

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 the prevalence of allergy continues to rise. Food allergenicity is caused by proteins either in their native form or in forms resulting from food processing. Progress in mass spectrometry greatly opened up 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. We review methods classified according to their ability to simultaneously quantify and identify allergenic proteins 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

BAT basophil activation test
BHR basophil histamine release
DIGE difference gel electrophoresis
EAST enzyme-allergosorbent test

ELISA enzyme-linked immunosorbent assay
ICAT isotope-coded affinity tag
ICPL isotope-coded protein labelling
ICP-MS inductively coupled plasma mass spectrometry
IgE immunoglobulin E
IgG immunoglobulin G
iTRAQ isobaric tag for relative and absolute quantification
LOD limit of detection
LOQ limit of quantification
MRM multiple reaction monitoring
MS mass spectrometry
MS/MS tandem mass spectrometry
PAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
RAST radio-allergosorbent test
SDS sodium dodecyl sulphate
SILAC stable isotope labelling with amino acids in cell culture

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Introduction

The prevalence of food allergy continuing to rise, especially in industrialized 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 USA (2.5–3%), whereas the major food allergens come from *Rosaceae* fruits for European adults (0.5%) or from shellfish for American adults (2%) [3–6]. Despite the importance of food allergies, considered to be the fourth most important

public health problem by the World Health Organization, allergy sufferers have no possibility of effective treatment other 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, 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 allergic consumers to be warned of the presence of allergens in foodstuffs [8, 9]. Since 2007, 14 substances have had to be mentioned on the label if they are present in a food product [10]. Moreover, cross-contamination of food products can occur. Allergens can be transferred to food that is not supposed to contain allergens, during production (unsuitable cleaning procedures for equipment), storage, shipment or later processing. Therefore, food producers very often use a so-called precaution labelling by mentioning on the packaging “*may contain traces of...*” or “*produced in a factory handling...*”.

There is urgent need to improve the robustness of the available analytical methods and to develop new standardized methods, 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 limit of detection, LOD), more accurate (better limit of quantification, LOQ) and more specific for better reliability to discriminate between similar sequences of allergenic proteins. They should ideally allow unambiguous identification of the allergens.

Monaci and Visconti [11] described all the aspects of separation and mass spectrometry (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 MS. The present review is concerned with classical methods in the first part, with only a brief description of the detection methods available for allergens in food because the paper of Poms et al. [12] reviews in detail all these methods with their advantages and drawbacks, and with MS-based methods in the second part. By classical methods, we mean indirect methods measuring allergen coding genes (polymerase chain reaction, PCR), antibody–antigen complexes (enzyme-linked immunosorbent assay, ELISA) or mediators released by cells (the basophil activation test, BAT). Mass-spectrometric methods allow one to both identify and quantify allergens in food independently of the individual sensitivity of each allergic consumer or independently of the use of serum.

Classical methods for the detection and quantification of food allergens

Food allergy is an adverse immune response to exposure to food allergens through the oral route. The allergic reaction is commonly mediated by key molecules, the allergen-specific immunoglobulins E (IgE), but there also exists a non-IgE-mediated mechanism. IgE have the capacity to bind specifically to antigens, to high-affinity Fc ϵ R1 receptors residing on mast cells, basophils and dendritic cells or to low-affinity Fc ϵ R2 and CD23 receptors expressed on monocytes and lymphocytes. In IgE-mediated allergy, the cross-linking 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 a few hours later, involving eosinophils and T lymphocytes secreting cytokines and interleukins that regulate IgE synthesis and are responsible for the inflammatory response [12–15].

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 for 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, with the severity depending on the allergen and 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 provide useful information about the allergenic potential of the food sample and the potential allergic reaction of the patient induced by ingestion of the foodstuff analysed. 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 quantification in the catering and food industry, whereas certain other methods are nowadays only applicable in the research field.

