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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods

Produits alimentaires — Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés — Méthodes quantitatives basées sur l'utilisation des acides nucléiques

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 21570 was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food Analysis — Horizontal methods*, in collaboration with Technical Committee ISO/TC 34, *Food products*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

The search for ingredients of genetically modified origin is performed by means of the following successive (or simultaneous) steps. After sample collection, nucleic acids are extracted from the test portion. Extracted nucleic acids can be further purified, simultaneously or after the extraction process. Afterwards, they are quantified (if necessary), diluted (if necessary) and subjected to analytical procedures (such as PCR). These steps are detailed in the present and in the following International Standards:

ISO 21569, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods*

ISO 21570, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods*

Further information about definitions and general items involving the steps cited above are collected in:

ISO 24276, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*.

The International Organization for Standardization (ISO) draws attention to the fact that it is claimed that compliance with this document may involve the use of a patent concerning the PCR technology.

ISO takes no position concerning the evidence, validity and scope of these patent rights.

ISO has been informed that Applied Biosystems, Roche Molecular Systems, Inc. and Hoffman-La Roche hold patent rights concerning PCR technology. The companies have assured the ISO that they are willing to negotiate licences under reasonable and non-discriminatory terms and conditions with applicants throughout the world. In this respect, the statements of the holders of these patent rights are registered with ISO. Information may be obtained from:

Licensing Department
Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404,
USA

and

Roche Molecular Systems, Inc.
Licensing Department
1145 Atlantic Avenue
Alameda, CA 94501,
USA

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Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Quantitative nucleic acid based methods

1 Scope

This International Standard provides the overall framework of quantitative methods for the detection of genetically modified organisms (GMOs) in foodstuffs, using the polymerase chain reaction (PCR).

It defines general requirements for the specific amplification of DNA target sequences, in order to quantify the relative GMO-derived DNA content and to confirm the identity of the amplified DNA sequence.

Guidelines, minimum requirements and performance criteria laid down in this International Standard are intended to ensure that comparable, accurate and reproducible results are obtained in different laboratories.

This International Standard has been established for food matrices, but is also applicable to other matrices, e.g. feed and plant samples from the environment.

Specific examples of methods are provided in Annexes A to D.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 21569:2005, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods*

ISO 21571, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction*

ISO 24276:—[1)], *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

ISO Guide 32, *Calibration in analytical chemistry and use of certified reference materials*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 24276 apply.

1) To be published.

4 Principle

4.1 General

Quantitative analysis consists of the quantitation of target DNA sequences in the test samples. Each method specifies the target sequences(s).

Quantitation may be performed using competitive [1],[2] or real-time PCR [3],[4].

A quantitative analysis should clearly express the quantity of the target genetic element, relative to the quantity of a specific reference, appropriate calibrants and controls, and be within the dynamic range of the analytical method used and the test portion analysed.

The analysis generally consists of

— amplification of one or more specific target sequences,

— detection and confirmation of the specificity of the PCR product(s), and

— quantitation of the amplified fragments relative to calibrants.

NOTE In the case of real-time PCR analysis, amplification, detection and confirmation occur simultaneously.

4.2 Amplification, detection and confirmation of PCR products

See ISO 21569 for the principles of amplification, detection and confirmation of the DNA sequences.

4.3 Quantitation of PCR products

The principle of quantitation is usually to determine the ratio (expressed as a percent) of two DNA target sequences; i.e. a sequence representing the genetically modified organism of interest and an (endogenous) target taxon-specific sequence. However, in some cases, quantitation can also be carried out relative to a specified amount of food matrix (e.g. when detecting GM microorganisms in foods).

Calibrants (calibration materials) used for quantitation should be traceable to certified reference materials (CRMs), if available. If not available, other suitable reference material should be used. Example guidance is provided in Reference [5]. Information on validation studies and measurement uncertainty has been gathered from international studies [6],[7],[8],[9].

5 Reagents

All reagents and materials used in the analysis should be identical, or equivalent, to those specified in the method. Otherwise, all reagents and materials should be of molecular biology grade. These reagents shall be stored and used as recommended by the supplier or according to the laboratory quality assurance specifications. For a list of reagents, see the specific annex.

6 Apparatus and equipment

See Annexes A to D and ISO 24276.

7 Guidelines concerning the procedure

7.1 General

General considerations relevant to PCR amplification for the detection of GMOs are described in ISO 21569.

Annexes A to D specify PCR detection methods together with details of their scope of application. The demonstrated performance characteristics for each method are detailed.

The concentration of the DNA sequence of interest should be within the dynamic range of the method.

NOTE A target taxon specific monitor run can be undertaken to determine whether the template DNA is of sufficient quality (length and structural integrity), purity and quantity to allow the detection and quantitation of a GMO belonging to the target taxon. This may be of particular relevance when DNA is extracted from composite or highly processed matrices.

