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Title: Quantitative methods for food allergens, a review.

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Abstract:
The quantitative detection of allergens in the food chain is a strategic health objective as allergy continues to rise. Food allergenicity is caused by proteins either in their native form or in forms resulting from food processing. The progress in mass spectrometry widely opened the field of proteomics. These advances are now available for the detection and the quantification of traces of allergenic proteins in complex mixtures, and complete the set of biological tests used until now, such as ELISA or PCR. The paper will review both families of methods and underline major advances in the mass spectrometric methods.

Keywords: absolute quantification, allergenic protein, cross contamination, ELISA, isotopically labelled peptide, Label free, mass spectrometry, PCR, tagging.

Abbreviations used:
AQUA , *heavy peptides: isotopically labelled peptides used as internal standards for MS*
BHR: *Basophil histamine release*
CID: *Collision induced dissociation*
DBPCFC: *Double-blind placebocontrolled food challenge*
DNA: *Desoxyribonucleic acid*
EAST: *Enzyme-allergosorbent test*
ELISA: *Enzyme-linked immunosorbent assay*
ICAT: *Isotope Coded Affinity Tag*
ICP-MS: *Inductively coupled plasma-mass spectrometry*
Ig: *Immunoglobulin*
ITRAQ: *Isobaric tag for relative and absolute quantitation*
LOD: *Limit of detection*
LOQ: *Limit of quantification*
MRM: *Multiple reaction monitoring*
MS/MS: *tandem mass spectrometry*

PCR: *Polymerase chain reaction*
PVDF: *Polyvinylidene difluoride*
RAST: *Radio-allergosorbent test*
RIE: *Rocket immuno-electrophoresis*
SILAC: *Stable isotope labeling with amino acids in cell culture*

Introduction

The prevalence of food allergy continues to rise, especially in industrialised countries where 2% of the adult population and 5-8% of children are affected [1, 2]. Cows' milk and egg allergies predominate among young children in Europe and in the United States (2,5-3%), whereas the major food allergens come from *Rosaceae* fruits for European adults (0,5%) or from shellfish for American adults (2%) [3, 4, 5, 6]. Despite the importance of food allergies, considered to be the 4th most important public health problem by the World Health Organisation, allergy sufferers have no other possibility of effective treatment than the total avoidance of allergen-containing food [7]. But avoidance is difficult when allergens are ubiquitous food proteins such as egg or milk proteins. In 2003, the European legislation (Directive 2003/89/EC amending Directive 2000/13/EC) established a list of ingredients with potential adverse (allergenic) effects. These ingredients have to be indicated on the label of food products by food producers. This obligation allows the allergic consumers to be warned of the presence of allergens in foodstuffs [7, 8]. Since 2007, 14 substances are to be mentioned on the label if they are present in a food product [9]. The risk of cross-contamination of food products is however still present. Indeed, allergens can be transferred to food that is not supposed to contain allergens, during production (unsuitable cleaning procedures of equipment), storage, shipment or preparation of meals in restaurants. The available detection and quantification methods for food allergens do not allow certifying the absence of cross-contamination. Therefore, food producers use very often a so called "precaution labelling" by mentioning on packaging « *may contain traces of…* » or « *produced in a factory handling…* ».

There is an urgent need to improve the robustness of the available analytical methods and to develop new standardized methods, in order to provide an appropriate tool for food and catering industries, and « allergen-free » foodstuffs for allergic consumers. The new or improved tests must be fast, more sensitive (lower LOD), more accurate (better LOQ), and more specific for a better reliability to discriminate close sequences of allergenic proteins. They should ideally allow unambiguous identification of the allergens

Monaci et al. [10] have described all the aspects of separation and MS-based methods that allow identification and characterization of allergenic food proteins in a recent review. Our review goes beyond the scope of that review and gives a status report of the current methods of quantification of allergens in food products, especially methods using proteomics and mass spectrometry.. The present review is concerned with classical methods in the first part, with only a brief description of the available detection methods for allergens in food because the paper of Poms et *al.* [10] reviews in detail all these methods with their advantages and drawbacks, and with mass spectrometry based methods in the second part. By classical methods, we mean indirect methods measuring allergen coding genes (PCR), antibody/antigen complexes (ELISA), or mediators released by cells (BAT). Mass spectrometric methods allow to both identify and quantify allergens in food independently of the individual sensitivity of each allergic consumer or independently of the use of serum.

