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SPECIAL GUEST EDITOR SECTION

Application of a Liquid Chromatography Tandem Mass Spectrometry Method for the Simultaneous Detection of Seven Allergenic Foods in Flour and Bread and Comparison of the Method with Commercially Available ELISA Test Kits

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To protect the allergic consumer, analytical methods need to be capable of detecting allergens in finished products that typically contain multiple allergens. An LC/MS/MS method for simultaneous detection of seven allergens was developed and compared with commercially available ELISA kits. The detection capabilities of this novel method were demonstrated by analyzing incurred material containing milk, egg, soy, peanut, hazelnut, walnut, and almond. Bread was chosen as a model matrix. To assess the influence of baking on the method's performance, analysis was done before and after baking. The same samples were analyzed with ELISA test kits from ELISA Systems, Morinaga, Neogen, and r-Biopharm. Peanut, hazelnut, walnut, and almond could be detected with both ELISA and LC/MS/MS regardless of whether the product was baked or not. LC/MS/MS clearly showed superior detection of milk in processed matrixes compared to ELISA, which exhibited significantly lower sensitivities when analyzing the baked products. Similar results were obtained when analyzing egg; however, one kit was capable of detecting egg in the processed samples as well.

Food allergy is a major public health concern that affects up to 8% of children and up to 2% of the adult population (1). It is an immunoglobulin E (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/or respiratory tract. There is no treatment available; patients need to avoid the offending food. More than 160 foods have been shown to evoke an allergic reaction; however, only eight of them account for more than 90% of all allergic reactions (2). These "big eight" are milk, egg, soy, peanut, tree nuts, crustaceans, fish, and wheat. The European Union Annex IIIa of the directive 2003/89/EC, with its latest amendment laid down in directive 2007/68/EC, lists 13 food allergen groups (the big eight plus celery, mustard, sesame seed, lupine, and molluscs) that are obligatory to label if used as an ingredient. Allergen labeling is also regulated in Australia and New Zealand, Canada, Japan, and the United States. (3). Despite these regulations, total avoidance might prove difficult for the allergic consumer. Processed food may be contaminated with an unintentionally added allergen, e.g., due to manufacturing on the same production lines. Allergen risk management, therefore, remains an important issue, and analytical methods for the detection of undeclared allergens are needed.

Two analytical techniques are mainly used for allergen detection: ELISA, based on antibodies that detect the protein portion of the allergenic food (or allergenic protein), and PCR methods that detect DNA. The former are commercially available for a number of target allergens. The advantages of ELISA kits are their relatively short analysis times and easy handling. In regard to multiplexing, an ELISA method for the simultaneous detection of peanut and tree nuts has been developed (4). Another multiplexing approach used a dot blot system with immobilized discrete spots of antibodies (5). However, none of these ELISA multiplexing methods is commercially available. When a sample needs to be tested for several allergens, analysis time and costs increase significantly, as each target has

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Table 1. Test kits used for allergen detection, the corresponding reporting unit, and the conversion factor to whole food, skim milk powder, and egg white protein[a]

ELISA test kit manufacturer	Allergenic food	Kit name	Conversion factor	Reporting unit
ELISA Systems, Windsor, Australia	Egg	Egg Residue	1.3	Egg white protein
	Hazelnut	Hazelnut Residue	8.3	Hazelnut protein
	Soy	Soy Residue Enhanced Assay	2.7	Soy flour protein
Morinaga, Tokyo, Japan	Egg	Morinaga Egg (Ovalbumin) ELISA Kit		
Neogen, Lansing, MI	Egg	Tepnel Biokits Egg Assay	1.3	Egg white protein
	Milk	Tepnel Biokits Casein Assay	2.9	Casein
	Peanut	Tepnel Biokits Peanut Assay	—	Peanut
	Soy	Tepnel Biokits Soya Assay	2.7	Soy protein
	Walnut	Tepnel Biokits Walnut Assay	—	Walnut
r-Biopharm, Darmstadt, Germany	Almond	RIDASCREEN® FAST Almond	—	Almond
	Egg	RIDASCREEN® FAST Egg Protein	0.2	Whole egg
	Hazelnut	RIDASCREEN® FAST Hazelnut	—	Hazelnut
	Milk	RIDASCREEN® FAST Casein	2.9	Casein
	Peanut	RIDASCREEN® FAST Peanut	—	Peanut

[a] Conversion according to the Souci, Fachmann, Kraut database (ref. 24).

to be tested with a different kit. Other disadvantages of ELISA are that results between different kits may not be comparable, as they may use antibodies with different specificity, and the occurrence of false positives due to cross-reactivity (6). Methods based on PCR techniques are capable of multiplexing, but they detect the DNA and not the allergenic protein itself. This might not correlate with the amount of allergenic proteins, especially in processed food matrixes (7).

