Development of a parallel reaction monitoring (PRM) method for milk allergens

Detection of both casein and whey allergens from baked cookies

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Goal

To develop a PRM workflow for detection of both casein and whey allergens and test its performance in a baked cookie matrix incurred with nonfat dry milk (NFDM).

Introduction

Milk allergy is one of the common food allergies presented in early childhood with an estimated prevalence ranging from 0.5% to 3% among children in developed countries[1]. Some cases persist into adulthood as well. Among the US population, an estimated 6.1 million report symptoms of allergic reactions to milk or milk-containing foods according to food allergy statistics released in 2020[2]. Milk is present as an ingredient in various foods as unfractionated milk, or as milk-derived ingredients enriched in casein or whey fraction. Since strict avoidance of milk and milk-containing foods is the only way to prevent allergic reactions in susceptible individuals, accurate declaration of intentional addition of or potential cross-contact with milk/milk-derived ingredients is critical to completely exclude milk from the diet. To ensure allergens are labeled properly, food manufacturers and regulators have to determine the presence of undeclared allergens in food products. This points to the importance of having reliable and sensitive methods for accurate detection of milk allergens present in foods, irrespective of the type of milk-derived ingredient

present. Conventional methods based on immunoassays face a challenge in accurate detection of milk allergens from processed complex food matrices, especially with whey allergens, which are more susceptible to thermal processing than the caseins. Mass spectrometry offers a possible solution here, as targeted detection of specific allergen proteins using its peptides as surrogates can be less susceptible to the changes in target proteins caused by food processing. Accurate and sensitive detection of many of the food allergens listed by US and European regulatory bodies employing mass spectrometry have been demonstrated[3]. Many of these studies employ widely established selected reaction monitoring (SRM) methods for target detection where pre-selected fragment ions of the target peptides are monitored using triple quadrupole mass spectrometers. Although SRM offers high sensitivity in detection, there could be challenges with specificity if the Q1 and Q3 quadrupole mass filters are set to unit resolution, reducing its ability to discriminate analytes from interfering matrix ions.

In this study, we have leveraged the advantages of high resolution and mass accuracy offered by the Thermo Scientific™ Orbitrap™ mass analyzer to develop a parallel reaction monitoring (PRM) method for targeted detection of both casein and whey milk allergens from complex food matrices. Towards this, we have selected a pool of peptides representing proteins from both casein and whey fractions of milk as targets through a discovery-driven target selection approach. Six milk-derived ingredients, varying in protein content and composition of casein and whey proteins, were used for target peptide selection employing bottom-up discovery proteomics. The applicability of these peptides in milk allergen detection from a complex matrix, which has undergone thermal processing, was evaluated using baked cookie and raw cookie dough incurred with NFDM as test matrices. The PRM method developed in this study demonstrates robust and reproducible detection of both casein and whey allergens from baked cookie matrix incurred with NFDM at 1 ppm concentration.

The experimental flexibility of Orbitrap mass spectrometers enable discovery-driven target selection and PRM acquisition on the same instrumentation platform. This workflow proved advantageous in hassle-free transfer of an untargeted discovery method to a targeted detection method (Figure 1).

