

MRM³-based LC-MS multi-method for the detection and quantification of nut allergens

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Received: 4 June 2016 / Revised: 4 August 2016 / Accepted: 16 August 2016
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Abstract Food allergies have become a global challenge to food safety in industrialized countries in recent years. With governmental monitoring and legislation moving towards the establishment of threshold allergen doses, there is a need for sensitive and quantitative analytical methods for the determination of allergenic food contaminants. Targeted proteomics employing liquid chromatography–mass spectrometry (LC-MS) has emerged as a promising technique that offers increased specificity and reproducibility compared to antibody and DNA-based technologies. As the detection of trace levels of allergenic food contaminants also demands excellent sensitivity, we aimed to significantly increase the analytical performance of LC-MS by utilizing multiple reaction monitoring cubed (MRM³) technology. Following a bottom-up proteomics approach, including a straightforward sample preparation process, 38 MRM³ experiments specific to 18 proteotypic peptides were developed and optimized. This permitted the highly specific identification of peanut, almond, cashew, hazelnut, pistachio, and walnut. The analytical performance of the method was assessed for three relevant food matrices with different chemical compositions. Limits of detection were around 1 µg/g or below in fortified matrix

samples, not accounting for the effects of food processing. Compared to an MRM-based approach, the MRM³-based method showed an increase in sensitivity of up to 30-fold. Regression analysis demonstrated high linearity of the MRM³ signal in spiked matrix samples together with robust intersample reproducibility, confirming that the method is highly applicable for quantitative purposes. To the best of our knowledge, we describe here the most sensitive LC-MS multi-method for food allergen detection thus far. In addition, this is the first study that systematically compares MRM³ with MRM for the analysis of complex foods.

Keywords Food allergens · Mass spectrometry · MRM³ · Marker peptides · Tree nut allergy

Introduction

Food allergies are currently one of the major issues in the field of food safety. Recent data demonstrate growing incidence rates in westernized countries, with nearly 5 % of the adult population and up to 8 % of young children suffering from an allergy to at least one allergenic food [1]. For many sensitized patients, exposure to the allergen results in dermatitis, gastrointestinal complications, or even life-threatening symptoms. The societal management of food allergies is therefore becoming increasingly important [2, 3] and an integral part of recent food law amendments [4].

At present, EU ([5]; regulation no. 1169) and US [6] regulations on food allergen labeling mainly target intentionally added ingredients, while the declaration of allergenic contaminants introduced during food production is based on voluntary “may contain” labeling by the manufacturer, who is responsible to guarantee product safety. Due to the limited long-term effectiveness of this precautionary labeling for the

Electronic supplementary material The online version of this article (doi:10.1007/s00216-016-9888-y) contains supplementary material, which is available to authorized users.

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protection of allergic consumers [7–9], the scientific focus has recently shifted towards the identification of “reference doses” of allergenic foods, following a NOAEL/LOAEL-based approach [10]. However, enforcing such regulations would require analytical methods that allow for the highly specific and sensitive detection and quantitation of allergens in the relevant concentration ranges, as currently discussed threshold levels are below 10 µg/g for peanut, hazelnut, and mustard, and below 1 µg/g for egg.

To date, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) have been the techniques most often applied to the analysis of food allergens [11]. Both methods, however, show significant disadvantages in terms of specificity and reproducibility: ELISA suffers from potential antibody cross-reactivity with matrix components, resulting in false positives [12, 13], and presents limitations when attempting to develop multiplexed methods [14, 15]. Furthermore, it has been shown multiple times that assays from different providers produce significantly different results, especially for the analysis of processed foods [16, 17]. PCR, on the other hand, does not directly detect the allergenic protein component, so it may give false results if proteins and DNA are separated or differentially degraded during food production, which is especially true for highly processed soy, peanut, or tree nut products [11, 18]. Due to these drawbacks, the development of methods based on liquid chromatography coupled to mass spectrometry (LC-MS) for the analysis of allergens is receiving increasing attention [19]. Several multi-methods that follow a bottom-up proteomics approach have been published, most of which use multiple reaction monitoring (MRM) [17, 20–22] or single-stage high-resolution mass spectrometry (HRMS) [23–25] to target proteotypic marker peptides. However, the weaker sensitivity of LC-MS for most allergens is still a disadvantage when compared to ELISA and PCR, which both reach limits of detection (LOD) of well below 10 µg/g, and in some cases below 1 µg/g [26–30].

With the implementation of the multiple reaction monitoring cubed (MRM³) technology in the latest QTRAP systems, a novel technique has become available for the advancement of targeted allergen analysis. In MRM³ mode, the Q3 quadrupole of the instrument is utilized as a linear ion trap (LIT), which permits the capture of MRM product ions and allows a second fragmentation step to be performed. Although a full MS³ spectrum can also be recorded, the MRM³ mode can also be run in a targeted fashion after specific experiments have been developed. In this context, MRM³ has repeatedly been employed for the quantification of single cellular proteins and peptides due to its ability to reduce background and unspecific signals relative to MRM analysis of enzymatic digests from complex protein mixtures [31–36]. It has also been proposed as a complementary or alternative

measurement mode for small molecule analysis in cases where MRM alone does not yield sufficient selectivity [37–39].

The potential of MRM³ for food analysis has recently been demonstrated for the speciation of red meat [40, 41] and the detection of crustacean allergens [42], but a multi-method approach targeting several analytes in one chromatographic run via MRM³ is yet to be reported. Here we describe just such an approach for the detection of allergens from five different nuts and peanut, and demonstrate its high specificity against commonly co-processed matrix materials (bread, chocolate, ice cream) and other allergens that would possibly interfere in ELISA- or MRM-based assays. Through the analysis of fortified food matrix samples (not accounting for the effects of food processing), we were able to demonstrate the potential of MRM³ to significantly improve LC-MS detection and quantitation of food allergens, even at concentration levels relevant to allergic patients with very low or near-zero tolerance to specific allergens.

Materials and methods

Sample material

All foodstuffs used for method development and analysis were purchased at local food stores. Hazelnuts and walnuts were bought unprocessed and shelled manually. All other nuts, grains, and seeds were industrially preshelled or ground. Synthetic peptides were obtained from JPT Peptide Technologies (Berlin, Germany) at SpikeTides quality (unpurified, unquantified).

