

STATEMENT

ADOPTED: 26 November 2020

doi: 10.2903/j.efsa.2021.6350

Statement on *in vitro* protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants

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Abstract

This statement supplements and updates the GMO Panel guidance document on allergenicity of genetically modified (GM) plants published in 2017. In that guidance document, the GMO Panel considered that additional investigations on *in vitro* protein digestibility were needed before providing any additional recommendations in the form of guidance to applicants. Thus, an interim phase was proposed to assess the utility of an enhanced *in vitro* digestion test, as compared to the classical pepsin resistance test. Historically, resistance to degradation by pepsin using the classical pepsin resistance test has been considered as additional information, in a weight-of-evidence approach, for the assessment of allergenicity and toxicity of newly expressed proteins in GM plants. However, more recent evidence does not support this test as a good predictor of allergenic potential for hazard. Furthermore, there is a need for more reliable systems to predict the fate of the proteins in the gastrointestinal tract and how they interact with the relevant human cells. Nevertheless, the classical pepsin resistance test can still provide some information on the physicochemical properties of novel proteins relating to their stability under acidic conditions. But other methods can be used to obtain data on protein's structural and/or functional integrity. It is acknowledged that the classical pepsin resistance test is embedded into international guidelines, e.g. Codex Alimentarius and Regulation (EU) No 503/2013. For future development, a deeper understanding of protein digestion in the gastrointestinal tract could enable the framing of more robust strategies for the safety assessment of proteins. Given the high complexity of the digestion and absorption process of dietary proteins, it is needed to clarify and identify the aspects that could be relevant to assess potential risks of allergenicity and toxicity of proteins. To this end, a series of research questions to be addressed are also formulated in this statement.

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Keywords: allergenicity assessment, pepsin test, *in vitro* digestion, protein safety, newly expressed proteins, GMO

Requestor: EFSA

Question number: EFSA-Q-2020-00314

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Acknowledgements: The Panel wishes to thank the members of the Working Group on Allergenicity, the members of the Stakeholder Consultative group 'Focus Group', the hearing experts Clare Mills and Henk van Loveren, and the EFSA staff Anna Lanzoni and Elisabeth Waigmann for the support provided to this scientific output.

Suggested citation: EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Naegeli H, Bresson J-L, Dalmay T, Dewhurst IC, Epstein MM, Firbank LG, Guerche P, Hejatko J, Moreno FJ, Mullins E, Nogué F, Rostoks N, Sánchez Serrano JJ, Savoini G, Veromann E, Veronesi F and Fernandez Dumont A, 2021. Statement on *in vitro* protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants. EFSA Journal 2021;19(1):6350, 16 pp. <https://doi.org/10.2903/j.efsa.2021.6350>

ISSN: 1831-4732

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Summary

This statement addresses *in vitro* protein digestibility tests in allergenicity and protein safety assessment of genetically modified (GM) plants. The main focus was to supplement and update the GMO Panel guidance document on the allergenicity of GM plants (EFSA GMO Panel, 2017) by reviewing the recent scientific literature on the usefulness of *in vitro* protein digestion in allergenicity risk assessment and protein safety, and data from the EFSA external report published by Mackie et al. (2019).

Data from the classical pepsin resistance test measuring resistance to degradation by pepsin has been used in a weight-of-evidence approach to assess the potential allergenicity and toxicity of newly expressed proteins in GM plants. However, there is debate on its predictive value for protein allergenicity, although the classical pepsin resistance test provides information on certain physicochemical properties of novel proteins with implications for their stability, structure and function. Nevertheless, other methods can be used to obtain data on protein's structural and/or functional integrity. The evidence supporting the resistance to degradation by pepsin as a direct predictor of allergy is weak with several publications describing the pepsin test as a poor predictor of allergenicity. It is also acknowledged that the classical pepsin resistance test is embedded into international guidelines, such as Codex Alimentarius and Regulation (EU) No 503/2013.

There is a need for more reliable systems to predict the fate of the proteins in the gastrointestinal (GI) tract and how they interact with the relevant cells in the human body. Resistance to protein digestion using GI models might contribute to the protein safety assessment because most dietary proteins are digested into small peptides and amino acids. Thus, a better understanding of protein–GI tract interactions might build a more robust assessment strategy. It is generally accepted that a protein which is completely digested in the GI tract would not be absorbed as an intact and functionally active molecule and is unlikely allergenic. Proteins that are resistant to digestive proteolysis might be more effective at stimulating the immune system and might be absorbed by small intestine epithelial cells in an active form that could be potentially toxic. However, a protein is not necessarily allergenic or toxic if it undergoes slow or limited protein digestibility.