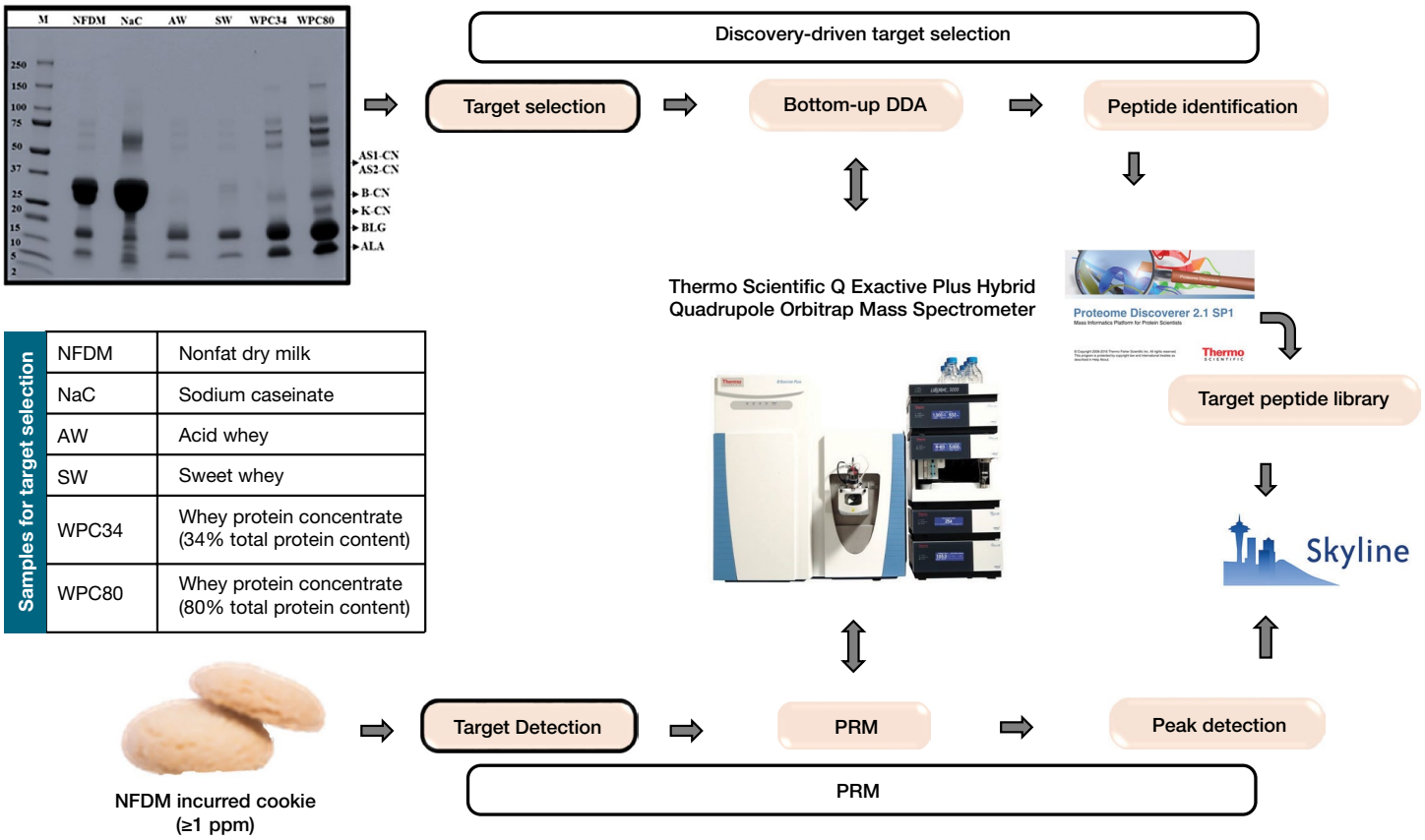

Figure 1. Workflow for discovery-driven target selection and PRM method development for targeted detection of milk allergens in baked cookie matrix

Experimental

Sample preparation

For target selection:

The soluble proteins from six milk-derived ingredients were extracted using an optimized multi-step extraction procedure under denaturing conditions for bottom-up data-dependent acquisition (DDA) experiments as described elsewhere[4]. In brief, 1 g of sample was extracted in 20 mL buffer containing 6 M urea, 50 mM Tris at pH 8.5, and 20 mM dithiothreitol (DTT) by subjecting to incubation at 60 °C for 10 min, vortexing for 1 min, sonication for 10 min, followed by re-incubation at 60 °C for 10 min. Finally, the solubilized proteins were collected by centrifugation at 2500 × g for 10 min. The supernatant was reduced with 5 mM DTT, alkylated with 10 mM iodoacetamide (IAA), and subjected to overnight in-solution tryptic digestion (1:100 trypsin:protein ratio). The digested peptides were desalted using Thermo Scientific™ Pierce™ C18 spin columns (P/N 89870) as per manufacturer's instructions and dried under vacuum.

For targeted PRM:

The proteins from cookie and dough samples were extracted as described above. Porcine gelatin was incorporated as a carrier protein in the extraction buffer to avoid non-specific loss of peptides in samples for PRM. To account for the dynamic complexity in analyte-to-matrix ratio in test samples, the sample preparation was modified to include filter-assisted sample preparation (FASP) for tryptic digestion and high peptide binding capacity (up to 5 mg) Pierce C18 spin columns (P/N 89852) for desalting[4].

Data acquisition

Bottom-up discovery:

A Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer coupled in-line with a Thermo Scientific™ UltiMate™ 3000 RS binary ultrahigh-performance liquid chromatography system was used for bottom-up discovery proteomics of milk-derived ingredients for target peptide selection. The dried peptides were resuspended in 50 µL of 0.1% formic acid (FA) containing 3% acetonitrile (ACN), and 2 µL (equivalent to 150 ng) were injected onto a Thermo Scientific™ Hypersil GOLD™ C18 Selectivity LC Column (100 x 1 mm, 1.9 µm), maintained at 35 °C. A binary gradient consisting of 0.1% FA in water as mobile phase A and 0.1% FA in ACN as mobile phase B was used. The peptides were separated on a linear gradient 2–40% B for 67 min at 60 µL/min flow rate. The static mixer of the UHPLC pump was replaced with the 10 µL inline filter (P/N 6044.3870, mixer set) to make it compatible with lower flow rates. The mass spectra of the eluted peptides were acquired in DDA mode using the parameters given in Table 1.

