



# Quantification of allergenic plant traces in baked products by targeted proteomics using isotope marked peptides



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## ABSTRACT

The right choice of analytical methods for plant allergen quantification is a deciding factor for the correct assessment and labeling of allergens in processed food in view of consumer protection. The aim of the present study was to develop a validated target peptide multi-method by LC/MS/MS providing high specificity and sensitivity for plant allergen protein detection, plant identification in vegan or vegetarian products using peptide markers for quantification. The methodical concept considers the selection of target peptides of thermostable allergenic plant proteins (*Gly m6* soy, *Ses i6* sesame and  $\beta$ -conglutinin from white lupine) by data base research, BLAST and *in silico* digestion using Skyline software. Different allergenic concentration levels of these proteins were integrated into our own reference bakery products and quantified with synthesized isotopically labeled peptides after in-solution digestion using LC/MS/MS. Recovery rates within the range of 70–113% and LOQ of 10 ppm–50 ppm (mg allergenic food/kg) could be determined. The results are independent of thermal processing applied during baking and of epitope binding site for the tested allergens.

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## 1. Introduction

In western industrialized nations around 8% of children and 2% of adults suffer from a food allergy type I (immediate type), which is mediated by immunoglobulin E antibody (IgE) (Sicherer & Sampson, 2014). In total, over 160 allergenic basic foods are known (Ballmer-Weber, 2011). It is assumed that the prevalence of food allergies in children continues to increase worldwide (Lack, 2012; Platts-Mills & Commins, 2013), and research efforts are directed to provide reliable alternatives for the diet composition. Older consumers will also be increasingly affected because of cross-reaction of food and inhalant allergens, e.g. secondary soy allergy resulting from primary birch pollen allergy (Berkner et al., 2009; Mehta, Groetch, & Wang, 2013; Mohrenschräger & Ring, 2011). Typical symptoms after ingestion are redness, hives, nausea,

vomiting and shortness of breath and under extreme conditions resulting in anaphylactic shock or death. The reason for such symptoms is often the accidental ingestion of products that had contact with allergen sources during processing. Even small amounts of allergens in the ppm range are sufficient to cause reactions in an allergic patient. For the consumer, it is therefore essential that food products are labeled with respect to the content of allergens. The safest option for consumers currently is to avoid labeled food and to eat those without any warnings at their own risk, although these products may have a particularly high rate of contamination, e.g. those typically offered by small scale food producers (Ford et al., 2010).

Substances or products, including non-prepacked foods, causing allergies or intolerances must be labeled since December 2014 in the EU, according to EU guidance on food information to consumers (“Regulation (EU) No 1169/2011 of the European Parliament and of the Council (2011)”). At present, it might be difficult for an allergic person to understand the food labeling for allergens because no assessment is available for the term “may contain” or “is free of” allergenic substances. The terminology for allergenic food has developed from threshold to action levels (Vital 2.0 of Allergen

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Bureau) towards reference doses (EFSA, 2014). The thresholds depend on the allergen and are in the range of 0.01 to 0.001% per allergenic food. This corresponds to 100 to 10 ppm of allergenic food or  $10^{-1}$  ppm of allergenic protein in food. Therefore, considering all legal and consumer related issues discussed above, the necessity to develop sensitive validated analytical methods to detect traces of allergenic substances becomes a high research priority.

Two methods are frequently used to identify the allergenic plant sources, ELISA (Enzyme-linked Immunosorbent Assay) and qPCR (real-time-PCR). Currently, ELISA is the method of choice for raw materials. Thermal processing of food destroys or may create structural epitopes, whereas sequence epitopes are usually retained and analytically detectable by various methods (Cuadrado et al., 2009; Kamath et al., 2014; Masthoff et al., 2013; Nowak-Węgrzyn & Fiocchi, 2009). The LC/MS/MS method distinguishes itself in this context by being independent of epitope structures. Real-time PCR has become a more popular alternative in utilizing certain advantages of DNA-detection in contrast to detection of proteins. Real-time PCR is a very sensitive and specific method (Costa, Mafra, Kuchta, & Oliveira, 2012; Lopez-Calleja et al., 2013), in most cases DNA is less affected by heat denaturation and false-positive results since cross reactions can be avoided (Herrero, Vieites, & Espineira, 2012). On the other hand, food ingredients can interfere with the DNA and as a result might affect an effective PCR reaction (Eischeid & Kasko, 2015; Waiblinger, Boernsen, Nümann, & Koepfel, 2014; Zhang, Cai, Guan, & Chen, 2015). This method also does not yet provide information on the actual content and amount of (allergenic) protein(s) in the sample due to a lack of calibrators needed for conversion of copy number, concentration or weight of DNA to ppm of protein (Platteau, De Loose, De Meulenaer, & Taverniers, 2011).

Finally, the emerging mass-spectrometry based proteomic approach is receiving attention due to its specific advantages against the above mentioned two options. Fast and easy sample preparation, high throughput processing, incorporation of post-translational and processing-dependent modifications and quantification of analytes have led to the introduction of MS-based techniques in clinical routine and application to allergen-detection research in foods (Johnson et al., 2011; Monaci, Losito, De, Pilolli, & Visconti, 2013). However, design and methodology need to be carefully applied (Johnson et al., 2011).