1. Classical methods for food allergens detection and quantification

Food allergy is an adverse immune response to an exposition to food allergens through the oral route. The allergic reaction is commonly mediated by key molecules, the allergen-specific immunoglobulins E (IgE), but it also exists a non IgE-mediated mechanism. IgE have the capacity to bind specifically to antigens, to high-affinity FcЄR1 receptors residing on mast cells, basophils and denditric cells or to low-affinity receptors FcЄR2 and CD23 expressed on monocytes and lymphocytes. In IgE-mediated allergy, the crosslinking by an allergen to a receptor-bound IgE triggers an immediate response characterized by the release of various potent cell derived mediators such as histamine, N-acetylhexoaminidase, proteases, leukotrienes or proinflammatory cytokines. A late-phase response follows in few hours, involving eosinophils and T lymphocytes secreting cytokines and interleukines that regulate IgE synthesis and are responsible for the inflammatory response [10, 11, 12, 13].
The diagnosis of food allergy of patients is based on several indirect detection tools using blood serum properties. The blood of allergic patients contains IgE antibodies that specifically recognize and bind to the antigen (allergen), and white blood cells that express active receptors and release mediators when the allergen is present. Therefore, most of the diagnosis tools are based on the immunochemical detection of IgE, receptors or mediators. On the other hand, to prevent contamination of the food chain by allergens, detection methods of allergens in foodstuffs have been developed. The challenge today is the detection and the quantification of trace amounts of allergens in miscellaneous food matrices, which are able to provoke an allergic reaction more or less severe according to the allergen and to the individual. The quantification of allergens in food firstly aims to guarantee with a high confidence level the absence of allergens in food for the allergic consumer. In parallel, the quantitative data obtained on patient serum can bring useful information about the allergenic potential of the food sample and the potential allergic reaction of the patient induced by ingestion of the analyzed foodstuff. In principle, the immunochemical methods used for diagnosis could be applied to the detection and quantification of hidden allergens in food. However, among the range of available methods for that purpose, only ELISA and PCR based tests are currently convenient for routine screening and semi-quantification in catering and food industry, whereas certain others methods are nowadays applicable in research field only.

1.1 Methods for large-scale food allergen quantification in catering and food industry

1.1.1 *ELISA, ELISA-ICP-MS*

Among available immunochemical methods, we can quote first the most commonly used method in laboratories to detect hidden allergens in food, the Enzyme-linked Immunosorbent Assay (ELISA). In an ELISA designed to screen allergenic proteins in food, antibodies mainly come from serum of an immunised animal serum. This serum contains immunoglobulins G able to bind to the allergen used to immunise the

animal. Whereas in tests used for clinical diagnostic, the properties of IgE present in human serum are used. The food extract is analysed in microplate wells. The quantification rests on the measure of the enzymatic activity of a second protein-specific antibody (anti-IgG, e.g. a rabbit anti-human antibody) coupled to an enzyme. This 2nd antibody binds to the allergen-primary antibody complex (Fig. 1).The quantification can also rest on the measure of the primary antibody wearing the enzyme label if any secondary antibody is used as it is the case in the direct ELISA. A reaction with the enzyme substrate produces a coloured product whose absorption is proportional (direct, indirect and sandwich ELISA) or inversely proportional (competitive ELISA) to the quantity of allergen in food sample. A multi-allergen immunoassay built starting from the ELISA model has been developed and allowed the simultaneous determination of at least 1 µg/g protein of each peanut and tree nuts allergens in chocolate, but a limit of quantification has not been established yet [14]. ELISA has recently been combined to Inductively coupled plasma-mass spectrometry (ICP-MS) in order to increase the sensitivity and the precision of the detection of a simple ELISA [15]. In ELISA-ICP-MS the secondary antibody is labelled with a stable isotope instead of an enzyme, which can be used for quantification with a mass spectrometer. Down to 2 µg of peanut allergens per gram of cereal-based matrix have been detected [15].