Table 1. Settings for MS parameters

Parameter	DDA settings	PRM settings
Ionization	ESI Positive mode	ESI Positive mode
Spray voltage	3.5 kV	3.5 kV
Ion transfer capillary temp.	320 °C	320 °C
Sheath gas	15	15
Aux gas	0	0
Sweep gas	1	1
S-Lens RF	60	60
Acquisition type	Full MS-DDA (Top 10)	Full MS-DIA
Precursor scan		
Resolution	70,000 at *m/z* 200	17,500 at *m/z* 200
AGC target	3e6	1e6
Max. injection time	100 ms	60 ms
Mass range	*m/z* 370–1400	*m/z* 370–1400
Spectrum data type	Profile	Profile
Product ion scan		
Resolution	70,000 at *m/z* 200	70,000 at *m/z* 200
AGC target	2.5e4	2e5
Max. injection time	60 ms	Auto
Loop count	NA	20
Isolation width	*m/z* 2	*m/z* 1.6 (+ *m/z* 0.2 offset)
Fragmentation mode	HCD	HCD
Normalized collision energy	27	27
Charge state rejection	Unassigned and >6	NA
Dynamic exclusion	3 s	NA
Spectrum data type	Profile	Profile

PRM:

PRM experiments were carried out using the same instrumentation platform as for the bottom-up discovery experiments, with certain modifications in method parameters (Table 1). The versatility in instrument setup available with the Orbitrap system was used to monitor

both the precursor and product ions of the targeted peptides. This is achieved by using a Full MS node followed by a Data Independent Acquisition (DIA) node with a narrow isolation window, in place of the predefined PRM node available with the instrument set up. In this mode a Full MS scan is acquired followed by consecutive acquisition of fragmentation spectra for all the target peptides in the inclusion list within the scheduled retention time. The candidate peptides were specified in an inclusion list within a 5 minute isolation window. A linear increase in B from 14% to 40% in 37 minutes was used for elution of target peptides. At the end of the gradient, the column was washed with 100% methanol (mobile phase D) and re-equilibrated in 2% ACN, at 150 µL/min flow rate, in order to avoid carry-over effects, if any. The flow was diverted to waste during the wash and re-equilibration steps. The LC gradient used for the PRM method is provided in Table 2.

Data acquisition:

Data acquisition for both bottom-up discovery and PRM runs were carried out using Thermo Scientific™ Xcalibur™ 4.1 SP1 software.

Data analysis

Identification of candidate targets:

The bottom-up DDA data was analyzed with Thermo Scientific™ Proteome Discoverer™ 2.1 SP1 software using the SEQUEST™ HT database search algorithm to identify proteins from milk-derived ingredients. Uniprot *Bos taurus* database (accessed on March 6, 2017), appended with the Global Proteome Machine common Repository of Adventitious Proteins database (https://www.thegpm.org/crap/) after excluding those for bovine entries, was used as a reference database for protein identification. The search parameters were as follows: mass error for precursors, up to 10 ppm; for fragments, up to 0.06 Da; maximum missed cleavages, 2; fixed modification, carbamidomethylation of cysteine; variable modification, oxidation of methionine and phosphorylation of serine, threonine, and tyrosine. False Discovery Rate (FDR) validation of peptide spectrum matches (PSM) was carried out using Percolator, and target FDR was kept at 0.01 for a confident identification of peptide and proteins. Candidate target peptides for casein and whey were identified based on the abundance of peptides across difference milk-derived ingredients. Towards this, the precursor ion area of the identified peptides was enumerated in Skyline software[5] using MS1 filtering mode.

Table 2. LC gradient method

Step	Time (min)	Flow rate (µL/min)	A 0.1% Formic acid in water	B 0.1% Formic acid in acetonitrile	D 100% Methanol
Equilibration	-3:00	60	98	2	0
Gradient elution	0:00	60	98	2	0
	3:00	60	98	2	0
	4:00	60	86	14	0
	7:00	60	86	14	0
	44:00	60	60	40	0
	50:00	60	60	40	0
Wash	54:00	60	2	98	0
	58.01	60	100	0	0
	58.02	60	0	0	100
	58.03	150	0	0	100
	64:00	150	0	0	100
Re-equilibration	64.01	150	100	0	0
	64:02	150	98	2	0
	74:00	150	98	2	0
	78:00	60	98	2	0
	80:00	60	98	2	0

Targeted detection of milk allergen:

The PRM data was analyzed in Skyline software. The peak detection and matching were performed with reference to the spectral library generated in the discovery experiments. Accuracy in peak detection is defined by several quantitative metrics. Peak Found Ratio (PFR) measures the number of fragments that match with the library spectra and a score of 1 indicates 100% match for all selected fragments. The correlation between intensities of matched fragments with that of the library spectra is measured as a dot product (dotp) and value of 1 indicates the highest correlation. In this study, a peak is considered detected when the top three intense fragments co-align with PFR =1, dotp value ≥ 0.9, and the average fragment mass error ≤ 5 ppm. A sum of extracted peak area from the top three matching fragments (Table 3) was used to determine the abundance of the peptides.

