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

iTeh **STANDARD PREVIEW**
AMENDMENT 1
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*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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AMENDEMENT 1



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Foreword

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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.

Amendment 1 to ISO 21570:2005 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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

AMENDMENT 1

No attempt has been made in this amendment to update the footnote numbering to fit in with the scheme adopted in ISO 21570:2005. The footnote numbers given are for use solely within this amendment.

Page 1, Clause 2

Update entries 1 to 3 as follows and delete footnote 1).

ISO 21569:2005 + AM1:2013, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Qualitative nucleic acid based methods*

ISO 21571:2005 + AM1:2013, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction*

ISO 24276:2006 + AM1:2013, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — General requirements and definitions*

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Page 2, 7.1, last paragraph; Page 8, A.1.5.1; Page 15, B.1.5.1; Page 23, C.1.5.1; Page 31, C.2.5.1; Page 38, C.3.5.1; Page 45, C.4.5.1; Page 53, C.5.5.1; Page 60, C.6.5.1; Page 68, C.7.5.1; Page 75, C.8.5.1; Page 83, C.9.5.1; Page 90, D.1.5.1; Page 96, D.2.5.1

Delete “ISO 24276:—”, insert “ISO 24276:2006”.

Page 4, Clauses 8–10

Replace the existing text with the following.

8 Interpretation

The PCR result will be either a) or b).

- a) Fit for quantification 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;
 - the analysis is calibrated in an acceptable way (see 7.3).
- b) Unfit for quantification of the target sequence if any of the conditions listed in a) are not fulfilled.

Interpretation of ambiguous results within the same test portion: in case of +/- results for the two replicates, repeat the two PCR for the relevant test portion. If the two novel replicates are tested +/- or -/-, the test portion is considered as negative.

Interpretation of ambiguous results between two test portions: in case of \pm results for the two test portions of a sample, the extractions and analysis of two new test portions shall be performed. If again the results are +/-, the sample is considered as negative according to ISO 24276:2006, 6.3.

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, e.g. 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 quantification, 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 coefficient of variation. Furthermore, the LOD and LOQ of the method and the practical LOD and LOQ should be reported. The indication that the result refers only to GMO targets should be reported. In the case of quantitative screening analysis on complex matrices, it is recommended to specify that the GMO signal can come from non target taxons.

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

The GMO-derived DNA content can 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) the 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) a reference to the methods used for the amplification of the DNA target sequences;
- e) the reference material used;
- f) the results expressed according to Clause 9;
- g) the PCR target and whether considered "event specific" or "construct specific" or "screening";
- h) the definition of the measurement uncertainty used.

NOTE For g) and h), information can figure in different documents (e.g. contract review, technical data sheets).

Table 1 — Expression of results

Result	Expression of the result
Target taxon-specific sequence is not detected.	“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 sample X, GM target sequence Y was not detected. The LOD of the method is x % determined with ABC (identify the reference material).” If it cannot be demonstrated that the test sample size and the amount of target DNA included in the PCR is sufficient for the LOD to be applicable, then the following sentence shall be added: “However, the amount of the target DNA extracted from species X can be/was insufficient for the LOD to be applicable for this sample. The LOD of sample is x %.” (Specify the unit used.) NOTE The LOD of the sample is determined by the quantity of DNA of the species included in the analytical reaction (copy number), and the ratio relative to the absolute LOD of the GM target (copy number), and in the case of grain and seeds, the number of grain or seeds in the portion that is ground.
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, below the practical limit of quantification” In addition, if applicable: “The practical limit of quantification is x %.” (Specify the 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 $x \pm u_{\text{meas}}$ %” where u_{meas} is the measurement uncertainty. (Specify the unit used.)

Page 11, Annex A

Add A.2 and A.3.