Unfortunately, in processed food the quantitative levels determined by using ELISA kits are generally below the actual values of allergens present. Therefore, the aim of our study was the validation of a multi plant allergenic parameter test method based on “targeted proteomics” utilizing UPLC/MS/MS (mass spectrometry) in line with DIN EN ISO/IEC 17025.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Allergens and chemical compounds

Wheat flour (type 550) from a local supermarket, soybeans (Rapunzel Naturkost GmbH, Legau, Germany), sesame seeds (Seberger GmbH, Ulm, Germany) and white lupine (Veggie's Delight; Düsseldorf, Germany) were used.

Ammonium bicarbonate (PubChem CID: 14013), 1,4-Dithiothreitol (DTT) (PubChem CID: 19001) and urea (PubChem CID: 1176) for the extraction buffer were purchased from Carl Roth GmbH, Karlsruhe, Germany, as well as acetonitrile LC-MS grade (PubChem CID: 6342) and formic acid (PubChem CID: 284) for LC-MS solvents. Isopropanol (PubChem CID: 3776) was ordered from Chemsolute, Renningen, Germany. Iodoacetamide (IAA) (PubChem

CID: 3727) as well as porcine pancreatic trypsin for enzymatic digestion were procured from Sigma Aldrich, St. Louis, Missouri, US. Bovine serum albumin (BSA) was used for calibrating the protein content and was purchased from Bio-Rad Laboratories, Hercules, California, US.

Standards (peptides of allergenic proteins) and their isotope labeled standards for quantification were synthesized by a subcontractor (peptides&elephants GmbH, Potsdam, Germany). Isotope labeling was applied to the amino acids Arginine [ $^{13}\text{C}(6)$   $^{15}\text{N}(4)$ C-term R] and Lysine [ $^{13}\text{C}(6)$   $^{15}\text{N}(2)$ C-term K] of the peptides.

#### 2.1.2. Reference materials

The aim of the method validation was focused on simultaneous detection of three selected plant allergens in processed food. Three matrix reference materials spiked with allergenic traces were prepared for the method validation using targeted proteomics-based analysis.

**2.1.2.1. Raw material – wheat flour (W).** To receive a non-processed food matrix, the required amounts of soybean, lupine and defatted sesame powders were suspended together with wheat flour in water to gain a 100 ppm allergenic mixture. After 2 h of homogenization, these were dried overnight at 40 °C (drying closet).

**2.1.2.2. Cookies (C).** The processed allergenic cookie matrix was made by adding different amounts of allergens to wheat flour. After homogenization, margarine and sugar were added. The mixture was molded into cookies and baked for 13 min at 190 °C. Spiked samples with a concentration of 100 ppm were produced.

**2.1.2.3. Soft bread (B).** The allergenic dough for soft bread was prepared in the same way as described above (100 ppm). Sugar, palm fat, salt, yeast and water were added and the dough was kneaded and let to rise for 50 min at 33 °C and 75% humidity. After baking for 30 min at 220 °C the breads were cooled down and then pre-dried over-night at 40 °C.

Homogenization of the reference materials (particle size of 0.8 mm) was tested by particle size analysis. Control samples free of allergenic substance were prepared for all materials and tested by LC/MS/MS, ELISA and qPCR. Spiked samples of 50 and 75 ppm were produced by mixing control samples with 100 ppm reference material (W, C, B). In the similar way lower concentration levels were also obtained for calibration purposes.

### 2.2. Methods

The general workflow is shown in Fig. 1 and described in the following sub sections.

#### 2.2.1. Selection of proteins and peptides by database research

After an extensive database research to find suitable protein markers using databases SDAP (Structural Database of Allergenic Proteins (Ivanciuc, Schein, & Braun, 2002), The University of Texas Medical Branch, available at: <https://fermi.utmb.edu/> (Johnson et al., 2011)) and UniProt (Universal Protein Resource, available: <http://www.uniprot.org/> (UniProt-Consortium, 2015)) specific amino acid sequences were selected using BLAST (Basic Local Alignment Search Tool) algorithm. First an *in silico* digestion was performed using the software Skyline (MacCoss Lab Software, University of Washington, available at: <https://skyline.gs.washington.edu> (MacLean et al., 2010)) to generate data for multiple reaction monitoring (MRM). Only peptides with a minimal length of 8 and maximal 25 amino acids were selected. The amino acids cysteine and methionine were excluded to avoid mass shifts caused by structural modifications. Precursor charges were set to

