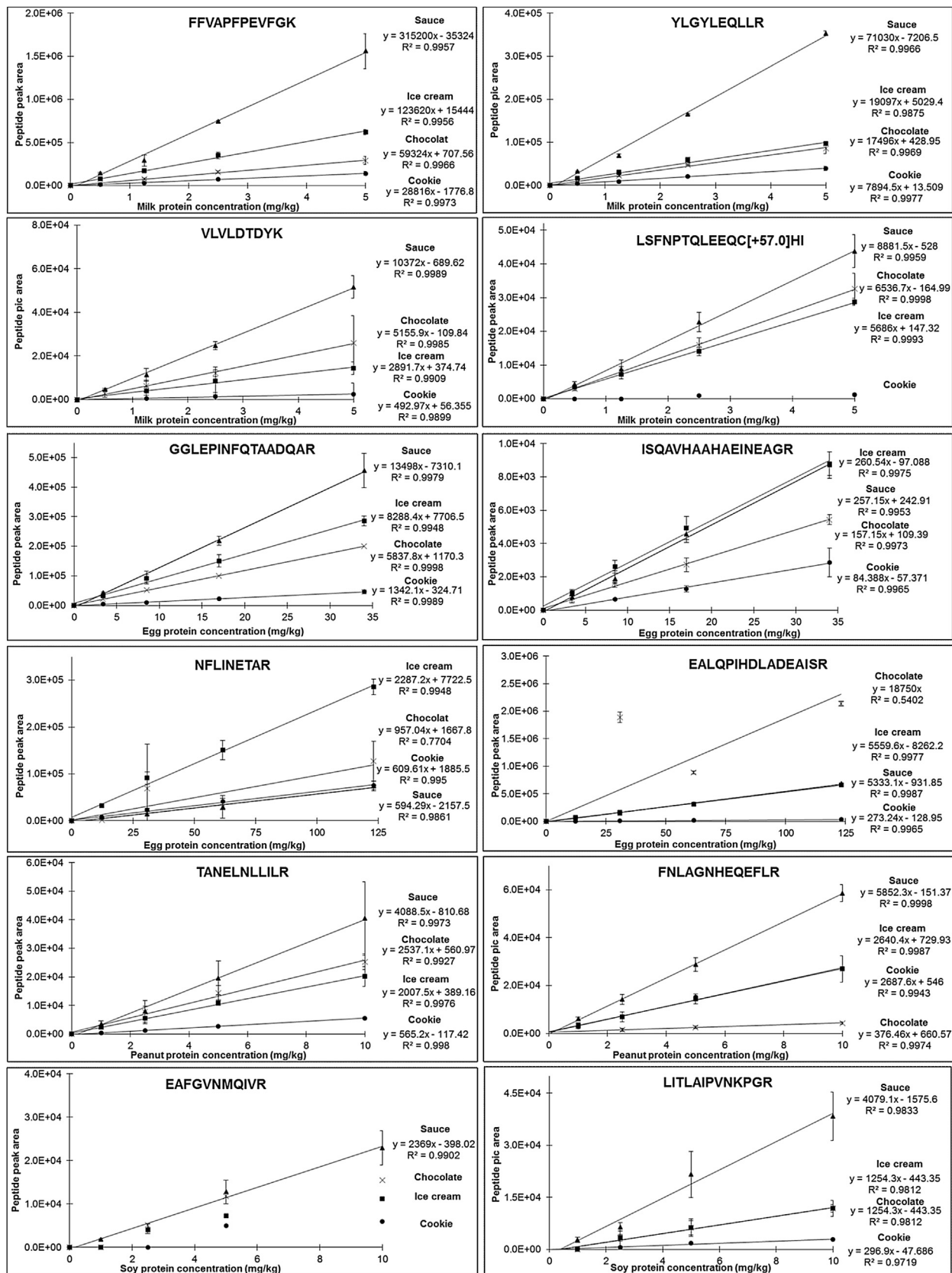


Fig. 2. Linear regression of peptide peak area of the higher MRM in function of the concentration of food allergen proteins performed in three independent replicates in incurred tomato sauce, chocolate, ice cream and processed cookies. The linearity was controlled for each food allergen: milk casein FFVAPFPEVFGK (692.9 > 920.5) and YLGYLEQLLR (634.4 > 771.5), whey milk VLVLDTDYK (533.3 > 853.4) and LSFNPTQLEEQC[+57]HI (858.4 > 928.4) (carbamidomethylation of cysteine amino acids by addition of iodoacetamide before an enzymatic digestion to block the onset of disulfur bridges), egg white GGLEPINFQTAADQAR (844.4 > 666.3) and ISQAVHAAHAEINEAGR (887.5 > 1067.5), egg yolk NFLINETAR (539.3 > 703.4) and EALQPIHDLADEAISR (593.3 > 668.8), peanut TANELNLLILR (635.4 > 741.5) and FNLAGNHEQEFLR (525.6 > 600.8), soybean EAFGVNMQIVR (632.9 > 760.4) and LITLAIPVNKPGR (464.6 > 583.4)".

