codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

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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.

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[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.

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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.