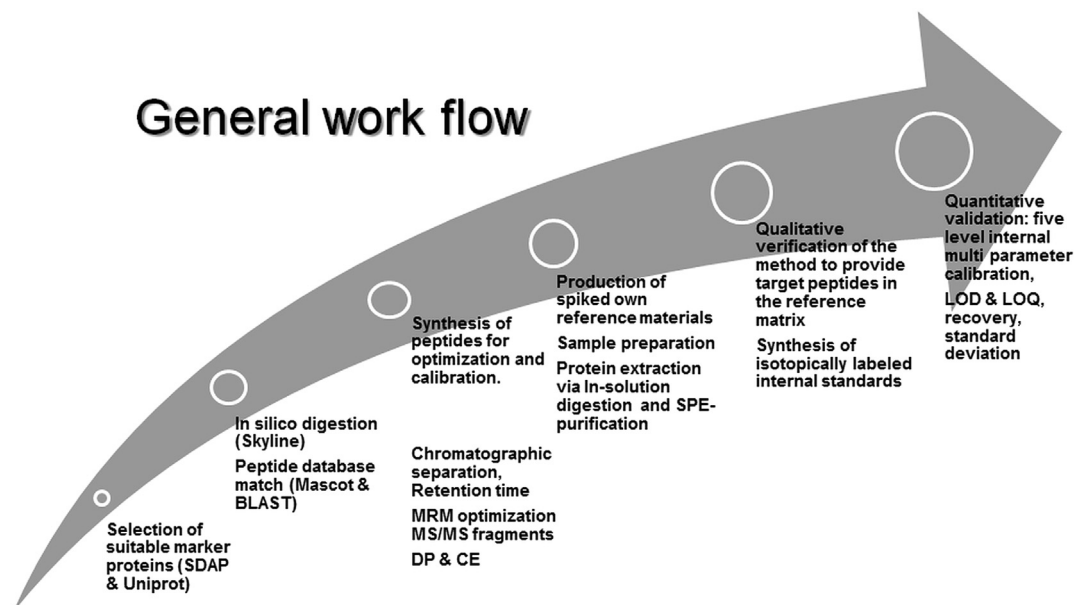


**Fig. 1.** General work flow: in solution digestion of allergenic plant proteins for target proteomics based analysis – the individual technical parameters for each step are given in detail in the methods sections and the corresponding practical/chemical considerations necessary discussed in the results section.

2<sup>+</sup> and 3<sup>+</sup> and ion charges to 1<sup>+</sup> and 2<sup>+</sup>. Only y-ions were considered with a mass-to-charge ratio (*m/z*) from 400 to 1250.

The list of peaks from the acquired MRM data was submitted to the search tool of the MASCOT database (Matrix Science Ltd.) and compared with the UniProt database (Fig. 1). One missed cleavage was allowed. Peptide tolerance and MS/MS tolerance were both set at 0.6 Da. Peptide charges were set to 2<sup>+</sup> and 3<sup>+</sup> and as instrument the ESI-TRAP was selected.

#### 2.2.2. Sample preparation – in solution digestion

One gram of allergen-spiked ground sample was incubated with extraction buffer, containing 100 mM ammonium bicarbonate, 4 M urea and 5 mM DTT at pH = 8.2 and shaken in a roller mixer for 30 min at room temperature. After two centrifugation steps for 5 min at 4000 and 7000 rcf, the isotope standard peptides were added to the supernatant. Afterwards, the extract was incubated in the dark with IAA for 20 min at 50 °C, allowing the splitting of the disulfide bonds into thiol groups. The digestion was performed with trypsin and digestion buffer (containing 100 mM ammonium bicarbonate, 15% acetonitrile in water). Following the overnight incubation, the digestion was stopped by adding formic acid to a final concentration of 2% (Johnson et al., 2011).

To remove matrix components from the extract, a conventional conditioned polymeric SPE cartridge (LiChrolut RP-18 standard PP-tubes; 40–63 µm; 1000 mg; 6 ml; Merck Millipore, Darmstadt, Germany) was used. The peptides were eluted from the cartridge using acidified acetonitrile (50% acetonitrile/water with 0.1% formic acid). After that, the extract was evaporated to complete dryness with nitrogen and reconstituted (5% acetonitrile/water with 0.1% formic acid). The optimization of the in solution digestion has been comprehensively described (Uhr, Wieland, Homann, Huschek, & Rawel, 2015).

#### 2.2.3. LC/MS/MS

For data acquisition a LC/MS/MS system was used, composed of Shimadzu Nexera XR UHPLC, Auto sampler PAL HTS-xt System of CTC Analytics and SCIEX QTRAP® 5500 MS/MS. Target peptide analysis was carried out on an Aeris Peptide XB-C18 analytical column, 100 × 2.1 mm; 1.7 µm (Phenomenex, Aschaffenburg,

Germany) with a flow rate of 0.3 ml/min and an oven temperature of 35 °C. Elution gradient program from 95% solvent A (0.1% formic acid in water) to 95% solvent B (0.1% formic acid in acetonitrile) was used over 20 min including 8 min re-equilibration time.

For peptide identification the MS parameters were optimized as follows: source temperature was set to 400 °C, ion spray voltage to 5500 V, curtain gas (CUR) to 40 units, collision gas (CAD) to “medium”. Ionization was performed in positive ESI mode. Full scan spectra were measured between 100 and 1250 Da. For applied IDA, criteria selected included: signal intensity greater than 500 counts per second (cps) and the 3 most intense peaks. Product ion spectra were measured between 230 and 1000 Da with the enhanced product ion (EPI) scan type.