Table 3. Candidate targets for milk allergen detection*

Protein	Target peptides**	Symbol	*m/z*	Charge state	Scheduling window‡	Fragments
α-Lactalbumin (ALA)	K.DDQNPHSSNICNISCDK.F	DDQ	668.6109	3+	19.58±2.5	896.3601(y7), 736.3294(y6), 509.2024(y4)
	K.VGINYWLAHK.A	VGI	400.8890	3+	31.47±2.5	654.3722(y5), 482.2929(y4), 355.2088(y3)
	K.LDQWLCEK.L	LDQ	546.2631	2+	27.66±2.5	735.3494(y5), 549.2701(y4), 436.1860(y3)
β-Lactoglobulin (BLG)	**-.LIVTQTMK.G†**	LIV	467.2755	2+	24.41±2.5	707.3756(y6), 608.3072(y5), 379.2010(y3)
	R.VYVEELKPTPEGDLEILLQK.W	VYV	771.7578	3+	37.63±2.5	1452.7944(y13), 1254.6940(y11), 388.2554(y3)
	K.IDALNENK.V	IDA	458.7404	2+	15.37±2.5	803.3984(y7), 688.3624(y6), 504.2413(y4)
	K.VLVLDTDYK.K	VLV	533.2950	2+	30.00±2.5	854.4302(y7), 754.3618(y6), 526.2508(y4)
	R.LSFNPTQLEEQCHI.-†	LSF	858.4045	2+	34.70±2.5	1254.5783(y10), 928.4193(y7), 815.3352(y6)
α_{S1}-Casein (AS1-CN)	K.HQGLPQEVLNENLLR.F†	HQG	587.3192	3+	33.17±2.5	871.4996(y8), 758.4155(y6), 436.2303(b4)
	R.FFVAPFPEVFGK.E	FFV	692.8686	2+	40.89±2.5	920.4876(y8), 676.3665(y6), 394.2125(b3)
	R.YLGYLEQLLR.L	YLG	634.3559	2+	39.55±2.5	991.5571(y8), 771.4723(y6), 658.3883(y5)
α_{S2}-Casein (AS2-CN)	K.ENLCSTFCK.E†	ENL	579.7495	2+	25.31±2.5	802.3222(y6), 642.2916(y5), 454.2119(y3)
	R.NAVPITPTLNR.E†	NAV	598.3433	2+	28.08±2.5	911.5309(y8), 701.3941(y6), 285.1557(b3)
	K.FALPQYLK.T†	FAL	490.2842	2+	33.64±2.5	761.4556(y6), 648.3715(y5), 423.2602(y3)
β-Casein (B-CN)	**K.VLPVPQK.A**	VLP	390.7525	2+	21.08±2.5	568.3453(y5), 372.2241(y3), 213.1598(b2)
	K.AVPYPQR.D	AVP	415.7296	2+	16.72±2.5	660.3464(y5), 400.2303(y3), 171.1128(b2)
	R.GPFPIIV.-	GPF	371.7285	2+	38.53±2.5	441.3071(y4), 344.2544(y3), 231.1703(y2)
κ-Casein (K-CN)	**K.YIPIQYVLSR.Y**	YIP	626.3584	2+	36.23±2.5	975.5622(y8), 765.4254(y6), 637.3668(y5)
	R.SPAQILQWQVLSNTVPAK.S	SPA	990.5494	2+	37.46±2.5	315.2027(y3), 384.1878(b4), 497.2718(b5)
			660.7019	3+	37.46±2.5	497.2718(b5), 738.4145(b7), 315.2027(y3)

*Peptides identified as robust, sensitive, and selective targets for milk allergen detection in baked cookie matrix are marked in bold

**Cysteines are modified by carbamidomethylation, which adds 57 Da to formula weight

†Peptides which are unique to domestic cow, *Bos taurus*

‡Scheduling windows are assigned in Skyline centered on the RT from training experiments

Results and discussion

Discovery-driven target selection

Bottom-up DDA analysis identified 309 peptides with high confidence (FDR < 0.01) from six milk-derived ingredients. These peptides represented 38 milk proteins including both major milk proteins (α_{S1}-, α_{S2}-, β-, and κ-caseins; β-lactoglobulin, and α-lactalbumin) and minor milk proteins (bovine serum albumin, lactoferrin, and immunoglobulins). From these, 91 peptides representing the four casein and two whey proteins were taken for further evaluation for candidate target selection. The distinction in peptide and protein identifications between casein- and whey-derived ingredients was not absolute as some whey peptides were identified in casein ingredients and vice versa. However, the relative abundances of casein and whey peptides were substantially different between different classes of milk ingredients (Figure 2). A series of compositional and performance criteria was applied to identify the candidate target peptides suitable for milk allergen detection from food matrices as follows:

- Should represent both casein and whey protein fractions.
- Should be unique to the candidate protein and should not have sequence homology with other proteins or proteins from unrelated species.
- Should be identifiable with good fragment matches (FDR ≤ 0.01).
- Should not have inherent post-translational modifications or be susceptible to atypical process induced modifications.
- Should be 7–20 amino acids long and should have a distinct isotopic pattern.
- Should not be part of another missed cleavage peptide.
- Should have a single dominant charge state.
- Fragments of target peptides should have consistent intensity profiles with a PFR of 1 and dotp value ≥0.9.

