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Draft guidance on allergenicity assessment of genetically modified plants

EFSA Panel on Genetically Modified Organisms (GMO)

Abstract

This document provides supplementary guidance on specific topics for the allergenicity risk assessment of genetically modified plants. The topics addressed are non-IgE-mediated immune adverse reactions to foods, *in vitro* protein digestibility tests and endogenous allergenicity. New scientific and regulatory developments on these three topics are described in this document and the necessity for their implementation in the risk assessment of genetically modified plants is discussed and recommended, when appropriate. This document will undergo a public consultation where stakeholders and general public are encouraged to contribute to enhance its quality and clarity. Following the public consultation process, the document will be revised accordingly.

Keywords: guidance, allergenicity assessment, GMO

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43 **Summary**

44 A summary will be provided after the public consultation.

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1. Introduction

1.1. Background as provided by EFSA

Allergenicity assessment of GM plants is performed following the recommendations laid down in the EFSA Guidance Document (2011). These recommendations are mainly based on considerations from the EFSA (2010) scientific opinion on allergenicity assessment of GM plants and microorganisms.

In 2012, EFSA launched a procurement call entitled: "Literature reviews on: i) non-IgE-mediated adverse reactions to foods, and ii) *in vitro* digestibility tests for allergenicity assessment". The aim of the project was to obtain relevant information related to these two topics to be used as background information for further discussion within the GMO Panel. The review on non-IgE-mediated immune adverse reactions to food identified relevant methodology (i.e. *in silico* and *in vitro*) that could be applied in the allergenicity assessment process (Mills et al., 2013a). The review dealing with *in vitro* digestibility testing for allergenicity assessment highlighted the need for better standardisation and harmonisation of the conditions used (e.g. pHs, ratios enzyme:substrate, controls) when performing *in vitro* digestibility studies (Mills et al., 2013b).

In addition, the new Implementing Regulation IR503/2013[1]) on applications for authorisation of GM food and feed has been in place since December 2013. One of the novelties in this new regulation is the inclusion of certain allergens (as defined in OECD Consensus documents) in the compositional analysis and consequently, the quantitative measurement of individual allergens. The development of supplementary guidelines on this topic would be very useful to assist both applicants as well as risk assessors in the practical implementation of this new requirement.

Therefore, the EFSA GMO Panel is of the opinion that supplementary guidelines on allergenicity assessment are needed to incorporate new developments in the area.

1.2. Terms of reference as provided by EFSA

The tasks of the Working Group of the GMO Panel are i) to develop supplementary guidelines for the allergenicity assessment of GM plants; ii) to participate in a workshop with stakeholders organised by EFSA; iii) to consult the public on the draft Scientific Opinion; and iv) to review and revise the draft Scientific Opinion accordingly.

2. Assessment

Food allergies represent an important public health problem affecting approximately 2-4% of the population. Essentially, the only way to avoid triggering of reactions in already allergic individuals is avoidance of the relevant food(s). A prerequisite for avoidance is knowledge of the allergen content of a food and corresponding labelling. Accordingly, EU regulatory measures are in place to ensure that foods containing common allergenic components are appropriately labelled and that new foods placed on the market are evaluated with regard to allergenicity. This applies to foods falling under the category of 'novel foods' as well as to foods derived from genetically modified organisms (GMOs). Controlling the introduction of new allergenic foods into the food supply has, in addition, the primary prevention goal of reducing the risk of novel allergic sensitisations.

Concerning potential allergenicity of novel proteins in GMOs there is, however, in practice no way to ensure full certainty, as there is no single test or parameter that on its own can provide sufficient evidence to predict allergenicity of a protein or peptide. This is the case because it is only insufficiently understood what causes a protein or peptide to be allergenic, and development of allergic disease additionally depends on other factors like genetic predisposition of the individual or environmental factors. Still, a high degree of confidence in the safety of GMOs can be reached using a weight-of-evidence approach. At any time, this approach must be based on the best and most up-to-date scientific knowledge and methodologies. The field of molecular biology is in rapid development, and

[1] Commission Implementing Regulation (EU) No. 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No. 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No. 641/2004 and (EC) No. 1981/2006. Off. J. Eur. Union L157, 1–48.

regulations and guidance documents need to be updated frequently to take scientific advances on board and to reduce remaining uncertainty after a weight-of-evidence evaluation.

To this end and following the outcome of an EFSA procurement call regarding literature reviews on i) non-IgE-mediated immune adverse reactions to food and ii) *in vitro* digestibility tests for allergenicity assessment of the newly expressed protein, the EFSA GMO Panel identified new scientific information available to be considered further in supplementary guidance documents for allergenicity assessment of GMOs. In addition, the more recent Implementing Regulation 503/2013 on applications for authorisation of GM food and feed incorporated a new element - the mandatory inclusion of certain allergens in the compositional analysis of genetically modified crops, as defined in OECD consensus documents. The development of supplementary guidance documents on this topic was also considered necessary to assist both applicants as well as risk assessors.

On this background, in 2014 a Working Group of the EFSA GMO Panel was established to develop a supplementary guidance document on allergenicity assessment of GMOs focusing on three topics: i) non-IgE-mediated adverse immune reactions to food ii) *in vitro* digestibility testing in the prediction of potential allergenicity of the newly expressed protein, and iii) quantification of endogenous allergens in the recipient plant. A stakeholder meeting to provide early input to the guidance development was held in Brussels in June 2015[2]. To further secure this input, a "Focus group" was established to give feedback to the Working Group during the guidance development[3]. Additional engagement with the stakeholders is foreseen since the aim of this document is to collect comments from the public. Following the public consultation, an EFSA info session to further address those comments is also envisioned. The final adoption of the document is foreseen in spring 2017.

2.1. Non-IgE-mediated immune adverse reactions to foods

Non-IgE-mediated adverse immune reactions to food antigens comprise a large group of diseases, mostly occurring during childhood. Of these, the best characterised diseases include food-protein induced enterocolitis (FPIES), as well as eosinophilic diseases of the gut, where food products play a role in the pathogenesis (eosinophilic oesophagitis, proctocolitis). However, the exact pathogenic mechanisms of these diseases are insufficiently understood, and the diagnosis mostly relies on positive food challenges. Thus, insights about the food components involved on the molecular level and knowledge on clearly recognised immune mechanisms for these diseases are currently lacking.

In contrast, celiac disease (CD) is a well characterised non-IgE mediated adverse immune reaction to food, and the food proteins involved have been described (Koning et al. 2015; van Bergen et al, 2015). The involvement of the immune system through cellular inflammation as well as production of various antibodies, including IgA against tissue transglutaminase, is well established. Gluten has been identified as the environmental trigger initiating the immune reaction. CD diagnosis is based on serological tests, with positive IgA against tissue transglutaminase being the most reliable. In addition, endoscopic findings and histopathological changes in CD have been well defined. Thus, allergenicity assessment of newly expressed proteins with regard to non-IgE mediated immune reactions should focus on CD.

2.1.1. Celiac Disease

CD is a disease of the small intestine characterised by flattening of the intestinal surface, resulting in a variety of clinical symptoms including malabsorption, failure to thrive, diarrhea and stomach ache. The disease is caused by an uncontrolled intestinal CD4+ T cell response to gluten proteins in wheat (*Triticum ssp*) and to the gluten-like hordeins and secalins in barley (*Hordeum vulgare)* and rye (*Secale cereale)* (Green and Cellier 2007). Oat (*Avena sativa)* is generally considered safe for patients (Garsed and Scott 2007), although exceptions have been reported (Lundin et al. 2003). The only available treatment is a lifelong gluten-free diet implying the exclusion of all food products that contain wheat, barley and rye or gluten and gluten-like proteins from these grains. Celiac disease affects approximately 0.5 to 1% of the population in the industrialised countries and on-going investigations indicate that a similar percentage may be affected in the Middle East and India.

[2] http://www.efsa.europa.eu/en/supporting/pub/899e
[3] http://www.efsa.europa.eu/sites/default/files/assets/shp_dg_guidance_document_allergenicity.pdf

Since CD is caused by an immune response to a foreign protein and all symptoms disappear upon withdrawal of gluten from the diet, the condition should not be regarded a true autoimmune disease. Autoantibodies specific for tissue transglutaminase appear to be secondary to the T cell driven immune response to gluten and disappear if gluten is eliminated from the diet (Rossjohn and Koning, 2016). Other conditions linked to wheat or gluten, summarized as "non celiac gluten sensitivity", are not part of this document because there are no known definite underlying pathomechanisms and the connection to gluten is unclear (Aziz et al. 2015).

CD has a strong genetic component. It is associated with particular immune response genes, i.e. of the class II major histocompatibility complex (MHC) molecules, called HLA in humans. Most CD patients express particular HLA-DQ-molecules. HLA-DQ molecules are dimers of an alpha- (DQA1) and a beta- (DQB1) chain. Like all HLA-molecules, HLA-DQ molecules bind short peptides and present these to T cells of the immune system. While T cells ignore HLA-bound peptides derived from harmless ("self") proteins, HLA-bound peptides derived from pathogens are specifically detected and this recognition leads to the generation of a protective T cell response and eradication of the pathogen. The large majority of CD patients express HLA-DQ2.5 (DQA1*05:01, DQB1*02:01) (Sollid et al. 2012) while the remainder are usually HLA-DQ8 positive (DQA1*03, DQB1*03:02). The few patients that express neither DQ2.5 nor DQ8, often express HLA-DQ molecules that contain only one of the DQ2.5-chains e.g., DQ2.2 (DQA1*02:01, DQB1*02:01) or DQ7.5 (DQA1*05, DQB1*03:01) (Karell et al. 2003). In patients, but not in healthy individuals, pro-inflammatory gluten-specific CD4+ T cells are present in the lamina propria of the affected duodenum. Importantly, these CD4+ T cells recognize gluten peptides only when presented by the disease associated HLA-DQ molecules (Lundin et al. 1993, 1994; Tye-Din et al. 2010; Vader et al. 2002b; van de Wal et al. 1998b). In essence, in patients with CD the immune system displays an aberrant response: the harmless gluten proteins in the food are recognised as hazardous, leading to a pro-inflammatory response as long as gluten is consumed. Elimination of gluten from the diet constitutes an effective treatment because the T cell stimulatory gluten peptides are no longer present. Unfortunately, once a gluten-specific T cell response has developed, this results in immunological memory. Therefore, every subsequent exposure to gluten will reactivate the gluten-reactive T cells and consequently lead to inflammation. A lifelong gluten-free diet is thus required.

Gluten is the cohesive mass that remains when starch has been removed from wheat dough (Shewry et al. 1992). Gluten consists of gliadin and glutenin subcomponents. The gliadins are subdivided into α-, γ- and ω-gliadins, and dozens of variants of each type are typically present in a single wheat variety. The glutenins are subdivided into high molecular weight (HMW) and low molecular weight (LMW) subunits. The most commonly used wheat varieties are bread wheats (*Triticum aestivum*), which are hexaploid species, and pasta wheats (*Triticum durum*), which are tetraploid species. Thus, in a single wheat variety up to a hundred different gluten proteins can be found, many of which are highly similar and only differ by a few amino acids from each other.

T cell epitopes derived from the α-, γ- and ω-gliadins as well as from the HMW- and LMW-glutenins have been reported (Arentz-Hansen et al. 2000; Shan et al. 2002; Sjöström et al. 1998; Vader et al. 2002b; van de Wal et al. 1998b, 1999). In addition, T-cell epitopes in both hordeins and secalins have been identified that are highly homologous or even similar to those found in wheat (Tye-Din et al. 2010; Vader et al. 2003a). The gluten-like avenins of oat are more distinct; however, avenin-specific as well as cross-reactive T cell responses have been described (Arentz-Hansen et al. 2004; Vader et al. 2003a).

