codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

JOINT OFFICE: Viale delle Terme di Caracalla 00100 ROME Tel: 39 06 57051 www.codexalimentarius.net Email: codex@fao.org Facsimile: 39 06 5705 4593

CX/FBT INF-1 (ENGLISH ONLY)

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX *AD HOC* INTERGOVERNMENTAL TASK FORCE ON FOODS DERIVED FROM BIOTECHNOLOGY

Fifth Session

Chiba, Japan, 19-23 September 2005

EXCERPT FROM ALINORM 05/28/23 AND CX/MAS 05/26/9

EXCERPT FROM ALINORM 05/28/23

CRITERIA FOR THE METHODS FOR THE DETECTION AND IDENTIFICATION OF FOODS DERIVED FROM BIOTECHNOLOGY (Agenda Item 7)[1]

108) The Committee recalled that at its 25th session it had agreed that the Delegations of the United Kingdom and Germany with the assistance of a Drafting Group would revise the document with a view to the elaboration of Guidelines for consideration at the next session.

109) The Delegation of Germany introduced the document and indicated that on the basis of the comments received the following major changes had been made: in the Section on Modular Approach to method validation it was explained how this method could be applied and in Annex V on Validation of a Protein-Based Method a new narrative was added.

110) The Delegation of the EC supported the development of the paper and expressed the view that it had been elaborated for the endorsement of methods for detection and identification of foods derived from biotechnology in the CCMAS and proposed to send this paper to the Task Force on Biotechnology for their information.

111) The Delegation of the Republic of Korea indicated that there were still some uncertainties in Table 1 on the Criteria for scoring Qualitative PCR analyses especially in expressing of the scoring of test when GM analyte in PCR was positive and endogenous PCR result was negative and proposed that the expression of "±" should be changed to "indeterminate" in the scoring of test expression.

112) The Delegation of the United States supported the view expressed by the Delegation of the Republic of Korea and indicated that it had provided general and detailed written comments presented in CX/MAS 05/26/9-Add.1. The Delegation proposed that this document should be retained in the Committee until it had been improved and technical issues resolved. This view was supported by several delegations.

---

[1] CX/MAS 05/26/9, CX/MAS 05/26/9-Add.1 (comments of the United States and AOCS), CRD 5 (comments of Chile), CRD 8 (comments of ILSI), CRD 17 (comments of the EC).

113) The Delegation of Malaysia proposed to include a wider description of protein based testing as it was less costly and wider applied, especially in developing countries.

114) The Delegation of Brazil urged the Committee to proceed with this work as a matter of urgency as the trade in GMO food was growing and governments needed to receive advice on this matter.

115) As regards to the status of the document, the Secretariat clarified that the Committee at its 24th session, following the request from the Committee on Food Labelling and the Task Force on Foods Derived from Biotechnology, had considered the methods of analysis for foods derived from biotechnology and had concluded that the criteria approach should be applied in the selection of methods of analysis for foods containing genetically modified material, and that the selection or endorsement of methods without appropriate provisions was not possible. It was further agreed to prepare recommendations for quality control measures in laboratories and criteria for method of analysis. The Secretariat also indicated that the Intergovernmental Task Force on Biotechnology and the Committee on Food Labelling would be informed about the work of the CCMAS in this area.

116) The Committee agreed that a Working Group led by Germany and the United Kingdom with the participation of all interested Members and Observers would revise the paper for consideration by the next Session of the Committee, especially in order to arrive at a common understanding on how to proceed on this matter.

codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

JOINT OFFICE: Viale delle Terme di Caracalla 00100 ROME Tel: 39 06 57051 www.codexalimentarius.net Email: codex@fao.org Facsimile: 39 06 5705 4593

Agenda Item 7 **CX/MAS 05/26/9**

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING
Twenty-sixth Session
Budapest, Hungary, 4-8 April 2005

CONSIDERATION OF THE METHODS FOR THE DETECTION AND IDENTIFICATION OF FOODS DERIVED FROM BIOTECHNOLOGY

GENERAL APPROACH AND CRITERIA FOR THE METHODS

BACKGROUND

At the Twenty-fourth Session of the Codex Committee on Methods of Analysis and Sampling, papers giving the methods that had been collated by the *ad hoc* Intergovernmental Task Force on Food Derived from Biotechnology (see CX/MAS 02/8) and outlining general considerations of methods of analysis for the detection and identification of foods derived from biotechnology (see CX/MAS 02/9) were discussed. It was noted that the presence of genetically modified organisms or their derivatives could be assessed by the detection of either DNA sequences present as a result of recombination or the protein coded by the inserted gene. It was pointed out that protein-based methods were cheap, offered high selectivity and sensitivity but that since proteins were denatured during processing these techniques were most suitable for the analysis of raw materials and were not generally applicable to highly processed foods. It was also noted that these methods cannot be used when no new protein is expressed in the food, and these methods cannot differentiate between genetic events that produce the same protein.

Methods of detection of DNA markers based on the polymerase chain reaction (PCR) have been used in a variety of food analyses and widely used for detection of GM derivatives in food for many years, and modifications of the PCR method were also widely used. A typical method involved several steps such as sampling, extraction and purification, amplification by PCR and detection/quantification. Specific questions arising in the area of proficiency testing, use of performance criteria and the necessity of quantification due to threshold settings since the results of investigations showed the difficulties in measuring low levels of GM material in processed foods were also discussed. Methods described in the collated documents could only be used successfully if all information about the sequence and certified reference materials were available.

GENERAL CRITERIA

In view of the absence of precise provisions for GMOs and of difficulties with the practical application of methodology in this area, the Committee proposed to develop recommendations with respect to criteria for methods of analysis and for quality control measures that should be introduced in laboratories offering GM analyses. It was agreed that a Working Group led by Germany and the United Kingdom would update and further develop the paper for this session and prepare recommendations for quality control measures in laboratories and criteria for methods of analysis for the Twenty-fifth Session of CCMAS.

The paper CX/MAS 04/10 was discussed at the Twenty-fifth Session of CCMAS, where the following comments were made or were noted:

- The Committee recalled that the last session had agreed that the Delegations of Germany and the United Kingdom in cooperation with a drafting group would prepare a revised document that would include recommendations for quality control measures in laboratories and criteria for methods of analysis.
- The Delegation of the United Kingdom introduced the document and indicated that it included recommendations on the criteria for methods of analysis and quality control measures that should be introduced in laboratories performing GM analysis, with specific focus on the detection of DNA markers based on PCR that were more commonly used.
- The Delegation of Germany referred to the list of methods developed by the Task Force on Foods Derived from Biotechnology and highlighted the importance of further work on guidelines that would provide guidance to governments to select methods for the detection of foods derived from biotechnology.
- The Delegation of the United States welcomed the paper that provided a good scientific basis for further discussion and drew the attention of the Committee to its comments in CRD 9. It noted in particular that the document developed criteria mostly for DNA-based methods but that alternative methods based on the detection of protein should also be addressed.
- The Delegation of Brazil expressed the view that the validation of immunoassay methods should be considered, and that in Annex 1 more information should be included on the description of the method, such as: complete description of the primer, number of cycles, composition of cycles, equipment, amplicon length, type of polymerase and reference material.
- The Delegation of Japan questioned the application of those criteria contained in the document to the detection of GMOs although they are applicable to chemical analysis.
- The Delegation of Norway proposed to amend the section on the modular approach to reflect that it should not be used "unless independence between the modules can be documented", since it should not be systematically avoided.
- The Delegation of Cuba drew the attention of the Committee to the issues related to consumer protection, that might need to be addressed by the Task Force in the future and in particular the level of transgenicity of the material.
- The Committee discussed whether new work should be initiated in the Step Procedure in order to circulate for comments as soon as possible the document in Appendix I: *Guidelines for the Validation and Quality Control Requirements for GMO Analyses*.
- Some delegations stressed the need to proceed rapidly as governments needed guidance on this very important and complex issue. Other delegations indicated that they had been part of the original Working Group but there had not been enough time to provide detailed comments and that it would be preferable to consider the text carefully before initiating the elaboration of specific guidelines.
- The Committee agreed that the document would be revised by the Delegations of the United Kingdom and Germany with the assistance of a Drafting Group for consideration at the next session, with a view to the elaboration of Guidelines.

The following countries and organisations expressed their willingness to participate in this work: Argentina, Australia, Brazil, Canada, Egypt, France, Iran, Ireland, Italy, Japan, Malaysia, The Netherlands, Norway, Philippines, United States, European Commission, AOAC International, AOCS, Bio, CROPLIFE International, EUROPABIO, and ISO.

These measures are given as Guidelines in the Appendix to this paper.

RECOMMENDATIONS

It is recommended that the draft Guidelines be discussed at the Twenty-sixth Session of CCMAS. If there is sufficient consensus, then the approaches described should be further refined and then sent to governments for comment and progress through the Codex system.

APPENDIX I: GUIDELINES FOR THE VALIDATION AND QUALITY CONTROL REQUIREMENTS FOR THE ANALYSIS OF FOODS DERIVED FROM BIOTECHNOLOGY

INTRODUCTION

Method Criteria

The Codex Alimentarius Commission places an emphasis on the acceptance of methods of analysis which have been "fully validated" through a collaborative trial conforming to an internationally accepted protocol. In a number of sectors, including the foods derived from biotechnology (GMO) sector, there are few methods of analysis which have been fully validated. As a result, Codex is also endorsing by reference single-laboratory validation protocols. In this area there may be pressure to adopt a formal single-laboratory validation as an interim measure in the absence of collaborative trial data. However, methods used for determination of the presence of GMO's are able to be, and intended to be performed at, multiple laboratories and should therefore be validated by multi-laboratory collaborative studies as soon as practicable.

In these Guidelines the term "GMO" has been used for "Foods Derived from Biotechnology".

Many methods are currently being developed for GMO detection, identification and quantification. Before they are accepted for use by Codex they must be validated to ensure that they are fit-for-purpose.

However, the two most common approaches are those based on DNA-based methods and those based on the detection of protein. The former is generally performed via PCR, although other methods that achieve measurement without a PCR step may be employed if properly validated. Both DNA and protein-based approaches are considered here, though it is the DNA-based PCR approach which is generally recognised as being the more widely applicable.

The conventional criteria that have been adopted by Codex for the evaluation of methods of analysis are:-

- accuracy
- applicability (matrix, concentration range and preference given to 'general' methods)
- limit of detection
- limit of determination
- precision; repeatability intra-laboratory (within laboratory), reproducibility inter-laboratory (within laboratory and between laboratories)
- recovery
- selectivity
- sensitivity
- linearity

These Guidelines address these requirements in the GMO sector, and anticipates that is likely that these will have to be further expanded (e.g. for PCR) by other items such as:-

- amplicon length
- whether the method is instrument specific
- whether there are differences between qualitative and quantitative PCR-based detection methods
- whether single- or multi-plex PCR amplifications are undertaken

for the DNA-based methods.

And

- equivalency of reagents over time

for the protein based methods

The method validation process accepted by Codex includes the definition of the requirements for the method, testing that the method meets these requirements when carried out, for instance, by different laboratories in different countries, and documentation of the method performance and measurement uncertainty.

Criteria Approach

Codex Alimentarius Commission has accepted the "criteria approach" for methods of analysis. This approach does not extend to Codex Type I empirical/defining, procedures. It is necessary to ensure that this approach is incorporated into Codex guidelines on the validation of GMO methods of analysis unless it is explicitly stated that all GMO methods of analysis are empirical, both theoretically as well as in practice.

Laboratory Quality

The Codex Alimentarius Commission has adopted guidelines for the "quality" of laboratories involved in the import and export of foods. These quality characteristics are based on accreditation to ISO/IEC Standard 17025, proficiency testing and internal quality control as well as the use of methods of analysis validated according to Codex requirements. These overarching guidelines provide information to and dictate requirements for laboratories working in the GMO sector.

Measurement Uncertainty

Codex is currently developing guidelines on Measurement Uncertainty. These guidelines, as well as the accreditation requirements cited above, require laboratories to estimate the uncertainty of their quantitative measurements. This is particularly important and has consequences for measurements in the GMO sector where analytical controls may not be as effective as found in other areas of analysis in the food sector. It is frequently not appreciated that the magnitude of the measurement uncertainty is considerably greater in this analytical sector than would normally be expected.

INFORMATION TO BE PROVIDED TO CODEX WHEN A METHOD FOR GMOs IS TO BE CONSIDERED FOR ENDORSEMENT BY CCMAS

The information that should be supplied to CCMAS when a method is to be considered for endorsement is given in Annex I. The annex lists both general considerations and specific requirements.

As GMO methodology becomes more developed the specific requirements will be converted to performance criteria to conform to the "criteria approach" already adopted by Codex.

DEFINITIONS

There are a number of Codex definitions applicable to GMO analysis. Suggested definitions are given in Annex II.

METHOD DEVELOPMENT TO FORMAL VALIDATION

Applicability of the Method

This is a particularly important criterion in GMO analysis. In principle the method should be applicable to the matrix of concern within the Codex system. If this is a specific product derived from GMO then there is merit in requiring those seeking endorsement to provide information on the method of analysis appropriate to the specific product and, ideally, the matrix in which it is likely to be used. In case of "general purpose" GMO methods, at least one extraction method applicable to a general matrix should be available.

As an example it is required from an extraction method, independent of matrix to which it is to be applied, that it yields DNA of sufficient quantity, structural integrity and purity to allow a proper evaluation of the performance of the subsequent method steps (e.g. adequate amplification of DNA during the PCR step) to be undertaken. This can be tested, for example, by setting up dilution series of the template DNA and determining that the ΔCT in a real-time PCR analysis between the dilutions corresponds to the dilution factor, e.g. if DNA is diluted 10X then the ΔCT should be approx. 3.32, if the DNA is diluted 4X, the ΔCT should be 2, etc. Deviations from this relationship may indicate that the extracted DNA contains PCR inhibitors, that the DNA solution is not homogenous or the DNA quantity so low that stochastic variation in copy numbers yield unreliable quantitative estimates.

Validation Process

Method validation is a process of establishing the performance characteristics and limitations of an analytical method and the identification of the influences, which may change these characteristics - and to what extent. The results of a validation process describe which analytes can be determined in what kind of matrices in the presence of which interference. The validation exercise results in precision and accuracy values of a certain analytical method under the examined conditions.

Formal validation of a method is the conclusion of a long process, which includes the following main steps:

- ***Method development and optimisation.*** Prior to any pre-validation, the method should be fully optimised so that an inter-laboratory transfer is possible. The protocol should be finalized so that no major changes are needed between the pre-validation and validation.
- ***Pre-validation of the method.*** Pre-validation should ensure that a method performs in a manner, which allows a successful conclusion of the validation study, i.e. it should provide evidence about the compliance with the regulations. Pre-validation should preferably be carried out by involving 2 – 4 laboratories.
- ***Full validation of the method.*** Full validation requires considerable resources and should be conducted only on methods which have received adequate prior testing.

A collaborative trial is expensive to undertake and usually follows only after the method has shown acceptable performance both in a single-laboratory and a pre-validation study.

Modular Approach to Method Validation

The "method" refers to all the experimental procedures needed to estimate the measurand in a particular matrix. For a particular material this may include the methods for DNA extraction and the final quantification in a PCR system. In such a case, the whole chain from extraction up to the PCR-method (or equivalent) constitutes a method, but the different method parts can be considered separately (i.e. modular validation). In practice this is difficult to achieve.

The theoretical advantage of a modular approach to method validation is that each section of a method or protocol can be validated separately, and once validated, can be combined with other sections in a flexible manner.

However, there are several disadvantages to a modular approach to method validation, particularly when GMO analysis is being considered. It has been found that the variability of GMO analysis is very significant, and this then reduces the effectiveness in comparing different approaches to the same module in a method. But most critically, a modular approach to method validation assumes that the modules in a method which form the whole are independent of each other. This is frequently not the case and where "official control" work is to be undertaken, a modular approach should not be taken unless independence between the modules can be clearly demonstrated and documented.

METHOD ACCEPTANCE CRITERIA

In order to evaluate a method prior to full validation, information concerning both the method and the method testing is required. Information on this is given in Annex I.

The method will be evaluated based on the information provided to Codex. The evaluation should verify that the principle preconditions for using the method for Codex purposes are fulfilled. This section describes the method acceptance criteria, which have to be fulfilled by the method in order to conduct further a pre-validation and full collaborative trial.

Principle Conditions

The provision of the detection method is aimed to serve mainly the requirements for the monitoring and labelling of GMOs, as set out in the specific regulations. To serve these purposes, the method should detect and quantify the specific GM event in the GM product; this may be achieved using either protein-based or DNA-based methods.

Currently, the DNA-based detection method typically consists of PCR methodology and includes:

- a protocol describing an extraction method which is applicable to a relevant matrix;
- a description of the oligonucleotide primer sequences which uniquely identify the GM event in the GM product[2];
- a description of the oligonucleotide primer sequences which amplify an endogenous gene sequence applicable to the specific host species;
- a protocol describing the conditions under which PCR can be used to detect the GM product;
- appropriate control samples.

The method provider should demonstrate that the method fulfils the principle method requirements:

[2] *Note: the fact that most event-specific sequences are not publicly disclosed should be discussed by CCMAS.*

(1) GMO Screening Methods. In the case of a method used for screening for the presence of multiple events, the method should be specific and allow for unequivocal detection/identification/ quantification of a specific DNA sequence in the case of DNA-based methods. In the case of Protein based methods, the method should be specific and allow for unequivocal detection/identification/ quantification of a specific protein.

(2) DNA-based event-specific methods should allow for unequivocal detection/identification/ quantification of a known target nucleotide sequence.

Currently, the best choice concerning event-specificity of a method, should PCR be the chosen technique, is to target an event-specific genomic region using a set of oligonucleotides (primers) that trigger the amplification of such a region. Among various types of event-specific genomic regions, the one relative to the junction between the transgenic insert and the host genomic DNA will probably be the location of choice. However, when a unique DNA sequence can be found within the transgenic insert, such a sequence can also be targeted by appropriate oligonucleotide primers and amplified through a PCR.

(3) All methods should be applicable to the material specified in their scopes, and to appropriate quality control and reference materials.

It should be noted that at present only relative quantitation can be carried out, which means that the transgenic material relative to the corresponding ingredient/species is measured.

COLLABORATIVE TRIAL REQUIREMENTS

General Information

The purpose of a collaborative trial is to fully validate the data provided by previous testing in a pre-validation or a single laboratory exercise and to determine methodological precision in terms of repeatability and reproducibility.

The values of any performance parameters reported from validation studies must be interpreted and compared with care. The exact values and their interpretation may depend – besides the performance of the method - on the extent of the method (e.g. a real-time quantitative PCR only versus a method chain ranging from extraction to the real-time PCR quantification), experimental design applied, exact calculation forms used to determine the parameters and the approach used to detect and analyse outliers. In order to have meaningful "minimum performance requirements" the above factors must be treated appropriately and in a standardized manner.

For Codex purposes the ISO/AOAC/IUPAC harmonized protocol[1] has been adopted.

Minimum Performance Requirements

In a collaborative trial, the method performance should comply with the relevant parts of the method acceptance criteria and fulfil the method performance requirements specifically set below for the collaborative trial. Thus, the collaborative trial confirms the results obtained during the previous method evaluation phases and provides additional information about the method performance in a multi-laboratory setting. In particular, the compliance with the criteria for sensitivity and repeatability standard deviation should be re-confirmed.