detection of egg yolk and whey peptides because of their allergenic properties.

3.4. Matrix effects

Linearity and matrix effects were tested by analyzing three independent foodstuff preparations (incurred chocolate and ice cream and processed cookies and sauce) containing different concentrations of milk, egg, soy, and peanut food allergen proteins (Fig. 2). For VLVLDTDYK and EAFGVNMQIVR in chocolate, cookies, and ice cream, it was not possible to draw a linear regression curve regarding to the LOQs. As shown in Fig. 2, the matrix effect and the effect of the thermal process were not the same for both targeted peptides from the same food allergen. For example, heating for 45 min at 95 °C was found to affect the ovalbumin peptide GGLEPINFQ-TAADQAR more weakly than the apovitellenin peptide NFLINETAR. Yet no matter how the matrix and thermal effects varied, the linear coefficient of regression supported the reliability of the method even the absence of an internal standard, except for the NFLINETAR peptide in chocolate ($R^2 = 0.77$) and EALQPIHDLADEAISR ($R^2 = 0.54$) from egg yolk proteins. As these egg yolk peptides can be detected with sufficient specificity and sensitivity in processed and incurred matrices, in future egg yolk protein quantifications one should mitigate the lack of linearity by introducing internal standards.

4. Conclusion

With a view to improving food allergen labeling and ultimately the quality of life of allergic people, a sensitive qualitative multi-allergen detection method was developed for two incurred and two –processed matrices. Sensitive detection of food allergens (milk casein, whey, egg white, egg yolk, peanut, and soybean) was achieved by analyzing food allergen peptides by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). In keeping with food production requirements, the targeted matrices were processed (tomato sauce, cookies) or incurred (chocolate, ice cream). The sensitivity of the method was determined on the basis of the signal-to-noise ratio. To our knowledge, this study is the first to identify a such high sensitivity whey and egg yolk marker peptides by UHPLC–MS/MS with very high sensitivity in both processed and incurred material. Our multi-allergen detection method has the lowest limits of quantification available to date (expressed in total proteins and not soluble proteins): 0.5 mg milk proteins/kg for caseins, 5 mg milk proteins/kg for whey, 3.4 mg egg proteins/kg for egg white, 30.8 mg egg proteins/kg for egg yolk, 2.5 mg peanut proteins/kg and 5 mg soybean proteins/kg. The method shows an LOQ below the VITAL threshold (portion size: 40 g) for milk casein, peanut, and soybean, but values 2, 4.5, and 45 times the VITAL threshold for whey milk, egg white, and egg yolk, respectively. With a view to developing a quantitative method, the introduction of internal standards and extension to other allergens are under investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2016.08.033>.