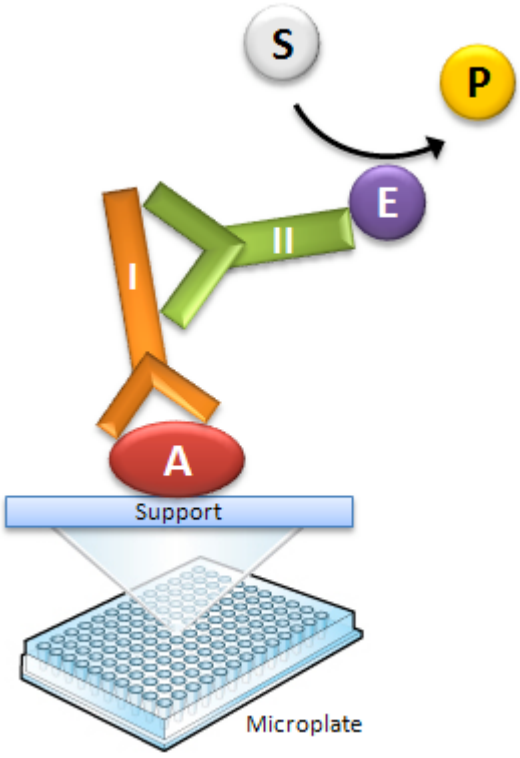

Fig. 1 Generalized ELISA scheme for detecting a target antigen (A = target antigen, I = Primary antibody, II = secondary antibody, E = enzyme linked to the secondary antibody, S = colorless substrate, P = colored product).

1.1.2 *PCR, RT-PCR, PCR-ELISA*

The Polymerase chain reaction (PCR), a tool based on nucleic acids, has been developed for the indirect analysis of allergenic ingredients in food. It consists in targeting a segment of the gene coding for the allergenic protein of interest and amplifying only this DNA fragment to make them detectable. This tool is highly specific and sensitive, showing a LOD <10 mg/kg for almond, hazelnut, soy, milk or peanut [16]. PCR is also available as Polymerase chain reaction coupled to ELISA (PCR-ELISA) and Real-Time Polymerase chain reaction (RT-PCR). In PCR-ELISA, the detection is gel-free since the amplified DNA fragments are hybridized to a protein probe and detected by ELISA. In RT-PCR, the detection is gel-free and performed in real-time, amplification of the PCR product results in the emission of fluorescence proportionally to the amount of the gene of interest in food sample. There is the possibility to perform quantification using a unique internal standard to compensate for the variability in DNA extraction and amplification efficiencies [17].

1.2 Methods for small-scale food allergen quantification in the research field

1.2.1 *Other immunoglobulin-based tests*

Three other immunochemical tests, the Enzyme-allergosorbent test (EAST), the Radio-allergosorbent test (RAST), and the Dot immunoblotting, function with a principle similar to ELISA. The food extract is analysed in microplate wells (RAST, EAST) or spotted on a PVDF, nitrocellulose or polyester cloth membrane (Dot blot). In case of RAST, the secondary antibody is labelled with a radioactive isotope instead of an enzyme, and the quantification is performed with a gamma counter (RAST). In case of EAST and Dot blot, the absorption of the coloured product is proportional (Dot blot) or inversely proportional (EAST) to the quantity of allergen in food sample. At last, RAST and EAST inhibition tests have been applied for the quantitative analysis of hazelnut in food products and milk in baby-food cereal flour with a LOD of 1 µg/g but no LOQ has been determined [18, 19]. A multiplex enzyme immunoassay system consisting in a reverse dot blot has also been developed for the multiple detection of allergens and shows a LOD of 0.1 µg/g for peanut allergens in various food, and for hazelnut and Brazil nut allergens in chocolate ice cream [20].

Two other immunochemical methods exist. Instead of binding allergens with antibodies in a complex matrix sample, the food proteins including allergens are beforehand separated on a 1D gel or 2D sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight (1D gel), or to their molecular weight and isoelectric point (2D gel). The immunoblotting is then performed on separated proteins. In the SDS-PAGE immunoblotting, proteins are transferred to a nitrocellulose of PVDF membrane and protein-specific radio- or enzyme-labelled antibodies are added after the blotting. Detected allergens appear like protein bands on 1D gel or like individualised spots on 2D gel. In the Rocket immuno-electrophoresis (RIE), antibodies are beforehand incorporated in the gel, so the antigen-antibody complexes precipitation occurs from the beginning of the migration. Detected allergens appear in the form of a rocket shape. A 1D SDS-PAGE

immunoblot technique using rabbit antisera and chemiluminescent detection has been developed for routine screening of low levels of potentially allergenic hazelnut and almond proteins in chocolate and allows the detection of less than 0.5 µg/g of chocolate [21]. However, gel-procedures are time-consuming and not well fit for the purpose of routine analysis.