High affinity binding of peptides to either HLA-DQ2.5 or -DQ8 depends on the presence of one or more negatively charged amino acids. As gluten proteins are virtually devoid of negatively charged amino acids, native gluten-derived peptides bind poorly to these HLA-DQ molecules. Due to the activity of the enzyme tissue transglutaminase 2 (TG2) in the gastrointestinal tract, the required negative charge(s) are introduced when this enzyme converts glutamine residues within gluten peptides into negatively charged glutamic acid (Molberg et al. 1998; Vader et al. 2002a; van de Wal et al. 1998a). These deamidated gluten peptides then bind with increased affinity to HLA-DQ2.5 or -DQ8, and this stronger binding enhances or causes immunogenicity (van de Wal et al. 1998; Arentz-Hansen et al. 2000; Henderson et al. 2007; Kim et al.2004; Moustakas et al. 2000; Quarsten et al. 1999).

The specificity of TG2 for particular target sequences in gluten proteins plays a crucial role in the generation of a relatively large number of gluten-derived peptides that bind to HLA-DQ2.5. Glutamine and proline are abundantly present in gluten proteins, together they comprise over 50% of the amino acids in gluten. Therefore, Q-X-P and Q-P sequences (where Q is glutamine; P is proline; X is any amino acid except P) are often found in gluten proteins and TG2 typically deamidates glutamine residues in Q-X-P sequences, but not in QP sequences (Vader et al. 2002a). Therefore, immunogenic gluten-derived peptides are typically found in the proline rich-regions of gluten proteins and usually contain a Q-X-P motif. "Classic" examples of such peptides are the immunodominant T cell epitopes present in the N-terminal part of the α-gliadins: PFPQPQLPY and PQPQLPYPQ. In fact, these sequences have a 7-amino acid overlap and in both sequences only one Q-residue is a target for TG2, the Q in the QLPY sequence which allows the introduction of a negative charge at either position 4 or position 6, respectively. In both instances this generates a gluten peptide that binds with high affinity to HLA-DQ2.5. The available crystal structures of HLA-DQ2.5-gliadin and the bound cognate T cell receptor demonstrate that the negatively charged glutamic acid serves as an anchor residue for peptide binding to HLA-DQ2.5 and does not contact the T cell receptor (Petersen et al. 2014). Additionally, the proline-rich nature of gluten renders these proteins resistant to degradation by enzymes in the gastrointestinal tract. Relatively long gluten fragments are, therefore, present in the small intestine. This likely contributes to the immunogenic nature of these peptides (Shan et al. 2002). Thus, at least three factors contribute to the immunogenicity of gluten: (a) resistance to proteolytic degradation, (b) specific recognition by TG2, and (c) peptide binding properties of HLA-DQ2.5 and HLA-DQ8.

The glutamic acid introduced by TG2 is usually in position 4 (p4) or p6 in HLA-DQ2.5 restricted epitopes and at position p1 and/or p9 in HLA-DQ8 restricted epitopes (Sollid et al. 2012). As a consequence, HLA-DQ2.5-restricted gluten epitopes carry a proline at either p6 or p8. This positioning of proline residues is less strict in the case of the DQ8 epitopes. In all cases the glutamic acid residues serve as anchors important for binding of the peptides to either HLA-DQ2.5 or -DQ8.

It is important to note that, while polyclonal T-cell responses to multiple T-cell epitopes are usually detected in CD patients, responses to the DQ2.5-glia-α1, DQ2.5-glia-α2 epitopes and homologues thereof in the ω-gliadins, hordeins and secalins are dominant in DQ2.5 positive patients (Arentz-Hansen et al. 2000; Tollefsen et al. 2006; Tye-Din et al. 2010). In DQ8-positive patients, responses to the DQ8-glia-α1 epitope are most frequently found (Tollefsen et al. 2006; van de Wal et al. 1998b).

The following criteria were used to define CD reactive epitopes in Sollid et al. 2012:

- Reactivity against the epitope must have been defined by at least one specific T-cell clone;
- The HLA-restriction element involved must have been unequivocally defined;
- The nine-amino acid core of the epitope must have been defined either by an analysis with truncated peptides and/or HLA-binding with lysine scan of the epitope, or a comparable approach. In a lysine scan all amino acids in the sequence of interest are individually replaced by a lysine and the impact of these single amino acids substitutions on HLA-binding is determined, information which usually reveals which amino acids in the sequence are required for binding to HLA.

Further details are listed in Annex A-1.

2.1.2. Risk assessment considerations

A large number of methods and tests can be used to determine potential detrimental properties of proteins and peptides under assessment for patients with CD, but in practice it will not be necessary to apply this full array to safeguard patients from undesired exposure to potential disease-inducing proteins. Rather, a stepwise approach is proposed in which firstly, *in silico* approaches can be employed, starting with identity searches. This includes database searches with known CD peptide sequences and motif searches to identify potential hazardous sequences. In a second step, *in silico* peptide modelling can be applied. When potentially hazardous sequences which cannot be disregarded by *in silico* testing are identified, more laborious tests such as HLA-DQ-peptide binding assays and/or testing with T cell clones derived from patients with CD (Fig.1, Fig.2) need to be performed to determine the safety profile of the protein/peptide under assessment.

A) *In silico* approaches

As it is well established that members of the prolamins and closely related families of proteins harbour the sequences that cause CD, the first step is to determine if the protein of interest belongs to this class of proteins. If this is the case, a potential hazard has been identified.

Identity search with known CD peptide sequences:

An identity search with known CD peptide sequences should be performed, allowing one or more amino acid mismatches. When this analysis results in hit(s) with only one amino acid mismatch, potential immunogenic sequence(s) are present and additional tests are required (Fig.2). When this analysis results in hit(s) with multiple amino acid mismatches, the position and nature of the mismatched and identical amino acids determine if the peptide sequence has the potential to be an immunogenic epitope. If concerns are raised, additional tests will be required.

Further details are listed in Annex A-1.

Identity search with Q/E-X1-P-X2 motif (Fig.3):

Examination of the list of epitopes currently identified (Annex A-1) reveals that a characteristic Q-X1-P-X2 motif is present in the large majority of HLA-DQ2 epitopes. This is a target for TG2 which yields E-X1-P-X2. Two options are possible:

- If not present, the probability of a T cell epitope is unlikely, given that no concern was raised during the identity search with known CD peptide sequences stated above.
- If present, a potential T cell epitope is detected and further investigation is required (Fig.2).

Further details are listed in Annex A-2.

HLA-DQ-peptide modelling:

Several HLA-DQ2-gliadin and HLA-DQ8-gliadin structures are publicly available. These structures can be used to model a peptide of interest into HLA-DQ2 or HLA-DQ8. This can then allow for a comparison and can indicate the likely position and orientation of the T cell receptor contact residues in the HLA-DQ-bound peptide. If a high degree of similarity is detected, this indicates potential cross-reactivity of the investigated peptide. Consequently, the immunogenicity of the peptide should be determined by additional *in vitro* approach(es) described below.

Further details are listed in Annex A-3.

B*) In vitro* approaches

In vitro protein digestibility:

Due to the proline-rich nature, gluten proteins are highly resistant to proteolytic degradation. This results in relatively long peptides that harbour one or more T cell-stimulatory epitopes. Further details are listed in Annex A-4 and in the chapter on *in vitro* protein digestibility testing (Section 2.2).

HLA-DQ peptide binding assays:

For peptides to evoke T cell responses, they must bind to HLA-molecules. HLA-DQ2- and HLA-DQ8-specific peptide binding assays have been developed and can be exploited to determine the likelihood that peptides under investigation might be immunogenic.

- When high affinity binding is detected, further testing is required.
- When no binding affinity is detected, the probability that the peptide is immunogenic is low.

Please see Annex A-5 for an overview of publications that have reported on HLA-DQ-peptide binding assays.

313 *T cell testing:*

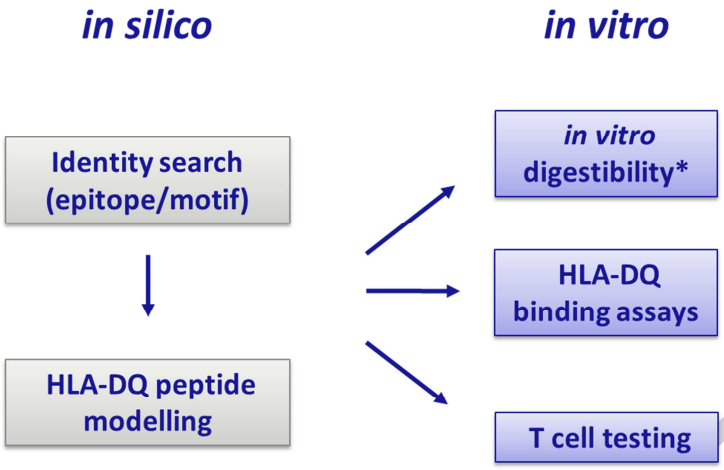
314 Recognition of gluten peptides by CD4+ T cells from one or more CD patients was a prerequisite for
315 defining toxic CD peptides (Sollid et al. 2012).

316 Such T cells have been isolated in a number of laboratories where the necessary expertise and
317 appropriate infrastructure are available. Hence, these T cells were used to provide conclusive evidence
318 on the capacity of a specific peptide sequence to stimulate CD-causative T cell responses.

319 Please see Annex A-6 for an overview of publications that have reported on T cells specific for HLA-
320 DQ-gluten complexes.

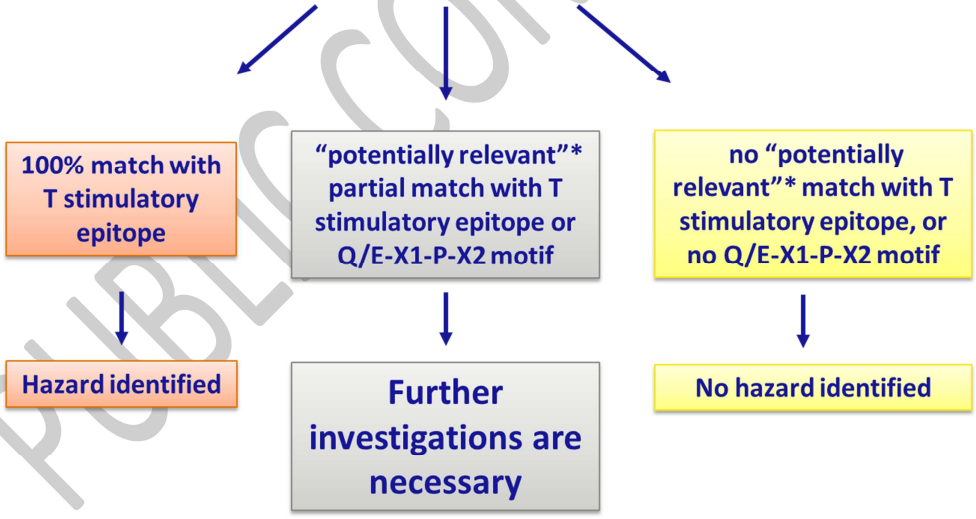
321

Fig 1. Stepwise approach for risk assessment



*for details, please see chapter on *in vitro* digestibility

Fig 2. Identity searches (epitope/motif)



*"potentially relevant" match: Match with a known T cell-stimulatory peptide which raises concern because of the position and nature of the identical amino acids.

