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Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry

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ABSTRACT

There is a raising demand for sensitive and high throughput MS based methods for screening purposes especially tailored to the detection of allergen contaminants in different food commodities. A challenging issue is represented by complex food matrices where the antibody-based kits commercially available might encounter objective limitations consequently to epitope masking phenomena due to a multitude of interfering compounds arising from the matrix. The performance of a method duly optimized for the extraction and simultaneous detection of soy, egg and milk allergens in a cookie food matrix by microHPLC–ESI-MS/MS, is herein reported. Thanks to the innovative configuration and the versatility shown by the dual cell linear ion trap MS used, the most intense and reliable peptide markers were first identified by untargeted survey experiment, and subsequently employed to design an ad hoc multi-target SRM method, based on the most intense transitions recorded for each selected precursor peptide. A sample extraction and purification protocol was optimized also including an additional step based on sonication, which resulted in a considerable improvement in the detection of milk allergen peptides. *Data Dependent*™ Acquisition scheme allowed to fill out a tentative list of potential peptide markers, which were further filtered upon fulfilling specific requirements. A total of eleven peptides were monitored simultaneously for confirmation purposes of each allergenic contaminant and the two most sensitive peptide markers/protein were selected in order to retrieve quantitative information. Relevant LODs were found to range from 0.1 μg/g for milk to 0.3 μg/g for egg and 2 μg/g for soy.

1. Introduction

Food allergy is a public health problem reported to be on the rise especially in industrialized countries and with a high prevalence in childhood [1]. This type of allergy referring to both Ig-E (type I) and non-Ig-E mediated reactions, is triggered by the ingestion of a food or food additive also known as "food allergen" [2]. The lethal risk associated with food allergies and the concern to protect public health have led the regulatory bodies to issue legislation concerning the labeling of the principal food allergens around the globe. Although a large number of foods are considered allergenic, legislation issued in several countries to protect allergic consumers is mainly targeted to a restricted list of food allergens. In the European Union the last 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 [3]. Nevertheless, some ingredients exempted from allergen declaration are also listed in Directive 2007/68/EC. Although threshold data could constitute the basis of regulatory thresholds, to date most regulatory agencies have focused on ingredients that must be reported in the label irrespective of the amounts in the food, the only exception being represented by gluten where limits have been set within EU.

Contamination of food by hidden allergens at the moment represents a major health problem for allergic patients. Allergens are defined hidden when they are not declared on the product label and unexpectedly might reach the end products through several routes. Apart from the intentional use of some allergenic ingredients in food manufacturing, a risk of an accidental contamination of foods by allergens is likely to exist.

Different analytical methods have been developed for monitoring food allergen contamination along the food chain. Methods address either the allergen itself or a marker contained in the allergenic food. To support allergen control within HACCP programs,

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laboratory immunoassays have been proposed due to the ease in use, the relatively high throughputs and the low detection limits [4]. On the other hand, an analysis might be required for confirmation of the results obtained. Therefore, a more direct analytical technique with a high level of specificity is needed. In the last few years mass spectrometry (MS) methods [5,6] have been considered a promising analytical strategy for food allergens monitoring thanks to the advances made in this technology that enables the reduction of the risk of false positives compared to ELISA methods.

As well known, there are a number of limitations presented by the antibody-based methods especially when applied to complex or processed food matrices where allergen detection might be hampered as a consequence of epitope modification or masking effect. Among them also the impossibility to run a multi-allergen analysis. To overcome such limitations, several MS based methods have been developed for multiallergen detection in foods [7–12]. A review of the recent literature yielded only a few reports mainly directed to the multiallergen determination of several classes of food allergens in different food commodities. Among the last papers published on this regard, the method authored by Heick et al. is one of the first MS approach using a triple quadrupole instrument, enabling the simultaneous detection of seven allergens in bread chosen as food model [10]. In their paper the authors reported LODs achieved of 5, 24 and 42 μg/g for milk, soy and egg, respectively, whereas a lower limit was reached for almonds. In another work Bignardi et al. developed a MS-based method for tracing 5 different nuts in fortified cookies with LODs ranging between 10 and 50 mg nut/kg matrix [13]. In the mentioned work a comparison between two different acquisition schemes, either MS^2 or MS^3 mode, was also carried out for detecting these classes of nuts, employing a linear ion trap based system. Other works reported methods mainly tailored to the detection of other allergenic categories including lupins, nuts and gliadin, milk, etc. in different food products with highly variable limits of detection achieved [14–17].

