codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

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Agenda Item 3 **CX/MAS 10/31/3-Add.1**

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING
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PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS, IN PARTICULAR IN FOODS DERIVED FROM MODERN BIOTECHNOLOGY

(Comments submitted by Argentina, Canada, Iran, Japan, Kenya, New Zealand, Panama, USA, BIO, IFT, ILSI and ISO)

ARGENTINA

First of all, we would like to express our appreciation to the delegations that have contributed during the elaboration of this draft document in the context of the CCMAS and the *ad hoc* electronic Working Group.

In regards to the bracketed text options of the document, we still advocate that this document is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. Certainly we do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an exaggerated emphasis of a particular field of application; unnecessary emphasis may hinder the wider applicability of the document for the general reader. In addition, we would like to offer suggestions on editorial and technical improvements to the draft text.

Therefore, our comments on individual paragraphs are included next:

Current Text **(stroked text and bold format have been introduced in order to highlight the sections that are commented in the following column)**	**Comments**	**Proposed Text** **(bold format is used only to highlight the proposed changes; this format is not in intended to remain in the final text)**
~~PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS, IN PARTICULAR IN FOODS DERIVED FROM MODERN BIOTECHNOLOGY.~~	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of	Alternative Title I: PROPOSED DRAFT GUIDELINES ON PERFORMANCE CRITERIA AND VALIDATION OF METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS IN FOODS.

[Alternative Title I: PROPOSED DRAFT GUIDELINES ON PERFORMANCE CRITERIA AND VALIDATION OF METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS IN FOODS] [Alternative Title II: ~~PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR THE DETECTION AND IDENTIFICATION OF FOODS DERIVED FROM MODERN BIOTECHNOLOGY]~~	the document for the general reader. Therefore, we support the "*Alternative title 1*" for the document.	
SECTION 1 – INTRODUCTION		
1. Molecular and immunological analytical methods are currently the recognized tools for determination of DNA and protein analytes in foods ~~[derived from modern biotechnology]~~. However, in order for the results obtained by such methods from different laboratories to gain wide acceptability and confidence as reliable, there is need for the analytical methods to satisfy certain quality criteria.	This guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance. We oppose to the bracketed text because it restricts the scope to cover only GM foods.	1. Molecular and immunological analytical methods are currently the recognized tools for determination of DNA and protein analytes in **foods. However**, in order for the results obtained by such methods from different laboratories to gain wide acceptability and confidence as reliable, there is need for the analytical methods to satisfy certain quality criteria.
SECTION 1.1 – PURPOSE AND OBJECTIVES		
4. The goal of this document is to support the establishment of molecular and immunological methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods [~~derived from modern biotechnology~~] that produce results with comparable reproducibility when performed at different laboratories	We oppose to the bracketed text because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance in any kind of food. Besides, the phrase "in foods derived from modern biotechnology" is used in a way that it would be interpreted as the matrix and not the analyte, as originally intended by its proponents.	4. The goal of this document is to support the establishment of molecular and immunological methods for detection, identification and quantification of specific DNA sequences and specific proteins in **foods, which produce** results with comparable reproducibility when performed at different laboratories
5. The guidelines are aimed to **give** guidance on how to establish methods to detect and identify specific DNA sequences and proteins in food by defining appropriate validation criteria, and whether or not a method complies	Editorial modifications: - The use of the verb "give" is colloquial. "Provide" is more adequate for this kind of document. - Similarly, it would be preferable to	5. The guidelines are aimed to **provide** guidance on how to establish methods to detect and identify specific DNA sequences and proteins in food by defining appropriate validation criteria, and whether or not a method complies

with these criteria based on the performance characteristics of a method. The guidelines **will** specify the relevant criteria and give explanations on how to consider these criteria, i.e.: -by **giving** the rationale for the most relevant criteria and -by showing how to find out whether or not a method fulfils the given criteria requirements.	drop the use of "will" for a finished document.	with these criteria based on the performance characteristics of a method. The **guidelines specify** the relevant criteria and give explanations on how to consider these criteria, i.e.: -by **providing** the rationale for the most relevant criteria and -by showing how to find out whether or not a method fulfils the given criteria requirements.
SECTION 1.2 SCOPE		
~~6. These guidelines provide information for the validation of methods for the detection, identification, and quantification of specific DNA sequences and specific proteins in foods derived from modern biotechnology. These Guidelines may also provide information on the validation of methods for other specific DNA sequences and proteins of interest in other foods.~~ 6 alternative [These guidelines provide information criteria for the validation of food analysis methods involving the detection, identification and quantification of specific DNA sequences and specific proteins of interest that may be present in foods and that will be used by laboratories responsible for food analysis. These methods can provide molecular and immunological approaches for, including among other uses, tests for food authenticity, and biomarkers for foods containing material derived from recombinant-DNA organisms.].	We support the paragraph "*6 alternative*", since it is clearer and more balanced. Please note that paragraph 6 alternative explicitly refers to biomarkers for foods derived from Modern Biotechnology. *Paragraph 6* is not appropriate, among other reasons, because the phrase "in foods derived from modern biotechnology" is used in a way that it would be interpreted as the matrix and not the analyte, as originally intended by its proponents.	6 alternative: These guidelines provide information criteria for the validation of food analysis methods involving the detection, identification and quantification of specific DNA sequences and specific proteins of interest that may be present in foods and that will be used by laboratories responsible for food analysis. These methods can provide molecular and immunological approaches for, including among other uses, tests for food authenticity, and biomarkers for foods containing material derived from recombinant-DNA organisms.
SECTION 3 – DEFINITIONS	Editorial modification: Section 3 should be renumbered to Section 2 (and so on…)	SECTION **2** – DEFINITIONS
7. There are a number of terms related to ~~the~~ methods of analysis in the Codex Procedural Manual and other sources, which may also be applicable to the analysis of [DNA sequences and proteins of interest in	We support the first option of bracketed texts, because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance.	7. There are a number of terms related **to methods** of analysis in the Codex Procedural Manual and other sources, which may also be applicable to **the analysis of DNA sequences and proteins of interest**

foods], [~~foods derived from modern biotechnology~~]. Suggested definitions of these terms are given in Annex II.		**in foods**. Suggested definitions of these terms are given in Annex II.
SECTION 4 – METHOD VALIDATION		
8. 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, 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 the analysis of [DNA sequences and proteins] [~~foods derived from modern biotechnology~~] 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~~.	Fourth sentence: We disagree with this speculation regarding non-technical issues. Fifth sentence: we support the first option of bracketed text because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance in any kind of food. As a corollary of our comment to the fourth sentence, we do not support the use of "as soon as practicable".	8. 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, there are few methods of analysis which have been fully validated. As a result, Codex is also endorsing by reference single-laboratory validation **protocols. However,** methods used **for the analysis of DNA sequences and proteins are** able to be, and intended to be performed at, multiple laboratories and should therefore be validated by multi-laboratory collaborative **studies**.
Section 4.1 – Criteria Approach		
9. Codex Alimentarius Commission has accepted the "criteria approach" for methods of analysis. It is necessary to ensure that this approach is incorporated into these guidelines.	Editorial modification: The second sentence is not appropriate for a final document.	9. Codex Alimentarius Commission has accepted the "criteria approach" for methods of analysis. **Therefore,** this approach is incorporated into these guidelines.
Section 4.3 – Validation Process		
13. Formal validation of a method is the conclusion of a long process, which includes the following main steps: • ***Pre-validation of the method***. Pre-validation ~~may be recommended but~~ should be performed on a case-by case as needed. […]	The use of the term "may be recommended" in the first dot point is confusing and, apparently, unnecessary.	13. Formal validation of a method is the conclusion of a long process, which includes the following main steps: • ***Pre-validation of the method***. **Pre-validation should** be performed on a case-by case as needed. […]
SECTION 5 – SPECIFIC CONSIDERATION FOR THE VALIDATION OF METHODS	We support the first option of bracketed texts, because this guideline is equally applicable and	**SECTION 5 – SPECIFIC CONSIDERATION FOR THE VALIDATION OF METHODS**

FOR THE DETECTION, IDENTIFICATION AND QUANTIFICATION OF [DNA SEQUENCES AND PROTEINS] ~~[FOODS DERIVED FROM MODERN BIOTECHNOLOGY]~~	useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance.	**FOR THE DETECTION, IDENTIFICATION AND QUANTIFICATION OF DNA SEQUENCES AND PROTEINS.**
Section 5.1.1 – Method Acceptance Criteria (Required condition for full validation)		
16. 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.	Editorial Modification: The repeated use of the word "information" and the colloquial style of the second sentence could be improved.	16. In order to evaluate a method prior to full validation, information concerning both the method and the method testing is required, **as detailed** in Annex I.
17. The method ~~will be evaluated based on the information provided. The~~ evaluation should verify that the principle preconditions for using the method for Codex purposes are fulfilled. […]	Editorial Modification: The use of "will" is not appropriate for this kind of document. Besides, the first sentence is unnecessary.	17. The **method evaluation** should verify that the principle preconditions for using the method for Codex purposes are fulfilled. […]
Section 5.1.2 – Applicability of the Method		
18. […]Especially, in analysis of the DNA sequences and protein, a method that can be applied to single raw matrix cannot be necessarily applied to **~~the~~** complex matrices and/or **~~the~~** processed food, since the DNA and protein will be denatured easily.	Editorial Modification: the use of "the" in the indicated instances is unnecessary.	18. […]Especially, in analysis of the DNA sequences and protein, a method that can be applied to single raw matrix cannot be necessarily applied **to complex** matrices and/**or processed** food, since the DNA and protein will be denatured easily.
19. [This is a particularly important criterion ~~in the analysis of foods derived from modern biotechnology~~]. In principle the method should be applicable to the matrix of concern within the Codex system. If [the method is used for analyzing DNA sequences and proteins, then information should be provided] [~~this is a specific food derived from modern biotechnology 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 the case of "general purpose" methods to identify and quantify [DNA sequences and proteins] [~~foods derived from modern biotechnology~~] in a range of food	This guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance. The guidance in this paragraph should be generalized for other analytes in the scope of the guideline.	19. This is a particularly important criterion. In principle the method should be applicable to the matrix of concern within the Codex system. If the method is used for analyzing DNA sequences and proteins, then information should be provided on the method of analysis appropriate to the specific product and, ideally, the matrix in which it is likely to be used. In the case of "general purpose" methods to identify and quantify DNA sequences and proteins in a range of food matrices, at least one extraction method applicable to a general food matrix should be available.

matrices, at least one extraction method applicable to a general food matrix should be available.		
Section 5.1.3 – Principle condition		
21. DNA-based methods should detect, identify and quantify the relative levels of specific target and taxon-specific DNA sequences whilst protein-based methods should detect and quantify the level of specific protein in the product. ~~[21 alt. The provision of the detection method is aimed to serve mainly the requirements for the measurement of products derived from modern biotechnology. To serve these purposes, the method can detect and quantify the specific target and taxon-specific DNA sequence or the protein derived there from in the product; this may be achieved in most cases using either protein-based or DNA-based methods.]~~	We support the first option for this paragraph, because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance.	21. DNA-based methods should detect, identify and quantify the relative levels of specific target and taxon-specific DNA sequences whilst protein-based methods should detect and quantify the level of specific protein in the product.
23. Currently, the DNA-based detection method typically consists of PCR methodology and includes: • […] • **a description of the method used to detect the DNA ~~when using a gel-based method~~**. • […]	7th dot point: a description of the method used to detect and quantify the DNA is necessary in any case.	23. Currently, the DNA-based detection method typically consists of PCR methodology and includes: • a protocol describing an extraction method […] • **a description of the method used to detect and quantify the DNA.** • […].
27. The method provider should demonstrate that the method fulfils the requirements below: • Protein-based methods should allow for unequivocal detection, identification and/or quantification of a specific antigen or epitope. • DNA-based screening methods that are used to detect multiple transformation events should allow for unequivocal detection and identification of a target DNA sequence which is common to a number of transformation events. • DNA-based target-specific methods that are used for detection or relative	We support the first option for this paragraph, because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance. In addition, the first option is technically more complete, and therefore constitutes better guidance. However the second and third dot points should be generalized considering a wider scope of the guideline.	27. The method provider should demonstrate that the method fulfils the requirements below: • Protein-based methods should allow for unequivocal detection, identification and/or quantification of a specific antigen or epitope. • **DNA-based screening methods that are used to detect multiple targets should allow for unequivocal detection and identification of a common DNA sequence.** • **DNA-based methods that are used for detection or relative quantification of a specific target which could be mixed with similar targets should**

quantification of a single transformation event should allow for unequivocal detection, identification and quantification of a target DNA sequence that is unique or specific to that transformation event. • DNA-based taxon-specific methods that are used for detection or relative quantification of target DNA should allow for unequivocal detection, identification and quantification of a DNA sequence that is unique or specific to that taxon • For target and taxon-specific methods used in relative quantification, identification of the amplified fragment, by e.g. probe hybridization or any appropriate equivalent method, is recommended. ~~[27 alt. Should PCR be selected as the analytical method, the method should target a DNA sequence which is not present in the food being examined. Currently, the best choice concerning event-specificity PCR should be the chosen technique, because it is targeting a 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 recombinant insert and the host genomic DNA will probably be the location of choice. However, when a unique DNA sequence can be found within the recombinant insert, such a sequence (generally called construct specific) can also be targeted by appropriate oligonucleotide primers and amplified through a PCR. Identification of the amplified fragment, by e.g. probe hybridization or any appropriate equivalent method, is recommended]~~		**allow for unequivocal detection, identification and quantification of a DNA sequence that is unique or specific to that target.** • DNA-based taxon-specific methods that are used for detection or relative quantification of target DNA should allow for unequivocal detection, identification and quantification of a DNA sequence that is unique or specific to that taxon • For target and taxon-specific methods used in relative quantification, identification of the amplified fragment, by e.g. probe hybridization or any appropriate equivalent method, is recommended.

