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# First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry

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ABSTRACT

The development of a multi-method for the detection of seven allergens based on liquid chromatography and triple-quadrupole tandem mass spectrometry in multiple reaction mode is described. It is based on extraction of the allergenic proteins from a food matrix, followed by enzymatic digestion with trypsin. The chosen marker peptides were implemented into one method that is capable of the simultaneous detection of milk, egg, soy, hazelnut, peanut, walnut and almond. This method has been used to detect all seven allergenic commodities from incurred reference bread material, which was baked according to a standard recipe from the baking industry. Detected concentrations ranged from 10 to 1000 μg/g, demonstrating that the mass spectrometric based method is a useful tool for allergen screening.


## 1. Introduction

Food allergy is an IgE mediated adverse reaction to certain food proteins. Symptoms occur immediately and can be diverse, the most severe reaction being anaphylaxis. Less severe symptoms affect the mouth, gut, skin and respiratory tract. So far, more than 160 foodstuffs were shown to provoke allergic reaction. However, only a few of them account for more than 90% of all food allergies. Up to 8% of children and up to 2% of adults are affected [1]. There is no treatment available; to prevent an allergic reaction patients need to avoid the offending food. Thus, they rely on accurate food labelling.

In the European Union a list of major food allergens that are obligatory to label is given in Annex IIIa of the directive 2003/89/EC, with its latest amendment laid down in directive 2007/68/EC. It includes 13 food allergen groups, with a total of 26 protein-based allergens [2]. Despite this regulation, total avoidance might be difficult for the allergic consumer. Processed food may be contaminated with allergens, e.g. due to manufacturing on the same production lines. Here analytical methods capable of detecting these so-called "hidden allergens" are needed.

Currently, the most frequently used analytical methods for allergen detection are either immunological based on antibodies or, to a lesser extend, based on polymerase chain reactions (PCR). Antibody based enzyme-linked immunosorbant assays (ELISA) are commercially available for different allergenic targets. They have the advantage of being fast and generally suitable for routine analysis. However, the target protein is detected by a structure so-called "epitope" that is recognized by an antibody, thereby being an indirect detection. In several matrices, antibodies recognize similar structures without relevance for allergy, still give a positive and indistinguishable signal from those for the target allergen. This fact is described by the term cross-specificity [3]. Another disadvantage is that ELISA test kits detect only one allergen per test. This economically presents a challenge when several allergens need to be analyzed in the same sample. On the other hand, methods based on PCR are capable of multiple allergen analysis, but detect not the allergenic protein itself but the corresponding, non-allergenic DNA [4]. This may not correlate with the amount of allergic protein in the food especially in unknown matrices. Thus, for an unambiguous identification confirmatory methods are required.

Mass spectrometry (MS) overcomes both the biggest problems of ELISA and PCR: it is a direct detection method and can detect multiple allergens in the same analysis. Monaci and van Hengel [5] have developed a method capable of detecting whey proteins in fruit juices. Here, the intact allergenic protein is tar-

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geted. Other approaches target the peptides of an allergen that result from a tryptic digest. So far, methods for the detection of milk and peanut have been developed. Weber et al. [6] and Monaci et al. [7] identified peptide markers for the detection of milk in cookies and casein in white wines, respectively. Shefcheck et al. [8] developed a method for the mass spectrometric detection of peanut in chocolate. So far, only methods for single target analysis have been published. Here we describe for the first time a real multi-screening method for seven allergenic targets in the same analysis, all of them in the so called group of major allergens. It is based on extraction of the allergen from the food matrix and followed by an enzymatic cleavage with trypsin. The resulting peptides are separated by HPLC and measured with a triple-quadrupole mass spectrometer in multiple reaction mode (MRM) to enhance sensitivity. Validation data are shown. The method is capable of detecting all targeted allergens in the incurred material, which was formulated according to a recipe commonly used in the baking industry.