Sample preparation

All sample materials were ground and homogenized prior to extraction with a cutting mill. One gram of the crude flour was added to 10 mL of extraction buffer (6 M urea, 1 M thiourea, 50 mM Tris-HCL adjusted to pH 8.0) in a 50-mL plastic tube and dispersed for 2 min using an Ultra Turrax T-25 (ika, Staufen, Germany) with a 10 N dispersing element at 9500 rpm, yielding a finely granulated suspension. After centrifugation for 60 min at 4 °C and 12000×g, a 100-µL aliquot of the supernatant was transferred to a 1.5-mL reaction tube and subjected to tryptic digestion according to a standard protocol [40]. To allow complete digestion, samples were incubated for at least 14 h at 37 °C under slow shaking using 20 µg of Serva (Heidelberg, Germany) sequencing-grade trypsin per sample.

For desalting and purification, the tryptic digest was subjected to solid-phase extraction using Strata-X 33 µm RP 30 mg/1 mL columns (Phenomenex, Aschaffenburg, Germany). The cartridges were activated and equilibrated

according to the manual. Samples were loaded onto the cartridge, washed with 1 mL of 1 % formic acid (FA), and eluted with 1 mL 90 % MeOH 1 % FA. Solvent was removed under a gentle nitrogen flow at 40 °C, and the samples were redissolved in 100 µL acetonitrile/water (3:97), corresponding to the initial HPLC conditions.

Preparation of fortified matrix samples

Fortified matrix extracts were prepared by first coextracting a mixture of 10 % (w/w) of ground allergenic food (almond, cashew, hazelnut, peanut, pistachio, walnut) in allergen-free matrix (multigrain bread, vanilla ice cream, dairy chocolate). These 10 % extracts were then combined and further diluted with pure matrix extract to produce extracts corresponding to food samples containing 100, 30, 10, 3, 1, and 0.3 µg/g of each allergenic food. Using this approach allowed the effects of the matrix on extraction to be sufficiently accounted for without the need to produce extremely low and probably inaccurate dilutions of ground nuts in matrix. Sample preparation was then performed as described above.

Liquid chromatography–mass spectrometry

Data were acquired on a QTRAP 6500 LC-MS/MS system (Sciex, Darmstadt, Germany) using an IonDrive™ Turbo V ESI source run in positive mode coupled to an Agilent 1260 Infinity HPLC. LC separation of tryptic digests was carried out on a Phenomenex Kinetex, 2.6 µm, C18, 100 Å (100 × 2.1 mm) column at a flow rate of 300 µL/min, employing a three-step acetonitrile/water gradient at a full duty cycle time of 34 min.

MS data were acquired under the control of Analyst software (v1.6.2) operating in MRM and MRM³ mode. Q1 and Q3 were both set to unit resolution (0.7 ± 0.1 amu) in MRM mode. In MRM³ mode, Q1 was also set to unit resolution while Q3 was used in LIT mode. The ion fill time and excitation time were set at 250 and 25 ms, respectively, and the scan rate was 10000 Da/s, resulting in a total scan time of 334–413 ms per MRM³ experiment. To reduce the cycle time, LC-MS runs were divided into multiple periods, with the acquisition of peptide-specific MRM³ experiments limited to its period of elution.

MRM and MRM³ parameters were tuned with synthetic peptides using syringe pump injection and chromatographic runs, respectively. Control samples of tryptic digests and/or synthetic peptides were routinely analyzed between sample injections to survey retention time stability and relative intensity of MRM transitions. Data evaluation was performed using Analyst v1.6.2. In the case of MRM³, signal integration was based on the extracted ion chromatograms (XIC) from −0.5 up to +1.0 Da around the m/z of the second product ion. All peaks were smoothed by three points before integration. For further

details regarding the the HPLC gradient, source parameters, and MS experiments, see Tables S1 and S2 in the “Electronic supplementary material” (ESM).

Results and discussion

MRM and MRM³ method development

Forty-four proteotypic marker peptides from allergenic proteins were identified in a previous study [25] by a shotgun proteomics approach and verified by a thorough investigation of specificity and sensitivity (ESM Table S3). For the purpose of sequence confirmation, all method development steps were performed using synthetic peptides. For each peptide, ≥ 10 (if possible) of the most intense b- and y-ion transitions were identified and tuned manually during syringe pump injection using synthetic peptides. The optimized MRM experiments were monitored during a chromatographic run, and for each peptide the five most sensitive MRM transitions (ESM Table S2) were considered for the subsequent development of MRM³ experiments. This broad approach ensured that there were a sufficiently large number of potential precursor ions for MRM³ optimization.

To identify suitable MRM³ transitions, MS³ fragmentation patterns were recorded for each peptide from chromatographic runs based on the five most sensitive MRM experiments. MRM³ experiments appropriate for the targeted analytical method were selected taking into account the following criteria: signal intensity; ion should be assignable to a sequence-specific b- or y-ion; where possible, no transitions should include fragmentation steps that apply to many tryptic peptides (e.g., $b_2^+ \rightarrow a_2^+$, $y_5^+ \rightarrow y_1^+$, with y_1 = lysine/arginine). A considerable number of the 220 MRM transitions from our initial selection showed only unspecific MS³ fragmentation in the linear ion trap (e.g., loss of H₂O or NH₃) and were therefore rejected. Following this approach, method development resulted in the selection of three proteotypic peptides for each allergenic food. Two MRM³ transitions per peptide were included in the multi-allergen method (Table 1). In the cases of marker peptides Peanut 2, Peanut 3, Pistachio 2, and Pistachio 3, one MRM³ transition for each had to be selected that was not attributable to a b- or y-ion but proved to be specific experimentally (see below). To further enhance specificity, we preferentially selected two MRM³ experiments from different second precursor ions. This was possible for most proteotypic peptides when the signal intensity for the second MRM³ was sufficiently high.