It is necessary to fully understand the complexity of digestion and absorption of dietary proteins to develop GI models to assess the usefulness of corresponding *in vitro* tests for protein allergenicity and toxicity. For example, it is necessary to understand what the usefulness of *in vitro* digestion is in the overall protein safety assessment; what the most suitable *in vitro* digestion models are; what constitutes an adequate test item (e.g. purified proteins vs whole food and/or representative food extracts); what criteria should be used to identify digestion fragments as relevant for the risk assessment (size, abundance, persistence, etc.); what follow-up actions would be required to assess the relevant proteins/fragments identified in previous steps; and, finally, how this information can be integrated into a weight-of-evidence approach. Addressing these and other issues are essential for considering *in vitro* digestion into the overall safety assessment of proteins and will be the basis of a subsequent EFSA scientific opinion which will be published in 2021.

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1. Introduction

1.1. Background

In 2017, the scientific Panel on Genetically Modified Organisms of the European Food Safety Authority (hereafter referred to as the 'GMO Panel') published a supplementary guidance document on allergenicity risk assessment of genetically modified (GM) plants addressing non-IgE-mediated adverse immune reactions to foods, *in vitro* protein digestibility tests and endogenous allergenicity of plant constituents (EFSA GMO Panel, 2017). In relation to *in vitro* protein digestibility, the GMO Panel considered that additional investigations were needed before providing any additional recommendations in the form of guidance to applicants. Thus, an interim phase was proposed to assess the utility of an enhanced *in vitro* digestion test, with a review undertaken in the final year. As a consequence, in July 2017, EFSA launched a procurement call entitled: '*In vitro* protein digestibility'. The aim of the project was to outsource the protocol development and the production of experimental data using a refined *in vitro* protein degradation test, following the principles described in the EFSA GMO Panel supplementary guidance document (Annex B of the EFSA GMO Panel, 2017). The outcome of the EFSA procurement has recently been published in the EFSA website¹ (Mackie et al., 2019).

In accordance with the procedure described in the EFSA GMO Panel supplementary guidance document (EFSA GMO Panel, 2017), an ad hoc Allergenicity working group of the GMO Panel has been established to address to what extent the *in vitro* digestion test adds value to the allergenicity risk assessment of GM plants and to the protein safety assessment in general, and what further actions are needed to move forward this field of risk assessment. Furthermore, the Allergenicity working group will also focus on current gaps in knowledge and on future development needs for the overall allergenicity and protein safety assessment. In this context, the first action of this working group is the publication of this current statement addressing the usefulness of *in vitro* protein digestion in allergenicity and protein safety assessment. Subsequently, a scientific opinion on recommendations for future developments in the field of allergenicity assessment (research needs), and protein safety, in general, will be delivered by the GMO Panel in 2021.

1.2. Terms of Reference

The European Food Safety Authority (EFSA) asked the Panel on Genetically Modified Organisms (GMO Panel) to develop a GMO Panel statement on *in vitro* protein digestibility tests in allergenicity and protein safety assessment of GM plants. This statement supplements and updates the GMO Panel guidance document on allergenicity of GM plants (EFSA GMO Panel, 2017), considering the information from the EFSA external report published last year (Mackie et al., 2019) and the state-of-the-art in science on the topic.

2. Data and methodologies

2.1. Data

In delivering this statement, the EFSA GMO Panel took into account the considerations on *in vitro* protein digestion described in its latest guidance document on allergenicity (EFSA GMO Panel, 2017) and information from the EFSA external report on *in vitro* protein digestion recently published (Mackie et al., 2019), including related scientific publications retrieved from the public domain. The GMO Panel also considered comments raised by a Stakeholder Consultative Group following the activities of the GMO Panel Allergenicity working group.²

2.2. Methodologies

The GMO Panel considered the principles described in its guidance documents and scientific opinions on allergenicity (EFSA GMO Panel, 2010, 2011, 2017), Regulation (EU) No 503/2013³ and other relevant international guidelines (Codex Alimentarius 2003, 2009).

¹ <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2019.EN-1765>

² <https://www.efsa.europa.eu/en/news/allergenicity-assessment-gm-plants-stakeholders-support-working-group>

³ 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 Text with EEA relevance OJ L 157, 8.6.2013, p. 1–48.