## 2. Materials and methods

### 2.1. Materials

Formic acid, hexane and iodoacetamide were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN), dithiotheritol (DTT), hydrochloric acid and tris(hydroxymethyl)aminomethane (TRIS) were from Sigma–Aldrich (St. Louis, USA), ammonium bicarbonate from AppliChem (Darmstadt, Germany). Modified porcine trypsin, sequencing grade, was obtained from Promega (Madison, USA). All chemicals were used without further purification and deionized water was used in all experiments. All standards were prepared using 100 mM $NH_4HCO_3$.

Skim milk powder, soy flakes, hazelnut, peanut, walnut, almond, wheat flour and yeast were obtained at the local retailer. Egg white powder was from Ovobest (Neuenkirchen-Vörden, Germany).

### 2.2. Preparation of standards

For the preparation of a mix standard 1 g of each defatted and grounded allergenic commodity was extracted with 10 ml TRIS–HCl, pH 8.2 at 60 °C for 3 h. The resulting extracts were centrifuged and their protein content was determined in triplicate with a Bradford assay (Sigma–Aldrich). The concentrations of total soluble protein were: milk 74 ± 3 mg/ml, egg 55 ± 4 mg/ml, soy 41 ± 4 mg/ml, hazelnut 35 ± 3 mg/ml, peanut 54 ± 12 mg/ml, walnut 98 ± 7 mg/ml and almond 74 ± 8 mg/ml. Standards were prepared by mixing these extracts, followed by the tryptic digestion as described in Section 2.4.2. Thus, all standard concentrations refer to the total soluble protein for each allergenic commodity as determined by Bradford.

### 2.3. Preparation of incurred reference material

For the preparation of incurred reference material wheat flour was spiked with 1000 μg/g (μg allergenic commodity/g wheat flour) of seven allergic commodities: milk, egg, soy, hazelnut, peanut, walnut and almond. For spiking, skim milk powder and egg white powder were used without further treatment. Soy flakes, hazelnut, peanut, walnut and almond were ground and defatted with hexane using soxhlet extraction (fat loss was 10.7%, 65.6%, 43.4%, 71.5% and 55.5%, respectively). After drying they were ground to fine flours which were used for spiking. To achieve a homogenous distribution the spiked flour was spun on a 360° shaker for 42 h. Afterwards the flour was used to bake breads according to a recipe commonly used in the baking industry [9]. The formulation was as follows: 500 g wheat flour, 300 ml $H_2O$, 9.6 g NaCl and 24 g yeast. Baking was done using the baking machine "Backmeister Modell 8690" from Unold Electro (Hockenheim, Germany). It was programmed to knead for 6 min and rise for 60 min. These steps were repeated followed by 60 min of baking at 200 °C. The breads were freeze-dried and milled to a fine powder. The same procedure was used to prepare breads containing no target analyte.

For the preparation of bread material containing 500, 100, 50 and 10 μg/g of milk, egg, soy, hazelnut, peanut, walnut and almond, the milled breads prepared using flour spiked with 1000 μg/g of the allergic commodities were mixed accordingly to a final amount of 100 g, adopting the procedure from Dumont et al. [10]. To ensure a homogenous distribution the mixtures were spun on a 360° shaker for 12 h.

### 2.4. Sample preparation

#### 2.4.1. Extraction

Extraction of the target analytes from 2 g sample was done with 20 ml TRIS–HCl buffer, pH 8.2 at 60 °C for 3 h. Samples were centrifuged and 10 ml of the extract were concentrated to approximately 1 ml via ultrafiltration (Amicon Ultra 15 ml, 5 kDa molecular weight cut-off from Millipore, Billerica, USA). The final volume was recorded and the total protein concentrations of the extracts were determined with Bradford microassay from Sigma–Aldrich according to the kit instructions.

