Explanatory note on the determination of newly expressed protein levels in the context of genetically modified plant applications for EU market authorisation

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Abstract

Genetically modified organisms are subject to a risk assessment and regulatory approval before entering the European market. According to legislation (Directive 2001/18/EC, Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013) and the EFSA guidance documents on the risk assessment of food and feed from genetically modified (GM) plants and on the environmental risk assessment of GM plants, applicants need to perform a molecular characterisation of any DNA sequence inserted in the GM plant genome. When a GM plant is designed to produce one or more newly expressed proteins, an aspect of the risk assessment process is to characterise these proteins and to reliably determine their levels in the GM plant tissues. A number of methods to measure protein expression levels are used and several experimental factors within these methods are of critical importance in order to obtain reliable results. This explanatory note provides details on key methodological aspects for the determination of newly expressed protein levels that should be considered and reported by applicants in order to harmonise the information in GM plant applications submitted to EFSA.

Key words: GM plant, protein extraction, protein quantification, molecular characterisation, risk assessment

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Summary

Genetically modified organisms (GMOs) are subject to a risk assessment and regulatory approval before entering the European market. According to legislation (Directive 2001/18/EC[1], Regulation (EC) No 1829/2003[2] and Regulation (EU) No 503/2013[3]) and the EFSA guidance documents on the risk assessment of food and feed from genetically modified (GM) plants (EFSA, GMO Panel 2011) and on the environmental risk assessment of GM plants (EFSA GMO Panel, 2010), applicants need to perform a molecular characterisation of any DNA sequence inserted in the GM plant genome. In many cases these inserted sequences are intended to express new protein(s). Knowledge on the levels of these proteins is required for their characterisation and to assess the exposure to these newly expressed proteins (NEPs) in the context of food/feed and environmental safety. In addition, such data are used as part of the assessment of potential interactions between events stacked in GM plants and in particular on interactions affecting NEP levels. A number of methods to measure protein expression levels are used and several experimental factors within these methods are of critical importance in order to obtain reliable results.

This explanatory note provides details on the key methodological aspects of the determination of NEP levels that should be considered and reported by applicants in order to harmonise the information in GM plant applications submitted to EFSA. However, this document is not intended to recommend any specific experimental approach.

The two main steps for a targeted protein quantification methodology are discussed: sample preparation and extraction of the NEP(s), and the analytical method employed to quantify the NEP(s). In addition, recommendations are presented as regards the description and reporting of the methodology used and data obtained.

[1] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 17.4.2001, p. 1-38.

[2] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23.

[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. OJ L157, 8.6.2013, p. 1–48.

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1. Introduction

According to relevant legislation (Directive 2001/18/EC, Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013) and the EFSA guidance documents on the risk assessment of food and feed from GM plants (EFSA GMO Panel, 2011) and on the environmental risk assessment of GM plants (EFSA GMO Panel, 2010), applicants need to perform a molecular characterisation of the genetically modified plant. In many cases the GM plants express new protein(s). As part of their characterisation, the levels of these proteins must be determined. A number of methods to measure protein expression levels are used and several experimental factors within these methods are of critical importance in order to obtain reliable results. In this explanatory note, EFSA provides details on what information should be submitted on the determination of NEP levels in GM plant applications submitted to EFSA.

2. Background

When a GM plant is designed to express one or more NEPs, an aspect of the risk assessment process is to characterise these NEPs and also to reliably determine their levels in the GM plant tissues. Knowledge on the levels of these proteins is required for their characterisation and to assess the exposure to these NEPs in the context of food/feed and environmental safety. In addition, such data are used as part of the assessment of potential interactions between events stacked in GM plants and in particular on interactions affecting NEP levels.

2.1. Terms of reference

This explanatory note is based on the risk assessment principles and data requirements outlined in guidance documents developed by EFSA and its GMO Panel (EFSA GMO Panel, 2010 and EFSA GMO Panel 2011), and relevant legislation, i.e. Regulation (EC) No 1829/2003 and Regulation (EU) No 503/2013 on GM food and feed, and Directive 2001/18/EC on the deliberate release of GMOs into the environment.

It provides details on the key methodological aspects of the determination of NEP levels that should be considered and reported by applicants in order to harmonise the information in GM plant applications submitted to EFSA. However, this document is not intended to recommend any specific experimental approach.