330 **Fig. 3: Q/E-X1-P-X2 motif:** Possible combinations for the Q/E-X1-P-X2 motif found in the large majority of identified
331 immunogenic gluten-derived epitopes. It was noted that, while position 1 is always either glutamic acid (E) or glutamine (Q)
332 and position 3 always consists of a proline (P), also positions 2 (X1) and 4 (X2) are restricted to certain amino acids.
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2.2. *In vitro* protein digestibility

2.2.1. Background

In vitro digestibility tests can provide useful data on the susceptibility of a protein to digestion which may reflect its digestibility in the human gut and subsequent presentation to the host's immune system (Foster et al. 2013). There is evidence that gastrointestinal digestion can affect the immunogenicity of dietary proteins in relation to both IgE and non-IgE-mediated adverse reactions to foods as discussed below. However, it should be noted that the ability of digestion-resistant dietary proteins or derived fragments to initiate diseases, such as IgE-mediated allergies or celiac disease (CD), also depends on the predisposition of the host. For CD, expression of certain HLA genotypes is a well described risk factor for developing the condition (See Chapter 2.1). However, whilst a genetic predisposition towards atopy is also thought to play a role in development of IgE-mediated allergies to a variety of environmental agents including foods, specific genetic and other risk factors have yet to be defined. This makes the risk assessment process less certain for IgE-mediated allergies compared to CD.

IgE-mediated adverse reactions to foods: The events leading to the breakdown of oral tolerance, allergic sensitisation, and development of food allergies in a subset of sensitised subjects are poorly understood. It is likely that multiple pathways could ultimately lead to a failure to develop or loss of oral tolerance (Chinthrajah et al., 2016). Notwithstanding, the role of route of exposure in sensitisation, understanding how proteins are presented to the gut mucosal immune system may provide insight into the mechanisms controlling the balance between tolerisation and sensitisation. Furthermore, oral exposure is also central to non-IgE assessments where gastrointestinal digestion has been demonstrated to be important in delivery of immunologically active fragments to gut mucosal segments. Impaired gastric digestion of food allergens has been associated with both development of IgE responses to foods (sensitisation) and modulating the severity of IgE-mediated reactions to foods (elicitation). A recent study of patients undergoing gastric-bypass surgery, a procedure known to reduce post-prandial gastric acidity and where the bulk of food ingested reaches the small intestine without prior gastric digestion, resulted in a significant increase in sensitisation to food (Shakeri-Leidenmuhler et al., 2015). Impaired digestion of cod fish proteins may also be a risk factor for severe reactions in fish allergic individuals (Untersmayr et al., 2007). Studies on animal models have shown that suppression of gastric acid secretion using widely-prescribed antacid medication increases propensity to sensitisation to proteins from cod fish (Untersmayr et al., 2003), celery (Untersmayr et al., 2008), hazelnut (Scholl et al. 2005) and egg (Diesner et al., 2008). Furthermore, an association between the use of anti-ulcer drugs and the induction of IgE-mediated allergy to a variety of foods such as milk, potato, celery, carrots, apple, orange, wheat, and rye flour has been reported (Untersmayr et al., 2005). Such studies indicate that elevated gastric pH, and the resulting reduction in peptic digestion, may enhance the potential of foods to cause allergies. Uptake of allergens into the circulation may play an important role in eliciting allergic reactions (Strait et al., 2011) but data in humans are sparse and conflicting as to the role of allergen uptake in triggering reactions, although uptake appears much greater in allergic than healthy individuals, as indicated in studies of wheat allergy (Brockow et al 2015; Matsuo et al 2005).

Non-IgE-mediated adverse reactions to foods: Studies seeking to define the structural basis of the toxicity of gluten in CD have made extensive use of *in vitro* gastrointestinal digestion, including the action of brush border proteases, to generate physiologically relevant fragments of gluten. These studies notably identified a 33mer peptide that is especially resistant to digestion and which is likely to persist in the brush border with a half-life estimated to be ~20h. This peptide is also an excellent substrate for tissue transglutaminase TG2 and was a potent stimulator of T-cells from celiac patients (Shan et al., 2002).

Such data support the premise that immunologically active fragments of food proteins persisting in the gut lumen can play a role in driving immune-mediated adverse reactions to foods. Persistent, soluble intact proteins and fragments are more likely to be sampled by the gut epithelium and are hence exposed to cells of the immune system as potentially immunogenic fragments, with particulates being sampled by M cells within the Peyer's patches. In animal models there is evidence that uptake of particulate peanut protein bodies by M cells may promote sensitisation by virtue of their size, with soluble material being taken up by the same route (Chambers et al 2004). Although the exact

mechanism(s) of antigen uptake in the intestine are still to be clearly elucidated (Pabst and Mowat, 2012), it is generally accepted that most food allergens should retain sufficient structural integrity throughout their pass through the human gastrointestinal tract to sensitize/elicit an allergic response (Metcalfe et al., 1996). After uptake by antigen presenting cells peptides generated through endosomal proteolysis bind to MHC class II molecules (Rudensky et al., 1991; Chicz et al., 1993). Peptides shorter than 9 amino acid residues are probably unable to bind to MHC class II molecules and to activate T-cells since there is a minimum length requirement - the so called "peptide binding register" (Mohan and Unanue, 2012). Thus, peptides shorter than 9 amino acid residues are not able to stimulate an immune response. However, studies on the sensitising capacity of digested proteins indicate larger peptide sizes are required for immunogenicity. Thus, for the allergen β-lactoglobulin, digestion into fragments of less than 3,000 Da abolished the proteins immunogenicity (Bogh et al 2013), whilst for Ara h 1 pepsin digested peptides of less than 1,500 Da had markedly reduced immunogenicity (Bogh et al 2012) which was completely lost when the digest was fractionated. This is because of the propensity of B cell receptors to require multivalent antigens for binding, and it has emerged that the way in which B cells encounter antigen *in vivo* depends on its properties, such as size (Harwood et al 2010). With regards elicitation of reactions, synthetic antigens have been used to investigate the mechanism of degranulation (Handlogten et al 2013). These studies have shown that a minimum of two distinct epitopes is required to trigger degranulation, with the epitopes separated by a maximum of 6.4 nm on the synthetic antigen. On this basis peptides of at least 3-5 kDa would be required to cross-link IgE bound to the surface of effector cells, such as mast cells and basophils. However, peptides as small as 791 Daltons resulting from the pepsinolysis of the avocado pear allergen Prs s 1, have been found to elicit reactions *in vivo* using skin testing (Diaz-Perales et al 2003). Such peptides are unlikely to carry multiple IgE epitopes, suggesting that peptide aggregation may play a role in triggering degranulation.

To either sensitise or trigger an allergic reaction in an already sensitised individual, a food protein needs to be "bioaccessible" by the hosts' immune system. "Bioaccessibility" describes the ability of a chemical entity (such as a protein) to be released from food during the digestive process, which can consequently interact with and/or be absorbed by the gut epithelium (Holst and Williamson, 2008). The EFSA Guidance Document for risk assessment of food and feed from genetically modified plants published in 2011 states that "the impact of the possible interaction between the protein and other components of the matrix as well as the effects of the processing should be taken into account in *in vitro* digestibility tests" (EFSA, 2011). *In vitro* digestibility tests have been applied to investigate the effects of digestion on the food matrix, and how processing conditions (including thermal treatment) may affect susceptibility to simulated gastrointestinal proteolysis (e.g. Minekus et al., 2014, Smith et al., 2015). However, given the diversity of food matrices and food processing procedures, our knowledge of their effects on susceptibility of proteins to digestion is limited. As a consequence, the effects of processing and of the food matrix on the susceptibility of a particular protein to digestion are difficult to predict. Because there is no effective animal model for food allergy, studies are often limited to investigating the impact of the food matrix on elicitation of allergic reactions in allergic human subjects while data on allergic sensitisation is very limited regarding this aspect (Ballmer-Weber et al 2002; Bartnikas et al., 2012; Brenna et al 2000; Grimshaw et al 2003; Mackie et al 2012; Netting et al., 2013; Worm et al 2009).

2.2.2. Types of *in vitro* digestibility tests

The pepsin resistance test, which is embedded in EFSA Guidance Document (EFSA 2011) and Codex Alimentarius (2003, 2009), is currently used for allergenicity risk assessment to provide additional information for the weight-of-evidence approach, as no single test is fully predictive of the allergenic potential of a protein.

The pepsin resistance test has several limitations (EFSA, 2010) including:

- The pH value usually employed in the assay is extremely acidic. Since pepsin activity is pH dependent, the pattern of proteolysis may not reflect the one likely to be found *in vivo*;
- Pepsin is added in a gross excess to the protein substrate, affecting the kinetics of the digestion;

- The correlation with allergenicity of proteins has been questioned. Studies comparing the digestibility of allergens with that of non-allergenic dietary proteins showed that food allergens were not always inherently more stable to pepsin digestion than non-allergenic proteins (Fu et al., 2002; Thomas et al., 2004; Herman et al., 2007).

Whilst this test may contribute to understanding the biochemical properties of newly expressed proteins, it does not reflect how these proteins behave under the physiological conditions encountered in the digestive tract. Furthermore, the test does not reflect changes in the digestive process that take place across the life course (Rémond et al., 2015). These limitations have been previously highlighted by EFSA (EFSA, 2010). In addition, the EFSA Guidance Document for risk assessment of food and feed from genetically modified plants (EFSA, 2011) indicates that "the digestibility of the newly expressed proteins in specific segments of the population such as infants and individuals with impaired digestive functions may be assessed employing *in vitro* digestibility tests using different conditions". Given these considerations, it is proposed to embed the pepsin resistance test within a suite of tests aimed to characterise how a newly expressed protein may behave during the digestive process (see Annex B).

Gastrointestinal digestion assays usually aim to simulate "normal" digestive function with regards digestive enzymes and acid secretion (Macierzanka et al., 2009; Moreno, 2007; Minekus 2014). However, alterations in the digestive milieu are often observed in patients with various gastrointestinal conditions (Kay and Jorgensen, 1994), in young infants with underdeveloped digestive system (Armand et al., 1996) and elderly with a weakened digestive function (Hosking et al., 1975). In an *in vitro* study assessing the impact of digestion on celery allergenicity in an aged population, decreased gastric proteolysis was identified (Untersmayr et al., 2008). Simulated infant digestion models (Dupont et al., 2010; Ménard et al., 2014) have been used to study the gastrointestinal tolerance of casein and α-lactalbumin and to optimize the milk processing and formula production. Experiments using simulated gastric fluid where the pH was raised, were used to assess the stability of allergens in fish, milk and hazelnut in patients taking antacids where intra-gastric pH is increased (Scholl et al., 2005; Untersmayr et al., 2005; Untersmayr et al., 2007). Another study developed a gastrointestinal model simulating the physicochemical conditions of the elderly's gastrointestinal tract which was applied to investigate the fate of bovine whey proteins (Levi and Lesmes, 2014). Finally, the COST Infogest network has recently proposed a standardised batch gastrointestinal digestion method based on physiologically relevant conditions that could be applied for various endpoints (Minekus et al., 2014).

In vitro models simulating the physiological or pathological conditions of gastrointestinal digestion by sequential addition of digestive enzymes, salts and fluids have been designed to understand the degradation of proteins and other constituents during digestion. Some *in vitro* models have also included biosurfactants such as phosphatidylcholine (PC), which can be found at low levels in gastric juice and which is also a component of bile (Moreno et al. 2005a; Mandalari et al., 2009a). Biosurfactants can have complex effects on the phase behaviour of lipid rich foods, since emulsification is an important aspect of lipid digestion (Macierzanka et al. 2009), but they may also have effects on digestion of proteins associated with lipids, and there is some evidence, although equivocal, that bile salts may affect the activity of pancreatic proteases.

The time course of simulated digestion tests can be based on the residence time of food in the stomach. This is dependent on the type of meal ingested. For example, liquid and solid meals display different gastric emptying rates after ingestion (Rémond et al., 2015). The halftime (t1/2), which indicates when 50% of an ingested meal is emptied, ranges from 10 to 60 min for liquid meals, whereas t1/2 values reported for solid foods ranges from 50 min to 115 min. Other factors, such as other meal components, meal volume, caloric content, ratio between liquid and solid in the meal or the type of dietary fibres also have an influence on the gastric emptying rate.