The DNA extracted from each test portion should be analysed at least in duplicate.

Appropriate controls shall be included (see ISO 24276:—, Table 1).

7.2 Target sequence stability

The allelic and copy number stability of the target sequence should be considered for cultivars of different geographic and phylogenic origins.

7.3 Calibration of the analysis

An appropriate number of calibration points and replicates covering the range of quantitation shall be applied [e.g. four calibration points with two replicates (altogether 4×2 values) or six calibration points with one measurement at each point (altogether 6 values)]. The quality of the calibration influences the measurement uncertainty [9].

As an alternative to genomic DNA calibration reference materials, for example, a dilution series of a plasmid or synthetic dsDNA containing the target sequence may be used, provided that it is demonstrated to perform in an equivalent way to the genomic DNA reference material and the genomic DNA extracted from the sample.

7.4 Quantitation considerations

PCR methods should be appropriately designed to minimize the variability.

NOTE Depending on the method used and/or the material analysed, the presence of stacked genes can lead to overestimation of the true GMO content.

For the determination of the limit of quantitation (LOQ), see ISO 24276.

Calculation of the GMO content based on copy numbers of target sequences per haploid genome is influenced by the homo- and heterozygosity of the species under investigation. For details, see Annexes A to D.

Use of the $\Delta\Delta C_t$ (cycle of threshold) method is only valid if the amplification efficiencies of the target taxon-specific assay and the GMO-specific assay are very similar.

7.5 Quality assurance requirements

Consistency between measurements is desirable to obtain reliable estimates of target sequence quantities. However, knowledge of the relative standard deviation of repeatability of the method is required to establish whether the measurements are consistent (see the ISO 5725 series for details). To calculate the relative standard deviation of repeatability, the number of separate measurements per laboratory sample may exceed what is feasible in practice in terms of acceptable costs. Consequently, if a specified GMO-derived DNA is to be reported (in percent), a feasible solution should require the following as a minimum:

a) within test portion consistency:

— through rejection of measurements <LOQ, and

— through maximum deviation observed between dilutions and individual measurements equals the value expected from the corresponding dilution factor ± 33 %;

b) between test portion consistency:

— estimated relative GMO-derived DNA concentrations obtained under a) for each test portion should not differ by values greater than −50 % to +100 % of the estimated quantity value (equal to a ΔC_t of 1 in real-time PCR) (i.e. for two test portions, measurements of 1,0 % and 2,0 % are acceptable, measurements of 0,9 % and 2,1 % are not).

In order to guarantee accuracy of the measurements, a reference material (RM), preferably certified (CRM), for the quantity of the event concerned, with an appropriate level of metrological reliability and with reasonable similarity of matrix shall be selected and analysed. In the absence of a CRM, in-house RM may be prepared by a procedure demonstrating stability, homogeneity and traceability, and ensuring the absence of bias. The quantified uncertainty shall fulfil the required uncertainty for the calibration (see ISO Guide 32).

8 Interpretation

The PCR result will be either

a) fit for quantitation of the target sequence provided

— the result is positive according to ISO 21569:2005, 8.1,

— the observable inhibition of the reaction is negligible,

— the analysis produces an unambiguous measurement value,

— the target sequence content is within the dynamic range of the method, and

— the analysis is calibrated in an acceptable way (see 7.3), or

b) not fit for quantitation of the target sequence if any of the conditions listed above are not fulfilled.

The measurement uncertainty shall be sufficiently small to enable the laboratory to draw the relevant conclusions.

Annexes A to D describe the measurement of the target DNA quantities. These quantities can be used to calculate the GMO content. These calculations usually take into consideration relevant biological factors, such as the homo- or heterozygosity of the target sequences.

If the GM target sequence content or the taxon-specific target sequence content is below the limit of quantitation, the result shall only be expressed qualitatively.

NOTE Stating that the GMO-derived DNA content is below the practical LOQ accompanied by a specification of that LOQ is considered to be a qualitative expression of the result.

9 Expression of results

The results shall clearly state the quantity of the GM target sequence relative to the target taxon-specific sequence. The results should also provide values for the measurement uncertainty, such as the standard

deviation or relative standard deviation. Furthermore, the LOD and LOQ of the method and the practical LOD and LOQ should be reported.

The target sequences may or may not be detected, or the quantity of at least one of them may be below the limit of quantitation. Table 1 describes the four alternative cases and the corresponding expression of the result to be included into the test report.

