

Accepted Manuscript

Allergenic and novel food proteins: State of the art and challenges in the allergenicity assessment

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PII: S0924-2244(17)30372-2

DOI: [10.1016/j.tifs.2018.03.007](https://doi.org/10.1016/j.tifs.2018.03.007)

Reference: TIFS 2186

To appear in: *Trends in Food Science & Technology*

Received Date: 15 June 2017

Revised Date: 16 February 2018

Accepted Date: 6 March 2018

Please cite this article as: Pali-Schöll, I., Verhoeckx, K., Mafra, I., Bavaro, S.L., Clare Mills, E.N., Monaci, L., Allergenic and novel food proteins: State of the art and challenges in the allergenicity assessment, *Trends in Food Science & Technology* (2018), doi: 10.1016/j.tifs.2018.03.007.

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Abstract**Background**

There is an increasing demand for animal-derived products in the developing countries. This poses major concerns for the sustainable production of safe and nutritious food. Consequently, to address these needs alternative sustainable sources of valuable dietary proteins are sought for.

Scope and approach

In this review, we discuss novel alternative protein sources for human food consumption such as novel foods derived from other animal sources like insects. Before these novel foods can enter the market place, they should demonstrate their safety for consumers. We herein provide an overview of the legislative framework currently in place across Europe, the key elements required for allergenicity assessment of novel foods, the tools at disposal for allergenicity prediction and the most advanced technologies available for food allergen detection and characterization.

Key finding and conclusions

Effective characterization of potential protein-based allergenic hazards in novel food ingredients is essential to support effective risk assessment. Development of a cost-effective, validated tool box to allow improved hazard characterization for allergenicity risk assessment is needed. Although novel methodologies, such as mass spectrometry, have great potential for allergen characterization and allergen detection in different food contributing to reduce the risk for allergic consumers, some work is still required for method validation and the creation of protein sequence databases for proteomic analysis.

Allergenic and novel food proteins: state of the art and challenges in the allergenicity assessment

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Conflict of interest:

The authors declare that they have no conflict of interest. CM acts as a scientific expert for the UK Food Standards Agency and for the European Food Safety Authority as part of a self-task group of the GMO panel.

Acknowledgements:

The authors are part of the COST Action FA1402 entitled: Improving Allergy Risk Assessment Strategy for New Food Proteins (ImpARAS).

Funding:

During research for this article, partial financial support was obtained by the Austrian Science Fund (grant SFB F4606-B28).

Key words:

Allergenicity assessment of food allergy; novel food allergens; allergen detection; management of food allergy;

Authors' contributions:

IPS and LM set up the outline of the MS; IPS wrote introduction and the part on novel allergen sources; KV wrote the part on current strategies to assess allergenicity of protein containing foods or novel proteins used for food production; CM revised the manuscript and contributed to the sections on application of MS methods and overall conclusions; IM wrote the part of allergen characterization; LM & SB contributed the section on methodologies for allergen detection; SB

47 prepared Figure 1. All authors approved the final version of MS.

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ACCEPTED MANUSCRIPT

Introduction

The growing world population with the increased demand for animal-derived dietary protein and in parallel the food insecurity especially in developing countries present major challenges for the sustainable production of safe and nutritious food. Alternative sources of dietary protein will be required to address the needs, which could range from the engineering of novel proteins to the introduction of novel protein sources to geographic regions where they have not been consumed before. Examples of novel foods include agricultural products from third countries like chia seeds or noni fruit products, as well as ingredients prepared either from natural sources (e.g. rapeseed protein isolate) or synthetic alternatives (e.g. zeaxanthin). It also includes foods and food ingredients from microorganisms, fungi or algae (e.g. docohexanoic-acid-rich microalgae oil and the fungal-protein source Quorn). Such foods can be considered to be novel foods within the European Union (EU), if they have not been consumed to a significant degree by humans in the EU prior to 1997. Further, novel foods can be newly developed foods, or food produced using new technologies and production processes (European Parliament & Council of the European Union, 1997). Before a novel food enter the market place, it must be demonstrated to be safe for the consumers with respect to its microbiological or chemical composition. Further, an allergenicity risk assessment is needed, which addresses the risk of sensitization and risk posed to the existing allergic population (EFSA Panel on Dietetic Products, 2016; van Huis, et al., 2013).