First an *in silico* digestion was performed with the Skyline software to generate data for the Multiple Reaction Monitoring (MRM) described above. After comparison with UniProt using the MASCOT algorithm the MRM data were submitted again to Skyline to determine the retention times for the identified peptides. For creating the sMRM method, retention times were incorporated together with the three most intense product ion signals (MRM transitions). This was pursued for three peptides per allergenic food.

For the statistical assessment, all experiments were conducted at least six times. All data are expressed as means with their standard deviations. The results were analyzed using GraphPad Prism 6 (Vers. 6.01, GraphPad Inc. CA, USA). Unpaired samples were analyzed by ANOVA one-way analysis of variance using Tukey's multiple comparisons test. Small letters were used to indicate significant differences in the recovery between the matrices for each protein and concentration level. Values of *p* < 0.05 were considered statistically significant.

### 3. Results and discussion

#### 3.1. Selection of proteins and digested peptides (amino acid sequences)

The selection of selective and sensitive allergenic target peptides was performed considering temperature stability of the

**Table 1**

Allergen-specific marker peptides with optimized LC parameter Retention time (Rt) and MS/MS parameters: MRM transitions, Declustering Potential (DP), Collision Energy (CE) of soy, sesame and white lupine.

| Allergenic food (target protein) | Marker peptide (amino acid sequence) | Rt [min] | Q1 (m/z) z = 2 | Q3 MRM fragments  | DP  | CE             |
|----------------------------------|--------------------------------------|----------|----------------|-------------------|-----|----------------|
| Soy: Gly m6                      | <b>VFDGELQEGR</b>                    | 5.7      | <b>575.3</b>   | 903.6/489.2/788.5 | 110 | 28/38/31       |
|                                  | LSAEFGSLR                            | 7.2      | 490.3          | 432.3/779.4/579.3 | 110 | 28/24/25       |
|                                  | LNALKPDNR                            | 2.9      | 520.8          | 742.4/629.3/501.2 | 110 | 31.6/29.6/28.6 |
| Sesame: Ses i6                   | <b>ISGAQPSLR</b>                     | 3.9      | <b>464.8</b>   | 472.3/728.4/671.4 | 110 | 22.6/20.6/20.6 |
|                                  | AFYLAGGVPR                           | 8.3      | 525.8          | 556.3/485.3/669.4 | 110 | 22.8/22.8/22.8 |
|                                  | SPLAGYTSVIR                          | 8.2      | 582.3          | 795.4/866.5/575.4 | 110 | 25.8/28.8/25.8 |
| White lupine: $\beta$ -conglutin | <b>LLGFGINADENQR</b>                 | 9.1      | <b>723.9</b>   | 846.4/661.3/797.4 | 110 | 40/49/38       |
|                                  | NTLEATFNTR                           | 6.4      | 583.8          | 951.5/838.4/709.4 | 110 | 30/33/31       |
|                                  | NPYHFSSQR                            | 3.4      | 568.3          | 761.4/624.3/477.2 | 110 | 34/36/38       |

allergenic plant proteins and using database research, as shown in Fig. 1 step one of general workflow: proteins for soy: <http://www.uniprot.org/uniprot/P04776>, sesame: <http://www.uniprot.org/uniprot/Q9XHP0> and lupine: <http://www.uniprot.org/uniprot/Q6EBC1> (Ivanciuc et al., 2002). The selected allergenic target peptides (amino acid sequences) were not influenced by temperature-induced denaturation of proteins or Maillard reaction (glycation of amino acids) thus formation of aggregates and altered allergenic

potential do not have to be taken into consideration (Cucu, De Meulenaer, Bridts, Devreese, & Ebo, 2012). Selected peptide markers documented in Table 1 provide high specificity ensured by database research, BLAST (Ivanciuc et al., 2002; UniProt-Consortium, 2015) and the comparison of generated mass spectra with libraries (MacLean et al., 2010), as well as showing low susceptibility towards degradation processes. This is due to the fact that the target analytes are peptides of selective plant proteins,