Digestibility studies using gastrointestinal conditions can provide useful data regarding persistency of newly expressed proteins and/or of digestion derived immunologically-active fragments in the gut lumen and hence pose a risk of causing an immune-mediated adverse reaction in a susceptible individual. The presence of digestion resistant fragments only provides an indication of exposure of the gut mucosal surface and is not on its own predictive of allergenicity, since this property is a function of the way in which the fragments interact with the individual. Consequently, resistance to gastrointestinal digestion of a newly expressed protein should be considered as part of the assessment for its potential to cause allergic reactions via the oral route. However, it should also be

noted that other routes of exposure such as respiratory or cutaneous have to be considered (EFSA 2010).

There is no internationally accepted model/protocol available to perform gastrointestinal *in vitro* digestibility tests for purified proteins although this has been developed for whole foods (Minekus 2014). This is a consensus model applied to several foods and based on available *in vivo* physiological data resulting from the COST Infogest network. This batch *in vitro* digestion assay includes an oral phase, as well as subsequent gastric and intestinal phases. Inter-laboratory trials have been performed at the European level to assess digestion of skim milk powder (Egger et al., 2016). Such *in vitro* digestibility methods will require adaptation to make them useful for analysis of purified proteins, taking into account the need for further standardisation and validation.

There are only two validated (ring-trialled) studies of digestibility tests for purified proteins which have been published up to date:

- Thomas et al. (2004). Modification of the seminal paper of Astwood et al. (1996) by lowering the pepsin:protein ratio and looking at the effect of very acidic pH values (1.2 and 2.0). *In vitro* pepsinolysis of 10 proteins (allergens and non-allergens) at pH 1.2 and 2.0 were evaluated by 9 laboratories. The authors observed that pH did not have an influence on the time of digestion of protein large-fragments but the detection by gel electrophoresis of low molecular weight proteolytic fragments was less consistent because the different fixation and staining methods used.
- Mandalari et al. (2009b). These authors evaluated the *in vitro* gastrointestinal digestibility of β-casein and β-lactoglobulin by using a low-protease assay with and without the addition of phospholipids (based on Moreno et al., 2005ab and Mandalari et al., 2009a) and the high-protease assay (based on Astwood et al., 1996 and Fu et al., 2002). Twelve laboratories tested the method without the addition of phospholipids and 5 labs studied the effect of the addition of surfactants. This study demonstrated that the low-protease assay was robust and reproducible although further validation should be undertaken using a more extensive panel of proteins. In addition, this inter-laboratory trial showed that the largest factor governing irreproducibility was the sampling and electrophoresis methods used to analyse digestion products.

2.2.3. Strategy for the allergenicity risk assessment

The proposed procedure for complementing the allergenicity risk assessment of newly expressed proteins is as follows.

EFSA previously highlighted the limitations of the pepsin resistance test for allergenicity risk assessment and recommended that resistance to digestion of novel proteins should be evaluated using other *in vitro* digestibility methods designed to simulate more closely the conditions of the human digestion process (EFSA, 2010). The current document proposes a novel strategy to reduce the uncertainty of both the IgE and the non-IgE allergenicity assessment. This strategy is based on state-of-the-art science, and foresees a refined *in vitro* digestion test that calibrates the conditions currently used in the pepsin resistance test to better reflect physiological conditions. This includes additional pH conditions that are more representative of the gastric environment and the addition of an intestinal digestion phase. In addition, more informative read-outs of the test are required that define the extent to which the intact protein or resistant fragments remain after *in vitro* digestion.

An interim phase is considered necessary to evaluate the efficacy of the proposed revisions to the *in vitro* gastrointestinal digestion test. An outline proposal for such revision is provided in Annex B to this document as a starting point to collect feedback from the public and stakeholders during the consultation period and the next EFSA workshop. EFSA is exploring how to practically perform such evaluation on the basis of data collected for such purpose, outside the regulated safety assessment responsibility frame.

During such interim phase and until the evaluation of the new approach is completed, EFSA will continue to follow the weight-of-evidence approach for allergenicity assessment as described by EFSA Guidance Document (EFSA, 2011) and Codex Alimentarius (Codex, 2003, 2009). Because the pepsin resistance test can provide information on the physicochemical stability of a protein (because

549 properties, such as the rigidity of the polypeptide backbone at low pH, determine susceptibility to
550 pepsinolysis), it may still provide useful data on the biochemical properties of newly expressed
551 proteins.

552

2.3. Endogenous allergenicity

According to the EFSA Guidance Document (EFSA, 2011) and in line with Codex Alimentarius (2003, 2009), the allergenicity assessment of GM plants includes two elements, the assessment of the newly expressed protein(s) and the assessment of endogenous allergenicity. The purpose of the assessment of endogenous allergenicity is to ensure that no unintended effect of the genetic modification affects the levels of endogenous allergens in a manner that would adversely impact on the human and animal health (EFSA, 2011, König et al., 2004; Metcalfe et al., 1996; Thomas et al., 2008). To this end, EFSA (2011) and Codex Alimentarius (2003, 2009) foresee the assessment of endogenous allergenicity when the plant receiving the new gene(s) is known to be allergenic. In these cases, any potential change in the overall allergenicity of the GM plant compared with that of its non-GM comparator(s) is foreseen to be analysed. Historically, this analysis was performed using sera from allergic individuals, limitations of which have been previously described (Fernandez et al., 2013).

EFSA and other scientists have previously recommended the inclusion of relevant endogenous allergens in the comparative compositional analysis as additional parameters to be measured and analysed (EFSA, 2010, 2011, Fernandez et al., 2013).

The Implementing Regulation (EU) No 503/2013 (IR503/2013) on applications for authorisation of GM food and feed has been in place since December 2013 (EC, 2013). Based on EFSA guidance document recommendation (EFSA, 2011), European Regulation incorporated as a mandatory requirement the inclusion of certain allergens in the compositional analysis and consequently, the quantitative measurement of individual allergens.

To assist both applicants as well as risk assessors in the practical implementation of this new mandatory requirement, this document provides further information on: i) the crops to analyse; ii) the relevant allergens to be quantified; iii) the methodology to be applied for quantification; and iv) interpretation of the resulting data.

2.3.1. Relevant crops for the analysis

According to IR503/2013 and in line with EFSA Guidance Document (EFSA 2011), an assessment of endogenous allergenicity should be performed on a case-by-case basis. When the recipient plant is known to be allergenic, the applicant should test any potential change in the allergenicity of the genetically modified food or feed by comparison of the allergen repertoire with that of its appropriate comparator(s).

To date, EFSA has performed endogenous allergenicity risk assessments based on experimental data for foods recognised to be common food allergens and, in this context, of public health importance by European Regulation for labelling purposes (EC, 2003, 2011). EFSA GMO Panel scientific opinions on GM soybean applications, including an endogenous allergenicity assessment, have been published previously (Annex C-1). To date, EFSA has not received any application involving recognised allergenic food other than soybean. For any other application received, specific experimental data on endogenous allergenicity are not requested by EFSA and the assessment is carried out using data on potential effects of the genetic modification on the general composition and on the molecular characteristics. However, this does not preclude that EFSA might request experimental data on endogenous allergenicity if considered necessary e.g., if the allergenic status of these foods changes. In addition, other plant-derived foods (e.g., fruits, nuts), which might be genetically engineered in the future, should be subjected to such assessments if they are recognised to be allergenic. For such decisions, risk assessors, risk managers, health professionals and stakeholders can provide valuable input.

2.3.2. Relevant allergens to be quantified

Soybean

Soybean is recognised as a common allergenic food by European Regulation (EC, 2003, 2011), and suggested as one of the foods accounting for approximately 90% of food allergies (FDA, 2004; OECD, 2012).

As described previously, the quantitative measurement of soybean allergens (as referred to in the relevant OECD consensus document) as part of the compositional analysis is now a mandatory requirement in the IR503/2013. Soybean proteins termed "potential allergens" are described in Table 20, Section III-C of the OECD consensus document on soybean (OECD, 2012). Nevertheless, in accordance with Article 5(2) and 5(3) of the IR503/2013: i) EFSA may accept derogations of specific requirements if they are demonstrated not to be scientifically necessary for food/feed safety assessment or technically not possible to perform; and/or ii) EFSA may request data not foreseen in OECD consensus documents anytime, if considered necessary based on new scientific findings.

In line with IR503/2013, the OECD allergen list should be taken as the starting point for the identification of "potential allergens" (Fig.1). In addition, this list should be complemented with a search in scientific and medical literature, and in various updated databases (see EFSA, 2010; Appendix 3.13, Table I for a list of relevant databases to be used). It is noted that on the one hand the OECD list of allergens might not be complete, e.g. it might be outdated and/or miss relevant entries. On the other hand, not all "potential allergens" listed in this OECD consensus document can currently be measured due to technical reasons (e.g. amino acid sequence not available) and/or their clinical relevance might not have been demonstrated. Once a comprehensive search of "potential allergens" in the literature and databases is conducted, the relevant allergens selected for quantification should be justified. As a complementary and/or alternative approach, a systematic review could be performed, aiming to identify clinically relevant allergens. A scientific rational, explaining why an allergen is not considered relevant should be provided.

A possible approach how to identify proteins relevant for the endogenous allergenicity assessment of soybean can be found in Annex C-2.

Other GM plants

For foods other than soybean which are recognised to be allergenic (risk assessors, risk managers, health professionals and stakeholders can provide invaluable input in such respect), a similar approach/strategy for the identification of relevant allergens as the one followed for soybean (see Annex C-2) should be applied, whenever considered necessary. To date, EFSA has not received any application involving a recognised allergenic food other than soybean.

2.3.3. Methodology to be applied for the quantification

Either ELISA or mass spectrometry (MS) approaches are appropriate methods for the quantification of endogenous allergens. Individual allergens quantified by ELISA should be measured using purified monoclonal or polyclonal antibodies raised against each purified allergen molecule together with calibrated standards, which can provide adequate information on allergen quantities in a sample. It is not recommended to use polyclonal antisera of animals raised against whole soy extract because of the variability of animal immune responses. MS approaches may allow specific detection and quantification of a single allergen. Further considerations on methodology are described in Annex C-3.

EFSA encourages the standardisation and harmonisation of the methods used to enhance measurement comparability. This would help the future establishment of an allergen database, including data on the natural variability, which would provide useful additional information to improve the robustness of the safety assessment.

2.3.4. Data interpretation and risk assessment

As indicated in IR503/2013, conclusions of the allergenicity assessment should indicate whether the GM food or feed is likely to be more allergenic than its appropriate comparator. The applicant should assess any potential change in the allergenicity of the GM food or feed by comparison of the allergen repertoire.

Allergens included in the compositional analysis should be measured and analysed according to the principles of the comparative assessment like already performed for all other compositional compounds (see Section 1.3.2 of IR503/2013). To this end, the starting point of the assessment should be the identification of statistically significant differences between the GM and its conventional counterpart. A further evaluation should investigate whether or not the differences observed fall

within or outside the range of natural variation – i.e. equivalent or not to non-GM reference varieties (IR503/2013). In the case that the levels of a specific allergen in a GM plant differs significantly from the levels observed in the appropriate comparator(s) and it falls outside the estimated range of natural variation, the biological relevance in relation to human and animal health needs to be assessed.

Additional considerations and/or experimental data might be needed on a case-by-case basis. As for other compounds included in the compositional analysis, the nature of these additional considerations and/or experimental data needed in the assessment might depend on the number and magnitude of the changes identified, as well as on the clinical/safety relevance of the specific allergen(s)/compound(s) involved.