Insects as novel protein sources

A novel source of proteins are edible insects, among them grasshopper, crickets and locusts, larvae from moths and beetles, flies, butterflies and many more (van Huis, et al., 2013). Due to differences in interpretation of the current EU legislation on novel foods (EC Regulation 258/97

(European Parliament & Council of the European Union, 1997) and 1169/2011 (European Parliament & Council of the European Union, 2011) by different member states, insect-derived food products have been placed on the market in Belgium, the UK, the Netherlands, and Denmark. However, the recent novel foods EC Regulation 258/97, which comes into force as of the 1st January 2018, clarifies the status of insects as novel foods across the EU and means that approvals will take place through a centralized procedure. Manufacturers of insect foods, which are already on the market, will need to gain the approvals they require for marketing such products until 2020.

A major concern for all novel foods, and particularly those containing proteins, is their potential to cause immune-mediated adverse reactions, commonly known as food allergy. Case reports of allergic reactions to novel protein sources include the edible algae spirulina in form of a tablet as nutritional supplement, a primary *de novo* sensitization (Le, Knulst, & Rockmann, 2014), and the fungal protein source Quorn (Hoff, Trueb, Ballmer-Weber, Vieths, & Wuethrich, 2003).

Other allergy-eliciting incidences include insects used as animal feed, in a case report these were grasshoppers for a bearded dragon inducing allergic asthmatic reactions in the pet keeper, leading to alerting patients who want to circumvent furred pets by introducing reptiles into their homes (Jensen-Jarolim, Pali-Scholl, Jensen, Robibaro, & Kinaciyan, 2015). There are also numerous instances in fish industry workers and recreational fisherman developing allergies to insects used in fish food or bait (Siracusa, et al., 2003) as well as laboratory workers handling insects such as *Drosophila* (Jones, et al., 2017).

Edible insects might pose the risk of causing cross-allergic reactions in patients allergic to shrimp or house dust mites, who reacted to yellow mealworm or cricket (Broekman, et al., 2016; Srinroch, Srisomsap, Chokchaichamnankit, Punyarit, & Phiriyangkul, 2015). The phylogenetic relationships

between the different classes of arthropods may explain sequence homologies and similarities in structure constituting B cell epitopes in common allergens (pan-allergens), responsible for possible cross-allergy between edible insects and other arthropods, mites (arachnids), crustaceans and non-edible insects (cockroaches) (EFSA Scientific Committee, 2015). Within arthropods, several pan-allergens were already identified, mainly tropomyosins and arginine kinase. Verhoeckx et al. showed that the cross-reactivity of yellow mealworm (*Tenebrio molitor*) with crustaceans and house dust mites was mostly caused by tropomyosins and arginine kinase (Verhoeckx, et al., 2014). Broekman and co-workers even showed that primary sensitization to mealworm in mealworm breeders was possible, leading to a food allergy to mealworm. None of these breeders were allergic to shrimp or any other food, indicating that new allergies may be introduced along with inclusion of novel proteins (Broekman, et al., 2016).

Current strategies to assess allergenicity of novel foods

Expert scientific bodies such as EFSA, *Codex Alimentarius* and FAO/WHO have produced different guidance to assess the allergenicity of proteins such as food enzymes, hydrolyzed infant formula and genetically modified food. In September 2016, a new guidance concerning novel foods was published by EFSA (EFSA Panel on Dietetic Products, 2016), describing amongst other safety issues the procedure how to assess the allergenicity of novel foods. Table 1 shows the key elements required for allergenicity assessment.

All foods containing proteins have the potential to be allergenic. Unfortunately there is no single property recognized that could predict for food allergy. For that reason all guidance recommend a weight of evidence approach (Verhoeckx, et al., 2016). This approach involves various tests to determine the allergenic potential of a protein. The outcome of the assessment is a conclusion

about the likelihood of the protein (source) being an allergen. The approach appeared to be well suited to protect consumers in case of GM crop commercialization. However, the strategy mainly focuses on cross-reactivity, but the new or modified proteins can also pose a risk of *de-novo* sensitization, leading to the development of new food allergies as was seen with mealworm. Moreover, many tests are not formally validated for their predictive ability, and allergenic risks of proteins may therefore be over- or underestimated (Remington, et al., 2017).

To date the focus of allergenicity risk assessment has been on IgE-mediated reactions, although EFSA have recently published a draft guidance relating to the assessment of non-IgE mediated food allergies in relation to GMOs (EFSA Panel on Dietetic Products, 2016)).

The approach adopted by EFSA for allergenicity risk assessment of novel foods is hazard- based and since almost all food allergens are proteins, the allergenicity risk assessment process is focused on the protein component. Unlike other allergenicity risk assessments, a default assumption has been taken for novel foods, that those containing protein have the potential to be allergenic. Applicants wishing to show a food is not allergenic have to provide proof.