Table 1 — Expression of results

Result	Expression of the result
Target taxon-specific sequence is not detected.	See ISO 21569. "For species x, DNA was not detected."
Target taxon-specific sequence is detected but GM target sequence is not detected.	According to ISO 21569. "For species x, GMO-derived DNA was not detected." In addition, if applicable, add: "The practical limit of detection is X %." (Specify unit used.)
The target taxon-specific sequence and the GM target sequence are both detected but the quantity is below the LOQ of at least one of the target sequences.	For each GMO, state: "GMO (specify the GMO) derived DNA as determined by detection of (specify target sequence) derived from (specify species) was detected." In addition, if applicable: "The practical limit of quantitation is X %." (Specify unit used.)
The target taxon-specific sequence and the GM target sequence are both detected and the quantity is above the LOQ for both target sequences.	For each GMO, state: "The content of GMO (specify the GMO) derived DNA as determined by detection of (specify target sequence) derived from (specify species) is X ± uncertainty %." (Specify unit used.)

The GMO-derived DNA content may also be reported as being above or below a specific value, taking into account the measurement uncertainty.

10 Test report

The test report shall be written in accordance with ISO 24276 and ISO 21569 and shall contain at least the following additional information:

a) LOQ of the method and the matrix used to establish it;

b) the practical LOQ;

c) a reference to the method which has been used for the extraction of DNA;

d) the reference material used;

e) the results expressed according to Clause 9.

Annex A
(informative)

Target taxon-specific methods

A.1 Target taxon-specific method for the absolute quantitation of the *adh*1 gene DNA of maize using real-time PCR

A.1.1 Introduction

This annex describes a method for the specific amplification and quantitation of the taxon-specific (housekeeping) maize (*Zea mays*) *adh*1 gene (coding for alcohol dehydrogenase 1) for determination of the content of maize DNA, or for testing for the presence/absence of detectable PCR inhibitors in DNA solutions extracted from products containing maize-derived DNA, e.g. foods.

For limitations, see A.1.8.

A.1.2 Validation status and performance characteristics

A.1.2.1 General

The method has been optimized for DNA extracted from pure ground maize kernels, maize leaves and certified reference materials (IRMM-411, IRMM-412, IRMM-413 series [10]) [11].

The reproducibility of the described method has been tested in a collaborative trial using unknown samples (U1 to U6) consisting of wild type maize DNA at different corresponding copy number of the target sequence (see A.1.2.2), and in other collaborative trials in combination with methods specific for GM maize events, e.g. Bt11 (see D.1).

The copy number of the target sequence per haploid genome is estimated to be 1 [11].

The allelic stability of the target sequence has been established [11].

A.1.2.2 Collaborative trial

The method has been validated in a collaborative trial organized by the European Commission's Joint Research Centre (EC-JRC), Institute for Health and Consumer Protection (IHCP), in agreement with the international harmonized protocol [12].

Six samples (S1-S6) of wild type maize DNA (extracted from leaf material [13] containing known absolute copy numbers (183 486, 61 162, 20 387, 6 796, 2 265, 755) of haploid maize genomes were used to establish a calibration curve for absolute quantitation of haploid maize genomes in unknown samples. The absolute copy numbers in the known samples were determined by dividing the sample DNA mass (determined by fluorometric quantitation of dsDNA with PicoGreen, Molecular Probes, Cat. Number P-7589) by the published average 1C value for maize genomes (2,725 pg) [14].

Six samples (U1-U6) of wild type maize DNA (extracted from leaf material [13]) were used as unknown samples. The expected copy numbers in the unknown samples were determined in the same way as those of the known samples.

The results of the collaborative trial validation are summarized in Table A.1

The method has also been validated in combination with event-specific methods for several maize GMO, e.g. for Bt11 sweet maize. See References [15] and [16] and D.1 for details on the combined (relative quantitation) trial.

Table A.1 — Validation data

	Sample					
	U1	U2	U3	U4	U5	U6
Number of participating laboratories	12	12	12	12	12	12
Number of laboratories having returned results	10	10	10	10	10	10
Number of invalid laboratories	1	1	1	1	1	1
Number of retained laboratories	9	9	9	9	9	9
Number of samples per laboratory	4	4	4	4	4	4
Number of Cochran outliers	1	1	1	1	—	—
Number of Grubbs outliers	—	1	1	1	1	1
Number of accepted samples	35	34	34	34	35	35
Expected copy number value	7 339	18 349	36 697	55 046	91 743	146 788
Mean copy number value	9 985	23 885	46 918	75 161	100 541	122 080
Bias of true value (%)	36,1	30,2	27,9	36,5	9,6	−16,8
Repeatability standard deviation s_r [a]	1 318,59	1 463,60	5 796,58	4 539,57	11 306,89	14 843,41
Repeatability relative standard deviation (%) [b]	13,21	6,13	12,35	6,04	11,25	12,16
Reproducibility standard deviation s_R [a]	2 013,12	2 083,57	6 145,39	6 806,85	14 592,04	17 777,70
Reproducibility relative standard deviation (%) [b]	20,16	8,72	13,10	9,06	14,51	14,56

[a] Expressed as copy number value.