#### 2.4.2. Enzymatic digestion

Enzymatic digestion was done with trypsin. Prior the extracts were diluted with $NH_4HCO_3$ solution (100 mM) to a final concentration of 1 mg total protein per ml as determined by Bradford assay. Aliquots of 100 μl were reduced with 50 μl DTT solution (200 mM) for 45 min at room temperature. Subsequently, an alkylation was performed by adding 40 μl of IA solution (1 M). The alkylation was left for 45 min in the dark at room temperature and was stopped by adding another 20 μl of the DTT solution. $NH_4HCO_3$ (50 μl, 100 mM) and trypsin (10 μl, 0.1 μg/μl in 50 mM acetic acid) were added and incubated for 12 h at 37 °C. The digestion was stopped by adding 2 μl concentrated formic acid. Samples were injected into the HPLC without further treatment.

### 2.5. Liquid chromatography

Separation of peptides was done with an Agilent 1200 HPLC (Santa Clara, USA), consisting of two quaternary pumps, a vacuum degasser, a temperature controlled autosampler kept at 15 °C and a thermostated column compartment kept at 35 °C. The injection volume was 10 μl. The analytical column used was an XBridge C18 3.5 μm (2.1 × 150 mm) from Waters (Milford, USA), the guard column was made of the same material. The mobile phase consisted of solvent A: 0.05% formic acid and 10% ACN in water; and solvent B: 0.05% formic acid in ACN. The LC run started with 0% B for 1 min, followed by a gradient to 20% B in 4 min, another gradient to 65% B in 10 min and a third gradient to 90% B in another minute. An isocratic step at 90% B continued for 1 min. At the end of the run the column was allowed to equilibrate at 100% A for 8 min. The flow rate was 300 μl/min. Prior to the mass spectrometer the flow was split and approximately 60 μl/min effluent was directed into the source.

### 2.6. Mass spectrometry

*Peptide identification* was carried out on an api 4000QTrap from Applied Biosystems/MDS SCIEX (Toronto, Canada). The following parameters were set: source temperature: 400 °C, ion

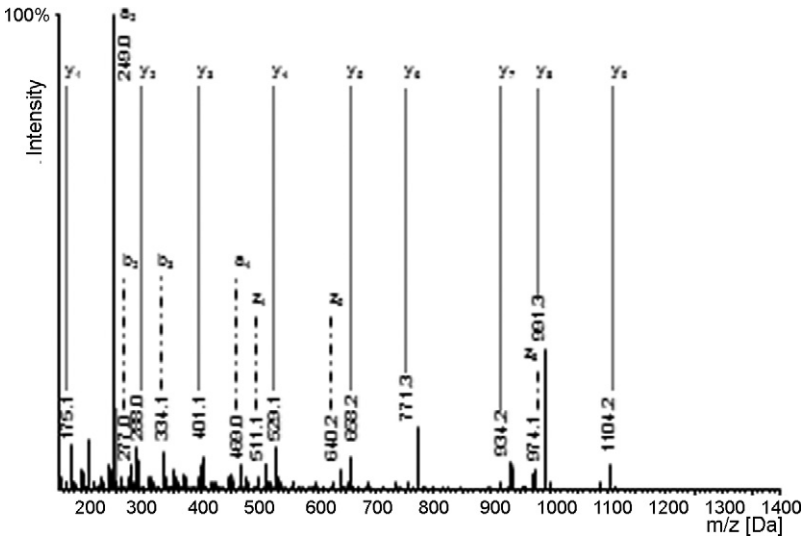


**Fig. 1.** Product ion spectra for the peptide YLGYLEQLLR, $m/z = 634.3$. The $a_2$ and $y_8$ fragments were chosen as product ion for the corresponding MRM transitions.

spray voltage: 5.5 kV, curtain gas flow 25. Information dependant acquisition (IDA) was used. Full scan spectra were measured between 400 and 1400 Da. Only signals that fulfilled the IDA criteria triggered fragmentation and a product ion scan. These criteria included: signal intensity greater than 5000 counts and charge state either between +2 and +4 or unknown. Product ion spectra were measured between 150 and 1400 Da. Peak lists from acquired MS/MS data were submitted to the online version of the MASCOT database search tool (www.matrixscience.com) [11]. The following parameters were set for the database search: databases searched were UniProt or NCBI. One missed cleavage was allowed. Peptide tolerance and MS/MS tolerance were set to 1.2 and 0.6 Da respectively. Fixed amino acid modification was carbamidomethyl.