In addition to the method acceptance criteria, at least the method performance requirements listed in Annex I should be evaluated from the experimental data of a collaborative trial. First, the definition and thereafter the requirements are described.

The endorsed methods and their associated validation data will be revised on a regular basis as the scientific knowledge and experience gained in Single-Laboratory validation and collaborative trials evolve. These Guidelines will also be complemented with practical information about the operational steps of the validation process.

Collaborative Trial Test Materials

In principle, the method should be applicable to and tested on the matrix of concern (i.e. on which any specification has been made).

In other fora recommendations have been made that in case of "general purpose" GMO procedures (in contrast to consideration of a specific product derived from GMO) that the validation of the detection

module is carried out using genomic DNA as the analyte (for a PCR-based method). This allows the detection step to be combined with various extraction methods applicable to different matrices. However, real materials/matrix typical of a type/group of matrices are preferred unless the effects of the materials/matrix on DNA quality in the extraction step is completely evaluated prior to applying a modular approach. Otherwise a modular approach is inappropriate when considering Codex specifications.

VALIDATION OF PCR METHODS

Specific information on the validation of quantitative and qualitative PCR methods is given in Annexes III and IV respectively.

Specific information on the validation of quantitative, semi-quantitative and qualitative protein-based methods is given in Annex V.

UNITS OF MEASUREMENT

Various countries have thresholds established for labelling of food and feed derived from modern biotechnology. These thresholds are explicitly or implicitly expressed as weight by relative percentage. However, none of the current detection methods (DNA – or protein-based) are able to measure this directly. Although there is a correlation between weight-% and the amount of DNA or protein, respectively, the very nature of this relationship is influenced by a number of biological factors and thus remains highly variable. This continues to cause considerable misunderstanding and requires significant technical guidance.

Based on the PCR technique used for GMO identification and quantification genome equivalents are measured.

Therefore it is not trivial to consider how the genetically modified material is calculated. For example, if a maize seed lot containing 2% genetically modified seeds with the "new" trait in a hemizygous state (coming from the pollen) is used to prepare a flour sample then, in theory, only 0.29% of the isolated genomic DNA copies will represent the genetically modified status. This is due to the different tissue types, the source from where the genomes in these tissue types are derived (maternal or paternal) and the contribution of the tissue types in the seed kernel. Consequently the amount of genetically material would be underestimated (on a seed basis) by a DNA based approach to express the content of material derived from genetically modified organisms.

Quantitation based on the "newly" expressed protein in the GMO would also lead to a significant contribution to the uncertainty of the analysis. For example the environment in which the material was grown can affect the amount of protein expressed. In addition, it is often the case that the protein is expressed at different levels in different tissue types of the plant. Consequently foods produced from different parts of a genetically modified plant would contribute a different amount of the "newly" expressed protein.

This issue needs to be appropriately addressed and performance and data reporting criteria established for these methods.

MEASUREMENT UNCERTAINTY

Analysts using methods which have been validated according to these guidelines will have available to them sufficient information to allow them to estimate the uncertainty of their result.

Guidance on the use of this measurement uncertainty estimation has been developed and adopted by Codex[2].

GUIDANCE ON LABORATORY SET-UP AND OPERATION

DNA-based methods for the analysis of foods derived from modern biotechnology apply techniques that are not considered as commonly available methods, as they require specific apparatus and handling techniques that differ from most chemical-analytical methods. It is therefore necessary to provide information and instructions on the essential differences in laboratory set-up and handling techniques. Examples are available[3].

REFERENCE MATERIALS

There are a number of matrices that can be used to develop reference materials or working standards for methods of detection of GM products. Each has its own advantages and drawbacks for particular purposes.

Codex may consider requiring the availability of suitable reference materials as part of the method endorsement procedure. However, it is recognised that there are specific problems with the development of reference materials, e.g. for maize materials should the maize event or the construct specific methods be considered.

A suitable reference material is generally required for validation of a method. Suitable reference materials are becoming available for many commercialized events. Where they are not available, the availability of quality control materials from proficiency testing schemes or from the use of Plasmid or amplicon DNA may be considered.

SAMPLING

In the area of GMO analysis it may be anticipated that sampling error can be expected to contribute significantly – if not dominate - the overall uncertainty of an analytical result, particularly when considering raw commodities. The combination of sampling and analytical uncertainties is now being addressed by a number of International Organisations, most notably EURACHEM which has set up a new Working Group dealing with uncertainty of sampling. Much work has been carried out on sampling generally by CCMAS[4] and of bulk sampling for GMOs by the EU JRC[5], ISO/CEN[6]and GIPSA.

REFERENCES

1. ISO/AOAC/IUPAC harmonized protocol (Protocol for the Design, Conduct and Interpretation of Method-Performance Studies, Ed. Horwitz, Pure & Appl. Chem. 331-343, **67**, 1995

2. Guidelines on the Use of Measurement Uncertainty Within Codex (being developed)

3. Draft ISO-standard (ISO/DIS 24276) or the corresponding French standard (AFNOR XP V03-020-2, tabled as room document CRD 5 in its previous version AFNOR XP V03-020-1 by the French Delegation at the 24th Session of CCMAS)

4. Codex General Guidelines on Sampling.

5. FP5 KeSTE project.

6. prEN ISO 21568

ANNEX I: INFORMATION TO BE PROVIDED TO CODEX WHEN A METHOD IS TO BE CONSIDERED FOR ENDORSEMENT BY CCMAS

In order to aid the endorsement of a proposed method of analysis in the GMO sector by Codex, and in particular CCMAS, the following should be provided:

DESCRIPTION OF THE METHOD

A complete and detailed description of all the components of the method should be provided. The use of multiple plates for PCR and protein methods, as an example, should be explicitly addressed. The information should also include information on the following:

Purpose and relevance of the method

The objective of the method and the relevance of the method with respect to relevant legislative requirements should be indicated. In particular, the proposer should indicate that the principle conditions for the method are fulfilled.

Scientific basis

An overview of the principles of how the method, such as DNA molecular biology based (e.g. for real-time PCR) information should be provided. References to relevant scientific publications are useful.

The prediction model adopted to interpret results and to make inferences must be described in complete detail.

Specification of the prediction model/mathematical model needed for the method

If the derivation of the results relies upon a mathematical relationship this must be outlined and recorded (e.g., a regression line or calibration curve obtained by other means). Instructions for the correct application of the model should be provided. These may include, depending on the method, a recommended number and range of levels to be analysed, minimum number of replicates to be included or the means to evaluate the goodness-of-fit.

Outline of the experimental design, including the details about the number of runs, samples, replicates etc. should be stated.

INFORMATION ABOUT THE METHOD OPTIMISATION

Primer pairs tested

For PCR methods, sufficient justification should be given of how and why the proposed primer pair has been selected, also for the reference gene (should this be part of the method).

Specificity testing

Empirical results from testing the method with non-target transgenic events and non-transgenic plants should be provided. This testing should include closely related events and cases were the limits of the sensitivity are truly tested. In addition it might be appropriate to test other plants to reduce the potential for obtaining a false positive.

Stability testing

Empirical results from testing the method with different varieties should be provided in order to demonstrate, for instance, the stability of the copy number of the reference gene.

Sensitivity testing

Empirical results from testing the method at different concentrations in order to test the sensitivity of the method. Limits of detection must be defined using samples comprising of single crops only, e.g. "the LOD for Roundup Ready® soy is 0.1 % of total soy *if the product is comprised of 100 % soy*". For food products made up of multiple ingredients, the actual sensitivity will be reduced, as total extracted DNA will be derived from more than one ingredient so that the starting amount of the actual measurand will be decreased. This dilution effect will depend on how much of the target ingredient (e.g. soy) is in the food product and the total quantity of DNA derived from the other ingredients. Some ingredients will contribute a large amount of DNA, such as wheat or maize flour and eggs, while other ingredients will not contribute any DNA, such as sugar, water or highly processed oils.

LOD should be determined in terms of genome equivalents for each PCR system separately.

Robustness testing

Empirical results from testing the method against small but deliberate variations in method parameters.

Cross-reactivity

The cross-reactivity, interferences and matrix effects should be evaluated, particularly for the protein-based methods of analysis.

PRACTICAL APPLICATION OF THE METHOD

Applicability

Indication of the matrix (e.g., processed food, raw materials, etc.), the type of samples (e.g., seeds, flour, pizza, cookies, etc.) and the range to which the method can be applied. Relevant limitations of the method should also be addressed (e.g. inference by other analytes or inapplicability to certain situations). Limitations may also include possible restrictions due to the costs, equipment or specific and non-specific risks implied for either the operator and/or the environment.

Operational characteristics and practicability of the method

The required equipment for the application of the method should be clearly stated, with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties, and of any other factor that could be of importance for the operators should be also indicated.

Operator skills requirements

A description of the practical skills necessary to properly apply the proposed method should be provided.

ANALYTICAL CONTROLS

The proper use of controls when applying the method should be indicated. Controls should be clearly specified and their interpretation recorded. These may include positive and negative controls, their detailed contents, the extent into which they should be used and the interpretation of the obtained values.

In particular the following should be stated:

- Positive and negative controls used
- Control samples, plasmids and alike used
- Reference materials used.

METHOD VALIDATION/PERFORMANCE

See the Codex "Check-list" (i.e. accuracy, applicability (matrix, concentration range and preference given to 'general' methods), detection limit, determination limit, precision, recovery, selectivity, sensitivity and linearity),

and in particular the following additional information should be supplied for DNA-based procedures:

- ***amplicon length***

The boundaries of the amplified product are formed by the primers at both sides. Therefore the selection of suitable primers is a crucial factor in the PCR analysis. The length of the amplified product does have a direct influence of the PCR performance. By increasing the product length, the PCR efficiency will decrease reciprocal as illustrated below (Fig. 1). In theory in every cycle the target DNA sequence is doubled (amplification factor of 2). In reality the PCR efficiency is less than 100% resulting in a decreased amount of amplified product. Moreover food processing will lead to a degradation of target DNA. Therefore the selection of shorter amplicon sizes (within reason) will increase the possibility to get a positive signal in the analysis of highly processed foodstuffs.

Amplification factor	2	1.65	1.60	1.55	1.50	1.45
Target copies after:						
10 cycles	10^3	150	110	80	58	41
20 cycles	10^6	$2.2x\ 10^4$	$1.2x10^4$	$6.4x10^3$	$3.3x10^3$	$1.7x10^3$
30 cycles	10^9	$3.3x10^6$	$1.3x10^6$	$5x10^5$	$1.9x10^5$	$7x10^4$
40 cycles	10^{12}	$5x10^8$	$1.5x10^8$	$4.1x10^7$	$1.1x10^7$	$2.8x10^6$
	100%	82.5%	80%	77.5%	75%	72.5%

Figure 1 PCR efficiency. A decrease of efficiency in PCR leads to lower amounts of amplified products being present after a certain number of cycles.

- ***whether the method is instrument specific***

At the moment a number of different types of real time instruments are available. These instruments may have different heating and cooling characteristics, which affects ramp rates and affects the time necessary for a whole PCR run.

Beside the differences in the heating and cooling system there are differences in the technique used to induce and subsequently to record the fluorescence. Some real time instruments use laser technique for inducing fluorescence, others are equipped only with a white lamp and filters for selecting a specific wave length. The detection of the fluorescence could also vary.

Taking all the differences into account it is impossible to change the instrument without adaptation of the PCR method. Thus, because the methods are generally instrument dependent they cannot be transferred to other equipment without evaluation and/or modification.

This is in many ways equivalent to the Codex Type I method and should be considered in the same light.

- ***whether single- or multi-plex PCR amplifications are undertaken***

Using more than one primer set in a single reaction is called multi-plex PCR. The aim of using such approach is to reduce costs and time for the analysis of different targets of a single sample (i.e. a GMO specific system is combined with a target taxon specific for relative quantitation). It must be emphasised that the unbalanced presence of one of the target sequences will lead in a preferred amplification by the polymerase during PCR. Moreover the combination of different primer sets is limited up to 7 to 10 in a single reaction.

The information provided should demonstrate the robustness of the method for inter-laboratory transferability. This means that the method should have been tested by at least one external laboratory besides the laboratory which has developed the method. This is an important pre-condition for the success of the validation of the method.

And for both protein and DNA based methods:

- ***whether there are differences between PCR-based and immunological methods concerning validation criteria***

The DNA and protein-based techniques used to detect and quantify a GMO derived material in foods are based on different principles. In PCR the targeted DNA is amplified in a exponential manner, in which a small difference in the beginning of the PCR process will lead to a big difference in the amplified amount of DNA after 35-45 cycles. In contrast to that immunological detection assays are based on the direct interaction with the target molecule and do not include an amplification step.

Moreover, the quantitation by real time PCR is often based on two independent PCR systems: one for the genetic modification and one for the taxon specific sequence.

ANNEX II: CODEX DEFINITIONS APPLICABLE TO GMO ANALYSIS

This Annex is concerned with the definitions needed in GMO analysis. ***(Note: a number of definitions have been grouped together in one heading; these may be contradictory and this needs to be resolved. The Codex definition given in the Procedural Manual should be used and amplified as necessary. Codex definitions have not been reproduced here if they need no further qualification for GMO analysis).***

Accuracy

The closeness of agreement between a reported result and the accepted reference value[3].

Applicability

The analytes, matrices and concentrations for which a method of analysis may be used[4].

The analytes, matrices, and concentrations should be appropriate for the control purposes for which the method has been proposed. The description may also include warnings to known inferences by other analytes, or inapplicability to certain matrices and situations.

It is not feasible to provide reference materials for every one of the many food matrices that are available, so that the use of a representative matrix reference will usually be necessary. The use of the method in a new matrix will need to be validated at a minimum via Single Laboratory validation, usually by spike and recovery experiments, and the reference material used should be described on the report to the customer.

Dynamic Range - Range Of Quantification

The interval of concentration within which the analytical procedure has been demonstrated by collaborative trial to have a suitable level of precision and accuracy.

Limit of Detection (LOD)

Limit of detection is the lowest concentration or content of the analytes that can be detected reliably, but not necessarily quantified, as demonstrated by collaborative trial or single-laboratory validation[5]. LOD is generally expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time (≤5% false negative results).

Limit of Quantification (LOQ)

The limit of quantification of an analytical procedure is the lowest amount or concentration of analyte in a sample, which can be quantitatively determined with an acceptable level of precision and accuracy as demonstrated by satisfactory collaborative trial or single-laboratory[6] validation[7].

Practicability

The ease of operations, in terms of sample throughput and costs, to achieve the required performance criteria and thereby meet the specified purpose[8].

Generally, the method should be practical for its intended purposes.

Repeatability standard deviation (RSD_r)

The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.[9]

Reproducibility standard deviation (RSD_R)

---

[3] Definition adopted from ISO 3534-1.

[4] Slightly modified from the definition provided in Codex CX/MAS 02/4: Proposed draft guidelines for evaluating acceptable methods of analysis. Version November 2002.

[5] Slightly modified from prEN ISO 24276:2002 (E).

[6] E.g. Thompson et al. 2002. IUPAC Technical Report: Harmonised guidelines fro single-laboratory validation of methods of analysis. Pure Appl. Chem. 74(5): 835-855.

[7] Slightly modified from prEN ISO 24276:2002 (E).

[8] Adopted from prEN ISO 24276:2002 (E).

[9] Definitions adopted from ISO 3534-1.

The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.[10]

Recovery

Proportion of the amount of analyte, present in or added to the analytical portion of the test material, which is extracted and presented for measurement.

Ruggedness (Robustness)

Robustness refers to variations in the method as performed in different laboratories by different technicians. The language used here is derived from "Ruggedness" which is the equivalent in the harmonized guidelines. Ruggedness should be demonstrated by the validation of the method in 8-12 laboratories as defined in the harmonized guidelines. It is preferable from a CODEX point of view, that these laboratories be distributed across several continent/trading blocks.

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage[11].

Sensitivity

The sensitivity of a method is a measure of the magnitude of the response caused by a certain amount of analyte.

The method should be sensitive enough in order to be able to detect/quantify with respect to the thresholds as provided in the relevant legislation.

Since sensitivity is method- and purpose-dependent it should be specified in the protocol. A reasonable goal for sensitivity is that required to meet levels specified in contracts, with a reasonable certainty that the level does not exceed the required limit.

Sensitivity as a term is used in two different ways - LOD and the slope of a curve. The use of "detection limit", or "limit of detection" is the preferred term to use as a measure of the ability of a method to detect a small amount of analyte. See also previous comments regarding sensitivity in this document.

Specificity

Property of a method to respond exclusively to the characteristic or analyte of interest.

Trueness

The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value[12].

The measure of trueness is usually expressed in terms of bias. Trueness has also been referred to as "accuracy of the mean".

[10] Definitions adopted from ISO 3534-1

[11] Definition adopted from ICH Topic Q 2 A "Validation of analytical methods: definitions and terminology." The European Agency for the evaluation of medicinal products. CPMP/ICH/381/95. Version November 1994. http://www.emea.eu.int/pdfs/human/ich/038195en.pdf

[12] Adopted from ISO 3534.

ANNEX III: VALIDATION OF A QUANTITATIVE PCR METHOD

INTRODUCTION

DNA-based analysis is commonly performed using Polymerase Chain Reaction (PCR). This technique amplifies a specific (short) segment of DNA to the extent that its quantity can be measured instrumentally (e.g., using fluorometric means). As DNA is a molecule that is easily degraded during food processing operations (e.g., due to heat, enzymes and mechanical shearing), we urge that this be considered in the performance criteria assessment of this technique. This is relevant as in most foods raw ingredients are not present, but are in a processed form, which has an effect on proteins and/or DNA present in food. Furthermore, these protein(s) and/or DNA may be degraded, or its total amount may be decreased due to processing. As a result, any current detection method (DNA- or protein-based) is affected.

It is often the case that the results of a determination are expressed in terms of percent of a sample that contains a particular biotechnology-derived sequence. In a quantitative test, this measurement actually involves two PCR-based determinations – that of the primary analyte (e.g. an inserted gene sequence) and that of the endogenous, or comparator sequence (e.g. an endogenous maize gene). Each of these determinations has its own uncertainties, and the two are likely to have different measurement characteristics. In most applications, the primary analyte will be present at low concentrations, and the comparator will be present at concentrations 10 to 1000 times higher. It is thus important that both measurements are properly validated. In cases where the measurement is expressed directly as a percentage (as in the use of ΔCT), these factors must be considered when validating the method.

The consequence is that the analysis of DNA, especially in processed foods, aims at detecting a very small amount of analyte. Although the result of a PCR analysis is often expressed in % as the relative amount of DNA specific for foods derived from modern biotechnology relative to the total amount of DNA for a specific species, the actual amount of DNA specific for foods derived from modern biotechnology is often in the nanogram/gram range or lower. Analysis of those low amounts of analyte is accompanied by a considerable measurement uncertainty; this needs to be appreciated by the users of analytical results.