During method optimization, we observed a linear correlation between signal intensity and fill time of the linear ion trap for selected MRM³ transitions (Fig. 1). The fill

Table 1 Peptide markers and MRM³ transitions

Peptide	Allergen	Sequence	Retention time (min)	Transitions (MRM ³)	
				Ions	<i>m/z</i>
Almond 1	Pru du 6.0101	GNLDFVQPPR	12.38 ± 0.32	M ⁺⁺ → y3 ⁺ → y1 ⁺	571.8 → 369.2 → 175.1
				M ⁺⁺ → y7 ⁺ → y6 ⁺	571.8 → 858.4 → 743.4
Almond 2	Pru du 6.0201	VQGQLDFVSPFSR	19.61 ± 0.48	M ⁺⁺ → y8 ⁺ → y7 ⁺	740.4 → 954.5 → 839.4
				M ⁺⁺ → y9 ⁺ → y7 ⁺	740.4 → 1067.6 → 839.4
Almond 3	Pru du 6.0201	ALPDEVLQNAFR	20.67 ± 0.40	M ⁺⁺ → y10 ²⁺ → y6 ⁺	686.9 → 594.8 → 748.4
				M ⁺⁺ → y10 ²⁺ → y8 ⁺	686.9 → 594.8 → 976.5
Cashew 1	Ana o 2	ADIYTPEVGR	8.78 ± 0.30	M ⁺⁺ → y5 ⁺ → y3 ⁺	560.8 → 557.3 → 331.2
				M ⁺⁺ → y6 ⁺ → y3 ⁺	560.8 → 658.4 → 331.2
Cashew 2	Ana o 2	EGQMLVVPQNFAVVK	20.86 ± 0.25	M ⁺⁺⁺ → y8 ²⁺ → y6 ⁺	553.6 → 451.8 → 677.4
				M ⁺⁺⁺ → y9 ²⁺ → y8 ²⁺	553.6 → 501.3 → 451.8
Cashew 3	Ana o 2	LTTLSLNLPIK	24.28 ± 0.86	M ⁺⁺ → y9 ⁺ → y4 ⁺	720.4 → 1011.6 → 470.3
				M ⁺⁺⁺ → y5 ⁺ → y4 ⁺	480.6 → 697.5 → 470.3
Hazelnut 1	Cor a 9	LNALEPTNR	6.23 ± 0.13	M ⁺⁺ → y5 ⁺ → y4 ⁺	514.1* → 616.3 → 487.3
				M ⁺⁺ → b3 ⁺ → b2 ⁺	514.3 → 299.2 → 228.1
				M ⁺⁺ → y7 ⁺ → y4 ⁺	514.1* → 800.4 → 487.3
Hazelnut 2	Cor a 9	VQVDDNGNTVFDDELK	15.87 ± 0.52	M ⁺⁺⁺ → y6 ⁺ → y2 ⁺	645.6 → 794.4 → 288.2
				M ⁺⁺⁺ → y6 ⁺ → y3 ⁺	645.6 → 794.4 → 417.2
Hazelnut 3	Cor a 9	QGQVLTIPQNFVAVK	18.34 ± 0.21	M ⁺⁺ → y10 ⁺ → y8 ⁺	807.5 → 1088.6 → 874.5
				M ⁺⁺⁺ → y8 ²⁺ → y6 ⁺	538.6 → 437.7 → 649.4
Peanut 1	Ara h 3	FNLAGNHEQEFLR	13.65 ± 0.32	M ⁺⁺⁺ → y11 ²⁺ → y3 ⁺	525.6 → 657.3 → 435.3
				M ⁺⁺⁺ → y10 ²⁺ → y3 ⁺	525.7 → 600.8 → 435.3
Peanut 2	Ara h 3	WLGLSAEYGNLYR	21.69 ± 0.37	M ⁺⁺ → y9 ⁺ → y6 ⁺	771.4 → 1072.5 → 785.4
				M ⁺⁺ → b2 ⁺ → unkn**	771.4 → 300.2 → 255.1
Peanut 3	Ara h 3	TANDLNLLILR	21.80 ± 0.33	M ⁺⁺ → y9 ⁺ → y7 ⁺	628.4 → 1083.7 → 854.6
				M ⁺⁺ → y6 ⁺ → unkn**	628.4 → 741.5 → 341.3
Pistachio 1	Pis v 5	AMISPLAGSTSVLR	17.52 ± 0.67	M ⁺⁺ → y10 ²⁺ → y8 ⁺	701.9 → 500.8 → 790.4
				M ⁺⁺ → y11 ⁺ → y7 ⁺	701.9 → 544.3 → 719.4
Pistachio 2	Pis v 5	ITSLNSLNLPIK	23.71 ± 0.81	M ⁺⁺ → y4 ⁺ → unkn**	713.4 → 470.3 → 183.4
				M ⁺⁺ → y9 ⁺ → y4 ⁺	713.4 → 1011.6 → 470.3
Pistachio 3	Pis v 5	GFESEEESEYER	6.92 ± 0.22	M ⁺⁺ → y7 ⁺ → y5 ⁺	745.8 → 941.4 → 683.3
				M ⁺⁺ → b2 ⁺ → a2 ⁺	745.8 → 205.1 → 177.1
Walnut 1	Jug r 2	FFDQQEQR	5.72 ± 0.22	M ⁺⁺ → y6 ⁺ → y5 ⁺ (- H ₂ O)	549.3 → 803.4 → 670.3
				M ⁺⁺ → y6 ⁺ → y2 ⁺	549.3 → 803.4 → 303.2
Walnut 2***	Jug r 2	ATLTLVSQETR	10.10 ± 0.26	M ⁺⁺ → y6 ⁺ → y2 ⁺	609.8 → 719.4 → 276.2
				M ⁺⁺ → y5 ⁺ → y2 ⁺	609.8 → 620.3 → 276.2
Walnut 3***	Jug r 4	ALPEEVLATAFQIPR	27.95 ± 0.25	M ⁺⁺ → y2 ⁺ → y1 ⁺	828.0 → 272.2 → 175.1
				M ⁺⁺⁺ → y13 ²⁺ → y2 ⁺	552.3 → 735.9 → 272.2
				M ⁺⁺⁺ → y13 ²⁺ → y8 ⁺	552.3 → 735.9 → 903.5

Notes: * Q1 mass differs from the actual peptide mass (513.3) to exclude signals from the homologous walnut peptide LDALEPTNR

** Structure of fragment is unknown

*** Interference from pecan (peptide also present)

time in the final method was consequently set to 250 ms for all MRM³ experiments. To avoid exceedingly high cycle times, the LC-MS method was divided into seven detection windows (periods) (Fig. 2). Notably, Jaffuel

et al. reported a saturation curve for the MRM³ analysis of Aquaporin peptides in urine samples [32], possibly because of higher peptide concentrations in the analyzed samples.