Methodologies for food allergen detection and characterization

An important part of novel food allergenicity risk assessment, is the characterization of the protein component. Many novel food ingredients some of which have already been approved contain small amounts of proteins. These include highly refined oils, polysaccharide ingredients and phytonutrients. This has been recognized in the EFSA guidance and hence the protein content of a novel food ingredient needs to be determined, using classical protein determination methods such as Kjeldahl or Dumas methods, or in addition on a case-by-case basis other protein determination

methods, such as the bicinchoninic acid (BCA) or the highly sensitive fluorimetric assay based on 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA), which has been used to determine residual protein in highly refined soybean oils (Rigby, et al., 2011).

Other methodologies have also been indicated to characterize the potential allergenic protein component, including SDS-PAGE, immunological methods and mass spectrometry (MS) (Sancho & Mills, 2010; Monaci & Visconti, 2010). Such tools provide information on the molecular weight and sequence, information useful in assessing the risk posed by a novel protein for the existing allergic population.

In the last decade, liquid chromatography coupled with MS has played a pivotal role in food allergen identification and characterization. Proteomic methods employing MS allowed to investigate protein-protein interactions, yielding precise information on critical epitopes spread along allergens or to detect post-translational modifications (PTMs) induced by food processing and occurring on the protein moiety that might alter the final allergenicity (Nony, Le Mignon, Brier, Martelet, & Moingeon, 2016).

Two types of MS analysis provide data that is of value in the hazard characterization aspects of novel protein allergenicity risk assessment. The first, known as untargeted mass spectrometry, allows profiling of complex mixtures of proteins in food ingredients. Annotation of proteins in such analysis is reliant on having either sequenced genomes or extensive libraries of protein sequences from a given organism. This is an acknowledged short-coming for foods and the need to apply sample preparation procedures that do not rely on trypsin, being especially important for food proteins poor in lysine and arginine residues, such as gluten (Johnson, et al., 2011). Even when many sequences are available for foods such as gluten, they require extensive data curation to make them usable for MS analysis (S. Bromilow, et al., 2017). Nevertheless the technology is being

increasingly used to profile allergens in foods including peanut (Johnson, et al., 2016; Sealey-Voyksner, Zweigenbaum, & Voyksner, 2016) and cereals and cereal-derived foods (Bromilow, et al., 2016; Fallahbaghery, Zou, Byrne, Howitt, & Colgrave, 2017).

It has also been applied in what is termed “targeted” analysis, which can provide absolute quantification of specific allergenic proteins, providing unambiguous identification, characterization, and quantification of allergens in a variety of food matrices including peanut and tree nuts amongst many (Monaci, Pilolli, De Angelis, & Mamone, 2015).

The general workflow required for protein analysis by MS is based on multiple steps (**Fig. 1**), including protein digestion to generate tryptic peptides.

The advantages offered by MS technology are (i) effective protein extraction protocol and (ii) multi-target analysis. Harmonization and validation of MS-based methods for multiple allergen detection is a current priority of international standardization bodies like AOAC.

Application of molecular methods in allergenicity risk assessment of novel foods

Novel methodologies, such as mass spectrometry, have much to offer for the hazard characterization process, but mass spectrometry in particular relies on having access to well curated protein sequence databases in formats suitable for proteomics analysis, which are currently lacking. It is also crucial that new test methodology is subject to multi-centre inter-laboratory trials to demonstrate its robustness and reproducibility. Ensuring that the data generated to support allergenicity risk assessments by regulatory authorities are well founded, will ensure the safety of novel foods and underpins consumer trust in the safety assessment process and acceptance of the novel foods that will be required to feed the world population in the coming decades.

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188 **Concluding remarks**

189 Characterization of potential protein-based allergenic hazards in novel food ingredients is essential
190 to support effective risk assessment. Development of a cost-effective, validated tool box to allow
191 improved hazard characterization for allergenicity risk assessment is needed. Currently, a
192 European network (COST Action) ImpARAS (www.Imparas.eu) is discussing possibilities how to
193 improve the risk assessment, preparing an inventory on methods that could help to predict
194 allergenicity of proteins, and trying to harmonize and validate methods.