Ultimately, when a potential increase in allergenicity due to the genetic modification cannot be excluded, the GM food or feed should be further characterised in the light of its anticipated intake, as requested by IR503/2013. Occupational allergy should also be considered with respect to inhalation or contact with potential allergens. In all cases, an exposure assessment should focus on the European population aiming at identifying particular groups at high risk which might be affected by a specific change of the allergen content.

Possible approaches for data interpretation and risk assessment of soybean endogenous allergenicity can be found in Annex C-4.

Fig. 1: Current requirements

OECD consensus soy 2012
Soy allergen list:
"potential soybean allergens"

Table 20. Potential soybean allergens

IgE-binding proteins	Allergen nomenclature	Molecular weight (kDa)	Family
Hydrophobic proteins	Gly m 1[1]	7.0-7.5	Lipid transfer protein
Defensin	Gly m 2[1]	8.0	Storage protein
Profilin	Gly m 3[1]	14	Profilin
SAM22	Gly m 4[1]	16.6	Pathogenesis related protein PR-10
P34	Gly m Bd 30 K	34	Protease
Unknown Asn-linked glycoprotein	Gly m Bd 28 K	26	Unknown
β-Conglycinin (vicilin, 7S globulin)	Gly m 5[1]	140–170	Storage protein (with subunits)
Glycinin (legumin, 11S globulin)	Gly m 6[1]	320–360	Storage protein (with subunits)
2S albumin	Not assigned	12	Prolamin
Lectin	Not assigned	120	Lectin
Lipoxygenase	Not assigned	102	Enzyme
Kunitz trypsin inhibitor	Not assigned	21	Protease inhibitor
Unknown	Not assigned	39	Unknown
Unknown	Not assigned	50	Homology to chlorophyll A-B binding protein
P22-25	Not assigned	22–25	Unknown

Source: adapted from L'Hocine and Boye, (2007); updated with information from WHO/IUIS (2011)

[1] WHO/IUIS (2011) Allergen nomenclature recognized by WHO and IUIS

Evidence check

Evaluation of literature for all single allergens
and
Comparison and complementation with databases (EFSA, 2010)
and/or
Systematic Reviews

Clinical relevance shown

Relevance for GMO risk assessment

3. Conclusions

Dietary proteins or their derived fragments are able to initiate diseases, which include IgE-mediated allergy as well as non-IgE-mediated adverse immune reactions. Onset of these diseases however depends to a large extent on the predisposition of the individual; e.g., the expression of certain HLA genotypes is a well-described risk factor for the development of celiac disease (CD). Also, a genetic predisposition towards atopy is strongly suspected to play a role in the development of IgE-mediated allergies. Nevertheless, also properties of food proteins or their derived fragments can be assessed to predict possible adverse immune reactions. Moreover, levels of endogenous allergens in foods might play a role for the onset and severity of IgE-mediated allergic disease.

CD is a well-characterised non-IgE mediated adverse immune reaction to specific food proteins. A stepwise approach for analysis of proteins is proposed in this guidance document, which parallels the classical weight-of-evidence approach conducted for the assessment of IgE-mediated allergenicity. In a first instance, *in silico* approaches searching for potentially hazardous peptide sequences are employed. Subsequently, *in vitro* tests may be necessary to determine the safety profile, such as HLA-DQ-peptide binding assays and/or testing with T cell clones derived from CD patients.

In vitro digestibility tests can provide useful data on the susceptibility of a protein to gastrointestinal digestion which may reflect its digestibility in the human gut and subsequently provide information on its immunogenicity. The pepsin resistance test can provide useful data on the biochemical properties of newly expressed proteins. Because of the limitations of this test for allergenicity risk assessment, resistance to digestion of novel proteins should also be evaluated using other *in vitro* digestibility methods designed to more closely simulate the conditions of the human digestion process. To further elucidate these aspects, an interim phase is foreseen. To this end, EFSA is exploring possibilities outside the regulated safety assessment responsibility frame.

The assessment of endogenous allergenicity ensures that a genetic modification does not significantly affect the levels of endogenous allergens in comparison to appropriate comparator(s). This allows to objectively establish *a priori* criteria for further assessment of the observed difference(s) with respect to its relevance for human and animal health.

Additional conclusions will be elaborated after the public consultation.

Documentation provided to EFSA

1. Proposal for a self-task mandate of the EFSA GMO Panel to establish a Working Group to develop supplementary guidelines for the allergenicity assessment of GM plants to incorporate new developments. May 2014. Submitted by the Chair of the ESA GMO Panel.

2. Acceptance of the self-task mandate of the EFSA GMO Panel to establish a Working Group to develop supplementary guidelines for the allergenicity assessment of GM plants to incorporate new developments. July 2014. Submitted by EFSA Executive Director.

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Annex A – Non-IgE-mediated immune adverse reactions to food

Annex A-1. T cell epitopes known in celiac disease

Several listings/databases of the known T cell stimulatory sequences identified in gluten, hordeins, secalins and avenins are available. These include the https://propepper.net database and the AllergenOnline database. Furthermore, an overview of the best characterized epitopes along with a unified nomenclature is presented in Sollid et al. (2012). Table A1 lists the epitopes described in the latter manuscript. This compilation lists both the HLA-DQ2 and HLA-DQ8 restricted epitopes and includes the known immunodominant epitopes present in the α- and ω-gliadins as well as the less commonly recognized epitopes in the γ-gliadins and the LMW- and HMW-glutenins.

The list contains a convenient overview of the most important and well defined epitopes. It is, nevertheless, important to note that due to the extreme variability of the gluten and gluten-like proteins in barley and rye, (single) amino acid variants of these epitopes do exist, some of which may also exhibit T cell stimulatory activity. Also, it cannot be fully excluded that additional immunodominant gluten epitopes may be identified in the future. This is however unlikely because large numbers of patients have already been extensively tested for gluten reactivity, including studies where gluten peptide libraries were employed. Thus, any protein containing one or more sequences that display high sequence identity to the epitope sequences present in this list will likely have the capacity to trigger gluten-specific T cells.

Any 9 amino acid-residue peptide which shows identity to a known T cell epitope might be able to induce an immune response in celiac disease patients. However, the necessary number of amino acids identical in a peptide to trigger a response is challenging to define, since the ability to bind to CD specific MHC molecules and the interaction with T cells is highly dependent on the nature and position of certain amino acids. Therefore, a definite size cut-off in respect to identity to a known epitope indicating potential hazardous peptides, for which further assessment would be needed, is demanding. As an example, a major alpha gliadin peptide of wheat (glia-α1a, Table A1) shares 5 out of 9 amino acids with a peptide in oat, which is highly suspected to induce immune responses in some celiac disease patients (Lundin et al 2003). However, single amino acids substitutions were described to abolish T cell reactivity in epitopes of α-2 gliadin (Ellis et al 2003, Mitea et al 2010). Following the public consultation process, further considerations will be elaborated.

Ellis HJ, Pollock EL, Engel W, Fraser JS, Rosen-Bonson S, Wieser H, Ciclitira PJ 2003. Investigation of the putative immunodominant T cell epitopes in coeliac disease. Gut 52: 212-7.

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Table A1. List of celiac disease relevant DQ2 and DQ8 restricted T-cell epitopes recognized by CD4+ T cells (taken from Sollid et al, 2012). The single letter code for amino acids is used. A characteristic Q-X1-P-X2 motif is present in the large majority of HLA-DQ2 epitopes (in bold). This sequence is a target sequence for TG2, which yields E-X1-P-X2. Due to the introduction of the negatively charged amino acid glutamate, the peptides become high affinity binders for HLA-DQ2. glia-α = α-gliadin; glia-γ = γ-gliadin; hor = hordein; sec = secalin; ave = avenin; glut-L = LWM-glutenin, glut-H = HMW-glutenin.

DQ2.5-glia-α1a	P F P Q P **Q L P Y**	(Arentz-Hansen et al. 2000)
DQ2.5-glia-α1b	P Y P Q P **Q L P Y**	(Arentz-Hansen et al. 2002)
DQ2.5-glia-α2	P Q P **Q L P Y** P Q	(Arentz-Hansen et al. 2000)
DQ2.5-glia-α3	F R P **Q Q P Y** P Q	(Vader et al. 2002b)
DQ2.5-glia-γ1	P Q Q S F P E Q Q	(Sjöström et al. 1998)
DQ2.5-glia-γ2	I Q P **Q Q P A** Q L	(Qiao et al. 2005; Vader et al. 2002b)
DQ2.5-glia-γ3	Q Q P **Q Q P Y** P Q	(Arentz-Hansen et al. 2002)
DQ2.5-glia-γ4a	S Q P E Q **Q F P Q**	(Arentz-Hansen et al. 2002)
DQ2.5-glia-γ4b	P Q P E Q **Q F P Q**	(Qiao et al. 2005)
DQ2.5-glia-γ4c	Q Q P **Q Q P F** P Q	(Arentz-Hansen et al. 2002)
DQ2.5-glia-γ4d	P Q P **Q Q P F** C Q	(Qiao, unpublished)
DQ2.5-glia-γ5	Q Q P F P **Q Q P Q**	(Arentz-Hansen et al. 2002)
DQ2.5-glia-ω1	P F P Q P **Q Q P F**	(Tye-Din et al. 2010)
DQ2.5-glia-ω2	P Q P **Q Q P F** P W	(Tye-Din et al. 2010)
DQ2.2-glut-L1	P F S E **Q E Q P V**	(Vader et al. 2002b)
DQ2.5-glut-L2	F S Q Q Q **Q S P F**	(Stepniak et al. 2005; Vader et al. 2002b)
DQ2.5-hor-1	P F P Q P **Q Q P F**	(Tye-Din et al. 2010; Vader et al. 2003a)
DQ2.5-hor-2	P Q P **Q Q P F** P Q	(Vader et al. 2003a)
DQ2.5-sec-1	P F P Q P **Q Q P F**	(Tye-Din et al. 2010; Vader et al. 2003a)
DQ2.5-sec-2	P Q P **Q Q P F** P Q	(Vader et al. 2003a)
DQ2.5-ave-1	P Y P E Q E E P F	(Arentz-Hansen et al. 2004; Vader et al. 2003a)
DQ2.5-ave-1b	P Y P E Q **Q Q P F**	(Arentz-Hansen et al. 2004; Vader et al. 2003a)

DQ8 restricted epitopes

DQ8-glia-α1	Q G S F Q P S Q Q	(van de Wal et al. 1998b)
DQ8-glia-γ1a	Q Q P Q Q P F P Q	(Tollefsen et al. 2006)
DQ8-glia-γ1b	Q Q P Q Q P Y P Q	(Tollefsen et al. 2006)
DQ8-glut-H1	Q G Y Y P T S P Q	(van de Wal et al. 1999)

Annex A-2. Typical motives found in celiac disease epitopes

Closer inspection of the Q/E-X1-P-X2 sequences demonstrates that only a limited number of amino acids are present at the X-positions: L, Q, F and S at position X1 and Y, F, A, V and Q at position X2 (Fig.3). Thus, a search motif that incorporates these amino acids should identify peptide sequences with the potential to bind to HLA-DQ2.5 and stimulate gluten-specific T cells.