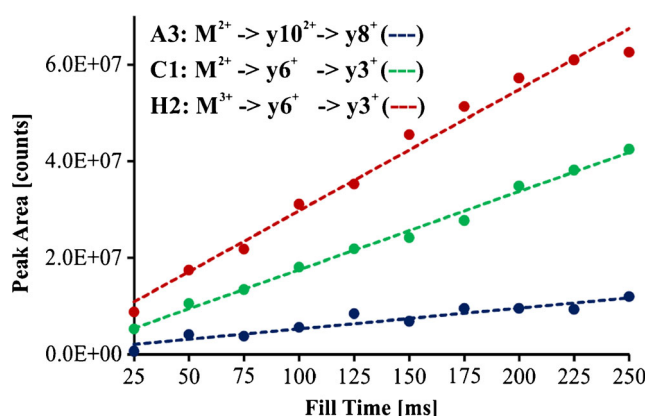


Fig. 1 Correlation between analyte peak area and fixed fill time of the linear ion trap (LIT) for selected MRM³ transitions analyzed from a tryptic digest of ground nuts (diluted 1:6000 for each nut)

MRM³ specificity

The risk of unspecific signals due to cross-reactivity is one of the major drawbacks of ELISA-based allergen detection [16]. In contrast to the majority of ELISA epitopes, the specificity of proteotypic peptides used in LC-MS analysis can be initially assessed *in silico* against protein databases. Importantly, there is still the possibility of false-positive detection due to *m/z*-overlapping MRM or MRM³ transitions from coeluting matrix-derived peptides. By applying MRM³, we were able to significantly reduce this matrix interference and increase the specificity of our method due to the second fragmentation step. Figure 3 demonstrates this for peptide Almond 2, which showed considerable matrix interference in the MRM analysis of ice cream and chocolate samples, probably due to milk protein, but was specific when using the MRM³ method. To eliminate the risk of false positives for our MRM³ method, we performed a comprehensive survey of specificity by analyzing all vegetable allergens currently regulated in the European Union (pecan, brazil nut, macadamia nut, soy beans, lupin flour, sesame, mustard, celery, oatmeal, wheat, rye, and barley (the latter three as ingredients of bread)). In addition, the most

relevant food matrices often processed with peanut or tree nuts were analyzed (bread, milk chocolate, chocolate confectionary, muesli with fruit and berries, ice cream). Aside from a few low abundant signals that interfered with one of the corresponding MRM³ transitions, no cross-reactivity was observed except for the peptides Walnut 2 and Walnut 3, which are also present in homologous allergenic proteins in pecan (Table 1). Matrix-specific interferences are exemplified for peptide Walnut 3 in Fig. 4a, where unspecific MRM³ signals at shifted retention times were observed in bread for the most sensitive transition (upper left lane). We included two additional MRM³ transitions for this peptide as independent qualifiers that showed no matrix interference to enhance specificity (middle and lower lane). Notably, no interference was observed in ice cream and chocolate matrices (Fig. 4a).

Cross-reactivity can also occur between specific proteotypic peptides from homologous allergens that differ only slightly in peptide structure when the mass difference between the polymorphic amino acids is small. Figure 4b demonstrates just such an “MRM³ crosstalk” effect for the Hazelnut 1 peptide (LNALPTNR, 1026.55 Da, *m/z* 514.2802). The spectral overlap of the *y*-ion series occurs with the homologous walnut peptide LDALEPTNR (1027.53 Da, *m/z* 514.7722) due to the limited mass resolution in the LIT and the charged state (*z* = 2). It can be avoided by either slightly shifting the Q1 mass of the *y*-ion series to 514.1 or by selecting the less intense *b*-ion series as a second qualifier, as the *b*-fragment ion contains the polymorphic site.

Analytical performance

As the presented MRM³ multi-method is meant to permit ultrasensitive detection of ppm or sub-ppm allergenic contaminants in food, we tested its analytical performance in three relevant food matrices. Fortified matrix extracts of multigrain bread, vanilla ice cream, and dairy chocolate containing almond, cashew, hazelnut, peanut,

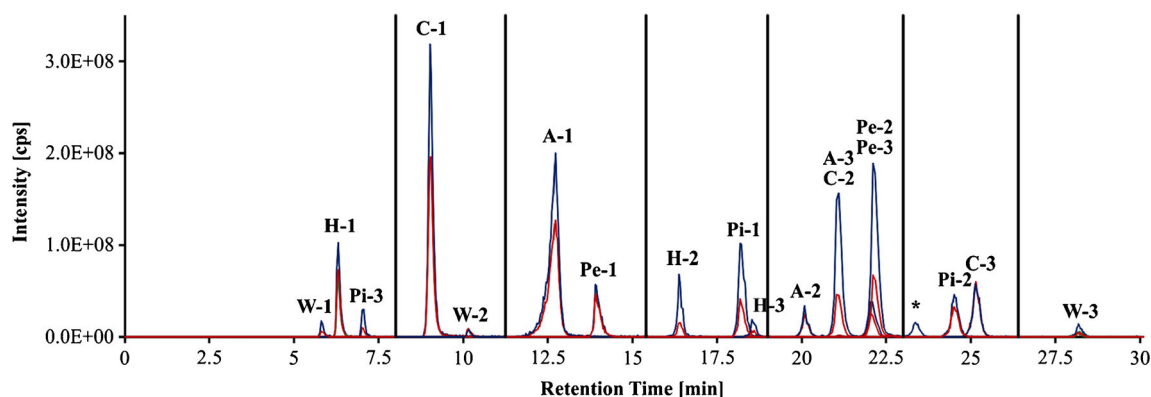


Fig. 2 LC-MS-MRM³ chromatogram from a mixture of ground nuts. Proteotypic peptides are labeled according to Table 1 by abbreviating species to their initial letters (e.g., A-1 = Almond 1). The most intense

transition is given in blue, the second most intense is given in red. Breaks between detection windows (periods), which were introduced to lower mass spectrometric cycle times, are indicated by black lines