Section 5.1.4 – Modular Approach to Method Validation		
28. […] However, it may be possible to use the same sample preparation (e.g. grinding) method in combination with the same DNA or protein isolation process for several different subsequent analyses (Chapela *et al.,* 2007; Holst-Jensen & Berdal, 2004; Turci *et al.,* **2009**) to achieve economic efficiencies as long as the validated method processes remain the same.	The year of the Turci et al reference should be corrected to 2010	28. […] However, it may be possible to use the same sample preparation (e.g. grinding) method in combination with the same DNA or protein isolation process for several different subsequent analyses (Chapela *et al.,* 2007; Holst-Jensen & Berdal, 2004; Turci *et al.,* **2010**) to achieve economic efficiencies as long as the validated method processes remain the same.
Section 5.2.2 – Minimum Performance Requirements		
35. The endorsed methods and their associated validation data will be revised on a regular basis as the scientific knowledge and experience gained in validation and collaborative trials evolve. These Guidelines **will also be complemented** with practical information about the operational steps of the validation process.	Editorial Modification: The phrase "will also be complemented" is not appropriate for a finished document.	35. The endorsed methods and their associated validation data will be revised on a regular basis as the scientific knowledge and experience gained in validation and collaborative trials evolve. These Guidelines **are** complemented with practical information about the operational steps of the validation process.
Section 5.2.4 – Specific Information on the Validation of Methods		
38. Specific information on the validation of quantitative and qualitative PCR methods is given in Annexes III and **IV** respectively.	Editorial Modification: The annex numbering should be updated	38. Specific information on the validation of quantitative and qualitative PCR methods is given in Annexes III and **V** respectively.
39. Specific information on the validation of quantitative and qualitative protein-based methods is given in Annex **V**.	Editorial Modification: The annex numbering should be updated	39. Specific information on the validation of quantitative and qualitative protein-based methods is given in Annex **VI**.
Section 5.3 – Unit of Measurement		
40. Appropriate units of measurement (e.g. target copy numbers per mg food/ molar equivalents, etc.), performance and data reporting criteria should be specified for each method prior to their use. For qualitative analysis, the results can be provided as present (+) or absent (-) and for this reason there is no unit of measurement.	Editorial Modification: the use of "/" could be misleading (it may be interpreted either as a separator of alternative, or as a division operator)	40. Appropriate units of measurement (e.g. target copy numbers per mg food **or** molar equivalents, etc.), performance and data reporting criteria should be specified for each method prior to their use. For qualitative analysis, the results can be provided as present (+) or absent (-) and for this reason there is no unit of measurement.
41. Measurements may be explicitly expressed as weight/weight or by relative percentage. However, none of the current methods (DNA or	The reference to the so-called "biological uncertainty" is not appropriate for this section. Moreover, we oppose to this	41. Measurements may be explicitly expressed as weight/weight or by relative percentage. However, none of the

protein based) are able to measure them directly. ~~In the case of a DNA-based method used for quantification of a specific DNA genome equivalents may typically be measured; notice that these may be influenced by a number of biological factors (Grothaus et al. 2007; Holst-Jensen & Berdal, 2004), depending on the part of the seed originally used for preparation of the flour or other components of the food (e.g. endosperm, germ), and whether the DNA or protein is retained in that portion. Protein methods measure the amount of a specific protein that is present and may be influenced by extraction efficiency.~~	misleading term since "uncertainty" is related to method error distribution, and not to other external factors. The choice of units of measurement should be restricted to those that represent a faithful interpretation of the analysis results, without resorting to other ambiguous factors outside of the technical determination. Moreover, the issue of "biological uncertainty", derived from different haploid level and parental contribution for different maize tissues is irrelevant for food purposes. Commercial hibrid SEEDS may have a different genetic distribution but they are planted. In contrast, what is used for food (codex) purposes is grain, i.e. the F1 from the hybrid self-pollination. The F1 grains will inherit the transgene randomly from the mother or the father (which supposedly are hemycigous hybrids for the transgene), so the genetic content will average to a 50% if the sample involves a significant number of grains, or flour or processed food (regardless of the tissue/s that contributed to the food material). Similarly, the reference to extraction efficiency in protein methods is irrelevant to the choice of units of measurement,.	current methods (DNA or protein based) are able to measure them directly.
Section 5.4 – Measurement Uncertainty		
42. […] Analysts using methods which have been validated according to these guidelines **will** have sufficient information to allow them to estimate the uncertainty of their result. ~~Quantification based on the protein expressed can also significantly contribute to the uncertainty of the analysis.~~	Second sentence: The use of "will" is not appropriate in this context Third sentence: this sentence is not clear and it seems to refer to factors that are outside of the analysis. The choice of units of measurement should be restricted to those that represent a faithful interpretation of the analysis results, without resorting to other ambiguous factors outside of the technical determination.	42. […] Analysts using methods which have been validated according to these guidelines **should** have sufficient information to allow them to estimate the uncertainty of their **result.**

43. For details, refer to the Codex Guideline on Measurement Uncertainty (CAC/GL54-2004), ~~and~~ the section entitled "*The Use of* […] from the Codex Procedural Manual and ~~(~~Trapman et al., 2009).	Minor editorial modifications.	43. For details, refer to the Codex Guideline on Measurement Uncertainty (CAC/GL54-2004), the section entitled "*The Use of* […]from the Codex Procedural Manual and Trapman *et al.* (2009).
Section 6.1 – Laboratory Quality		
44. […] These overarching guidelines provide information to and dictate requirements for laboratories working in the sector dealing with [DNA sequences and proteins] ~~[foods derived from modern biotechnology]~~.	We support the first option of bracketed texts, because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance. However, option 1 may require some slight editorial adjustment.	44. […] These overarching guidelines provide information to and dictate requirements for laboratories working in the sector dealing with **the analysis of** DNA sequences and proteins
45. It is required for laboratories to have standardized quality control practices in place, in order to avoid cross-contamination of material, which could result in false positive results. Several examples are available (Dieffenbach & Dveksler, 1993; Kwok & Higuchi, 1989; Mifflin, 2007; Newton, 1995). Other relevant guidance for DNA-based methods may be ISO 21569:2005 and ISO 21570:2005.	The risk of cross contamination is much a more critical issue for PCR methods than for protein methods or other DNA methods. In addition, the reference to uracyl-n-glycosylase in paragraph 27 of annex III requires further guidance on the use of this enzyme. Therefore a new paragraph for this purpose is included.	45. It is required for laboratories to have standardized quality control practices in place, in order to avoid cross-contamination of material, which could result in false positive results. Several examples are available (Dieffenbach & Dveksler, 1993; Kwok & Higuchi, 1989; Mifflin, 2007; Newton, 1995). Other relevant guidance for DNA-based methods may be ISO 21569:2005 and ISO 21570:2005. **45 bis In PCR protocols, the reaction mix could include the enzyme uracyl-n-glycosylase and the replacement of dTTP with dUTP. This aims to prevent cross-contamination from earlier amplifications (and, in quantitative PCR, to have a higher control over the real number of amplification cycles). However, the inclusion of this feature in the protocol should not justify any reduction in the stringency of the practices applied to avoid cross contamination.**
Section 6.2 – Guidance on the Laboratory Set-up and Operation		
46. ~~DNA-based~~ methods for the analysis of [DNA sequences and proteins] ~~[foods derived from modern biotechnology]~~ require specific apparatus and handling techniques that differ from most	We support the first option of bracketed texts, because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance.	46. **Methods** for the analysis of DNA sequences and proteins require specific apparatus and handling techniques that differ from most chemical-analytical methods. The use of **these** methods is

chemical-analytical methods. The use of DNA based methods is consistently growing in other detection fields such as microbiology of food pathogens. It is necessary to provide information and instructions on the essential differences in laboratory set-up and handling techniques. Examples are available (ISO/DIS 24276:2006).	There is no basis for the differentiations that this paragraph and the next one aim to introduce between nucleic acid and protein-based methods.	consistently growing **for the analysis of food**. It is necessary to provide information and instructions on the essential differences in laboratory set-up and handling techniques. Examples are available (ISO/DIS 24276:2006).
47. Immunological (protein-based) methods of analysis are well understood, and used in many laboratories for a number of analyses, and often come in kit form, simplifying their use; however, it is to be noticed that protein-based detection limits are below those of DNA-based methods**.**	Currently there is no basis to the idea implied in this phrase, which is that there is more familiarity or ease of use with protein-based methods compared to nucleic acid methods.	47. Immunological (protein-based) **and PCR** methods of analysis are well understood, and used in many laboratories for a number of analyses, and often come in kit form, simplifying their use; however, it is to be noticed that protein-based detection limits are below those of DNA-based methods**.**
~~48. In addition to the cross-contamination topic considered in the preceding section, compliance with minimal necessary biosafety directions (WHO, 2004) is recommended.~~	The WHO biosafety manual deals with directions for laboratories handling or researching on dangerous organisms, particularly microorganisms. In contrast, this guideline is applied to the validation of methods of analysis applied to food materials, focused on the methods applied after the extraction of DNA or proteins. Therefore, there is no reason to introduce a reference to the WHO biosafety manual.	(PARAGRAPH SHOULD BE DELETED)
Section 6.3 – Reference Material	No comments	
49. A suitable reference material is generally required for validation of a method. There are a number of matrices that can be used to develop reference materials or working standards for methods of detection of [DNA sequences and proteins] ~~[foods derived from modern biotechnology]~~. Each has its own advantages and drawbacks for particular purposes. […].	We support the first option of bracketed texts, because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance.	49. A suitable reference material is generally required for **the** validation of a method. There are a number of matrices that can be used to develop reference materials or working standards for methods of detection of DNA sequences and proteins. Each has its own advantages and drawbacks for particular purposes. […]
SECTION 7 – REFERENCES		
~~WHO (2004). Laboratory Biosafety Manual, 3rd Edition. Geneva: World Health Organization.~~	It is proposed to delete this reference. It belongs to paragraph 48, which we also propose to delete.	(REFERENCE SHOULD BE DELETED)

ANNEX I: REQUIRED INFORMATION WHEN METHODS ARE TO BE CONSIDERED FOR USE		
DESCRIPTION OF THE METHOD		
4. The DNA and protein-based techniques used to detect and quantify [DNA sequences and proteins] [foods derived from modern biotechnology] are based on different principles. In PCR the targeted DNA is amplified in an 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. Moreover, the quantification by real-time PCR is often based on two independent PCR systems: one for the target DNA and one for the taxon specific sequence. In contrast to PCR, immunological detection assays do not include multiple cycles in which the product of the previous amplification step is itself amplified.	We support the first option of bracketed texts, because this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis for any analyte of Codex relevance. ELISA does include certain "cyclic" amplification, since several primary antibodies may bind the target protein, then several secondary antibodies may bind the primary (and the secondary antibody may be linked to several enzymes.)	4. The DNA and protein-based techniques used to detect and **quantify DNA sequences and proteins are** based on different principles. In PCR the targeted DNA is **replicated** in an 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. Moreover, the quantification by real-time PCR is often based on two independent PCR systems: one for the target DNA and one for the taxon specific sequence. In contrast to PCR, **ELISA involve binding enzyme molecules to each initial target molecule, and amplification of the signal is proportional to the number of bound enzymes and the enzymatic reaction time**.
SPECIFIC INFORMATION REQUIRED FOR DNA-BASED METHODS		
~~10. Qualitative methods may employ (for example) a gel-based system for interpreting results. In addition,~~ qualitative methods generally tend to be less instrument-specific than quantitative methods.	Some quantitative PCR methodologies use gel systems, while some qualitative PCR methods may also use capillary electrophoresis and photometers. Therefore, there is no need for the first sentence	Qualitative methods generally tend to be less instrument-specific than quantitative methods. (THE SENTENCE SHOULD BE MERGED WITH THE PREVIOUS PARAGRAPH)
11. Taking all the differences into account it is not appropriate to change the instrument without adaptation of the PCR method. Thus, because the methods are generally instrument and chemistries dependent they cannot be transferred to other equipment and chemistries without evaluation and/or modification. 12. This is ~~in many ways~~ equivalent to the Codex Type I method and should be considered in the same	Editorial Modification: paragraph 12 contains a single sentence which is the corollary of paragraph 11, therefore they should be merged. The sentence "in many ways" is colloquial and not significantly informative.	11. Taking all the differences into account it is not appropriate to change the instrument without adaptation of the PCR method. Thus, because the methods are generally instrument and chemistries dependent they cannot be transferred to other equipment and chemistries without evaluation and/or modification. This is equivalent to the Codex Type I method and should be considered in the same light.

light.		
SPECIFIC INFORMATION REQUIRED FOR PROTEIN-BASED METHODS		
Confirming method 18. For a ~~quantitative~~ immunoassay, antibodies may cross-react with other proteins present in the matrix; thus, it is necessary to demonstrate the credibility of assays. Another method such as HPLC, LC/MS, ~~western blot~~ or biological assay may be used as a confirming method. Empirical results from testing both methods with aliquots of the same analytical samples of known concentration may be provided.	The issue of cross reactivity can also affect qualitative immunoassay methods. In addition, western blot would not be an adequate confirming method, since it is also based on antibodies and it is mostly of qualitative nature. On the other side, the lack of reaction with target-free matrix material may be also acceptable. A new paragraph is proposed, inspired on paragraph 26 of annex VI.	**Confirming method** 18. For immunoassays, antibodies may cross-react with other proteins present in the matrix; thus, it is necessary to demonstrate the credibility of assays. Another method such as HPLC, LC/MS, or biological assay may be used as a confirming method. Empirical results from testing both methods with aliquots of the same analytical samples of known concentration may be provided. **18 bis Another approach to discard cross-reaction with other proteins from the matrix would be to carry the assay on extracts from free-analyte matrix; and then compare with a sample-free assay, where a corresponding volume of pure water is added to the reaction instead of the sample.**
INFORMATION ABOUT THE METHOD *OPTIMISATION*	"Optimization" would be the process of modifying the method parameters, reagents, etc. in order to improve it. In contrast, this section refers to information about the final configuration of the method. Therefore, the word optimization is not adequate.	**INFORMATION ABOUT THE METHOD *PERFORMANCE.***
Primer pairs 19. General methods have to provide the defined primer pairs and the sequence they target, as well as different sets of primers if available. Recommendations as to the efficiency/use of primer set have to be clearly stated, including if the primers are suitable for screening and/or quantification.	This is general information that is only applicable to PCR. Therefore, it should be moved at the beginning of SPECIFIC INFORMATION REQUIRED FOR DNA-BASED METHODS. Primer characteristics are crucial for PCR methods. Therefore, more information should be provided so the evaluator can have a better idea of the primer pair suitability.	(Title and paragraph should be moved above, after paragraph 6.) 19. General methods have to provide the defined primer pairs and the sequence they target, as well as different sets of primers if available. Recommendations as to the efficiency/use of primer set have to be clearly stated, including if the primers are suitable for screening and/or quantification. **19 bis. Information regarding the primer design rationale should be supplied. This includes the rationale behind the selection of**

		the amplified region boundaries, the selection of the primers length and 3´ ends, etc. In addition, results from preliminary computational analysis should also be provided; this includes oligonucleotide folding potential, binding stability and theoretical melting temperature, as well as the results of checking against relevant sequence databases.
Stability testing 23. Empirical results from testing the methods (to detect both reference and target DNA sequences, or proteins) with different **varieties**, as appropriate, may be provided in order to demonstrate, for instance, the stability of the copy number and sequence conservation of the reference taxon-specific gene DNA, or the stability of expression of the protein.	Given the wide applicability of these methods, the origin of the reference and/or target protein could be derived from plants, animals or microorganisms.	23. Empirical results from testing the methods (to detect both reference and target DNA sequences, or proteins) with different **varieties, races or strains,** as appropriate, may be provided in order to demonstrate, for instance, the stability of the copy number and sequence conservation of the reference taxon-specific gene DNA, or the stability of expression of the protein.
Sensitivity testing		
26. LOD should be determined for each method separately 27. In the case of protein-based analyses, the limit of detection should be ascertained according to established procedures for immunoanalyses for each matrix..	Matrix can also have an effect on the LOD for DNA methods. Paragraph 26 should also make reference to matrices; in turn, this would render paragraph 27 unnecessary.	26. LOD should be determined for each method **and matrix** separately. 27. (PARAGRAPH SHOULD BE DELETED)
Cross-reactivity 29. The cross-reactivity, interferences and matrix effects should be evaluated.	This paragraph does not provide any useful guidance on HOW to evaluate cross-reactivity. In addition, these issues are already considered in other sections of the guideline, including section on selectivity testing (paragraphs21-22), section on matrix effects of annex VI, etc.	29. (PARAGRAPH SHOULD BE DELETED)
Extraction efficiency 30. Empirical results from testing **the protein method** for its extraction efficiency in each matrix should be provided to demonstrate the extraction is sufficient and reproducible. For quantitative detection, the method of calibration for incomplete extraction may need to be provided.	Extraction efficiency is also an issue for quantitative DNA analysis	*Extraction efficiency* 30. Empirical results from testing **the method** for its extraction efficiency in each matrix should be provided to demonstrate the extraction is sufficient and reproducible. For quantitative detection, the method of calibration for incomplete extraction may need to be provided.

