INTERNATIONAL STANDARD

ISO 21571

First edition
2005-02-15

AMENDMENT 1
2013-03-15

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

AMENDMENT 1

Produits alimentaires — Méthodes d'analyse pour la détection des organismes génétiquement modifiés et des produits dérivés — Extraction des acides nucléiques

AMENDEMENT 1

Reference number
ISO 21571:2005/Amd.1:2013(E)

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Published in Switzerland

Foreword

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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 21571:2005 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

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

AMENDMENT 1

Page v, Introduction

Delete the reference to ISO 21568.

Replace the reference to ISO 24276 with the following:

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

Page 1, Clause 2

Replace the reference to ISO 24276:—with the following:

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

Delete the reference to Footnote 1) and the footnote itself.

Delete the reference to ISO 21568.

Page 2, 5.1.1, paragraph 3, line 2

Delete "(e.g. 3 000 particles at an LOD of 0,1 %) to allow a statistically valid conclusion to be made (see ISO 21568)".

Page 3, 5.1.2, paragraph 1, line 2

Replace "procedures described in ISO 24276" by "laboratory design specified in ISO 24276:2006, 5.3.2".

Page 3, 5.1.2, paragraph 2

Replace by: "Laboratory samples shall be sufficiently homogeneous before reducing or grinding and before taking the test portion."

Page 3, 5.1.2, paragraph 4, lines 3 and 6

Delete "as specified in ISO 21568" and "as defined ISO 21568".

Page 3, 5.1.2, paragraph 8, 1st sentence

Replace by “After such treatment, homogenize the whole laboratory sample.”

Page 4, 5.2.1

After paragraph 7, beginning “Re-suspend …”, insert an example as follows:

EXAMPLE A tris/EDTA (TE, 1× or 0,1×) buffer adjusted to pH 8,0 is appropriate for re-suspending or diluting DNA.

Page 5, 5.3.1, paragraph 1

Add a new final sentence:

The total amount of DNA used in the PCR should be determined as well as the total amount of target taxon DNA shall be considered, as non-target taxon DNA can affect the efficiency of the PCR.

Page 6,Clause 6, paragraph 2

Replace the phrase in parentheses by: “(e.g. if the analysis to be performed is PCR, the quality of the extracted DNA should be assessed using adequate PCR controls).”

Page 9, A.1.1.6.5

Replace 2) by 1) and renumber the footnote.

Page 10, A.1.1.8

Replace 3) by 2) and renumber the footnote.

Page 19, A.1.4.2

Delete paragraph 2: “This method applied to microbial pellets or mycelium mats has been submitted for interlaboratory validation exercises[17].”

Page 33, after A.5.1.8, add A.5.2

A.5.2 Guanidine chloroform method: Protocol for soybean lecithin

A.5.2.1 Purpose, relevance and scientific basis

Soybean lecithin is a frequent ingredient and is used in many food products as an emulsifier. Soybean lecithin can be either produced using genetically modified (GM) or non-modified soybeans. The method described here can be used to extract DNA present in the sample in order to perform subsequent PCR analyses for detection of genetically modified DNA sequences derived from GM soybeans.

The method is based on Reference [44], and a very similar procedure has been validated in a collaborative trial in Switzerland. In that study, for the purpose of interpretation, the quantity of extracted DNA was spectrometrically determined (Reference [35], section 1.3). The Chemical and Veterinary Institute Freiburg, Germany, conducted an additional collaborative trial with 12 participating laboratories where the amount of extractable and amplifiable soybean DNA was determined by means of quantitative real-time PCR. For the results, see Reference [45].

A.5.2.2 Scope

This method describes a procedure for DNA extraction from soybean lecithins in raw and cold-pressed vegetable oils, respectively. If the DNA content of the sample material is low, the real-time PCR approach is used for quantitation of the amount of DNA isolated from a test portion and for the calculation of the practical limit of detection achievable in PCR analysis of the extracted DNA, respectively.

A.5.2.3 Validation status and performance criteria

A.5.2.3.1 Validation criteria

The method described in this subclause has been validated in a collaborative trial by determining the quantity of extractable, amplifiable soybean DNA by means of quantitative real-time PCR. The collaborative study was performed in accordance with the IUPAC protocol (Rererence [46]).

A.5.2.3.2 Robustness of the method

The method has been routinely used in enforcement and private GMO testing laboratories in Germany and Switzerland for more than 10 years and no problems have been reported. Although specific robustness data (e.g. by modifying method parameters) are not available, the experiences of the laboratories showed that small variations in conditions does not interfere with the performance of the method.