VALIDATION

A quantitative PCR assay should be validated for the intended use or application. A harmonized ISO/IUPAC/AOAC protocol was developed for chemical analytical methods. This defines the procedures necessary to validate a method (Horwitz W; Protocol for the design, conduct and interpretation of method-performance studies. Pure and Applied Chemistry, 67, 331 (1995)). It is important to emphasize that all the principles and rules of the harmonized protocol are applicable to quantitative PCR methods.

A number of the parameters involved in validation of the performance of a quantitative PCR assay will be discussed in detail. These are scope, LOD and LOQ, accuracy, precision, sensitivity and ruggedness (robustness). Other important factors are acceptance criteria and interpretation of results, and the issue of the units in which results are expressed.

It is important to note that a quantitative PCR assay typically consists of two assays, one determines the amount of DNA specific for the transgenic product, while the other is specific for the amount of plant specific DNA. Each of these assays has to be considered separately, as these assays can be considered as independent analytical procedures. Thus, all parameters listed below, including specificity and sensitivity, have to be assessed individually for each of the assays involved. These are given alphabetically, not necessarily in order of importance.

Accuracy

As for any method, the accuracy of a method should be compared to known values derived from reference materials, ideally the best characterised. Precision will be determined in the usual way from single laboratory (repeatability) and multi-laboratory (reproducibility) studies.

Recommendation: The accuracy should be within [± 35%] of the accepted reference value over the whole dynamic range.

Applicability

The analytes, matrices and concentrations for which a method of analysis may be used must be stated.

Dynamic Range - Range Of Quantification

The scope of the methods defines the concentration range over which the analyte will be determined. Typically the range for a GM product will range from a tenth of a percent up to a few percent and for the endogenous control the range will be close to 100%, unless the testing of complex mixtures is envisioned. This desired concentration range defines the standard curves and a sufficient number of standards must be used to adequately define the relationship between concentration and response. The relationship between response and concentration should be demonstrated to be continuous, reproducible and should be linear after suitable transformation.

The range of a quantitative method is typically designed to be in the range 0.1% - 100% (DNA %w/w). However, it is common to validate a method for a range of concentrations that is relevant to the scope of the application. If a method is validated for a given range of values, the range may not be extended without validation. For certain applications (e.g., seed or grain analysis) the use of genomic DNA for the preparation of the standard curve (see discussion on the use of plasmid DNA below) may be considered. While it is easy to establish a nominal 100% standard (limited only by the purity of the materials used) it is difficult to reliably produce standard solutions below 0.1%. This is due to the uncertainties involved in measuring small volumes and the error propagation if serial dilution steps are applied. Additionally, the number of target sites (DNA sequence to be amplified) becomes so small that stochastic errors will begin to dominate and no reliable analysis is possible[1, 2]. If genomic DNA is chosen to be used as calibrator, it is important that this calibrator needs to be traced back (in its metrological meaning) to a reference of highest metrological order, e.g. a certified reference material. The range will be established by confirming that the PCR procedure provides an acceptable degree of linearity and accuracy when applied to samples containing amounts of analyte within or at the extremes of the specified range of the procedure.

Recommendation: The dynamic range of the method should cover at least [20% and 5] times the target concentration, where practicable. Target concentration should be understood here as the threshold relevant for a certain regulation.

Example: 0.1% and 2.0% for a 1% GMO concentration or 50 and 1000 genome copies if the target is 500 copies.

There is a general scientific discussion still going on about the interpretation of the percentage values (e.g. dynamic range from 10% to 5 times the target value). Although the experts agreed that – at least for PCR – copy number is desired over weight/weight percentage, it was recognised that so far there is no reliable weight/copy number relationship because of inter-variety fluctuation of the 1C value and because of uncertainty in the correlation of weight of ingredient to weight of DNA. For the time being, both the w/w and copy number/copy number calculations are acceptable.

The unique characteristics of quantitative PCR impose particular restrictions on the low end of the dynamic range of a quantitative PCR. This is due to the difficulty in determining LOD and LOQ values due to the non-normal distribution of variances in the values in this range. Thus it may not be appropriate to require a range extending to 10% of the measured value. The suggestion of a dynamic range that ranges from 10 to 200% can be problematic. For example, capability to analyse a foodstuff composed of more than 50% (w/w) of a biotechnology-derived material (as might be the case for a nutraceutical) would require a dynamic range exceeding 100% (w/w). This is clearly not possible.

Limit of Detection (LOD)

If the validation of the quantitative PCR assay shows that the assay can measure transgenic plant DNA at (for example) 0.1% with acceptable trueness and precision, then it is often not necessary to determine the LOD and LOQ, as the method is only being applied above the range where these are relevant. However, if the method is being used at concentrations close to the limit of detection and limit of quantification (typically 0.01-0.05%), then the assessment of the LOD and LOQ will become part of the validation procedure.

It is worth noting that a determination of an LOD or LOQ is not necessarily needed to establish the validity of a method for a given application. For example, it does not add much value if an LOD is determined to be 1ng/kg, while the scope of the method validation extends only for concentrations ranging in g/kg. In this and similar cases the reliability of the method will be proven by the other parameters and no efforts are included in the method validation to assess the LOD. However, the LOQ shall always be established and included in the validation study.

If the LOD is required, it is common practice to assume that it is the signal strength of a blank increased by three times the standard deviation of the blank. However, this method gives at best an estimate, relies on normal Gaussian distribution of the blank measurements around zero, and may give a lower value than the actual LOD. Its use is not valid in methods such as Quantitative PCR, in which the distribution of measurement values for blanks is typically truncated at zero and is thus not normally distributed. Thus the LOD need to be experimentally determined unless the targeted concentrations are well above the LOD and the LOD therefore becomes irrelevant. For quantitative methods the LOD is the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time (≤5% false negative results). This, and the false positive rate, are the only parameters required for a qualitative method other than specificity.

For a quantitative method, it is important to know whether the LOQ for a particular matrix is close to the values to be measured. Using the traditional approach, the LOQ can be expressed as the signal strength of a blank equal to the LOD increased by 6-10 times the standard deviation of the blank, unless it is known from other sources that the measured values range so high above the LOQ that its knowledge becomes irrelevant. However, this method to determine the LOQ leads only to an estimate of the true LOQ that may be an artificially high or low approximation.

In practice, two procedures have been employed to determine the LOQ. The first approach is to assay a number of negative samples that have been supplemented (spiked) with known amounts of analyte. The LOQ is then the level at which the variability of the result and percent recovery of the analyte meet certain preset criteria. For small molecules, these criteria have typically been a CV of <20% and 70-120% recovery. DNA recovery, however, may be difficult from some matrices, e.g. starches or ketchup, and lower recovery efficiencies may have to be accepted. When recovery efficiencies are low, this must be stated in the validation data and in the analytical report. A more complete approach is to test the method using a number of samples that contain known amounts of the GM material. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of concentrations of the GM event of interest. Procedures for assessing LOD and LOQ during the validation of quantitative PCR methods are discussed in Annexes III and IV..

Validation of methods consists of two phases. The first is an in-house validation of all of the parameters above except reproducibility. The second is a collaborative trial, the main outcome of which is a measure of the repeatability and reproducibility together with detailed information on the transferability of methods between laboratories. It is strongly recommended that a small-scale collaborative trial be performed to test the general ruggedness of a particular method before the expense of organizing a large-scale trial is incurred. In case any improvement of the method or the method description are needed, only limited expenses are incurred through the pre-trial, while a failure of a full interlaboratory method validation due to a ambiguous method description is a very costly failure. Additionally, it may be pointed out that the implementation of an already validated method in a laboratory needs to include necessary experiments to confirm that the implemented method performs as well under local conditions as it did in the interlaboratory method validation. It is important to note that a method should be validated using the conditions under which it will be performed.

Recommendation: Limit of detection is to be < 10% of the value of specification. The value of specification should be understood here as the threshold relevant for a certain application.

Note: limits of detection must be defined using samples comprising of single crops only, e.g. "the LOD for Roundup Ready® soy is 0.1 % of total soy if the product is comprised of 100 % soy". For food products made up of multiple ingredients, the actual sensitivity will be reduced, as total extracted DNA will be derived from more than one ingredient so that the starting amount of the actual measurand will be decreased. This dilution effect will depend on how much of the target ingredient (e.g. soy) is in the food product and the total quantity of DNA derived from the other ingredients. Some ingredients will contribute much DNA, such as wheat or maize flour and eggs, while other ingredients will not contribute any DNA, such as sugar, water or highly processed oils.

Limit of Quantification (LOQ)

See introduction above for limit of detection.

Recommendation: The limit of quantification is to be < 20% of the value of specification with an $RSD_r \leq$ [25%] or as close as is practicable. The value of specification should be understood here as the threshold relevant for a certain regulation

Example: For a 1 % nominal value LOQmin = 0.1 % or for 500 copies LOQmin = 50 copies.

For a quantitative method, it is important to know whether the LOQ for a particular matrix is close to the values to be measured. Traditional methods of approximating the LOQ (zero value plus 6-10 standard deviations) rely on normal Gaussian distribution of the blank measurements around zero. This approach is not valid in methods such as Quantitative PCR, in which the distribution of measurement values for blanks is typically truncated at zero and is thus not normally distributed. Thus the LOQ needs to be experimentally determined.

Practicability

The practicability of the method must be demonstrated.

Repeatability standard deviation (RSD_r)

Recommendation: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Reproducibility standard deviation (RSD_R)

Recommendation: The relative reproducibility standard deviation should be below 35% at the target concentration and over the majority of the dynamic range. RSD_R < 50% at the limit of quantification/lower end of the dynamic range.

Ruggedness (Robustness)

The evaluation of ruggedness (robustness) demonstrates the reliability of a method with respect to inadvertent variation in assay parameters. Variations that may be included are reaction volumes (e.g., 25 vs. 30µl), annealing temperature (e.g., plus and minus 1°C) and/or other relevant variations. The experiments need to be performed at least in triplicates and the recovery needs to be calculated. The response of an assay with respect to these small changes should not deviate more than ±30% from the response obtained under the original conditions.

The adequacy of the robustness testing needs to be analysed on method-by-method basis. For instance, for a real-time PCR method, the following factors should ideally be taken into account: different thermal cycler models, DNA polymerase, uracil-n-glycosylase, magnesium chloride concentration, primer forward and reverse concentration, probe concentration, temperature profile, time profile, dNTP including dUTP concentrations.

Sensitivity

For a quantitative PCR method, a linear relationship of the CT as a function of the logarithm of the concentration of the target of the individual target should be obtained across the range of the method. The correlation coefficient, y-intercept, slope of the regression line and % of residual should be reported. The % of residual for each of the calibrators should preferably be ≤=30%.

In order to obtain a standard curve for event specific quantitative assays, standard DNA mixtures can be prepared by combining purified genomic DNA from transgenic and non-transgenic plants material such as seed or leaves. The content of transgenic plant DNA in the mixtures might be 100, 50, 10, 5, 1, 0.5, 0.1, and 0% or as appropriate for a smaller concentration range. Three replicates must be analysed for each point on the standard curve.

For quantitative assays on plant endogenous genes, standard DNA mixtures can be prepared by combining purified genomic DNA from the target plant species and that of a non-target plant species. For example, for validation of a maize ADH1 quantitative assay, the target plant species is maize and the non-target plant species could be soybean or another species. The content of DNA of the target plant species in the mixtures is typically 100, 50, 25, 10, 5, 1 and 0% or as appropriate. Three replicates must be analysed for each point on the standard curve. Alternatively the 5% CRM used and further diluted without target DNA.

In cases where the ΔCT-method is employed, it will be the responsibility of the analyst to ensure that the overall amount of DNA is well within the range for which the assay was validated.

Recommendation: Typical sensitivities are in the range of 0.1% biotechnology-derived material by weight if the material is not highly processed.

Specificity

The specificity should be demonstrated by showing experimental results from testing the method with non-target transgenic events and non-transgenic plants. This testing should include closely related events and cases where the limits of the detection are truly tested. As the method should be event-specific it should only be functional with the GMO or GM based product considered and ought not to be functional if applied to other events already authorised. In addition, if a reference gene system is a part of the method this should not recognize any gene corresponding to even phylogenetically related species, and should give similar CT-values when amplifying equal amounts of DNA from different cultivars of the same species.

The adequacy of the testing needs to be analysed on a method-by-method basis. It will be necessary to obtain information about the specificity testing in case of stacked genes at some stage.

Recommendation: Specificity is the starting point for a method and needs to be considered during primer design. Primers should be checked against the known sequence of the event insert and pertinent databases for possible matches. Specificity must also be demonstrated experimentally. The following suggests a reasonable approach and the experiments should be performed during pre-validation of an assay.

For event-specific assays:

- *Analyse at least a total of ten non-target transgenic events and any non-transgenic plants that may commonly be found as contaminants in the commodity.*
- *Test on sample from each source (total of at least 10 DNA samples).*
- *Analyse two replicates for each DNA sample.*

Test results shall clearly indicate that no significant instrument reading is observed.

For assays on plant endogenous genes:

- *Analyse at least a total of ten different plant samples comprising different varieties of the same plant species as well as other plants species important for food production (such as wheat, rice, corn, potato, and soybean) and that may commonly be found as contaminants in the commodity.*
- *Test one sample from each source (total of at least 10 DNA samples).*
- *Analyse two replicates for each DNA sample.*

Test results shall clearly indicate that no significant instrument reading is observed.

Trueness

Recommendation: The trueness should be within ± [30%] of the accepted reference value over the whole dynamic range. This refers to the PCR-step provided that a modular validation approach has been applied.

ANALYTICAL CONTROL ACCEPTANCE CRITERIA AND INTERPRETATION OF RESULTS

A validated method also includes criteria on which the validity of an observed measurement result can be accepted as valid. It is important to follow these criteria and to observe the rules for data interpretation. In the case that it may be desired to deviate from said criteria and rules a new method validation study would be needed in order to demonstrate the validity of the new rules and procedures.

At a minimum, the following acceptance criteria are common to all quantitative PCR methods and applicable to each PCR run:

- The result of the positive DNA target control, with, for example 1% transgenic DNA, the mean of the replicates deviates less than 3 standard deviations from the assigned value. A target DNA control is defined as reference DNA or DNA extracted from a certified reference material or known positive sample representative of the sequence or organism under study. The control is intended to demonstrate what the result of analyses of test samples containing the target sequence will be.

- The amplification reagent control is ≤ LOD. The amplification control is defined as control containing all the reagents, except extracted test sample template DNA. Instead of the template DNA, a corresponding volume of nucleic acid free water is added to the reaction.
- The % of residual for each of the standards should be <30%

To accept the result of an unknown sample, the relative standard deviation of the sample replicates should be <[35]%.

REFERENCES FOR ANNEX III

1. Huebner P, Waiblinger H U, Pietsch K, Bordmann P (2001) Validation of PCR methods for quantitation of genetically modified plants in food. Journal of AOAC International 84(6) 1855-1864;

2. Kay S, Van den Eede G, The limits of GMO detection, Nature Biotech. 19(5) 504 (2001)).

3. Residue Chemistry Test Guidelines OPPTS 860.1340 "Residue Analytical Method" United States Environmental Protection Agency, August 1996, (Mihaliak & Berberich, 1995).

ANNEX IV: VALIDATION OF A QUALITATIVE PCR METHOD

Introduction

A qualitative PCR must be validated in the same way as it is intended to be used – that is the sensitivity of the method must be shown to be such that it can reliably detect one positive particle (seed) in a pool, and does not give rise to a significant number of false positives. A concept of using false-positive and false-negative rates to describe the accuracy and precision of a qualitative assay has been developed for microbial assays[1]. This concept can be applied to qualitative PCR assays. A critical issue in the validation of this type of method is the availability of test materials that are known to be positive and negative. The provision of negative reference materials is particularly important and critical in the case of a qualitative method. Any impurities must be present only at levels so low that they become negligible.

By their very nature, qualitative test results refer to the identification above/below a limit. The measures of precision and accuracy are the frequencies of false negative and/or false positive results at the detection limit. False negative results indicate the absence of a given analyte when in fact the analyte is present in the sample, while false positive results indicate the presence of an analyte that is not present in the sample. Due to the inherent nature of the analytical technique, an increase in false negative results will be observed when the amount of analyte approaches the LOD of the method. Like the limit of detection for quantitative methods, the limit of detection for a qualitative method can be defined as the concentration at which a positive sample yields a positive result at least 95% of the time. This results in a rate of false negative results of 5% or less. During validation of a qualitative PCR assay, it is also important to determine the number of false positive results (a positive result obtained using a sample that is known to be negative). This is also expressed as a rate.

False Positive Rate

This is the probability that a known negative test sample has been classified as positive by the method. The false positive rate is the number of misclassified known negatives divided by the total number of negative test samples (misclassified positives plus the number of correctly classified known negatives) obtained with the method:

For convenience this rate can be expressed as percentage:

$$\% \text{ false positive results} = \frac{\text{number of misclassified known negative samples}}{\text{total number of negative test results [incl. misclassified]}}$$

Note: different sectors use different definitions here.

False Negative Rate

This is the probability that a known positive test sample has been classified as negative by the method. The false negative rate is the number of misclassified known positives divided by the total number of positive test samples (misclassified positives plus the number of correctly classified known positives) obtained with the method.

For convenience this rate can be expressed as percentage:

$$\% \text{ false negative results} = \frac{\text{number of misclassified known negative samples}}{\text{total number of positive test results [incl. misclassified]}}$$

Note: different sectors use different definitions here.

In order to demonstrate the false negative rate for qualitative assay, a series of samples (e.g. grain/seed pools) with a constant, known concentration of positive material in a pool of negative material (e.g., 1 positive kernel in 199 conventional corn kernels) have to be analysed and the results evaluated. It is important to note that the concept of confidence intervals and statistical uncertainty needs to be applied to the risk of false positive and/or false negative results as well. The desired level of confidence determines the size and number of pools that need to be tested. For example, 100 positive test results obtained from 100 independent measurements on truly positive samples lead to the conclusion that the level of false negative results is below 4.5% at a confidence level of 99% for the tested concentration of positive kernels (expressed as the number of positive kernels in a pool of negative kernels).

Ruggedness

As with any validated method, reasonable efforts must be made to demonstrate the ruggedness of the assay. This involves careful optimisation and investigation of the impact of small modifications that could occur for technical reasons.

Acceptance Criteria and Interpretation of Results

A validated method includes criteria on which the validity of an observed measurement result can be accepted as valid. It is important to follow these criteria and to observe the rules for data interpretation. It is therefore important to make sure that the result of the positive DNA target control, is positive. Similarly the amplification reagent control must be negative. In addition to these controls, it is desirable to carry out a parallel reaction on the same DNA sample using a primer set which detects an endogenous single copy sequence. This reaction is carried out on every DNA sample, and can either be in the same reaction (multiplexed) or as a separate reaction. In the case of multiplexed reactions, it is important that the endogenous reaction does not out compete the event specific reaction for reagents, as the endogenous sequence is likely to be present at up to 1000 fold the amount of the target sequence. The control reaction with the endogenous sequence gives an indication of the quality of the DNA as a template for the PCR reaction. Table 1 sets out the accept/reject criteria for the PCR reactions on a per lane basis, using the results of the PCR reaction with the endogenous sequence.