PRACTICAL APPLICATION OF THE METHOD		
Applicability 31. 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 should be given. Relevant limitations of the method should also be addressed (e.g. **inference** by other analytes or inapplicability to certain situations). […].	Since this guideline is intended for food purposes, it would be more appropriate to refer to "grain" instead of "seeds". In addition, there is a typo on the use of "inference" instead of "interference".	***Applicability*** 31. Indication of the matrix (e.g., processed food, raw materials, etc.), the type of samples (e.g., **grains**, flour, pizza, cookies, etc.) and the range to which the method can be applied should be given. Relevant limitations of the method should also be addressed (e.g. **interference** by other analytes or inapplicability to certain situations). […]
METHOD VALIDATION/PERFORMANCE	The whole guideline deals with validation of methods, while the specific criteria listed in this section are performance parameters.	**METHOD PERFORMANCE**
37. See the Codex "Check-list" (i.e. accuracy, applicability (matrix, concentration range and preference given to 'general' methods), detection limit, quantification limit, precision, recovery, selectivity, sensitivity, extraction efficiency and parallelism/linearity) and an assessment that the methods will be fit for purpose.	The wording is confusing, and the reference to a "check list" is unclear.	Data on the parameters listed in Section 4.2, "General Method Criteria" should be provided, as well as a general assessment that the method is fit for its intended purpose.
ANNEX II: DEFINITIONS APPLICABLE TO THE ANALYSIS OF [DNA SEQUENCES AND PROTEINS] [FOODS DERIVED FROM MODERN BIOTECHNOLOGY]	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader. Therefore, we support the first bracketed option.	**ANNEX II: DEFINITIONS APPLICABLE TO THE ANALYSIS OF DNA SEQUENCES AND PROTEINS.**
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. Alternatively it may be taken from the last value with reliable data used to determine the LOD. LOD is generally expressed as the amount of analyte	The second sentence should refer to LOQ, or it would be superfluous. Third sentence: LOD is the LOWEST amount of analyte that can be reliably detected.	**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. Alternatively it may be taken from the last value with reliable data used to determine the LO**Q**. LOD is generally expressed as the **lowest** amount of

at which the analytical method detects the presence of the analyte at least 95% of the time (≤5% false negative results).		analyte at which the analytical method detects the presence of the analyte at least 95% of the time (≤5% false negative results).
Practicability The ease of operations, in terms of sample throughput and costs, to achieve the required performance criteria and thereby meet the specified purpose. ~~Generally, the method should be practical for its intended purposes.~~	The second sentence is not adequate for a definition, and it is superfluous.	**Practicability** The ease of operations, in terms of sample throughput and costs, to achieve the required performance criteria and thereby meet the specified purpose.
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.	There is incongruence on which term is preferred. The term "Harmonized Guidelines" is unclear for the general reader. There are only a few continents, and geographical distribution of the test is not as important as trading block distribution, from a Codex perspective.	**<u>Robustness (Ruggedness)</u>** 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. **Robustness** should be demonstrated by the validation of the method in 8-12 laboratories as defined in the **AOAC/IUPAC** harmonized guidelines. It is preferable**,** from a CODEX point of view, that these laboratories be distributed across **different 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.
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.~~	The second and third sentences are not appropriate for a definition, and are beyond the guideline scope.	**Sensitivity** The sensitivity of a method is a measure of the magnitude of the response caused by a certain amount of analyte. Sensitivity as a term is used in two different ways: LOD and instrument response. 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.

Sensitivity as a term is used in two different ways: LOD and instrument response. 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.~~		
Trueness Trueness is defined as […]. *In practice the accepted reference value is substituted for the true value.* ~~*Expectation is the expected value of a random variable, e.g. assigned value or long term average (ISO 5725-1).*~~	Last sentence: It is not necessary to define "expectation"	**Trueness** Trueness is defined as […]. *In practice the accepted reference value is substituted for the true value.*
Uncertainty Parameter associated with the outcome of a measurement to characterize the dispersion of the values that could reasonably be attributed to **him** by measuring the magnitude.	Editorial modification regarding the use of a pronoun	**Uncertainty** Parameter associated with the outcome of a measurement to characterize the dispersion of the values that could reasonably be attributed to **it** by measuring the magnitude.
ANNEX III: VALIDATION OF A QUANTITATIVE PCR METHOD		
INTRODUCTION		
1. 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 in which case the minimal length of the segment that can be measured instrumentally has to be specified. Food processing […]	- DNA segments amplified by PCR can be of varied length scales, and "short" is a relative adjective. - Editorial modification: There is one closing parenthesis missing	1. DNA-based analysis is commonly performed using polymerase chain reaction (PCR). This technique amplifies a specific segment of DNA to the extent that its quantity can be measured instrumentally (e.g., using fluorometric means in which case the minimal length of the segment that can be measured instrumentally has to be specified**)**. Food processing […]
2. ~~It is often the case that the results of~~ a determination are expressed in terms of percent of a target-specific DNA sequence relative to a taxon-specific DNA sequence. In such a relative quantitative test, this measurement actually involves two PCR-based determinations – that of the target-specific DNA sequence (e.g. a DNA sequence from another species) and that of the endogenous, or taxon-specific sequence (e.g. an	The beginning of the first sentence is can be misleading, since it is not clear if it states that quantitative determinations are more widely used than quantitative ones (which is not necessarily true), or if it states that quantitative determinations are usually expressed in terms of a percentage.	2. **Quantitative determinations are often** expressed in terms of percent of a target-specific DNA sequence relative to a taxon-specific DNA sequence. In such a relative quantitative test, this measurement actually involves two PCR-based determinations – that of the target-specific DNA sequence (e.g. a DNA sequence from another species) and that of the endogenous, or taxon-specific

endogenous maize gene sequence). […]		sequence (e.g. an endogenous maize gene sequence). […]
4. […] 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. […]	"Ruggedness" should be substituted with "Robustness", since it is the main term chosen and defined in the guideline.	4. […] It is strongly recommended that a small-scale collaborative trial be performed to test the general **robustness** of a particular method before the expense of organizing a large-scale trial is incurred. […]
VALIDATION		
6. […] These are scope, LOD and LOQ, trueness, precision, sensitivity and **ruggedness (robustness).** […]	"Ruggedness" should be substituted with "Robustness", since it is the main term chosen and defined in the guideline.	6. […] These are scope, LOD and LOQ, trueness, precision, sensitivity and **robustness.** […].
*7. ~~Note:~~ There is a general scientific discussion about the interpretation of the percentage values. It is recognised that so far there is no reliable weight/copy number relationship because of uncertainty in the correlation of weight of ingredient to number of molecules of DNA. Both the w/w and copy number/copy number calculations are acceptable provided **it** is clearly stated when reporting results.*	- There is no need of giving this paragraph a different format and a "note" heading. -In addition, an editorial change is suggested.	7. There is a general scientific discussion about the interpretation of the percentage values. It is recognised that so far there is no reliable weight/copy number relationship because of uncertainty in the correlation of weight of ingredient to number of molecules of DNA. Both the w/w and copy number/copy number calculations are acceptable provided **this** is clearly stated when reporting results.
Trueness	Editorial modification: This title should be moved below to its alphabetically correct location.	(TITLE AND PARAGRAPHS 9-10 SHOULD BE MOVED TO THE END OF THIS SECTION)
10. Recommendation: The trueness should be within ± 25% of the accepted reference value over the whole dynamic range. This refers to the PCR-step provided that the modular approach has been applied.	- There is no need of giving this paragraph a different format and a "Reference" heading. Everything in a Codex Guideline is an optional recommendation for governments. - "Value". Should be substituted by "values" since it refers to each value of different reference samples along the dynamic range. - "Provided that" should be substituted with "if", or the sentence could be misleading and interpreted in the sense that it only applies to that particular case.	10. The trueness should be within ± 25% of the accepted reference **values** over the whole dynamic range. This refers to the PCR-step **if** the modular approach has been applied.
Applicability		
12. As an example it is required from an extraction method, independent of matrix to which it is to be applied, that it yields DNA or protein of sufficient quantity, structural integrity and purity to allow a proper evaluation of the performance of the subsequent method steps (e.g. adequate	The year of the Turci et al reference should be corrected to 2010. The full reference is present in the list of references for the main text, but it is not included in the list of references for this annex.	12. As an example it is required from an extraction method, independent of matrix to which it is to be applied, that it yields DNA or protein 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, detection of the protein) to be undertaken (e.g. Chapela et al. 2007; Turci et al. **2009**).		amplification of DNA during the PCR step, detection of the protein) to be undertaken (e.g. Chapela et al. 2007; Turci et al. **2010**).
Dynamic Range - Range Of Quantification		
16. **If 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, […]	Only DNA can be used as a calibrator for the purposes of this annex.	16. **The DNA used as calibrator should** be traced back (in its metrological meaning) to a reference of highest metrological order, […]
Limit of Detection (LOD) and Limit of Quantification (LOQ)		
18. If the validation of the quantitative PCR assay shows that the assay can measure [DNA] ~~[foods derived from modern biotechnology]~~ 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. […].	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader. Therefore, we support the first bracketed option.	18. If the validation of the quantitative PCR assay shows that the assay **can measure 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. […]
19. 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** […].	The first and last sentence are contradictory in regards to the need of establishing the LOQ. The units used at the end of the second sentence should be corrected for more realism	19. It is worth noting that a determination of **an LOD 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. […]
20. […]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). […]	Editorial change: "time" should be substituted by "times"	20. […]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 **times** (≤5% false negative results). […]
22. […] A more complete approach is to test the method using a number of samples that contain known amounts of analyte. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of concentrations of the [DNA sequences or proteins] ~~[foods~~	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an	22. […]A more complete approach is to test the method using a number of samples that contain known amounts of analyte. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of **concentrations of the DNA sequences or proteins of**

~~derived from modern biotechnology]~~	emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader. Therefore, we support the first bracketed option.	**interest.**
Practicability 23. The practicability of the method should be demonstrated.	This paragraph does not provide useful guidance on how to do it. In addition, "Practicability" is a criteria that inevitably involves subjective judgment, therefore it cannot be "demonstrated".	**The practicability of the method should be assessed by considering parameters such as: the quantity of samples that can be processed within a given time, estimated fixed costs to implement the method and the approximate cost per sample, practical difficulties on daily use or under particular conditions, as well as other factors that could be of importance for the operators.**
Repeatability standard deviation (RSD_r) 24. ~~Recommendation~~: The relative repeatability standard deviation should be ≤25% ~~or as close as is practicable~~ over the whole dynamic range of the method.	- There is no need of giving this paragraph a different format and a "Recomendation" heading. Everything in a Codex Guideline is an optional recommendation for governments. - The use of the clause "or as close as practicable" is unnecessary given the generous limit <u>recommended</u> for the RSDr	**Repeatability standard deviation (RSD_r)** 24. The relative repeatability standard deviation should be ≤25% over the whole dynamic range of the method.
Reproducibility standard deviation (RSD_R) 25. ~~Recommendation:~~ The relative reproducibility standard deviation should be below 35% ~~or as close as is practicable~~ at the target concentration and over the majority of the dynamic range. $RSD_R \leq 50\%$ or ~~as close as is practicable~~ at the limit of quantification lower end.	There is no need of giving this paragraph a different format and a "Recomendation" heading. Everything in a Codex Guideline is an optional recommendation for governments. - The use of the clause "or as close as practicable" is unnecessary given the generous limit <u>recommended</u> for the RSDR and the 50% extension for the lower end	**Reproducibility standard deviation (RSD_R)** 25. The relative reproducibility standard deviation should be **≤** 35% at the target concentration and over the majority of the dynamic range, excepting the the limit of quantification lower end, where it could be ≤ 50%.
Ruggedness (Robustness) 26. 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., 29 vs. 30µl), annealing temperature (e.g., +/-1°C) and/or other relevant variations. The experiments need to be performed at least in triplicates. […]	- "Ruggedness" should be substituted with "Robustness", since it is the main term chosen and defined in the guideline. - The reference to "inadvertent variations" and "variations that may be included" could be confusing for the general reader. - This kind of test is an assessment of potential reliability, but not a "demonstration".	**Robustness** 26. The evaluation of robustness **estimates** the reliability of a method with respect to inadvertent variation in assay parameters **during future routine use**. **This evaluation consists on purposely introducing variations of parameters such as** reaction volumes (e.g., 29 vs. 30µl), annealing temperature (e.g., +/-1°C) and/or other relevant variations. The experiments need to be performed at least in triplicates. […]

Specificity	In section 4.2 of the main text and the definition annex the name previously chosen for this criteria is "Selectivity". It should be repeated in this title to avoid confusing the general reader.	**Selectivity**
32. A taxon-specific assay should not recognize any sequence corresponding to phylogenetically related **species**, and should give similar Ct-values, not statistically different, when amplifying equal amounts of DNA from different **varieties/cultivars** of very different origins of the same taxon.	- It was agreed to replace the word "species" with "taxon" along the text, for the purposes of paragraphs like this one. - Given the wide applicability of these methods, the origin of the reference and/or target protein could be derived from plants, animals or microorganisms.	32. A taxon-specific assay should not recognize any sequence corresponding to phylogenetically related **taxon**, and should give similar Ct-values, not statistically different, when amplifying equal amounts of DNA from different **varieties, races or strains** of very different origins of the same taxon.
~~33. A species-specific assay should not recognize any sequence corresponding to closely related species, and should give similar Ct-values, not statistically different, when amplifying equal amounts of DNA from different varieties/cultivars of very different origins of the same species.~~	- Paragraph 33 is a repetition of paragraph 32.	33. (PARAGRAPH SHOULD BE DELETED)
34. The adequacy of the testing needs to be analysed on a method-by-method basis.	The sentence is unclear and provides little guidance.	34. The adequacy of the **specificity** needs to be analysed **according to the purpose of the method**. **For instance, a method for raw materials may have less chances of cross reacting compared to methods intended to be applied to processed foods composed of several ingredients.**
35. Primer and probe design is the starting point for a method. Primers and probes should be checked against the known sequence of the target that the assay is designed to detect and pertinent sequences databases for possible homologies ~~or against known target organism/pathogen sequences~~. After such a theoretical ~~specificity~~ assessment, selectivity should then be demonstrated experimentally.	- the end of the second sentence does not provide adequate guidance. - the third sentence seems to introduce a differentiation between "specificity" and "selectivity"	35. Primer and probe design is the starting point for a method. Primers and probes should be checked against the known sequence of the target that the assay is designed to detect and pertinent sequences databases for possible homologies **with other sequences potentially present in the expected matrices, according to the intended use.** After such a **theoretical assessment,** selectivity should then be demonstrated experimentally.
REFERENCES FOR ANNEX III		
ORPHAN REFERENCES	Full references from paragraph 12 should be inserted	Chapela MJ, Sotelo CG, Pérez-Martín RI, Pardo MA, Pérez-Villareal B, Gilardi P and Riese J (2007). Comparison of DNA extraction methods from muscle of canned tuna for species

		identification. *Food Control.* 18(10):1211-1215. Turci M, Sardaro MLS, Visioli G, Maestri E, Marmiroli, M and Marmiroli N (2010). Evaluation of DNA extraction procedures for traceability of various tomato products. Food Control. 21(2):143-149.
ANNEX IV ANALYTICAL CONTROL ACCEPTANCE CRITERIA , PROFICIENCY TESTING AND INTERPRETATION OF RESULTS FOR QUANTITATIVE PCR METHODS		
1. A validated method also includes values of criteria on which the validity of an observed measurement result can be assumed. It is important to follow these criteria and to observe the decision support system for data analysis and 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 decision support system and procedures.	Editorial modification: a comma should be introduced after the word "rules", in the last sentence.	1. A validated method also includes values of criteria on which the validity of an observed measurement result can be assumed. It is important to follow these criteria and to observe the decision support system for data analysis and 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 decision support system and procedures.
• The % of residual for each of the standards should be ≤30% ~~or as close as is practicable.~~	- The use of the clause "or as close as practicable" is unnecessary given the generous limit recommended and the optional nature of the Codex guidance.	- The % of residual for each of the standards should be ≤30%.
3. To accept the result of an unknown sample, the relative standard deviation of the sample replicates should be ≤35 % ~~or as close as is practicable.~~	The use of the clause "or as close as practicable" is unnecessary given the generous limit recommended and the optional nature of the Codex guidance	3. To accept the result of an unknown sample, the relative standard deviation of the sample replicates should be ≤35 %.
4. The amplification process used in quantitative PCR determinations often commences with a small number of copies of the target DNA and the sampling process at this initial stage, together with the log-linear nature of the PCR calibration function, leads to a positive skew in results. ~~As predicted, the~~ study results consistently follow a positively skewed distribution	The phrase "as predicted, the study…" is not adequate for this document.	4. The amplification process used in quantitative PCR determinations often commences with a small number of copies of the target DNA and the sampling process at this initial stage, together with the log-linear nature of the PCR calibration function, leads to a positive skew in results. **In one** study**,** results consistently follow**ed** a positively skewed distribution (Thompson et