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

ELISA, ELISA inductively coupled plasma MS

Among the available immunochemical methods, we first mention the most commonly used method in laboratories to detect hidden allergens in food, the ELISA. In an ELISA designed to screen allergenic proteins in food, antibodies mainly come from the serum of an immunized animal. This serum contains immunoglobulin G (IgG), which is able to bind to the allergen used to immunize the animal, whereas in tests used for clinical diagnostics, the properties of IgE present in human serum are used. The food extract is analysed in microplate wells. The principle of the quantification is based on the measurement of the enzymatic activity of a second protein-specific antibody (anti-IgG, e.g. a rabbit anti-human antibody) coupled to an enzyme. This second antibody binds to the allergen–primary antibody complex (Fig. 1). The quantification can also be based on the measurement of the primary antibody bearing the enzyme label if any secondary antibody is used as is the case in the direct ELISA. A reaction with the enzyme substrate produces a coloured product, for which the absorption is proportional (direct, indirect and sandwich

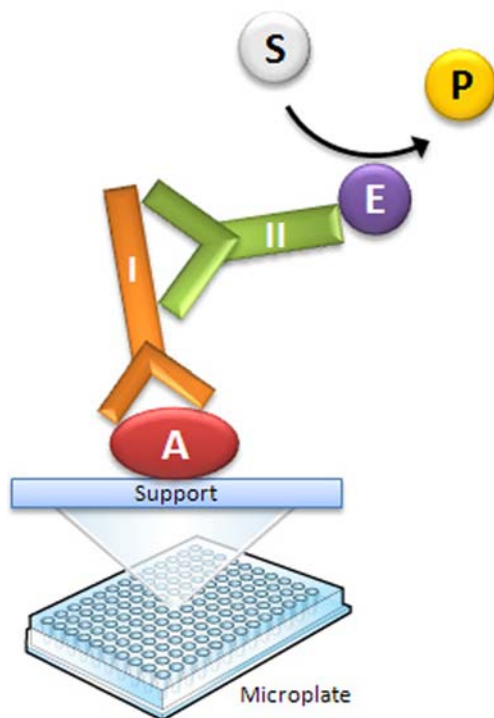


Fig. 1 Generalized enzyme-linked immunosorbent assay scheme for detecting a target antigen. *A* target antigen, *I* primary antibody, *II* secondary antibody, *E* enzyme linked to the secondary antibody, *S* colourless substrate, *P* coloured product

ELISA) or inversely proportional (competitive ELISA) to the quantity of allergen in the food sample. A multiallergen immunoassay built from the ELISA model has been developed and allowed the simultaneous determination of at least 1 µg/g protein of each peanut and tree nut allergen in chocolate, but a LOQ has not been established yet [16]. ELISA has recently been combined with inductively coupled plasma MS (ICP-MS) to increase the sensitivity and the precision of the detection of a simple ELISA [17]. In ELISA-ICP-MS the secondary antibody is labelled with a stable isotope instead of an enzyme, and can be used for quantification with a mass spectrometer. As little as 2 µg of peanut allergens per gram of cereal-based matrix has been detected [17].

PCR, real-time PCR, PCR-ELISA

The PCR, a tool based on nucleic acids, has been developed for the indirect analysis of allergenic ingredients in food. It involves targeting a segment of the gene coding for the allergenic protein of interest and amplifying only this DNA fragment to make the protein detectable. This tool is highly specific and sensitive, having a LOD of less than 10 mg/kg for almond, hazelnut, soy, milk or peanut [18]. PCR is also available as PCR coupled to ELISA and as real-time 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 real-time PCR, the detection is gel-free and is performed in real time; amplification of the PCR product results in fluorescence proportional to the amount of the gene of interest in the 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 [19].

Methods for food allergen quantification in the research field

Other immunoglobulin-based tests

Three other immunochemical tests, the enzyme-allergosorbent test (EAST), the radio-allergosorbent test (RAST), and the dot immunoblotting test, function with a principle similar to that of ELISA. The food extract is analysed in microplate wells (RAST, EAST) or spotted onto a poly(vinylidene difluoride), nitrocellulose or polyester cloth membrane (dot blot). In the case of the RAST, the secondary antibody is labelled with a radioactive isotope instead of an enzyme, and the quantification is performed with a gamma counter. In the case of the EAST and the dot blot, the absorption of the coloured product is proportional (dot blot) or inversely proportional (EAST) to the quantity of allergen in the food sample. RAST and EAST inhibition tests have been

applied to 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 [20, 21]. A multiplex enzyme immunoassay system consisting of a reverse dot blot has also been developed for the multiple detection of allergens and has a LOD of 0.1 µg/g for peanut allergens in various food, and for hazelnut and Brazil nut allergens in chocolate ice cream [22].