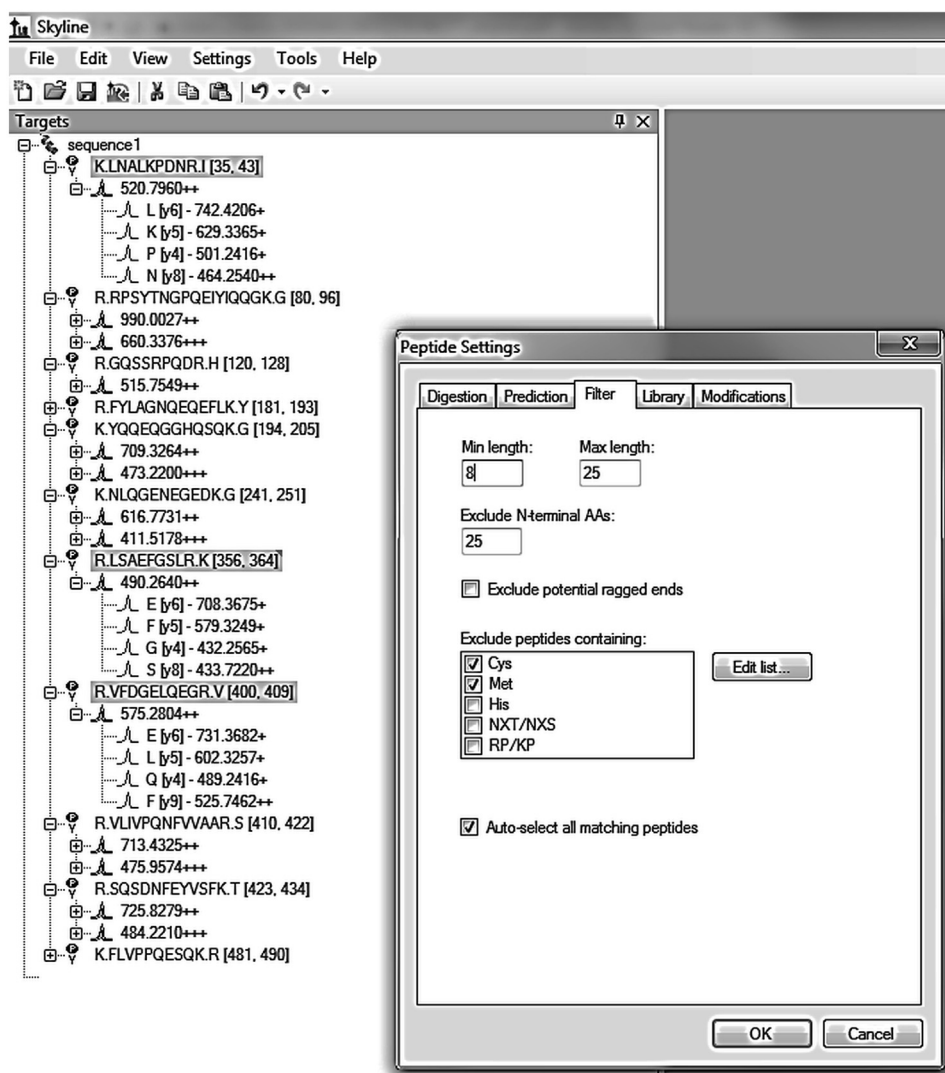


**Fig. 2.** The pre-selection of specific and sensitive allergenic target peptide is exemplary shown at the hand of database results for the thermostable soy protein Gly m6 after *in silico* trypsin digestion by Skyline (MacLean et al., 2010).