(Thompson et al., 2006). […]		al., 2006). […].
ANNEX V: VALIDATION OF A QUALITATIVE PCR METHOD		
False Positive Rate 3. […] For convenience this rate can be expressed as percentage: % false positive results = x 100 number of misclassified known negative samples / total number of negative test results [incl. misclassified]	Please see our comment below on false negative rate	3. […] For convenience this rate can be expressed as percentage: % false positive results = 100 **x** number of misclassified known negative samples / total number of **known negative samples**
False Negative Rate 4. 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: % false negative results = x 100 number of misclassified known **negative** samples / total number of positive test results [incl. misclassified]	- In the equation, it should state "number of misclassified known POSITIVE samples" - This definition is not coherent with sentences 5 and 6 of paragraph 2. If a known positive sample is assayed 100 times, obtaining 95 positive results and 5 negative results, then the % false negatives would be 5 / 95 x 100 = 5.26%, which is higher than 5%. The definition of the divisor of the equation is not very clear. Therefore, we propose using a clearer definition where the divisor is the total of known positive samples.	4. 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: % false negative results = 100 **x** number of misclassified known **positive** samples / total number of **known positive samples**
Note: different sectors use different definitions here.	The purpose of this clarification could be confusing for the general reader.	Note: since there are different definitions in use for the false positive and false negative rates, the validation report should clarify which one has been used.
5. […] 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.~~	The statistical basis or rationale sustaining the last sentence is not provided, therefore it is suggested to delete it.	5. […] The desired level of confidence determines the size and number of pools that need to be tested.

Ruggedness 6. As with any validated method, reasonable efforts should be made to demonstrate the ruggedness of the assay. This involves careful optimisation and investigation of the impact of small modifications made to the method due to technical reasons.	- "Ruggedness" should be substituted with "Robustness", since it is the main term chosen and defined in the guideline. - The paragraph does not contain enough guidance on how to assess ruggedness. In addition, the repetition of this issue in separate annexes may mislead the general reader to believe that robustness is assessed differently.	**Robustness** 6. As with any validated method, reasonable efforts should be made to demonstrate the r**obustness** of the assay. This involves careful optimisation and investigation of the impact of small modifications made to the method due to technical reasons**, as described in the annex for quantitative PCR.**
Applicability 7. In the case of qualitative PCR methods that use gels as the data delivery method, it is advisable to operate the methods at levels well above the LOD if possible, to ensure that the data interpretation is easy and as objective as possible.	The method use to generate results, like gel electrophoresis for visual analysis, is part of the method and contributes to its LOD, therefore the sentence is confusing.	**Applicability** 7. In the case of qualitative PCR methods **that rely on visual inspection to generate results after a gel electrophoresis of the amplification products, it is advisable to establish a conservative LOD based on results from different operators in order to account for differences in human visual sensibility and expertise. However, the use of digital photography and image analysis software is recommended instead, given its greater sensibility and objectivity, its wider availability, low cost and due to the increased operator safety.**
NEW PARAGRAPH	Some important concepts mentioned earlier are not mentioned for qualitative PCR, this could be intriguing for the general reader.	For qualitative methods, selectivity can be defined as equal to (= 1 − false positive rate/100), while sensitivity can be defined as (= 1 - false negative rate/100). General concepts on applicability and practicability provided for quantitative PCR are also applicable, *mutatis mutandis*, to quailtative PCR.
ANNEX VI: VALIDATION OF A PROTEIN-BASED METHOD		
QUANTITATIVE TESTING		
2 The following description of the procedure is only one of several possibilities to carry out an immunological detection assay for proteins [of interest] ~~[expressed in organisms derived from recombinant DNA] .~~	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but	2 The following description of the procedure is only one of several possibilities to carry out an immunological detection assay for **proteins of interest.**

	there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader. Therefore, we support the first option of bracketed text.	
3. In typical immunological detection assay for proteins, the optical density (OD) of the product of immunological reaction is measured. The standard curve is generated by […].	The description of the first sentence corresponds to ELISA, while other immunological methods can rely on other detection methods. In addition, the description is not completely accurate.	3. In **a** typical **ELISA** assay for proteins, the optical density (OD) of **the product of enzymatic reaction** is measured. **A** standard curve is generated by […]
REFERENCE MATERIALS		
6. When applicable, the reference material consists of the same matrix as the target analytical sample to be tested. It typically includes negative control and positive reference materials. For example, if the matrix to be tested is soybean flour the standardized positive reference material would be soybean flour containing a known proportion of [protein of interest] ~~[foods derived from modern biotechnology]~~. Alternatively, a pure sample or extract of the protein of interest may be used, […]	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader. Therefore, we support the first option of bracketed text.	6. When applicable, the reference material consists of the same matrix as the target analytical sample to be tested. It typically includes negative control and positive reference materials. For example, if the matrix to be tested is soybean flour the standardized positive reference material would be soybean flour containing a known proportion of protein of interest. Alternatively, a pure sample or extract of the protein of interest may be used, […]
VALIDATION OF A QUANTITATIVE PROTEIN-BASED METHOD		
8. The principles of method validation defined in the harmonized ISO/IUPAC/AOAC standard (Horwitz 1995) apply to ~~both PCR and~~ protein methods. ISO has developed specific international guidelines for validation of immunoassays for the detection and quantification of GM foodstuffs (ISO21572:2004). These guidelines are equally applicable to other foodstuffs.	This annex only deals with protein methods	8. The principles of method validation defined in the harmonized ISO/IUPAC/AOAC standard (Horwitz 1995) **apply to protein methods**. ISO has developed specific international guidelines for validation of immunoassays for the detection and quantification of GM foodstuffs (ISO21572:2004). These guidelines are equally applicable to other foodstuffs.
10. 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	The term "fortified" and "spiked" are used in the document, referring to the same kind of samples. We suggest harmonizing the text to the use of "spiked", since "fortified" is more adequate for particular issues like food nutritional enhancement.	10. Accuracy: Accuracy is demonstrated by measuring the recovery of analyte from **spiked** 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).		measured recoveries at each fortification level (Mihaliak & Berberich, 1995).
11. The recovery of proteins ~~[expressed in organisms derived from recombinant DNA]~~ should be determined by comparing results obtained from analysis of a reference material with the known or assigned value for that reference material. The impact of sample matrix effects, particularly when the sample matrix differs from that of the reference material, should be considered.	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader..	11. The recovery of **proteins of interest** should be determined by comparing results obtained from analysis of a reference material with the known or assigned value for that reference material. The impact of sample matrix effects, particularly when the sample matrix differs from that of the reference material, should be considered.
~~*Recommendation:*~~ *The Recovery should be between 70 and 120%* 12. Repeatability standard deviation (RSDr) *Recommendation: The relative repeatability standard deviation should be ≤25% or as close as is practicable over the whole dynamic range of the method.* 13. Reproducibility standard deviation (RSDR) *Recommendation: The relative reproducibility standard deviation should be below 35% or as close as is practicable at the target concentration and over the majority of the dynamic range. $RSD_R \leq 50\%$ or as close as is practicable at the limit of quantification lower end.*	- There is no need of giving these paragraphs a different format and a "Recomendation" heading. Everything in a Codex Guideline is an optional recommendation for governments. The use of the clause "or as close as practicable" is unnecessary given the generous limits recommended and the optional nature of the Codex guidance.	11 bis. The Recovery should be between 70 and 120%. 12. The relative repeatability standard deviation should be ≤25% over the whole dynamic range of the method. 13. The relative reproducibility standard deviation should be **≤** 35% at the target concentration and over the majority of the dynamic range, excepting the the limit of quantification lower end, where it could be ≤ 50%.
Dynamic Range - Range Of Quantification		
18. The scope of the methods **defines** the concentration range over which the analyte will be accurately determined. […]	Editorial modification for clarity	18. The scope of the methods **should include** the concentration range over which the analyte will be accurately determined. […]
19. Quantitative protein methods generally give an estimate of the concentration of the protein of interest in the matrix . ~~For GMO~~ the interpretation of the percentage values (e.g. dynamic range from 10% to 500% the target value) can be difficult when using quantitative methods, due to variations in the expression of the amount of protein	The second sentence should be adapted to the generalized scope of the document. There is no technical reason to retrain it to GMO-derived products.	19. Quantitative protein methods generally give an estimate of the concentration of the protein of interest in the matrix . **T**he interpretation of the percentage values (e.g. dynamic range from 10% to 500% the target value) can be difficult when using quantitative methods, due to variations in the expression of the amount of

in different **tissues of plants or among cultivars, and within the same tissue at different locations**. Care should be taken to employ a method which can detect the specific protein in the analyzed matrix. […].		**protein in different tissues, varieties/races/strains, and/or at different locations**. Care should be taken to employ a method which can detect the specific protein in the analyzed matrix. […].
20. It is worth noting that if the LOD or LOQ is established to be much lower than the range in which the method is intended to be used, a precise determination is not necessary. This would be the case, for example, when the LOD is in the range of 1 ng/kg, while the range of the method validation extends only for concentrations ranging in **g/kg**.	The units used at the end of the last sentence should be corrected for more realism	20. It is worth noting that if the LOD or LOQ is established to be much lower than the range in which the method is intended to be used, a precise determination is not necessary. This would be the case, for example, when the LOD is in the range of 1 ng/kg, while the range of the method validation extends only for concentrations ranging in **g/kg.**
Limit of Detection (LOD)		
21. LOD is defined in annex II. Proteins are usually present in foods ~~[derived from modern biotechnology]~~ 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.	We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader. Therefore we oppose to the bracketed text	21. LOD is defined in annex II. Proteins are usually present in foods at higher concentrations than the target DNA for PCR methods. Thus stochastic effects have less influence on the determination of the LOD **compared to** using PCR.
Limit of Quantification (LOQ)		
24. In practice, two procedures have been employed to determine the LOQ. The first approach is to assay a number of conventional 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 RSDr of ≤20% and 70-120% recovery (Mihaliak & Berberich, 1995). 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	- A Codex Guideline should not prejudge over the government's decisions. - We consider that this guideline is equally applicable and useful for the validation PCR or ELISA methods of analysis applied to the detection of any analyte of Codex relevance. We do not oppose to its applicability to biomarkers for GM foods, but there is no need of making an emphasis of it; unnecessary emphasis may hinder the wider applicability of the document for the general reader. Therefore we oppose to the bracketed text	24. In practice, two procedures have been employed to determine the LOQ. The first approach is to assay a number of conventional 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 RSDr of ≤20% and 70-120% recovery (Mihaliak & Berberich, 1995). Protein recovery, however, may be difficult from some matrices, e.g. starches or oils, **and methods for such matrices may reach lower recovery efficiencies**.

should 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 target. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of concentrations of the [protein of interest] ~~[foods derived from modern biotechnology]~~. Procedures for assessing LOD and LOQ during the validation of quantitative PCR methods are also discussed in annexes III and IV.		When recovery efficiencies are low, this should 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 target. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of concentrations **of the protein of interest**. Procedures for assessing LOD and LOQ during the validation of quantitative PCR methods are also discussed in annexes III and IV.
Cross-reactivity 25. The cross-reactivity or assay specificity is the degree to which analogs or other molecules can bind to the detection antibodies and therefore should be characterized and described in the method. **Cross-reactivity should be demonstrated** by showing experimental results from testing the method with proteins or molecules from non-target and closely related species/variety, and purified target protein and/or reference positive control materials. The potential for interferences from reagents and labware can be evaluated by assaying extracts from analyte free material.	In this case, assay specificity is the contrary idea of cross-reactivity. The list of criteria from previous sections already established "selectivity" as one of the criteria to be analyzed for each method. Of course, cross reactivity is undesirable and presumed to be low or absent from the method.	**Selectivity** 25. The **cross-reactivity is the degree** to which analogs or other molecules can bind to the detection antibodies and therefore should be characterized and described in the method. **The absence of** cross-reactivity should be **assessed using** experimental results from testing the method with proteins or molecules from non-target and closely related species/variety, and purified target protein and/or reference positive control materials. The potential for interferences from reagents and labware can be evaluated by assaying extracts from analyte free material.
Ruggedness (Robustness) 27. 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~~^{o}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	- "Ruggedness" should be substituted with "Robustness", since it is the main term chosen and defined in the guideline. - The reference to "inadvertent variations" and "variations that may be included" could be confusing for the general reader. - This kind of test is an assessment of potential reliability, but not a "demonstration". - A range of -/+10 degrees is excessive for "inadvertent" variations.	**Robustness** 27. The evaluation **of robustness estimates** the reliability of a method with respect to inadvertent variation in assay parameters **during future routine use**. **This evaluation consists on purposely introducing variations of parameters such as** reaction volumes**,** incubation temperature **(e.g., +/- 1^{o}C for oven incubations and +/- 4^{o}C for incubations at "room temperature")** 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

be performed to establish ruggedness include repeated analysis of a sample or samples on several days and measurement of trueness and precision in **fortified** samples using control material from several sources.	- The term "fortified" and "spiked" are used in the document, referring to the same kind of samples. We suggest harmonizing the text to the use of "spiked", since "fortified" is more adequate for particular issues like food nutritional enhancement.	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 trueness and precision in **spiked** samples using control material from several sources.
QUALITATIVE ~~(THRESHOLD)~~ TESTING	The use of the term threshold was unnecessary for PCR methods and there is no reason to introduce it here. Moreover, it can introduce confusion with regulatory thresholds, which are not necessarily in the same levels.	**QUALITATIVE TESTING**
28. Lateral flow devices are useful tools for on-site or field **threshold** testing. Traditional ELISA methods can also be used for qualitative testing. In order to ensure reliable results, the manufacturers of such assays should conduct a method validation and provide a description of the performance characteristics of the product in the package insert, including sensitivity, specificity, applicability, and hook-effect. If this has been completed there is generally no need for validation studies to be performed by users of Lateral Flow devices for implementation of the technique within their laboratory as long as the method is performed according to the manufacturer's instructions. […]	- The use of the term threshold was unnecessary for PCR methods and there is no reason to introduce it here. Moreover, it can introduce confusion with regulatory thresholds, which are not necessarily in the same levels. - The second paragraph gives for granted that lateral flow devices are provided by a commercial manufacturer, which may not necessarily be true. Moreover, if the manufacturer does not provide a full validation data set, the operator could do it instead. - Three important criteria for this kind of assays are missing from the list: limit of detection, ruggedness and matrix effects.	28. Lateral flow devices **(LFD)** are useful tools for on-site or **field testing although** traditional ELISA methods can also be used for qualitative testing. In order to ensure reliable results, the **developers** of such LFD should conduct a method validation and provide a description of the performance characteristics of the product in the package insert, including sensitivity, specificity, applicability, **limit of detection, ruggedness, matrix effects,** and hook-effect. If this has been completed there is generally no need for validation studies to be performed by users of Lateral Flow devices for implementation of the technique within their laboratory as long as the method is performed according to the manufacturer's instructions. […]
~~29. In order to establish an on-site procedure for **threshold** testing, the **threshold** level should first be established. To establish that the lateral flow device is able to differentiate between samples containing target protein above or below the **threshold**, both a negative reference and a **threshold** reference containing a known proportion of matrix with target~~	- The interpretation of a positive/negative result (including the LOD of the method) in regards to any regulatory threshold of a national legislation is beyond the scope of the guideline.	29. PARAGRAPH SHOULD BE DELETED

~~protein 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 (e.g. USDA, 2004) 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 PROTEIN-BASED METHOD		
Applicability		
~~33. For determination of the LOD of qualitative assays, fortification levels near to the LOD may be used, as long as one of the levels used meets the criteria of being above but close to the LOD. While such procedures can give an indication of the performance of the method, incurred samples with well known characteristics (if available) are the best matrix on which to establish the applicability of a method.~~	The paragraph is unclear, LOD is obviously not known before it is determined, so samples cannot be chosen close to it unless a iterative approach (not described) is used.	33. (PARAGRAPH SHOULD BE DELETED)
Practicability		
34. The practicability of the method should be demonstrated.	This paragraph does not provide useful guidance on how to do it. In addition, "Practicability" is a criteria that inevitably involves subjective judgment, therefore it cannot be "demonstrated".	34. The practicability of the method should be **assessed by considering parameters such as: the quantity of samples that can be processed within a given time, estimated fixed costs to implement the method and the approximate cost per sample, practical difficulties on daily use or under particular conditions, as well as other factors that could be of importance for the operators.**
35. 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	There is no reference to support the last sentence of this paragraph, whose utility is unclear; moreover, it may induce the general reader to disregard the need of assessing the	35. 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.~~	applicability of the LOD for a particular intended use. The last sentence should be deleted.	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).