Two other immunochemical methods exist. Instead of binding allergens with antibodies in a complex matrix sample, one separates the food proteins including allergens beforehand using 1D gel or 2D sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) according to their molecular weight (1D gel), or according to their molecular weight and isoelectric point (2D gel). Immunoblotting is then performed on separated proteins. In SDS-PAGE immunoblotting, proteins are transferred to a nitrocellulose or poly(vinylidene difluoride) membrane and protein-specific radio- or enzyme-labelled antibodies are added after the blotting. The allergens detected appear like protein bands on a 1D gel or like individualized spots on a 2D gel. In the rocket immunoelectrophoresis, antibodies are incorporated in the gel beforehand, so the antigen–antibody complex precipitation occurs from the beginning of the migration. The allergens detected appear in the form of a rocket shape. A 1D SDS-PAGE immunoblot technique using rabbit antisera and chemiluminescence 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 [23]. However, gel procedures are time consuming and are not very well adapted for routine analyses.

Cell-based methods

Among other immunochemical methods, the basophil histamine release (BHR) assay 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 binding of the allergen 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 [24, 25] or to control the standardization of allergen extracts from different manufacturers [26], and show high sensitivity and reproducibility. The BAT, also called the “flow-cytometric allergen stimulation test”, targets mediators released (e.g. histamine, leukotriene C₄, interleukin-4, interleukin-13) and surface receptors (e.g. CD63, CD203c) appearing on activated basophils coming from allergic patients after

exposure to allergens. The quantification is performed thanks to dye-labelled antibodies, which bind to active receptors and are detected by flow cytometry [27–29]. The principle of the quantification of the allergen of interest is based on the measured fluorescence. The BAT has been shown to have better sensitivity and specificity than BHR tests in food allergy diagnosis [30, 31]. Roasted and native hazelnut extracts have been analysed by the BAT to prove the reduction in allergenicity after processing of hazelnut; 8.2 µg/mL of roasted extract is needed to induce 50% basophil activation against 0.15 µg/mL for nonprocessed extract [32]. Other researchers 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 the results are obtained with high analytical sensitivity (Ebo et al., personal communication).

Limitations of immunochemical methods and nucleic acid 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, quantification depends on the quality of this recognition and might be distorted by several things, but mainly by the immunoglobulin specificity. The epitope of the allergen involved in the immunoglobulin binding is either linear or conformational. The linear epitope, also called “sequential”, is a continuous string of amino acids and the recognition is specific to the amino acid sequence (primary structure). The conformational epitope can be a continuous or discontinuous string of amino acids and the recognition depends on the 3D shape of the protein (tertiary structure). As antibodies only recognize epitopes, rather than the whole molecule, the specificity of an antibody depends on the uniqueness of the epitope. A lack of specificity leads to false positives and negatives owing to cross-reactions between closely related proteins. Moreover, naturally present IgG is likely to compete with IgE for binding to the same allergen [33]. 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 other patients.

In the case of cell-based tests, in vitro activated basophils also suffer from the use of human or animal cells, which implies a broad variability in basophil activity between the different basophil donors and an extremely heterogeneous response between individuals [34]. Moreover, basophil in vitro activation relies upon the use of natural allergen extracts which might be heterogeneous with varying composition [29]. Despite these pitfalls, the BAT has potential and possibilities for quantifying allergens in food and for assessing the allergenic potency of a food

extract. However it is important to keep in mind that a large-scale application of the BAT could be limited owing to the need for a sizeable quantity of human cells. It is not possible today to collect and store enough cells to constitute a collection representative of a population of allergic patients. The BAT should be used as a complement to classical immunochemical tests such as ELISA and PCR.