The latest additions in the field of allergen analysis are methods based on MS. They overcome the major disadvantages of both ELISA and PCR methods: MS targets the allergen not the DNA, and it is highly specific, thus eliminating false-positives. Also, it is capable of multiplexing. So far, only a few allergens have been targeted using MS, and no method for the simultaneous detection of allergens from several allergenic foods has been reported. However, there are methods for the detection of milk (8–10), peanut (11), egg (12), and gluten (13). None of these methods targets the intact allergenic protein, but rather marker peptides derived from tryptic digests of these.

To protect consumers from allergic reactions, methods must be capable of detecting unintentionally added allergens in a broad variety of foods, some of them highly processed. The performance of all analytical methods for the detection of allergens can be highly influenced by food processing. In particular, heat treatment might reduce the solubility of the protein due to aggregation and reaction with other food components (e.g., the Maillard reaction; 14–16). Therefore, the extraction of the allergen from the food matrix becomes a crucial step regardless of which detection method is used. Heating also causes a loss of almost all of the secondary and tertiary structure of proteins and might, therefore, lead to reduced binding of antibodies when the epitope that is recognized by a particular ELISA kit is conformational. This might lead to an underestimation of the allergen or even to false negatives (17). Another reason for reduced method performance is the possibility of chemical modifications, e.g., when a marker peptide of an MS method is modified during the process, it might not be detected even though the allergen was present in the food. Therefore, the use of incurred reference material for method validation is highly desirable (18).

This paper presents a novel MS method capable of simultaneous screening for seven allergens, all of them listed in Annex IIIa of the directive 2003/89/EC. After extraction of the allergens from the food matrix they are digested with trypsin, and the resulting peptides are separated by LC. Tandem MS is used to monitor four marker peptides for each allergenic food. This is done in the multiple reaction monitoring mode (MRM), in which the MRM transitions consist of the peptide of interest (the precursor ion) and one of its fragments. This approach is sensitive and specific, and thus, capable of monitoring the proteins of interest in the presence of a large amount of matrix proteins. To prove its applicability for processed food, an incurred bread material was analyzed. This incurred reference material contained all seven target allergens and was formulated according to a recipe commonly used in the German baking industry (19). To assess the influence of baking on the target allergens, and thus, its influence on the method, unbaked flours were analyzed in parallel. For evaluation of whether ELISA

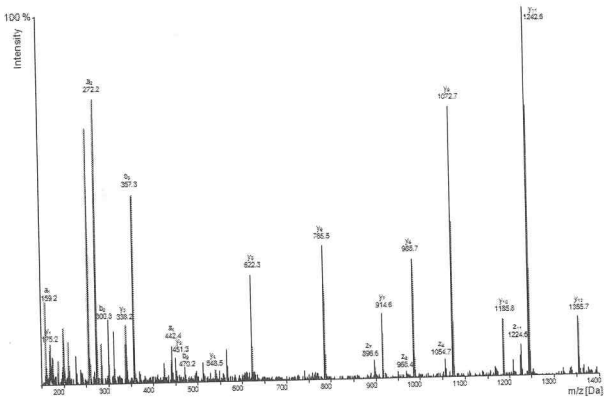

Figure 1. Product ion spectrum for the peptide WLGLSAEYGNR, *m/z* = 771.5. The fragments a_2 and y_{11} were chosen as product ions for the corresponding MRM transitions.

and MS are affected to the same extent, flour and bread samples were analyzed with different commercially available ELISA test kits. Five out of the seven targets were analyzed with at least two different kits, allowing for a comparison of the different test kits when analyzing a processed matrix.

Experimental

Chemicals and Reagents

Formic acid, hexane, and iodoacetamide (IA) were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN), dithiotheritol (DTT), hydrochloric acid, and tris(hydrohymethyl)aminomethane (TRIS) were from Sigma-Aldrich (St. Louis, MO) and ammonium bicarbonate from AppliChem (Darmstadt, Germany). Modified porcine trypsin, sequencing grade, was obtained from Promega (Madison, WI). 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 solution.