Table 1: Criteria for scoring Qualitative PCR analyses

PCR result (GM analyte)	PCR result (endogenous)	Scoring of test
+	+	+
-	+	-
+	-	[+][-]
-	-	Reject

A further complication is however introduced by the fact that qualitative PCR reactions are typically carried out in duplicate. Thus it can occur that the duplicates do not agree. It is common practice to repeat PCR reactions once on DNA samples that are rejected. A repeated indeterminate result is indicative that the analyte cannot be reliably detected. (Table 2), and that the assay is operating below the limit of detection as, by definition, a 95% or better detection rate would be achieved at the limit of detection. The sample is therefore scored negative. Similar criteria apply if more replicates are carried out on each DNA sample.

Table 2: Criteria for scoring duplicate qualitative PCR analyses

Lane 1	Lane 2	Scoring of test
+	+	Positive
-	+	Repeat/Indeterminate
+	-	Repeat/Indeterminate
-	-	Negative

REFERENCES FOR ANNEX IV

1. AOAC® Official Methods℠ Program Manual, Appendix X p14f, May 2002, AOAC International; http: www.aoac.org/vmeth/omamanual/htm.

ANNEX V: VALIDATION OF A PROTEIN-BASED METHOD[1]

QUANTITATIVE TESTING

Quantitative immunoassays are used to determine levels of the protein analyte in specific parts of the plant (e.g. seed, leaf, root, stalk etc). Typical applications are given in Table 1. In order to perform a microplate ELISA for quantitative determination of a protein analyte in plant tissue, it is first necessary to obtain a representative sample of the plant material. The sample amount will influence the detection limit or sensitivity of the assay. The analyte is then extracted from the plant material by adding a solvent and blending, agitating, or applying sheering or sonic forces. Typical solvents used are water or buffered salt solutions. Sometimes detergents or surfactants are added. Some proteins require more rigorous procedures like homogenisation or boiling in solvents, detergents, salts etc.

After the capture antibody has been immobilized on the microplate well surface, a precise volume of the standard or sample extract solution is added to each well. The analyte in the test solution binds to the capture antibody. The enzyme-labelled second antibody is then added and also binds to the analyte, forming a sandwich. At this point, the well is washed to remove unbound analyte and antibodies, leaving only the antibody-analyte-antibody complex bound to the well surface. A colorimetric substrate is added which reacts with the enzyme label and produces a coloured product. The reaction is stopped after a set period of time and the colour absorbance at a given wavelength is measured on a photometer. The standard curve is generated by plotting the optical density (OD) on the *y*-axis (linear scale) against the concentration on the *x*-axis (log scale) which produces a sigmoidal dose response curve Figure 4.

To obtain an accurate and precise quantitative value, the OD for the sample solutions must fall on the linear portion of the standard curve. If the OD is too high, the sample solution must be diluted until the OD falls within the quantitative range of the assay. The concentration of the protein analyte in the original sample of plant material is calculated by correcting for any dilution factor that was introduced in preparing the sample for application to the microplate. The initial weight of the sample and the volume of extraction solvent, as well as any subsequent dilutions are used to calculate the dilution factor.

Various assay controls can be employed to demonstrate the performance of the assay. A blank sample such as an empty well or buffered solution can be run in the assay to determine any background response which can be subtracted from sample and standard responses if desired. A negative control sample (i.e. matrix extract solution known to contain no analyte) can be used to demonstrate whether a non-specific response or matrix effect is occurring in the assay. A positive control or matrix extract spiked with a known amount of the analyte can be run to demonstrate accuracy. Standards and samples can be run in replicate to demonstrate precision. Blanks, negative controls, positive controls, fortified sample extracts, standardized reference material extracts, and replicates are typically run on each microplate to control for plate-plate variation.

STANDARDIZED REFERENCE MATERIALS

The standardized reference material consists of the same matrix as the actual agricultural commodity to be tested. For example, if the matrix to be tested is soybean seed, the standardized reference material would be soybean seed containing a known proportion of transgenic seed. Alternatively, a pure sample or extract of the protein of interest may be used, providing the use of such protein reference materials has been validated against the matrix in question. Access to standardized reference materials is important during the development, validation, and use of immunoassays for analysis of introduced proteins in transgenic agricultural commodities. The best available reference material should be used in order to comply with regulations and testing needs.

In the case of commodities such as grain or seed, where the commodity consists of discrete units, it is fairly straightforward to make a reference sample with a known proportion of transgenic material. In other cases, generating reference samples for certain matrices and analytes can be difficult. Stability and uniformity are important considerations. For example, if the matrix to be tested consists of a mixture of materials, it would be difficult to combine transgenic and non-transgenic material in such a way as to achieve a homogeneous reference sample with a known proportion of transgenic material. The stability of these materials would need to be evaluated under storage and test conditions. In any case, it is useful to have non-transgenic and transgenic material available to use as negative and positive controls.

During assay development, the reference material is used to help select assay parameters which would minimize any interfering effects of the matrix (e.g. non-specific binding of sample components to the

antibodies). During validation and use of the assay, the reference materials can be extracted and analysed alongside the test samples so that the results can be directly compared.

VALIDATION OF A QUANTITATIVE PROTEIN-BASED METHOD

The principles of method validation described in appendices III and IV for PCR methods also apply to protein methods. For commercially available immunoassay kits, assay performance is generally validated by the manufacturer and is documented in the product user's guide.

Quantitative protein-based methods are better characterized as a class than PCR-based methods. Validation should be conducted according to the harmonized ISO/IUPAC/AOAC protocol was developed for chemical analytical methods. This defines the procedures necessary to validate a method[2].

Accuracy: Accuracy is demonstrated by measuring the recovery of analyte from fortified samples and is reported as the mean recovery at several levels across the quantitative range. Ideally, quantitative methods will have demonstrated recoveries between 70 and 120% and a coefficient of variation (CV) of less than 20% for measured recoveries at each fortification level (Mihaliak & Berberich, 1995).

Extraction efficiency: Extraction efficiency is a measure of how efficient a given extraction method is at separating the protein analyte from the matrix. It is expressed as percent analyte recovered from the sample. Since the introduced protein expressed is endogenous to the plant, it can be difficult to demonstrate efficiency of the extraction procedure. There may not be an alternate detection method against which to compare the immunoassay results. One approach to addressing extraction efficiency is to demonstrate the recovery of each type of introduced protein analyte from each type of food fraction by exhaustive extraction, i.e. repeatedly extracting the sample until no more of the protein is detected (Stave, 1999).

Precision: Intra-assay precision describes how much variation occurs within an assay. it can be evaluated by determining the variation (%CV) between replicates assayed at various concentrations on the standard curve and on the pooled variation (%CV) derived from absorbance values in standards from independent assays performed on different days. Interassay precision describes how much variation occurs between separate assays and can be measured by analysis of quality control samples on every microplate. The quality control samples required would consist of two pools of extracts, one extract from transgenic plant tissue and one from conventional plant tissue. These extracts would be stored frozen and a portion would be thawed and assayed on every microplate. Interassay precision could be evaluated over time and expressed as % CV (Rogan *et al*, 1999). The precision of protein-based quantitative methods is in general higher than PCR-based methods.

Recommendation: The accuracy should be within [± 25%] of the accepted reference value over the whole dynamic range.

Sensitivity: The sensitivity of the assay could be defined as the amount of analyte that can be measured by an absorbance reading of two standard deviations above background absorbance (Rogan *et al*, 1992). The detection limit could be expressed as the lowest dilution of transgenic crop that could be detected when transgenic and non-transgenic crop are combined (Rogan *et al*, 1999).

Dynamic Range - Range Of Quantification

The scope of the methods defines the concentration range over which the analyte will be determined. In most cases the analytical range for a GM product will range from a tenth of a percent up to a few percent. This desired concentration range defines the standard curves and a sufficient number of standards must be used to adequately define the relationship between concentration and response. The relationship between response and concentration should be demonstrated to be continuous, reproducible and should be linear after suitable transformation.

Interpretation of the percentage values (e.g. dynamic range from 10% to 5 times the target value) can be difficult when using quantitative methods. Quantitative protein methods generally give an estimate of the concentration of the GM protein in the matrix, due to variations in the expression of the amount of protein in different tissues of plants, and within the same tissue at different locations. The use of qualitative protein-based methods is thus much more prevalent. In addition, care must be taken to employ a method which can detect the protein in the matrix. For example, it is believed that proteins undergo modification or degradation due to processing to a greater degree than DNA, and thus loss of signal due to processing effects must be considered.

It is worth noting that a determination of an LOD or LOQ is not necessarily needed to establish the validity of a method for a given application. For example, it does not add much value if an LOD is determined to be 1ng/kg, while the scope of the method validation extends only for concentrations ranging in g/kg. In this and similar cases the reliability of the method will be proven by the other parameters and no efforts are included in the method validation to assess the LOD. However, the LOQ shall always be established and included in the validation study.

Limit of Detection (LOD)

LOD is defined in annex II. Proteins are present in GM foods at higher concentrations than the target DNA for PCR methods. Thus stochastic effects have less influence on the determination of the LOD than when using PCR.

It is common practice when estimating the LOD to assume that it is the signal strength of a blank increased by three times the standard deviation of the blank. This method gives at best an estimate, and relies on normal Gaussian distribution of the blank measurements around zero. This can generally be assumed for methods such as ELISA, but the LOD is best determined experimentally. Alternatively the LOD is commonly defined as a concentration equal to the lowest standard used in the assay, should a positive value be consistently obtained with that standard.

Limit of Quantification (LOQ)

For a quantitative method, it is important to know whether the LOQ for a particular matrix is close to the values to be measured. Using the traditional approach, the LOQ can be expressed as the signal strength of a blank equal to the LOD increased by 6-10 times the standard deviation of the blank, unless it is known from other sources that the measured values range so high above the LOQ that its knowledge becomes irrelevant. However, this method to determine the LOQ leads only to an estimate of the true LOQ that may be an artificially high or low approximation.

In practice, two procedures have been employed to determine the LOQ. The first approach is to assay a number of negative samples that have been supplemented (spiked) with known amounts of analyte. The LOQ is then the level at which the variability of the result and percent recovery of the analyte meet certain preset criteria. For small molecules, these criteria have typically been a CV of <20% and 70-120% recovery[3]. Protein recovery, however, may be difficult from some matrices, e.g. starches or oils, and lower recovery efficiencies may have to be accepted. When recovery efficiencies are low, this must be stated in the validation data and in the analytical report. A more complete approach is to test the method using a number of samples that contain known amounts of the GM material. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of concentrations of the GM event of interest. Procedures for assessing LOD and LOQ during the validation of quantitative PCR methods are also discussed in annexes III and IV.

Specificity

The specificity is the degree to which analogs or other molecules bind to the antibodies and should be characterized and described in the method. Specificity should be demonstrated by showing experimental results from testing the method with non-target transgenic events and non-transgenic plants. This testing should include closely related events and cases where the limits of the detection are truly tested. As the method should be protein-specific it should only be functional with the GMO or GM based products considered and ought not to be functional if applied to events which do not express the protein in question. Interferences: the potential for interferences from reagents and labware can be evaluated by assaying extracts from non-transgenic plant material.

Matrix effects: if the response of the method is affected by a substance in the final extract other than the specific protein analyte, the non-specific response is referred to as a matrix effect. One way to manage matrix effects is to demonstrate that the analytical method gives identical results with or without sample matrix present in the extract. In this approach, freedom from matrix effects would have to be demonstrated in all matrices for which the assay is to be used. Another approach (although less desirable) to managing matrix effects would be to prepare the standard solutions in extracts from non-transgenic matrix, i.e. matrix-matched standards. This would ensure that any matrix effects would be consistent between the standards and the samples.

Ruggedness (Robustness)

The evaluation of ruggedness (robustness) demonstrates the reliability of a method with respect to inadvertent variation in assay parameters. Variations that may be included are reaction volumes incubation temperature (e.g., plus and minus 5-10°C) and/or other relevant variations. The experiments need to be performed at least in triplicates and the recovery needs to be calculated. The response of an assay with respect to these small changes should not deviate more than ±30% from the response obtained under the original conditions. Experiments which may be performed to establish ruggedness include repeated analysis of a sample or samples on several days and measurement of accuracy and precision in fortified samples using control material from several sources.

QUALITATIVE (THRESHOLD) TESTING

Lateral flow devices are useful tools for on-site or field threshold testing. This type of testing requires a quick, accurate and cost-effective approach. In order to ensure reliable results, the manufacturer of the lateral flow device must conduct a method validation and provide a description of the performance characteristics of the product in the package insert. If this has been completed there is generally no need for validation studies to be performed by users. Each lateral flow device is an individual stand-alone unit, capable of performing to the standards described in the product package insert.

In order to establish an on-site procedure for threshold testing, the threshold level must first be established. To establish that the lateral flow device is able to differentiate between samples containing transgenic protein above or below the threshold, both a negative reference and a threshold reference containing a known proportion of transgenic grain should be assayed concurrently. The negative reference is a sample of the test matrix known to contain none of the protein analyte and is assayed to demonstrate that the method can distinguish between zero and the threshold level. A sufficient number of these samples are run to ensure that assay sensitivity is adequate to determine whether the level in the test sample is greater or less than the threshold level. During routine testing of bulk commodity samples, the lateral flow devices would typically be used without running the concurrent negative and threshold reference samples.

VALIDATION OF A QUALITATIVE (THRESHOLD) PROTEIN-BASED METHOD

The same principles apply to qualitative protein-based testing as to qualitative PCR testing. These approaches, including calculation of false positive and false negative rates, can therefore be applied to protein-based methods. In general, due to the more reliable nature of protein-based lateral flow strip methods, they are not performed in duplicate on each sample. However, if threshold ELISA testing is performed, duplicate wells should be used.

The same types of control samples, and criteria for acceptance/rejection of the result can be used as for qualitative PCR methods. The LOD is expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time (<5% false negative results). However, lateral flow strip tests are generally applied at test concentrations that are at least two fold (or more) above the LOD.

REFERENCES FOR ANNEX V

1. Guidelines for the Validation and Use of Immunoassays for Determination of Introduced Proteins in Biotechnology Enhanced Crops and Derived Food Ingredients. Lipton et al., Food and Agricultural Immunology, 2000, 12, 153-164.

2. Horwitz W; Protocol for the design, conduct and interpretation of method-performance studies. Pure and Applied Chemistry, 67, 331 (1995).

3. Residue Chemistry Test Guidelines OPPTS 860.1340 "Residue Analytical Method" United States Environmental Protection Agency, August 1996, (Mihaliak & Berberich, 1995).

codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

JOINT OFFICE: Viale delle Terme di Caracalla 00100 ROME Tel: 39 06 57051 www.codexalimentarius.net Email: codex@fao.org Facsimile: 39 06 5705 4593

CX/FBT INF-2 (E)

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX *Ad HOC* INTERGOVERNMENTAL TASK FORCE ON FOODs DERIVED FROM BIOTECHNOLOGY

Fifth Session

Chiba, Japan, 19-23 September 2005

PROPOSED DRAFT GUIDELINES FOR THE LABELLING OF FOOD AND FOOD INGREDIENTS OBTAINED THROUGH CERTAIN TECHNIQUES OF GENETIC MODIFICATION/GENETIC ENGINEERING

(At Step 3 of the Procedure)

APPENDIX VI of ALINORM 04/27/22

PURPOSE OF THE GUIDELINES

To provide guidelines to ensure that the labelling of food and food ingredients obtained through certain techniques of genetic modification/genetic engineering provides factual, verifiable, understandable and non-misleading information to protect consumer's health and to ensure fair practices in food trade. Food labelling plays an important role in providing information to consumers and thereby facilitating consumer choice.

These guidelines set out a number of approaches and related information that could be used for the labelling of food and food ingredients obtained through certain techniques of genetic modification/genetic engineering.

1.0 SCOPE

These guidelines recommend procedures for the labelling of food and food ingredients obtained through certain techniques of genetic modification/genetic engineering.

1.1 These guidelines apply to the labelling of such food and food ingredients:

1.1.1 when it is demonstrated, through an appropriate analysis of data, that the composition, nutritional value, or intended use of the food or food ingredient differ in comparison to that of corresponding conventional counterparts, having regard to accepted limits of natural variation[1]; and /or

---

1 This would include products such as oils with altered fatty acid levels, but would not include products such as those with agronomic modifications which contain recombinant DNA and/or protein but no further overall change to composition, nutritional value or intended use.

1.1.2 when they are composed of or contain a genetically modified / engineered organism or contain protein or DNA resulting from gene technology[2]; and/or

1.1.3 when they are produced from, but do not contain, genetically modified / engineered organisms, protein or DNA resulting from gene technology.

2.0 DEFINITION OF TERMS[3]

(At Step 7 of the Procedure)

For the purpose of these Guidelines:

"Food and food ingredients obtained through certain techniques of genetic modification / genetic engineering" means food and food ingredients composed of or containing genetically modified / engineered organisms obtained through modern biotechnology, or food and food ingredients produced from, but not containing genetically modified / engineered organisms obtained through modern biotechnology.

"Organism" means any biological entity capable of replication, reproduction or of transferring genetic material.

"Genetically modified / engineered organism" means an organism in which the genetic material has been changed through modern biotechnology in a way that does not occur naturally by multiplication and/or natural recombination.

"Modern biotechnology" means the application of:

a. In vitro nucleic acid techniques[4], including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or

b. Fusion of cells[5] beyond the taxonomic family,

that overcome natural physiological, reproductive or recombination barriers and that are not techniques used in traditional breeding and selection.

3.0 LABELLING PROVISIONS

In adopting a specific approach to the labelling of food and food ingredients obtained through certain techniques of genetic modification/genetic engineering the following provisions could be used:

3.1 When food and food ingredients obtained through certain techniques of genetic modification/genetic engineering, as defined in Section 2 are [no longer equivalent to / differ significantly] from the corresponding existing food and food ingredients, as regards:

-composition; and/or

-nutritional value; and/or

-intended use;

the characteristics or properties which make it different from the corresponding existing food and food ingredients should be clearly identified on the label as described in Subsection 6.1 on label declarations.

3.2 The presence in any food or food ingredients obtained through certain techniques of genetic modification/genetic engineering of an allergen transferred from any of the products listed in Section

---

2 [Gene Technology: Means a collection of techniques which are used to alter the heritable genetic material of living cell or organisms in a way that does not occur naturally by multiplication an/or recombination]

3 The terminology used in this section on definitions should not determine the terminology which is appropriate for use on food labels

4 These include but are not limited to: recombinant DNA techniques that use vector systems and techniques involving the direct introduction into the organism of hereditary materials prepared outside the organism such as micro-injection, macro-injection, chemoporation, electroporation, micro-encapsulation and liposome fusion

5 Fusion of cells (including protoplast fusion) or hybridization techniques that overcome natural physiological, reproductive, or recombination barriers, where the donor cells/protoplasts do not fall within the same taxonomic family

4.2.1.4 of the General Standard for the Labelling of Prepackaged Foods (CODEX STAN 1-1985 (Rev.1-1991) shall be declared[6]

3.3 [The presence of substances which may result in physiological or metabolic disorders for certain sections of the population and that are absent in corresponding existing foods[should][shall] be labelled].

3.4 In addition to the provisions of Subsection 3.1 to 3.3, when food and food ingredients obtained through certain techniques of genetic modification/genetic engineering as defined in Section 2, are labelled to indicate method of production, labelling declarations should apply (some examples of which are described in Subsection 6.2):

(a) When they are composed of or contain a genetically modified / engineered organism or contain protein or DNA resulting from gene technology; and/or

(b) When they are produced from, but do not contain, genetically modified /engineered organisms, protein or DNA resulting from gene technology even when they do not differ in composition, nutritional value and, intended use.