1.2.2 *Cell-based methods*

Among others immunochemical methods, the Basophil histamine release assay (BHR) and the β-hexosaminidase release assay are based on the quantification of two mediators released by blood cells from allergic patients named basophils and mast cells respectively following the allergen binding to the cell receptors. The quantity of histamine or β-hexosaminidase is proportional to the concentration of the specific allergen. Several kinds of *in vitro* mediator release assays have been used to test the allergenicity of soybean allergens [22, 23] or to control the standardization of allergen extracts from different manufacturers [24], and show a high sensitivity and reproducibility. The Basophil activation test (BAT), also called flow-cytometric allergen stimulation test (FAST), targets mediators released (e.g., histamine, leukotriene C4, interleukin IL-4 and IL-13) and surface receptors (e.g., CD63, CD203c) appearing on activated basophils coming from allergic patients after allergen exposure. The quantification is performed thanks to dye-labelled antibodies, which bind to active receptors and are detected by flow cytometry [25, 26, 27]. The quantification of the allergen of interest rests on the measured fluorescence. The BAT has been shown to have a better sensitivity and specificity than BHR tests in food allergy diagnosis [28, 29]. Roasted and native hazelnut extracts have been analyzed by BAT in order to prove the reduction of allergenicity after processing of hazelnut ; 8.2 µg/mL of roasted extract are needed to induce 50% of basophils activation against 0.15 µg/mL for non processed extract [30]. Others authors are also developing an *in vitro* BAT to quantify trace amounts of hazelnut and soy allergens in food in the framework of the ALLERRISK project and results are obtained with a high analytical sensitivity (Ebo et *al.*, work in progress, personal communication).

1.3 Limitations of immunochemical methods and nucleic acids based methods

The similarity between all the immunochemical methods is the use of biological sera and the fact that the detection is based on the antigen-antibody recognition. Thus, the quantification depends on the quality of this recognition and might be distorted by several things but mainly by the Ig specificity. The epitope of the allergen involved in the Ig-binding is either linear, or conformational. The linear epitope, also called sequential, is a continuous string of aminoacids and the recognition is specific to the aminoacid sequence (primary structure). The conformational epitope can be a continuous or discontinuous string of aminoacids and the recognition depends on the three-dimensional shape of the protein (tertiary structure). As antibodies do not recognize the whole molecule but only epitopes, the specificity of an antibody depends on the uniqueness of the epitope. A lack of specificity leads to false positives and negatives due to cross-reaction between closely related proteins.

Moreover, the natural presence of IgG is susceptible to compete with IgE for the binding to the same allergen [31]. On the other hand, the variability of human or animal sera means a variability of IgE and IgG between individuals which limits the validity of results for others patients.

In case of cell-based tests, *in vitro* activated-basophils also suffer from the use of human or animal cells that implies a broad variability in basophil activity between the different basophil donors and an extremely heterogeneous response between individuals [32]. Moreover, basophils *in vitro* activation relies upon the use of natural allergen extracts which might be heterogeneous with varying composition [27]. Despite these pitfalls, BAT offers potentials and perspectives in quantifying allergens in food and in assessing the allergenic potency of a food extract. However it is important to keep in mind that a large scale application of BAT could be limited due to the need of a sizeable quantity of human cells. It is not possible today to collect and store cells enough in order to constitute a collection representative of a population of allergic patients. BAT should be used in complement of classical immunochemical tests such as ELISA and PCR.

PCR and RT-PCR methods are not based on the use of serum or cells but the quantification remains indirect and semi-quantitative like immunochemical methods. The presence of the target in food, a DNA fragment corresponding to the gene of a protein (the allergenic protein or a protein specific to the source species), does not necessarily prove the presence of the allergen itself but indicates the source species in case of contamination. PCR methods are suitable to know the origin (taxonomy) of the contaminating species.

Two additional phenomena are the adsorption of allergens on solid matrices such as cellulose or nitrocellulose, and the food processing, which may destroy epitopes by altering their three-dimensional structure or modifying their accessibility [24]. In case of PCR, food processing and biological variability differently affect a nucleic acid than a protein marker.

In summary, despite the great diversity of Ig-, cell- and DNA-based methods, the quantification is indirect because it does not target the food allergen itself.

1.4 Threshold issue and perspectives in routine analysis

A pivotal issue in food allergen quantification is the impossibility to define a useful threshold (a limit below which a stimulus causes no reaction) and valuable limits of quantification. The sensitivity of a patient to a given allergen varies from a patient to another and over the years. Accordingly, it is difficult to define threshold doses for allergenic foods. Some authors tried to established threshold values for some ingredients, using published data from low-dose challenges from the clinical literature and assessing them statistically [33]. Defined threshold values that would protect 99% of allergic individuals were 8.6 mg (milk), 3.4 mg (egg), 1.2 mg (peanut) and 2.2 mg (soybean). However, this approach is complicated by the uncertainties associated with failure to identify a NOAEL in most existing observations, the effects of differences in the protocols, and other factors. Moreover, no international agreement has been reached on an acceptable level of risk for allergic individuals. Thus, a

