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A rapid solid-phase extraction combined with liquid chromatography-tandem mass spectrometry for simultaneous screening of multiple allergens in chocolates

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ABSTRACT

Allergen derived from food is a world-wide problem to specific consumers. The regulation for accurate control of risk demands a fast and sensitive method for comprehensive screening and confirmation for potential allergens in foods. In this work, an optimized and sensitive LC-MS/MS method for multiplex detection of eight allergenic ingredients in chocolate matrix is proposed. Improved throughput and enhanced sensitivity were obtained by introduction of a rapid solid-phase extraction step using MonoSpin PBA spin column. The most intense and reliable peptide markers were first identified by untargeted survey experiment using quadrupole-orbitrap high resolution mass spectrometer, and subsequently employed to design a multi-target MRM method, based on the most intense transitions recorded for each selected precursor peptide. The MRM quantitative analysis was performed on triple quadrupole mass spectrometer with optimized sensitivity and reproducibility. Protein sequence coverage of 70.8–93.3% were obtained for confirmation of each allergenic ingredient and two or three most sensitive peptide markers were selected in order to retrieve quantitative information. Limits of quantification shown as 0.2–0.4 μg/g for milk, 1.0–4 μg/g for soy, 2.5–4 μg/g for peanut, 1–3 μg/g for tree nuts respectively as well as recoveries ranged from 60.1(±2.7)% to 92.4(±5.6)% for chocolate samples proved the excellent capabilities of the exploited sample treatment method combined with the LC-MS/MS analysis. Finally, this method was successfully applied to the investigation of multiple allergens in commercially available chocolates of different brands aiming to ascertain possible discrepancies between allergen content and food allergen labelling.

1. Introduction

Food allergy is an IgE mediated adverse reaction to certain food proteins which were also known as “food allergens”. It has emerged as a global health issue over the past few decades affecting up to 5% of the adult population and 8% of young children (Sanchiz et al., 2017). Typical allergic symptoms include urticaria, vomiting, asthma, and life-threatening anaphylaxis (Ciardiello et al., 2013). So far, there is no treatment available. Avoiding the offending food is the best choice for these allergic consumers. Legislation on food labeling has been put in place to protect the increasing numbers of food allergic individuals. In the European Union Directive 2007/68/EC regulates the mandatory labeling of a total of 14 allergenic ingredients that, whenever used, must be declared on the respective food label (European Commission, 2007). The US Food Labeling and Consumer Protection Act (FALCPA) identified eight foods or groups of foods that were considered to be “priority allergens” and required the label of these allergens (Food Allergen Labeling and Consumer Protection, 2004). Though different countries mandate a different selection of allergens for food labeling, eight major food allergen groups including milk, soy, egg, peanut, tree nuts, wheat, fish, and shellfish are often on the list (Ciardiello et al., 2013;

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Koeberl, Clarke, & Lopata, 2014). Despite these regulations, total avoidance might be difficult for the allergic consumer. Apart from the intentional use of some allergenic ingredients in food manufacturing without labelling, a risk of an accidental contamination with allergens of processed foods is more likely to occur due to manufacturing on the same production lines. The USA and EU legislations on food labelling only refer to the allergenic foods added as known ingredients and do not prevent the unintentional presence of trace allergens (Careri et al., 2008). Thus, analytical methods capable of simultaneous screening of multiple allergens, especially these so-called"hidden allergens" are needed.

Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are currently the most commonly used techniques for the analysis of food allergens, although both show significant limitations. For ELISA, a lack of reproducibility between assays from different vendors as well as cross-reactivity, leading to false-positive results and/or by unpredictable effects caused by food processing that could mainly generate epitope masking (Lamberti et al., 2016). PCR methods are capable of distinguishing different species by specific DNA markers but provide only an indirect allergen detection (Heick, Fischer, & Pöpping, 2011). In addition, the selective fractionation for proteinaceous material makes PCR nonquantitative and of questionable utility when assessing exposure doses for products (Cho, Nowatzke, Oliver, & Garber, 2015). Mass spectrometry (MS) overcomes both the biggest problems of ELISA and PCR, which is a direct detection method and can detect multiple allergens simultaneously in a single analysis (Monaci & Visconti, 2009; Picariello, Mamone, Addeo, & Ferranti, 2011). In the last years, the number of applications of MS techniques in this field has considerably increased for unambiguous identification and accurate quantification of allergenic proteins and peptides (De Angelis, Pilolli, & Monaci, 2017; Julka et al., 2012; Sealey-Voyksner, Zweigenbaum, & Voyksner, 2016; Wang, Wang, & Cai, 2013). Thanks to their high specificity, sensitivity, and accuracy, MS-based methods have been suggested as confirmatory tools for food allergen analysis (Ji et al., 2017; Koeberl et al., 2014).