*For the MRM method* the two most intense signals from the product ion spectra of two peptides per allergen were taken for MRM transitions. Scheduled MRM was used with a target scan time of 1 s and a peak detection window of 60 s.

## 3. Results and discussion

For the development of the MRM method, milk, egg, soy, hazelnut, peanut, walnut and almond were extracted and digested with trypsin without further purification. These digests containing only one allergenic food were injected to identify suitable marker peptides that could be implemented into the final method. To minimize the amount of product ion spectra and to avoid product ion scans of precursor ions that were no peptides, information dependant

**Table 1**
Overview of all 27 peptides from the final method, the protein they originate from, as well as the product ions used for the MRM transitions.

| Allergic food | Protein | Peptide | Retention time [min] | Precursor (charge state) [$m/z$] | Product 1 (fragment)/product 2 (fragment) [$m/z$] |
|---|---|---|---|---|---|
| Almond | Prunin | GNLDFVQPPR | 8.24 | 571.9 (+2) | 369.4 (y3)/858.6 (y7) |
| | Prunin | GVLGAFSGCPETFEESQQSSQQGR | 9.43 | 896.1 (+3) | 662.4 (y6)/790.4 (y7) |
| | Prunin | ALPDEVLANAYQISR | 9.52 | 830.4 (+2) | 922.5 (y8)/1035.5 (y9) |
| | Prunin | NGLHLPSYSNAPQLIYIVQGR | 9.94 | 780.8 (+3) | 735.7 (y6)/1154.7 (b11) |
| Egg | Ovalbumin | HIATNAVLFFGR | 9.07 | 673.4 (+2) | 223.2 (a2)/1095.6 (y10) |
| | Ovalbumin | YPILPEYLQCVK | 9.47 | 761.6 (+2) | 810.5 (y6)/1036.4 (y8) |
| | Ovalbumin | DILNQITKPNDVYSFSLASR | 9.80 | 761.6 (+3) | 201.1 (a2)/930.5 (y8) |
| | Ovalbumin | ELINSWVESQTNGIIR | 9.89 | 929.5 (+2) | 1017.5 (y9)/1116.5 (y10) |
| Hazelnut | 11S globulin | ADIYTEQVGR | 5.89 | 576.3 (+2) | 689.4 (y6)/852.5 (y7) |
| | 11S globulin | INTVNSNTLPVLR | 8.54 | 720.9 (+2) | 484.4 (y4)/1013.6 (y9) |
| | 11S globulin | QGQVLTIPQNFAVAK | 9.02 | 807.5 (+2) | 874.6 (y8)/1088.6 (y10) |
| | 11S globulin | ALPDDVLANAFQISR | 10.78 | 815.5 (+2) | 906.6 (y8)/1019.5 (y9) |
| Milk | Casein α S1 | YLGYLEQLLR | 11.29 | 634.3 (+2) | 249.2 (b2)/991.3 (y8) |
| | Casein α S1 | FFVAPFPEVFGK | 12.10 | 692.9 (+2) | 920.3 (y8)/991.3 (y9) |
| | Casein α S2 | NAVPITPTLNR | 7.80 | 598.3 (+2) | 158.3 (b2)/911.4 (y8) |
| | Casein α S2 | FALPQYLK | 9.11 | 490.3 (+2) | 120.1 (a1)/648.4(y5) |
| Peanut | Ara h1 | DLAFPGSGEQVEK | 8.18 | 688.8 (+2) | 300.2 (a3)/930.6 (y9) |
| | Ara h1 | GTGNLELVAVR | 8.33 | 564.4 (+2) | 557.5 (y5)/686.6 (y6) |
| | Ara h3/4 | RPFYSNAPQEIFIQQGR | 8.54 | 684.5 (+3) | 748.6 (y6)/836.5 (b7) |
| | Ara h3/4 | WLGLSAEYGNLYR | 9.84 | 771.4 (+2) | 272.2 (a2)/1242.6 (y11) |
| Soy | Glycinin | NLQGENEGEDKGAIVTVK | 6.66 | 634.3 (+3) | 200.2 (a2)/356.2 (b3) |
| | Glycinin | VFDGELQEGR | 7.26 | 575.2 (+2) | 219.2 (a2)/903.2 (y8) |
| | Glycinin | SQSDNFEYVSFK | 8.52 | 725.7 (+2) | 381.2 (y3)/1235.4 (y10) |
| | Glycinin | EAFGVNMQIVR | 8.90 | 632.3 (+2) | 760.6 (y6)/916.4 (y8) |
| Walnut | Jug r1 | DLPNECGISSQR | 5.72 | 688.2 (+2) | 477.2 (y4)/1147.4 (y10) |
| | Jug r1 | QCCQQLSQMDEQCQCEGLR | 7.31 | 820.2 (+3) | 345.5 (y3)/1294.3 (y10) |
| | Jug r1 | GEEMEEMVQSAR | 7.67 | 698.3 (+2) | 820.5 (y7)/949.4 (y8) |