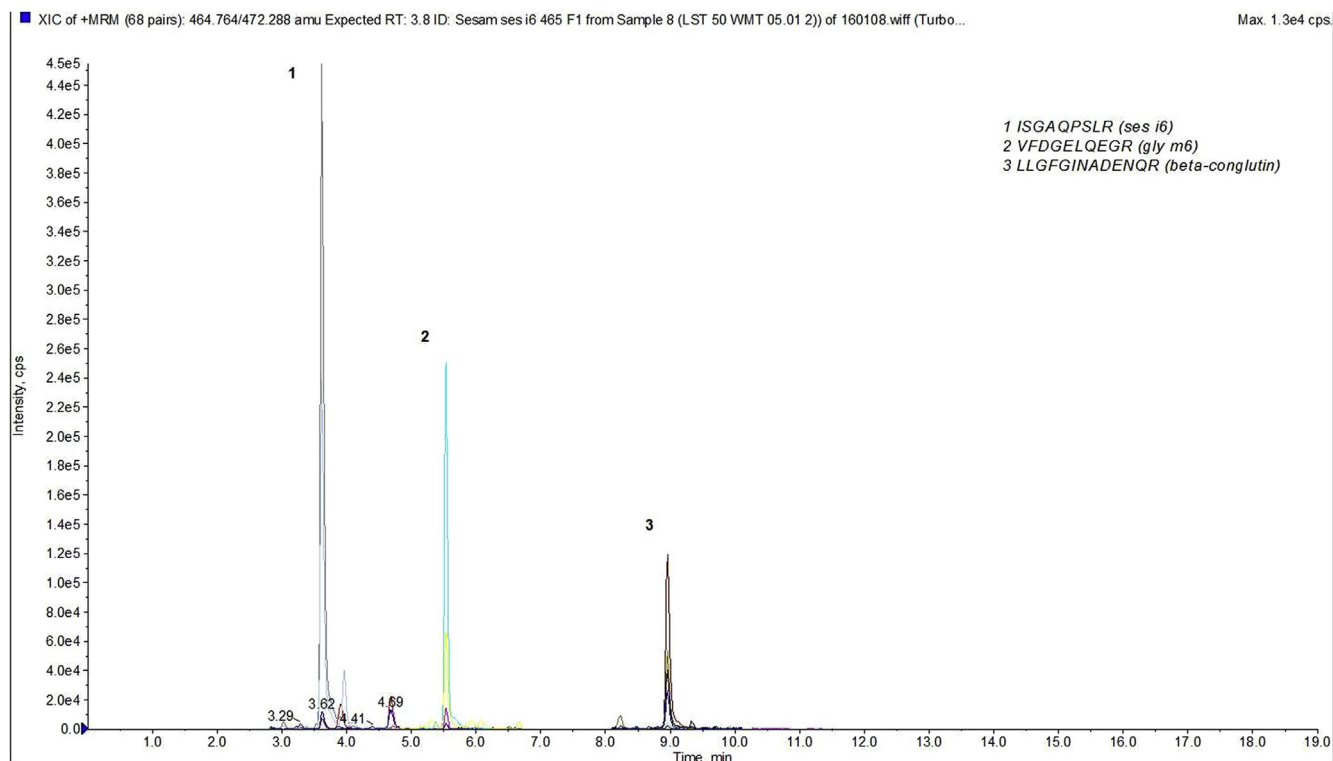


**Fig. 3.** An exemplary mass spectrum showing optimized quantifier peptides and isotope synthesized marker peptides of soy, white lupine and sesame in wheat flour spiked with 50 ppm allergenic food.

which are small fragments, unaffected by structural alterations, the latter being ensured by selecting thermostable peptides as preferable precursors. In Fig. 2 results of *in silico* digestion by trypsin are exemplary highlighted for the thermostable soy protein Gly m6 by Skyline (MacLean et al., 2010), step 2 of the workflow.

### 3.2. LC/MS/MS optimization

According to the *in silico* digested and identified proteins and mass fragments generated by Skyline (MacLean et al., 2010), three specific peptides and their three relevant MRM fragments per allergenic plant protein were selected. Marker peptides were synthesized and LC/MS/MS measurement conditions were optimized as in step 3 of the general workflow. In Table 1 the allergenic plant target peptides of thermostable plant proteins are reported together with the MRM fragments and optimized MS/MS parameters: Decustering Potential (DP) and Collision Energy (CE). The bold marked amino acid sequences in Table 1 are the quantifier and the other peptides the qualifiers of the allergenic plant protein. Chromatographic separation of target peptides and their respective isotopically labeled standards is shown exemplarily in Fig. 3. The results of LC/MS/MS parameter optimization summarized in Table 1 reflects step 3 of the workflow.

### 3.3. Quantification of allergenic food, based on allergenic plant proteins

No certified processed reference material for allergenic food is available. Thus, three different reference materials were prepared, a blank which is allergen free and those spiked with 100 ppm allergenic food. Corresponding lower concentrations were prepared as described in section 2.1.1. For the validation process wheat flour (W) and two processed reference materials soft bread (B) and

cookies (C), based on wheat flour were used, which is illustrated as step four of the work flow (Fig. 1). The reference materials were also tested by ELISA and qPCR (data not shown). Multi-Parameter LC/MS/MS method validation was adapted in respect to Guidance document on analytical quality control and validation procedures for pesticide residue analysis in food and feed SANCO/12571/2013 (EC, 2013).

For each concentration level of reference material examined, at least six replicates were analyzed, reported in Table 2. According to step five of the workflow – qualitative verification, the reproducibility of the selected target peptides was checked, one quantifier and two qualifiers with three MRM fragments each (Table 1), in the reference materials. For internal calibration isotopically labeled peptide standards were synthesized.

### 3.4. Calibration for quantification

Method calibration, step six of the work flow, was carried out in the range of 0.001–0.02 ppm per selective allergenic plant peptide, based on synthesized analyte quantifier peptides and their isotopically labeled internal standards using weighted linear regression ( $1/x$ ) for internal multi-level calibration (five levels). Data analysis was performed with SCIEX software Analyst 1.6.2,  $R^2$  was above 0.998 for all analytes in the calibration range. Dissolved calibration standards are stable for 1 month at  $-20^\circ\text{C}$ .

Quantification should be based on an assessment for the allergenic foods and food ingredients for labeling purposes (BFR, 2009). Neither the German Federal Institute for Risk Assessment (BfR) nor the EFSA define thresholds or set reference doses of allergens for food labeling (BFR, 2009; EFSA, 2014). Therefore, for the validation process “internal action levels for allergenic food” of German food legislation ([http://www.bvl.bund.de/SharedDocs/Downloads/01\\_Lebensmittel/ALS\\_ALTS/ALTS\\_Beschluesse\\_74\\_Arbeitstagung\\_Dez\\_](http://www.bvl.bund.de/SharedDocs/Downloads/01_Lebensmittel/ALS_ALTS/ALTS_Beschluesse_74_Arbeitstagung_Dez_)

**Table 2**

Average recovery values of allergenic target peptides in relation to allergenic food of own reference material wheat flour (W), cookies (C) and soft bread (B).