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

For the preparation of standards, extracts of the allergenic foods were mixed. Skim milk powder and egg white powder were extracted with TRIS-HCl (pH 8.2, 60°C) for 3 h. Soy flakes, peanut, hazelnut, walnut, and almond were defatted using Soxhlet extraction with hexane (fat loss was 10.7, 65.5, 43.4, 71.5, and 55.5%, respectively). After drying they were ground to a fine powder, which was extracted with TRIS-HCl. The total soluble protein concentration was determined with a Bradford microassay kit from Sigma-Aldrich according to the kit's instructions.

Preparation of Incurred Reference Material

For the incurred bread material, wheat flour was spiked with 1000 µg/g of seven allergenic foods: milk, egg, soy, peanut, hazelnut, walnut, and almond. For spiking, skim milk powder and egg white powder were used without further treatment. Soy flakes, peanut, hazelnut, walnut, and almond were defatted and grounded to fine powders as described in the previous section, which were used for spiking. To achieve a homogeneous distribution, the spiked flour was spun on a 360° shaker for 42 h. The formulation of the bread was as follows: 500 g spiked wheat flour, 300 mL H_2O, 9.6 g NaCl, and 24 g yeast. Baking was done using a baking machine (Backmeister Model 8690, Unold Electro, Hockenheim, Germany). The program was kneading for 6 min and rising for 60 min at room temperature. Both steps were repeated followed by baking for 60 min at 200°C. The finished breads were freeze-dried and ground to fine powders. The same procedure was used to prepare breads containing no added allergen.

For the preparation of breads containing different levels of allergenic foods, the 1000 µg/g bread was serial diluted with the "allergen-free" bread at the powder level. To ensure homogeneous distribution, all dilutions were spun on a 360° shaker for 12 h. Four levels were prepared: 500, 100, 50, and 10 µg/g. The homogeneity of the material was tested at the 50 µg/g level with a commercially available almond ELISA (r-Biopharm, Darmstadt, Germany). Six replicates were measured, and the RSD was 14%, proving the material to be homogenous.

Analysis of Samples with ELISA

For the analysis of the samples with ELISA, commercially available test kits were used. The protein sample extractions and the detection procedure were done according to the manufacturers' instructions. In summary, the protein fraction was extracted with the kits' buffer, diluted, and added onto an antibody-coated ELISA well plate. After a washing step, the enzyme conjugate was added and incubated. Following another washing step, the reaction substrate was added. After development of the color, a stop solution was added, and the color intensity was measured using the ELISA plate reader GENion Pro (Tecan, Männedorf, Switzerland). Analysis was done in triplicate. The kits used, their reporting unit, and their conversion factor to whole food, skim milk powder, and egg white protein are listed in Table 1.

MS Analysis

Sample preparation.—The protein fraction from 2 g sample was extracted using 20 mL TRIS-HCl buffer, pH

Table 2. Overview of the peptide markers and product ions used for the MRM transitions in the final MS method