A.2 Target-taxon-specific method for the detection of DNA derived from rice

A.2.1 Principle

Rice *SPS* gene has been described as being suitable for use as an endogenous reference gene in GM rice identification and quantification (Reference [59]). The GMO Detection Laboratory of Shanghai Jiao Tong University (GMDL-SJTU) organized a collaborative trial for validation of the applicability of the rice sucrose phosphate synthase (*SPS*) gene as an endogenous gene for quantitative analysis of genetically modified (GM) or non-GM rice. The study involved 12 laboratories from Spain, Korea, Lithuania, Slovenia, Japan, Italy, and China.

The operational procedure of the collaborative study comprised the following modules.

Quantitative real-time PCR for quantification of blind rice DNA samples used to construct standard curves.

Quantitative real-time PCR for the quantification of blind rice DNA samples using the constructed standard curves.

The interlaboratory test was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725;[51]–[56]
- the IUPAC protocol for the design, conduct and interpretation of method-performance studies (Reference [12]).

The results of the collaborative study as well as the related protocol are given in A.2.3.3.

A.2.2 Scope

The method has been optimized for rice grain and its processed products containing mixtures of rice and other matrices, e.g. maize and soybean. The applicability of the *SPS* gene was tested through collaborative trials using DNA samples extracted from rice grains.

A.2.3 Validation status and performance criteria

A.2.3.1 Robustness of the method

The robustness of the *SPS* gene quantitative real-time PCR system was tested by the method developer on different temperature–time programmes (i.e. two-step and three step) and on three different DNA samples containing known amounts of rice DNA (10 ng, 1 ng, 0,1 ng rice genome DNA samples). There were three repetitions per sample. The quantitative real-time PCR systems had the expected ruggedness and worked well at different temperature–time programmes and three concentrations of the rice DNA samples.

The quantitative PCR system for the *SPS* gene was also tested on different real-time PCR instruments (Rotor gene 3000A,¹⁾ Corbett Research and ABI7700,¹⁾ Applied Biosystems), with three different reaction volumes (20 µl, 25 µl, and 30 µl; three repetitions per volume). The quantitative real-time PCR system demonstrated appropriate ruggedness, working well on the different real-time PCR instruments and with the different reaction volumes. [ISO 21570:2005/Amd 1:2013](https://standards.iteh.ai/catalog/standards/sist/87c2eba2-0b40-4c94-8277-0451dc063025/iso-21570-2005-amd-1-2013)

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A.2.3.2 Intralaboratory trial

For sample preparation, all the DNA samples were extracted using the cetyl(trimethyl)ammonium bromide (CTAB) method adopted from ISO 21571. Spectrometric quantification of the amount of total DNA extracted was performed using a method adopted from ISO 21571:2005, B.1. After the DNA quantification, a quantitative real-time PCR run was carried out to provide data about possible PCR inhibition.

The *SPS* gene PCR system was tested by three researchers using the rice genome DNA, and gave satisfactory results; in particular, in quantitative PCR, the bias was below 25 % over the dynamic range (i.e. 0,05 ng to 1,00 ng).

A.2.3.3 Collaborative trial

Standard curves were constructed using serially diluted DNA samples extracted by the GMDL-SJTU from four rice cultivars by means of quantitative PCR. The PCR efficiency, calculated from the slope of the standard curve as $(10^{-1/a} - 1) \times 100$, where a is the slope, of the *SPS* gene PCR system ranging from 0,846 3 to 1,223 3, and the linearity (regression coefficient, R_c^2) was on average equal to 0,997.

The results of the eight blind DNA samples are reported in Table A.5. These are evaluated with respect to the method acceptance criteria and to the method performance requirements, as established by the European Network of GMO laboratories (ENGL) and adopted by the European Reference Laboratory for GM Food and Feed (EU-RL GMFF) (Reference [60]). In Table A.5, estimations of both repeatability and reproducibility for each rice concentration level are reported, after identification and removal of outliers according to Cochran's test.