PCR and real-time PCR methods are not based on the use of serum or cells, but the quantification remains indirect like in the case of 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 the case of contamination. PCR methods are suitable for determining the origin (taxonomy) of the contaminating species.

Two additional phenomena are the adsorption of allergens on solid matrices such as cellulose or nitrocellulose, and food processing, which may destroy epitopes by altering their 3D structure or modifying their accessibility [26]. In the case of PCR, food processing and biological variability differently affect a nucleic acid rather than a protein marker.

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

Threshold issue and prospects 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 LOQs. The sensitivity of a patient to a given allergen varies from one patient to another and over the years. Accordingly, it is difficult to define threshold doses for allergenic foods. Some authors tried to establish threshold values for some ingredients, using published data from low-dose challenges from the clinical literature and assessing them statistically [35]. 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 no observable adverse effect level 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 double-blind placebo-controlled food challenge has been proposed to standardize data that would improve these estimates [36, 37]. 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 ELISA is presently used as the official screening method for 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. The usefulness of 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 MS.

Mass-spectrometric methods for the simultaneous identification and quantification of food allergens

MS has long been used for the study of proteins. The first experiments were designed for their identification and experiments are now routinely used in high-throughput proteomics. Hyphenated methods coupling separation techniques and MS allow the direct and absolute identification and quantification of allergens. 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 3D 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 consisting in the comparison of MS signal intensities of the analytes with those of references. The standard should have physicochemical properties similar 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 with the qualities of the internal standard. The best internal standard is an isotopically labelled version of the analyte, as it will have a similar extraction recovery, chromatographic elution, ionization response and spectrum. 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: in the first one, the analyte, and therefore the standard, is the intact protein; in the second one, the analyte is a peptide resulting from the protein digestion by proteolytic enzymes, such as trypsin. Selection of the analyte is based on experiments like those described in the review of Monaci and Visconti [11]. 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.

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 of a series of peaks corresponding to charge state distributions of the protein. This technique, however, has 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 are eluted 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 different studies to be conducted, all aiming at the quantification of proteins. Milk allergy is one of the best known and is triggered by milk proteins. There are two groups of milk proteins. One group consists of the 80% caseins, and the other of the 20% whey proteins. The latter includes α -lactalbumin, β -lactoglobulins (A and B), bovine serum albumin and immunoglobulins. Huber and Premstaller [38] did the first quantitative experiments on all the whey proteins. 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 concentrations measured were 0.684, 1.839 and 1.599 mg/ml for α -lactalbumin, β -lactoglobulin B and β -lactoglobulin A, respectively. Czerwenka et al [39] introduced the concept of internal standards for the quantification of β -lactoglobulin in different cows' milk products. After sample preparation (lipid removal and casein precipitation), proteins were separated by liquid chromatography using a C₈ column. The mass spectrometer was in full-scan mode to acquire the entire charge state

distributions of the proteins. Quantification was done after deconvolution. Two internal standards, species variants of bovine β -lactoglobulin, were used, one to determine the recovery, the other for MS quantification. Calibration curves were constructed (without the matrix) and displayed good linearity over a range of 25–1,000 μ g/ml for bovine β -lactoglobulin and 12.5–500 μ g/ml for caprine β -lactoglobulin (internal standard for recovery) proteins. A good correlation was found between bovine β -lactoglobulin concentration in the whole milk analysed (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 -lactoglobulin with increasing heat treatment.

Monaci and van Hengel [40] developed a method using solid-phase extraction to detect traces of these three allergenic cows' milk proteins in mixed-fruit juice samples. Proteins were separated by liquid chromatography using a C₅ column. Two different acquisition modes were used and compared: full-scan and multiple ion monitoring modes. For the latter, the 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 analysed. External standards were already used, but this time with a matrix-matched calibration curve. Their method was linear in a range from 5 to 40 μ g/ml and the LOD and the LOQ were estimated at 1 and 4 μ g/ml, respectively.