3. Assessment

Resistance to degradation by pepsin using the classical pepsin resistance test (Astwood et al., 1996) has been considered as an additional element, together with other relevant information, in a weight-of-evidence approach for the assessment of novel proteins introduced into GM plants (Metcalf et al., 1996). The pepsin resistance test was intended to evaluate the susceptibility of the protein to digestion under fixed conditions *in vitro*, as a relevant parameter for the identification of potential allergens in risk assessment (FAO/WHO, 2001). Codex Alimentarius initially introduced this test in an international context in 2003 where stability to degradation by representative gastric/intestinal proteases and model systems was considered relevant not only for the allergenicity but also the toxicity assessment (Codex Alimentarius, 2003, 2009). As a consequence, this test has been embedded in other international guidance documents^{3,4} (EFSA GMO Panel, 2011). Since 1996, adaptations to the original classical pepsin resistance test have been implemented to provide information on the susceptibility of a protein to digestion in a more reproducible manner (Thomas et al., 2004). However, the direct correlation between resistance to pepsin digestion and allergenicity *per se* is uncertain (Kenna and Evans, 2000; Fu, 2002; Fu et al., 2002; Herman et al., 2007; EFSA GMO Panel, 2017; Costa et al., 2021). Furthermore, the principles of the classical pepsin digestion protocol do not mimic the physiological conditions of the human gastric digestion (EFSA GMO Panel, 2011, 2017; Bøgh and Madsen, 2016). All these elements have raised questions on the overall utility of the classical pepsin resistance test in the risk assessment of proteins. At the time, Codex Alimentarius (2003, 2009) indicated that alternative protocols to the classical pepsin resistance test, aimed to assess susceptibility to digestion by proteases, could be used when adequate justification is provided.

EFSA previously highlighted the limitations of the classical pepsin resistance test in protein safety assessment, and recommended that *in vitro* protein digestibility could be evaluated using other *in vitro* digestibility methods designed to more closely simulate the conditions of the human digestion process (EFSA GMO Panel, 2010, 2011, 2017). This has prompted the development of alternative protocols aimed at improving the knowledge of proteins behaviour during the digestive process (EFSA GMO Panel, 2017; Mackie et al., 2019; Torcello-Gómez et al., 2020a,b).

In this statement, the GMO Panel considers the information on *in vitro* protein digestion described in its latest guidance document on allergenicity (EFSA GMO Panel, 2017) and information from an EFSA external report on *in vitro* protein digestion (Mackie et al., 2019). In addition, the GMO Panel also reviews recent scientific literature dealing with the usefulness of *in vitro* protein digestion in allergenicity risk assessment and the overall protein safety assessment, framing recommendations for future development of a field with great potential.

3.1. Protein digestibility and relevance to risk assessment

The intestinal tract is the largest mucosal surface of the human body. It is lined by a single layer of columnar intestinal epithelial cells that forms a barrier between the intestinal lumen and host connective tissue and strongly participates in innate immunity (Artis, 2008). Virtually all nutrients from the diet are absorbed into the bloodstream across the epithelial cell layer forming the small intestinal mucosa (Kiela and Ghishan, 2016). Oral exposure to dietary proteins, as a general class of substances, is a necessary physiological process. Nevertheless, a very small number of food proteins have been identified to be capable of causing adverse effects, in terms of allergy or toxicity when consumed and these effects may be observed within the gut (Markell et al., 2017) and/or at extraintestinal sites, such as those food allergens responsible for the oral allergy syndrome (Carlson and Coop, 2019).

Resistance to digestion by pepsin and pancreatic endoproteases is informative for protein safety assessment because the normal fate of most dietary proteins is digestion into small peptides and individual amino acids which pass into the systemic circulation via transcellular or paracellular routes (Caspary, 1992). This reinforces the fact that digestion affects exposure to intact protein. Codex Alimentarius (2003, 2009) refers to protein *in vitro* digestion tests as relevant studies for assessing protein safety, mainly allergenicity but also protein toxicity. On the one hand, the generally accepted position is that if a protein is completely digested in the GI tract, the potential for its absorption from the digestive tract as an intact and functionally active macromolecule would be in most cases negligible, minimising the likelihood that the protein could exert adverse effects such as allergenicity (Pali-Schöll et al., 2018) and/or toxicity (Hammond et al., 2013; Tafazoli et al., 2019). On the other

⁴ <http://www.fao.org/food/food-safety-quality/gm-foods-platform/en>

hand, evidence of slow or limited protein digestibility does not indicate on its own that the protein is necessarily allergenic or toxic, but proteins that are at least partially resistant to digestive proteolysis might be more effective at stimulating the immune system because possible immunogenic structures are persisting longer in the gut (Shan et al., 2002; Fernandez et al., 2019; Pilolli et al., 2019) and might be absorbed by epithelial cells of the small intestine in an active form in terms of potential toxicity (Delaney et al., 2008). Therefore, studies on resistance to digestion are considered as additional evidence together with other elements in a weight-of-evidence approach, mainly informing the hazard identification step, for the allergenicity and toxicological assessment of newly expressed proteins (Codex Alimentarius, 2003, 2009; EFSA GMO Panel, 2011, 2017; European Commission, 2013). Protease resistance of food proteins can also be a useful tool for assessing exposure (Akkerdaas et al., 2018).