Annex A-3. Considerations for HLA-DQ peptide binding modelling

Recent studies have determined the T cell receptor repertoire used by CD4+ T cells specific for immunodominant gluten epitopes and crystal structures of such T cell receptors bound to the HLA-DQ-gluten complexes have been determined. As a consequence, detailed knowledge is available indicating which amino acids in the gluten peptides are responsible for the high-affinity binding to

HLA-DQ and which amino acids mediate the specific interaction with the T cell receptor. Several publications describing the binding of CD peptides to HLA-DQ molecules are available. The coordinates of all these structures are available through public databases (Broughton et al 2011; Hensdersen et al 2007, Kim et al 2004; Petersen et al 2014; Petersen et al 2015). As an example, the x-ray coordinates of HLA-DQ8 bound to a gliadin peptide were deposited in the Protein Data Bank with the accession number 2NNA. This allows modelling studies in which the gluten peptide can be replaced by any peptide sequence of choice (Moustakis et al 2000; Wiesner et al 2008; van Heemst et al 2015). It can be anticipated that, if the selected peptide is likely to bind to HLA-DQ in a way that it resembles the known structure of gluten peptides bound to HLA-DQ, it might have the capacity to stimulate gluten-specific T cells (Fig. A1, Wiesner et al 2008). Thus, molecular modelling can be employed to aid the determination of potential T cell stimulatory properties of peptide sequences.

A non-exhaustive list of selected publications that have reported on HLA-DQ-modelling assays:

Broughton SE, Petersen J, Theodossis A, Scally SW, Loh KL, Thompson A, van Bergen J, Kooy-Winkelaar Y, Henderson KN, Beddoe T, Tye-Din JA, Mannering SI, Purcell AW, McCluskey J, Anderson RP, Koning F, Reid H & Rossjohn J. (2012) Biased T cell receptor usage directed against human leukocyte antigen DQ8-restricted gliadin peptides is associated with Celiac Disease. Immunity 37: 611-21

Henderson KN, Tye-Din JA, Reid HH, Chen Z, Borg NA, Beissbarth T, Tatham A, Mannering SI, Purcell AW, Dudek NL, van Heel DA, McCluskey J, Rossjohn J, Anderson RP (2007) A structural and immunological basis for the role of human leukocyte antigen DQ8 in celiac disease. Immunity. 27:23-34

Kim CY, Quarsten H, Bergseng E, Khosla C, Sollid LM (2004) Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. Proc Natl Acad Sci U S A 101:4175-4179

Moustakas AK, van de WY, Routsias J, Kooy YM, Van VP, Drijfhout JW, Koning F, Papadopoulos GK (2000) Structure of celiac disease-associated HLA-DQ8 and non-associated HLA-DQ9 alleles in complex with two disease-specific epitopes. Int. Immunol. 12:1157-1166

Petersen J, Montserrat V, Mujico JR, Loh KL, Beringer DX, van Lummel M, Thompson A, Mearin ML, Schweizer J, Kooy-Winkelaar Y, van Bergen J, Drijfhout JW, Kan WT, La Gruta NL, Anderson RP, Reid HH, Koning F, Rossjohn J. (2014) T-cell receptor recognition of HLA-DQ2-gliadin complexes associated with celiac disease. Nat Struct Mol Biol. 21: 480-8

Petersen J, van Bergen J, Loh K, Kooy-Winkelaar Y, Beringer DX, Thompson A, Bakker SF, Mulder CJ, Ladell K, McLaren JE, Price DA, Rossjohn J, Reid HH, & Koning F. Determinants of Gliadin-specific T Cell Selection in Celiac Disease. J. Immunol 2015; 194: 6112-22

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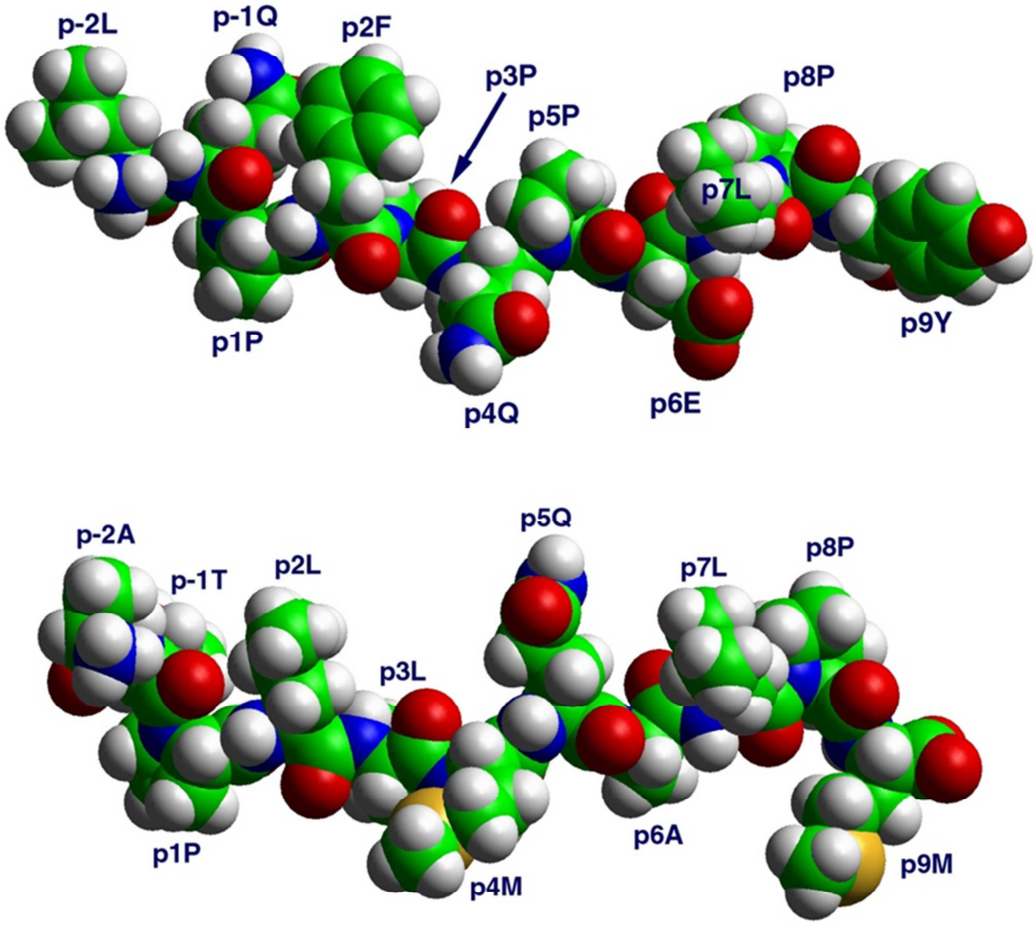
Wiesner M, Stepniak D, de Ru AH, Moustakis AK, Drijfhout JW, Papadopoulos GK, van Veelen PA and Koning F (2008). Dominance of an alternative CLIP-sequence in the celiac disease associated HLA-DQ2 molecule. Immunogenetics 60: 551-555

Figure A1: Shown is a side view of two peptides, one a gliadin T cell epitope (top panel) while the other is a peptide derived from a self-antigen (lower panel). The side chains of the amino acids that point downwards anchor the peptide to the HLA-DQ-molecule, the upward-pointing amino acids can be contacted by the T cell receptor. Even though the peptide share some sequence similarity (P at p1, L at P7, P at p8, large amino acid at p2) there are several features that are predicted to prohibit a functional interaction between a gliadin specific T cell receptor and the self-peptide, like an overall different conformation and the presence of a large amino acids at p5 (Q) instead of the much smaller proline (P) in the gliadin peptide. (taken from Wiesner et al 2008).



Annex A-4. Considerations for in vitro digestibility tests

As specified above and because a nine amino acid core is almost invariably required for efficient peptide binding to HLA-DQ, proteins that are easily degraded into fragments shorter than 9 amino acids are unlikely to harbour T cell stimulatory epitopes. Thus, determination of proteolytic resistance of proteins can aid in the identification of proteins with potential harmful potential. For more details please see chapter on *in vitro* protein digestibility in this document.

Annex A-5. Considerations for HLA-DQ peptide binding assays - Non-exhaustive list of publications that have reported on HLA-DQ-peptide binding assays.

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Annex B – *In vitro* protein digestibility tests for risk assessment of GM plants

As specified in Section 2.2 of this document, an interim phase (~2 years duration), with a review undertaken in the final year is proposed. During this phase the involved laboratories will define and apply the refined digestion test methodology. After that period, EFSA will discuss whether the test adds value and, if so, what further steps are needed for its final implementation.

Principles of *in vitro* protein digestibility tests for risk assessment

A) Test conditions:

i) Material to be used:

With regard to the test protein to be used for the revised/refined *in vitro* digestibility assays, it is considered important that the test protein comes from the parts of the plant commonly considered as edible, at present mostly the seeds/grain, since this material will be consumed.

- The testing of the newly expressed proteins purified from the edible raw plant material is a more realistic scenario.
- Should strong evidence be provided that the protein cannot be purified from plant material, use of recombinant protein may be justified. Such recombinant protein should be produced which displays the same structural, biochemical and functional properties – e.g. post-translational modifications (glycosylation, phosphorylation, hydroxylation, carboxylation, disulphide bond formation) as the protein expressed in a plant tissue.

ii) Digestion conditions:

Digestion conditions should be selected based on the range of conditions found *in vivo* and which encompass the needs of special groups and those receiving medication, such as antacids. The current document does not specify a set protocol but rather requests that the conditions employed in the models reflect the different conditions experienced *in vivo*.

Different situations combining low and high pepsin concentrations with low and high pH values could be performed to potentially cover the range found physiologically (Fig. B1). One set of these conditions could be the current pepsin test. Different concentrations for proteolytic enzymes and/or biosurfactants have also been described (Dupont et al., 2010; Mandalari et al., 2009b; Minekus et al., 2014, Thomas et al., 2004) and they could be considered to be varied in the gastric and intestinal phases according to the specific situations.

B) Data interpretation:

Criteria such as length and persistence of peptides derived from *in vitro* gastrointestinal digestion of newly expressed proteins will play key roles in identifying potential hazards within the weight-of-evidence approach for the allergenicity assessment. Using concepts such as "half-life" is a possible principle to define "transient" and "persistent" peptides. This has been used in other assessments of the allergenic risk of novel proteins, such as the ice structuring protein (Baderschneider et al 2002). However, further evidence to support this concept needs to be provided. Figure B2 displays different example scenarios and possible subsequent data interpretation:

- If a protein digest is composed of peptides < 9 amino acid residues in length, the allergenic potential is low;
- If a protein digest is composed of peptides ≥ 9 amino acids residues in length but transient, the allergenic potential can be assumed to be low;
- If digestion fragments of ≥ 9 amino acids or longer are identified and are persistent, further considerations are required. In that case, the proportion of the stable peptides ≥ 9 amino acids in length within the whole protein digesta should be considered in the risk assessment process. If the proportion of the stable peptides ≥ 9 amino acids in length is considered to be

significant, further assessment may be requested on a case-by-case basis. This may include information on the potential of such digestion resistant fragments to interact with the immune system. For non-IgE-mediated allergic reactions (celiac disease), specific HLA-DQ-peptide modelling and binding assays, as well as T cell testing are proposed in this document (see section 2.1 describing *Non-IgE-mediated adverse immune reactions to foods*). For IgE-mediated allergic reactions, IgE reactivity and formation of immune complexes may depend on the proximity and number of IgE epitopes (Huby et al., 2000; Gieras et al., 2016). Therefore, in contrast to T-cell epitopes, the minimum size of peptides which might act as B-cell receptor epitopes and cause IgE cross-linking is less clear, and will require the presence of multiple epitopes (at least two) which can only be accommodated in peptides greater than 9 amino acids in length (Harwood et al 2010; Handlogten et al 2013).

Finally, an appropriate exposure assessment, on a case by case basis, is envisaged to allow a more reliable and accurate allergenicity assessment

Interim phase for refining the conditions of *in vitro* digestion tests

General considerations:

During the interim phase, an objective measurement of the extent of digestion should provide more comparable results. This includes the definition of transient and persistent digestion fragments together with (at least) semi-quantitative assessment of the proportion of such fragments in a digest. The determination of persistent peptide fragments which are ≥ 9 amino acids in length is critical, since these may indicate that further assessment is required.