As a reminder, the classical difference gel electrophoresis (DIGE) technique can also be used. It allows multiple samples to be coseparated and visualized on a single 2D gel through the use of multiple fluorescent dyes to label intact proteins prior to 2D PAGE. Relative quantification can be performed, followed by peptide mass fingerprinting, and tandem MS (MS/MS) is subsequently used to identify the proteins extracted from the gel. Hobson et al. [41] used DIGE to identify protein biomarkers of food allergy in mice exposed to ovomucoid, a major food allergen found in chicken egg white. Alm et al. [42] used DIGE to determine the proteomic variation within and between different strawberry varieties, to breed a red strawberry with a low amount of allergen. Although the quantification of intact proteins gives good results, fragments or derived peptides that may still have immunological activity are not included. The second approach could solve this problem.

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 based on signal intensity was introduced. The final analyte is the peptide; therefore, all of these methods have to achieve a digestion step to obtain the peptides to be analysed. In addition, the sequence of the peptides must be determined to ensure identification. MS/MS is mandatory.

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 to find biomarkers, to detect changes in protein abundances, for example, before and after the roasting of peanuts. They can be classified into metabolic labelling (stable isotope labelling with amino acids in cell culture, SILAC), chemical labelling (isotope-coded affinity tag, ICAT; isotope-coded protein labelling, ICPL; isobaric tag for relative and absolute quantification iTRAQ; etc.) and enzymatic labelling (H_2^{16}O , H_2^{18}O). Ong et al. [43] introduced the SILAC method in 2002. Two cell populations are generally studied. All the proteins in each cell population are metabolically labelled with a light or heavy, nonradioactive 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 [44] or the DIGE dyes, or lysines or N-terminals in the case of iTRAQ and ICPL reagents [45]. iTRAQ was developed by Darryl Pappin and colleagues at Applied Biosystems in 2004 [46]. With iTRAQ, four (or eight) independent reagents of the same mass, upon fragmentation in MS/MS, give rise to four (or eight) unique reporter ions ($m/z = 114\text{--}117$) 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 application has been made for the analysis of allergens using this technique [47]. Most of these techniques result in the same peptides labelled heavy or light. The same peptide acts therefore as an internal standard.

Reynolds and Fensleau [48] have developed an isotope coding approach that uses “normal” water (^{16}O) or “heavy” water (^{18}O) as the solvent for proteolytic digestion of the proteins in two different cell states. 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 mass displacement of 4 Da.

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 evaluated well: the selection of the peptide, the design of the MS analysis and the digestion step.

The first step, the selection of the peptide, is obviously crucial, 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, such as methionine and cysteine, which can be irregularly oxidized. At least the peptide must be analysed well by the system (liquid chromatography and especially MS). It must be what is called a “proteotypic peptide”. If these three conditions are not fulfilled, the protein concentration will be underestimated and this will 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 a 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 present in the amino acid sequence of the processed allergen. It allows the two forms of the allergenic protein to be detected and all traces of allergen in a processed foodstuff to be quantified.

The second step, the design of the MS 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 reaction 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 collision-induced dissociation in the second quadrupole. The third quadrupole only transmits the fragmented ions of a selected m/z ratio to the detector. This mode increases the

selectivity of the analysis. As this spectrometer is a low-resolution mass analyser, 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 [49, 50], 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 concentrations of both are 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 (Fig. 2). Pratt et al. [51] designed artificial QConCat proteins that are concatemers of tryptic peptides for several proteins. Although in this method 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 owing to the

surrounding different amino acids. Another concept has been used in our laboratory [52]. As the proteins (IGF-1 and IGF-3) studied were commercially available, calibration curves were built on the basis of digestions carried out on samples of serum fortified with increasing concentrations of the proteins of interest. The amount of standard added was low compared with 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 MS signal. Brun et al. [53] developed the protein standard for absolute quantification (PSAQ) concept. The internal standard is the labelled protein, enabling all the systematic variations due to the sample process to be taken into account. In the field of food allergens, few developments of this method were investigated and only for two types of allergens, casein and peanut proteins.