3.5 [Notwithstanding Section 4.2.2.2 of the General Standard[6], the presence of substances that are absent in corresponding existing food and food ingredients that could be the subject of dietary restrictions, based on religious objections or cultural practices, may be labelled. Where such labelling is used, member countries should establish criteria on how labelling decisions, based on dietary restrictions, will be decided and implemented in a manner that is fair, transparent and consistent.]

[4.0 THRESHOLD LEVELS

4.1 Where food and food ingredients obtained through certain techniques of genetic modification/genetic engineering, are labelled to declare the method of production, consideration may be given to:

[Establishment of a threshold level in food and food ingredients for the presence of food and food ingredients obtained from certain techniques of genetic modification/genetic engineering, below which labelling would not apply[7]] and/or

[Establishment of a de minimis threshold level for adventitious or accidental inclusion in food and food ingredients, of food and food ingredients obtained through certain techniques of genetic modification/genetic engineering, below which labelling would not apply]]

[5.0 EXEMPTIONS

5.1 Notwithstanding the provisions of Subsection 3.1 to 3.3, consideration may be given to the exemption from labelling of specific categories (for example highly processed food ingredients, processing aids, food additives, flavours) of food and food ingredients obtained through certain techniques of genetic modification / genetic engineering.]

6.0 LABEL DECLARATIONS

In accordance with the General Principles section of the Codex General Standard for the Labelling of Prepackaged Foods and the Codex General Guidelines on Claims, prepackaged food shall not be described on any label or in any labelling or presented in a manner that is false, misleading or deceptive or is likely to create an erroneous impression regarding its character or safety in any respect.

6.1 Where food and food ingredients obtained through certain techniques of genetic modification/genetic engineering are labelled to indicate final product characteristics, the following

---

6 This provision was adopted at Step 8 by the Codex Alimentarius Commission at its 24rd Session (July, 2001)

7 Consideration of a threshold must address existing provisions of the *Codex General Standard for the Labelling of Prepackaged Foods*, e.g. Section 4.2.1.3 (Compound Ingredients)

requirements should apply:

(a) if the composition or nutritional value of food and food ingredients is [no longer equivalent to/ differs significantly] from the corresponding existing food and food ingredients, the label should provide, in conjunction with, or in close proximity to, the name of the food and food ingredients, such additional words or phrases as necessary to inform the consumer as to its changed composition or nutrient content in conformity with Sections 4.1 and 4.2.2 of the General Standard. In addition, nutrient declaration should be provided in conformity with the Codex Guidelines on Nutrition Labelling.

(b) if the mode of storage, preparation or cooking is [no longer equivalent to / differs significantly] from the corresponding existing food and food ingredients, clear instructions for use should be provided.

6.2 In accordance with Section 6.0 and in addition to the provisions in Subsection 6.1, food labels should be meaningful to the [intended] consumer. Where food and food ingredients obtained through certain techniques of genetic modification/genetic engineering are labelled to declare the method of production, examples of label declaration(s) include but are not limited to:

(a) ["Produced from genetically modified (naming the source)"] e.g. "produced from genetically modified soya"

(b) If the ingredient is already listed as produced from the source, ["genetically engineered (naming the food)"], e.g. "genetically engineered maize flour"

(c) ["Grown from seeds obtained through [modern] plant biotechnology"]

(d) If the ingredient is designated by the name of a category, ["contains (name of the ingredient) produced from genetically modified (source)"], e.g. starch ("contains starch produced from genetically modified maize")

(e) ["Genetically engineered (naming the characteristic) (naming the food)"] e.g. "genetically engineered high oleic soybean oil"

(f) ["Product of plant / animal biotechnology"]

(g) ["Naming the food/food ingredient (genetically modified)"] e.g. "soybean (genetically modified)"

(h) ["Naming the food/food ingredient (genetically modified food/food ingredient (not segregated)"] e.g. "soybean (genetically modified soybean not segregated)"

(i) ["Product of gene technology"]

6.3 Where the presence of food and food ingredients obtained through certain techniques of genetic modification/genetic engineering is declared on the label, the following would apply:

(a) In the case of single-ingredient foods, or where there is no list of ingredients, the information should appear clearly on the label of the food; or

(b) In the case of a food ingredient(s) in a multi-ingredient food, the information should be shown in the list of ingredients or in parentheses immediately following the ingredient(s). Alternately, the ingredient(s) may be identified by an asterisk and the required wording should appear in a statement immediately following the list of ingredients.

[7.0 IMPLEMENTATION

Consistent with the approach(es) adopted under Section 3, additional consideration should be given to procedures and methodologies for the identification of food and food ingredients produced using certain techniques of genetic modification/genetic engineering and verification of label declarations. These include, but are not limited to: development of validated detection methods; establishment of verification (for example, documentation) systems; and efforts for the development of supporting capacity and infrastructure.]

ANNEX

[Optional Labelling: Without prejudice to the acceptance of the approach to method of production labelling as a "legitimate concern"* of governments in establishing their national legislation, the following is provided
as optional considerations to member countries:]

[*Statements of Principle Concerning the Role of Science in the Codex Decision-Making Process and the Extent to Which Other Factors are Taken Into Account]

Food Derived from Biotechnology (BOOKLET)

PREFACE

The Codex Alimentarius Commission and the FAO/WHO Food Standards Programme

1. The Codex Alimentarius Commission implements the Joint FAO/WHO Food Standards Programme, the purpose of which is to protect the health of consumers and to ensure fair practices in the food trade. The *Codex Alimentarius* (Latin, meaning Food Law or Code) is a collection of internationally adopted food standards presented in a uniform manner. It also includes provisions of an advisory nature in the form of codes of practice, guidelines and other recommended measures to assist in achieving the purposes of the Codex Alimentarius. The Commission has expressed the view that codes of practice might provide useful checklists of requirements for national food control or enforcement authorities. The publication of the Codex Alimentarius is intended to guide and promote the elaboration and establishment of definitions and requirements for foods, to assist in their harmonization and, in doing so, to facilitate international trade.

Principles for Risk Analysis and Guidelines for Safety Assessment of Foods derived from Modern Biotechnology

2. The Codex Alimentarius Commission at its 26th session in 2003 adopted Principles and Guidelines on foods derived from biotechnology. These are overarching principles on the risk analysis of foods derived from modern biotechnology and guidelines for food safety assessment of foods derived from recombinant-DNA plants and microorganisms. It is hoped that this compact format will allow wide use and understanding of the risk analysis and safety assessment of food derived from biotechnology and that it will encourage their use by governments, regulatory authorities, food industries and all food handlers, and consumers.

3. Further information on these texts, or any other aspect of the Codex Alimentarius Commission, may be obtained from:

The Secretary, Codex Alimentarius Commission,
Joint FAO/WHO Food Standards Programme,
FAO, Viale delle Terme di Caracalla,
00100, Rome Italy

fax: +39(06)57.05.45.93
email: *codex@fao.org*

http://www.codexalimentarius.net

TABLE OF CONTENTS

PRINCIPLES FOR THE RISK ANALYSIS OF FOODS DERIVED FROM MODERN BIOTECHNOLOGY

CAC/GL 44-2003

SECTION 1 - INTRODUCTION

1. For many foods, the level of food safety generally accepted by the society reflects the history of their safe consumption by humans. It is recognised that in many cases the knowledge required to manage the risks associated with foods has been acquired in the course of their long history of use. Foods are generally considered safe, provided that care is taken during development, primary production, processing, storage, handling and preparation.

2. The hazards associated with foods are subjected to the risk analysis process of the Codex Alimentarius Commission to assess potential risks and, if necessary, to develop approaches to manage these risks. The conduct of risk analysis is guided by general decisions of the Codex Alimentarius Commission[1] as well as the Codex Working Principles for Risk Analysis[2].

3. While risk analysis has been used over a long period of time to address chemical hazards (e.g. residues of pesticides, contaminants, food additives and processing aids), and it is being increasingly used to address microbiological hazards and nutritional factors, the principles were not elaborated specifically for whole foods.

4. The risk analysis approach can, in general terms, be applied to foods including foods derived from modern biotechnology. However, it is recognised that this approach must be modified when applied to a whole food rather than to a discrete hazard that may be present in food.

5. The principles presented in this document should be read in conjunction with the Codex Working Principles for Risk Analysis to which these principles are supplemental.

---

1 These decisions include the *Statements of principle concerning the role of science in the Codex decision-making process and the extent to which other factors are taken into account* and the *Statements of principle relating to the role of food safety risk assessment* (Codex Alimentarius Commission Procedural Manual; Thirteenth edition).

2 "Working Principles for Risk Analysis for Application in the Framework of the Codex Alimentarius"(adopted by the 26th Session of the Codex Alimentarius Commission, 2003; Codex Alimentarius Commission Procedural Manual; Thirteenth edition)

6. Where appropriate, the results of a risk assessment undertaken by other regulatory authorities may be used to assist in the risk analysis and avoid duplication of work.

SECTION 2 – SCOPE AND DEFINITIONS

7. The purpose of these Principles is to provide a framework for undertaking risk analysis on the safety and nutritional aspects of foods derived from modern biotechnology. This document does not address environmental, ethical, moral and socio-economic aspects of the research, development, production and marketing of these foods[3].

8. The definitions below apply to these Principles:

"Modern Biotechnology" means the application of:

i) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or

ii) Fusion of cells beyond the taxonomic family,

that overcome natural physiological reproductive or recombinant barriers and that are not techniques used in traditional breeding and selection[4].

"Conventional Counterpart" means a related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food[5].

SECTION 3 – PRINCIPLES

9. The risk analysis process for foods derived from modern biotechnology should be consistent with the Codex Working Principles for Risk Analysis.

RISK ASSESSMENT

10. Risk assessment includes a safety assessment, which is designed to identify whether a hazard, nutritional or other safety concern is present, and if present, to gather information on its nature and severity. The safety assessment should

---

3 This document does not address animal feed and animals fed such feed except insofar as these animals have been developed by using modern biotechnology.

4 This definition is taken from the Cartagena Biosafety Protocol under the Convention on Biological Diversity.

5 It is recognized that for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts.

include a comparison between the food derived from modern biotechnology and its conventional counterpart focusing on determination of similarities and differences. If a new or altered hazard, nutritional or other safety concern is identified by the safety assessment, the risk associated with it should be characterized to determine its relevance to human health.

11. A safety assessment is characterized by an assessment of a whole food or a component thereof relative to the appropriate conventional counterpart:

A) taking into account both intended and unintended effects;

B) identifying new or altered hazards;

C) identifying changes, relevant to human health, in key nutrients.

12. A pre-market safety assessment should be undertaken following a structured and integrated approach and be performed on a case-by-case basis. The data and information, based on sound science, obtained using appropriate methods and analysed using appropriate statistical techniques, should be of a quality and, as appropriate, of quantity that would withstand scientific peer review.

13. Risk assessment should apply to all relevant aspects of foods derived from modern biotechnology. The risk assessment approach for these foods is based on a consideration of science-based multidisciplinary data and information taking into account the factors mentioned in the accompanying Guidelines[6].

14. Scientific data for risk assessment are generally obtained from a variety of sources, such as the developer of the product, scientific literature, general technical information, independent scientists, regulatory agencies, international bodies and other interested parties. Data should be assessed using appropriate science-based risk assessment methods.

15. Risk assessment should take into account all available scientific data and information derived from different testing procedures, provided that the procedures are scientifically sound and the parameters being measured are comparable.

RISK MANAGEMENT

16. Risk management measures for foods derived from modern biotechnology should be proportional to the risk, based on the outcome of the risk assessment

[6] Reference is made to the Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (CAC/GL 45-2003) and the Guideline for the Conduct of Food Safety Assessment of Foods Produced using Recombinant-DNA Microorganisms (CAC/GL 46-2003).

and, where relevant, taking into account other legitimate factors in accordance with the general decisions of the Codex Alimentarius Commission[7] as well as the Codex Working Principles for Risk Analysis.

17. It should be recognised that different risk management measures may be capable of achieving the same level of protection with regard to the management of risks associated with safety and nutritional impacts on human health, and therefore would be equivalent.

18. Risk managers should take into account the uncertainties identified in the risk assessment and implement appropriate measures to manage these uncertainties.

19. Risk management measures may include, as appropriate, food labelling[8] conditions for marketing approvals and post-market monitoring.

20. Post-market monitoring may be an appropriate risk management measure in specific circumstances. Its need and utility should be considered, on a case-by-case basis, during risk assessment and its practicability should be considered during risk management. Post-market monitoring may be undertaken for the purpose of:

A) verifying conclusions about the absence or the possible occurrence, impact and significance of potential consumer health effects; and

B) monitoring changes in nutrient intake levels, associated with the introduction of foods likely to significantly alter nutritional status, to determine their human health impact.

21. Specific tools may be needed to facilitate the implementation and enforcement of risk management measures. These may include appropriate analytical methods; reference materials; and, the tracing of products[9] for the purpose of facilitating withdrawal from the market when a risk to human health has been identified or to support post-market monitoring in circumstances as indicated in paragraph 20.

[7] See footnote 1.

[8] Reference is made to the CCFL in relation to the Proposed Draft Guidelines for the Labelling of Foods and Food Ingredients obtained through certain techniques of genetic modification/genetic engineering at Step 3 of the Codex Elaboration Procedure.

[9] It is recognised that there are other applications of product tracing. These applications should be consistent with the provisions of the SPS and TBT Agreements. The application of product tracing to the areas covered by both Agreements is under consideration within Codex on the basis of decisions of 49th Session of Executive Committee.

RISK COMMUNICATION

22. Effective risk communication is essential at all phases of risk assessment and risk management. It is an interactive process involving all interested parties, including government, industry, academia, media and consumers.

23. Risk communication should include transparent safety assessment and risk management decision-making processes. These processes should be fully documented at all stages and open to public scrutiny, whilst respecting legitimate concerns to safeguard the confidentiality of commercial and industrial information. In particular, reports prepared on the safety assessments and other aspects of the decision-making process should be made available to all interested parties.

24. Effective risk communication should include responsive consultation processes. Consultation processes should be interactive. The views of all interested parties should be sought and relevant food safety and nutritional issues that are raised during consultation should be addressed during the risk analysis process.

CONSISTENCY

25. A consistent approach should be adopted to characterise and manage safety and nutritional risks associated with foods derived from modern biotechnology. Unjustified differences in the level of risks presented to consumers between these foods and similar conventional foods should be avoided.

26. A transparent and well-defined regulatory framework should be provided in characterising and managing the risks associated with foods derived from modern biotechnology. This should include consistency of data requirements, assessment frameworks, the acceptable level of risk, communication and consultation mechanisms and timely decision processes.

CAPACITY BUILDING AND INFORMATION EXCHANGE

27. Efforts should be made to improve the capability of regulatory authorities, particularly those of developing countries, to assess, manage and communicate risks, including enforcement, associated with foods derived from modern biotechnology or to interpret assessments undertaken by other authorities or recognised expert bodies, including access to analytical technology. In addition capacity building for developing countries either through bilateral arrangements

or with assistance of international organizations should be directed toward effective application of these principles[10].

28. Regulatory authorities, international organisations and expert bodies and industry should facilitate through appropriate contact points including but not limited to Codex Contact Points and other appropriate means, the exchange of information including the information on analytical methods.

REVIEW PROCESSES

29. Risk analysis methodology and its application should be consistent with new scientific knowledge and other information relevant to risk analysis.

30. Recognizing the rapid pace of development in the field of biotechnology, the approach to safety assessments of foods derived from modern biotechnology should be reviewed when necessary to ensure that emerging scientific information is incorporated into the risk analysis. When new scientific information relevant to a risk assessment becomes available the assessment should be reviewed to incorporate that information and, if necessary, risk management measures adapted accordingly.

---

[10] Reference is made to technical assistance of provisions in Article 9 of the SPS Agreement and Article 11 of the TBT Agreement.

GUIDELINE FOR THE CONDUCT OF FOOD SAFETY ASSESSMENT OF FOODS DERIVED FROM RECOMBINANT-DNA PLANTS

CAC/GL 45-2003

SECTION 1 - SCOPE

1. This Guideline supports the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology. It addresses safety and nutritional aspects of foods consisting of, or derived from, plants that have a history of safe use as sources of food, and that have been modified by modern biotechnology to exhibit new or altered expression of traits.

2. This document does not address animal feed or animals fed with the feed. This document also does not address environmental risks.

3. The Codex principles of risk analysis, particularly those for risk assessment, are primarily intended to apply to discrete chemical entities such as food additives and pesticide residues, or a specific chemical or microbial contaminant that have identifiable hazards and risks; they are not intended to apply to whole foods as such. Indeed, few foods have been assessed scientifically in a manner that would fully characterise all risks associated with the food. Further, many foods contain substances that would likely be found harmful if subjected to conventional approaches to safety testing. Thus, a more focused approach is required where the safety of a whole food is being considered.

4. This approach is based on the principle that the safety of foods derived from new plant varieties, including recombinant-DNA plants, is assessed relative to the conventional counterpart having a history of safe use, taking into account both intended and unintended effects. Rather than trying to identify every hazard associated with a particular food, the intention is to identify new or altered hazards relative to the conventional counterpart.

5. This safety assessment approach falls within the risk assessment framework as discussed in Section 3 of the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology. If a new or altered hazard, nutritional or other food safety concern is identified by the safety assessment, the risk associated with it would first be assessed to determine its relevance to human health. Following the safety assessment and if necessary further risk assessment, the food would be subjected to risk management considerations in accordance with the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology before it is considered for commercial distribution.

6. Risk management measures such as post-market monitoring of consumer health effects may assist the risk assessment process. These are discussed in paragraph 20 of the Principles for the Risk Analysis of Foods derived from Modern Biotechnology.

7. The Guideline describes the recommended approach to making safety assessments of foods derived from recombinant-DNA plants where a conventional counterpart exists, and identifies the data and information that are generally applicable to making such assessments. While this Guideline is designed for foods derived from recombinant-DNA plants, the approach described could, in general, be applied to foods derived from plants that have been altered by other techniques.

SECTION 2 - DEFINITIONS

8. The definitions below apply to this Guideline:

"***Recombinant-DNA Plant***" - means a plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

"***Conventional Counterpart***" - means a related plant variety, its components and/or products for which there is experience of establishing safety based on common use as food[1].

SECTION 3 - INTRODUCTION TO FOOD SAFETY ASSESSMENT

9. Traditionally, new varieties of food plants have not been systematically subjected to extensive chemical, toxicological, or nutritional evaluation prior to marketing, with the exception of foods for specific groups, such as infants, where the food may constitute a substantial portion of the diet. Thus, new varieties of corn, soya, potatoes and other common food plants are evaluated by breeders for agronomic and phenotypic characteristics, but generally, foods derived from such new plant varieties are not subjected to the rigorous and extensive food safety testing procedures, including studies in animals, that are typical of chemicals such as food additives or pesticide residues that may be present in food.