consensus protocol based on low-dose DBPCFC has been proposed in order to standardize data that would improve these estimates above [34, 35]. For the moment, without well-defined thresholds, the quantification methods must be as sensitive, accurate and reliable as possible. This demand level can be achieved by targeting directly the allergen rather than a marker of the presence of the allergen. Although the colorimetric-based enzyme-linked immuno-sorbent assay (ELISA) is presently used as the official screening method of food samples for allergen detection, several problems such as selectivity, accuracy and cross-reactivity lead to severe limitations in the applicability of this screening technique. The robustness of the commercially available immunochemical methods must be improved to cope with the problem of the high variability among allergens, and to guarantee safe food for the consumer. Immunochemical methods applied today in the research field might be applied in the future for routine analysis. Current ELISA and PCR screening methods should be confirmed by more reliable methods of molecular identification and quantification of allergens. Such confirmatory methods have necessarily to be based on mass spectrometry.

2: Mass spectrometric methods for quantification of food allergens

Mass spectrometry has long been used for the study of proteins. The first experiments were designed for their identification and are now routinely used in high throughput proteomics. Hyphenated methods coupling separation techniques and mass spectrometry allow to identify and quantify allergens on a direct and absolute way. The development of such quantification methods for food allergens in trace amounts will improve the safety of the food chain. Food products could be certified « allergen-free » and be consumed in total safety. The quantification is independent of the allergic sensitivity of patients. Finally, for protein allergens, mass spectrometric methods can be performed at the peptide scale making the quantification independent of the three-dimensional structure of the allergen and the marker peptide chosen can still be valuable after food processing.

Simultaneous quantification and identification rapidly appeared to be a priority issue. In the last few years, routine analytical methods used for small molecules were adapted for protein quantification. These methods are based on the principle of external or better, internal standards (IS), consisting in the comparison of mass spectrometry signal intensities of the analytes to those of references. The standard should have similar physicochemical properties to those of the analyte. With external calibration, the standard can be the analyte itself, thus avoiding problems with response factors. Nevertheless this advantage is minimal compared to the qualities of the internal standard. The best internal standard is an isotopically labelled version of the analyte, as it will have similar extraction recovery, chromatographic elution, ionization ionisation response, and spectral similarity. In practice, the internal standard is added in a constant known amount to samples (blank, analyte, calibration standard). It can be used for calibration by plotting the ratios of signals for the analyte and the internal standard as a function of the analyte concentration. Two approaches of this global concept are available, the first one where the analyte and therefore the standard, is the intact protein and the second one, where the analyte is a peptide resulting from the protein digestion by proteolytic enzymes, such as trypsine. Selection of the analyte is based on experiments like those described in the review of Monaci et al. [10]. Methods proposed in their paper enable the characterization of the proteins that will subsequently be quantified, taking into account, for example, the presence of isoforms or protein modifications.

2.1 Quantification at the protein level

The analyte is the protein itself, so no modification of the protein during the quantification process is involved. Spraying directly intact proteins from solutions using electrospray yields MS spectra consisting in a series of peaks corresponding to charge state distributions of the protein. This technique however presents strong limitations. The identification of targeted proteins in complex mixtures is hindered by two factors. The first one is the ion suppression that appears when different proteins elute at the same time. The second is the superposition of numerous peaks in the

mass spectra, corresponding to different proteins that may not be resolved even using deconvolution algorithms.

Unlike for serum where there is very large number of different proteins and where there is a very large dynamic range of concentrations, food matrices are not too complex allowing conducting different studies, all aiming to the quantification of proteins. Milk allergy is one of the most known and is triggered by milk proteins. There are two groups of milk proteins, made up of 80 % caseins, and 20 % whey proteins. The latter includes α-lactalbumin (α-LA), β-lactoglobulins (β-LG A and B), bovine serum albumin and immunoglobulins. Huber et al. did the first quantitative experiments on all the whey proteins [36]. Selected ion monitoring was used to follow the most abundant ions. An external calibration curve (0.01-1 mg/ml) allowed the concentrations of the three proteins to be determined in a commercial whey drink. The measured concentrations were 0.684, 1.839 and 1.599 mg/ml) for α-lactalbumin, β-lactoglobulins B and A, respectively. Czerwenka et al introduces the concept of internal standards for the quantification of the β-lactoglobulin in different cows' milk products [37]. After sample preparation (lipid removal and casein precipitation), proteins were separated by liquid chromatography using a C_8 column. The mass spectrometer was in full scan mode in order to acquire the entire charge state distributions of the proteins. Quantification was done after deconvolution. Two internal standards, species variants of bovine β-LG, were used, one to determine the recovery, the other for MS quantification. Calibration curves were constructed (without matrix) and displayed good linearity over a range of 25-1000 µg/ml for bovine β-LG and 12.5-500 µg/ml for caprine β-LG (IS for recovery) proteins. A good correlation was found between bovine β-LG concentration in the analyzed whole milk (3.25 ± 0.15 g/l) and previous literature reports. Recovery rates ranged from 107.2% for whole milk to just 53.5% for processed milk products. The influence of processing was investigated, showing an increasing loss of β-LG with increasing heat treatment.