Allergic food	Protein	Peptide	Precursor (charge state), *m/z*	Product 1 (fragment) / product 2 (fragment), *m/z*	Signal ratio
Milk	Casein α S1	YLGYLEGLLQR	634.3 (+2)	249.2 (b2) / 991.3 (y8)	1.2
	Casein α S1	FFVAPFPEVFGK	692.9 (+2)	920.3 (y8) / 991.3 (y9)	3.0
	Casein α S2	NAVPITPTLNR	598.3 (+2)	158.3 (b2) / 911.4 (y8)	8.9
	Casein α S2	FALPQYLK	490.3 (+2)	120.1 (a1) / 648.4 (y5)	1.1
Egg	Ovalbumin	HIATNAVLFFGR	673.4 (+2)	223.2 (a2) / 1095.6 (y10)	1.2
	Ovalbumin	YPILPEYLQCVK	761.6 (+2)	810.5 (y6) / 1036.4 (y8)	0.1
	Ovalbumin	DILNQITKPNDVYSFSLASR	761.6 (+3)	201.1 (a2) / 930.5 (y8)	2.9
	Ovalbumin	ELINSWVESQTNGIIR	929.5 (+2)	1017.5 (y9) / 1116.5 (y10)	1.3
Soy	Glycinin	NLQGENEGEDKGAIVTVK	634.3 (+3)	200.2 (a2) / 356.2 (b3)	6.7
	Glycinin	VFDGELQEGR	575.2 (+2)	219.2 (a2) / 903.2 (y8)	0.9
	Glycinin	SQSDNFEYVSFK	725.7 (+2)	381.2 (y3) / 1235.4 (y10)	0.2
	Glycinin	EAFGVNMQIVR	632.3 (+2)	760.6 (y6) / 916.4 (y8)	1.3
Peanut	Ara h1	DLAFPGSGEQVEK	688.8 (+2)	300.2 (a3) / 930.6 (y9)	1.2
	Ara h1	GTGNLELVAVR	564.4 (+2)	557.5 (y5) / 686.6 (y6)	1.3
	Ara h3/4	RPFYSNAPQEIFIQQGR	684.5 (+3)	748.6 (y6) / 836.5 (b7)	1.3
	Ara h3/4	WLGLSAEYGNLYR	771.4 (+2)	272.2 (a2) / 1242.6 (y11)	0.9
Hazelnut	11S globulin	ADIYTEQVGR	576.3 (+2)	689.4 (y6) / 852.5 (y7)	0.9
	11S globulin	INTVNSNTLPVLR	720.9 (+2)	484.4 (y4) / 1013.6 (y9)	0.8
	11S globulin	QGQVLTIPQNFAVAK	807.5 (+2)	874.6 (y8) / 1088.6 (y10)	2.6
	11S globulin	ALPDDVLANAFQISR	815.5 (+2)	906.6 (y8) / 1019.5 (y9)	1.1
Walnut	Jug r1	DLPNECGISSQR	688.2 (+2)	477.2 (y4) / 1147.4 (y10)	1.1
	Jug r1	QCCQQLSQMDEQCQCEGLR	820.2 (+3)	345.5 (y3) / 1294.3 (y10)	1.8
	Jug r1	GEEMEEMVQSAR	698.3 (+2)	820.5 (y7) / 949.4 (y8)	0.9
Almond	Prunin	GNLDFVQPPR	571.9 (+2)	369.4 (y3) / 858.6 (y7)	1.8
	Prunin	GVLGAFSGCPETFEESQQSSQQGR	896.1 (+3)	662.4 (y6) / 790.4 (y7)	2.0
	Prunin	ALPDEVLANAYQISR	830.4 (+2)	922.5 (y8) / 1035.5 (y9)	1.0
	Prunin	NGLHLPSYSNAPQLIYIVQGR	780.8 (+3)	735.7 (y6) / 1154.7 (b11)	1.0

8.2, at 60°C for 3 h. Following centrifugation, 10 mL extract was concentrated to approximately 1 mL using ultrafiltration (Amicon Ultra 15 mL, 5 kDA MW cut-off; Millipore, Billerica, MA). The final volume was recorded, and the total protein concentration determined.

For the enzymatic digestion with trypsin, samples were diluted with 100 mM NH_4HCO_3 solution to a concentration of 1 mg total protein. Aliquots of 100 µL were reduced with 50 µL DTT solution (200 mM) for 45 min at room temperature. Subsequently, an alkylation step was performed using 40 µL IA solution (1 M). The alkylation was left in the dark at room temperature for 45 min and was stopped by adding another 20 µL DTT solution. NH_4HCO_3 solution (100 µL, 100 mM) and trypsin (10 µL, 0.1 µg/µL in 50 mM acetic acid) were added, and samples were incubated at 37°C for 12 h. The digestion was stopped by adding 2 µL concentrated formic acid. Samples were injected into the LC system without further treatment. Analysis was done in triplicate.

HPLC

Separation of peptides was done with an Agilent 1200 (Santa Clara, CA) 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 particle size, 2.1 × 150 mm) from Waters Corp. (Milford, MA), and 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 chromatographic 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 1 min. 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 reaching the mass spectrometer the flow was split, and approximately 60 µL/min effluent was directed into the source.

MS

Peptide identification was carried out on an API 4000 QTrap from Applied Biosystems/MDS SCIEX (Toronto, Canada). The following parameters were set: source temperature 400°C, ion spray voltage 5.5 kV, and curtain gas flow 25 psig. Information dependent acquisition (IDA) was applied. 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 (20). The following parameters were set for the database search: databases searched were UniProt (www.uniprot.org) or National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). 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 four peptides/allergenic food protein were taken for MRM transitions. For walnut only three peptides were monitored.