acquisition (IDA) was applied. This ensured fragmentation only of precursor ions that exceeded a threshold and that were multiple charged. The recorded MS/MS spectra were submitted to database searches with the online version of MASCOT. Aim was to find peptides from allergens that reproducibly occurred in every digest and therefore could be used as protein marker in the MRM method. A set of possible marker peptides from every allergenic food was identified. An example of a product ion spectrum that was assigned to the peptide YLGYLEQLLR from the milk allergen casein α S1 is shown in Fig. 1.

Reproducible occurring peptides that were possible candidates for the final method had to meet a set of criteria; most important was the specificity of their amino acid sequence to the allergen they were derived from. In MRM measurements no full product ion spectra are recorded, but only a precursor ion/product ion pair, the MRM transition. Only if the amino acid sequence of the peptide is specific to the protein, the MRM transition is specific as well. Thus, BLAST searches were performed to verify that the peptide's amino acid sequences cannot be found in other proteins and thereby lead to false-positives [12]. Other chosen criteria were the absence of cystein in the peptides and the avoidance of miscleavages. In total, 24 of the candidate peptides met all criteria. Since the signals of the peptides RPFYSNAPQEIFIQQGR from Ara h3/4, DILNQITKPNDVYSF-SLASR from ovalbumin and NLQGENEGEDKGAIVTVK from glycinin were very abundant, they were included into the final method even though they contained one miscleavage. An overview of all 27 peptides implemented into the final method and the allergen they originate from is given in Table 1. Peptides used as precursor in the final method were either double or triple charged. For the selection of product ions, the two most abundant fragments from the product ion spectra were chosen for the precursor/product ion pair (the MRM transition), preferably with a higher $m/z$ than the $m/z$ of the precursor ion. All fragments used and their $m/z$ are shown in Table 1 as well.

For the evaluation of the method performance, extracts of the allergenic foods prepared according to 2.2 were mixed in different concentrations with $NH_4HCO_3$ solution and digested. These mix standards were used to evaluate the linearity and the relative standard deviation of the analytical response of the method. All transitions gave a linear response (correlation coefficient greater than 0.995) in the chosen concentration range (between 0.7 and 760 μg/ml total soluble protein, 6 data points per transition, each digested in triplicate). For the most intense MRM transition per allergenic food, the correlation coefficient, the slope and the LOD (signal to noise ration = 3) are given in Table 2. Relative standard deviations were lower than 10%. Exceptions were one transition each from the peptides ALPDE-VLANAYQISR (from almond), DILNQITKPNDVYSFSLASR (egg), YPILPEYLQCVK (egg) and QCCQQLSQMDEQCQCEGLR (walnut). Here the relative standard deviations were below 20% (data not shown).