[b] Expressed as percentage of the mean value.

A.1.2.3 Molecular specificity

A.1.2.3.1 General

The method has been designed to target a part of the sequence described in EMBL/GenBank/DDBJ[2)] accession number X04050. This sequence is unique to *Zea mays* (maize/corn) and *Zea mays* subsp. *diploperennis* (teosinte) [11].

A.1.2.3.2 Theoretical specificities

The theoretical specificities of the primers and probes were assessed through a search of the GenBank/EMBL/DDBJ databases [2)] using the nucleotide sequences as query sequences with the BLASTN programme [2)] at http://www.ncbi.nlm.nih.gov/blast/ [October 9, 2003]. The result of the search confirmed a complete identity only with the expected target sequences.

2) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.1.2.3.3 Experimental determination of specificity

The specificity of the method was tested against a wide range of non-target taxa and 20 different maize lines representing geographically and phylogenetically diverse samples [11]. No cross-reactivity was observed with the non-target taxa (except with teosinte *Zea mays* subsp. *diploperennis*, the wild ancestor of cultivated maize) [11], [17]. The copy number and allelic stability of the target sequence across different maize lines has been established [11].

A.1.2.4 Optimization

This was carried out for the ABI PRISM 7700® sequence detection system (SDS) 3) and TaqMan® chemistry 3). Primer and probe design were done with Primer Express® software (Applied Biosystems) 3).

A.1.2.5 Limit of detection (LOD)

According to method developer, the absolute LOD is 10 copies of the target sequence [11].

The lowest number of copies of the target sequence included in the collaborative trial was 7 399 copies of the target sequence.

A.1.2.6 Limit of quantitation (LOQ)

According to the method developer, the absolute LOQ is 100 copies of the target sequence [11].

The lowest number of copies of the target sequence included in the collaborative trial was 7 399.

A.1.3 Adaptation

No specific information is available.

A.1.4 Principle

A 134 bp fragment of the *adh*1 gene is amplified using two maize *adh*1-specific primers (see Table A.2). Accumulation of PCR products is measured at the end of each PCR cycle (real-time) by means of a maize *adh*1-specific oligonucleotide probe (ADH1-MDO, see Table A.2) labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher. For that purpose, TaqMan® 3) chemistry was employed.

The measured fluorescence signal crosses a user-defined threshold value after a certain number of cycles. This number is called the C_t-value. For quantitation of the amount of maize *adh*1-DNA in an unknown sample, the C_t-value is converted into a corresponding copy number value by comparison with a calibration curve whose C_t-values are directly linked with known copy numbers (regression analysis).

A.1.5 Reagents

A.1.5.1 General

For quality of reagents to be used, see ISO 24276:—, 6.6.

A.1.5.2 Water.

A.1.5.3 PCR buffer (without $MgCl_2$), 10-fold.

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3) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.1.5.4 $MgCl_2$ solution, $c(MgCl_2)$ = 25 mmol/l.

A.1.5.5 dNTP solution, c(dNTP) = 2,5 mmol/l (each).

A.1.5.6 Oligonucleotides

Details of the oligonucleotides are listed in Table A.2.

Table A.2 — Oligonucleotides

Name	Oligonucleotide DNA sequence	Final concentration in PCR
ADH-FF3	5'-CgT CgT TTC CCA TCT CTT CCT CC-3'	300 nmol/l
ADH-RR4	5'-CCA CTC CgA gAC CCT CAg TC-3'	300 nmol/l
ADH1-MDO	5'-FAM-AAT CAg ggC TCA TTT TCT CgC TCC TCA-TAMRA-3' [a]	200 nmol/l
[a] FAM: 6-carboxyfluorescein; TAMRA: 6-carboxytetramethylrhodamine.		

The length of the *adh*1 amplicon is 134 bp.

A.1.5.7 Thermostable DNA polymerase

AmpliTaq Gold® DNA polymerase [4].

A.1.5.8 Uracil *N*-glycosylase (optional).

A.1.5.9 Amplification reaction mixture

Details of the amplification reaction mixture are listed in Table A.3.

Table A.3 — Amplification reaction mixture in the final volume/concentration per reaction vial

Total reaction volume		25 µl
Template DNA (maximum 250 ng)		5 µl
Taq-DNA-polymerase	TaqMan® Universal Master Mix 2X	12,5 µl (1 X)
Decontamination system (dUTP included uracil *N*-glycosylase)		
Reaction buffer (containing passive reference ROX) [a]		
dNTP mix		
Primers	see Table A.2	see A.1.5.6
Probe	see Table A.2	see A.1.5.6
[a] ROX = carboxy-X-rhodamine.		

4) These are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.