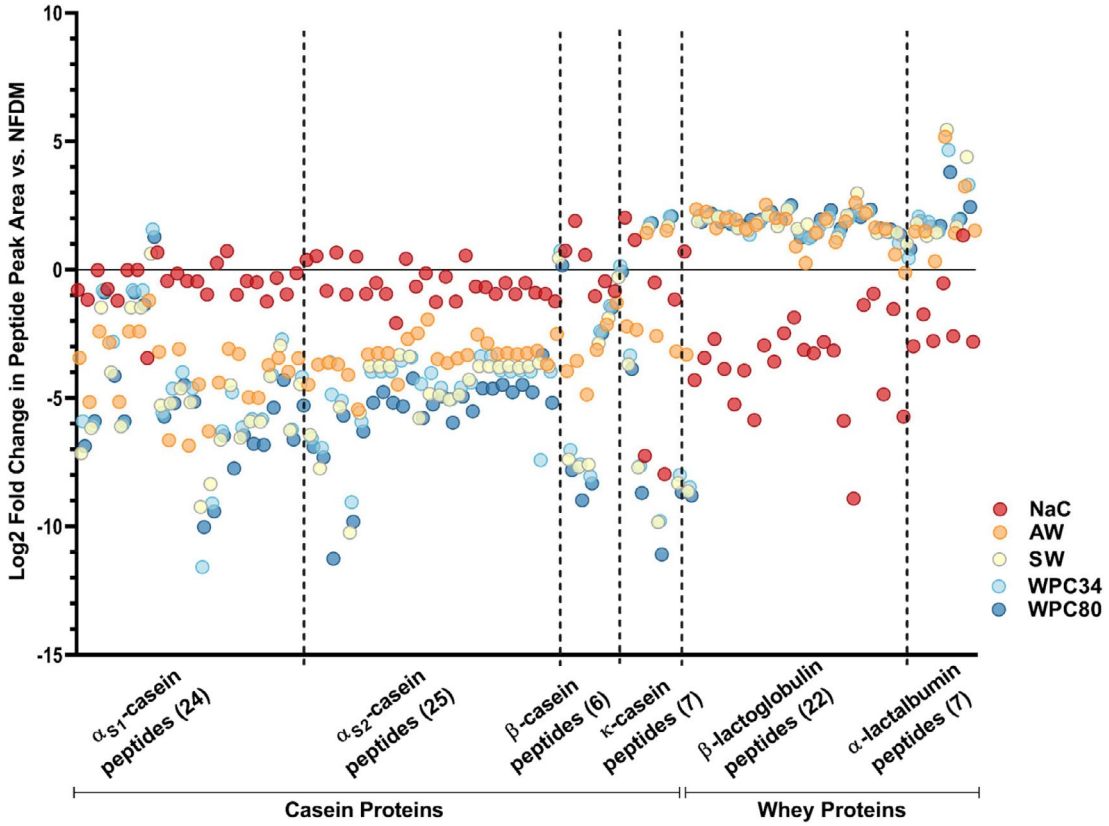

Figure 2. A quantitative comparison of peptides representing major milk proteins identified from milk-derived ingredients: sodium caseinate (NaC), acid whey (AW), sweet whey (SW), whey protein concentrate 34 (WPC34), and whey protein concentrate 80 (WPC80). The average peak areas of the peptides in respective milk-derived ingredients were calculated using MS1 filtering mode in Skyline. The graph depicts the log2 fold change in peptide peak area normalized to that of NFDM (n=4). A clear distinction in abundance of casein and whey peptides among casein-rich ingredient NaC and whey-rich ingredients AW, SW, and WPCs is observable.

The refined candidate target list included 19 peptides representing four major casein and two major whey proteins (Table 3). Sequence analysis revealed that six of these candidate peptides were unique to domestic cow, *Bos Taurus,* which could potentially help in distinguishing milk from cow and other closely related species such as goat, sheep, buffalo, etc. These peptides were further taken for PRM method development.

PRM method development

The spectral library for PRM data processing was constructed from the discovery proteomics data. The top three intense fragments in the library for each precursor, without any background interference, were used for peak detection. The gradient length for the PRM method was set between 14% and 47% B based on the training experiments. There was minimal to no overlap in elution of targets (Figure 3a), and there was a good agreement between the predicted and observed retention time of the target peptides (Figure 3b). The targets were scheduled within 5 minutes isolation windows and the maximum number of possible concurrent precursors was 6–7 (Figure 3c). The candidate target peptides were evaluated for their sensitivity in targeted detection from NFDM extracts of varying concentrations (0, 1, 5, 10, 20, 100, and 1000 ppm). Porcine gelatin was incorporated as a carrier protein to avoid loss of peptides at lower concentrations due to non-specific adsorption. Thirteen of the candidate targets, representing both casein and whey proteins, were sensitive to detect milk allergens from 1 ppm NFDM in presence of gelatin (Figure 4a). Interestingly, the use of carrier protein was found to enhance the sensitivity for many of the candidate target peptides monitored. Moreover, the addition of the carrier protein also rendered a more linear response-to-concentration correlation for the candidate targets (Figure 4b).