Monaci et al. developed a method using solid-phase extraction to detect traces of these three allergenic cows' milk proteins in mixed-fruit juice samples [38]. Proteins were separated by liquid chromatography using a C_5 column. Two different acquisition modes were used and compared: full scan and multiple ion monitoring modes. For this last one, most abundant specific masses, corresponding to different protonated states of the same protein, were recorded for each protein. This mode allowed the selectivity of the method to be increased when more complex matrices were analyzed. External standards were already used but this time with matrix-matched calibration curve. Their method was linear in a range of 5-40 µg/ml and the limits of detection (LOD) and quantification (LOQ) were estimated at 1 and 4 µg/ml respectively.

Although good results are obtained by this method, fragments or derived peptides that may still have immunological activity are not included. The second approach could solve this problem.

The classical DIGE technique can also be used. It allows multiple samples to be co-separated and visualized on one single 2-D gel through the use of multiple fluorescent dyes to label intact proteins prior to 2-D PAGE. Relative quantification can be performed followed by PMF

(peptide mass fingerprinting) and MS/MS are subsequently used to identify the proteins extracted from the gel. Hobson et al. used DIGE to identify protein biomarkers of food allergy in mice exposed to ovomucoid (OVM), a major food allergen found in chicken egg white [44]. Alm et al. used DIGE to determine the proteomic variation within and between different strawberry varieties, in order to breed a red strawberry with low amount of allergen [45].

2.2 Quantification at the peptide level

Quantification at the peptide level can be classified in methods involving stable isotopes: tagging by light (^{12}C) and heavy (^{13}C labelled) tags and using isotopically labelled synthetic peptide to achieve respectively relative or absolute quantification. More recently the so-called label free quantitative method has been introduced based on signal intensity. The final analyte is the peptide; therefore all of these methods have to achieve a digestion step in order to obtain the peptides to be analyzed. In addition, the sequence of the peptides must be determined to insure identification. Tandem MS is mandatory.

2.2.1 *Tagging methods*

Many strategies have been developed during the last decade to label proteins or peptides with stable isotopes. These methods are mainly used for relative quantification purposes; however, most can also be used for absolute quantification as well. All of these strategies incorporate isotopically labelled chemical moieties into the samples. They are useful in order to find biomarkers, in order to detect changes in protein abundances, for example, before and after the roasting of peanuts. They can be classified into: metabolic labelling (SILAC); chemical labelling (ICAT, ICPL, iTRAQ,…) and enzymatic labelling ($H_2{}^{16}O$, $H_2{}^{18}O$). Ong et al. introduced the SILAC method in 2002 [39]. Two cell populations are generally studied. All the proteins in each cell population are metabolically labelled with a light or heavy, non-radioactive isotope form of an essential amino acid. For ICAT and ICPL, the tagging reaction occurs on the protein level, whereas for iTRAQ, it is the peptides that are labelled. These tags are specifically designed to react chemically with a particular amino acid: cysteine residues in the case of the ICAT reagent [40] or the DIGE dyes, or lysines or N-terminals in the case of iTRAQ and ICPL reagents [41]. iTRAQ was developed by Darryl Pappin and colleagues at Applied Biosystems in 2004 [42]. With iTRAQ, four (or eight) independent reagents of the same mass that, upon fragmentation in MS/MS, give rise to four (or eight) unique reporter ions (m/z =114–117) that are subsequently used to quantify the four (or eight) different samples, respectively. Because this region is free of other common fragment ions, signals found in this region are due only to contributions from the reporter ions from the corresponding labelled sample digests. A patent has been deposed for the analysis of allergens using this technique [43]. Most of these techniques result in the same peptides labelled heavy or light. The same peptide act therefore as an internal standard.