2.2. Transition period

In line with the indicative timelines for submitting molecular datasets that require the generation of plant material (EFSA, 2014), the recommendations described in this explanatory note will be applicable for GM plant applications submitted 24 months after its publication. However, applicants are recommended to take into account the elements described in this document for all applications submitted after its publication.

A transition period of two months applies to those elements for which only the provision of information is recommended, such as:

- a description of how the plant material was collected including the number of analysed plants
- a description of all the experimental protocols including those for method validation
- information on all the critical methodological parameters and any associated limitations and method deviations
- the provision and description of representative standard curves (see Section 4.2.1.1)
- information on the reference standards used (see Section 4.2.2.1)
- information on the antibodies used for immuno-based analytical methods (see Section 4.2.2.3).

3. Data and methodologies

In developing this explanatory note, EFSA consulted experts from its molecular characterisation working group and took into account the principles and requirements defined in the relevant legislation and the GMO Panel guidelines for the risk assessment of GM plants and derived food and feed (EFSA GMO Panel, 2011) and the environmental risk assessment of GM plants (EFSA GMO Panel,

2010) for the expression of the inserted/modified sequence(s) leading to the production of NEPs. In addition, guidance documents on bioanalytical method validation parameters published by other regulatory authorities, as well as data from published scientific literature, and experience from previously assessed EFSA GM plant applications were considered.

4. Required information

The determination of NEP levels in GM plant tissues requires the use of an appropriate analytical method to detect and quantify the NEP(s). The NEP analytical methods discussed in this document are antibody-based methods such as ELISA and immunoblotting/western blot analysis as well as mass spectrometry (MS) methods, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) (e.g. Andreasson et al., 2015; Crespo et al., 2008; Grothaus et al., 2006; Kruve et al., 2015a,b; Liebler and Zimmerman, 2013; Pan et al., 2009; Schmidt and Alarcon, 2011; Settlage et al., 2017). These methods are the most commonly used approaches for NEP quantification reported in dossiers submitted for GM market authorisation in the EU. More extensive reference is made to the ELISA methodology because the majority of datasets on NEP quantification are produced by this experimental approach. Even though the principles and considerations described in this document are discussed in the frame of the above-mentioned experimental approaches, many of these are also valid for other bioanalytical methods.

The two main steps of a targeted protein quantification methodology are discussed: sample preparation and extraction of the NEP (Section 4.1), and the analytical method employed to quantify the NEP (Section 4.2). In addition, recommendations for the description and reporting of the methodology used and resulting data are presented in Section 4.3.

4.1. Extraction of the NEP(s) from GM plant tissues

The adequate extraction of proteins from GM plant tissues is a critical step for a reliable determination of NEP levels in the GM plant. The extraction process should be robust and should ensure that the NEP is not unintentionally biochemically modified (e.g. degraded or aggregated) so it can be reliably quantified by the chosen analytical method, and its efficiency must be determined. With this in mind, a number of experimental and data quality parameters are discussed below.

4.1.1. Protein extraction efficiency

In order to comprehensively inform the GM plant risk assessment, the total amount of the NEP in the analysed tissues must be determined. However, complete extraction of the NEP present in a tissue can be difficult to achieve and the efficiency of the chosen extraction method is often limited by factors such as the molecular properties of the NEP and choice of extraction buffer. Consequently, a significant amount of the NEP may remain in the insoluble fraction after extraction and should be quantified to estimate the extraction efficiency of the chosen method. Extraction efficiency is defined as the amount of extracted protein relative to the total amount in the plant tissue, expressed as a percentage. To estimate the total amount of protein in the plant tissue, the NEP present in the insoluble fraction should be extracted under strong denaturing ('harsh') conditions (e.g. Laemmli buffer and extensive sample heating and/or acidic/alkaline treatments) and quantified by western blot analysis (Matsuo et al., 2006; Martínez-García et al., 1999; Schmidt and Alarcon, 2011). Once the extraction efficiency is determined it must be taken into account when calculating the final NEP levels present in the plant tissues.

4.1.2. Tissue disruption/cell lysis

The choice of tissue disruption/cell lysis protocol and equipment used depends on factors such as particle size (after tissue grinding), tissue-to-buffer ratios, plant species or type of tissue to be analysed (Grothaus et al., 2006). The applicant should justify the chosen protocol.