To evaluate matrix effects, similar dilutions were prepared by spiking the mix standards into allergen free bread extracts prepared according to Sections 2.2 and 2.4. Again all transitions gave a linear response, with correlation coefficients above 0.993, remaining similar to those obtained by diluting the mix standards with $NH_4HCO_3$. The correlation coefficients, the slope and LOD of the most intense MRM transitions per allergenic commodity are given in Table 2. A comparison of the slopes of the calibration curves with and without matrix shows that matrix effects lead to a decrease in signal intensities between 10 and 30%, depending on the allergenic commodity. An exception was walnut were the intensities increased. Matrix effects also lead to higher LOD.

The developed method was used to analyze incurred bread material containing between 0 and 1000 μg/g of the seven allergic commodities, prepared according to Section 2.3. Bread was cho-

**Table 2**
Comparison of correlation coefficients, slopes and LOD for three sample types: the standards produced from extracts of the allergenic commodities; allergen free bread extract spiked with mix standards of the allergenic extracts; and the incurred bread material prepared according to Section 2.3.

| Allergenic food | Peptide | *Correlation coefficient* standard | *Correlation coefficient* allergen free bread extract spiked with standards | *Correlation coefficient* incurred bread material | *Slope* standard [cps × ml/μg] | *Slope* allergen free bread extract spiked with standards [cps × ml/μg] | *Slope* incurred bread material [cps × g/μg] | *LOD* standards [μg/ml] | *LOD* allergen free bread extract spiked with standards [μg/ml] | *LOD* incurred bread material [μg/g] |
|---|---|---|---|---|---|---|---|---|---|---|
| Milk | YLGYLEQLLR | 0.9983 | 0.9989 | 0.9998 | 7890 ± 160 | 5100 ± 90 | 237 ± 2 | 0.11 | 0.14 | 5 |
| Egg | YPILPEYLQCVK | 0.9966 | 0.9998 | 0.8985 | 2900 ± 90 | 2300 ± 20 | 20 ± 4 | 0.58 | 0.45 | 42 |
| Soy | VFDGELQEGR | 0.9999 | 0.9998 | 0.9879 | 2600 ± 10 | 2100 ± 10 | 21 ± 1 | 0.24 | 0.42 | 24 |
| Hazelnut | INTVNSNTLPVLR | 0.9994 | 1.0000 | 0.9998 | 2800 ± 40 | 2300 ± 7 | 188 ± 2 | 0.32 | 0.42 | 5 |
| Peanut | DLAFPGSGEQVEK | 1.0000 | 0.9995 | 0.9977 | 2900 ± 10 | 2600 ± 30 | 73 ± 2 | 0.20 | 0.63 | 11 |
| Walnut | DLPNECGISSQR | 0.9995 | 0.9988 | 0.9986 | 90 ± 1 | 100 ± 2 | 37 ± 1 | 10 | 16 | 70 |
| Almond | GNLDFVPPR | 0.9993 | 0.9992 | 0.9996 | 14,900 ± 190 | 11,600 ± 170 | 803 ± 9 | 0.13 | 0.19 | 3 |