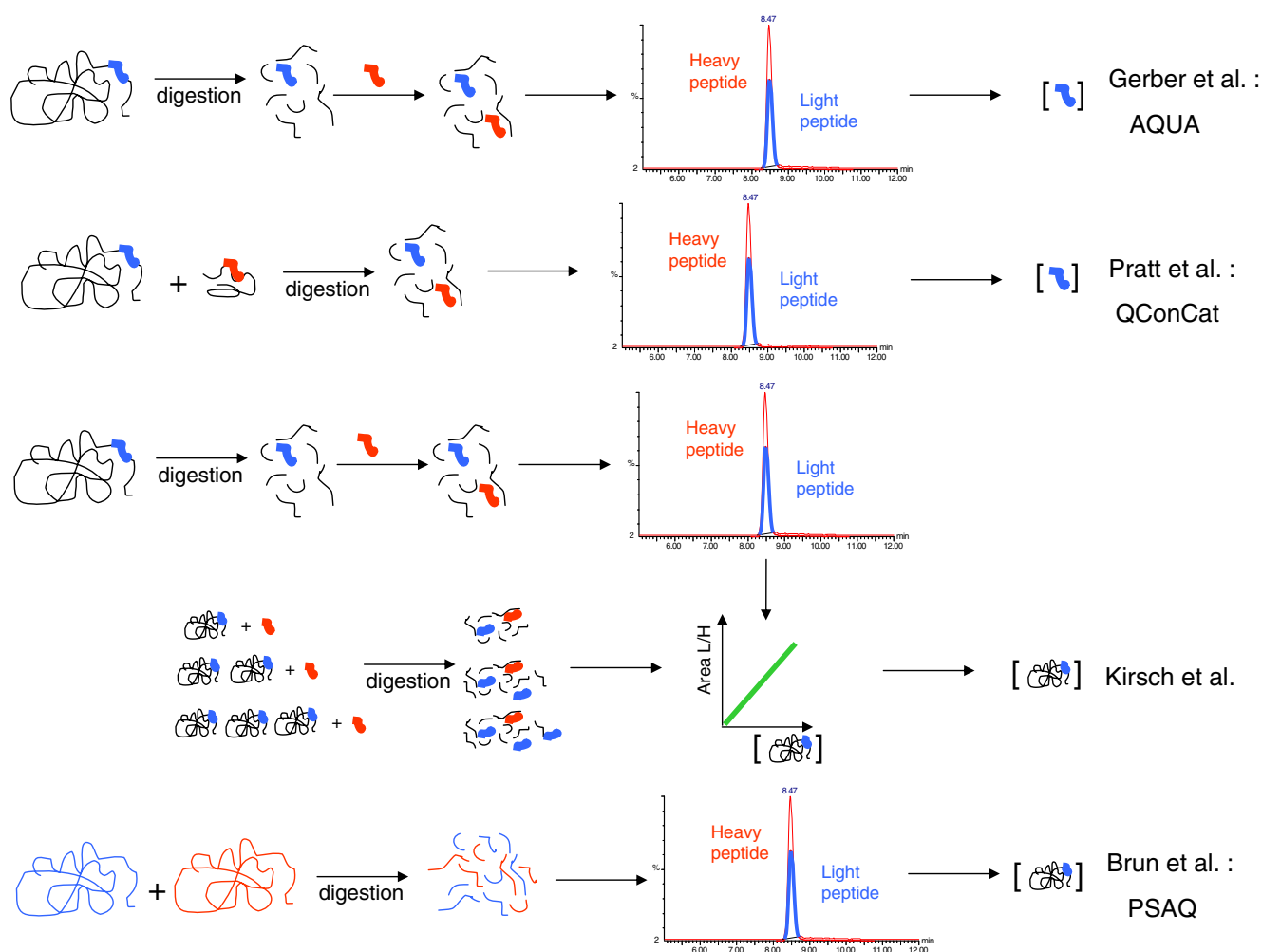


Fig. 2 Workflows of the isotopically labelled synthetic peptides method. Methods proposed by Gerber et al. [49] and also by Pratt et al. [51] allow one to obtain the concentration of the targeted peptide.