As regards tissue-to-buffer ratio, an appropriate volume of extraction buffer should be applied to reduce the effects of the tissue matrix on the molecular stability of the target NEP (Laing and Christeller, 2004) (see Section 4.2.1.2).

The use of either fresh or lyophilised tissue for protein extraction is acceptable. However, protein extraction from fresh tissues with high water content (e.g. fresh leaves or roots typically contain 80–90% water) might result in significant molecular instability (e.g. degradation or aggregation) of the NEP(s) or increased sample heterogeneity. Thus, extracting proteins from lyophilised material is recommended.

Additional steps (e.g. an enrichment step) may be needed in some specific cases, taking into account aspects such as NEP sub-localisation (e.g. membrane-associated proteins or nuclear proteins such as transcription factors). If such additional steps are taken, they should be clearly described and justified.

4.1.3. Extraction buffer

The buffer used for the protein extraction should not significantly interfere with the downstream analytical method.

The possible underestimation of NEP levels caused by protein degradation due to the presence of proteases is considered important and therefore protease inhibitors (typically a protease inhibitor cocktail) should be included in the extraction buffer (e.g. Herman and Shan, 2011; Laing and Christeller, 2004). If protease inhibitors are not used, the justification should be provided. Sufficient information including the justification for the choice of other buffer components (e.g. other additives) should also be given.

4.2. Quantification of the NEPs from GM plant tissues

The analytical method to quantify the extracted NEPs from the different GM plant tissues should be robust and 'fit for purpose'. To confirm this, a number of method parameters (Section 4.2.1) should be validated to demonstrate the suitability of the method and sufficient information on these parameters should be provided.

Some additional elements associated with the analytical method should also be considered (Section 4.2.2) and sufficient information on these elements should also be provided. Any modifications made to the developed method should be discussed and considered for the validation experiments.

In cases where a commercially available assay has been used, some of the validation experiments may be omitted. However, sufficient explanation should be provided on which parameters were validated by the manufacturer.

4.2.1. Validation and quality parameters of the analytical method

A number of international guidelines have already been published containing extensive details on the experimental parameters that should be assessed in order to validate a bioanalytical method (e.g. FAO, 2010; EMA, 2011; FDA, 2018; Grothaus et al., 2006; ICH, 2005; ISO, 2013; Jenkins et al., 2015; Lipton et al., 2000). Where applicable, the principles described in these documents should be considered by the applicant. The sections below provide details on some of these validation parameters that are considered the most relevant when assessing the analytical method used for the quantification of NEP(s) produced in GM plants.

4.2.1.1. Sensitivity

The sensitivity of the analytical method is determined by establishing the limit of detection as well as the lower and upper limits of quantification (LLOQ and ULOQ, respectively) that define the method's quantitative range. How these experimental factors were defined and validated should be described and information on the following elements should be provided:

- Quality of the standard (calibration) curve: the produced standard curve forms the basis for determining the quantity of the NEP extracted from the GM plant tissue(s). A complete description of how the calibration curve was derived, including any identified critical parameters, should be provided.
- Establishing the quantitative range: information should be provided on how the standard curve was used to define the quantitative range (LLOQ and ULOQ) for each analysed tissue matrix. Measurements of the extracted NEP levels from the GM plant tissues should normally fall within the middle of the quantitative range and the applicant should clearly indicate any

measured values that are close to the LLOQ or ULOQ and discuss the impact of such results. Dilutional linearity should be demonstrated for tested samples above the ULOQ.

- Limit of detection: can be determined theoretically or empirically and a number of validation methods are reported in the literature (e.g. Armbruster and Pry, 2008; EMA, 2011). Either approach is acceptable as long as an adequate description is provided of how it was defined for each tissue matrix analysed.

The applicant should also discuss any other possible limitations such as those arising from the quality of the used reagents (e.g. antibody, extraction buffer) as well as the magnitude and possible impact of false positive and false negative measurements.

4.2.1.2. Matrix effects

The analytical method may be significantly influenced by components in the tissue extract resulting in a non-specific response commonly referred to as a 'matrix effect' which is one of the parameters that could affect the method accuracy (EMA, 2011; FDA, 2018; Jenkins et al., 2015; Herman and Shan, 2011). In order to assess whether there are any matrix effects, it should be demonstrated that the analytical method is not significantly affected by the presence of matrix components. To do this, the established NEP-specific standard curve should be verified for each tissue matrix analysed. If significant matrix effects are identified, appropriate matrix dilutions should be applied. If dilution does not eliminate matrix effects, alternative methods should be explored. The acceptance criteria for an observed matrix effect should be clearly described, including an adequate justification for any applied dilution factors for the tested plant matrices.