10. The use of animal models for assessing toxicological endpoints is a major element in the risk assessment of many compounds such as pesticides. In most

[1] It is recognized that for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts.

cases, however, the substance to be tested is well characterised, of known purity, of no particular nutritional value, and, human exposure to it is generally low. It is therefore relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels, in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

11. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds, often characterised by a wide variation in composition and nutritional value. Due to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects which are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

12. Due to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods derived from food plants, including recombinant-DNA plants. This has been addressed by the development of a multidisciplinary approach for assessing safety which takes into account both intended and unintended changes that may occur in the plant or in the foods derived from it, using the concept of substantial equivalence.

13. The concept of substantial equivalence is a key step in the safety assessment process. However, it is not a safety assessment in itself; rather it represents the starting point which is used to structure the safety assessment of a new food relative to its conventional counterpart. This concept is used to identify similarities and differences between the new food and its conventional counterpart[2]. It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of

[2] The concept of *substantial equivalence* as described in the report of the 2000 joint FAO /WHO expert consultations (Document WHO/SDE/PHE/FOS/00.6, WHO, Geneva, 2000).

foods derived from recombinant-DNA plants. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the new product can be considered relative to its conventional counterpart.

UNINTENDED EFFECTS

14. In achieving the objective of conferring a specific target trait (intended effect) to a plant by the insertion of defined DNA sequences, additional traits could, in some cases, be acquired or existing traits could be lost or modified (unintended effects). The potential occurrence of unintended effects is not restricted to the use of *in vitro* nucleic acid techniques. Rather, it is an inherent and general phenomenon that can also occur in conventional breeding. Unintended effects may be deleterious, beneficial, or neutral with respect to the health of the plant or the safety of foods derived from the plant. Unintended effects in recombinant-DNA plants may also arise through the insertion of DNA sequences and/or they may arise through subsequent conventional breeding of the recombinant-DNA plant. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA plant would have an unexpected, adverse effect on human health.

15. Unintended effects can result from the random insertion of DNA sequences into the plant genome which may cause disruption or silencing of existing genes, activation of silent genes, or modifications in the expression of existing genes. Unintended effects may also result in the formation of new or changed patterns of metabolites. For example, the expression of enzymes at high levels may give rise to secondary biochemical effects or changes in the regulation of metabolic pathways and/or altered levels of metabolites.

16. Unintended effects due to genetic modification may be subdivided into two groups: those that are "predictable" and those that are "unexpected". Many unintended effects are largely predictable based on knowledge of the inserted trait and its metabolic connections or of the site of insertion. Due to the expanding information on plant genome and the increased specificity in terms of genetic materials introduced through recombinant-DNA techniques compared with other forms of plant breeding, it may become easier to predict unintended effects of a particular modification. Molecular biological and biochemical techniques can also be used to analyse potential changes at the level of gene transcription and message translation that could lead to unintended effects.

17. The safety assessment of foods derived from recombinant-DNA plants involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety. A variety of data and information are necessary to assess unintended effects because no individual test can detect all possible unintended effects or identify,

with certainty, those relevant to human health. These data and information, when considered in total, provide assurance that the food is unlikely to have an adverse effect on human health. The assessment for unintended effects takes into account the agronomic/phenotypic characteristics of the plant that are typically observed by breeders in selecting new varieties for commercialization. These observations by breeders provide a first screen for plants that exhibit unintended traits. New varieties that pass this screen are subjected to safety assessment as described in Sections 4 and 5.

FRAMEWORK OF FOOD SAFETY ASSESSMENT

18. The safety assessment of a food derived from a recombinant-DNA plant follows a stepwise process of addressing relevant factors that include:

A) Description of the recombinant-DNA plant;

B) Description of the host plant and its use as food;

C) Description of the donor organism(s);

D) Description of the genetic modification(s);

E) Characterization of the genetic modification(s);

F) Safety assessment:

   a) expressed substances (non-nucleic acid substances);

   b) compositional analyses of key components;

   c) evaluation of metabolites ;

   d) food processing;

   e) nutritional modification; and

G) Other considerations.

19. In certain cases, the characteristics of the product may necessitate development of additional data and information to address issues that are unique to the product under review.

20. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, Good Laboratory Practice. Primary data should be made available to regulatory authorities at request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

21. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use. The expected endpoint of such an assessment will be a conclusion regarding whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. In essence, therefore, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed and if so to make well-informed and appropriate decisions.

SECTION 4 - GENERAL CONSIDERATIONS

DESCRIPTION OF THE RECOMBINANT-DNA PLANT

22. A description of the recombinant-DNA plant being presented for safety assessment should be provided. This description should identify the crop, the transformation event(s) to be reviewed and the type and purpose of the modification. This description should be sufficient to aid in understanding the nature of the food being submitted for safety assessment.

DESCRIPTION OF THE HOST PLANT AND ITS USE AS FOOD

23. A comprehensive description of the host plant should be provided. The necessary data and information should include, but need not be restricted to:

A) common or usual name; scientific name; and, taxonomic classification;

B) history of cultivation and development through breeding, in particular identifying traits that may adversely impact on human health ;

C) information on the host plant's genotype and phenotype relevant to its safety, including any known toxicity or allergenicity; and

D) history of safe use for consumption as food.

24. Relevant phenotypic information should be provided not only for the host plant, but also for related species and for plants that have made or may make a significant contribution to the genetic background of the host plant.

25. The history of use may include information on how the plant is typically cultivated, transported and stored, whether special processing is required to make the plant safe to eat, and the plant's normal role in the diet (e.g. which part of the plant is used as a food source, whether its consumption is important in particular subgroups of the population, what important macro- or micro-nutrients it contributes to the diet).

DESCRIPTION OF THE DONOR ORGANISM(S)

26. Information should be provided on the donor organism(s) and, when appropriate, on other related species. It is particularly important to determine if the donor organism(s) or other closely related members of the family naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health (e.g. presence of anti-nutrients). The description of the donor organism(s) should include:

A) its usual or common name;

B) scientific name;

C) taxonomic classification;

D) information about the natural history as concerns food safety;

E) information on naturally occurring toxins, anti-nutrients and allergens; for microorganisms, additional information on pathogenicity and the relationship to known pathogens; and

F) information on the past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g. possible presence as contaminants).

DESCRIPTION OF THE GENETIC MODIFICATION(S)

27. Sufficient information should be provided on the genetic modification to allow for the identification of all genetic material potentially delivered to the host plant and to provide the necessary information for the analysis of the data supporting the characterization of the DNA inserted in the plant.

28. The description of the transformation process should include:

A) information on the specific method used for the transformation (e.g. Agrobacterium-mediated transformation);

B) information, if applicable, on the DNA used to modify the plant (e.g. helper plasmids), including the source (e.g. plant, microbial, viral, synthetic), identity and expected function in the plant; and

C) intermediate host organisms including the organisms (e.g. bacteria) used to produce or process DNA for transformation of the host organism.

29. Information should be provided on the DNA to be introduced, including:

A) the characterization of all the genetic components including marker genes, regulatory and other elements affecting the function of the DNA;

B) the size and identity;

C) the location and orientation of the sequence in the final vector/construct; and

D) the function.

CHARACTERIZATION OF THE GENETIC MODIFICATION(S)

30. In order to provide clear understanding of the impact on the composition and safety of foods derived from recombinant-DNA plants, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out.

31. Information should be provided on the DNA insertions into the plant genome; this should include:

A) the characterization and description of the inserted genetic materials;

B) the number of insertion sites;

C) the organisation of the inserted genetic material at each insertion site including copy number and sequence data of the inserted material and of the surrounding region, sufficient to identify any substances expressed as a consequence of the inserted material, or, where more appropriate, other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food; and

D) identification of any open reading frames within the inserted DNA or created by the insertions with contiguous plant genomic DNA including those that could result in fusion proteins.

32. Information should be provided on any expressed substances in the recombinant-DNA plant; this should include:

A) the gene product(s) (e.g. a protein or an untranslated RNA);

B) the gene product(s)' function;

C) the phenotypic description of the new trait(s);

D) the level and site of expression in the plant of the expressed gene product(s), and the levels of its metabolites in the plant, particularly in the edible portions; and

E) where possible, the amount of the target gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the accumulation of a specific endogenous mRNA or protein.

33. In addition, information should be provided:

A) to demonstrate whether the arrangement of the genetic material used for insertion has been conserved or whether significant rearrangements have occurred upon integration;

B) to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function;

C) to demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are expressed and inherited in a manner that is stable through several generations consistent with laws of inheritance. It may be necessary to examine the inheritance of the DNA insert itself or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly;

D) to demonstrate whether the newly expressed trait(s) are expressed as expected in the appropriate tissues in a manner and at levels that are consistent with the associated regulatory sequences driving the expression of the corresponding gene;

E) to indicate whether there is any evidence to suggest that one or several genes in the host plant has been affected by the transformation process; and

F) to confirm the identity and expression pattern of any new fusion proteins.

SAFETY ASSESSMENT

Expressed Substances (non-nucleic acid substances)

Assessment of possible toxicity

34. In vitro nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in plants. The new substances can be conventional components of plant foods such as proteins, fats, carbohydrates, vitamins which are novel in the context of that recombinant-DNA plant. New substances might also include new metabolites resulting from the activity of enzymes generated by the expression of the introduced DNA.

35. The safety assessment should take into account the chemical nature and function of the newly expressed substance and identify the concentration of the substance in the edible parts of the recombinant-DNA plant, including variations

and mean values. Current dietary exposure and possible effects on population sub-groups should also be considered.

36. Information should be provided to ensure that genes coding for known toxins or anti-nutrients present in the donor organisms are not transferred to recombinant-DNA plants that do not normally express those toxic or anti-nutritious characteristics. This assurance is particularly important in cases where a recombinant-DNA plant is processed differently from a donor plant, since conventional food processing techniques associated with the donor organisms may deactivate, degrade or eliminate anti-nutrients or toxicants.

37. For the reasons described in Section 3, conventional toxicology studies may not be considered necessary where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary.

38. In the case of proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins and anti-nutrients (e.g. protease inhibitors, lectins) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies[3] may need to be carried out in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food, and taking into account its biological function in the plant where known.

39. Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity and biological function in the plant of the substance and dietary exposure. The type of studies to be performed may include studies on metabolism, toxicokinetics, sub-chronic toxicity, chronic toxicity/carcinogenicity, reproduction and development toxicity according to the traditional toxicological approach.

40. This may require the isolation of the new substance from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case, the material should be shown to be biochemically, structurally, and functionally equivalent to that produced in the recombinant-DNA plant.

---

[3] Guidelines for oral toxicity studies have been developed in international fora, for example, the OECD Guidelines for the Testing of Chemicals.

Assessment of possible allergenicity (proteins)

41. When the protein(s) resulting from the inserted gene is present in the food, it should be assessed for potential allergenicity in all cases. An integrated, stepwise, case-by-case approach used in the assessment of the potential allergenicity of the newly-expressed protein(s) should rely upon various criteria used in combination (since no single criterion is sufficiently predictive on either allergenicity or non-allergenicity). As noted in paragraph 20, the data should be obtained using sound scientific methods. A detailed presentation of issues to be considered can be found in the Annex to this document[4].

42. The newly expressed proteins in foods derived from recombinant-DNA plants should be evaluated for any possible role in the elicitation of gluten-sensitive enteropathy, if the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains.

43. The transfer of genes from commonly allergenic foods and from foods known to elicit gluten-sensitive enteropathy in sensitive individuals should be avoided unless it is documented that the transferred gene does not code for an allergen or for a protein involved in gluten-sensitive enteropathy.

Compositional Analyses of Key Components

44. Analyses of concentrations of key components[5] of the recombinant-DNA plant and, especially those typical of the food, should be compared with an equivalent analysis of a conventional counterpart grown and harvested under the same conditions. In some cases, a further comparison with the recombinant-DNA plant grown under its expected agronomic conditions may need to be considered (e.g. application of an herbicide). The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. The comparator(s) used in this assessment should ideally be the near isogenic parental line. In practice, this may not be feasible at all times, in which case a

[4] The FAO/WHO expert consultation 2001 report, which includes reference to several decision trees, was used in developing the Annex to these guidelines.

[5] Key nutrients or key anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates as nutrients or enzyme inhibitors as anti-nutrients) or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes if the level is increased, selenium in wheat) and allergens.

line as close as possible should be chosen. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that are nutritionally important or that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

45. The location of trial sites should be representative of the range of environmental conditions under which the plant varieties would be expected to be grown. The number of trial sites should be sufficient to allow accurate assessment of compositional characteristics over this range. Similarly, trials should be conducted over a sufficient number of generations to allow adequate exposure to the variety of conditions met in nature. To minimise environmental effects, and to reduce any effect from naturally occurring genotypic variation within a crop variety, each trial site should be replicated. An adequate number of plants should be sampled and the methods of analysis should be sufficiently sensitive and specific to detect variations in key components.

Evaluation of Metabolites

46. Some recombinant-DNA plants may have been modified in a manner that could result in new or altered levels of various metabolites in the food. Consideration should be given to the potential for the accumulation of metabolites in the food that would adversely affect human health. Safety assessment of such plants requires investigation of residue and metabolite levels in the food and assessment of any alterations in nutrient profile. Where altered residue or metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (e.g. procedures for assessing the human safety of chemicals in foods).

Food Processing

47. The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps.

Nutritional Modification

48. The assessment of possible compositional changes to key nutrients, which should be conducted for all recombinant-DNA plants, has already been

addressed under 'Compositional analyses of key components'. However, foods derived from recombinant-DNA plants that have undergone modification to intentionally alter nutritional quality or functionality should be subjected to additional nutritional assessment to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

49. Information about the known patterns of use and consumption of a food, and its derivatives should be used to estimate the likely intake of the food derived from the recombinant-DNA plant. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage.

50. The use of plant breeding, including *in vitro* nucleic acid techniques, to change nutrient levels in crops can result in broad changes to the nutrient profile in two ways. The intended modification in plant constituents could change the overall nutrient profile of the plant product and this change could affect the nutritional status of individuals consuming the food. Unexpected alterations in nutrients could have the same effect. Although the recombinant-DNA plant components may be individually assessed as safe, the impact of the change on the overall nutrient profile should be determined.

51. When the modification results in a food product, such as vegetable oil, with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e. foods or food components whose nutritional composition is closer to that of the food derived from recombinant-DNA plant) as appropriate comparators to assess the nutritional impact of the food.

52. Because of geographical and cultural variation in food consumption patterns, nutritional changes to a specific food may have a greater impact in some geographical areas or in some cultural population than in others. Some food plants serve as the major source of a particular nutrient in some populations. The nutrient and the populations affected should be identified.

53. Some foods may require additional testing. For example, animal feeding studies may be warranted for foods derived from recombinant-DNA plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods.

SECTION 5 – OTHER CONSIDERATIONS

POTENTIAL ACCUMULATION OF SUBSTANCES SIGNIFICANT TO HUMAN HEALTH

54. Some recombinant-DNA plants may exhibit traits (e.g., herbicide tolerance) which may indirectly result in the potential for accumulation of pesticide residues, altered metabolites of such residues, toxic metabolites, contaminants, or other substances which may be relevant to human health. The safety assessment should take this potential for accumulation into account. Conventional procedures for establishing the safety of such compounds (e.g., procedures for assessing the human safety of chemicals) should be applied.

USE OF ANTIBIOTIC RESISTANCE MARKER GENES

55. Alternative transformation technologies that do not result in antibiotic resistance marker genes in foods should be used in the future development of recombinant-DNA plants, where such technologies are available and demonstrated to be safe.

56. Gene transfer from plants and their food products to gut microorganisms or human cells is considered a rare possibility because of the many complex and unlikely events that would need to occur consecutively. Nevertheless, the possibility of such events cannot be completely discounted[6].

57. In assessing safety of foods containing antibiotic resistance marker genes, the following factors should be considered:

A) the clinical and veterinary use and importance of the antibiotic in question;

---

[6] In cases where there are high levels of naturally occurring bacteria which are resistant to the antibiotic, the likelihood of such bacteria transferring this resistance to other bacteria will be orders of magnitude higher than the likelihood of transfer between ingested foods and bacteria.

(Certain antibiotics are the only drug available to treat some clinical conditions (e.g. vancomycin for use in treating certain staphylococcal infections). Marker genes encoding resistance to such antibiotics should not be used in recombinant-DNA plants.)

B) whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficacy of the orally administered antibiotic; and

(This assessment should provide an estimate of the amount of orally ingested antibiotic that could be degraded by the presence of the enzyme in food, taking into account factors such as dosage of the antibiotic, amount of enzyme likely to remain in food following exposure to digestive conditions, including neutral or alkaline stomach conditions and the need for enzyme cofactors (e.g. ATP) for enzymatic activity and estimated concentration of such factors in food.)

C) safety of the gene product, as would be the case for any other expressed gene product.

58. If evaluation of the data and information suggests that the presence of the antibiotic resistance marker gene or gene product presents risks to human health, the marker gene or gene product should not be present in the food. Antibiotic resistance genes used in food production that encode resistance to clinically used antibiotics should not be present in foods.

REVIEW OF SAFETY ASSESSMENTS

59. The goal of the safety assessment is a conclusion as to whether the new food is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. Nevertheless, the safety assessment should be reviewed in the light of new scientific information that calls into question the conclusions of the original safety assessment.

ANNEX: ASSESSMENT OF POSSIBLE ALLERGENICITY

SECTION 1 – INTRODUCTION

1. All newly expressed proteins[7] in recombinant-DNA plants that could be present in the final food should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals.

2. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case by case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

3. The endpoint of the assessment is a conclusion as to the likelihood of the protein being a food allergen.

SECTION 2 - ASSESSMENT STRATEGY

4. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability and/or, acid and enzymatic treatment.

5. As there is no single test that can predict the likely human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins from the recombinant-DNA plant, or the synthesis or production of the substance from an alternative source, in which case the

[7] This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing gluten-sensitive or other enteropathies. The issue of enteropathies is already addressed in Assessment of possible allergenicity (proteins), paragraph 42 of the Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants. In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

material should be shown to be structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA plant. Particular attention should be given to the choice of the expression host, since post-translational modifications allowed by different hosts (i.e.: eukaryotic vs. prokaryotic systems) may have an impact on the allergenic potential of the protein.

6. It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

SECTION 3 – INITIAL ASSESSMENT

SECTION 3.1 - SOURCE OF THE PROTEIN

7. As part of the data supporting the safety of foods derived from recombinant-DNA plants, information should describe any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and frequency of allergic reactions; structural characteristics and amino acid sequence; physicochemical and immunological properties (when available) of known allergenic proteins from that source.

SECTION 3.2 – AMINO ACID SEQUENCE HOMOLOGY

8. The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether that protein has an allergenic potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results.[8] Validated search and

[8] It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segments in searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives, inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

evaluation procedures should be used in order to produce biologically meaningful results.

9. IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35% identity in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

10. Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding themselves specifically with IgE antibodies.

11. A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

SECTION 3.3 – PEPSIN RESISTANCE

12. Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential.[9] Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen.

[9] The method outlined in the U.S. Pharmacopoeia (1995) was used in the establishment of the correlation (Astwood *et al.* 1996).

13. Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided[10].

SECTION 4 – SPECIFIC SERUM SCREENING

14. For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals[11] In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic, and which do not exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available as described in paragraph 17.