| Reference material spiked with allergenic food         |   | Concentration of allergenic food [ppm]     |                           |                            | LOQ [n = 6]<br>ppm allergenic food | Allergen reference dose* |         | Action level** achieved by 100 g consumption<br>ppm allergenic food |
|--------------------------------------------------------|---|--------------------------------------------|---------------------------|----------------------------|------------------------------------|--------------------------|---------|---------------------------------------------------------------------|
|                                                        |   | 100                                        | 75                        | 50                         |                                    | mg protein               | mg food |                                                                     |
| Allergenic food (target protein)                       |   | Recovery rates [%] with standard deviation |                           |                            |                                    |                          |         |                                                                     |
| Soy ( <i>Gly m6</i> )                                  | W | 99.5 [±3.2] <sup>a</sup>                   | 94.9 [±13] <sup>a</sup>   | 100.8 [±4.5] <sup>a</sup>  | 10                                 | 1.0                      |         | 25                                                                  |
| Reported factor for soy based on protein: 6***         | C | 83.9 [±2.9] <sup>b</sup>                   | 84.6 [±11.8] <sup>a</sup> | 94.4 [±10.9] <sup>ab</sup> | 10                                 | 2.5                      |         | >20**                                                               |
| Sesame ( <i>Ses i6</i> )                               | B | 87.6 [±2.6] <sup>a</sup>                   | 82.0 [±3.5] <sup>a</sup>  | 85.3 [±2.8] <sup>ab</sup>  | 20                                 |                          |         |                                                                     |
| Empiric factor for sesame based on protein: 9.5        | W | 102.6 [±3.9] <sup>a</sup>                  | 111.0 [±6.1] <sup>a</sup> | 83.4 [±2.8] <sup>b</sup>   | 20                                 | 0.2                      |         | 11.8                                                                |
| White lupine ( <i>β-conglutin</i> )                    | C | 70.0 [±4.8] <sup>b</sup>                   | 75.0 [±5.9] <sup>b</sup>  | 69.4 [±9.1] <sup>a</sup>   | 10                                 | 1.18                     |         | >10**                                                               |
| Empiric factor for white lupine based on protein: 10.8 | B | 79.3 [±3.1] <sup>b</sup>                   | 85.4 [±5.9] <sup>b</sup>  | 81.1 [±7.4] <sup>b</sup>   | 50                                 |                          |         |                                                                     |
|                                                        | W | 112.9 [±8.0] <sup>a</sup>                  | 84.9 [±12.1] <sup>a</sup> | 102.5 [±4.4] <sup>a</sup>  | 20                                 | 4.0                      |         | 100                                                                 |
|                                                        | C | 91.4 [±17.0] <sup>b</sup>                  | 82.1 [±12.9] <sup>a</sup> | 80.9 [±3.3] <sup>b</sup>   | 10                                 | 10.0                     |         | >50**                                                               |
|                                                        | B | 71.7 [±8.5] <sup>c</sup>                   | 70.6 [±3.7] <sup>a</sup>  | 85.0 [±7.4] <sup>ab</sup>  | 50                                 |                          |         |                                                                     |

The results are presented as the mean of six replicates (n = 6). Different small letters in each sub-column box depicting each different concentration in ppm indicate significantly different values (p < 0.05, Tukey's test) for each protein sample. LOQ = Limit of Quantification; \* according to (Taylor et al., 2014) – the upper values in the column represent the reference dose in mg protein and the lower values corresponding mg allergen food; \*\* not allergen free, must be labeled as allergen in respect to German legislation: [http://www.bvl.bund.de/SharedDocs/Downloads/01\\_Lebensmittel/ALTS\\_ALTS/Beschluesse\\_74\\_Arbeitstagung\\_Dez\\_2014.pdf?\\_\\_blob=publicationFile&v=2](http://www.bvl.bund.de/SharedDocs/Downloads/01_Lebensmittel/ALTS_ALTS/Beschluesse_74_Arbeitstagung_Dez_2014.pdf?__blob=publicationFile&v=2).

\*\*\*According to (Adachi et al., 2001; Capriotti et al., 2014; Taski-Ajdukovic et al., 2010).

2014.pdf?\_\_blob=publicationFile&v=2), see Table 2, based on reference doses of allergenic protein (Taylor et al., 2014) was used. This approach shows that the lowest calibration levels selected are below the action levels and Limit of Quantification (LOQ), for soy for example 0.6 ppm allergenic food. For matrix effects compensation 1 µg/ml of every isotopically labeled peptide standard were generically added to samples and calibration standards likewise. It was also used to facilitate the identification of analytes, having the same behavior for retention time and peak shape.