1) Product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Table A.5 — Results of quantitative real-time PCR

Parameter	Blind samples							
	0,5 ng	0,5 ng	1ng	1 ng	2 ng	5 ng	5 ng	10 ng
Laboratories returning results	12	12	12	12	12	12	12	12
Samples per laboratory	1	1	1	1	1	1	1	1
Total data no.	108	108	108	108	108	108	108	108
Data excluded	4	2	0	8	0	0	0	0
Reason for exclusion	Cochran's test	Cochran's test	—	Cochran's test	—	—	—	—
Mean value	0,407 8	0,412 1	0,814 6	0,734 4	1,857 5	5,261 0	5,445 7	10,889 6
Repeatability standard deviation	0,058 3	0,071 9	0,143 5	0,111	0,336 2	0,936 4	1,142 7	1,867 7
Repeatability coefficient of variation, %	14,29	17,44	17,62	15,11	18,10	17,80	20,98	17,15
Reproducibility standard deviation	0,130 2	0,061 8	0,249 6	0,092 7	0,201 3	0,810 9	0,989 6	1,617 5
Reproducibility coefficient of variation, %	31,92	14,99	30,65	12,63	10,84	15,41	18,17	14,85
Bias, absolute value	0,092 2	0,087 8	0,185 3	0,265 5	0,142 4	-0,261 0	-0,445 8	-0,889 6
Bias, %	-18,44	-17,57	-18,54	-26,53	-7,12	5,22	8,92	8,90

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A.2.3.4 Molecular selectivity ISO 21570:2005/Amd 1:2013 <https://standards.iteh.ai/catalog/standards/sist/87c2eba2-0b40-4c94-8277-0451dc063025/iso-21570-2005-amd-1-2013>

A.2.3.4.1 General

The primers and probe targeting the 81 bp *SPS* gene DNA fragment are listed in Table A.6.

Table A.6 — Oligonucleotide primers and probe sequences

Name	Oligonucleotide DNA sequence (5' to 3')
Quantitative-real time PCR primer and probe sequence	
<i>SPS</i> primer F	TTgCgCCTgAACggATAT
<i>SPS</i> primer R	CggTTgATCTTTTCgggATg
<i>SPS</i> probe	HEX- TCCgAgCCgTCCgTgCgTC -TAMRA

A.2.3.4.2 Experimental

DNA samples extracted from 11 different plant materials (including rice) were analysed using the *SPS* gene PCR method. Among the 11 samples, only rice DNA gave positive results. The 10 other samples (i.e. bamboo, green bristlegrass, barley, wheat, foxtail millet, rapeseed, tomato, potato, soybean and *Arabidopsis*) gave negative results.

DNA samples extracted from 12 different rice cultivars were analysed by the specific PCR method developed for the detection of the *SPS* gene. All 12 samples gave positive results.

A.2.3.4.3 Theoretical

The theoretical specificity of the *SPS* gene primers and probe was assessed through a similarity search using the BLASTN 2.0MP-WashU program (Reference [64], search date: 2010-01-09). The 81 bp sequence

used as query is part of the NCBI accession number U33175 (nucleotides 1055–1135). The results of the blast search confirmed the complete identity of the query sequence with rice *SPS* gene sequence, and no similarity with other genes and species.

A.2.4 Principle and summary

An 81 bp fragment of the *SPS* gene is amplified using two rice *sps*-specific primers. Accumulation of PCR products is measured at the end of each PCR cycle (real-time) by means of a rice *sps*-specific oligonucleotide probe labelled with two fluorescent dyes: FAM as reporter dye and TAMRA as quencher (see Table A.6). For that purpose, TaqMan®¹ chemistry is employed. The fluorescence signal measured crosses a user-defined threshold value after a certain number of cycles. This number is called the C_t value. For quantification of the amount of rice *sps*-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.2.5 Terms and definitions

For the purposes of this clause, the terms and definitions of ISO 5725-1^[51] and ISO 24276 apply.

A.2.6 Sample type and amounts

DNA samples extracted from the grains of four rice cultivars, were used to construct the standard curves in this collaborative study. Then, eight blind samples were analysed using the four standard curves constructed.