Specific and key considerations:

A) Digestion model conditions: Since the physiological process of digestion is inherently dynamic, *in vitro* batch models of digestion inevitably restrict the range of conditions that exist *in vivo*. To better reflect the range of conditions found *in vivo*, without overly increasing the complexity of the test, it is proposed that a minimum of two gastrointestinal test conditions should be used to reflect the range found *in vivo*. The classical pepsin-resistance test could form one part of this testing scenario, having the added value of providing a comparative data on digestion resistance of newly expressed proteins. The pH conditions employed in the *in vitro* digestibility tests range from 1.2-4.2 with many using pH conditions of 1.2 or 2.0, the former based on the simulated gastric fluid conditions detailed in the US Pharmacopeia (1995) and used for drug dissolution tests. However, intragastric pH conditions found *in vivo* tend only to go down to around pH 2 towards the end of gastric emptying, and are generally much higher because of the buffering capacity of many foods (Kalantzi et al., 2006). Concerning pepsin:protein ratio, many reports only describe it on a (w:w) basis and do not take differences in enzyme activity into account. However, the two available ring-trialled studies of digestibility tests for purified proteins used a ratio of 10 U (Thomas et al., 2004) and 0.165 U (Mandalari et al., 2009b) of pepsin per μg of tested protein, respectively. With regards the duration, test material is typically exposed to gastric digestion for 60 min followed by intestinal digestion for 30-60 min with corresponding intermediate sampling time points (Mandalari et al., 2009b; Dupont et al., 2010). Different durations of digestion have been proposed, based on digestion of whole foods (Minekus et al., 2014). Adaptation and integration of these approaches could be undertaken to provide the conditions to be used in a refined *in vitro* digestion test.

Although a flexible framework for performing the digestion tests is considered in line with the current considerations, the following recommendations should ensure consistency between the different laboratories undertaking the tests:

i) Specification of digestive enzymes (e.g., pepsin and intestinal endoproteases). Source, purity and specific activities should be determined using standardised protocols. Individual intestinal enzymes (i.e., trypsin and chymotrypsin) should be prioritised over the use of pancreatin. In the case of using pancreatin, proteolytic, lipolytic and amylolytic activity of

the extract should be determined and the amount of pancreatin added should be based on the trypsin activity. The use of brush border enzymes such as amino/carboxypeptidases could also be considered;

ii) Enzyme concentrations used in digestion tests should be specified and could be based on those previously used interlaboratory studies performed with purified proteins (Thomas et al., 2004; Mandalari et al., 2009b);

iii) Different gastric pH values should be specified which reflect those found in infants, adults, elderly or people with impaired digestive functions;

iv) The addition of bile salts and biosurfactants (phospholipids) at physiologically relevant levels should be considered.

It is proposed that, at a minimum, two gastrointestinal digestion test conditions should be considered. These should encompass the most extensive (including the current pepsin test conditions) and the less extensive digestion conditions, reflective of those found in children or individuals taking antacid medication.

1.- Low pH / High [pepsin] (these conditions could include those used in the classical pepsin resistance test) followed by intestinal conditions.

2.- High pH / Low [pepsin] followed by intestinal conditions.

Replication of the *in vitro* digestion experiments should also be implemented to obtain more reliable and statistically significant results.

B) Control proteins: Digestion studies should be performed including control proteins to demonstrate the effectiveness of the digestion system employed and allow benchmarking of different digestion models. The control proteins are not to provide an indication of allergenicity, but rather reflect the different susceptibilities of proteins to gastrointestinal digestion.

For proteins to act as appropriate controls in the *in vitro* digestion tests they must be:

1.- Either commercially available, and/or purified in reasonable quantities using published methods and made available for use by the community (e.g., either as quality control (QC) or reference materials).

2.- Well characterised with regards their primary sequence, post-translational modifications (if any) and physicochemical state (e.g. size, oligomerisation, pI, hydrophobicity, ligand binding/prosthetic group).

3.- Previously subjected to *in vitro* digestion tests allowing their susceptibility to digestion to be classified as either highly resistant, moderately resistant or labile to the action of digestive enzymes.

One protein which meets these criteria, has been extensively tested in digestion tests and was found to be extremely resistant to gastric digestion is bovine β-lactoglobulin (Reddy et al., 1988; Schmidt et al., 1995; Astwood et al., 1996; Yagami et al., 2000; Fu et al., 2002; Takagi et al., 2003; Thomas et al., 2004; Sanz et al., 2007; Herman et al., 2007; Lucas et al., 2008; Ofori-Anti et al., 2008; Macierzanka et al., 2009; Mandalari et al. 2009a, 2009b; Misra et al., 2009; Dupont et al., 2010; Zheng et al., 2010; Bogh et al., 2013). β-lactoglobulin has also been reported to be relatively stable under gastrointestinal digestion conditions (Mandalari et al. 2009a, 2009b; Dupont et al., 2010; Borgh et al., 2013). On the other hand, phosphofructokinase and/or sucrose synthetase could also be recommended as control proteins because both enzymes have been described to be rapidly digested under simulated gastric (Astwood et al., 1996; Fu et al., 2002) and intestinal conditions (Fu et al., 2002).

C) Digestion end-points and read-out considerations: The terms "persistent" and "transient" are used for classification of proteins and peptides with different kinetic behaviour and for fragments in relation to their rate of formation as well as their rate of further degradation.

Kinetic studies are funded on following the time course of a reaction or process and hence there is a necessity for the digestion tests to take the form of time-course experiments. Consequently, sampling should be undertaken at various time points during the gastric and intestinal digestion steps, to allow the evolution of peptide fragments to be monitored. Sampling time points should be selected which are appropriate and will allow transient and persistent peptides to be distinguished based on kinetic parameters.

Standardised methodology for monitoring protein digestion needs to be used which is suitable for profiling of both large resistant fragments and lower molecular weight peptides of ~1,000 Da (the average mass of a 9 residue peptide fragment). Techniques should also allow at least semi-quantitative profiling of residual intact protein and digestion products. Traditionally, protein digestibility has been measured using SDS-PAGE. However, whilst providing valuable data especially for intact proteins and large resistant fragments, this technique is essentially qualitative in nature, can provide inconsistent results between laboratories and is not an appropriate technique to carry out reliable quantification of peptide fragments. Tandem mass spectrometry, even with caveats with regards peptide ionisation efficiency, is a more effective tool to carry out a comprehensive peptide mapping of digesta and identify stable digestion fragments ≥ 9 amino acids in length. Since at present no single methodology can readily characterise the digestion of both proteins and peptides effectively, a combination of the best available methodology, such as SDS-PAGE and mass spectrometry, should be used. These techniques can provide at least a semi-quantitative output of digestion following disappearance of the intact protein and appearance of digestion resistant fragments.

Replicate analysis should be undertaken using two biological replicates (i.e. digestion tests) being analysed in duplicate, which is considered the minimum. Best laboratory practice should be addressed for any methodology such as use of standards, use of appropriate protein stains able to provide a broad dynamic range and stain many proteins, molecular weight markers (SDS-PAGE) or internal peptide standards spanning range of masses (mass spectrometry).

Special attention should be paid to the pre-treatment of the digesta before analysis (e.g., avoidance of reducing conditions or derivatization of the sulfhydryl groups of cysteine residues, if possible) to remain as close as possible to physiological conditions. It is necessary to not only take the presence of covalent linkages (such as disulphide bridges) into account, but also non-covalent interactions, which may lead to smaller peptides assembling to complexes of higher molecular weight and size.

D) Classification of digestion resistant proteins/peptides: A consensus definition of transient and persistent proteins and peptides is required that can apply to the different *in vitro* digestion test conditions. Control proteins accepted to be highly resistant, moderately resistant and highly digestible could be used to support development of such a definition. To do that, two approaches (individually or in combination) based on the objective measurement of the extent of digestion could be explored:

- The half-life of the intact protein and resulting peptide fragments could be determined and used to establish definitions of transient and resistant peptides (Shan et al., 2002; Baderschneider et al., 2002, Herman et al., 2007; Ofori-Anti et al., 2008; Macierzanka et al., 2009; Defernez et al., 2010; Yao et al., 2013; Smith et al., 2015).

and/or

- Based on the mass spectrometric/gel electrophoresis detection/visualisation of any peptide generated throughout the digestion monitoring (performed at every sample point). In this case, there is no need of peptide quantification (only its constant detection and/or visualisation). Factors to be considered for persistence: i) detection/visualisation of the target peptide(s) in at least two sampling points (that is, constant mass/band) and ii) detection/visualisation of the target peptide(s) in the intestinal phase.

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1508 **Figure B1.** Illustrative examples of *in vitro* gastrointestinal test conditions and proposed gastric conditions.
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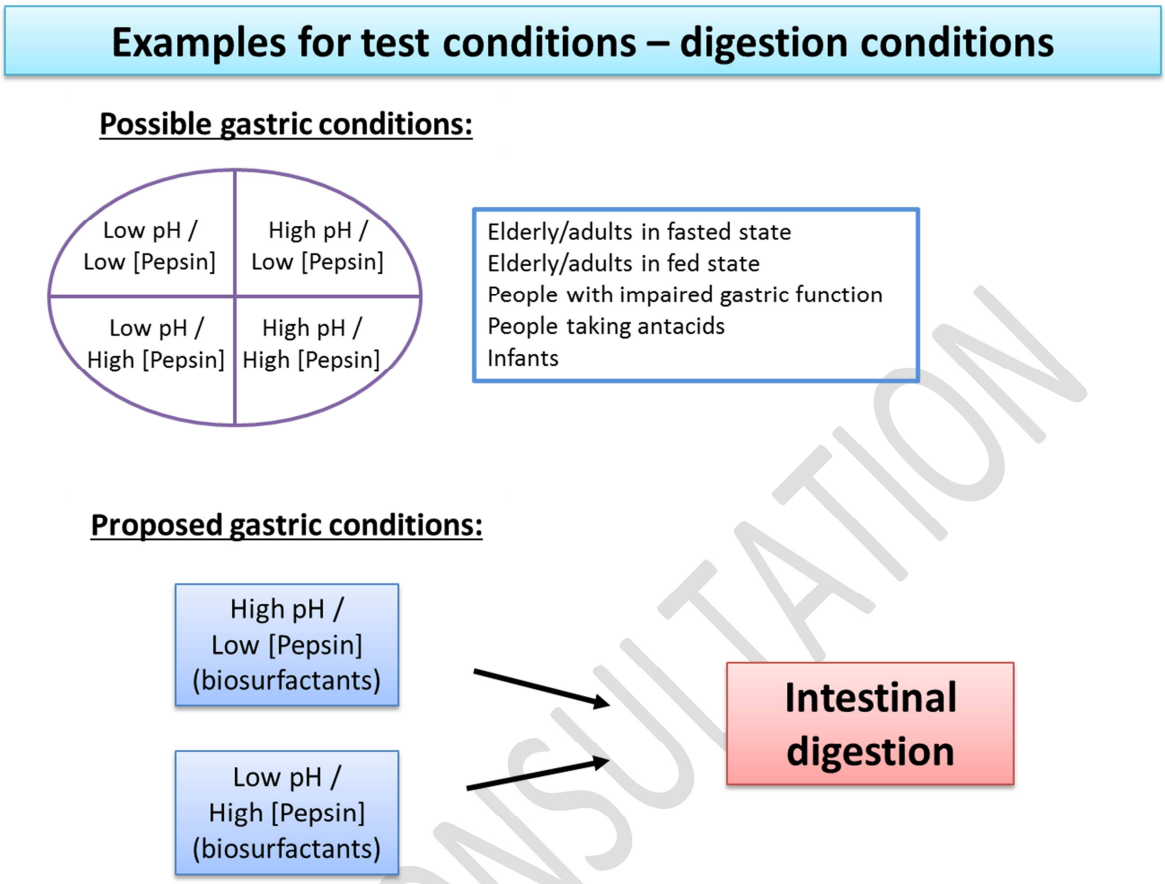