Digestion and absorption involve complex, highly integrated and regulated physiological processes. Key host factors include, for instance, the interaction of the digesta with the gut epithelial cells and the integrity of the intestinal epithelial barrier which determine the transport of nutrients. Likewise, a number of extrinsic factors, such as the presence of particular matrix components (e.g. fibre, lipids, polyphenols, anti-nutritive factors), food microstructure or food-processing conditions, may affect protein digestibility in the GI tract and these may be of importance when designing an *in vitro* model (Ekmay et al., 2017). Therefore, the simulation of such a complex system in its entirety using static unresponsive *in vitro* methods is very challenging (Butts et al., 2012), and the application of *in vitro* digestibility assays within a risk assessment frame should be tempered.

Given the marked differences between the mechanisms underlying allergen- and toxin-induced adverse reactions, as well as between the inherent properties of potential allergens and toxins, the relevance of protein digestibility in the allergenicity and toxicity risk assessment processes will be separately discussed below.

3.1.1. Digestibility and the allergenicity risk assessment process

Food allergy consists of two separate phases: i) sensitisation, a phase where the immune system develops hyperreactivity to the allergen, but no symptoms occur; and ii) elicitation, a phase where clinical manifestations are triggered (Fernandez et al., 2013; Selb et al., 2017). One of the main arguments questioning the relevance of protein stability and resistance to proteolytic digestion to the allergenicity risk assessment process, is that the evidence demonstrating that *de novo* sensitisation takes place through oral exposure via the gut immune system is weak or even missing (Herman and Ladics, 2018). Therefore, this could make digestive protein stability a poor predictor of sensitisation (Herman et al., 2020). Although it has been shown that impairing gastric digestion exerted by different means (e.g. suppression of gastric acid secretion, bariatric surgery) increases the risk for allergic sensitisation (Pali-Schöll et al., 2018), *de novo* allergies to plant and animal food allergens can be induced by inhalation (Ramirez and Bahna, 2009) or the dermal route (Kimber et al., 2014; Brough et al., 2020).

Nevertheless, the distinction of IgE antibody-mediated responses to ingested food proteins compared to those responses induced by inhalation or dermal contact has been considered artificial. This is because, although there may be route-dependent differences in response, there appears to be a substantial overlap in allergens of significance across routes of exposure (Blackburn et al., 2015). This is in line with the fact that allergic sensitisation to food proteins can be achieved by any physiologic route of exposure in the presence of an appropriate environment (exogenous adjuvant) (Dunkin et al., 2011). Likewise, it has been shown that non-GI exposure to food proteins, i.e. via the cutaneous or respiratory route, may facilitate a subsequent sensitisation via the oral route without the use of adjuvants (Wavrin et al., 2014, 2015). On the one hand, survival in the GI tract of stable protein/peptide fragments would facilitate the maintenance of epitope expression and thereby facilitate the development of sensitisation (Pekar et al., 2018; Krutz et al., 2020); however, this attribute can be ascribed to some food allergens, but not to all of them (Bøgh and Madsen, 2016; EFSA GMO Panel, 2017; Fernandez et al., 2019; Verhoeckx et al., 2019). On the other hand, recent evidence has shown the capacity of the intestinal microbiota to metabolise food protein antigens or allergens, thereby, altering their immunogenicity (Caminero et al., 2019) or to produce metabolites that directly affect tolerogenic responses (Smith et al., 2013).

Notwithstanding the role of route of exposure in sensitisation as described above, understanding how proteins are presented to the gut mucosal immune system may provide insight into how the balance between tolerance and sensitisation is regulated (Dearman et al., 2002; EFSA GMO Panel,

2017). When characteristics of known food allergens are examined, one of the most prominent traits attributed to food allergens is protein stability (Helm, 2001; Breiteneder and Mills, 2005; Costa et al., 2021). The term protein stability encompasses several properties, including not only proteolytic stability but also thermal stability, pH-dependent stability and physical stability (e.g. tendency to aggregate), among others (Li et al., 2019). Consequently, protein stability to digestion is one of several relevant parameters to consider in the weight-of-evidence approach (EFSA GMO Panel, 2010, 2011, 2017). The allergenicity of proteins is likely determined by several factors, such as the relationship between physicochemical and structural properties governing protein stability (James et al., 2018), abundance, size, point in time of exposure and duration of exposure to the immune system. Environmental conditions also need to be considered, including microbial exposure, immune-modulating components (adjuvant effect) of allergenic sources facilitating T helper 2 (Th2) immune responses, as well as the intrinsic effects of proteins on the innate and adaptive immune system (Scheurer et al., 2015).