Fig. 3 LC-MS-MRM³ analysis of peptide Almond 2 in ice cream. While considerable matrix interference is observed for five out of six MRM transitions in the blank sample, the MRM³ transitions show high sensitivity. Comparable results were obtained in milk chocolate but not for the bread matrix (data not shown)

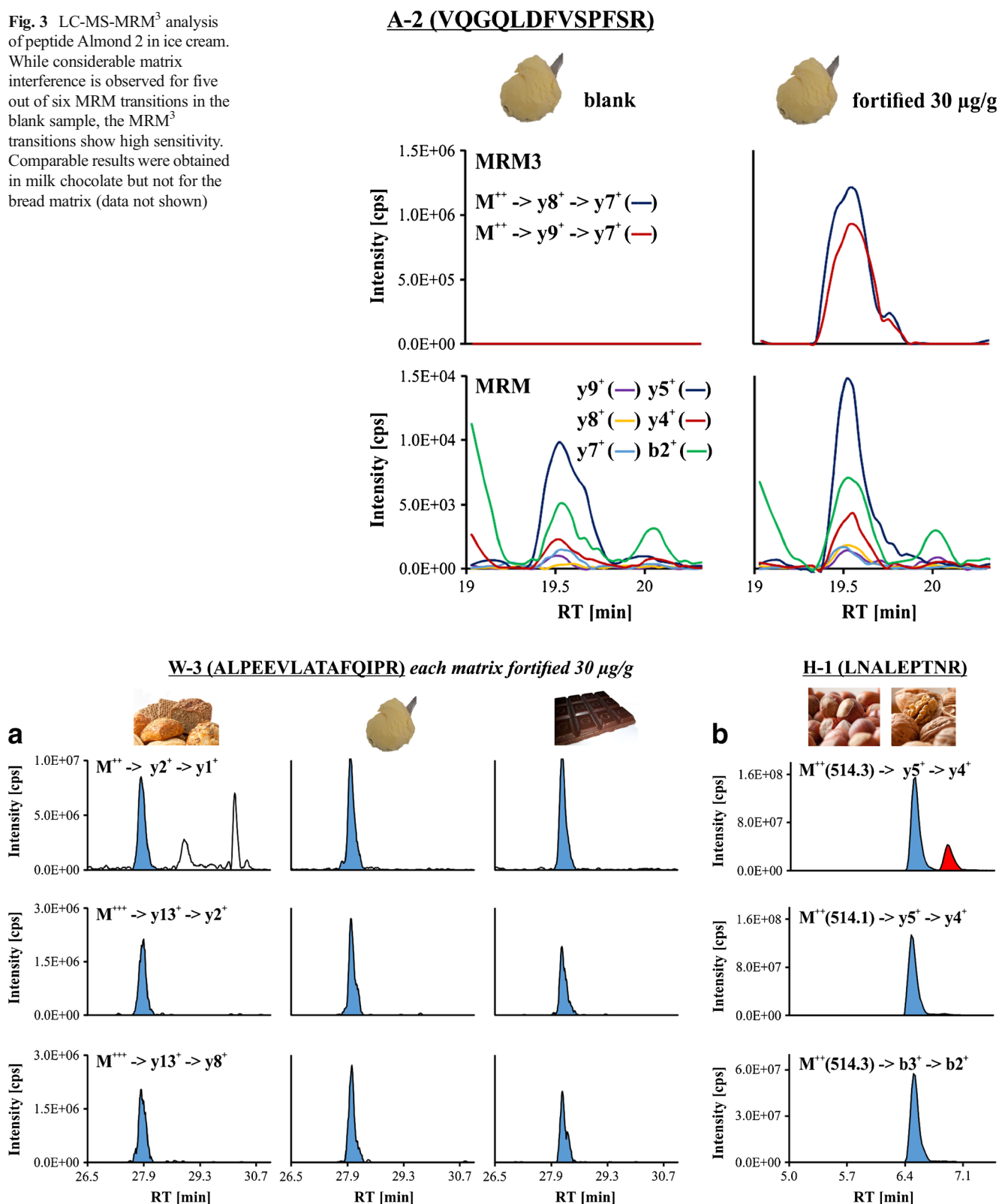


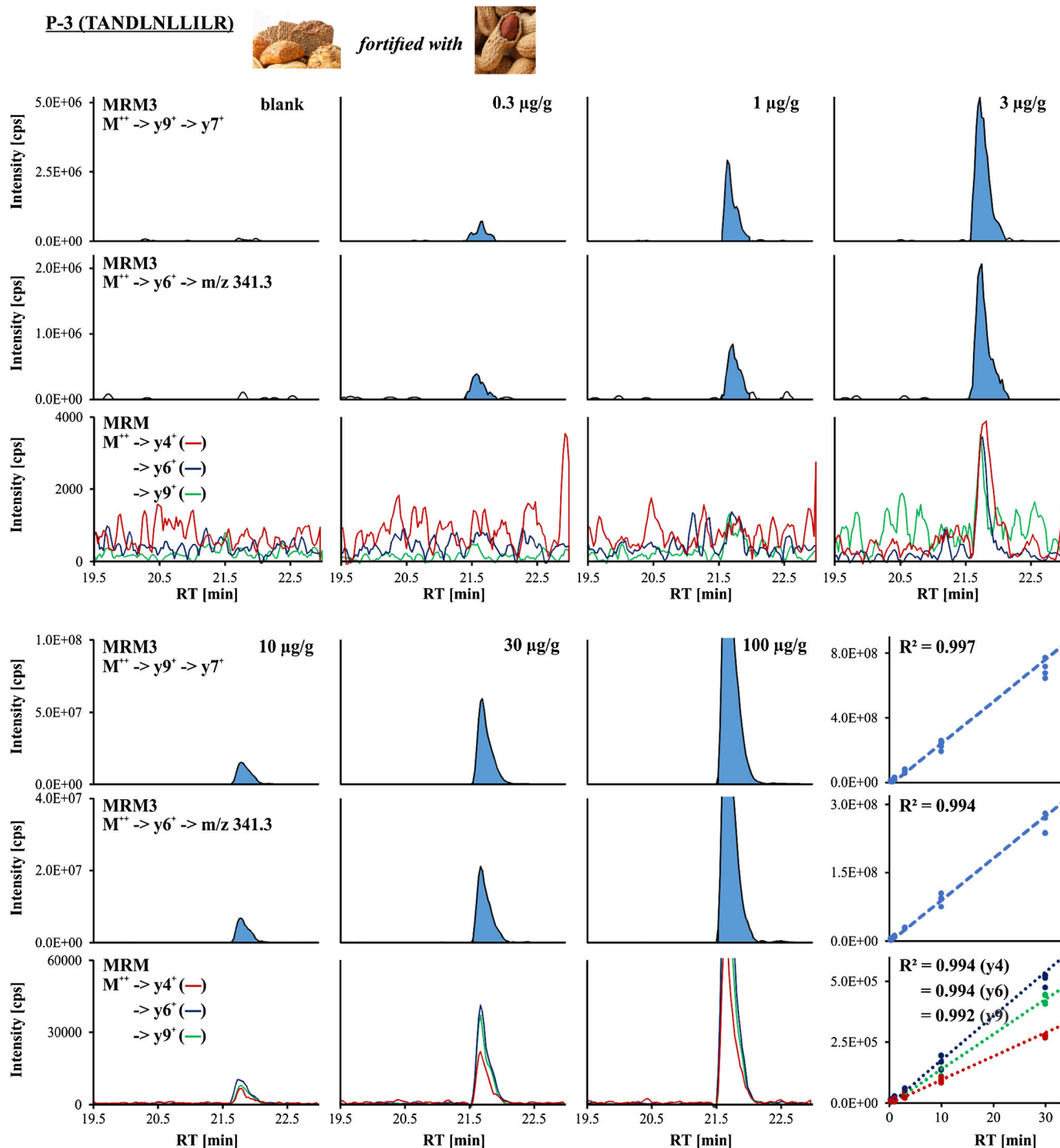
Fig. 4 a LC-MS-MRM³ analysis of peptide Walnut 3 in different food matrices fortified with 30 µg/g walnut flour prior to analysis. While the bread matrix shows unspecific interferences with one MRM³ experiment, the detection remains specific on the basis of the further MRM³ experiments. **b** An “MRM³ crosstalk” effect that arises for peptide Hazelnut 1 when walnut is present in the sample. If the correct m/z (514.3) is used to isolate

the M^{2+} precursor ion, a crosstalk signal resulting from the homologous walnut peptide LDALEPTNR ($m/z = 514.8$) is observed for the $y5 \rightarrow y4$ MRM³ transition due to insufficient mass resolution. Shifting the Q1 to m/z 514.1 solves the issue at the cost of a minor decrease in signal intensity. No crosstalk signal is observed for the $b3 \rightarrow b2$ MRM³ transition, as the fragment ions contain the SNP

pistachio, and walnut at concentrations of 100, 30, 10, 3, 1, and 0.3 $\mu\text{g/g}$ were prepared as described above and analyzed. Four replicates (two independent interday sample preparations, each measured twice) per sample were analyzed for MRM and MRM³ transitions in the same run to allow direct comparison. Notably, no analyte

carryover was observed in solvent-only runs after any of the 100- $\mu\text{g/g}$ samples.

Figure 5 demonstrates the strongly increased sensitivity of the MRM³ measurements compared to MRM-based detection using the example of peptide Peanut 3. While even the most sensitive MRM experiments drop under the noise level at



yield increased sensitivity compared to MRM at a near-zero noise level. Both MRM and MRM³ exhibit high linear correlation over the analyzed concentration range (100 $\mu\text{g/g}$ not shown in the calibration curves)