Nonetheless, besides the detection method, a crucial target analyte purification step in the pretreatment procedure needs to be considered, especially when analysing complex foodstuffs (Mattarozzi et al., 2014). The food matrix can have a considerable effect on the purification and determination of allergenic food residues as matrix components are prone to form covalent, ionic, and hydrogen bonds or hydrophobic interactions with target proteins (Alves et al., 2017; Khuda, Jackson, Fu, & Williams, 2015). Chocolate is one of the most challenging food matrices. Indeed, chocolate contains a large amount of sugars, tannin and particularly polyphenolic compounds which can react with food proteins, thus masking the target proteins under investigation (Bignardi et al., 2013; Khuda et al., 2015). These are crucial issues, since in some studies on allergen detection in chocolates, protein purification using solid-phase extraction (SPE) and/or ultrafiltration takes up to 60 min or even longer, which greatly reduces the analysis throughput (Planque et al., 2016; Shefcheck, Callahan, & Musser, 2006). Also, it has been reported that trypsin digestion was carried out without any further protein purification, but the method has not been applied to real chocolate samples, which makes the method's validation incomplete (Costa, Ansari, Mafra, Baumgartner, & Oliveira, 2014).

Taking into account the complexity of chocolate matrix, and that milk, soybean, peanut and tree nuts are among the major eight food allergen groups, the aim of the present work was to establish a high sensitive and throughput LC-MS/MS method through the introduction of a rapid solid-phase extraction step for sample clean-up, and demonstrate its application in chocolate samples for multi-allergen determination. Validation of the method was performed in terms of sensitivity, linearity, repeatability and recovery in the chocolate matrices under investigation. The method was then applied for the analysis of eight allergen ingredients (milk, soybean, peanut, hazelnut, walnut, almond, cashew and pistachio) in commercially available chocolates.

2. Materials and methods

2.1. Materials and reagents

All solvents and chemicals used were of reagent grade unless otherwise mentioned. Water was obtained with a MilliQ element A10 System (S. Francisco, CA, USA). Acetonitrile (HPLC purity), formic acid (analytical reagent grade), dithiotheritol (DTT), iodoacetamide (IAA), calcium chloride dehydrate, ammonium bicarbonate, ovalbumin from chicken egg white were purchased from Sigma-Aldrich (St. Louis, USA). Protein LoBind tube (1.5 mL), low adsorption pipette tip (10–100 μL, 100–1000 μL), and acetate cellulose membrane (0.45 μm) were obtained from Eppendorf, Brand and Agela Techologies, respectively. Modified trypsin (sequencing grade) was obtained from Promega (Madison, USA). Milk powder, soybean (Glycine max), roasted peanuts (Arachis hypogea), walnuts (Juglans regia), hazelnuts (Corylus avellana), almonds (Prunus dulcis), cashewnuts (Anacardium occidentale), pistachio (Pistacia vera) and chocolates were obtained at a local food store. Chocolates that did not report the precautionary label "may contain trace of nuts, milk and soy" were used as blank samples.

For the preparation of stock solutions, 1 g of each grounded allergenic commodity was extracted with 20 mL of 40 mM Tris-HCl buffer (pH 8.2) at 60 °C for 3 h (Heick et al., 2011). The resulting extracts were centrifuged, collected and employed as stock solutions for the preparation of standards and artificially contaminated samples. The protein content of the stock solutions was evaluated by colorimetric Bradford assay (Sigma-Aldrich, St. Louis, USA) as described previously (Monaci, Pilolli, Angelis, Godula, & Visconti, 2014), and the described concentrations in the following text were referred to the content of proteins.

2.2. Preparation of blank matrices and spiked matrices

Food allergen free chocolate, containing mainly cacao and sugar were finely ground. Incurred matrices were prepared in three independent replicates by adding raw food allergens to blank matrices to obtain a theoretical allergen protein concentration of 0.2, 0.5, 1, 2, 4, 8, 20, 80, 160, 400 μg/g for milk powder, 0.4, 1, 2, 4, 8, 16, 40, 160, 320, 800 μg/g for peanut, hazelnut, cashew, and pistachio, 0.6, 1.5, 3, 6, 12, 24, 60, 240, 480, 1200 μg/g for soybean, walnut and almond.

2.3. Sample preparation

2.3.1. Extraction and purification

Extraction of the target analytes from 1 g sample was done with 20 mL of 40 mM Tris-HCl buffer, pH 8.2, and left shaking for 3 h at 60 °C. The extract was centrifuged at 4000 rpm for 10 min and the supernatant filtered through 0.45 μm acetate cellulose membranes. Protein purification from the sample extracts was performed by a solid-phase extraction step using MonoSpin PBA spin column (GL Science, Tokyo). Firstly, the column was activated and conditioned with 500 μL H_2O followed by 500 μL 50 mM ammonium acetate solution (The pH value was adjusted to 11 using ammonia water) and centrifuged for 1 min at 10,000×g. Aliquots of 200 μL extract were loaded directly on the column and centrifuged for 1 min. Two