CANADA

Canada thanks the Co-Chairs (Argentina, United Kingdom and Germany) of the electronic working group for leading the activities. We commend the Co-Chairs for leading the e-WG in a very effective and transparent manner and are pleased to offer the following comments:

GENERAL COMMENTS

Canada believes that the development of guidelines on criteria for methods for the detection, identification and quantification of specific DNA sequences and specific proteins in foods are necessary. As stated during the e-WG deliberation, we are supportive of refocusing the guidelines and expanding their applicability beyond methodology for specific DNA sequences and specific proteins in food derived from modern biotechnology. We are of the view that the expansion of the scope will make these guidelines more useful to member countries, since there are many applications that would benefit from such guidance. These methodologies may be used for other purposes besides detection of foods derived from modern biotechnology and should be subject to the guidelines as well. Canada is supportive of further developing guidelines that are generally applicable to all DNA and protein based methodologies for food in general.

SPECIFIC COMMENTS

Canada supports the document structure as proposed. However, the text will need to be consistent throughout the document and harmonized with the broadened Scope and the Title.

Title:

For reasons outlined in our General Comments, Canada supports the following alternative title: **"Proposed Draft Guidelines on Performance Criteria and Validation of Methods for Detection, Identification and Quantification of Specific DNA Sequences and Specific Proteins in Foods"**

The use of this Title would be in line with the broadened objectives of the guidelines.

Section I - Introduction:

Paragraph 1: Canada supports deletion of the text in square brackets [derived from modern biotechnology], as it is not in line with a broadened Scope.

Section 1.1- Purpose and Objectives

Paragraph 4: Canada supports deletion of the text in square brackets [derived from modern biotechnology], as it is not in line with a broadened Scope.

Section 1.2- Scope

Canada supports paragraph 6 alternative in square brackets. The alternative text would be more consistent with the Title that we are supporting and would be an acceptable compromise as it supports the concept of the broadened objectives. We want to re-iterate that an expanded scope would make the guidelines more useful to member countries, since there are many applications that would benefit from such guidance.

Paragraphs 7, 8, 44, 46 and 49

For consistency purpose, we support the first bracket statement [DNA sequences and proteins of interest in foods]. This would make these paragraphs consistent with the revised Title and Scope of the guidelines.

Section, 5.1.2- Applicability of the Method

Paragraph 19: Canada supports deletion of the following statements in square brackets, also for purposes of text consistency with the revised Title and Scope:

[This is a particularly important criterion in the analysis of foods derived from modern biotechnology];

[this is a specific food derived from modern biotechnology then there is merit in requiring those seeking endorsement to provide information]; and

[foods derived from modern biotechnology].

Section 5.1.3- Principle condition

Paragraph 21 Canada supports the text in the current paragraph 21 as it would further promote consistency, and therefore, supports the deletion of the alternative paragraph 21 in square brackets: [The provision of the detection method is aimed to serve mainly the requirements for the measurement of products derived from modern biotechnology. To serve these purposes, the method can detect and quantify the specific target and taxon-specific DNA sequence of the protein derived there from in the product; this may be achieved in most cases using either protein-based or DNA-based methods].

Paragraph 27: Canada supports the text in the current paragraph 27 and the deletion of the alternative paragraph 27 in square brackets, as the alternative text would be inconsistent the Title and Scope.

Annex I

Paragraph 4: For consistency purpose, we support the first bracket statement [DNA sequences and proteins of interest in foods]. This would make the paragraph consistent with the revised Title and Scope.

Annex II

Title: For consistency purpose, we support the first bracket statement [DNA sequences and proteins of interest in foods]. This would make it consistent with the revised Title and Scope.

Annex VI

Paragraphs 2, 6 and 24: We support the first bracket statement [DNA sequences and proteins of interest in foods] in each of these paragraphs, for consistency purpose.

Paragraphs 11 and 21: Canada supports deletion of the text in square brackets as it is not in line with a broadened Scope.

IRAN

1- General comment:

1-1 It is recommended that the document be prepared in two separate parts, one part for the criteria for DNA based methods, and the second part for protein based methods. While some criteria are common for both methods, there are some criteria that are completely different, and stating both of them in same paragraph makes it less understandably.

2- Title:

2-1 The Iranian committee agrees with the alternative title 1, and since the text has been prepared focusing on the "FOODS DERIVED FROM BIOTECHNOLOGY", it is recommended the mentioned phrase be added to the title.

If the above comment be accepted, then the phrase should be added to the other parts such as Introduction.

3- Method validation:

3-1 It is recommended that **section 4.3** on "validation process" be expanded and a protocol for validation of DNA based methods as well as protein based methods, containing documentation and keeping the records, be stated in detail. Such a protocol could be added as an annex to the guideline.

4- section 5:

4-1 In **Section 5.1.2**- "Applicability of the method", #19. it is recommended that the paragraphs in the second brackets [this is a specific food derived from modern biotechnology ….] and [foods derived from modern biotechnology] be deleted. We also suggest eliminating same phrases in the entire of the document.

4-2 Section **5.1.3**- "Principle and condition", #21. We agree with the first option, so the alternative paragraph should be deleted.

4-3 In this section, particularly # 23, #24 and # 25, it is suggested to state all of the details needed for a method, either DNA based or protein based. In the current paragraphs just some examples of the matters are reviewed and some crucial details such as "number of cycles and time and temperature" have been ignored.

4-4 A critical detail should be inserted in the section "describing the oligonucleotide primer sequences" is "the system used for fluorescence reading".

4-5 "The criteria for acceptance of sample" both for DNA based and protein based methods should also be stated.

4-6 Since many items such as the following items are not included, the paragraph regarding protein based methods and quantification should be completely revised.

- Description of the extraction method (if applicable),

- Description of the operation condition including the apparatus, washing system (manually or automatically), stop solution diluents, etc.

JAPAN

The government of Japan appreciates the efforts of Argentine, the United Kingdom and Germany in leading the electronic Working Group to prepare the Proposed draft Guidelines for consideration at the forthcoming 31st Session of the Codex Committee on Method of Analysis and Samplings (CCMAS).

Japan would like to submit the following comments:

1. General comment

Japan, acknowledging that the proposed Draft Guidelines is quite comprehensive and informative, is of the opinion that the document contains too much detail. Japan suggests that the document needs to retain only essential points and be shortened by deleting some explanations of analytical methods, which are already available elsewhere, for conciseness and readability.

For example, paragraph 1 of Annex VI which is just providing what ELISA methods are, should be deleted. In this moment, Japan would like to present some proposed deletion, in line with it comments expressed during the electronic working group, to shorten Sections 5.1.2 and 5.1.3 of the main document by completely deleting paragraphs 19, 20, 23, 24 and 26 and removing square brackets in paragraph 27 so as to better focus on what should be done for determining applicability of methods and principle condition.

Japan notices that there are some other sections and paragraphs which are also just providing basic information of analytical methods, etc. Japan may wish to prepare additional comments in a later stage, prior to the plenary of the next CCMAS, to indicate proposed modification.

2. Text in square brackets

Japan, recalling that long discussion was held at the previous CCMAS on the new scope and title of the document, suggests that the next CCMAS should not reopen the same discussion held at previous meeting for the sake of time economy. On the condition that the current title remains unchanged. Japan is of the opinion that it is not necessary to refer to "derived from modern biotechnology" every time at relevant part of the main text. Validations of methods for the detection of DNA and proteins should be applicable not only to GMOs but also to all other foods (conventional foods and/or foods produced through other techniques/technologies) unless

guidance is only applicable to food derived from biotechnology.

3. Other specific comments

i) Annex III, paragraph12

The term "protein" should be deleted because this annex addresses issues on the validation of quantitative PCR methods in which DNAs are analytes, but not proteins.

> 12. As an example it is required from an extraction method, independent of matrix to which it is to be applied, that it yields DNA ~~**or protein**~~ 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, ~~**detection of the protein**~~) to be undertaken (e.g. *Chapela et al*. 2007; *Turciet al* 2009).

ii) Annex VI, paragraph10

According to the discussion in the electronic working group, it had been decided to replace paragraph 10 with paragraph 11, which was proposed as a substitution. So, paragraph 10 should be deleted.

KENYA

Section 5.1.3 – Principle condition

Comment on para 21

We would like to inform the committee that we have propose to retain alternative 1 as stated below accepting the word "food derived from modern biotechnology" at the end of the title and subsequent sections in the text. The sentence should read ´5.1.3.21 alt. *The provision of the detection method is aimed to serve mainly the requirements for the measurement of products derived from modern biotechnology. To serve these purposes, the method can detect and quantify the specific target and taxon specific DNA sequence or the protein derived there from in the product, this may be achieved in most cases using either protein-based or DNA-based methods.*

We therefore recommend the deletion of para 21 that states "21. DNA-based methods should detect, identify and quantify the relative levels of specific target and taxon specific DNA sequences whilst protein-based methods should detect and quantify the level of specific protein in the product.

Justification

1. We retain the word "food derived from Biotechnology" because once the genes are introduced or inserted artificially in the food to be modified, the genes naturally immutate automatically to adapt to the environment.

2. So when we talk of the word "Biotechnology" then we are more specific to the artificial manipulation of the gene or DNA sequences.

2.1 [Alternative Title I:

PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR THE DETECTION AND IDENTIFICATION OF FOODS DERIVED FROM MODERN BIOTECHNOLOGY

Section 5.1.3 – Principle condition

27. The method provider should demonstrate that the method fulfils the requirements below.

Comment

We propose the PCR method which is the most appropriate as stated in "alternative para 27 clause 5.1.3 to fulfill the requirement in our country. We recommend that the closing and opening square brackets be removed, to read as follows:

[27 alt. *Should PCR be selected as the analytical method, the method should target a DNA sequence which is not present in the food being examined. Currently, the best choice concerning event specificity PCR should be the chosen technique, because it is targeting 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 recombinant insert and the host genomic DNA will probably be the location of choice. However, when a unique DNA sequence can be found within the recombinant insert, such a sequence (generally called construct specific) can also be targeted by appropriate oligonucleotide primers and amplified through a PCR. Identification of the amplified fragment by e.g. probe hybridization or any appropriate equivalent method is recommended.]

Justification

1. The PCR method is more efficient and can target a particular DNA sequence that has been inserted in the gene.

NEW ZEALAND

New Zealand notes the following points in the draft that require clarification:

1) LOD for validation of quantitative PCR methods (Annexes III and IV)

These annexes seem to contain conflicting advice on whether LOD is needed for validation of quantitative PCR methods. Annex III, paragraphs 18 & 19 (page 29) states that often the LOD need not be estimated if it is outside the range of the proposed applications. Yet Annex IV, paragraph 2 states (2nd bullet), "(Therefore it is important to always determine LOD for validation of the method.)" This apparent conflict needs to be resolved.

2) LOQ in validation studies (Annex III, paragraphs 18 & 19)

This annex recommends always including LOQ in validation studies, even though the LOQ may be outside the relevant range. This is in contrast to the LOD, which, in the same circumstances, need not be established. The distinction seems unwarranted. If the trueness and precision are satisfactory over the relevant range, then the LOQ necessarily lies below this range, almost by definition, and that seems adequate.

3) Sensitivity (Annex III, paragraph 28)

The correlation coefficient does not seem to be much use in this context. The standard deviation of measured values about the regression line would be much more useful, and much easier to interpret. The correlation coefficient would require attention if criteria for it formed part of the validation.

PANAMA

Lo siguiente es el comentario al Anteproyecto de Directrices sobre los criterios para métodos de detección, identificación y cuantificación de secuencias específicas de ADN y proteínas específicas, en particular en alimentos obtenidos por la biotecnología moderna (CX/MAS 10/31/3).

Comentario:

Reconocemos el trabajo del grupo electrónico liderizado por Argentina, estamos de acuerdo en que el ámbito del documento sea ampliado, que el mismo además de incluir la validación de métodos para OGM, incluya pruebas de identificación de patógenos de riesgo y pruebas basadas en proteínas (PCR y ELISA).

Resaltamos la necesidad de que el titulo del documento sea negociado de tal manera que el alcance del mismo sea más amplio y no solo incluya métodos de detección para alimentos obtenidos biotecnológicamente.

USA

General Comments

The United States commends the Electronic Working Group, led by Argentina, for the recently completed review of this document. This was a difficult assignment and Argentina did an excellent job of keeping members of the Electronic Working Group engaged and focused on the task. With the recommended changes, the United States believes this document has been improved and can provide more useful information and criteria that will enable laboratories engaged in molecular testing to design and conduct collaborative studies to demonstrate the acceptability of methods for an even wider variety of DNA-based and Protein-based applications.

The 31st Session of the Codex Alimentarius Commission (CAC) approved the Guidelines as New Work, and, in response to concerns expressed about the limited scope of this document, the CAC issued the following statement:

> "The Commission approved new work on the Guidelines on the Criteria and recommended that the Committee consider the concerns and recommendations regarding the scope expressed at the current session" (ALINOM 08/31REP, Paragraph 97)."

Additionally, at the 2009 meeting of the CCMAS, this document was returned to Step 2 and it was agreed to create another intersession Electronic Working Group to work on this document and try to resolve differences. Argentina, Germany and the United Kingdom co-chaired this Electronic Working Group, and as a result of the Electronic Working Group's efforts, the document has been significantly improved and almost all of the language was acceptable to all of the participants. However, the Electronic Working Group was unable to achieve consensus on the Title and Scope of the document, as reflected in the document now before the CCMAS.