concentrations below 3–10 µg/g, the corresponding MRM³ signals are still observed at about tenfold lower concentrations, and for many peptides even at sub-µg/g levels (see also Tables 2 and 3). This increase in sensitivity is caused by the accumulation of analytes in the linear ion trap and the strongly reduced background signal compared to MRM due to the implementation of two sequence-specific fragmentation steps.

The determination of the limits of detection (LOD) and limits of quantification (LOQ) was not performed using the signal-to-noise ratio (which is common practice for analytical MRM-based methods) because of the near-zero noise of the MRM³ method. Instead, the LOD of a MRM³ transition was defined as the lowest concentration yielding a peak area above the 99 % confidence interval of the noise in a blank sample in at least three of four replicate measurements. The upper limit of the 99 % confidence interval was calculated as $\bar{x} + 2.33 s$ (i.e., the mean peak area plus 2.33 times the standard deviation) of ≥ 10 replicates of the respective blank matrix, thus assuming a normally distributed background signal due to instrument and matrix noise. LOQ was defined as the concentration yielding three times the signal required for LOD. Validation parameters for selected MRM³ transitions in the bread matrix are given in Table 2 (for full validation tables in all three analyzed matrix materials, see Tables S4, S5, and S6 in the ESM), demonstrating the high sensitivity of the MRM³ approach, with an LOD ≤ 1 µg/g for at least one proteotypic peptide of each allergenic nut. Correlation coefficients (R^2) were above 0.95 for most analytes, demonstrating the high linearity in the analyzed concentration range (0.3–

100 µg/g), which covers the currently discussed allergen threshold levels [10]. Intersample reproducibility, expressed as the precision of the method, was found to be well below 25 % for most analytes, even at low concentrations. Compared to recent PCR methods [28], the reproducibility is significantly increased. The accuracy values demonstrate the quantitative capability of the method at concentrations of 3–10 µg/g and above. At extremely low concentrations of 1 µg/g and below, we observed higher deviations, leading to the overestimation of allergen concentrations. However, it should be noted that preliminary threshold levels are not to be expected at these low concentrations.