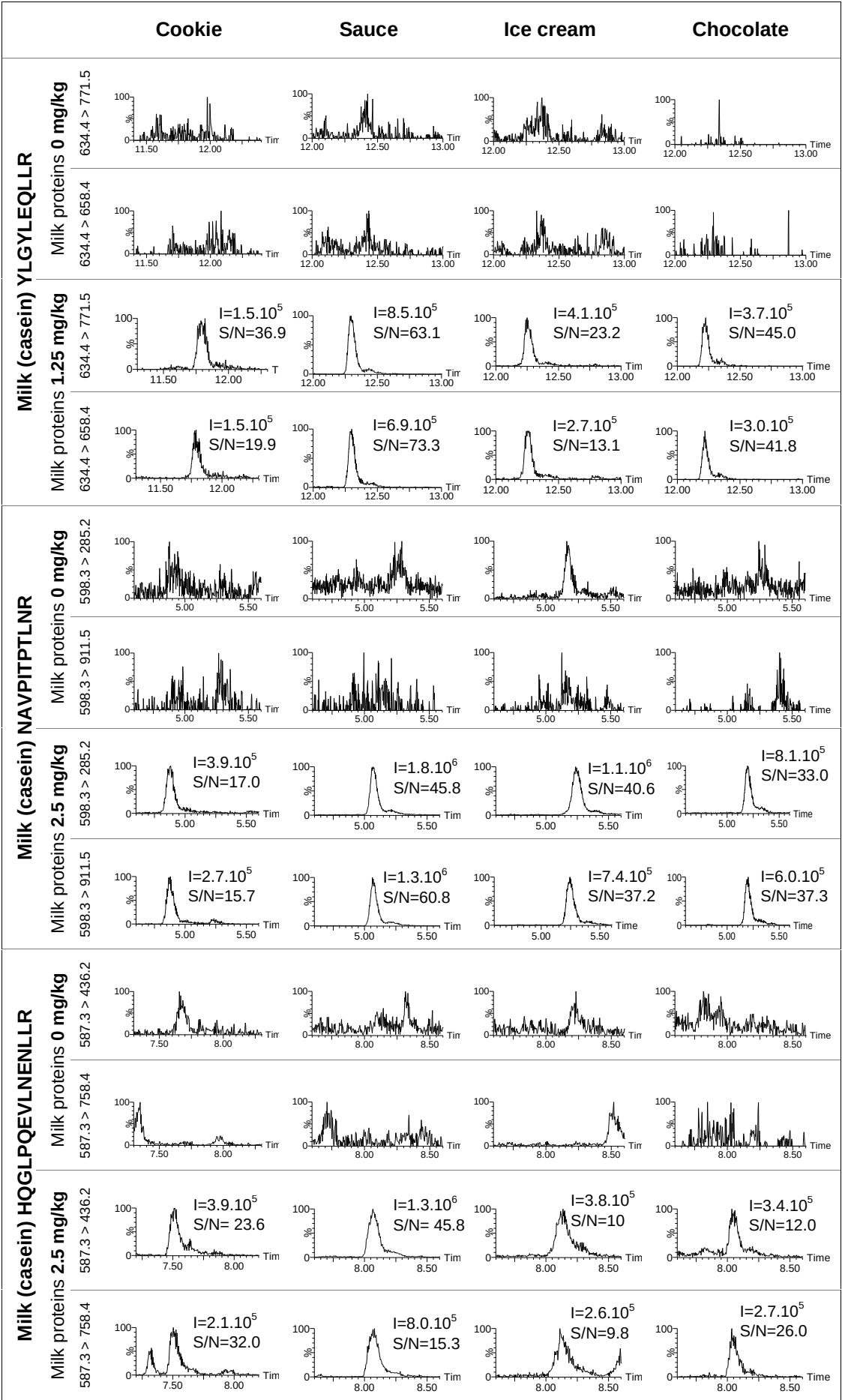
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Figure 1: Chromatograms of the two higher multiple reaction monitoring MRM transtion of egg, milk, soy and peanut allergens. Data of non-contaminated matrices (0 mg/kg), incurred or processed matrices at the limit of quantification are presented without any data treatment.

Supplementary figure



		Cookie	Sauce	Ice cream	Chocolate
Milk (whey) VYVEELKPTPEGDLEILLQK	Milk proteins 0 mg/kg 771.8 > 627.9				
	Milk proteins 0 mg/kg 771.8 > 790.9				
	Milk proteins 5 mg/kg 771.8 > 627.9				
	Milk proteins 5 mg/kg 771.8 > 790.9				
Milk (whey) LSFNPTQLEEQCHI	Milk proteins 0 mg/kg 858.4 > 627.8				
	Milk proteins 0 mg/kg 858.4 > 928.4				
	Milk proteins 5 mg/kg 858.4 > 627.8				
	Milk proteins 5 mg/kg 858.4 > 928.4				
Peanut RPYSNAPQEIFIQQR	Peanut proteins 0 mg/kg 684.4 > 748.4				
	Peanut proteins 0 mg/kg 6684.4 > 836.4				
	Peanut proteins 10 mg/kg 684.4 > 748.4				
	Peanut proteins 10 mg/kg 6684.4 > 836.4				