Results and Discussion

Development of the MS Method

For the development of a multiscreening MS, seven allergenic foods were chosen. These were milk, egg, soy, peanut, hazelnut, walnut, and almond. All of them are subject to the European allergen labeling directive and might be found in baked matrixes.

To find suitable marker peptides for each of the allergenic foods, IDA was used to analyze extracts of them. In the IDA approach, the mass spectrometer is programmed to automatically select ions that are fragmented for a product ion scan, if these fulfill a given set of criteria, e.g., the charge state of the ion. Because peptides usually obtain double or higher charge states when ionized with an electrospray ionization source (21), this approach minimizes the number of product ion spectra, as singly-charged ions that are not peptides are excluded prior to fragmentation. The obtained MS/MS spectra were submitted to a database search using the online version of MASCOT. Figure 1 is an example

Table 3. Correlation coefficient, slope, change in slope (Δ slope), and LOD for the standard and spiked bread extract (n = 3)

Allergenic food	Milk	Egg	Soy	Peanut	Hazelnut	Walnut	Almond
Peptide	YLGYLEQLLR	YPILPEYLQCVK	VFDGELQEGR	DLAFPGSGEQVEK	INTVNSNTLPVLR	DLPNECGISSQR	GNLDFVPPR
RSD, % (standard/spiked extract)	8/3	14/6	5/3	7/1	17/4	6/17	5/3
Correlation coefficient (standard/spiked extract)	0.9983/0.9989	0.9966/0.9998	0.9999/0.9998	1.0000/0.9995	0.9994/1.0000	0.9995/0.9988	0.9993/0.9992
Slope, cps × mL/µg (standard/spiked extract)	7890 ± 160/ 5100 ± 90	2900 ± 90/ 2270 ± 20	2580 ± 10/ 2050 ± 10	2910 ± 10/ 2550 ± 30	2830 ± 40/ 2300 ± 10	90 ± 1/ 100 ± 2	14920 ± 190/ 11590 ± 170
Δ slope, %[a]	35	22	21	12	19	–11	22
LOD, µg/mL (standard/spiked extract)	0.1/0.1	0.6/0.5	0.2/0.4	0.2/0.6	0.3/0.4	10/16	0.1/0.2

[a] The change in slope was calculated from the following formula: Δ slope [%] = ($\text{slope}_{\text{standard}}$ [cps] – $\text{slope}_{\text{spiked extract}}$ [cps]) × 100%/$\text{slope}_{\text{standard}}$.

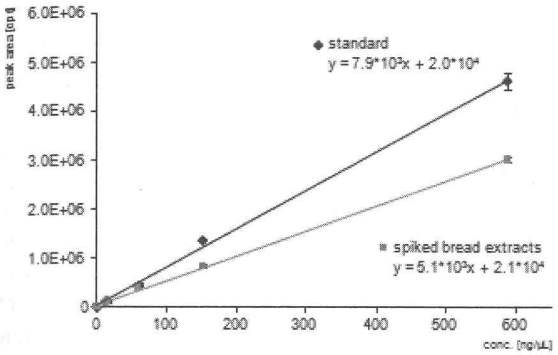

Figure 2. Calibration curve of YLGYLEQLLR (transition 634.3/249.2) from milk for the mixed allergen extracts (standard) and the standard spiked into "allergen-free" bread extracts.

of a typical product ion spectrum. It was identified by the database as the peptide WLGLSAEYGNR from peanut.

Peptides that were reproducibly found in every digest were considered as markers for the final method. However, to ensure the selectivity of the final method, the peptide markers need to be unambiguously related to the allergenic food from which they have been derived. To ensure this specificity, the peptides were submitted to BLAST searches. This tool enables comparison of a given amino acid sequence with the amino acid sequences of the proteins in a database (e.g., SwissProt; 22) and, therefore, allows determination of whether the amino acid sequence is unique to a protein. Other applied criteria for the marker were the absence of cystein and the avoidance of miscleavages. Table 2 shows all peptides chosen for the final method. The two most intense fragments of each peptide were chosen as product ions for the