4.2.1.3. Specificity

If there are proteins present in the matrix that are similar to the target NEP (e.g. endogenous protein or another NEP produced in the GM plant) they might interfere with the NEP-specific quantification method thereby affecting its specificity (e.g. EMA, 2011; Grothaus et al., 2006). In the case of antibody-based methods, the NEP quantification may be impacted by cross-reactivity of the used antibodies with such proteins, whereas in the case of MS-based methods, the quantification may be impacted by the presence of interfering peptides. This should be quantified using appropriate approaches and must be taken into account to estimate the levels of the NEP.

For antibody-based methods in particular, information should also be provided on the antibody's ability to detect certain forms of the NEP in the test samples (e.g. if only the native form of the NEP can be detected).

4.2.1.4. Repeatability

Method repeatability should be validated by determining the variation between replicate NEP level measurements (e.g. ISO, 2013; Schmidt and Alarcon, 2011). It should be assessed 'within-run (intra) assay' and 'between-run (inter) assay' to determine the coefficient of variation for the measured NEP levels. Intra- and inter-assay coefficients of variation are related to the variation occurring within an assay and to the variation occurring between separate assays, respectively. The acceptance criteria for the observed coefficient of variation should be clearly described and discussed in the context of the repeatability of the methods used.

4.2.2. Additional considerations for the analytical method

In addition to the aspects described above for the validation of the analytical method, sufficient information on a number of additional elements should be provided by the applicant so that EFSA can comprehensively perform its assessment on the provided NEP quantification data.

4.2.2.1. Reference standards

Sufficient information should be provided to demonstrate that the reference protein (reference standard) used as a surrogate of the NEP expressed in the GM plant is suitable for its intended use.

The reference protein used (usually produced in alternative expression systems such as bacteria) should be equivalent to the NEP expressed in the GM plant with respect to its molecular and

biochemical properties. Methods such as western blot analysis, MS or activity assays can be used to evaluate equivalence in terms of molecular mass, amino acid sequence, immunoreactivity, function, etc. For antibody-based analytical approaches such as ELISA and western blot analysis, equivalence in immunoreactivity is critical for method validation.

For MS-based analytical methods such as LC-MS/MS, additional reference standards are used such as stable-isotope-labelled analogues of the full-length or any surrogate peptide(s) of the target NEP (internal standard). Sufficient information on these reference standards including a justification for the choice should be provided.

In addition, information should be provided on the purity and concentration of the reference standard(s) and the methods used to determine them. Protein concentration determination using two independent methods is recommended for reference proteins (e.g. a non-colorimetric method such as amino acid analysis and a colorimetric method such as a Bradford assay, e.g. Noble and Bailey, 2009).

Proteins (or peptides) used as reference standards may denature, aggregate or degrade during the different experimental steps or during storage. Thus, information on their stability under testing and storage (short- and/or long-term) conditions should be provided.

4.2.2.2. Reference plant materials

Reference plant materials used as control samples (e.g. non-GM plant matrix spiked with a reference protein or non-GM plant matrix alone) should be treated under the same testing and storage conditions as the GM plant materials intended for analysis.

4.2.2.3. Antibodies

For the determination of NEP levels in GM plants by antibody-based methods, information should be provided on the used antibody(ies) including:

- Type of antibody and production system: information should be provided on the nature of the antibody(ies) (i.e. monoclonal or polyclonal) as well as on the production host. The suitability of the antibodies should be justified, in particular for sandwich ELISAs that require the use of two antibodies.
- Antigen used for antibody production: information on the antigen (e.g. similarity to the GM plant-produced NEP) should be provided. If a peptide is used as the antigen, its sequence should be provided including a justification for the peptide choice (e.g. length, physicochemical properties, amino acid composition). In addition, when possible, information should be provided on the sample used as the antigen (e.g. purity, denatured protein extracted from a sodium dodecyl sulfate gel or purified protein in solution).
- Antibody stability: information should be provided on the functional stability of the chosen antibodies under the experimental conditions of the analytical method (e.g. assay buffer, temperature, or incubation times).