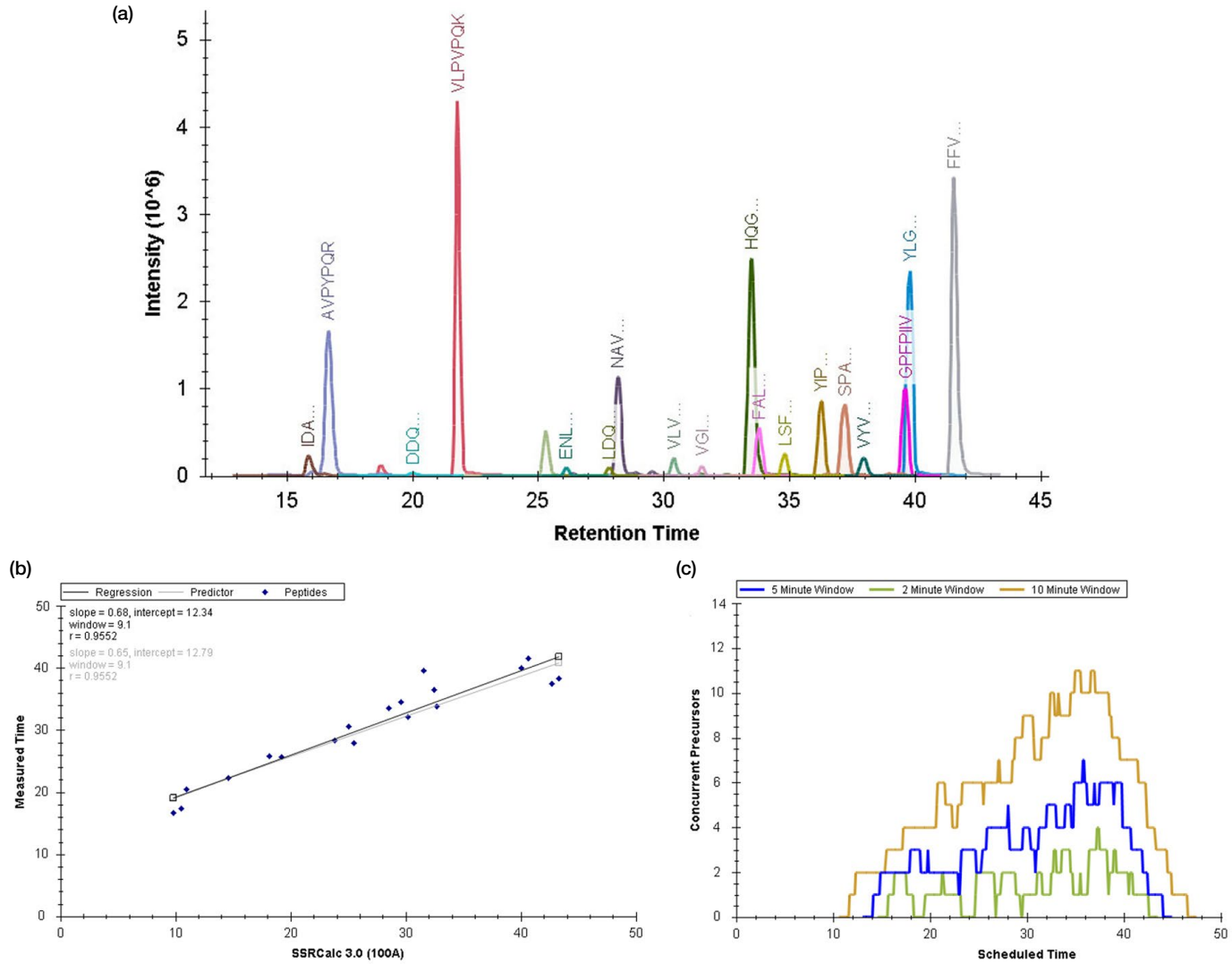

Figure 3. Elution profile of target peptides. (a) The intensity trace from the target peptide fragments across the gradient; (b) The regression curve showing the correlation between measured and theoretical retention time of target peptides; (c) The number of possible concurrent precursors with 2, 5, and 10 minute isolation windows. A five-minute window was used for PRM experiments.