Food allergens with high stability to digestion and processing are able to reach the GI immune system in their IgE-reactive form and to elicit more severe reactions (Valenta et al., 2015). However, recent studies have demonstrated that some food allergens, when digested to small peptide fragments, may still retain both the sensitising and the IgE-reacting potential. This is because of the peptides' ability to hold together and to adopt three-dimensional structures similar to that of the native allergen under certain conditions (Prodic et al., 2018). For instance, post-translational modifications, such as disulphide bond formation (Apostolovic et al., 2016) or hydroxylation of proline residues (Bernard et al., 2015), as well as physicochemical properties that promote peptide aggregation (Bøgh et al., 2009, 2012; Radosavljević et al., 2020) have been identified as important determinants for preserving allergenic properties in digestion-resulting peptides derived from allergens found in plant- and animal-based food sources.

Finally, protein digestibility is also central for the risk assessment of celiac disease where GI digestion is important in the delivery of immunologically active fragments to gut mucosal segments (Shan et al., 2002; Pilolli et al., 2019).

3.1.2. Digestibility and the toxicity risk assessment process

Tools to investigate novel protein oral toxicity from GM plants include *in silico*, *in vitro* and *in vivo* studies, and information on the history of safe consumption of the proteins under assessment, combined in a weight-of-evidence approach. Noteworthy, the *in vivo* toxicity studies requested (i.e. 28-day toxicity studies, EFSA GMO Panel, 2011) are based on protocols developed for small molecules, but they can provide some information on the potential toxicity following ingestion of proteins. However, the studies normally will provide no information on the digestibility of the protein or whether any effects observed were due to the intact protein or particular domains or peptides derived from it. Therefore, information on the fate of the protein after ingestion is an important element to take into consideration in the toxicological (and allergenic assessment) of proteins in a weight-of-evidence approach. According to Codex Alimentarius (2003, 2009), the assessment of potential toxicity considers, among others stability of the protein to heat or food processing procedures and to degradation in appropriate representative gastric and intestinal model systems. The EFSA guidance document (EFSA GMO Panel, 2011) also refers to the need for data on resistance of the novel proteins to proteolytic enzymes using appropriate and standardised tests, as not only the intact protein but also stable fragments might pose concerns (Bethune and Khosla, 2008). In recent years, there has been increasing pressure to move away from animal models for the toxicological assessment because of ethical considerations (Krutz et al., 2020). In addition, the inherent properties of the so-called intractable proteins (e.g. proteins associated with lipid membranes) may prevent the performance of the traditional high-dose *in vivo* toxicology studies because of the technical limitations to produce a sufficient amount of high purity protein in a functionally active form as a part of overall safety assessment (Bushey et al., 2014; Eaton et al., 2017). This panorama could put the focus on the reinforcement of all weight-of-evidence elements used for the safety assessment, such as the concept of history of safe use, bioinformatic analysis, mode of action and specificity, *in vitro* digestibility, stability, expression levels and dietary intake evaluation.

In relation to *in vitro* digestibility, while the use of a single enzyme (e.g. pepsin) in the context of risk assessment is clearly an oversimplification, it is also acknowledged that the implementation of *in vitro* GI investigations using appropriate, validated and standardised tests is currently not feasible. Ideally, stable breakdown protein fragments should be characterised and evaluated with regard to the

potential to cause adverse health effects linked to their biological activity (Bøgh and Madsen, 2016; EFSA GMO Panel, 2017); however, the appropriate methodology is not currently available.