Fensleau's group developed an isotope coding approach that uses 'normal' water (^{16}O) as the solvent for proteolytic digestion of proteins from one cell state, and 'heavy water' (^{18}O) as the solvent for proteolytic digestion of the proteins in the

second cell state. The use of heavy water results in the incorporation of two ^{18}O atoms in the C-terminal carboxy moiety of each proteolytic peptide, giving a 4 Da isotope code [46].

2.2.2 *Isotopically labelled synthetic peptides method*

When the identity of the protein to be quantified is known in advance, this is currently the method of choice. This method uses a reference analyte, which is an isotopically labelled peptide. This reference peptide incorporates ^{13}C and ^{15}N stable isotopes on one of its amino acids leading to a known mass difference with the endogenous peptide. There are three critical steps in the development of this method, each leading to bad results if they are not well evaluated: the selection of the peptide, the design of the mass spectrometry analysis and the digestion step.

The selection of the peptide is obviously a crucial point as this peptide will be the analyte (endogenous or reference) that will be quantified. This peptide must be unique to the protein of interest. If this is not the case, the protein of interest might be overestimated, this can lead to false positives. The selected peptide must also be efficiently liberated by digestion of the protein. This peptide must be stable in solution during the whole process. Some amino acids should therefore be avoided like methionine and cysteine that can be irregularly oxidized. At least the peptide must be well analyzed by the system (liquid chromatography and especially mass spectrometry). It must be what is called a proteotypic peptide. If these three conditions are not fulfilled, the protein concentration would be underestimated and this would give rise to false negative results.

Another major advantage lies in the choice of the reference peptide regarding the issue of modifications induced by industrial processes. Roasting, boiling or different kind of cooking may spoil the quaternary structures of the allergen and prevent antibodies from recognizing conformational epitopes, leading to false negatives. In the AQUA method, the reference peptide can be chosen to be both present in the amino acid sequence of the native allergen and in the amino acid sequence of the processed allergen. It allows to detect the two forms of the allergenic protein and to quantify the entirety of traces of allergen in a processed foodstuff.

The design of the mass spectrometric analysis is also important. Different mass spectrometers can be employed for such analyses but the most dedicated for this kind of analysis is the triple quadrupole running in the multiple reactions monitoring (MRM) mode. The first quadrupole only allows the precursor ions of a selected m/z ratio to pass. These selected precursor ions are fragmented by CID in the second quadrupole. The third quadrupole only transmits the fragmented ions of a selected mass to charge ratio to the detector. This mode increases the selectivity of the analysis. As this spectrometer is a low resolution mass analyzer, more than one MRM transition is required to ensure the specificity of the signal.

The last critical point is the digestion step. With the traditional AQUA concept [47, 48], where the internal standard is the isotope labelled peptide, we deduced the concentration of the protein from the measurement of the concentration of the peptide. To ensure that the molar concentration of both is equal, the digestion must

be complete. It is well known that this is hard to accomplish. To circumvent this problem, different strategies have been employed. Pratt et al. designed artificial QConCat proteins that are concatemers of tryptic peptides for several proteins [49]. Although by this way the internal standard undergoes the digestion step, in reality the sequence is not exactly the same as that in the endogenous proteins. There are still different cleavage kinetics due to the surrounding different amino acids. Another concept has been used in our laboratory [50]. As the studied proteins (IGF-1 and IGFBP-3) were commercially available, calibration curves were built based on digestions carried out on samples of serum fortified with increasing concentrations of the proteins of interest. The amount of standard added was low compared to the total amount of proteins in the samples. Therefore completeness of the digestion should be the same in the calibration standard as in unknown samples. Synthetic isotopically labelled peptides were only used to correct the mass spectrometry signal. Brun et al. developed the PSAQ concept, protein standard for absolute quantification [51]. The internal standard is the labelled protein, enabling all the systematic variations due to the sample sample process to be taken into account.

Many studies have used these different concepts with success.

In the field of food allergens, few developments of this method were investigated and only for two types of allergens, casein and peanut proteins.

As previously said, casein is the most abundant milk protein. Weber et al. investigated the applicability to detect this protein in cookies [52]. The mass spectrometer was operated using data-directed analysis. Using the reconstructed ion chromatograms of two peptides, it was possible to detect 1.25 ppm in the spiked food. Comparisons with ELISA results were done on 27 samples (positive and negative) and good agreements were obtained.