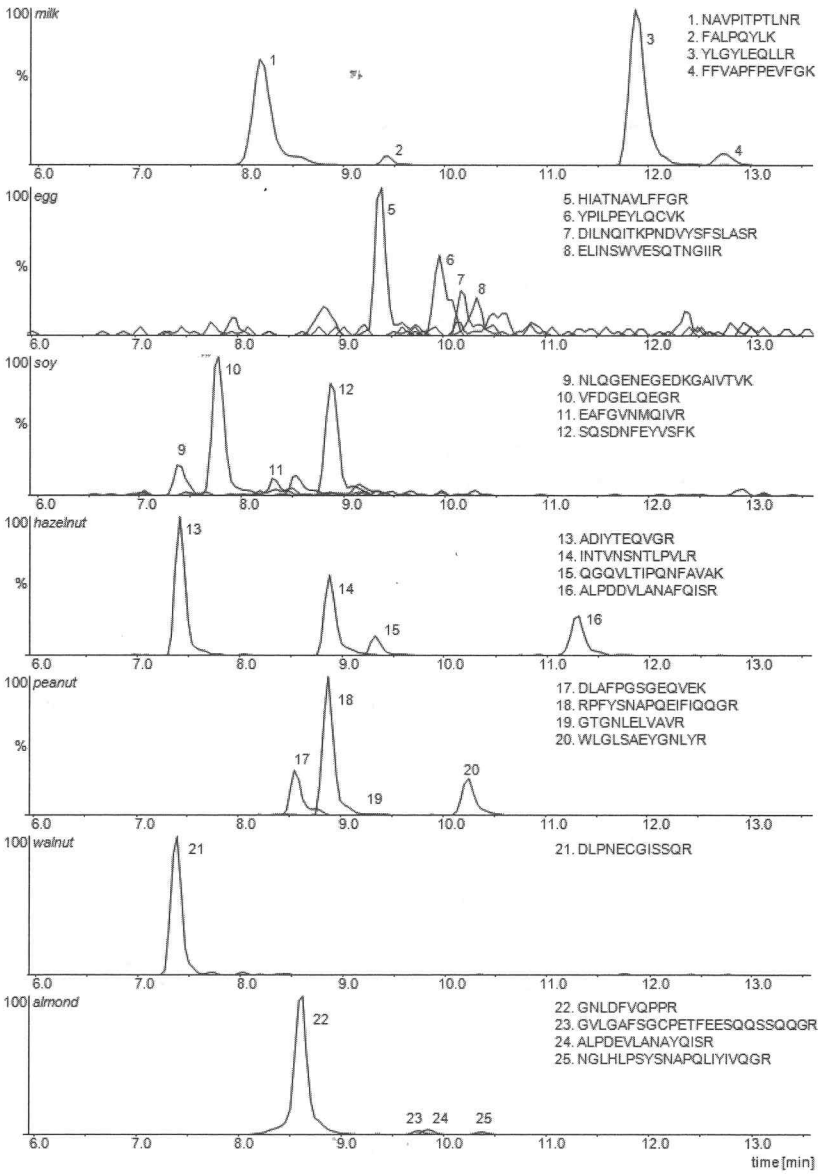

Figure 3. Extracted MRM transitions from bread containing 1000 µg/g of seven allergenic foods.

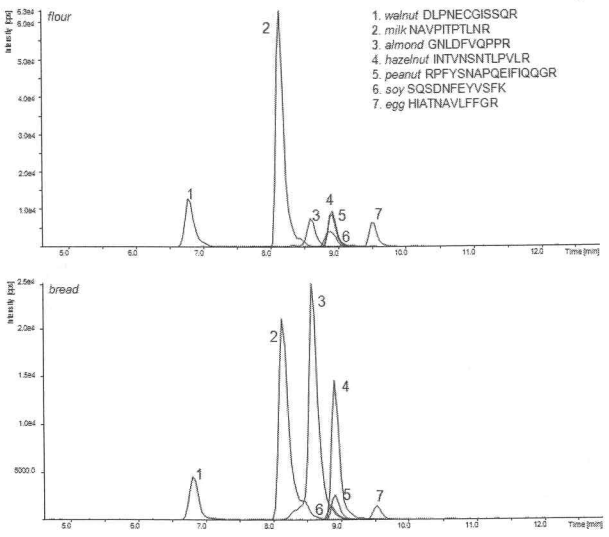

Figure 4. MRM transitions for the most intense marker for each allergenic food obtained when measuring flour and bread (1000 µg/g).

MRM transition, preferably with a higher *m/z* than the precursor ion. For the peptide WLGLSAEYGNR shown in Figure 1, the two selected product ions are 272.2 (a_2) and 1242.6 (y_{11}). All chosen fragments, their *m/z*, and the ratio between the two transitions for each peptide are listed in Table 2.