3.2. Current state-of-the-art of digestibility tests in the protein safety assessment of GM plants

3.2.1. Risk assessment practice and controversies

The classical pepsin resistance test defined by Codex Alimentarius (2003, 2009) is currently used to provide information on protein digestibility into the risk assessment and a relevant element in the allergenicity prediction *per se*, as hypothesised by FAO/WHO (2001) and Codex Alimentarius (2003, 2009). However, several studies have not supported a correlation between resistance to pepsin digestion and allergenicity (Kenna and Evans, 2000; Fu et al., 2002; Takagi et al., 2003; Thomas et al., 2004; Herman et al., 2007; Ofori-Anti et al., 2008; Costa et al., 2021). This is in contrast with other recent articles claiming that the classical pepsin resistance assay and simple sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, as developed by Astwood et al. (1996), remains the most useful assessment to distinguish digestible proteins from non-digestible proteins within the weight-of-evidence approach to assess protein safety, including the allergenic risk of GM crops (Wang et al., 2017, 2020). Furthermore, Foster et al. (2013) reported that analysis of pepsin-resistant fragments could improve the power of the pepsin test to discriminate between allergens and non-allergens, when studied in their native form. Unfortunately, there is no consensus about how to interpret specific characteristics of digestion products (e.g. molecular size, persistence and abundance) within the context of the safety assessment of newly expressed proteins. Moreover, the criteria for classifying a protein as resistant or labile to digestion are not defined, which impairs the setting of appropriate limits for digestibility in assessing the safety of a protein. Initial steps in this direction have been undertaken (see Section 3.2.2); however, further research and discussion are needed to reach a more consolidated knowledge.

Nevertheless, the classical pepsin resistance test still may provide information on physicochemical properties of proteins, relating to how compact the protein is folded and, thus, on the stability of a protein under acidic conditions (Helm, 2001; Thomas et al., 2004). Information on stability of the protein also includes effects of temperature and pH on protein structure and function. Overall, information on stability of the newly expressed proteins (e.g. pepsin resistance test, effects of temperature and pH) can be used for the safety assessment of proteins in a weight-of-evidence approach.

Concerns have been also raised on the test item used in *in vitro* digestibility studies of GM plants performed for regulatory purposes. These studies are mostly carried out on purified newly expressed proteins because their expression levels *in planta* are usually very low, imposing limitations to the assessment. In addition, the safety assessment should cover any use of GM plants for food/feed purposes, which makes the overall assessment a challenge if all possible food matrices and food processing conditions that the GM plants might undergo when released into the market were to be tested. However, it is recognised that exposure to pure dietary allergens is rare and, in fact, the stability of proteins to digestion can be altered due to the presence of components in the food matrix by inhibiting or activating digestion and due to different processing conditions (EFSA GMO Panel, 2011, 2017). Overall, this aspect underlines the importance of the food matrix and food processing in the digestibility of food allergens and in their potential ability to trigger an immune response (Ćirković Veličković and Gavrović-Jankulović, 2014). *In vitro* digestibility tests have been applied to investigate the effects of the food matrix on digestion, and how processing conditions may affect susceptibility to simulated GI proteolysis (e.g. Minekus et al., 2014; Di Stasio et al., 2017; Brodkorb et al., 2019). However, given the diversity of food matrices and food processing procedures, knowledge of their effects on susceptibility of proteins to digestion is limited. Consequently, the effects of processing and of the food matrix on the susceptibility of a particular protein to digestion are difficult to predict (EFSA GMO Panel, 2017).

In this context, a test where GI conditions are considered could provide more relevant information on the fate of the protein in the GI tract than the classical pepsin resistance test alone. However, no GI *in vitro* digestibility assay is currently available for risk assessment purposes, raising the urgent need for standardisation and validation of a test that provides reliable knowledge about the fate of the novel protein in the GI tract (EFSA GMO Panel, 2017; Fernandez et al., 2019; Verhoeckx et al., 2019).

3.2.2. Use of alternative *in vitro* digestibility tests and comparison with the classical pepsin resistance test

Alternative *in vitro* digestibility protocols aim to more closely simulate (within the inherent limitations of *in vitro* models, as explained above) the physiological conditions of GI digestion. This is achieved by sequential addition of digestive enzymes (i.e. gastric digestion conditions are followed by an intestinal *in vitro* digestion) and biosurfactants (bile salts and phospholipids) at physiologically relevant levels (EFSA GMO Panel, 2017). Since the publication of the GMO Panel guidance document in 2017, several studies have investigated *in vitro* protein digestibility of proteins and considered its usefulness as a predictor of the fate of the protein in the GI tract and of allergenicity. However, it is important to stress that the purpose of such tests is not to represent the broad range of GI conditions found *in vivo*.