In general, sensitivity of a method strictly depends on different factors including the complexity of the food matrix, the extractability of the targeted proteins from the allergen-containing-food and the detection performance of the instrument used. On this regard, in order to assist the scientific community in properly designing MS-based methods for detection of allergens, some guidelines have been reported [18]. Among the recommendations, a preliminary proper selection of reliable and stable candidate peptide-markers tracing for the allergenic protein must be accomplished and the most reliable transitions effectively designed for each selected peptide. However, the effective validation of methods through the use of naturally incurred reference materials for each food matrix investigated should be accomplished for the harmonization of the final results.

In this work, we focus on three allergenic food categories including egg, milk and soy that might accidentally contaminate cookies chosen as food model. Beyond optimization of the best extraction conditions, different instrumental parameters were carefully investigated and evaluated in the work performed by using a linear ion trap MS instrument based on a differential pressure cell system and able to deliver a rapid and sensitive multi-allergen detection method.

Notably, while the first cell held at higher pressure than in a usual linear ion trap system produced an improvement in the efficiency of trapping, isolating and fragmenting ions of interest reducing the time required for precursor isolation, the second cell held at a lower pressure, instead, allowed for a faster mass analysis with increased resolution. This novel method for controlling the ion population in the trap produced a dramatic increase in the practical scan rate achievable in a typical data dependent tandem MS experiment. The features offered by this system have been in this work exploited to run a survey experiment followed by a multiple selected reaction monitoring (SRM) experiment based on the best transitions selected to set up a sensitive multi-target method.

2. Materials and methods

2.1. Reagents

Acetonitrile (LC–MS grade), formic acid, acetic acid, ammonium bicarbonate, trizma base, Tween 20, hydrochloric acid, iodoacetamide (IAA), dithiothreitol (DTT), egg powder (EP) and skimmed milk powder (MP) were obtained from Sigma–Aldrich (Milan, Italy). Trypsin (proteomic grade) was purchased by Promega (Milan, Italy); Rapigest™ surfactant was purchased by Waters (Milford, MA, USA). Cellulose acetate syringe filters, 0.45 μm (size 30 mm), and polytetrafluoroethylene syringe filters, 0.45 μm (size 4 mm) were purchased by Sartorius (Stedim Biotech GmbH, Goettingen, Germany). Quick Start™ Bradford protein assay was purchased by Bio-Rad (Segrate, MI, Italy). Disposable cartridges PD-10 were purchased from GE Healthcare Life Sciences (Milan, Italy), while ultrafiltration (UF) tubes with 9 kDa cut-off membranes were from Thermo Fisher Scientific (Illkirch Cedex, France). Pre-cooked soy flour (SF) was purchased from a local retailer.

2.2. Stock solutions

Stock solutions of EP and MP were prepared dissolving 1 mg of commercial powder into 1 mL of 50 mM ammonium bicarbonate solution. SF extract was prepared by incubation for 2 h at 60 °C of 1 g of commercial flour with 20 mL of 20 mM Tris–HCl buffer added with 0.01% of Tween 20. The SF extract was collected and employed as stock solution together with EP and MP solutions for the preparation of artificially contaminated cookies. In order to align the concentration values among EP, MP and SF extract, the protein content of the aforementioned three stock solutions was evaluated by colorimetric Quick Start™ Bradford assay. Thus, all concentration reported in the following text were referred to μg of spiked proteins. A mixture of stock solutions EP, MP, and SF extract in 50 mM ammonium bicarbonate solution was employed for the identification of the best peptide markers.

2.3. Bradford assay for protein quantification

Protein quantification of stock solutions and cookie extracts were performed with the commercial Quick Start™ protein assay based on colorimetric Bradford assay. The Bradford assay involves the binding of Comassie Brilliant Blue G-250 dye to proteins. First a calibration curve with BSA (bovine serum albumin), selected as reference protein, was obtained by serial dilution of BSA within the 0.125–1 μg/mL concentration range. 20 μL of each BSA solution were mixed to 1 mL of dye reagent, previously warmed up at room temperature. After 5 min incubation at room temperature, the absorbance at 595 nm was recorded. A standard curve was created by plotting the $Abs_{(595)}$ values versus their concentration in μg/mL. The unknown samples were analogously treated and the relevant concentration determined by extrapolation of the calibration curve. At least three replicates for each sample were analyzed.

2.4. Preparation of spiked samples

Commercial cookies declared egg, soy and milk-free were purchased from a local retailer. Cookies were milled coarsely for 30 s in a blender at 17000 rpm and passed through a sieve of 1 mm (Sterilmixer 12 model 6805-50; PBI International). 1 g of ground allergen free cookie powder was fortified with standard solutions of egg powder, milk powder and SF extract in order to obtain in the final