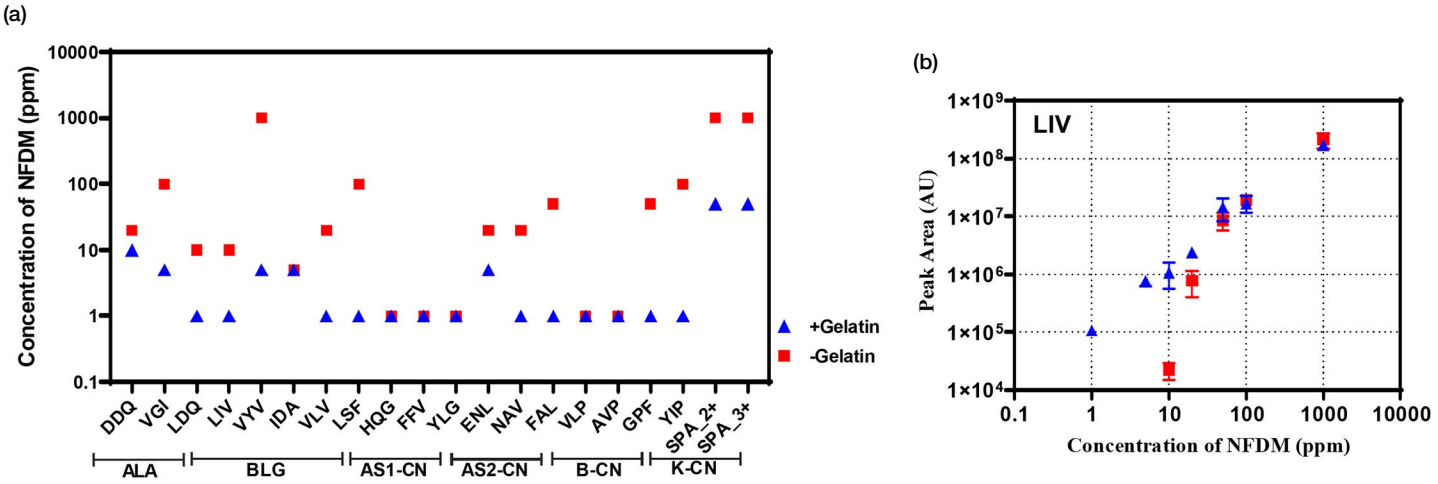

Figure 4. Sensitivity in detection of target peptides. (a) The lowest detection point for each of the peptides from varying concentrations of NFDM is plotted (n=4). Presence of gelatin as carrier protein improves the sensitivity in detection of 13 of the candidate targets tested. (b) Linearity in response with increase in concentration of analyte is depicted with a peptide LIV as an example.

Evaluation of milk allergen detection in baked cookies

Thermal processing during baking can alter the proteins present in foods. The proteins could get denatured, modified, or be subjected to non-specific proteolysis. This poses a challenge to conventional immunochemical methods as retaining intact conformation of the target proteins is mandated for detection. However, some of the peptides of these proteins might have escaped from the processing-induced alterations and could be used as targets for detection from a processed food matrix. Hence, it is very important to identify peptides that are resistant to food processing as candidate targets. Cookie samples, incurred with 1, 10, and 100 ppm of NFDM, were prepared[6] to evaluate the performance of candidate milk allergen targets in baked cookies. Meanwhile, the detection of allergen peptides can be influenced by the matrix as well, since the cookies are rich in protein, fat, and carbohydrates contributed by the ingredients in the cookie formulation. The candidate targets were also evaluated in cookie dough, which has not undergone thermal processing, to understand the matrix effects in target detection.

All 19 target peptides were detectable in cookie and dough samples incurred with 100 ppm NFDM, indicating these targets are impervious to both thermal processing and matrix effects at 100 ppm levels. However, the sensitivity of detection varied among cookie and dough at lower levels. At 10 ppm incurred levels, 16 of the 19 targets were detectable from baked cookies. Ten candidate targets were detectable from cookies incurred with 1 ppm NFDM (Figure 5), making these peptides suitable targets for sensitive detection of milk allergen from baked food matrices. These included one or more peptides (YLG, NAV, FAL, VLP, AVP, YIP, SPA_2+) representing each of the four casein allergens. Whey proteins are generally more sensitive to thermal processing. However, three peptides (LIV, IDA, LSF) representing whey protein, BLG, were detectable at 1 ppm incurred levels. At the same time, candidate peptides used as surrogate for second whey protein ALA, were not detectable from 1 ppm incurred cookies, indicating that these peptides are more amenable to thermal processing to be used as sensitive targets. However, evaluating a pool of targets as candidates ensured that sensitive candidates for both casein and whey proteins could be identified. In food allergen analysis, it is important to avoid both false negatives and false positives. Two of the AS1-CN peptides, HQG and FFV, have shown an occasional carryover effect due to its high hydrophobicity. These peptides were removed from the final target list without affecting the sensitivity, specificity, or throughput of the method. This further points to the advantage of having an exhaustive list of candidate peptides in the method development stage for evaluation.