For the validation of the method without bread matrix, extracts of the allergenic foods were prepared, and the concentration of total soluble protein was determined in triplicate with a Bradford microassay. Mixtures of these extracts (the standards) were digested to determine the linearity, RSD, and LOD. The results for the most intense transition for each allergenic food are given in Table 3. The calibration curve of the transition 634.3/249.2 (milk) is shown in Figure 2. All concentrations of the mix standards refer to the total soluble protein. To assess the influence of the bread matrix on these parameters, the same concentrations of mixed standards were spiked into extracts of allergen-free bread. The matrix had no influence on the linearity and the RSD. However, the decrease in the slopes of the calibration curves shows the influence of the bread matrix on signal intensities. An example of this is shown in Figure 2. All results for matrix-matched evaluation of the method performance are given in Table 3.

MS Analysis of Incurred Bread Material

To evaluate how food processing influences the developed MS method, incurred reference material was analyzed. Bread containing all seven allergenic foods was chosen as a model matrix because of the heat processing during baking. Different spiking levels were prepared according to the procedure described in the *Experimental* section.

MS was used to analyze the unbaked flour (0 and 1000 µg/g), as well as the baked products in different concentrations (0, 10, 50, 100, 500, and 1000 µg/g). All seven allergenic foods could be detected in the incurred material. However, the tryptic digestion of milk, egg, soy, and peanut was influenced by the matrix, as the relative intensities of the markers for each allergenic food changed compared to the standard. Figure 3 shows the signals for a 1000 µg/g bread sample, with the MRM transitions sorted by the allergenic foods. Two peptides from walnut, QCCQQLSQMDEQCQCEGLR and GEEMEEMVQSAR, could not be detected. LOD values were determined at S/N = 3 for the most intense signals. The LOD was around 10 µg/g for almond, milk, hazelnut, and peanut (3, 5, 5, and 11 µg/g, respectively); below 50 µg/g for egg and soy (42 and 24 µg/g, respectively); and 70 µg/g for walnut.

Comparing the results for the flour and bread samples, two different effects were observed: an increase of the signal (and thus the peak area) and a decrease. Figure 4 shows the most intense marker for a flour and a bread sample. The numerical values of the peak areas for these transitions, as well as the percentage of the marker peak area in bread compared to the peak area in flour, are given in Table 4. The latter value is an indicator of the influence of baking. In the case of milk, egg, soy, peanut, and walnut, peak areas decreased. In this group, milk was least affected (decrease by 45%). A significant change was found for egg, soy, peanut, and walnut, for which signal intensities decreased between 70 and 80% when the processed bread samples were compared to the unprocessed flours. This might be caused by the loss of extractability during heat processing and/or the heat-induced chemical modification of the allergen during the baking process, resulting in a mass shift that is not detected in a method based on MRM transitions. Influence of the matrix on the tryptic digestion might also lead to this decrease. This processing effect does not apply to hazelnut and almond. Here, the peak areas were increased by 340 and 140%, respectively, indicating signal suppression in the flour matrix. The application of a more selective sample cleanup, e.g., SPE or size exclusion chromatography, might lead to a reduction of matrix effects and, thus, to higher sensitivities and less signal suppression (23).

Comparison of the MS Method with Commercially Available ELISA Test Kits

For comparison with the developed MS method and to further assess the influence of heat on the target allergens, flour and bread samples (0 and 1000 µg/g) were analyzed with commercially available ELISA test kits. An overview of the obtained results is given in

Table 4. Overview of the ELISA and MS results[a]