1510 **Examples: Blanquet et al. 2001, Dupont et al. 2010, Moreno et al. 2005, Mandalari et al. 2008, Mandalari et al. 2009b, Mercuri et al. 2011, Minekus et al. 2014**

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1513 **Figure B2.** Example of possible scenarios resulting from the *in vitro* digestibility tests and subsequent data interpretation.
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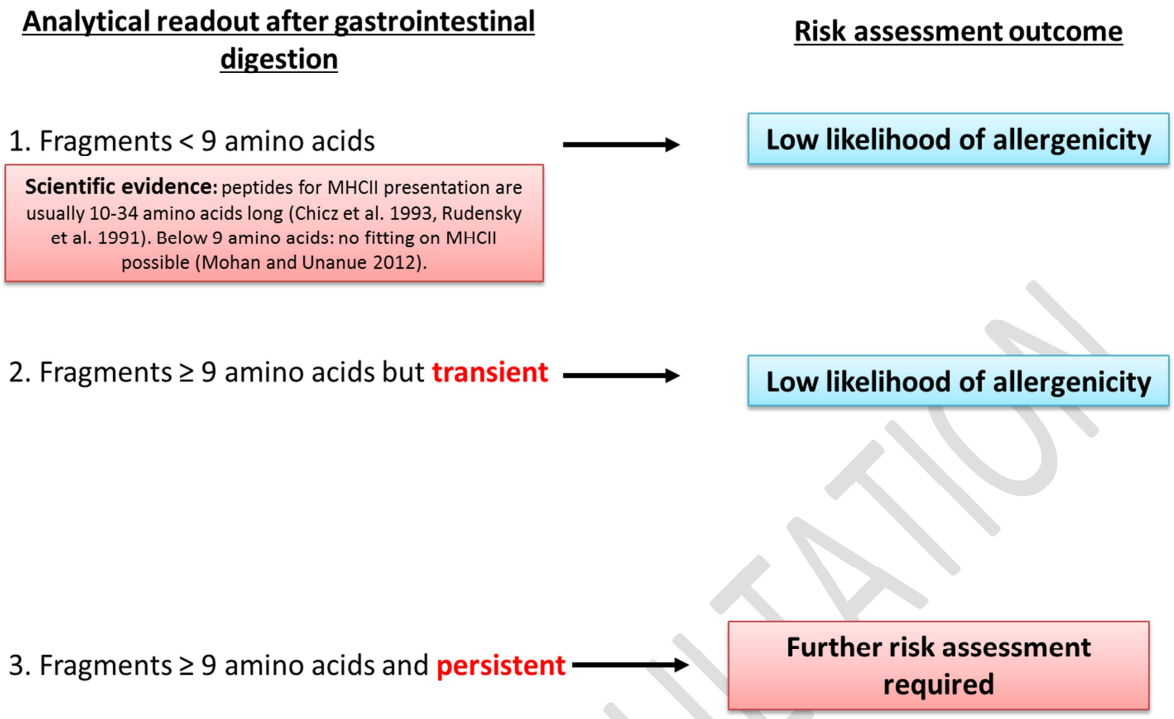

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Annex C – Endogenous allergenicity

Annex C-1. History of endogenous allergenicity assessments

Scientific Opinions including an assessment of endogenous allergenicity have been published by the EFSA GMO panel (e.g. EFSA, 2007, 2011, 2015). Historically, the assessment was carried out using sera from allergic individuals, e.g. using 2D gel electrophoresis (immunoblot) in combination with spot quantification. More recently however, novel absolute quantification methods have been developed and applicants employed those in the latest applications submitted to EFSA. Until now and considering the EFSA GMO Panel Scientific Opinions published, no significant qualitative or quantitative change in the allergen repertoire has been observed in the GM plants assessed. So far it was concluded that there was no evidence that the genetic modification might significantly change the overall allergenicity of the GM plant when compared with that of its conventional counterpart.

Annex C-2. Possible approach for selection of soybean allergens to be analysed

The OECD list of "potential allergens" (Table 20 of OECD, 2012) which is based on allergens listed in the WHO/IUIS database (2011 www.allergen.org) and a review (L'Hocine and Boyne 2007) was taken as a basis, and further complemented with the updated WHO/IUIS-, as well as other allergen databases, including the FAARP allergen database of the University of Nebraska-Lincoln (www.allergenonline.org) and Allergome (http://allergome.org/). Sequence information was additionally reviewed using the NCBI and Uniprot databases. Other allergen databases, listed in EFSA 2010 scientific opinion (EFSA, 2010), were considered, but not screened in detail since they did not provide any additional information or were no longer available.

Using these sources, data on individual "potential allergens" and on the allergic individuals tested were retrieved. Furthermore, evidence was collected connecting clinical reactivity to total soy preparations with data on specific allergens, as well as possible clinical reactions to single purified allergens (either isolated from the plant or produced as recombinant allergens). To date, clinical data for soy allergy have been obtained by double blind placebo controlled food challenge (DBPCFC) using whole soybean protein extract or soy flour (Ballmer-Weber et al. 2007), while only little clinical research is available for single proteins and it is mostly restricted to component resolved diagnostics (Tuano and Davis 2015). The available information retrieved on the soybean "potential allergens" can be found in Table C1.

It is challenging to unequivocally connect clinical data obtained on soy extracts by DBPCFC with reactivity to single proteins. In this particular example, taking the information collected for Table C1 as starting point and following the strategy proposed above, defined criteria were applied for a possible selection of relevant allergens. WHO/IUIS defines criteria that a protein should meet to be included in the allergen list. Primary criteria to include a protein among the allergens in the WHO/IUIS database are i) a minimum of 5 sera from allergic individuals (allergic to the allergen source in question) have to contain IgE which binds to the protein in question, or ii) at least 5% of the allergic individuals' sera tested react with the allergen in question by IgE binding. It is noteworthy that WHO/IUIS was the main basis for listing allergens in the OECD document on soybean. WHO/IUIS is also mentioned as a key reference for listing allergens in the EFSA NDA Panel opinion on food allergens (See section 8 of EFSA NDA Panel opinion 2014). WHO/IUIS committee meetings deciding on the inclusion of a new allergen are held in the frame of EAACI and AAAAI international allergy meetings. It is noted that since an application has to be filed in order to include a protein into the WHO/IUIS database, not all proteins potentially fulfilling the requirements are included in the current database. In our example approach following the current WHO/IUIS criteria, the following allergens listed in Table C1 are also part of the WHO/IUIS database and should be measured accordingly: Gly m 1, Gly m 2, Gly m 3, Gly m 4, Gly m 5, Gly m 6, Gly m 7 and Gly m 8. Out of these, Gly m 2 can currently not be measured since the sequence is unknown. The following potential allergens fulfil the primary WHO/IUIS criteria of required tested allergic individuals, but did not undergo an expert peer-review by the committee of WHO/IUIS: Gly m Bd 28 K, Gly m Bd 30 K, soybean lectin, lipoxygenase, Kunitz trypsin inhibitor and Gly m 50 KD. Out of these proteins, for Gly m Bd 28 K, Gly m Bd 30 K and Kunitz trypsin inhibitor considerable peer-reviewed literature is available and endogenous allergenicity measurement was

previously suggested by other scientists (Ladics et al. 2014). In contrast, for soybean lectin and lipoxygenase, evidence for involvement in soy allergy is more scarce and further revision by an independent expert panel is suggested. Gly m 50KD is currently not measurable since the sequence is unknown. The other listed proteins, Gly m 39KD, P22-25, Gly m CPI, Gly m EAP and the "unknown possible allergen" currently do not fulfil the primary WHO/IUIS criteria and measurement would therefore not be performed at the current time. However, more evidence might be available in the future. It should be noted that the above stated considerations i) might be incomplete and ii) cannot exclude a revision of the progress in science at any time in the future, which might considerably change the potential allergens suitable for assessment.

Even though food allergy to soybean is known in animals (Suto 2015, Kang 2014) it is noted that only little data is available on relevant individual soybean allergens in animals. However, certain proteins known to cause soybean allergy in humans (e.g. Gly m 5, Gly m 6) were also causing reactions in calves, piglets, dogs and other animals (Taliercio 2014, Lalles 1996, Dreau 1994).

Annex C-3. Methodology

As described, it is not recommended to use polyclonal antisera of animals raised against whole soy extract. It should be considered that limited immunogenicity of some allergens as well as restricted specificity, affinity and concentration of the antibodies present in serum samples might lead to over- or underrepresentation of some allergens, and some might not even be detected at all.

Helpful mass spectrometry protocols for the assessment of endogenous allergenicity of soybean have been developed for some potential allergen molecules (Kuppannan 2014, Stevenson 2012, Houston 2011). Further development and standardisation of protocols is encouraged, including analysis of all allergens posing a potential risk.

Annex C-4. Data interpretation and risk assessment considerations

According to IR503/2013, after comparison to a conventional counterpart within the comparative analysis, natural variability is currently considered the main tool to identify significant and potential relevant changes in allergen content. The European Commission clarified this issue further[4].

Currently, the experimental field design of an application for comparative compositional analysis requires at least six non-GM reference commercial varieties - the selection of which should be justified according to defined criteria (see Section 1.3.2 of IR503/2013). These varieties are used to estimate the overall natural variability to which consumers are routinely exposed. The application of such an approach allows an objective comparative evaluation independently of the absolute content of endogenous allergens ensuring a high degree of protection for consumers.

It is recognised that the natural variability of any given endogenous allergen content estimated from the reference commercial varieties, even if appropriately selected, may not capture the full range of its variability, which is currently unknown. Further efforts in such respect are encouraged, as described in Section 2.3.3.

Finally, specific considerations associated with the particular allergenic risk have to be taken into account. As an example, it could be considered:

- Exposure considerations to a certain allergen might be taken into account as a last step in the risk assessment process with particular interest in understanding levels of allergens in foods derived from soybean varieties consumed in Europe by humans and animals at a given time;
- Efforts to reduce the uncertainty could be anticipated according to considerations based on the single allergen in question for which enhanced allergen content has been encountered and for which there is a potential increase in allergenicity to be communicated to risk managers. The relevance of increase might be evaluated with the help of clinicians, concerning a) the

[4] Letter from European Commission to EuropaBio (Ref. SANCO/El/SP/mb sanco.ddg2.e.l(2014)l 140685) where it was clarified that "allergens included in the compositional analysis should be treated as any other compound meaning that allergens in the reference varieties included in the assessment should also be analysed (so that an equivalence test as well as a different test can be performed)".

magnitude of the increase, b) the potency of the increased potential allergen in question, c) the overall percentage of the allergen in the non-GM crop and the consequent possible impact of an increase, d) the route how the allergen is encountered by the allergic individual, e) how frequent the potential allergen in question is present in various products;

- Depending on the level of uncertainties identified and, in the case of need, an allergenicity comparison of a GM plant versus its appropriate comparator might be carried out by DBPCFC. This would imply to perform a clinical study comparing the reactivity of selected allergic individuals to the varieties under assessment. Therefore, well-characterised allergic individuals reacting with the allergen in question would have to be challenged;
- As a future perspective, the probability of elicitation of an allergic reaction might be further investigated with the help of dose-distribution curves (Ballmer-Weber 2015) obtained by DBPCFC to single allergens (Kinaciyan 2016) and with reference values (e.g., threshold of elicitation) more precisely evaluated.

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