In Table 2, validated and statistically proven quality assurance parameters: recovery, standard deviation and LOQ are reported in relation to the spiked allergenic plants of the three own reference materials. Recovery for all analytes and concentration levels are in

the range of 70–113% for the proved materials, therefore it was not necessary to compensate with matrix calibration complying with SANCO/12571/2013 (EC, 2013). For allergen quantification using ELISA and qPCR in processed food an undervaluation of allergenic food occurred in dependence of the processing grade (data not shown). The LOQ was estimated using a signal-to-noise-ratio (S/N) of ten (EC, 2013) by the auto integrator tool of software Analyst 1.6.2 of SCIEX. All S/N results are above 10 for the reported LOQ. Limit of Detection (LOD) was estimated by S/N ratio >3 (Shrivastava & Gupta, 2011). Statistical evaluation of the data (Table 1) indicates that the medium concentration level (75 ppm) has the least variation in recovery for the different matrices (W, C, B) and the different allergenic foods (p < 0.05). Acceptable mean

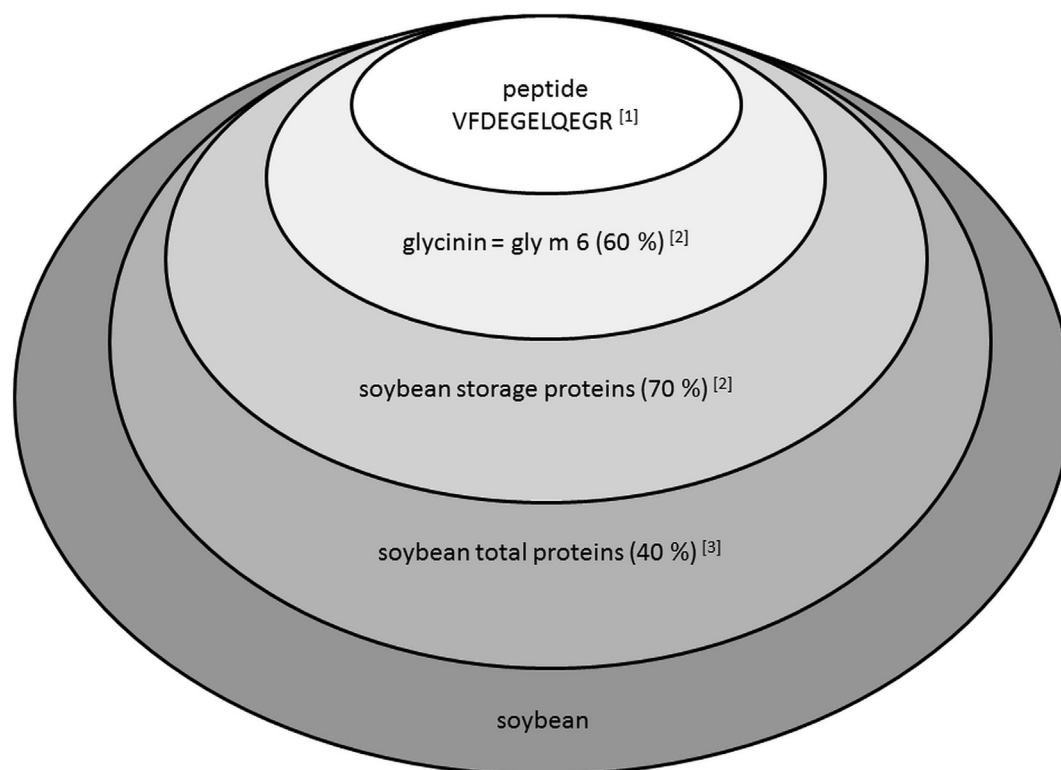


**Fig. 4.** Reported calculation factors for soybean based on peptide VFDEGELQEGR of soy *Gly m6*. [1] = (Adachi et al., 2001); [2] = (Taski-Ajdukovic et al., 2010); [3] = (Capriotti et al., 2014).

recoveries for all levels and matrices within the range 70–113% (mean =  $87.1 \pm 11.9$  with an associated repeatability CV = 13.7% (n = 27)) for all analytes were determined within the scope of the method.

### 3.5. Calculation of results

The results are calculated as ppm allergenic food with respect to EU legislation (EFSA, 2014) and international assessment (Taylor et al., 2014). In Fig. 4 reported calculation factors of allergenic food are documented for soy bean based on peptide VFDEGELQEGR (Adachi, Takenaka, Gidamis, Mikami, & Utsumi, 2001; Capriotti et al., 2014; Taski-Ajdukovic, Djordjevic, Vidic, & Vujakovic, 2010). For sesame and lupine no references for calculation factors are available. Own factors were used, empirically determined for unprocessed wheat flour, for calculation, see Table 2.

## 4. Conclusion

The developed and accredited (with regard to DIN EN ISO/IEC 17025) target peptide method by LC/MS/MS provides high specificity for plant allergen detection, plant identification and quantification of peptide markers in food. The selected peptides are relatively thermostable under baking conditions, were confirmed by database research, BLAST, comparison of generated mass spectra with libraries, and finally they also show little susceptibility towards degradation processes. Quantification of target allergenic plant proteins could be developed using synthesized isotopically labeled standards. Furthermore, this method enables the simultaneous extraction and subsequent analysis of several plant allergens in a sample. Another advantage of this target peptide method is its relative independence of the grade of food processing (in this specific case related to baking) or the complexity of composition. The method has also been tested for screening of other plant allergenic protein candidates.

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