15. In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient, but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols.[12] A positive result in such tests would indicate a potential allergen.

SECTION 5 – OTHER CONSIDERATIONS

16. The absolute exposure to the newly expressed protein and the effects of relevant food processing will contribute toward an overall conclusion about the potential for human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the

---

10 Report of Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (2001): Section "6.4 Pepsin Resistance".

11 According to the Joint Report of the FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (22-25 January 2001, Rome, Italy) a minimum of 8 relevant sera is required to achieve a 99% certainty that the new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

12 *Ex vivo* procedure is described as the testing for allergenicity using cells or tissue culture from allergic human subjects (Report of Joint FAO/WHO Expert Consultation on Allergenicity of Foods derived from Biotechnology).

types of processing which would be applied and its effects on the presence of the protein in the final food product.

17. As scientific knowledge and technology evolves, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly-related categories of foods); the development of international serum banks; use of animal models; and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.

GUIDELINE FOR THE CONDUCT OF FOOD SAFETY ASSESSMENT OF FOODS PRODUCED USING RECOMBINANT-DNA MICROORGANISMS

CAC/GL 46-2003

SECTION 1 – SCOPE

1. This Guideline supports the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology and addresses safety and nutritional aspects of foods produced through the actions of recombinant-DNA microorganisms.[1] The recombinant-DNA microorganisms that are used to produce these foods are typically derived using the techniques of modern biotechnology from strains that have a history of safe, purposeful use in food production. However, in instances where the recipient strains do not have a history of safe use their safety will have to be established.[2] Such food and food ingredients may contain viable or non-viable recombinant-DNA microorganisms or may be produced by fermentation using recombinant-DNA microorganisms from which the recombinant-DNA microorganisms may have been removed.

2. Recognizing that the following issues may have to be addressed by other bodies or other instruments, this document does not address:

- safety of microorganisms used in agriculture (for plant protection, biofertilizers, in animal feed or food derived from animals fed the feed etc.);
- risks related to environmental releases of recombinant-DNA microorganisms used in food production;
- safety of substances produced by microorganisms that are used as additives or processing aids, including enzymes for use in food production[3];

[1] The microorganisms included in these applications are bacteria, yeasts, and filamentous fungi. (Such uses could include, but are not limited to, production of yogurt, cheese, fermented sausages, natto, kimchi, bread, beer, and wine.)

[2] The criterion for establishing the safety of microorganisms used in the production of foods where there is no history of safe use is beyond the scope of the current document.

[3] The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is revising guidelines for General Specifications and Considerations for Enzyme Preparations used in food processing. These guidelines have been used to evaluate enzyme preparations derived from genetically modified microorganisms.

- specific purported health benefits or probiotic effects that may be attributed to the use of microorganisms in food; or
- issues relating to the safety of food production workers handling recombinant-DNA microorganisms.

3. A variety of microorganisms used in food production have a long history of safe use that predates scientific assessment. Few microorganisms have been assessed scientifically in a manner that would fully characterize all potential risks associated with the food they are used to produce, including, in some instances, the consumption of viable microorganisms. Furthermore, the Codex principles of risk analysis, particularly those for risk assessment, are primarily intended to apply to discrete chemical entities such as food additives and pesticide residues, or specific chemical or microbial contaminants that have identifiable hazards and risks; they were not originally intended to apply to intentional uses of microorganisms in food processing or in the foods transformed by microbial fermentations. The safety assessments that have been conducted have focused primarily on the absence of properties associated with pathogenicity in these microorganisms and the absence of reports of adverse events attributed to ingestion of these microorganisms, rather than evaluating the results of prescribed studies. Further, many foods contain substances that would be considered harmful if subjected to conventional approaches to safety testing. Thus, a more focused approach is required where the safety of a whole food is being considered.

4. Information considered in developing this approach includes:

A) uses of living microorganisms in food production;

B) consideration of the types of genetic modifications likely to have been made in these organisms;

C) the types of methodologies available for performing a safety assessment; and

D) issues specific to the use of the recombinant-DNA microorganism in food production, including its genetic stability, potential for gene transfer, colonization of the gastrointestinal tract and persistence[4] therein, interactions that the recombinant-DNA microorganism may

[4] Persistence connotes survival of microorganisms in the gastrointestinal tract longer than two intestinal transit times (International Life Science Institute, *The safety assessment of viable genetically modified microorganisms used as food*, 1999, Brussels; the Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology- *Safety assessment of foods derived from genetically modified microorganisms*, 24-28 September, 2001, Geneva, Switzerland).

have with the gastrointestinal flora or the mammalian host, and any impact of the recombinant-DNA microorganism on the immune system.

5. This approach is based on the principle that the safety of foods produced using recombinant-DNA microorganisms is assessed relative to the conventional counterparts that have a history of safe use, not only for the food produced using a recombinant-DNA microorganism, but also for the microorganism itself. This approach takes both intended and unintended effects into account. Rather than trying to identify every hazard associated with a particular food or the microorganism, the intention is to identify new or altered hazards relative to the conventional counterpart.

6. This safety assessment approach falls within the risk assessment framework as discussed in Section 3 of the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology. If a new or altered hazard, nutritional or other food safety concern is identified by the safety assessment, the risk associated with it would first be assessed to determine its relevance to human health. Following the safety assessment and, if necessary, further risk assessment, the food or component of food, such as a microorganism used in production, would be subjected to risk management considerations in accordance with the Principles for the Risk Analysis of Foods Derived from Modern Biotechnology before it is considered for commercial distribution.

7. Risk management measures such as post-market monitoring of consumer health effects may assist the risk assessment process. These are discussed in paragraph 20 of the Principles for the Risk Analysis of Foods derived from Modern Biotechnology.

8. The Guideline describes approaches recommended for making safety assessments of foods produced using recombinant-DNA microorganisms, using comparison to a conventional counterpart. The safety assessment will focus on the safety of the recombinant-DNA microorganisms used in food production, and, where appropriate, on metabolites produced by the action of recombinant-DNA microorganisms on food. The Guideline identifies the data and information that are generally applicable to making such assessments. When conducting a comparison of a recombinant-DNA microorganism or a food produced using recombinant-DNA microorganism with their respective conventional counterparts, any identified differences should be taken into account, whether they are the result of intended or unintended effects. Due consideration should be given to the interactions of the recombinant-DNA microorganism with the food matrix or the microflora and to the safety of any newly-expressed protein(s) and secondary metabolic products. While this Guideline is designed for foods produced using recombinant-DNA microorganisms or their components, the approach described could, in general,

be applied to foods produced using microorganisms that have been altered by other techniques.

SECTION 2 – DEFINITIONS

9. The definitions below apply to this Guideline:

"***Recombinant-DNA Microorganism***" - means bacteria, yeasts or filamentous fungi in which the genetic material has been changed through *in vitro* nucleic acid techniques including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

"***Conventional Counterpart***"[5] – means:

- a microorganism/strain with a known history of safe use in producing and/or processing the food and related to the recombinant-DNA strain. The microorganism may be viable in the food or may be removed in processing or rendered non-viable during processing; or
- food produced using the traditional food production microorganisms for which there is experience of establishing safety based on common use in food production.

SECTION 3 - INTRODUCTION TO FOOD SAFETY ASSESSMENT

10. Most foods produced as a result of the purposeful growth of microorganisms have their origins in antiquity, and have been deemed safe long before the emergence of scientific methods for assessing safety. Microorganisms possess properties, such as fast growth rates, that enable genetic modifications, whether employing conventional techniques or modern biotechnology, to be implemented in short time frames. Microorganisms used in food production derived using conventional genetic techniques have not customarily been systematically subjected to extensive chemical, toxicological, epidemiological, or medical evaluations prior to marketing. Instead microbiologists, mycologists, and food technologists have evaluated new strains of bacteria, yeasts and filamentous fungi for phenotypic characteristics that are useful in relation to food production.

11. Safety assessments of recombinant-DNA microorganisms should document the use of related microorganisms in foods, the absence of properties known to be characteristic of pathogens in the recombinant-DNA microorganisms or the

[5] It is recognized that for the foreseeable future, microorganisms derived from modern biotechnology will not be used as conventional counterparts.

recipient strains used for constructing the recombinant-DNA microorganisms, and known adverse events involving the recipient or related organisms. In addition, when a recombinant DNA microorganism directly affects or remains in the food, any effects on the safety of the food should be examined.

12. The use of animal models for assessing toxicological effects is a major element in the risk assessment of many compounds, such as pesticides. In most cases, however, the substance to be tested is well characterized, of known purity, of no particular nutritional value, and human exposure to it is generally low. It is therefore relatively straightforward to feed such compounds to animals at a range of doses some several orders of magnitude greater than the expected human exposure levels, in order to identify any potential adverse health effects of importance to humans. In this way, it is possible, in most cases, to estimate levels of exposure at which adverse effects are not observed and to set safe intake levels by the application of appropriate safety factors.

13. Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixtures of compounds, and often characterized by a wide variation in composition and nutritional value. Due to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects that are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole food. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information.

14. Animal studies typically employed in toxicological evaluations also cannot be readily applied to testing potential risks associated with ingestion of microorganisms used for food production. Microorganisms are living entities, containing complex structures composed of many biochemicals, and therefore are not comparable to pure compounds. In some processed foods, they can survive processing and ingestion and can compete and, in some cases, be retained in the intestinal environment for significant periods of time. Appropriate animal studies should be used to evaluate the safety of recombinant-DNA microorganisms where the donor, or the gene or gene product do not have a history of safe use in food, taking into account available information regarding the donor and the characterization of the modified genetic material and the gene product. Further, appropriately designed studies in

animals may be used to assess the nutritional value of the food or the bioavailability of the newly expressed substance in the food.

15. Due to the difficulties of applying traditional toxicological testing and risk assessment procedures to whole foods, a more focused approach is required for the safety assessment of foods produced using recombinant-DNA microorganisms. This has been addressed by the development of a multidisciplinary approach for assessing safety that takes into account the intended effect, the nature of the modification and detectable unintended changes that may occur in the microorganism or in its action on the food, using the concept of substantial equivalence[6].

16. While the focus of a safety assessment will be on the recombinant-DNA microorganism, additional information on its interaction with the food matrix should be taken into consideration when applying the concept of substantial equivalence, which is a key step in the safety assessment process. However, the concept of substantial equivalence is not a safety assessment in itself. Rather it represents the starting point that is used to structure the safety assessment of both a recombinant-DNA microorganism relative to its conventional counterpart and the food produced using recombinant-DNA microorganism relative to its conventional counterpart. This concept is used to identify for evaluation similarities and differences between a recombinant-DNA microorganisms used in food processing as well as the food produced using the recombinant-DNA microorganisms and their respective conventional counterparts as defined in paragraph 9. It aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy to date for safety assessment of foods produced using recombinant-DNA microorganisms. The safety assessment carried out in this way does not imply absolute safety of the new product; rather, it focuses on assessing the safety of any identified differences so that the safety of the recombinant-DNA microorganism and the food produced using recombinant-DNA microorganism can be considered relative to their respective conventional counterparts.

UNINTENDED EFFECTS

17. In achieving the objective of conferring a specific target trait (intended effect) to a microorganism by the addition, substitution, removal, or

---

[6] The concept of *substantial equivalence* as described in the Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology- Safety aspects of genetically modified plants, 29 May – 2 June, 2000, Geneva, Switzerland, and Section 4.3 of the Joint FAO/WHO Expert Consultation of Foods Derived from Biotechnology, - Safety assessment of foods derived from genetically modified microorganisms, 24-28 September, 2001, Geneva, Switzerland.

rearrangement of defined DNA sequences, including those used for the purpose of DNA transfer or maintenance in the recipient organism, additional traits could, in some cases, be acquired or existing traits could be lost or modified. The potential for occurrence of unintended effects is not restricted to the use of *in vitro* nucleic acid techniques. Rather, it is an inherent and general phenomenon that can also occur in the development of strains using traditional genetic techniques and procedures, or from exposure of microorganisms to intentional or unintended selective pressures. Unintended effects may be deleterious, beneficial, or neutral with respect to competition with other microorganisms, ecological fitness of the microorganism, the microorganism's effects on humans after ingestion, or the safety of foods produced using the microorganism. Unintended effects in recombinant-DNA microorganisms may also arise through intentional modification of DNA sequences or they may arise through recombination or other natural events in the recombinant-DNA microorganism. Safety assessment should include data and information to reduce the possibility that a food derived from a recombinant-DNA microorganism would have an unexpected, adverse effect on human health.

18. Unintended effects can result from the insertion of DNA sequences new to a microorganism into the microbial genome; they may be compared with those observed following the activity of naturally occurring transposable genetic elements. Insertion of DNA may lead to changes in expression of genes in the genome of the recipient. The insertion of DNA from heterologous sources into a gene may also result in the synthesis of a chimeric protein, also referred to as a fusion protein. In addition genetic instability and its consequences need to be considered.

19. Unintended effects may also result in the formation of new or changed patterns of metabolites. For example, the expression of enzymes at high levels or the expression of an enzyme new to the organism may give rise to secondary biochemical effects, changes in the regulation of metabolic pathways, or altered levels of metabolites.

20. Unintended effects due to genetic modification may be subdivided into two groups: those that could be predicted and those that are "unexpected." Many unintended effects are largely predictable based on knowledge of the added trait, its metabolic consequences or of the site of insertion. Due to the expanding knowledge of microbial genomes and physiology, and the increased specificity in function of genetic materials introduced through recombinant-DNA techniques compared with other forms of genetic manipulation, it may become easier to predict unintended effects of a particular modification. Molecular biological and biochemical techniques can also be used to analyse changes that occur at the level of transcription and translation that could lead to unintended effects.

21. The safety assessment of foods produced using recombinant-DNA microorganisms involves methods to identify and detect such unintended effects and procedures to evaluate their biological relevance and potential impact on food safety. A variety of data and information is necessary to assess unintended effects, because no individual test can detect all possible unintended effects or identify, with certainty, those relevant to human health. These data and information, when considered in total, should provide assurance that the food is unlikely to have an adverse effect on human health. The assessment of unintended effects takes into account the biochemical, and physiological characteristics of the microorganism that are typically selected for improving strains for commercial food or beverage uses. These determinations provide a first screen for microorganisms that exhibit unintended traits. Recombinant-DNA microorganisms that pass this screen are subjected to safety assessment as described in Section 4.

FRAMEWORK OF FOOD SAFETY ASSESSMENT

22. The safety assessment of a food produced using a recombinant-DNA microorganism is based on determining the safety of using the microorganism, which follows a stepwise process of addressing relevant factors that include:

A) Description of the recombinant-DNA microorganism;

B) Description of the recipient microorganism and its use in food production;

C) Description of the donor organism(s);

D) Description of the genetic modification(s) including vector and construct;

E) Characterization of the genetic modification(s);

F) Safety assessment:

   a) expressed substances: assessment of potential toxicity and other traits related to pathogenicity;

   b) compositional analyses of key components;

   c) evaluation of metabolites;

   d) effects of food processing;

   e) assessment of immunological effects;

   f) assessment of viability and residence of microorganisms in the human gastrointestinal tract;

   g) antibiotic resistance and gene transfer; and

h) nutritional modification.

23. In certain cases, the characteristics of the microorganisms and/or the foods produced/processed using these microorganisms may necessitate generation of additional data and information to address issues that are unique to the microorganisms and/or food products under review.

24. Experiments intended to develop data for safety assessments should be designed and conducted in accordance with sound scientific concepts and principles, as well as, where appropriate, Good Laboratory Practice. Primary data should be made available to regulatory authorities upon request. Data should be obtained using sound scientific methods and analysed using appropriate statistical techniques. The sensitivity of all analytical methods should be documented.

25. The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food will not cause harm when prepared or consumed according to its intended use, nor should the organism itself cause harm when viable organisms remain in the food. Safety assessments should address the health aspects for the whole population, including immuno-compromised individuals, infants, and the elderly. The expected endpoint of such an assessment will be a conclusion regarding whether the new food and/or microorganisms are as safe as the conventional counterparts taking into account dietary impact of any changes in nutritional content or value. Where the microorganism is likely to be viable upon ingestion, its safety should be compared to a conventional counterpart taking into account residence of the recombinant-DNA microorganism in the gastrointestinal tract, and where appropriate, interactions between it and the gastrointestinal flora of mammals (especially humans) and impacts of the recombinant-DNA microorganism on the immune system. In essence, the outcome of the safety assessment process is to define the product under consideration in such a way as to enable risk managers to determine whether any measures are needed to protect the health of consumers and if so to make well-informed and appropriate decisions in this regard.

SECTION 4- GENERAL CONSIDERATIONS

DESCRIPTION OF THE RECOMBINANT-DNA MICROORGANISM

26. A description of the bacterial, yeast, or fungal strain and the food being presented for safety assessment should be provided. This description should be sufficient to aid in understanding the nature of the organism or food produced using the organism being submitted for safety assessment. Recombinant-DNA microorganisms used in food production or contained in food should be conserved as stock cultures with appropriate identification using molecular

methods, and preferably, in established culture collections. This may facilitate the review of the original safety assessment. Such stock cultures should be made available to regulatory authorities upon request.

DESCRIPTION OF THE RECIPIENT MICROORGANISM AND ITS USE IN FOOD PRODUCTION

27. A comprehensive description of the recipient microorganism or micro-organism subjected to the modification should be provided. Recipient microorganisms should have a history of safe use in food production or safe consumption in foods. Organisms that produce toxins, antibiotics or other substances that should not be present in food, or that bear genetic elements that could lead to genetic instability, antibiotic resistance or that are likely to contain genes conferring functions associated with pathogenicity (i.e., also known as pathogenicity islands or virulence factors) should not be considered for use as recipients. The necessary data and information should include, but need not be restricted to:

A) identity: scientific name, common name or other name(s) used to reference the microorganism, strain designation, information about the strain and its source, or accession numbers or other information from a recognized culture repository from which the organism or its antecedents may be obtained, if applicable, information supporting its taxonomical assignment;

B) history of use and cultivation, known information about strain development (including isolation of mutations or antecedent strains used in strain construction); in particular, identifying traits that may adversely impact human health;

C) information on the recipient microorganism's genotype and phenotype relevant to its safety, including any known toxins, antibiotics, antibiotic resistance factors or other factors related to pathogenicity, or immunological impact, and information about the genetic stability of the microorganism;

D) history of safe use in food production or safe consumption in food; and

E) information on the relevant production parameters used to culture the recipient microorganism.

28. Relevant phenotypic and genotypic information should be provided not only for the recipient microorganism, but also for related species and for any extra-chromosomal genetic elements that contribute to the functions of the recipient strain, particularly if the related species are used in foods or involved in pathogenic effects in humans or other animals. Information on the genetic

stability of the recipient microorganism should be considered including, as appropriate, the presence of mobile DNA elements, i.e. insertion sequences, transposons, plasmids, and prophages.

29. The history of use may include information on how the recipient microorganism is typically grown, transported and stored, quality assurance measures typically employed, including those to verify strain identity and production specifications for microorganisms and foods, and whether these organisms remain viable in the processed food or are removed or rendered non-viable as a consequence of processing.