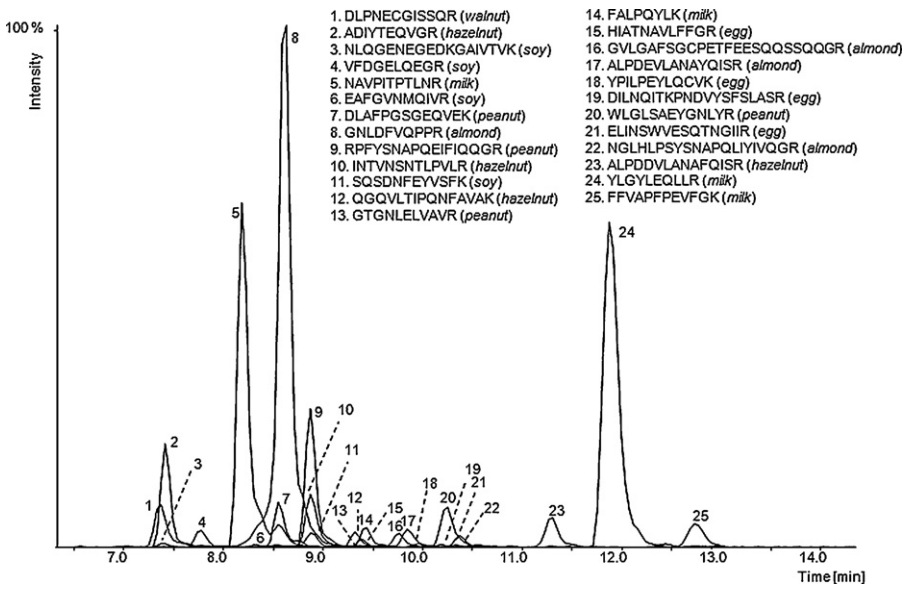


**Fig. 2.** MRM transitions for the incurred bread reference material containing 1000 ppm of all seven allergenic foods.

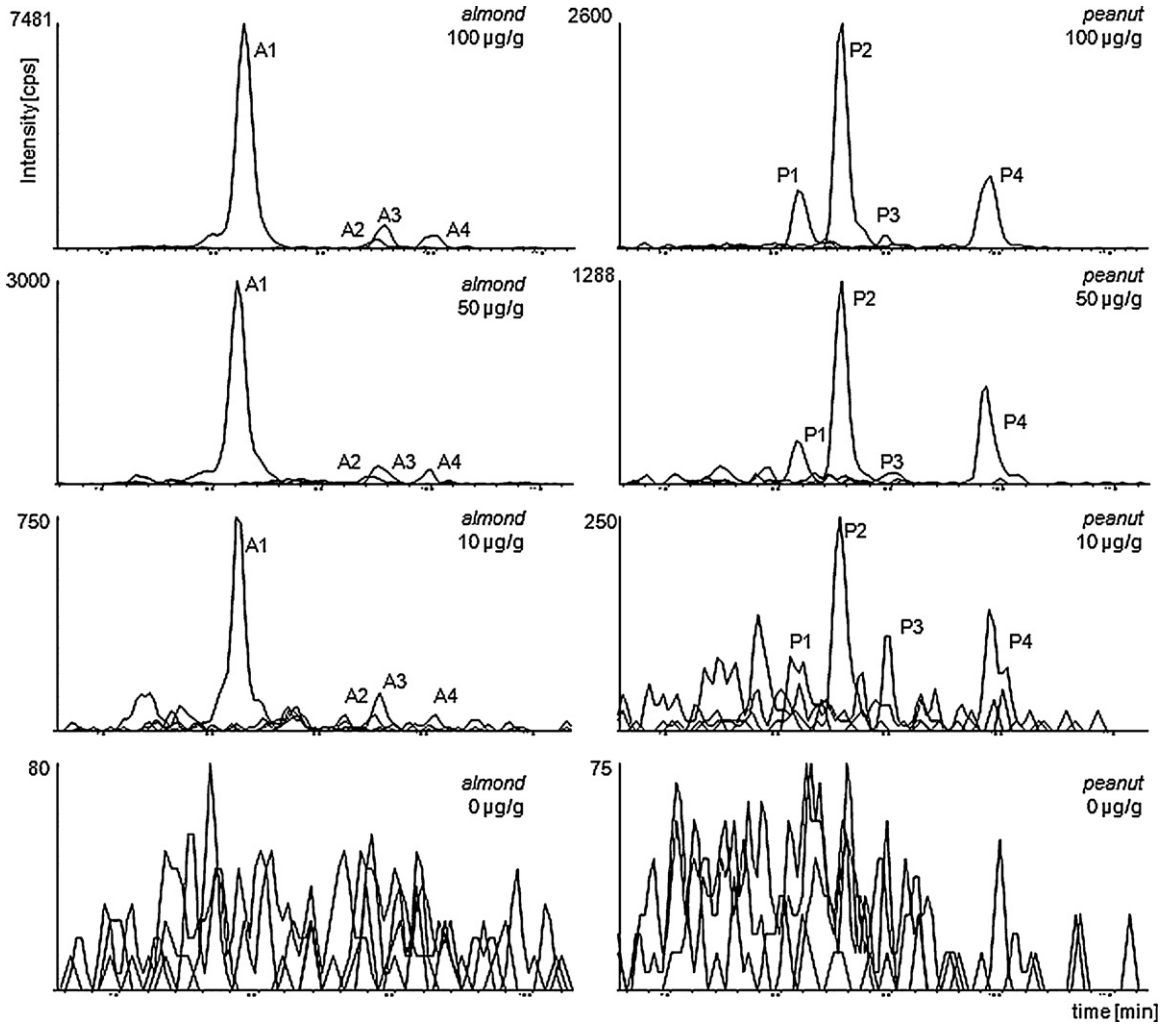


**Fig. 3.** Extracted MRM transitions for almond and peanut measured from different concentrations of the incurred bread material. The signals correspond to the following peptides: almond: A1: GNLPVQPPR, A2: GVLGAFSGPETFEESQQSSQQGR, A3: ALPDEVLANAYQISR and A4: NGLHLPSYSNAPQLIYIVQGR. Peanut: P1: DLAFPGSGEQVEK, P2: RPFYSNAPQEIFIQQGR, P3: GTGNLELVAVR and P4: WLGLSAEYGNLYR.

sen as a model matrix, as its contamination with nuts or milk may provide a problem for the allergic consumer. More important, it is an incurred reference material that exposes the allergens to some degree of processing, which is typical for products usually found on supermarket shelves. As processing might lead to chemical modifications or partial destruction of the protein structure [13], the limits of detection from incurred material may be somewhat higher than the limits of detection calculated from standards spiked into blank extracts. The resulting chromatogram for bread prepared using flour spiked with 1000 μg/g of each of the allergic commodities is given in Fig. 2. It shows one MRM transition for each of the peptides, except for two peptides from walnut, QCCQQLSQMDEQCQCEGLR and GEEMEEMVQSAR, which could not be detected. For almond and peanut the corresponding chromatograms for the different concentrations of the incurred material are given in Fig. 3. For clarity only one transition per peptide is shown, even though two were measured.

The lowest detectable concentration was determined from the signal to noise ratio ($s/n = 3$) of the most intense peptide. It was around 10 μg/g for milk, hazelnut, peanut and almond (5, 5, 11 and 3 μg/g, respectively), below 50 μg/g for egg and soy (42 and 24 μg/g) and 70 μg/g for walnut. These, as well as the correlation coefficients and the slopes of the corresponding regression