From the United States' perspective, the Title and Scope of this document are critically important for informing governments of the broad range of its applicability, and our comments focus primarily on ensuring that it is clear that this document includes guidelines on criteria for all methodologies utilizing DNA- and Protein-based testing technologies. Including the phrase "foods derived from modern biotechnology" in the Title and throughout the text of the document is unnecessary and potentially confusing as it may lead some users to conclude that its applicability is limited to only foods derived from biotechnology. This would be a disservice not only to governments, but also to the hard work of the Electronic Working Group. Furthermore, removing the phrase "foods derived from modern biotechnology" would result in a more comprehensive and accessible guidance document to be used by governments for a variety of DNA- and Protein-based methods, regardless of the ultimate utilization of the method.

Finally, we would like to note that issuing a comprehensive document with a broadened scope would be an effective use of the committee's time as it would eliminate the need for future time consuming CCMAS work on preparation of additional documents for each food application of these methods.

Specific Comments

1. Modify the Title and Scope of the document. The United States believes that the current Title and Scope place an unnecessary emphasis on foods derived from biotechnology given the broad applicability of the document from a scientific, safety and health perspective. Protein-based and DNA-based methods have applications that are well beyond the detection of foods derived from biotechnology, including applications such as the identification of specific varieties of plants for authenticity purposes, and the identification of molecular markers important to conventional breeding. Furthermore, modifying the Title and Scope to reflect a broader scope is consistent with the Commission's recommendation to CCMAS in that it would provide recommendations with respect to criteria for methods of analysis and for quality control measures that would assist laboratories offering protein-based and DNA-based analyses for many food applications.

The United States endorses the following:

Alternative Title I as stated in the document:

> **"PROPOSED DRAFT GUIDELINES ON PERFORMANCE CRITERIA AND VALIDATION OF METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS IN FOODS"**

Alternative Scope as stated in the document:

> "These guidelines provide information criteria for the validation of food analysis methods involving the detection, identification and quantification of specific DNA sequences and specific proteins of interest that may be present in foods and that will be used by laboratories responsible for food analysis. These methods can provide molecular and immunological approaches for, including among other uses, tests for food authenticity, and biomarkers for foods containing material derived from recombinant-DNA organisms."

2. Revise the text and pertinent Annexes to reflect the broader scope of the document, and eliminate unnecessarily limiting text, such as the phrase "foods derived from modern biotechnology".

The changes involved in broadening the scope would be minor. We strongly believe these changes can be accomplished at the upcoming 31st Session of CCMAS, and therefore these changes would not delay the advancement of the document.

Should these recommendations be adopted by the Committee, the United States would support moving this document forward in the Codex step process.

BIOLOGY INDUSTRY ORGANIZATION (BIO)

On behalf of the Biotechnology Industry Organization (BIO), we welcome the opportunity to provide comments on the document "Proposed Draft Guidelines on Criteria for Methods for Detection, Identification and Quantification of Specific DNA Sequences and Specific Proteins, in Particular in Foods Derived from Modern Biotechnology" that is under development in Codex Committee on Methods of Analysis and Sampling (CCMAS).

BIO is a recognized NGO before the Codex Alimentarius Commission and represents more than a 1,000 biotechnology companies, academic institutions, state biotechnology centers and related organizations across the United States and in 31 countries. BIO members are involved in the research and development of healthcare, agriculture, industrial and environmental biotechnology products.

BIO applauds the work of the Chairman of the electronic working group (e-WG) to invite collaborative input in order to attempt to achieve consensus on the scope and objective of the document. The e-WG made significant progress in agreeing the scientific rationale to broaden the scope of the document to include protein and/or DNA detection for all foods – and not specifically to foods derived from modern biotechnology – as indicated by the Codex Commission at their 2008 CAC meeting.

Broadening the scope of the document is appropriate for a number of reasons:

1. Criteria for methods of detection work in the Codex Procedural Manual do not include areas relevant to protein and/or DNA detection generally;
2. A method of detection guidance document focused on foods derived via a process, such as biotechnology, inappropriately characterizes the product as unique in composition, nutritional quality or end use. Foods and food ingredients derived from modern biotechnology are not different from their conventional counterparts in composition, nutritional quality and/or end use. Therefore, there is no scientifically-based justification for specific guidance related to detection of DNA/protein from use of r-DNA technology;
3. Broader guidance would be of greater use for foods produced for the entire food and feed trade than guidance directed to the more narrow use of foods derived from modern biotechnology; and
4. The 2002 FAO/WHO Evaluation of Codex, a major recommendation was that Codex devote resources to standards more horizontal in nature. Thus, guidance with a broader scope is more in line with the report's recommendation to Codex and more useful for member governments.

Given the rationale provided, BIO therefore recommends the use of Alternative Title 1 and the Alternative Scope Paragraph 6 currently listed as bracketed text options. The alternative title and scope are consistent with a more scientifically-based approach, with resulting guidance likely to be more useful for detection of DNA/protein generally.

Again, BIO appreciates the opportunity to participate in the work of the e-WG and all the efforts in developing the document to date. We also appreciate the opportunity to provide these comments and look forward to the continuing work of the Committee.

INSTITUTE OF FOOD TECHNOLOGISTS (IFT)

IFT recognizes the diligent work of those participating in the electronic working group, co-chaired by the delegations of Argentina, United Kingdom and Germany, that led to this important document. IFT respectfully reiterates the view that IFT expressed during the electronic working group deliberations that there is scientific merit in moving forward with guidelines that are broad in scope, for broad applicability to DNA and protein-based analytical methodologies. Therefore, in the Proposed Draft Guidelines presented as Annex II of CX/MAS 10/31/3, IFT respectfully supports Alternative Title 1: "PROPOSED DRAFT GUIDELINES ON PERFORMANCE CRITERIA AND VALIDATION OF METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS IN FOODS" and removal of bracketed text that specifies applicability to foods derived from modern biotechnology (i.e., removal of "[derived from modern biotechnology]"or "[foods derived from modern biotechnology]" and selection instead of the broadly descriptive text (i.e., "[DNA sequences and proteins of interest in foods]" and "[DNA sequences and proteins]"). Similarly, IFT supports use of alternative paragraph 6 instead of paragraph 6, and use of paragraph 21 and paragraph 27 instead of paragraph 21 alt. and paragraph 27 alt.

INTERNATIONAL LIFE SCIENCES INSTITUTE (ILSI)

1. List of Additional References (with Abstracts where available)

Allmann, M; Candrian, U; Hoefelein, C; Luethy,n J. (1993) "Polymerase Chain Reaction (PCR) : a possible alternative to immunochemical methods assuring safety and quality of food Z". Lebensm. Unters. Forsch. 196:248-251.

A rapid, sensitive and specific analysis of food samples determining wheat contamination was established using polymerase chain reaction (PCR) technology. First, primers specific for highly conserved eukaryote DNA sequences were used to prove isolated nucleic acid substrate accessibility to PCR amplification. Subsequently, a highly repetitive and specific genomic wheat DNA segment was amplified by PCR for wheat detection. This assay was tested with 35 different food samples ranging from bakery additives to heated and processed food samples. In addition, the PCR method was compared to an immunochemical assay that detected the wheat protein component gliadin. Combination of both assays allowed a detailed characterization of wheat contamination. Hence, wheat flour contamination could be distinguished from gliadin used as a carrier for spices as well as from wheat starch addition.

Asensio, L. (2007). "Review: PCR-based methods for fish and fishery products authentication". Trends in Food Science & Technology. 18(11): 558-566

doi:10.1016/j.tifs.2007.04.016

This work intends to provide an updated and extensive overview on the PCR-based methods for fish and fishery products authentication. Various techniques such as PCR-sequencing, Multiplex-PCR, PCR-RFLP, PCR-SSCP, RAPD, Real-Time PCR and PCR lab-on-a-chip are described and discussed. Moreover, commercial PCR kits for fish species identification are provided in this review. These methods could allow consumers protection against fraudulent practices in the fishery industry and enforce national and trans-national laws and regulations.

Asensio, L; Gonzalez, Isabel; Garcia, Teresa; Martin, Roasrio. (2008). "Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA)." Food Control. 19:1-8.

This work intends to provide an updated and extensive overview on the applications of ELISA techniques for meat, fish and milk species discrimination; fruit juice labeling authentication; genetically modified and irradiated food detection; feedstuffs origin and allergen ingredients identification. These methods have been widely used because they reduce the use of costly, sophisticated equipment and time of analysis and are suitable for routine analysis of a large number of samples. Therefore, ELISA could allow, together with other analytical methods such as DNA-based methods, consumer protection and confidence, and an accurate implementation of the traceability for successful regulatory food controls.

Ayaz, Y., Ayaz, N.D. and Erol, I. (2006) "Detection of Species in Meat and Meat Products Using Enzyme-Linked Immunosorbent Assay". Journal of Muscle Foods 17 214-220.

Detection of species adulteration in meat products is important for consumer protection and food labeling law enforcement. In this study, samples of 28 fermented sausages; 14 cooked salami; 11 frankfurters; 9 raw meats; 16 raw ground meats and meat balls; 3 pastramis, 2 hams and 5 bacons; 7 cooked meats; and 5 canned products resulting in a total of 100 meat and meat products were analyzed for species determination by enzyme-linked immunosorbent assay test kits prepared with monoclonal antibody technique. Results showed that 11 of 28 fermented sausages (39.2%), 5 of 14 cooked salami (35.7%), 3 of 11 frankfurters (27.2%), 2 of 9 raw meat (22.2%) and 1 of 16 raw ground meat and meat ball (6.2%) samples were found to contain undeclared species. Fermented sausage, cooked salami and frankfurter samples that had been declared as beef only contained poultry meat. Raw meat samples that were declared as beef were determined as horse and deer meat. One meatball sample declared as beef was found to be poultry meat. These results indicate that 22.0% of the samples were not in compliance with Turkish Food Codex violating consumer rights and presenting a potential public health risk. A broad national control program by central authority is required to protect consumers and to prevent unfair competition.

Ballin, N.Z. (2008). "Analytical methods for authentication of fresh vs. thawed meat – A review." Meat Science. 80(2):151-8.

Proper labeling of meat products is important to ensure fair-trading and to enable consumers to make informed choices. Different investigations indicate that wrong labeling where thawed meat is labeled as fresh meat is present in 8–15% of analyzed samples. Enforcement of regulations requires adequate analytical methods where enzymatic-, DNA based-, spectroscopic-, bio imaging- and sensory techniques constitute the majority of published papers. The molecular changes that these techniques detect are described. The capability of both discrimination between fresh and thawed meat, and determination of frozen storage time are discussed for each of the analytical techniques. The products included in this review are primarily whole meat from Bos taurus (cow), Sus scrofus (pig) and Gallus gallus (chicken). The best analytical choice in the discrimination of fresh vs. thawed meat is concluded to be a combination of analytical methods.

Bonwick, G. A. & Smith, C. J.. (2004) “Immunoassays: their history, development and current place in food science and technology”. International Journal of Food Science & Technology 39, 817–827

The purpose of this paper is to introduce the reader to immunoassays. This paper is the first in a themed issue of the Journal in which a number of papers have been brought together in order to demonstrate the types and variety of immunoassays, which are currently available. Indeed it might be said that all an analyst needs to do is to name a molecule and somewhere there will now be an immunoassay for the detection of that molecule. This obviously is not entirely accurate, however immunoassays do provide a powerful tool, which can be used in the analysis and quality control of food materials. For both the novice and the experienced worker the specialist terminology of a subject presents an initial barrier, which must be overcome before full understanding is achieved. In this paper an attempt is made to introduce the important terms with which the reader should be familiar and to try to set the various technologies in context. The various basic methods are described and the theoretical and practical basis of more sophisticated assays now being devised are introduced.

Carnegie, P. R. (1994). "Quality control in the food industries with DNA technologies." Australas Biotechnol. 4(3):146-9.

An important part of quality control in the food industry are tests to determine the origin of materials used in processed products. Because of the stability of DNA, tests which make use of DNA in the product can be used to authenticate the species used in meat pies, tinned fish and even the variety of oats used in porridge. Tests based on hybridization, sequencing of oligonucleotides and amplification of DNA are likely to find increasing application with the only inhibition being the unpredictable nature of the market which makes commercial development difficult.

Chapela, María José; Sotelo, Carmen G; Pérez-Martín, Ricardo I; Pardo, Miguel Ángel; Pérez-Villareal, Begoña; Gilardi, Patricia; Riese, Juan. (2007). “Comparison of DNA extraction methods from muscle of canned tuna for species identification.” Food Control. 18(10):1211-1215

Four DNA extraction methods from canned tuna in different liquid media were compared. Wizard DNA Clean Up system with proteinase K previous digestion, Nucleospin (Clontech), GenomicPrep (Amersham Pharmacia Biotech) and the CTAB precipitation method were employed. DNA was extracted from four different canned tuna of the same tuna species, light tuna (Thunnus albacares): light tuna in brine, oil, vinegar and with a tomato sauce. Three samples of each type of product were analysed with the different methods. Quantity and quality of DNA extracted was evaluated using the ratio A260/A280. Also, quality was evaluated by using a set of designed primers amplifying fragments of cytochrome b of increasing size ranging from 100 bp to 300 bp. Results show that DNA extraction from canned tuna preserved in different liquid media could be optimized employing a specific DNA extraction method in each case. Best results were obtained with CTAB method for canned tuna in oil and for canned tuna in vinegar, with Wizard method for canned tuna in brine and with Genomic Prep method for canned tuna with tomato sauce.

Colgan, S; O'Brien, L. O; Maher, M; Shilton, N; McDonnell, K; Ward, S. (2001). "Development of a DNA-based assay for species identification in meat and bone meal." Food Research International. 34(5):409-414.

Restrictions on the use of meat and bone meal (MBM) as a feed ingredient were introduced by the European Commission as a result of the BSE crisis. This paper describes the development of species specific PCR assays to detect bovine, ovine, porcine and poultry species content in MBM. A simple lysis based extraction procedure and a commercial DNA extraction kit were evaluated using simulated samples of rendered material for performance in terms of yielding DNA that is amenable to PCR amplification. From a small cohort of commercially produced samples (n=7) the application of species specific PCR assays was used to determine the species content of these samples returning an accuracy of 72.5 and 60% for the two selected extraction procedures. Using the simple lysis method, with single species rendered material, a inclusion rate of 0.3% for the bovine and ovine primers and 1% for the porcine primers in MBM containing another reference species was detectable.

Dahinden, Isabelle; von Büren, Michael; Lüthy, Jurg. (2001). "A Quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for coeliac patients." European Food Research and Technology. 212(2):228-233.

The only treatment in coeliac disease is a lifelong avoidance of wheat, barley and rye (WBR) in the daily nutrition. According to the Codex Alimentarius Commission of the Joint Food and Agricultural Organisation and the World Health Organisation of the United Nations, 100 ppm gliadin (10 mg gliadin/100 g dry weight) is the maximum allowed in food labelled as gluten-free'. The present study describes the evaluation of a quantitative competitive polymerase chain reaction (QC-PCR) system as an indication of contamination of gluten-free food with the coeliac-toxic cereals. The QC-PCR system simultaneously detects WBR-DNA on the basis of a non-coding region of chloroplast trnL gene. An internal DNA standard was constructed by adding 20-bp to the original PCR product. This standard was calibrated to 0.02% and 0.2% wheat DNA corresponding to 10 ppm and 100 ppm gliadin, respectively. The QC-PCR system was applied to 15 commercially available products labelled as gluten-free'. QC-PCR and ELISA yielded identical results for most cases. By QC-PCR as well as by ELISA one sample was shown to contain more than the allowed maximum limit to be labelled as gluten-free'. Through the application of both methods, the origin of a contamination can be identified. A positive QC-PCR signal and a negative ELISA result indicates a possible gliadin-free wheat starch addition and vice versa a possible addition of wheat-free gliadin as a food additive. Both methods support each other in testing gluten-free products.