	ELISA results					MS results		
Allergenic food	ELISA test kit	0 µg/g flour	0 µg/g bread	1000 µg/g flour ($\times 10^2$ µg/g)	1000 µg/g bread ($\times 10^2$ µg/g)	Peak area (PA) flour (×104 cps)	PA bread (×104 cps)	$(PA_{bread}/PA_{flour}) \times 100\%$
Milk	A	< 0.5	< 0.5	4 ± 0.2	< 0.005	51 ± 2	23 ± 1	45
	B	< 2	< 2	3 ± 0.3	0.5 ± 0.02			
Egg	C	< 0.5	< 0.5	4 ± 0.5	< 0.005	6 ± 0.05	1 ± 0.4	17
	D	< 1	< 1	8 ± 0.8	< 1			
	E	< 0.5	< 0.5	8 ± 0.8	< 0.005			
	F	< 0.312	< 0.312	11 ± 0.05	7 ± 0.2			
Soy	G	< 2.5	< 2.5	0.8 ± 0.05	< 0.025	5 ± 0.3	1 ± 0.05	20
	H	<1.25	< 1.25	8 ± 0.5	1 ± 0.2			
Peanut	I	< 2.5	< 2.5	34 ± 3	9 ± 0.6	8 ± 0.3	2 ± 0.2	25
	J	< 1	< 1	20 ± 3	12 ± 1			
Hazelnut	K	< 0.5	< 0.5	16 ± 5	1 ± 0.3	8 ± 0.4	11 ± 0.5	138
	L	< 2.5	< 2.5	15 ± 4	3 ± 0.2			
Walnut	M	< 3	< 3	53 ± 4	4 ± 0.07	14 ± 0.9	4 ± 0.5	29
Almond	N	< 2.5	< 2.5	13 ± 2	2 ± 0.4	8 ± 0.2	27 ± 1	338

[a] ELISA results are given in whole food, with the exception of egg for which the results are given as egg white. Conversion was done according to nutrition tables from the Souci, Fachmann, Kraut database (ref. 24).

Table 4. If necessary, results were converted to whole food using nutrition tables from the Souci, Fachmann, Kraut databank (24). Milk, soy, peanut, and hazelnut were analyzed with two different kits, egg with four, and walnut and almond with one.

Analogous to the MS method, peanut, hazelnut, walnut, and almond could be detected with ELISA in flour and bread samples. For peanut, 900 and 1200 µg/g were found in the processed samples with kits I and J (Table 4), respectively. Here, heat does not seem to influence the ELISA kits' performance, as was the case when peanut was analyzed with MS, indicating that the decrease in MS peak areas was induced by an effect that influences the MS method only. When analyzing hazelnut and almond, the opposite effect was observed. The amount found by ELISA was less in the processed bread samples than in the unprocessed flours, whereas the MS peak areas increased in the processed breads. The latter could result from signal suppression in flour matrix in the case of hazelnut and almond. Reduced ELISA recoveries for these two allergens after processing may be due to a loss of extractability for these allergens. However, this would affect the MS method as well. An effect induced by heating that influences ELISA only could be a reduced antibody binding due to at least partially destroyed epitopes. For hazelnut, kit K found 40% less in the baked samples than kit L, which may be a sign that the antibodies in kit K recognize native proteins, while the antibodies in kit L also recognize the processed (heat-treated) allergens.

When comparing the two detection methods, as well as the ELISA kits among each other, the biggest differences were found when analyzing milk, egg, and soy. The detection of these allergens with MS was influenced by the processing, leading to a decrease in signal intensities up to 80% for egg and soy and 50% for milk. Nevertheless, the MS method was capable of detecting all allergenic foods in the processed matrix. This was not the case when analyzing the samples with ELISA. For milk and soy, only kits B and H were able to determine the allergens in the processed food. These kits found 17 and 13% milk and soy, respectively, of the amount found in flour. Kit A (milk) and kit G (soy) did not detect the targeted allergens in the processed matrix. Both also underestimated the concentration in flour. In the case of egg, only kit F could detect the allergen in the processed bread. However, all the kits detected egg in the unprocessed matrix, indicating that heat destroys, at least partially, the structures recognized by the kits' antibodies. Another heat-induced effect might be the loss of extractability of the allergens. This would influence the performance of both MS and ELISA, whereas the former would affect ELISA only. ELISA kits allow semiquantitation of the target analyte. For MS, this can be achieved by the standard addition method or by using isotopically labeled standards.

Conclusions

A novel MS method for the simultaneous detection of seven allergenic foods has been developed and applied to unprocessed and processed samples (flour and bread). The influence of the baking process was assessed and the results were compared to those obtained with commercially available ELISA test kits. Peanut, hazelnut, walnut, and almond could be detected with both methods regardless of whether the product was baked or not. Major differences could be seen between the two methods when analyzing milk, egg, and soy. For egg, only one kit was capable of detecting the allergen in the processed product. For milk and soy the sensitivities were decreased when analyzing bread, or the allergen could not be detected at all. For these allergens, the MS method showed superior detection capability for the processed samples. Another advantage of the MS method is its capability of multiplexing. A disadvantage of the developed MS method is that, so far, it is only qualitative. However, to overcome this problem, isotopic labeled peptides could be used.

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