One study undertaken was commissioned by EFSA (procurement on *in vitro* protein digestibility) and followed the principles described in the EFSA GMO Panel supplementary guidance document (EFSA GMO Panel, 2017). The study assessed three gastric digestion scenarios simulating early phase (i.e. Infogest protocol (Minekus et al., 2014), pH 3.0 and 0.8 U pepsin/ μ g test protein) and a late phase (i.e. as that described by Thomas et al. (2004), pH 1.2 and 10 U pepsin/ μ g test protein), as well as an infant gastric digestion (pH 5.3 and 85 U pepsin/mg test protein), all of which were followed by intestinal phases (i.e. pH 7, trypsin (100 U/mL), chymotrypsin (25 U/mL) and 10 mmol/L bile in early and late phases; pH 6.6, trypsin (16 U/mL), chymotrypsin (4 U/mL) and 3.1 mmol/L bile in the infant model) (Mackie et al., 2019). The authors selected 10 different control proteins from plant and animal origin with distinctly different digestibility and allergenic potential that were subjected to the different digestion scenarios. A simple SDS-PAGE readout alone was considered insufficient for risk assessment of newly expressed proteins since it cannot provide a readout of the peptide fragments arising from digestion. Inclusion of liquid chromatography-mass spectrometry (LC-MS) data addressed this shortcoming showing several correlations between some regions of high peptide abundance and the location of known epitopes, although the correlation was overall limited. This study concluded that the approach used was considered relevant to inform the risk assessment process, but more targeted research is needed to link these results to immunological outcomes. For instance, the use of multivariate analytic and machine learning approaches to provide statistical analysis of all persistent peptides and using a broad range of known allergens and their epitopes as training sets was proposed. Thus, rules could be developed that link LC-MS data analysis of digesta to the probability of allergenicity (Mackie et al., 2019).

More assertive conclusions were presented in two other studies that tested up to four pairs of control proteins under a total of nine combinations of assay conditions. One study included three different pH conditions (1.2, 2.5 and 4.0) and three pepsin-to-protein ratios (10, 1 and 0.1 U/ μ g) during simulated gastric digestion followed by pancreatin digestion in the presence of bile salts (Akkerdaas et al., 2018). A second study analysed two pairs of control proteins under two pepsin digestion conditions (optimal pepsin digestion condition: pH 1.2, 10 U pepsin/ μ g test protein; suboptimal pepsin digestion condition: pH 5.0, 1 U pepsin/10 mg test protein), followed by trypsin and chymotrypsin digestion in the absence or presence of bile salts (Wang et al., 2020). These authors reported that under the conditions tested, the addition of a sequential intestinal phase to follow the pepsin digestion phase did not improve the power to discriminate allergens from non-allergens. However, Akkerdaas et al. (2018), based on the susceptibility profile of parvalbumin (a well-established fish allergen), considered valuable to extend the current practice of pepsin digestion protocols with a stand-alone pancreatin digestion protocol. Akkerdaas et al. (2018) also considered that protease resistance of food proteins can be a useful tool for assessing exposure, being this an interesting aspect for further investigation in the future recommendations of the GMO Panel.

Wang et al. (2020) concluded that, despite the lack of representation of dynamic physiological conditions, the classical pepsin resistance test and SDS-PAGE analysis are applicable and provide useful information on the likelihood of exposure to structurally intact and functionally active proteins as part of a weight-of-evidence approach to assess the allergenic risk of GM crops. For these reasons, some authors consider the classical pepsin resistance test a valid solution until a more informative test has been developed to support allergenicity risk assessment (Wang et al., 2017, 2020).

Finally, the number of studies that use more physiological conditions of GI digestion and the number of control proteins tested are still scarce to infer consolidated conclusions in the risk assessment process. Likewise, given that these alternative protocols may provide more comprehensive information about the progress of the protein degradation, it is currently unclear the most adequate

read-outs for analyses of digestion products including their statistical analysis, as well as the interpretation of the outcome of such alternative studies to be considered in a weight-of-evidence approach.

4. Conclusions and Recommendations

There is evidence that digestion in the GI tract can affect the biological properties of dietary proteins. A deeper understanding of protein digestion in the GI tract could enable risk assessors to frame and build a more robust strategy in the safety assessment. Thus, the development and validation in robustness and reliability of testing tools targeting the fate of dietary proteins along the digestive tract would provide relevant evidence on the overall protein safety assessment.

By considering the complexity and variety of factors involved in food allergy, as well as the current state-of-the-art, it is unrealistic to consider that a single test, including resistance to simulated GI digestion, will be identified in the short-term to be completely predictive of the allergenic potential of a protein. Although more robust tools are available for the toxicity assessment of proteins, similar principles as those described for allergenicity are also envisaged here if the use of animals is to be substantially reduced.