		Cookie	Sauce	Ice cream	Chocolate
Peanut FNLAGNHEQEFLR	Peanut proteins 0 mg/kg 525.6 > 600.8				
	Peanut proteins 0 mg/kg 525.6 > 565.3				
	Peanut proteins 10 mg/kg 525.6 > 600.8				
	Peanut proteins 10 mg/kg 525.6 > 565.3				
Peanut NTLEAAFNAEFNEIR	Peanut proteins 0 mg/kg 869.9 > 1139.5				
	Peanut proteins 0 mg/kg 869.9 > 992.5				
	Peanut proteins 10 mg/kg 869.9 > 1139.5				
	Peanut proteins 10 mg/kg 869.9 > 992.5				
Soy ISTNLNSLTLPALR	Soy proteins 0 mg/kg 699.9 > 984.6				
	Soy proteins 0 mg/kg 699.9 > 870.5				
	Soy proteins 10 mg/kg 699.9 > 984.6				
	Soy proteins 10 mg/kg 699.9 > 870.5				

		Cookie	Sauce	Ice cream	Chocolate
Soy ELINLATMCR	Soy proteins 0 mg/kg 610.8 > 638.3				
	Soy proteins 0 mg/kg 610.8 > 865.4				
	Soy proteins 10 mg/kg 610.8 > 638.3				
	Soy proteins 10 mg/kg 610.8 > 865.4				
Soy LITAIPVKNKPR	Soy proteins 0 mg/kg 464.6 > 583.4				
	Soy proteins 0 mg/kg 464.6 > 767.5				
	Soy proteins 10 mg/kg 464.6 > 583.4				
	Soy proteins 10 mg/kg 464.6 > 767.5				
Egg (white) ISQAVHAAHAEINEAGR	Soy proteins 0 mg/kg 887.5 > 1067.5				
	Egg proteins 0 mg/kg 887.5 > 1138.6				
	Egg proteins 17 mg/kg 887.5 > 1067.5				
	Egg proteins 17 mg/kg 887.5 > 1138.6				

		Cookie	Sauce	Ice cream	Chocolate
Egg (white) LTEWTSSNVMEER	Egg proteins 0 mg/kg 791.3 > 951.4				
	Egg proteins 0 mg/kg 791.3 > 1052.5				
	Egg proteins 17 mg/kg 791.3 > 951.4	 $I=2.3 \cdot 10^5$ $S/N=12.4$	 $I=5.9 \cdot 10^5$ $S/N=41.0$	 $I=4.9 \cdot 10^5$ $S/N=29.1$	 $I=3.6 \cdot 10^5$ $S/N=33.2$
	Egg proteins 17 mg/kg 791.3 > 1052.5	 $I=1.1 \cdot 10^5$ $S/N=6.2$	 $I=3.2 \cdot 10^5$ $S/N=22.9$	 $I=2.6 \cdot 10^5$ $S/N=79.4$	 $I=2.2 \cdot 10^5$ $S/N=60.0$
Egg (yolk) EALQPIHDLADEAISR	Egg proteins 0 mg/kg 593.3 > 668.8				
	Egg proteins 0 mg/kg 593.3 > 761.4				
	Egg proteins 61.6 mg/kg 593.3 > 668.8	 $I=2.9 \cdot 10^5$ $S/N=14.5$	 $I=4.5 \cdot 10^6$ $S/N=130.5$	 $I=2.7 \cdot 10^6$ $S/N=82.3$	 $I=31 \cdot 10^4$ $S/N=$
	Egg proteins 61.6 mg/kg 593.3 > 761.4	 $I=5.3 \cdot 10^4$ $S/N=5.4$	 $I=4.0 \cdot 10^5$ $S/N=26.1$	 $I=2.6 \cdot 10^5$ $S/N=12.4$	 $I=5.1 \cdot 10^4$ $S/N=$
Egg (yolk) NIPFAEYPTYK	Egg proteins 0 mg/kg 671.8 > 558.3				
	Egg proteins 0 mg/kg 671.8 > 1115.5				
	Egg proteins 61.6 mg/kg 671.8 > 558.3	 $I=3.7 \cdot 10^5$ $S/N=16.1$	 $I=3.1 \cdot 10^6$ $S/N=35.9$	 $I=2.0 \cdot 10^6$ $S/N=15.6$	 $I=8.4 \cdot 10^5$ $S/N=19.7$
	Egg proteins 61.6 mg/kg 671.8 > 1115.5	 $I=9.4 \cdot 10^4$ $S/N=18.2$	 $I=6.1 \cdot 10^5$ $S/N=62.7$	 $I=3.8 \cdot 10^5$ $S/N=21.4$	 $I=1.7 \cdot 10^5$ $S/N=30.5$