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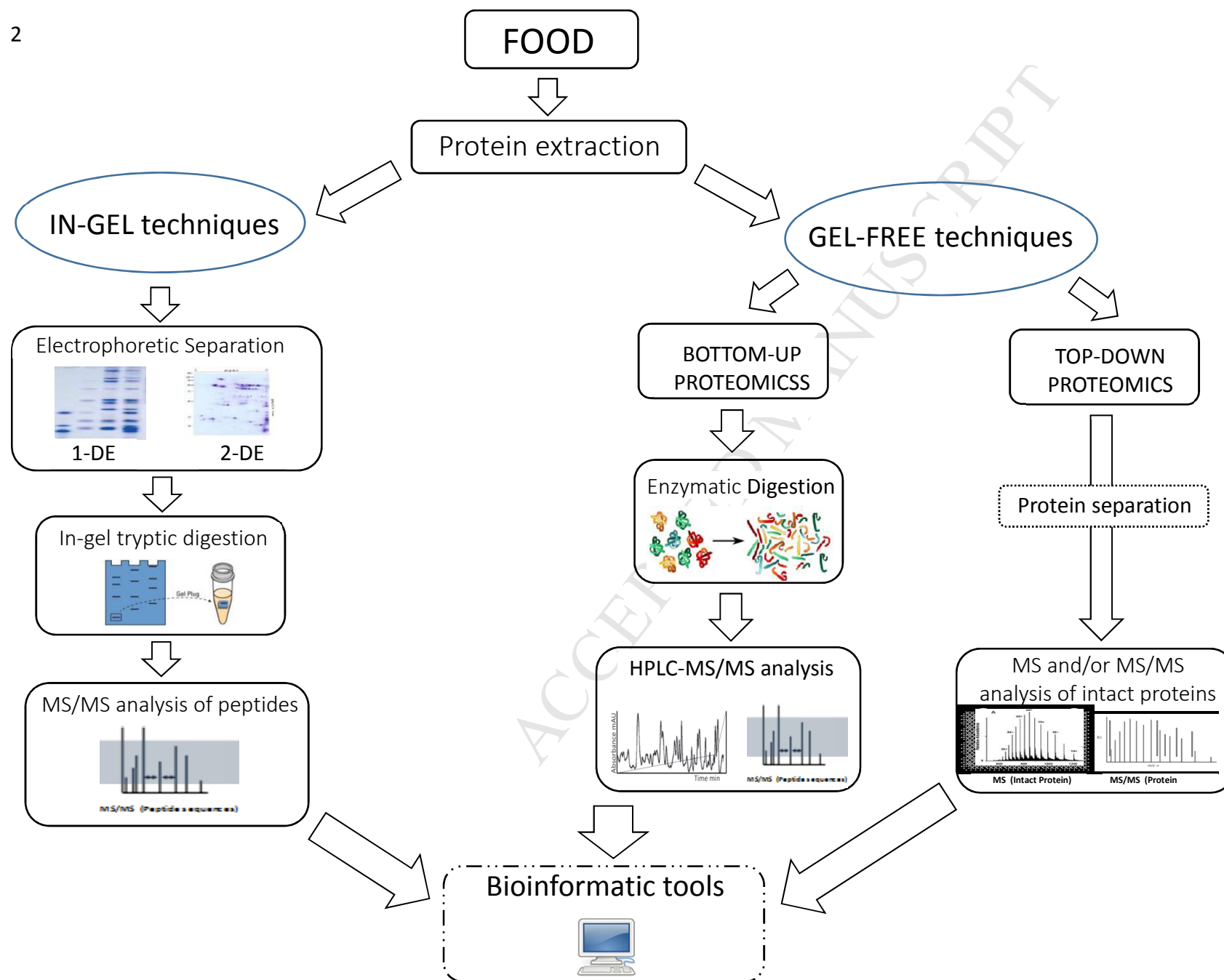
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Table 1: Key elements required for allergenicity assessment of novel foods.

Background	• Comprehensive literature review • Food composition, source (taxonomic relationships), production process, available experimental and human allergenicity data (e.g. case reports, <i>in vitro</i> and <i>in vivo</i> allergenicity studies, cross-reactivity)
Protein analysis	• Protein content (total, specific) • Degree of sequence homology with known allergens • Immunological tests (western blotting) • Molecular weight, stability to heat, pH and digestion by gastrointestinal proteases
Human testing	• Detection of specific IgE • Skin prick tests • Double-blind placebo-controlled food challenge studies

1 Figure 1: General workflow based on Mass Spectrometry for allergen analysis

2



1. Characterization of potential protein-based allergenic hazards in novel food ingredients is essential to support effective risk assessment
2. Development of a cost-effective, validated tool box to improve hazard characterization for allergenicity risk assessment is needed.
3. Effort at EU level are directed to improve the risk assessment, develop methods for allergenicity prediction and method harmonization.