The LOD of a peptide was defined as the LOD of its second most sensitive MRM³ transition, following the quantifier–qualifier approach. A comparison of the different matrix materials demonstrates differences in analytical performance, with LODs and LOQs tending to be lowest in the bread matrix (Table 3). Milk chocolate was the most challenging matrix, with approximately four- to fivefold-increased LODs. It must be kept in mind, however, that thermal processing (as used in bakery products) can lead to significant changes in allergen recovery rates in LC-MS due to reduced protein extractability for example [17, 43]. These matrix effects should be thoroughly investigated in further studies to allow absolute quantification in processed foods.

Compared to the values obtained for MRM (analyzed in the same run), the LODs for MRM³ were on average about 17-fold lower for the analysis of fortified bread and ice cream samples (sevenfold for fortified milk chocolate). In addition, MRM³ showed increased specificity due to the strongly

Table 2 Validation parameters for selected MRM³ transitions in spiked multigrain bread rolls (for full validation tables in all three matrix materials, see Tables S4, S5, and S6 in the ESM)

Peptide	Transition	0.3 µg/g		1 µg/g		3 µg/g		10 µg/g		30 µg/g		100 µg/g		LOD (µg/g)	LOQ (µg/g)	R^2
		p*	a**	p*	a**	p*	a**	p*	a**	p*	a**	p*	a**			
Almond 1	M ²⁺ → y ³⁺ → y ¹⁺			4.9	37.4	8.4	3.2	20.7	−1.7	11.4	−2.6	9.0	0.2	≤ 1	≤ 3	0.990
	M ²⁺ → y ⁷⁺ → y ⁶⁺	13.6	132.7	11.4	47.7	5.1	5.3	17.2	0.1	7.1	−4.9	4.4	0.4	≤ 0.3	≤ 1	0.997
Cashew 1	M ²⁺ → y ⁵⁺ → y ³⁺	16.3	333.7	11.1	97.7	17.1	18.9	19.2	−2.2	11.8	−11.0	9.3	1.0	≤ 0.3	≤ 0.3	0.988
	M ²⁺ → y ⁶⁺ → y ³⁺	23.5	118.6	6.1	36.0	7.0	−1.8	13.9	2.5	7.8	−4.2	6.3	0.4	≤ 0.3	≤ 1	0.995
Hazelnut 1	M ²⁺ → y ⁵⁺ → y ⁴⁺			17.4	95.2	12.1	17.3	16.0	0.6	9.8	−12.8	9.9	1.1	≤ 1	≤ 10	0.987
	M ²⁺ → b ³⁺ → b ²⁺			42.3	101.2	25.1	14.0	12.4	−8.7	4.4	−9.4	9.2	0.9	≤ 1	≤ 10	0.989
Peanut 3	M ³⁺ → y ^{9²⁺} → y ⁷⁺	26.7	251.8	12.3	80.4	14.3	16.3	12.4	−5.0	7.9	−7.5	4.2	0.7	≤ 0.3	≤ 1	0.997
	M ³⁺ → y ^{6²⁺} → unkn	56.4	141.4	9.7	44.0	4.5	12.8	13.6	1.9	8.8	−6.7	6.5	0.6	≤ 0.3	≤ 1	0.994
Pistachio 1	M ²⁺ → y ^{10²⁺} → y ⁸⁺	38.2	235.6	11.9	62.0	10.7	18.1	24.8	−3.8	16.9	−7.2	11.1	0.7	≤ 0.3	≤ 1	0.985
	M ²⁺ → y ¹¹⁺ → y ⁷⁺	57.7	303.3	36.7	96.6	15.1	17.6	19.9	−2.8	24.9	−10.1	12.3	0.9	≤ 0.3	≤ 1	0.979
Walnut 2	M ²⁺ → y ⁶⁺ → y ²⁺			15.5	68.8	20.8	5.9	23.9	−4.2	8.0	−4.6	6.4	0.4	≤ 1	≤ 3	0.995
	M ²⁺ → y ⁵⁺ → y ²⁺			52.4	119.6	27.8	36.3	27.6	−6.9	6.8	−14.1	2.8	1.3	≤ 1	≤ 3	0.995

Notes: * Precision (p) given as relative standard deviation [$s(\bar{x})/\bar{x}$] in %

** Accuracy (a) given as the difference [(concentration found – concentration added)/concentration added] in %

reduced background signal, and permitted the analysis of several peptides that coeluted with matrix compounds triggering several MRMs in the first place (see Table 3). The benefits of increased sensitivity and specificity come, however, at the cost of greatly increased MS duty cycle time due to the long fill time of the LIT. The application of a comprehensive multi-allergen method based on MRM³ for routine analysis that would include all currently regulated allergens would thus require a reduction in the number of experiments per allergen. This could be achieved by, for example, performing a multi-allergen scan focusing on only one primary proteotypic peptide per allergenic food and a subsequent verification of candidate allergens in a confirmatory run. A software-based scheduling of MRM³ experiments might be a further possibility to improve the flexibility of such a multi-method.

Table 3 MRM and MRM³ limits of detection and quantification in different matrices