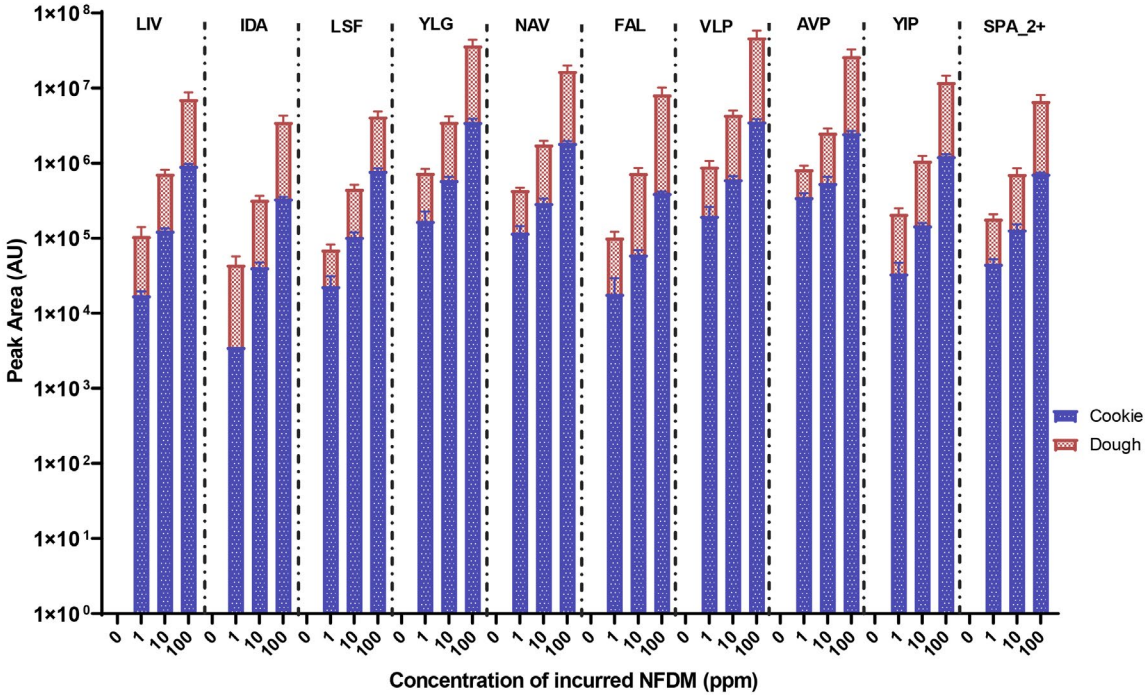

Figure 5. Detection of milk allergen from baked cookie sample. The peptides detected from cookies incurred with 1 ppm NFDM are plotted. LIV, IDA, and LSF represent whey protein BLG and YLG, NAV, FAL, VLP, AVP, YIP, and SPA represent casein proteins. The sum of peak areas from three fragment ions are compared as surrogates for abundance across 1, 10, and 100 ppm incurred cookie samples. The variation in abundance between cookie and dough samples represent the impact of thermal processing in cookie samples. Each bar represents mean ± standard error from eight data sets.

The composite peak areas of fragments, used as measure of peptide abundance, was relatively high in dough samples at each incurred level because of the difference in susceptibility of these peptides to matrix effects and thermal processing effects. A consistent reduction in recovery from thermally processed samples was observed for all targets. The robustness of this method was evaluated by testing eight replicates of cookies incurred with 10 ppm NFDM. The replicates accounted for possible variations arising from extraction, digestion, and injection steps. The relative standard deviation for the composite fragment peak area was measured for variability (Figure 6). The overall variation observed was below the upper threshold (≤20%) proposed for food allergen detection.[7]

In summary, we have developed a PRM method and identified 10 milk allergen target peptides for robust detection of both casein and whey allergens with high sensitivity and specificity.

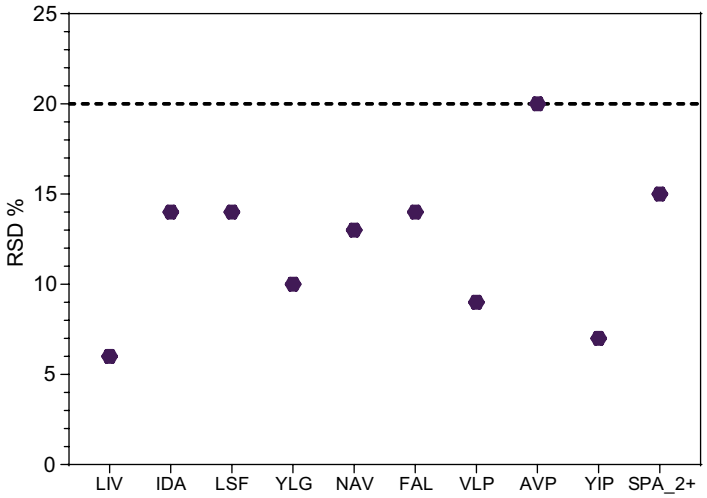

Figure 6. Robustness of the PRM method. Relative standard deviation in peak area of fragments detected from eight replicate runs of cookies incurred with 10 ppm NFDM

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Conclusion

Since milk is used in multiple forms in the food industry and conventional allergen detection methods often target either casein or whey proteins independently, identifying the source of milk becomes mandatory to have accurate detection of milk allergens. Targeting both casein and whey proteins in a single PRM method makes the requirement for prior knowledge on the source of milk or milk-derived ingredient unnecessary. The list of target peptides proposed in this study is a subset of all the plausible targets tested for milk allergen detection either independently or in combination in food matrices.[8] By incorporating the whole panel of peptides as targets in this study, all the six major milk allergens were detectable at 1 ppm incurred levels from dough matrix and at 10 ppm incurred levels from baked cookies. The applicability and adoption of this method in food allergen analysis can be improved in the future by developing a quantification strategy. Towards this, a set of matrices with varying protein, fat, and carbohydrate composition should be evaluated for target detection and matrix effects. Lastly, as laboratories are often focused on method throughput, the sample preparation procedures and LC gradients can be evaluated to find opportunities to streamline the workflow and reduce the turnover time. Also, the sensitivity and specificity of the targets identified in this study could be evaluated for a triple quadrupole platform for transferability into SRM workflows.

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