The classical pepsin resistance test, as currently used, is not an *in vitro* digestibility test designed to mimic the physiologic conditions of gastric digestion. It can be used to determine the biochemical character of the subject protein by determining whether it is stable to pepsin degradation under acidic conditions. However, it does not reflect the entirety of physiological conditions encountered in the digestive tract and cannot indicate how the protein might behave *in vivo* to allow the systemic exposure to the intact protein to be assessed. Furthermore, there is still controversy on its usefulness as a predictor of protein allergenicity *per se* (see Section 3.2). The evidence supporting the resistance to degradation by pepsin as a direct predictor of allergy is weak with several publications describing the pepsin test as a poor predictor of allergenicity (see Sections 3.1 and 3.2). The information that the classical pepsin resistance test can provide to the risk assessment is on the stability of the proteins. In this context, this test may be a useful tool offering knowledge on the biochemical and physicochemical properties of proteins as additional information in a weight-of-evidence approach for the overall safety assessment. However, other methods can be used to obtain data on a protein's structural and/or functional integrity (see Section 3.2). It is acknowledged that the classical pepsin resistance test is embedded into international guidelines such as Codex Alimentarius and Regulation (EU) No 503/2013.

For future development, there is a need for more reliable systems to predict digestion, to better understand the fate of the protein/fragments in the GI tract and how they interact with the relevant cells in the human body. Regarding the use of alternative *in vitro* digestion test methodologies, an EFSA external report was published in 2019⁵ and more research is still needed to assess their potential utility to reduce uncertainty and improve the reliability of predictions. Given the high complexity of the digestion and absorption process of dietary proteins, it is needed to clarify and identify those aspects that could be relevant to assess potential risks of allergenicity and toxicity of proteins. Thus, the following questions could be addressed to pave the way for the scientific opinion on recommendations for future developments in the field of allergenicity assessment, and protein safety, to be adopted by the GMO Panel in 2021:

General questions:

- what is the usefulness of *in vitro* digestion in the overall protein safety assessment?
- what are the most suitable *in vitro* digestion models?
- what is the most adequate item to test in such models?
- what follow up actions would be required to assess the relevant proteins/fragments identified in previous steps?
- how this information can be integrated into a weight-of-evidence approach?

Specific questions:

i) Protocol for digestibility assays:

- is it more appropriate and informative using relevant physiological conditions in the digestion process than using a fixed and suprphysiological concentration of pepsin?

⁵ <https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2019.EN-1765>

- if so, and considering that physiological conditions are very variable and dependent on many factors in the digestion process, what parameters should be considered (e.g. multi-enzyme system (which ones and at what levels? should other intestinal lumen proteases such as elastase and carboxypeptidases, as well as additional brush border enzymes such as aminopeptidases be considered?), biosurfactants, other GI components or fluids)? should subsequent steps of digestion be considered (e.g. gut epithelial cell-based approaches)?
- what type of test material would be more relevant and feasible? (i.e. purified protein vs whole plant extracts and considerations of food matrix and food-processing conditions); what characteristics of the test material should be considered (e.g. post-translational modifications, other biochemical and/or physicochemical properties related to protein stability)?

ii) *Monitoring of digestion reactions:*

- are readouts evaluating the fragments generated upon digestion relevant and needed?
- if so, what analytical techniques could be more appropriate (e.g. LC-MSⁿ, MALDI-ToF, Size Exclusion Chromatography and/or SDS-PAGE with staining or western blot, consideration of reducing and non-reducing conditions)?

iii) *Interpretation and expected outcomes:*

- what endpoints should be considered (fragments *versus* intact protein)? what is meant by fate of the protein in the GI tract?
- if fragments are considered, what properties should be considered (i.e. size, persistence, abundance, others)? could appropriate cut-off values be set for risk assessment purposes and fit into the sensitisation and elicitation scenarios?
- are static *in vitro* digestion methods suitable for detailed kinetic analysis? If so, what kinetic approach would be useful to assess proteins not fully degraded?
- what follow-up actions would be required to assess the relevant proteins/fragments identified in previous steps?
- would it be feasible setting acceptable/unacceptable limits for digestibility in assessing the safety of a protein?
- if so, could these limits be used for a ranking of proteins according to their *in vitro* digestibility?
- how could this information be integrated into a weight-of-evidence approach to inform the allergenicity and toxicity assessment?

5. 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 EFSA 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.
- 3) Acceptance of the self-task mandate of the EFSA GMO Panel to establish a Working Group to develop a statement of the GMO Panel updating its latest guidance document on Allergenicity assessment of GM plants (EFSA GMO Panel, 2017). May 2020. Submitted by EFSA Executive Director.

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Abbreviations

CD	coeliac disease
GI	gastrointestinal
GM	genetically modified
GMO	genetically modified organisms
LC–MS	liquid chromatography–mass spectrometry
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Th2	T helper type 2 cells