As an improvement, methods proposed by Kirsch et al. [52] and also by Brun et al. [53] allow the concentration of the protein to be obtained directly

Many studies have used these different concepts with success.

As previously said, casein is the most abundant milk protein. Weber et al. [54] investigated the applicability to detect this protein in cookies. The mass spectrometer was operated using data-directed analysis. Using the reconstructed ion chromatograms of two peptides, they could 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 [55]. Selected ion chromatograms of a product ion from a MS/MS scan obtained from each of four peptides allowed the detection of Ara h1 in vanilla ice cream at a level of 10 ppm. The method was improved by using MRM [56]; 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 on the basis of the signal intensity, retention time position, deficiency of missed cleavages and overlap with immunologically active epitope. Optimization of the sample preparation permitted a LOD of 2 ppm to be achieved in dark chocolate. As the authors said, the intention was to develop an appropriate internal standard. Chassaigne et al. [57] 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. Multiple ion monitoring was used and the 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. [58] introduced the concept of an internal standard for the quantification of Ara h2 and Ara h3/4 in chocolate rice crispy based snacks. The main selection criterion for the peptides was the presence in the different isoforms. Two peptides were selected for each protein. Compared with the previous study of Shefcheck et al., there was only one peptide in common. Peptides for Ara h2 did not overlap with immunologically active epitopes. MRM was achieved with one transition for each peptide, so there were four transitions in total. The LOD and the LOQ were 5 and 14 μg protein per gram of matrix for Ara h2 and better results were obtained for Ara h3/4, with a LOD and a LOQ of 1 and 3.7 μg protein per gram of matrix, respectively. The internal standard chosen for this study was leucine-enkephalin, a peptide (YGGFL) with five amino acids. As said by the authors, this internal standard did not completely overcome the matrix suppression effect. To improve their method, a completely different sample treatment was developed [59]. An immunomagnetic bead

based method was used to extract Ara h3/4 from breakfast cereals. An ion-trap mass analyser was used 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 a LOD and a LOQ of 3 and 10 μg peanuts g^{-1} matrix to be obtained, respectively. This seems to be higher than reported in the previous article; however, here the values include the extraction yield. Commercial samples were analysed and the results were consistent with those obtained by ELISA.

Shefcheck's and Careri's groups compared two different sample preparations. Both studies were somewhat similar; however, the results obtained from each respective study were different. Careri's group performed the protein extraction step before the digestion, whereas Shefcheck's group performed 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 to obtain good performances from the method in terms of the LOQ and the LOD.

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 [60, 61] or on the ion signal intensity. In spectral counting, the intensity is estimated through the number of times a MS/MS transition of a peptide belonging to the quantified protein is chosen. Even though this relation may be questioned, it has a link with the chromatographic peak intensity and thus with 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 to be constant, an internal standard is used, based on a known amount of an external protein mixture [62].

Very recently, to overcome the difficulties encountered with external standards, the use of proteins of constant quantity in the mixtures has been proposed as a pseudo internal standard [63]. This allows "standard peptides" to be found at retention times close to that of the "analyte" 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 semiquantification, in view of the experimental simpli-

fication they bring. The bioactive proteins in lupin were analysed recently using a label-free method [64].

Conclusion

Allergen detection can be direct or indirect. When dealing with diagnostics, a large panel of well-established indirect methods exist which do not detect the allergen but the reaction of the patients. The amplification of the markers of the allergen to which the patient was exposed does not take place when dealing with the quantitative analysis of the allergens themselves and therefore the protein levels to be detected may be very low. In that perspective, amplification methods such as PCR and enzymatic tests can be very useful once specific reagents are available. Progress in MS largely improved the sensitivity and the specificity of the method. A LOD of 1 µg of protein per gram of matrix has been obtained for a peanut protein with a MS-based method [58]. This value seems to be better than the value of 2 µg/g obtained for the same protein in the same matrix but with ELISA-ICP-MS [17]. MS will certainly make a major contribution through the simultaneous identification of allergens and their quantification. Once identified, the allergens are best quantified by absolute methods benefiting 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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