The participants received the following samples.

- Four DNA samples from different rice varieties (3M, Indica variety from US; Balilla, Japonica variety from Italy; Guangluai, Indica variety from Southern China, and Shennong265, Chinese Japonica variety), 50 ng/ μ l, 30 μ l each. Each rice cultivar DNA was diluted and used to generate the corresponding standard curve.
- Eight blind rice DNA samples from four different rice varieties with different concentrations (0 ng/ μ l to ~50 ng/ μ l), 50 μ l each.
- Negative DNA target control (labelled N): salmon sperm DNA (20 ng/ μ l).
- Positive DNA target control (labelled P): (Guangluai4) genomic DNA (20 ng/ μ l). All the DNA samples were purified using the CTAB method by-GMDL-SJTU. The negative and positive DNA target controls were used for each PCR plate.
- Primers and probes for the *SPS* gene PCR system (see Table A.6) and further reaction reagents as follows:
 - real-time PCR master mixture (1 ml \times 6);
 - DNA dilute solution (0,1 \times TE, 1,2 ml).

A.2.7 Limit of quantification (LOQ), range of use

According to the developed method, the absolute LOQ of the method is 0,01 ng/ μ l. The relative LOQ of quantitative PCR has not been assessed in a collaborative trial.

A.2.8 Estimation of measurement uncertainty

The global uncertainty of the method is given by the results of the collaborative trial (see Table A.5).

A.2.9 Interferences

The amount and the ability for amplification of the nucleic acid used as template for the real-time PCR is of major importance for the sensitivity of the method. In addition to this general point, no specific interferences are known for this method.

A.2.10 Physical and environmental conditions

The procedures require experience of working under sterile conditions.

Maintain strictly separated working areas for DNA extraction, PCR set-up and amplification.

Any residual DNA should be removed from equipment prior to its use.

In order to avoid contamination, filter pipette tips (A.2.11.6) protected against aerosol should be used.

Use only powder-free gloves (A.2.11.8) and change them frequently.

Clean laboratory benches and equipment periodically with sodium hypochlorite (10 % active chloride) solution (bleach).

Pipettes should be calibrated regularly, if necessary.

A.2.11 Apparatus and equipment

Usual laboratory equipment and in particular the following.

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- A.2.11.1 **Microcentrifuge.**
 - A.2.11.2 **Freezer** maintained at $-20\text{ }^{\circ}\text{C}$ and **refrigerator** maintained at $4\text{ }^{\circ}\text{C}$.
 - A.2.11.3 **Micropipettes.**
 - A.2.11.4 **Vortex mixer.**
 - A.2.11.5 **Tubes**, of capacities 0,2 ml, 1,5 ml, 2,0 ml.
 - A.2.11.6 **Tips** and **filter tips** for micropipettes.
 - A.2.11.7 **Rack** for reaction tubes.
 - A.2.11.8 **Vinyl or latex gloves.**
 - A.2.11.9 **Vacuum dryer** suitable for drying DNA pellets, optional.
 - A.2.11.10 **Real-time PCR system** with plastic reaction vials suitable for fluorescence measurement.
 - A.2.11.11 **Software:** Sequence Detection System¹⁾ version 1.7 (Applied Biosystems Part No 4311876¹⁾) or equivalent versions.
 - A.2.11.12 **Optical 96 well reaction plates**, MicroAmp^{®1)} (Applied Biosystems Part No N801-0560¹⁾).
 - A.2.11.13 **Optical adhesive covers**, MicroAmp^{®1)} (Applied Biosystems Part No 4311971¹⁾).
 - A.2.11.14 **Optical caps**, MicroAmp^{®1)} (Applied Biosystems Part No. No 801-0935¹⁾).

A.2.12 Reagents and materials

A.2.12.1 General

Unless otherwise stated, use only reagents that conform to the specifications of ISO 24276 and only sterile distilled or demineralized water or water of equivalent purity.