	Bread			Ice cream			Milk chocolate		
	MRM ³		MRM	MRM ³		MRM	MRM ³		MRM
	LOD*	LOQ*	LOD*	LOD	LOQ	LOD	LOD	LOQ	LOD
A-1	≤ 1	≤ 1	≤ 3	≤ 1	≤ 1	≤ 10	≤ 1	≤ 1	—**
A-2	≤ 3		≤ 100	≤ 3		—**	≤ 10		—
A-3	≤ 1		≤ 30	≤ 0.3		≤ 10	≤ 10		≤ 30
C-1	≤ 0.3	≤ 0.3	≤ 3	≤ 0.3	≤ 1	≤ 3	≤ 1	≤ 1	≤ 10
C-2	≤ 1		≤ 10	≤ 1		≤ 10	≤ 10		≤ 10
C-3	≤ 0.3		≤ 10	≤ 1		≤ 3	≤ 1		≤ 10
H-1	≤ 1		≤ 10	≤ 3		≤ 10	≤ 3		≤ 10
H-2	≤ 1	≤ 3	≤ 30	≤ 1	≤ 3	≤ 10	≤ 10		—
H-3	≤ 3		≤ 10	≤ 1		≤ 10	≤ 3	≤ 3	≤ 10
Pe-1	≤ 0.3	≤ 1	≤ 3	≤ 3		≤ 3	≤ 1	≤ 1	≤ 10
Pe-2	≤ 3		≤ 10	≤ 3		≤ 3	≤ 3		≤ 10
Pe-3	≤ 0.3		≤ 10	≤ 0.3	≤ 1	≤ 3	≤ 1		≤ 10
Pi-1	≤ 0.3	≤ 1	≤ 3	≤ 1	≤ 3	≤ 10	≤ 3	≤ 3	≤ 30
Pi-2	≤ 1		≤ 10	≤ 1		≤ 10	≤ 3		≤ 30
Pi-3	≤ 10		≤ 30	≤ 30		≤ 30	≤ 30		≤ 100
W-1	≤ 3		≤ 100	≤ 3		≤ 30	≤ 10		≤ 100
W-2	≤ 1	≤ 3	≤ 30	≤ 3	≤ 3	≤ 30	≤ 10		≤ 100
W-3	≤ 3		≤ 30	≤ 3		≤ 10	≤ 3	≤ 10	≤ 10

Notes: Proteotypic peptides labeled according to Table 1; species are abbreviated by their initial letters (e.g., A-1 = Almond 1)

* LOD (MRM³) given as the limit of detection of the second most sensitive MRM³ experiment, in µg/g

* LOQ (MRM³) given as the limit of quantification of the most sensitive MRM³ experiment, in µg/g

* LOD (MRM) given as the limit of detection (S/N = 3) of the second most sensitive MRM experiment, in µg/g

** Not suitable as a marker due to matrix interferences (Fig. 3)

Application to commercially available samples

We assessed the applicability of the MRM³ method by analyzing a selection of commercially available products from a local supermarket. In total, 23 samples were analyzed, including packaged nuts ($n = 10$), ice cream ($n = 2$), bread ($n = 3$), oatmeal ($n = 2$), muesli ($n = 2$), and chocolate products ($n = 4$). We detected significant trace contaminations of allergens in two samples (milk chocolate contaminated with hazelnut, macadamia nuts contaminated with cashew), which were both verified by abundant signals for all proteotypic peptides in two biological replicates (Fig. 6). The concentration of allergenic contamination was roughly estimated as >50 µg/g in each case based on the external calibration for LOD determination. These contamination levels were above the advisory threshold levels recently suggested by German food authorities. Both results were verified by a validated real-time PCR analysis in a national food authority laboratory (LGL Oberschleißheim, Germany; no quantitative information). In addition, we identified four minor contaminations ($2 \times$ almond in milk chocolate, pistachio in almonds, and cashew in peanuts), each also verified by replicated detection of all proteotypic peptides and roughly estimated at <10 µg/g each. Only one of these findings could be reproduced by the abovementioned real-time PCR, possibly owing to the higher sensitivity of the MRM³ method. All of the samples found to be positive were labeled “may contain traces of” for the respective nut.

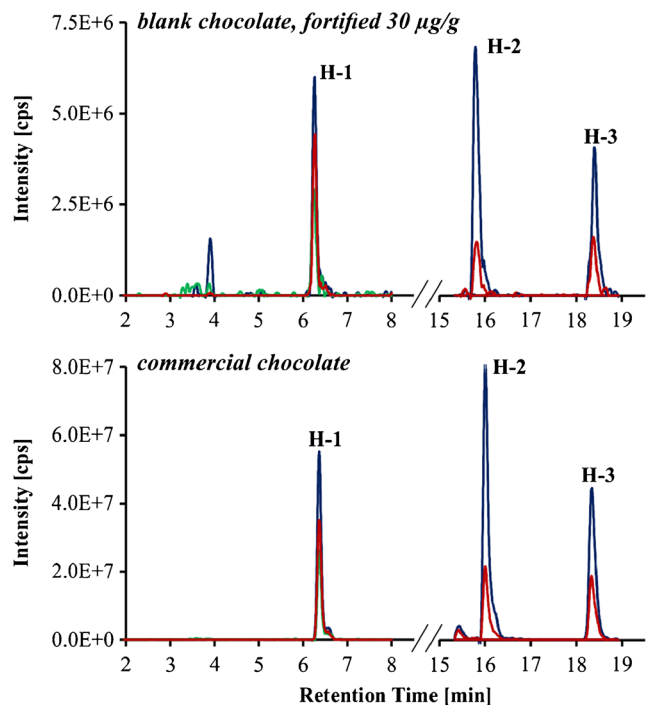


Fig. 6 Detection of hazelnut in a fortified milk chocolate sample (30 µg/g) and in commercially available milk chocolate obtained from a local supermarket. The presence of the allergenic contaminant is confirmed by three specific hazelnut markers showing two corresponding MRM³ transitions each (three for peptide H-1)

Conclusions

We present in this study a novel approach to the multiplexed detection and quantification of food allergens by LC-MS, using a quick and simple sample preparation workflow. For the first time, MRM³ technology was applied systematically to allergen analysis, allowing for the highly specific determination of allergens down to the µg/g and even sub-µg/g level of contamination with allergenic nuts. To the best of our knowledge, this is the first comprehensive MRM³ multi-method in the field of food analysis.

The quantitative performance and the excellent sensitivity of the method demonstrate the huge potential of this method and mass spectrometry in general for the reliable and sensitive absolute quantification of allergenic contaminants in processed food, which is a major task for future studies. A thorough investigation of the effect of food processing on analyte recovery as well as the development of suitable calibration strategies are also both needed.

Acknowledgments We thank Imke Westkamp for excellent technical assistance and the Bavarian Agency for Health and Food Safety (LGL) Oberschleißheim for the real-time PCR analyses. We also acknowledge support from the German National Academic Foundation (Studienstiftung des Deutschen Volkes) for funding this work by providing a Ph.D. scholarship.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of financial or nonfinancial interest in the work that is presented in this study.

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