4.2.2.4. Stability of plant material and tissue extracts

Information on the stability of the NEP(s) in analysed plant tissue extracts should be provided as regards storage and assay conditions (e.g. time, temperature, freeze/thaw cycles). In addition, information should be provided on the stability of NEPs in the plant material intended for analysis. Any possible impact due to plant tissue extract/plant material instability on NEP level determination (e.g protein degradation or aggregation) should be discussed.

4.2.2.5. Specific information requirements for MS-based methods

In addition to the information on the internal standard(s) used, information on some additional elements should be provided for MS-based methods. These include information on the choice and performance characteristics of the surrogate and monitoring peptides used (biochemical properties, detectability, etc), enzymatic digestion efficiency, protein/peptide enrichment steps (e.g. affinity capture), 'carry over' and ionisation/fragmentation efficiency (Kruve et al., 2015a,b; Jenkins et al., 2015, Parker and Borchers, 2014, Rauh, 2012; van den Broek et al., 2013). Some of these aspects are

associated with the peptide/protein recovery which should be thoroughly evaluated. In addition, sufficient information should be provided on the computational methods used (Gatto et al., 2016).

4.2.2.6. Analysis of processed food/feed

In cases where the analytical method aims to quantify NEP levels in processed food and/or feed products, additional/alternative information may be needed. Processed plant products can be derived after various chemical, physical or enzymatic treatments which may cause significant biochemical, compositional and/or structural changes to the target NEP(s). Thus, adequate information should be provided to demonstrate that the chosen analytical method is 'fit for purpose' (e.g. choice of suitable antibodies used in the case of antibody-based methods).

4.3. Description/reporting of the information

This section outlines the type of information that should be included in a GM plant application submitted to EFSA as regards the description and reporting of information on the samples, methodology and resulting data on the determination of NEP levels in GM plants. It is recommended that when possible, data should be summarised (e.g. tabulated):

Information on the samples

- A description of how the plant material was collected including the number of analysed plants.
- A description of the nature of the test materials (e.g. crop variety, developmental stage).
- A description of all plant/tissue treatments (e.g. herbicide treatment).
- Any relevant certificates of analysis (e.g. characterisation of the reference standard(s)).
- Any sample contamination and a discussion of possible impact on the results.
- A description of the stability of the NEP(s) in analysed tissue extracts under storage and assay conditions and of plant material intended for analysis.

Information on the experimental methods

- A description of all the experimental protocols including those for method validation.
- A description of all the equipment and reagents used.
- All critical methodological parameters with any associated limitations and method deviations.
- Any relevant standard operating procedures (SOPs) should be referenced; if any SOPs contain information not sufficiently described in the provided information then these SOPs should also be submitted.

Information on data reporting and data analysis methods

- A description of all data analysis methods including the number of replicates for all the steps and the model fitting method(s) to produce standard curves.
- Representative standard curves should be included and sufficiently described.
- A description of how the protein concentrations used for the standard curve (e.g. ng/ml) were used to deduce the values for NEP levels in the analysed tissues (e.g. µg/g tissue).
- Data should be clearly summarised (i.e. tabulated) for each NEP measured in the different tissues of the GM plant. NEP levels should be calculated taking into account the determined extraction efficiency for each tissue and these calculations should be clearly described.
- Besides those cases for which it may not be technically possible (e.g. for pollen tissue), NEP levels should be reported both on a fresh and on a dry weight basis. A description of the step(s) to convert the values from fresh weight- to dry weight-based should also be provided.
- Data reporting should include those generated from method validation (e.g. data produced from inter- and intra-assay runs) and should be summarised (e.g. tabulated) and included as appendices to the main method validation report.

- Summarised NEP level values derived from analysis using descriptive statistics.
- The number of test samples within the quantitative range and total sample number used in data analysis (i.e. 'N' number) should be clearly indicated.
- All the acceptance criteria defined for the different experimental steps (e.g. coefficient of variation to assess repeatability) and discussion of any possible impact resulting from data that fell outside of these criteria.

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Abbreviations

GM	Genetically modified
GMO	Genetically modified organism
LC-MS	Liquid chromatography mass spectrometry
LLOQ	Lower limit of quantification
MS	Mass spectrometry
NEP	Newly expressed protein
SOP	Standard operating procedure
ULOQ	Upper limit of quantification