DESCRIPTION OF THE DONOR ORGANISM(S)

30. Information should be provided on the donor organism(s) and any intermediate organisms, when applicable, and, when relevant, related organisms. It is particularly important to determine if the donor or intermediate organism(s) or other closely related species naturally exhibit characteristics of pathogenicity or toxin production, or have other traits that affect human health. The description of the donor or intermediate organism(s) should include:

A) identity: scientific name, common name or other name(s) used to reference the organism, strain designation, information about the strain and its source, or accession numbers or other information from a recognized culture repository from which the organism or its antecedents may be obtained, if applicable, and information supporting its taxonomic assignment;

B) information about the organism or related organisms that concerns food safety;

C) information on the organism's genotype and phenotype relevant to its safety including any known toxins, antibiotics, antibiotic resistance factors or other factors related to pathogenicity, or immunological impact; and

D) information on the past and present use, if any, in the food supply and exposure route(s) other than intended food use (e.g., possible presence as contaminants).

DESCRIPTION OF THE GENETIC MODIFICATION(S) INCLUDING VECTOR AND CONSTRUCT

31. Sufficient information should be provided on the genetic modification(s) to allow for the identification of all genetic material potentially delivered to or modified in the recipient microorganism and to provide the necessary

information for the analysis of the data supporting the characterization of the DNA added to, inserted into, modified in, or deleted from the microbial genome.

32. The description of the strain construction process should include:

A) information on the specific method(s) used for genetic modification;

B) information on the DNA used to modify the microorganism, including the source (e.g., plant, microbial, viral, synthetic), identity and expected function in the recombinant-DNA microorganism, and copy number for plasmids; and

C) intermediate recipient organisms including the organisms (e.g., other bacteria or fungi) used to produce or process DNA prior to introduction into the final recipient organism.

33. Information should be provided on the DNA added, inserted, deleted, or modified, including:

A) the characterization of all genetic components including marker genes, vector genes, regulatory and other elements affecting the function of the DNA;

B) the size and identity;

C) the location and orientation of the sequence in the final vector/construct; and

D) the function.

CHARACTERIZATION OF THE GENETIC MODIFICATION(S)

34. In order to provide clear understanding of the impact of the genetic modification on the composition and safety of foods produced using recombinant-DNA microorganisms, a comprehensive molecular and biochemical characterization of the genetic modification should be carried out. To facilitate the safety assessment, the DNA to be inserted should be preferably limited to the sequences necessary to perform the intended functions.

35. Information should be provided on the DNA modifications in the recombinant DNA microorganism; this should include:

A) the characterization and description of the added, inserted, deleted, or otherwise modified genetic materials, including plasmids or other carrier DNA used to transfer desired genetic sequences. This should include an analysis of the potential for mobilization of any plasmids or other genetic elements used, the locations of the added, inserted, deleted, or otherwise modified genetic materials (site on a chromo-

somal or extra-chromosomal location); if located on a multi-copy plasmid, the copy number of the plasmid;

B) the number of insertion sites;

C) the organisation of the modified genetic material at each insertion site including the copy number and sequence data of the inserted, modified, or deleted material, plasmids or carrier DNA used to transfer the desired genetic sequences, and the surrounding sequences. This will enable the identification of any substances expressed as a consequence of the inserted, modified or deleted material;

D) identification of any open reading frames within inserted DNA, or created by the modifications to contiguous DNA in the chromosome or in a plasmid, including those that could result in fusion proteins; and

E) particular reference to any sequences known to encode, or to influence the expression of, potentially harmful functions.

36. Information should be provided on any expressed substances in the recombinant-DNA microorganism; this should include:

A) the gene product(s) (e.g., a protein or an untranslated RNA) or other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food;

B) the gene product's function;

C) the phenotypic description of the new trait(s);

D) the level and site of expression (intracellular, periplasmic - for Gram-negative bacteria, organellar - in eukaryotic microorganisms, secreted) in the microorganism of the expressed gene product(s), and, when applicable, the levels of its metabolites in the organism;

E) the amount of the inserted gene product(s) if the function of the expressed sequence(s)/gene(s) is to alter the level of a specific endogenous mRNA or protein; and

F) the absence of a gene product, or alterations in metabolites related to gene products, if applicable to the intended function(s) of the genetic modification(s).

37. In addition, information should be provided:

A) to demonstrate whether the arrangement of the modified genetic material has been conserved[7] or whether significant rearrangements have occurred after introduction to the cell and propagation of the recombinant strain to the extent needed for its use(s) in food production, including those that may occur during its storage according to current techniques;

B) to demonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function;

C) to demonstrate whether the intended effect of the modification has been achieved and that all expressed traits are expressed and inherited in a manner that is stable for the extent of propagation needed for its use(s) in food production and is consistent with laws of inheritance. It may be necessary to examine the inheritance of the inserted or modified DNA or the expression of the corresponding RNA if the phenotypic characteristics cannot be measured directly[8];

D) to demonstrate whether the newly expressed trait(s) is expressed as expected and targeted to the appropriate cellular location or is secreted in a manner and at levels that is consistent with the associated regulatory sequences driving the expression of the corresponding gene;

E) to indicate whether there is any evidence to suggest that one or more genes in the recipient microorganism has been affected by the modifications or the genetic exchange process; and

F) to confirm the identity and expression pattern of any new fusion proteins.

SAFETY ASSESSMENT

38. The safety assessment of the modified microorganism should be performed on a case by case basis depending on the nature and extent of the introduced changes. Conventional toxicology studies may not be considered necessary

---

7 Microbial genomes are more fluid than those of higher eukaryotes; that is, the organisms grow faster, adapt of changing environments, and are more prone to change. Chromosomal rearrangements are common. The general genetic plasticity of microorganisms may affect recombinant DNA in microorganisms and must be considered in evaluating the stability of recombinant DNA microorganisms.

8 Modified strains should be maintained in a manner to enable verification of the genetic stability.

where the substance or a closely related substance has, taking into account its function and exposure, been consumed safely in food. In other cases, the use of appropriate conventional toxicology or other studies on the new substance may be necessary. Effects of the recombinant-DNA microorganism on the food matrix should be considered as well. If the characterisation of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal or *in vitro* studies with the recombinant-DNA microorganism and/or the food produced using it could be considered necessary.

Expressed Substances: Assessment of Potential Toxicity and Other Traits Related to Pathogenicity

39. When a substance is new to foods or food processing, the use of conventional toxicology studies or other applicable studies on the new substance will be necessary. This may require the isolation of the new substance from the recombinant-DNA microorganism, the food product if the substance is secreted, or, if necessary, the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally, and biochemically equivalent to that produced in the recombinant-DNA microorganism. Information on the anticipated exposure of consumers to the substance, the potential intake and dietary impact of the substance should be provided.

40. The safety assessment of the expressed substance should take into account its function and concentration in the food. The number of viable microorganisms remaining in the food should be also determined and compared to a conventional counterpart. All quantitative measurements should be analysed using appropriate statistical techniques. Current dietary exposure and possible effects on population sub-groups should also be considered.

- In the case of proteins, the assessment of potential toxicity should take into account the structure and function of the protein and should focus on amino acid sequence similarity between the protein and known protein toxins and anti-nutrients (e.g., protease inhibitors, siderophores) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies[9] may be carried out in cases where the protein is present in the food, but is not closely similar to proteins that have been safely consumed in food, and has not previously been consumed safely in food, and taking into account its biological function in microorganisms where known.

[9] Guidelines for oral toxicity studies have been developed in international fora, for example the OECD Guidelines for the Testing of Chemicals.

- Potential toxicity of non-protein substances that have not been safely consumed in food should be assessed in a case-by-case basis depending on the identity, concentration, and biological function of the substance and dietary exposure. The type of studies to be performed may include evaluations of metabolism, toxicokinetics, chronic toxicity/ carcinogenicity, impact on reproductive function, and teratogenicity.

41. The newly expressed or altered properties should be shown to be unrelated to any characteristics of donor organisms that could be harmful to human health. Information should be provided to ensure that genes coding for known toxins or anti-nutrients present in the donor organisms are not transferred to recombinant-DNA microorganisms that do not normally express those toxic or anti-nutritious characteristics.

- Additional *in vivo* or *in vitro* studies may be needed on a case-by-case basis to assess the toxicity of expressed substances, taking into account the potential accumulation of any substances, toxic metabolites or antibiotics that might result from the genetic modification.

Compositional Analyses of Key Components

42. Analyses of concentrations of key components[10] of foods produced by recombinant-DNA microorganisms should be compared with an equivalent analysis of a conventional counterpart produced under the same conditions. The statistical significance of any observed differences should be assessed in the context of the range of natural variations for that parameter to determine its biological significance. Ideally, the comparator(s) used in this assessment should be food produced using the near isogenic parent strain. The purpose of this comparison, in conjunction with an exposure assessment as necessary, is to establish that substances that can affect the safety of the food have not been altered in a manner that would have an adverse impact on human health.

Evaluation of Metabolites

43. Some recombinant-DNA microorganisms may be modified in a manner that could result in new or altered levels of various metabolites in foods produced

[10] Key nutrients or key anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major nutritional constituents (fats, proteins, carbohydrates), enzyme inhibitors as anti-nutrients, or minor compounds (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be produced by the microorganism, such as those compounds whose toxic potency and level may be significant to health. Microorganisms traditionally used in food processing are not usually known to produce such compounds under production conditions.

using these organisms. Where altered metabolite levels are identified in foods, consideration should be given to the potential impacts on human health using conventional procedures for establishing the safety of such metabolites (e.g., procedures for assessing the human safety of chemicals in foods).

44. New or altered levels of metabolites produced by a recombinant-DNA microorganism may change the population of microorganisms in mixed culture, potentially increasing the risk for growth of harmful organisms or accumulation of harmful substances. Possible effects of genetic modification of a microorganism on other microorganisms should be assessed when a mixed culture of microorganisms is used for food processing, such as for production of natural cheese, miso, soy sauce, etc.

Effects of Food Processing

45. The potential effects of food processing, including home preparation, on foods produced using recombinant-DNA microorganisms should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food. For example, in the case of yoghurt, information should be provided on the growth of the organism and culture conditions.

Assessment of Immunological Effects

46. When the protein(s) resulting from an inserted gene is present in the food, it should be assessed for its potential to cause allergy. The likelihood that individuals may already be sensitive to the protein and whether a protein new to the food supply will induce allergic reactions should be considered. A detailed presentation of issues to be considered is presented in the Annex to this guideline.

47. Genes derived from known allergenic sources should be assumed to encode an allergen and be avoided unless scientific evidence demonstrates otherwise. The transfer of genes from organisms known to elicit gluten-sensitive enteropathy in sensitive individuals should be avoided unless it is documented that the transferred gene does not code for an allergen or for a protein involved in gluten-sensitive enteropathy.

48. Recombinant-DNA microorganisms that remain viable in foods may interact with the immune system in the gastrointestinal tract. Closer examination of these interactions will depend on the types of differences between the recombinant-DNA microorganism and its conventional counterpart.

Assessment of Viability and Residence of Microorganisms in the Human Gastrointestinal Tract

49. In some foods produced using recombinant-DNA microorganisms, ingestion of these microorganisms and their residence[11] may have an impact on the human intestinal tract. The need for further testing of such microorganisms should be based on the presence of their conventional counterpart in foods, and the nature of the intended and unintended effects of genetic modifications. If processing of the final food product eliminates viable microorganisms (by heat treatment in baking bread, for example), or if accumulations of end-products toxic to the microorganism (such as alcohol or acids) eliminate viability, then viability and residence of microorganisms in the alimentary system need no examination.

50. For applications in which recombinant-DNA microorganisms used in production remain viable in the final food product, (for example, organisms in some dairy products), it may be desirable to demonstrate the viability (or residence time) of the microorganism alone and within the respective food matrix in the digestive tract and the impact on the intestinal microflora in appropriate systems. The nature of intended and unintended effects of genetic modification and the degree of differences from the conventional counterpart will determine the extent of such testing.

ANTIBIOTIC RESISTANCE AND GENE TRANSFER

51. In general, traditional strains of microorganisms developed for food processing uses have not been assessed for antibiotic resistance. Many microorganisms used in food production possess intrinsic resistance to specific antibiotics. Such properties need not exclude such strains from consideration as recipients in constructing recombinant-DNA microorganisms. However, strains in which antibiotic resistance is encoded by transmissible genetic elements should not be used where such strains or these genetic elements are present in the final food. Any indication of the presence of plasmids, transposons, and integrons containing such resistance genes should be specifically addressed.

---

[11] Permanent life-long colonization by ingested microorganisms is rare. Some orally administered microorganisms have been recovered in faeces or in the colonic mucosa weeks after feeding ceased. Whether the genetically modified microorganism is established in the gastrointestinal tract or not, the possibility remains that it might influence the microflora or the mammalian host (Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology – *Safety assessment of foods derived from genetically modified microorganism*, 24-28 September, 2001, Geneva, Switzerland).

52. Alternative technologies, demonstrated to be safe, that do not rely on antibiotic resistance marker genes in viable microorganisms present in foods should be used for selection purposes in recombinant-DNA microorganisms. In general, use of antibiotic resistance markers for constructing intermediate strains should pose no significant hazards that would exclude the use of the ultimate strains in food production, provided that the antibiotic resistance marker genes have been removed from the final construct.

53. Transfer of plasmids and genes between the resident intestinal microflora and ingested recombinant-DNA microorganisms may occur. The possibility and consequences of gene transfer from recombinant-DNA microorganisms and food products produced by recombinant-DNA microorganisms to gut microorganisms or human cells should also be considered. Transferred DNA would be unlikely to be maintained in the absence of selective pressure. Nevertheless, the possibility of such events cannot be completely discounted.

54. In order to minimize the possibility of gene transfer, the following steps should be considered:

A) chromosomal integration of the inserted genetic material may be preferable to localization on a plasmid;

B) where the recombinant-DNA microorganism will remain viable in the gastrointestinal tract, genes should be avoided in the genetic construct that could provide a selective advantage to recipient organisms to which the genetic material is unintentionally transferred; and

C) sequences that mediate integration into other genomes should be avoided in constructing the introduced genetic material.

NUTRITIONAL MODIFICATION

55. The assessment of possible compositional changes to key nutrients, which should be conducted for all foods produced using recombinant-DNA microorganisms, has already been addressed under 'Compositional analyses of key components.' If such nutritional modifications have been implemented, the food should be subjected to additional testing to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

56. Information about the known patterns of use and consumption of a food and its derivatives should be used to estimate the likely intake of the food produced using the recombinant-DNA microorganism. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential

for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing, and storage.

57. The use of modern biotechnology to change nutrient levels in foods produced using microorganisms could result in broad changes to the nutrient profile. The intended modification in the microorganism could alter the overall nutrient profile of the product, which, in turn, could affect the nutritional status of individuals consuming the food. The impact of changes that could affect the overall nutrient profile should be determined.

58. When the modification results in a food product with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e., foods whose nutritional composition is closer to that of the food produced using the recombinant-DNA microorganism) as appropriate comparators to assess the nutritional impact of the food.

59. Some foods may require additional testing. For example, animal-feeding studies may be warranted for foods produced using recombinant-DNA microorganisms if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits, may require an assessment beyond the scope of these guidelines such as specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole food.

REVIEW OF SAFETY ASSESSMENTS

60. The goal of the safety assessment is a conclusion as to whether the food produced using a recombinant-DNA microorganism is as safe as the conventional counterpart taking into account dietary impact of any changes in nutritional content or value. Nevertheless, the safety assessment should be reviewed in the light of new scientific information that calls into question the conclusions of the original safety assessment.

ANNEX: ASSESSMENT OF POSSIBLE ALLERGENICITY

SECTION 1 – INTRODUCTION

1. All newly expressed proteins[12] produced by recombinant-DNA micro-organisms that could be present in the final food should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals.

2. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case by case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

3. The endpoint of the assessment is a conclusion as to the likelihood of the protein being a food allergen.

SECTION 2 - ASSESSMENT STRATEGY

4. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability and/or, acid and enzymatic treatment.

5. As there is no single test that can predict the likely human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins produced by recombinant-DNA microorganisms, or the synthesis or production of the substance from an alternative source, in which

[12] This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing gluten-sensitive or other enteropathies. The issue of enteropathies is already addressed in Assessment of immunological effects, paragraph 47 of the Guideline for the Conduct of Food Safety Assessment of Foods Produced using Recombinant-DNA Microorganisms. In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

case the material should be shown to be structurally, functionally and biochemically equivalent to that produced by recombinant-DNA microorganisms. Particular attention should be given to the choice of the expression host, since post-translational modifications allowed by different hosts (i.e.: eukaryotic vs. prokaryotic systems) may have an impact on the allergenic potential of the protein.

6. It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

SECTION 3 – INITIAL ASSESSMENT

SECTION 3.1 - SOURCE OF THE PROTEIN

7. As part of the data supporting the safety of foods produced using recombinant-DNA microorganisms, information should describe any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated oral, respiratory or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and frequency of allergic reactions; structural characteristics and amino acid sequence; physicochemical and immunological properties (when available) of known allergenic proteins from that source.

SECTION 3.2 – AMINO ACID SEQUENCE HOMOLOGY

8. The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is similar in structure to a known allergen. This information may suggest whether that protein has an allergenic potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results[13]. Validated search and

[13] It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segment searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false

evaluation procedures should be used in order to produce biologically meaningful results.

9. IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35% identity in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

10. Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding themselves specifically with IgE antibodies.

11. A negative sequence homology result indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein is likely to be allergenic. If the product is to be considered further, it should be assessed using serum from individuals sensitized to the identified allergenic source.

SECTION 3.3 – PEPSIN RESISTANCE

12. Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential[14]. Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen.

---

positives, inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

[14] The method outlined in the U.S. Pharmacopoeia (1995) was used in the establishment of the correlation (Astwood *et al.*, 1996).

13. Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used where adequate justification is provided[15].

SECTION 4 – SPECIFIC SERUM SCREENING

14. For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed where sera are available. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in *in vitro* assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals[16]. In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result. For proteins from sources not known to be allergenic, and which do not exhibit sequence homology to a known allergen, targeted serum screening may be considered where such tests are available as described in paragraph 17.

15. In the case of a newly expressed protein derived from a known allergenic source, a negative result in *in vitro* immunoassays may not be considered sufficient, but should prompt additional testing, such as the possible use of skin test and *ex vivo* protocols[17]. A positive result in such tests would indicate to a potential allergen.

SECTION 5 – OTHER CONSIDERATIONS

16. The absolute exposure to the newly expressed protein and the effects of relevant food processing will contribute toward an overall conclusion about the potential for human health risk. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of processing which would be applied and its effects on the presence of the protein in the final food product.

---

[15] Reference to Joint FAO/WHO Expert Consultation (2001).

[16] According to the Report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (22-25 January 2001, Rome, Italy) a minimum of 8 relevant sera is required to achieve a 99% certainty that the new protein is not an allergen in the case of a major allergen. Similarly, a minimum of 24 relevant sera is required to achieve the same level of certainty in the case of a minor allergen. It is recognized that these quantities of sera may not be available for testing purposes.

[17] Reference to Joint FAO/WHO Expert Consultation (2001) on description of *ex vivo*.

17. As scientific knowledge and technology evolves, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These methods should be scientifically sound and may include targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly-related categories of foods); the development of international serum banks; use of animal models; and examination of newly expressed proteins for T-cell epitopes and structural motifs associated with allergens.