There are three major peanut proteins that cause allergic reactions, Ara h1, Ara h2 and Ara h3/4. As Ara h1 accounts for 20 % of the total proteins and is therefore the major protein. It was the first that was studied by Shefcheck et al. In a preliminary study, data-dependent MS/MS was used to determine specific Ara h1 peptides [53]. Selected ion chromatograms of a product ion from MS/MS scan obtained from each four peptides allowed the detection of Ara h1 in vanilla ice cream at a value of 10 ppm. The method was improved by using MRM [54]; three product ions were monitored for each selected parent mass. The two targeted peptides were different from those followed in the first experiment. Peptide selection was done based on the signal intensity, retention time position, deficiency of missed cleavages and overlap with immunologically active epitope. Optimisation of the sample preparation permitted to reach a LOD of 2 ppm in dark chocolate. As the author said, the perspectives were to develop an appropriate internal standard. The group of Chassaigne undertook a big study to determine the best peptides that can serve as markers for the detection of Ara h1, Ara h2 and Ara h3/4 [55]. Multiple ion monitoring was used and identity was verified by MS/MS. Peptide selection included among others overlap with epitopes and stability during the heat process of peanuts. Careri et al. introduces the concept of internal standard for the quantification of Ara h2 and Ara h3/4 in chocolate rice crispy-based snacks [56]. The main selection criterion of the peptides was the

presence in the different isoforms. Two peptides were selected for each protein. Compared to the previous study of Shefcheck, only one peptide is in common. Peptides for Ara h2 didn't overlap immunologically active epitope. Multiple reaction monitoring was achieved with one transition for each peptide, so 4 transitions in total. LOD and LOQ were 5 and 14 µg protein g^{-1} matrix for Ara h2 and better results were obtained for Ara h3/4 with the LOD and LOQ at 1 and 3.7 µg protein g^{-1} matrix, respectively. The internal standard chosen for this study was leucine-enkephalin, a five aminoacids peptide (YGGFL). As said by the authors, this internal standard did not completely overcome the matrix suppression effect. In order to improve their method, a completely different sample treatment was developed [57]. An immunomagnetic bead-based method was used to extract Ara h3/4 from breakfast cereals. The type of analyser used was changed for an IT mass analyser in order to allow simultaneous acquisition in product ion and MRM mode, permitting therefore the unambiguous identification of the peptides. All the modifications of the sample treatment allowed an LOD and an LOQ of 3 and 10 µg peanuts g^{-1} matrix to be obtained, respectively. This seems to be higher than in the previous article, however, here the values include the extraction yield. Commercial samples were analysed and results were consistent with those obtained by ELISA.

Shefcheck's and Careri's groups compare two different sample preparations. Both studies were somewhat similar, however, the results obtained from each respective study were diverging. Careri's group performed the protein extraction step before the digestion, whereas Shefcheck's group preferred the digestion step before the extraction. Both groups based their conclusions on the results from the experiments, however, the results from Shefcheck's group were also in agreement with the fact that there are strong interactions between proteins and the tannin from the chocolate. This shows that the sample preparation is important in order to obtain good performances from the method in terms of LOQ and LOD.

2.2.3 *The label free method*

A more recent method is the label free approach. It was designed to simplify the experimental procedure and avoid the use of stable isotopes. The quantification relies either on the measurement of the so-called spectral counting [57, 58] or on the ion signal intensity. In spectral counting the intensity is estimated through the number of times an MS/MS transition of a peptide belonging to the quantified protein is chosen. Even if this relation may be questioned, it has a link with the chromatographic peak intensity and thus to the protein's abundance.

The signal intensity can also be used as in classical analytical methods. As the response of the mass spectrometer is not considered constant, an internal standard is used, based on a known amount of an external proteins mixture [59].

Very recently, in order to overcome the difficulties encountered with external standards, the use of proteins of constant quantity in the mixtures has been proposed as pseudo internal standard [60]. This allows finding "standard peptides" at retention times close to that of the "analytes" peptides.

In both cases, the method is very demanding in terms of retention time quality and mass spectrometric duty cycle but the new generation of instruments and the availability of adapted software make those methods attractive at least for semi-quantification, in view of the experimental simplification they bring. The bioactives proteins in lupin were analysed recently using a label free method [61].

Conclusion

Allergens detection can be direct or indirect. For diagnosis, a large panel of well-established indirect methods exists. For the direct quantitative detection of the presence of allergens in the food chain, the amplification of the markers of the allergen contact by the patient has not taken place and the levels may be very low. Mass spectrometric methods will certainly help by a major contribution: the simultaneous identification of allergens and their quantification. Once identified, the allergens are best quantified by absolute methods taking benefit from the use of stable isotope standards. Screening tests using label free methods will certainly play a part in the overall strategy provided the peptides from allergens are univocally identified, mostly when processed foodstuffs are analysed.

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