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 *β-conglutin* 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; Mohrenschlager & 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

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-Wegrzyn & 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, Näumann, & Koeppel, 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 (Seeberger 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}C(6) ^{15}N(4)C-term R] and Lysine [^{13}C(6) ^{15}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, marge 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