Davidson, W.S. Chapter 7: DNA/PCR Techniques, p 182-199. Analytical methods of food authentication eds.: P. R. Ashurst, M. J. Dennis. Springer-Verlag, New York. 2007.

Fukal, L. (1991) "Modern immunoassays in meat-product analysis" Nahrung / Food. 35, 431-448

The increased regulation of foodstuffs in modern society requires analytical methods which are easy to perform, sensitive, specific and relatively inexpensive. The basic antigen-antibody reaction provides means for very specific analytical procedures. Immunoassays are powerful analytical tools that permit the specific and rapid detection or measurement of antigens and haptens to which antibodies can be

produced. Sensitive recognition of the interaction is made possible by labelling the analyte or antibody, mainly with radioisotope (RIA) and enzyme (ELISA). Wide applications of these modern immunoassays to food analysis began about 1980.

The paper reviews investigations, where various types of RIA and ELISA were developed for the use in meat product analysis. Detection and determination of various meat species, non-meat proteins; microorganisms and bacterial toxins, drugs, anabolic hormones, pesticides, mycotoxins, and other contaminants in meat and meat products by the means of immunoassays is described. Now, the commercial kits are available for most of these compounds. They make possible to perform analysis in different laboratories under standard conditions. The reason of an enthusiastic acceptance of this technology is related to its inherent specificity, high sensitivity, and the facility of application. In fact, immunoassays compete with other analytical technics. They have the advantage of economy when screening large numbers of samples.

Giovannacci, I., Guizard, C. Carlier, M. Duval, V., Martin, J. and Demeulemester, C. (2004). "Species identification of meat products by ELISA", International Journal of Food Science & Technology 39, 863–867.

ELISA methods used in this study are proved to detect low contents of animal species (pork, beef, sheep and poultry), even in highly processed foods. They present the advantages of being robust, cheap and easy to perform. Nevertheless, F factors, determining the threshold values of the test, need to be validated for each species.

Hurley, I.P. Coleman, R.C. Ireland, H.E. and Williams, J.H.H. (2006) "Use of sandwich IgG ELISA for the detection and quantification of adulteration of milk and soft cheese". International Dairy Journal 16, 805–812.

The aim of this work was to develop an assay capable of detecting adulteration of soft goat, sheep and buffalo milk cheese with bovine milk from cheaper sources. A previously developed indirect competitive ELISA had a lower sensitivity when applied to cheese, compared with milk. A sandwich ELISA was developed utilising the same monoclonal antibody in combination with a polyclonal goat anti-bovine IgG antibody. Once optimised, the ELISA was found to be highly specific. Detection limits in milk were 0.001% cows' milk adulteration of sheep or buffalo milk, and 0.01% cows' milk adulteration of goat milk. Detection limits in soft cheese were 0.001% in goat cheese and 0.01% in sheep or buffalo cheese. The assay was highly reproducible with both intra- and inter-assay coefficient of variation <10%. The ELISA performance makes it suitable for development as a kit for use in routine surveillance of milk and soft cheese.

Liu-Stratton, Y; Roy, S; Sen, C.K. (2004). "DNA microarray technology in nutraceutical and food safety." Toxicol Lett. 150(1):29-42.

The quality and quantity of diet is a key determinant of health and disease. Molecular diagnostics may play a key role in food safety related to genetically modified foods, food-borne pathogens and novel nutraceuticals. Functional outcomes in biology are determined, for the most part, by net balance between sets of genes related to the specific outcome in question. The DNA microarray technology offers a new dimension of strength in molecular diagnostics by permitting the simultaneous analysis of large sets of genes. Automation of assay and novel bioinformatics tools make DNA microarrays a robust technology for diagnostics. Since its development a few years ago, this technology has been used for the applications of toxicogenomics, pharmacogenomics, cell biology, and clinical investigations addressing the prevention and intervention of diseases. Optimization of this technology to specifically address food safety is a vast resource that remains to be mined. Efforts to develop diagnostic custom arrays and simplified bioinformatics tools for field use are warranted.

Lockley, A. K; Bardsley, R.G. (2000). "DNA-based methods for food authentication." Trends in Food Science & Technology. 11(2):67-77

doi:10.1016/S0924-2244(00)00049-2

A variety of methods exist whereby the residual DNA content of food material can be used to unequivocally identify the nature of the product. Various techniques are described and discussed, mainly with reference to meat and fish. An attempt has been made to collate the species identified,

comprehensively but not exhaustively. Emerging technologies have the potential to simplify the protocols involved with food authentication testing in the future, although the uptake of these by the food industry is liable to be limited by factors such as cost.

Martlbauer, W. Chapter 9: Antibody Techniques, p 241-265. Analytical methods of food authentication eds.: P. R. Ashurst, M. J. Dennis. Springer-Verlag, New York. 2007.

Meyer, R; Candrian, U. (1996). "PCR-based DNA Analysis for the Identification and Characterization of Food Components." Lebensmittel-Wissenschaft und-Technologie. 29(1-2):1-9.

Analysis of specific nucleic acids in food allows control laboratories to determine the presence or absence of certain ingredients in complex products or the identification of specific characteristics of single food components. These analyses are based on nucleic acids probes, including the polymerase chain reaction (PCR), which made the detection of minute amounts of degraded nucleic acids and their sequence determination possible. In this review, we describe the approaches that have been taken to detect low levels of contaminants such as wheat in dietary food for coeliac patients and pork meat or fat in sausages. In addition, these methods may also be used for the identification of meat or fish species and the recognition of genetically altered foods, including the FlavrSavr™ tomato. These examples indicate that a comprehensive description of food products based on the analysis of nucleic acids will be feasible.

Olexova, L; Dovičovičová, L; Švec, M; Siekel, P; Kuchta, T. (2006). “Detection of gluten-containing cereals in flours and “gluten-free” bakery products by polymerase chain reaction.” Food Control. 17(3):234-237

A polymerase chain reaction-based method for the detection of gluten-containing cereals in flours and “gluten-free” bakery products was optimized and its intralaboratory validation was carried out. The optimized method involved DNA isolation by chaotropic solid-phase extraction and PCR with primers of Dahinden et al. [Dahinden I., von Büren M., Lüthy J., 2001. A quantitative competitive PCR system to detect contamination of wheat, barley and rye in gluten-free food for coeliac patients. European Food Research and Technology 212, 228–233]. Using purified DNA, intrinsic detection limit of 42 ± 12 pg was determined, which corresponds to 10° genome copies. By the analysis of a panel of 26 European wheat cultivars and flours from six non-gluten-containing plants, which are commonly used for the production of gluten-free bakery products, inclusivity of 100% and exclusivity of 100% were determined. By the analysis of model samples of soya flour and cakes, detection limit of 0.1% (w/w) of fine wheat flour was determined, which is suitable for the analysis of “gluten-free” food products, as it is approximately equivalent to the limit of 10 mg per 100 g for gluten stated by Codex Alimentarius. The method was successfully applied to four samples of flours and 13 brands of biscuits designated “gluten-free”, out of which two flours and one brand of biscuits were found positive for gluten-containing cereals. The method proved to be suitable for routine use, it was relatively straightforward and could be completed in one working day.

Pasqualone, Antonella; Montemurro, Cinzia; Caponio, Francesco; Blanco, Antonio. (2004). “Identification of Virgin Olive Oil from Different Cultivars by Analysis of DNA Microsatellites.” J. Agric. Food Chem. 52(5):1068–1071

DOI: 10.1021/jf0348424 Publication Date (Web): February 3, 2004

DNA analysis enables genome fingerprinting with consequent identification of different individuals. In the agro-food industry, this can have interesting applications for the identification of species and cultivars of both raw materials and processed food. In this investigation, the efficiency of DNA microsatellite analysis in identifying virgin olive oils from different cultivars was evaluated. Ten virgin oils were obtained in the laboratory from olives of 10 different cultivars and the DNA extracted from the cell residues, recovered by oil centrifugation, was used as a template with seven different primer pairs of microsatellite sequences. The electrophoretic patterns showed an adequate level of amplification and were identical to those obtained from leaves and drupes of the same cultivar. By analyzing all the patterns obtained, the smallest number of microsatellites able to distinguish the examined oils was established and an identification key for the different oils was developed.

Poms, R. E; Klein, C. L; Anklam, E. (2004). "Methods for allergen analysis in food: a review." Food Addit Contam. 21(1):1-31.

Food allergies represent an important health problem in industrialized countries. Undeclared allergens as contaminants in food products pose a major risk for sensitized persons. A proposal to amend the European Food Labelling Directive requires that all ingredients intentionally added to food products will have to be included on the label. Reliable detection and quantification methods for food allergens are necessary to ensure compliance with food labelling and to improve consumer protection. Methods available so far are based on protein or DNA detection. This review presents an up-to-date picture of the characteristics of the major food allergens and collects published methods for the determination of food allergens or the presence of potentially allergenic constituents in food products. A summary of the current availability of commercial allergen detection kits is given. One part of the paper describes various methods that have been generally employed in the detection of allergens in food; their advantages and drawbacks are discussed in brief. The main part of this review, however, focuses on specific food allergens and appropriate methods for their detection in food products. Special emphasis is given to allergenic foods explicitly mentioned in the Amendment to the European Food Labeling Directive that pose a potential risk for allergic individuals, namely celery, cereals containing gluten (including wheat, rye and barley) crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products, mustard, tree-nuts, sesame seeds, and sulphite at concentrations of at least 10 mg kg(-1). Sulphites, however, are not discussed.

Samarajeewa U; Wei CI; Huang TS; *et al* (1991) "Application of immunoassay in the food industry" Food Science and Nutrition 29(6) 403-434.

Immunoassay techniques using the highly specific and sensitive nature of immunological reactions have been developed and applied in the food industry for detecting the naturally occurring constituents, antibiotics, pesticide residues, micro-organsims, and fragments of microbial constituents related to food analysis, food production, food processing, and food safety. Both polyclonal and monoclonal antibodies are employed for the development of the various immunoassay systems, including enzyme-linked immunoassay (ELISA) and radioimmunoassay (RIA). Immunoassay techniques provide complementary and/or alternate approaches in reducing the use of costly, sophisticated equipment and analysis time, but still maintaining reliability and improved sensitivity. Immunoassay techniques in their most simple forms provide excellent screening tools to detect adulteration and contaminations qualitatively. The application of immunoassay techniques contributes tremendously to the quality control and safety of our food supply.

Teletchea, Fabrice; Maudet, Celia; Hänni, Catherine. (2005) "Food and forensic molecular identification: update and challenges." Trends in Biotechnology. 23(7):359-366

doi:10.1016/j.tibtech.2005.05.006

The need for accurate and reliable methods for animal species identification has steadily increased during past decades, particularly with the recent food scares and the overall crisis of biodiversity primarily resulting from the huge ongoing illegal traffic of endangered species. A relatively new biotechnological field, known as species molecular identification, based on the amplification and analysis of DNA, offers promising solutions. Indeed, despite the fact that retrieval and analysis of DNA in processed products is a real challenge, numerous technically consistent methods are now available and allow the detection of animal species in almost any organic substrate. However, this field is currently facing a turning point and should rely more on knowledge primarily from three fundamental fields – paleogenetics, molecular evolution and systematics.

Turci, Manuela; Sardaro, Maria Luisa Savo; Visioli, Giovanna; Maestri, Elena; Marmiroli, Marta; Marmiroli, Nelson. (2009). "Evaluation of DNA extraction procedures for traceability of various tomato products." Food Control. In Press,

Globalization of food trade requires the development of integrated approaches, such as traceability of origin, quality and authenticity, to ensure food safety and consumers satisfaction. In this study, different

genomic DNA extraction procedures were evaluated for their applicability to internal traceability of different products in the tomato food chain. Quality, quantity and amplificability by SSR markers of extracted DNA tallied the methods performances; times and costs were considered too. The results were processed with “fuzzy-logic” approach. “Wizard” (Promega) scored the best performance in methods final ranking. This work demonstrated the value of genomic methodologies for internal traceability of tomato-derived goods.

Williams, Rachael. (2005). “Gene tests served up to tell fine foods from fakes”. Nature. 434:262

doi:10.1038/434262b; Published online 16 March 2005

Japan plans to ensure local delicacies are the real thing.

Japan is set to crack down on imitations of its local delicacies — from seaweed to crab — by using genetic testing to check the origins of foodstuffs. Many Japanese products use their place of origin as a sign of quality, in the same way that real champagne must come from the region in France of the same name.

Woolfe, Mark; Primrose, Sandy. (2004). "Food forensics: using DNA technology to combat misdescription and fraud." Trends in Biotechnology. 22(5):222-6.

The fraudulent misdescription of food contents on product labels is a widespread problem, particularly with high added-value products commanding a premium price. Proving conclusively that fraud has occurred requires the detection and quantification of food constituents. These are often biochemically similar to the materials they replace, making their identification and measurement extremely difficult. Despite the fact that food matrices are extremely complex and variable, a variety of the molecular markers used to physically map genomes have now been successfully adapted for detection of food substitution. These successes include the speciation of meats, fish and fruit in processed food products, the identification of the geographical origin of olive oil, the detection of dilution of Basmati rice with non-Basmati varieties and the quantitative detection of neuronal tissue and offal in processed meat.

2. ILSI proposals on Annex I for the CCMAS electronic working group on the 'Proposed draft guidelines for methods for detection, identification and quantification of specific DNA sequences and specific proteins, in particular in foods derived from modern biotechnology'

Para	Sentence	Reason	Current wording	Proposed wording
8		Add wording for protein methods		Similarly, for protein methods, empirical results from testing the method with proteins from non-target and closely relevant species/varieties/traits, and purified target protein and/or reference positive control materials should be provided.
9		Add wording for protein methods	Empirical results from testing the methods (to detect both reference and target DNA sequences, or proteins) with different varieties, as appropriate, may be provided in order to demonstrate, for instance, the stability of the copy number and sequence conservation of the reference DNA, [or the stability of expression of the protein. {AOCS}]	For protein methods, empirical results from testing the methods with target material and its derived/processed products, as appropriate, should be provided to demonstrate the stability of the immunoreactive form of the protein.
11	1	Add wording for protein methods		For protein methods, LOD should be determined for each matrix. Alt, based on 11bis of Mexico: In the case of protein-based analyses, the limit of detection must be ascertained according to established procedures for immunoanalyses for each matrix.

19		To include specific criteria for Protein Method	(i.e. accuracy, applicability (matrix, concentration range and preference given to 'general' methods), detection limit, quantification limit, precision, recovery, selectivity, sensitivity and linearity)	(i.e. accuracy, applicability (matrix, concentration range and preference given to 'general' methods), detection limit, quantification limit, precision, recovery, selectivity, extraction efficiency and parallelism/linearity)
		Add one section "**Extraction efficiency**" after "**Cross-reactivity**" section.		Empirical results from testing the protein method for its extraction efficiency in each matrix should be provided to demonstrate the extraction is sufficient and reproducible. For quantitative detection, the method of calibration for incomplete extraction may need to be provided.
		Add three paragraphs after para 29.		The following additional information should be supplied for protein-based procedures: **Assay applicability.** Food processing will generally lead to degradation or denaturation of the target protein, which may result in a substantial change in immuno-reactivity. Immunoassays should be evaluated for applicability to the target processed products. Empirical results from testing the method for applicability for target processed foods should be provided. **Hook Effect.** In an antibody-based lateral flow device and plate format assay, a hook effect could lead to a false negative result. A thorough demonstration that the working concentration range comfortably covers the practical need of target analytical samples is necessary.

				Therefore, empirical results from testing for a hook effect in target matrices should be provided. **Confirming method** For a quantitative immunoassay, antibodies may cross-react with other proteins present in the matrix; thus, it is necessary to demonstrate the credibility of assays. Another method such as HPLC, LC/MS, western blot or biological assay may be used as a confirming method. Empirical results from testing both methods with aliquots of the same analytical samples of known concentration may be provided.