curves are given in Table 2. With the exception of egg and soy, the correlation coefficients are similar, regardless whether standards, standards spiked into matrix extracts or incurred reference material are analyzed. However, the slopes appear significantly lower for all allergenic commodities in the incurred reference materials. This finding could be due, at least partly, to the fact that 1 μg/g of allergenic commodity in the flour used to prepare a bread sample correspond to less than 1 μg/ml of allergen protein in the final extract. Moreover, when allergens are heated, they may be subjected to chemical modifications, like the Maillard reaction or aggregations of denatured proteins [13]. This could influence the extractability of the processed protein from food matrix. However, lower concentrations might be detected with different sample preparation procedures, e.g. solid phase extraction.

## 4. Conclusion

In this work, a method based on liquid chromatography/triple-quadrupole mass spectrometry in multiple reaction mode for the simultaneous detection of seven allergens is presented. The seven allergenic foods implemented into one method were: milk, egg, soy, hazelnut, peanut, walnut and almond. The method is based on extraction of the allergens from the food matrix, followed by an enzymatic digestion with trypsin. Marker peptides that were specific to the allergen they arise from, and fragments of these were used as precursor/product ion pair in the MRM transition. Mixed standards were used to determine the limits of detection.

The MS method was used to detect all seven allergens from an incurred bread matrix. This matrix was chosen as it exposes the allergens to some kind of processing. Concentrations as low as 10 μg/g could be detected for milk, peanut and almond. To the best of our knowledge, this is the first time a mass spectrometric method has been used to simultaneously detect seven allergens from an incurred reference material.

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