3. ILSI proposals on Annex V for the CCMAS electronic working group on the 'Proposed draft guidelines for methods for detection, identification and quantification of specific DNA sequences and specific proteins, in particular in foods derived from modern biotechnology'

Para	Sentence	Reason	Current wording	Proposed wording
1	All	It was too specific for plant materials. It is necessary to expand to more general matrices.	Quantitative immunoassays are used to determine levels of the protein analyte in specific parts of the plant (e.g. seed, leaf, root, stalk etc) of a cultivar or a mix of cultivars**.** Typical applications are given in Table 1. In order to perform any immunological detection method such as a microplate ELISA for quantitative determination of a protein analyte in plant tissue(s), it is first necessary to obtain a representative sample of the plant material. The sample amount and procedure to prepare test portions will influence the detection limit or sensitivity of the assay. The analyte is then extracted from the plant material by adding the appropriate liquid and blending, agitating, or applying sheering or sonic forces. Typical liquids used are water or buffered salt solutions. Sometimes detergents or surfactants are added according to the validated test and matrices. Some proteins require more rigorous procedures like homogenization or boiling in solvents, detergents, salts etc.	Quantitative immunoassays are used to determine levels of the target protein in food products. In order to perform any immunological detection method such as a microplate ELISA for quantitative determination of a protein analyte in food sample, it is first necessary to obtain a representative sample of the target matrix. The sample amount and procedure to prepare test portions will influence the detection limit or sensitivity of the assay. The analyte is then extracted from the sample by adding the appropriate liquid and blending, agitating, or applying sheering or sonic forces. Typical liquids used are water or buffered salt solutions. Sometimes surfactants or additives such as BSA (bovine serum albumin) are added according to the validated test and matrices. It is important that the extraction methods do not denature the proteins so as to make them unrecognisable by the antibodies.

Para	Sentence	Reason	Current wording	Proposed wording
2	All	Simplify the paragraph	The following description of the procedure is only one of several possibilities to carry out an immunological detection assay for proteins expressed in [****allusion to target/analyte****].	The following description of the procedure is only one of several possibilities to carry out an immunological detection assay for proteins.
3	1	Clarification of the language	After more or less specific parts of the detection tests such as the capture antibody's immobilization on the microplate well surface, a precise volume of the standard or sample extract solution is added to each well.	After the immobilization of capture antibody on the microplate well surface, a precise volume of the standard or sample extract solution is added to each well.
3	Last	Clarity	The standard curve is generated by plotting the optical density (OD) on the *y*-axis against the concentration on the *x*-axis, which produces a dose response curve using quadratic equation or other required curve fit model from the method.	The standard curve is generated by plotting the optical density (OD) on the *y*-axis against the concentrations of the standards on the *x*-axis, which produces a dose response curve using quadratic equation or other required curve fit model from the method.
5	Last	Clarity	Blanks, negative controls, positive controls, fortified sample extracts when necessary, reference materials, reference material extracts, and replicates are typically run on each microplate to control for plate-plate variation.	Blanks, negative controls, positive controls, reference materials, and replicates can be run on each microplate to control for plate-plate variation.
6	1	To be more specific and fit the scope of section.	The reference material consists of the same matrix as the actual agricultural commodity to be tested.	The reference material consists of the same matrix as the target analytical sample to be tested. It typically includes negative control and positive reference materials.

Para	Sentence	Reason	Current wording	Proposed wording
6	2	Soybean flour is a better example and is commonly used for the preparation of reference materials.	For example, if the matrix to be tested is soybean seed, the standardized reference material would be soybean seed containing a known proportion of [***allusion to target/analyte****].	For example, if the matrix to be tested is soybean flour, the standardized positive reference material would be soybean flour pre-mixed with proportion of pure positive soybean flour containing the known target protein.
6	5	Clarity and address scope	Access to reference materials is important during the development, validation, and use of immunoassays for analysis of introduced proteins in recombinant-DNA agricultural commodities.	Access to reference materials is important during the development, validation, and use of immunoassays for analysis of proteins in food matrix.
7	1	Provides guidance rather than opinion.	In the case of products such as grain or seed, where the commodity consists of discrete units, it is fairly straightforward to prepare a reference sample with a known proportion of [***allusion to target/analyte****].	Where the positive and negative commodities are available, it is fairly straightforward to prepare a reference sample with a known proportion of the target material.
7	3	Improve to be applicable to all applications Generation of reference material is not necessarily difficult.	For example, if the matrix to be tested consists of a mixture of materials, it will be difficult to combine recombinant- and non-recombinant-DNA material in such a way as to achieve a homogeneous reference sample with a known proportion of recombinant-DNA material.	For example, if the matrix to be tested consists of a mixture of materials, the operator will need to combine negative control and positive reference materials in such a way as to achieve a homogeneous reference sample with a known proportion of target analyte-containing material.
7	5	Clarity and scope	In any case, it is useful to have non-recombinant- and recombinant-DNA material available to use as negative	In any case, it is useful to have negative and positive reference materials available to use as negative and positive controls

Para	Sentence	Reason	Current wording	Proposed wording
			and positive controls.	
8	All	It is covered in Para 7.	In case of lack of continuity of standards furniture, such as the withdrawal of the market of a plant derived from recombinant DNA, appropriate solutions have to be found to get positive controls.	Delete paragraph
8	all	Alternative if retained - simplify	In case of lack of continuity of standards furniture, such as the withdrawal of the market of a plant derived from recombinant DNA, appropriate solutions have to be found to get positive controls.	In case of lack of continuity of standards, appropriate solutions should be found to provide positive controls.
9	1	Clarity;	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 or enzyme inhibitors).	During assay development, the reference material is used to help optimize assay parameters that minimize any interfering effects of the matrix (e.g. non-specific binding of sample components to the antibodies or enzyme inhibitors).
10	1	Combined with para 11	The principles of method validation described in appendices III and IV for PCR methods also apply to protein methods. 11 Validation should be conducted according to the harmonized ISO/IUPAC/AOAC protocol for chemical analytical methods. This document defines the procedures	The principles of method validation defined in the harmonized ISO/IUPAC/AOAC standard (Horwitz 1995) apply to both PCR and protein methods. ISO (ISO21572:2004) has developed specific international guidelines for validation of immunoassays for the detection and quantification of GM foodstuffs. These guidelines are equally applicable to other foodstuffs. Quantitative method validation parameters include accuracy/trueness, specificity, extraction efficiency, sensitivity, range of quantitation, precision, ruggedness, applicability, practicability and parallelism

Para	Sentence	Reason	Current wording	Proposed wording
			necessary to validate a method (Horwitz 1995).	(Grothaus et al., 2006).
11b	2	Clarity	Since the introduced protein expressed is endogenous to the plant, it can be difficult to truly demonstrate efficiency of the extraction procedure.	It can be difficult to truly demonstrate efficiency of the extraction procedure.
11b	5	Clarity	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	One approach to addressing extraction efficiency is to demonstrate the recovery of the target 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
11c	4,5	Clarity	The quality control samples required would consist of two pools of extracts, one extract from recombinant-DNA plant tissue and one from the non-recombinant-DNA plant tissue. These extracts would be stored frozen and a portion would be thawed and assayed on every microplate.	The quality control samples required would consist of two pools of extracts, one extract from target analyte-containing samples and one from the control samples. If the protein is stable in extract, it can be stored frozen and a portion can be thawed and assayed on every microplate.
	New	Add dilution agreement or parallelism under precision section (para 11c)		Dilution agreement or parallelism is used to evaluate that the assay is capable of giving equivalent results regardless of where in the quantitative range of the standard curve the sample OD interpolates. To conduct these experiments, samples that are positive for the target protein are ideally diluted such that at least three of the dilutions result in values that span the

Para	Sentence	Reason	Current wording	Proposed wording
				quantitative range of the curve. The CV of the adjusted results from several dilutions of a single sample extract should ideally be ≤ 20%..
11d	3-6	This should not affect the sensitivity of the assay. If purified protein is used to determine the assay sensitivity, this type of discrepancy can be avoided. On the other hand, protein-based method usually is used to quantify a specific protein in samples. In the case mentioned here a DNA based method might be the method of choice.	Discrepancies may arise when the protein of interest is the same for several transformation events yet they have different rates of protein expression. For example two transformation events may express the same protein but the protein expression rates are different in the harvested grain (as well as in other parts of the plant). In a similar way, there is probably substantial variability in protein expressions under various growing conditions. If the reference material (used for calibrating an ELISA method) happens to have a fairly high expression rate, the test will under-report the presence in plant material coming from plants grown under conditions that induce lower expression levels	Delete
11d	Alternate 3-6 (if retained)	This should not affect the sensitivity of the assay. If purified protein is used to determine the assay sensitivity, this type of discrepancy can be avoided. On the other hand, protein-	Discrepancies may arise when the protein of interest is the same for several transformation events yet they have different rates of protein expression. For example two transformation events may express the same protein but the protein	Discrepancies may arise when the protein of interest is present in multiple sources. For example two sources may express the same protein but the protein expression rates are different in the two sources. If the reference material (used for calibrating an ELISA method) has a different protein expression level, the test may not be accurate as to the presence of

Para	Sentence	Reason	Current wording	Proposed wording
		based method usually is used to quantify a specific protein in samples. In the case mentioned here a DNA based method might be the method of choice.	expression rates are different in the harvested grain (as well as in other parts of the plant). In a similar way, there is probably substantial variability in protein expressions under various growing conditions. If the reference material (used for calibrating an ELISA method) happens to have a fairly high expression rate, the test will under-report the presence in plant material coming from plants grown under conditions that induce lower expression levels	the target material.
14	3	This information is covered in the validation of the method. This is a debatable statement (same to the PCR method). Suggest to delete. For any quantitative method, assay sensitivity is always a key parameter and should be defined and validated during the method validation.	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 1 ng/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.	Delete

Para	Sentence	Reason	Current wording	Proposed wording
19	1-3	To be more specific and differentiate from next paragraph. Here is about cross-reactivity, which is a main parameter measuring the assay specificity.	The target specificity is the degree to which analogs or other molecules bind to the antibodies and should be characterized and described in the method. Target specificity should be demonstrated by showing experimental results from testing the method with non-target recombinant-DNA transformation events and non-recombinant-DNA plants. This testing should include closely related transformation events and cases where the limits of the detection are truly tested.	Cross-reactivity: The cross-reactivity or assay specificity is the degree to which analogs or other molecules bind to the antibodies and should be characterized and described in the method. Cross-reactivity should be demonstrated by showing experimental results from testing the method with proteins or molecules from non-target and closely related species/variety/trait, and purified target protein and/or reference positive control materials**.**
	4	Not necessary to add this statement. It should have been covered and considered for the assay development.	As the method should be protein-specific it should only be functional with the specific food derived from biotechnology considered and ought not to be functional if applied to transformation events which do not express the protein in question.	Delete.
	5	To fit the general scope	The potential for interferences from reagents and labware can be evaluated by assaying extracts from non-recombinant-DNA plant material.	The potential for interferences from reagents and labware can be evaluated by assaying extracts from target matrix.
20	2	It is not likely to give identical result in the presence of matrix in the assay system.	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.	One way to manage matrix effects is to demonstrate that the analytical method gives similar results with or without sample matrix present in the extract.

Para	Sentence	Reason	Current wording	Proposed wording
22	2	To be more specific.	In order to ensure reliable results, the manufacturers of such assays must conduct a method validation and provide a description of the performance characteristics of the product in the package insert.	In order to ensure reliable results, the manufacturers of such assays must conduct a method validation and provide a description of the performance characteristics of the product in the package insert, including sensitivity, specificity, applicability, and hook-effect.
23	2	Use widely applicable language.	To establish that the lateral flow device is able to differentiate between samples containing protein derived from [***allusion to target/analyte****] above or below the threshold, both a negative reference and a threshold reference containing a known proportion of [***allusion to target/analyte****] should be assayed concurrently.	To establish that the lateral flow device is able to differentiate between samples containing a specific protein above or below the threshold, both a negative reference and a threshold reference containing a known proportion of the protein or target material should be assayed concurrently.
23	4 (if retained)	Add a reference since it contains the specific number of test needed for LFD validation.	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.	A sufficient number of these samples (e.g. USDA/GIPSA Directive 9181.2, 2004) 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.
24	3, 4 (if retained)	Both validated LFD and ELISA are reliable. Use of single strip is more cost related.	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 ELISA testing is performed, duplicate wells should be used.	In general, due to the reliable nature of protein-based lateral flow strip methods, they are not performed in duplicate on each sample. However, in ELISA testing (due to its quantitative nature), duplicate wells are typically used.

Para	Sentence	Reason	Current wording	Proposed wording
New		To be consistent with PCR method (for both quantitative and qualitative methods)		**Applicability** The analytes, matrices and concentrations for which a method of analysis may be used must be stated.
New		To be consistent with PCR method (for both quantitative and qualitative methods)		Practicability The practicability of the method must be demonstrated.
New	Reference			U.S. Department of Agriculture/Grain Inspection, Packers and Stockyards Administration (2004) Directive 9181.2, http://archive.gipsa.usda.gov/reference-library/directives/9181-2.pdf
New	Reference			Grothaus, G. D. , Bandla, M., Currier, T., Giroux, R.., Jenkins, G. R., Lipp, M., Shan, G., Stave, J. W., Pantella, V.. Immunoassay as an Analytical Tool in Agricultural Biotechnology. *AOAC International.* **2006**, 89: 913-928
New	Reference			ISO 21572 (2004) Foodstuffs-Methods for the detection of genetically modified organisms and derived products-protein based methods, ISO, Geneva, Switzerland.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION (ISO)

ISO/TC 34/SC 16 wishes to thank the Codex Secretary for the opportunity to comment on the results of the electronic Working Group. A narrative summary of the comments received follows:

There is presently no guidance in Codex for analysis of large molecules. Within the context of the criteria approach, existing criteria in the 18th Edition of the Codex Procedural Manual do not adequately address guidance for CCMAS to address protein or DNA testing, thus we support such draft guidelines applied broadly to all food products (not specifically or exclusively those derived from r-DNA technology).

The expanded scope of the Guidance to be developed will enable Codex text to objectively and distinctly cover all types of DNA and proteins, including those present in biotechnology-derived foods. Guidance on such analyses would be particularly useful in dealing with authenticity.

The present scope of the document emphasizes its application only to DNA/proteins derived through biotechnology. Making the scope of the CCMAS document detection of all types of DNA/proteins would be scientifically correct, in line with other work at ISO and would increase the value and usefulness of this document. It will avoid Codex having to later develop new documents to cover analysis of these biomolecules (DNA and proteins), and help harmonize approaches to the analysis of proteins for authenticity, food quality, etc.

Within ISO TC34 (foods and food products), SC16, which is horizontal, covers biomolecular methods (proteins, DNA, etc.), and this ISO activity is consistent with the CCMAS document which covers broader use of these analytical methods.

ISO should support the following version of the title and scope of the document:

The preferred title and scope are:

“PROPOSED DRAFT GUIDELINES ON PERFORMANCE CRITERIA AND VALIDATION OF METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS IN FOODS” (alternative title I in square brackets in the present draft).

“These guidelines provide information criteria for the validation of food analysis methods involving the detection, identification and quantification of specific DNA sequences and specific proteins of interest that may be present in foods and that will be used by laboratories responsible for food analysis. These methods can provide molecular and immunological approaches for, including among other uses, tests for food authenticity, and biomarkers for foods containing material derived from recombinant-DNA organisms.” (alternative paragraph 6 in brackets in the present draft).