A.5.2.3.3 Intralaboratory trial

The materials used in the collaborative trial study were tested in the method developers' laboratory regarding the intralaboratory precision of the method.

For estimation of the precision, five DNA extractions from each of five soybean lecithins were prepared and measured by real-time PCR under repeatability conditions using a soybean lectin gene specific method according to ISO 21570:2005,[41] C.2. The results are given in Table A.6.

Table A.6 — Intralaboratory validation of the DNA extraction method with five soybean lecithin samples (n = 5 extractions each)

Lecithin sample No.	Mean lectin copy No.	Repeatability standard deviation, s_r copy No.	Coefficient of variation of repeatability, $C_{V,r}$ %
1	20 464	5 367	26
2	3 203	620	19
3	2 005	306	15
4	6 978	331	5
5	14	8	61

Prior to the collaborative trial, DNA was extracted from five commercial soybean lecithins and tested for PCR inhibition. The DNA prepared from each sample was further diluted using TE buffer (1 volume DNA diluted with 4 volumes of TE; 1 volume of first DNA dilution diluted with 4 volumes of TE, 1 volume of second DNA dilution diluted with 4 volumes of TE). The difference between the calculated (extrapolated) C_t-value of the undiluted DNA sample and the measured C_t-value of each DNA dilution was below 0,1, showing that DNA preparations were free of PCR-inhibitors.

A.5.2.3.4 Collaborative trial

A collaborative trial (validation study) was carried out in 12 laboratories (Reference [45]). Five commercial soybean lecithin samples were used. Each laboratory received 15 coded samples and a standard DNA for calibration. The samples were distributed in a way that each participant received three identical samples of each of the five soybean lecithins. A single DNA extraction was performed per

sample by each laboratory. The returned results were assigned to the five different lecithin samples for evaluation of the collaborative trial.

The extracted DNAs were tested in a real-time PCR amplifying a DNA sequence of the soybean lectin gene according to the method in ISO 21570:2005,[41] C.2. Standard-DNA for calibration was prepared from soybean flour [ERM BF410a[1)]] by using the Plant Mini Kit[3)] (Qiagen, Hilden/Germany) starting with a CTAB extraction (see A.3). The concentration of DNA was estimated fluorimetrically with the PicoGreen[3)] dsDNA method (Reference [17]). DNA-standards were defined by the copy number (cp) of haploid genome equivalents per microlitre. For the calculation, a haploid genome mass for soybean of 1,13 pg was assumed. A dilution series was prepared ranging from about 50 000 cp/5 µl to 80 cp/5 µl. Seven laboratories used ABI[3)] real-time PCR equipment (ABI 7000, 7500, 7700), and five laboratories used the Light Cycler[3)] (Roche) with the following modifications to the protocol described in ISO 21570:2005,[41] C.2: PCR was performed in a 20 µl final volume containing QuantiTect Probe PCR Master Mix[3)] (Qiagen), primers GM1-F and GM1-R at 500 nmol/l each and 150 nmol/l of probe GM1. Real-time PCR program was: initial step with 900 s at 95 °C, then 45 cycles with 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Fluorescence signal acquisition was done within the elongation step. The temperature ramping rate was set to 2°C/s.

As a criterion for the suitability of the method, the practical limit of detection, LOD_{prac}, for genetically modified soya was determined in accordance with ISO 24276. The value was calculated individually for each sample using Formula (A.1):

$$LOD_{prac}\ \% = \frac{LOD_{abs}}{c_{s,DNA}} \times 100 \qquad \text{(A.1)}$$

where

LOD_{abs} is the LOD of the event-specific real-time PCR method used for quantitation, in copies per PCR;

NOTE For the calculations in Table A.7, an LOD of 10 copies is assumed.

$c_{s,DNA}$ is the amount of amplifiable soybean DNA in the sample, in copies per PCR, determined by means of real-time PCR method specific for a soybean reference gene.

Table A.7 summarizes the results of the collaborative trial. In four out of five lecithin samples, mean values of LOD_{prac} below 0,9 % (example for an existing legislative threshold) were obtained. For lecithin sample 2, the LOD_{prac} was above 0,9 % in only one laboratory, for lecithin sample 3 in two laboratories. For the lecithin sample 5, all laboratories amplified fewer than 80 copies of the lectin gene, and thus an LOD_{prac} of 0,9 % or less could not be achieved. In addition, the coefficient of variation of reproducibility for the lectin copy numbers reported for the five lecithin samples were calculated. In total, real-time PCR data of 36 extractions per lecithin sample were used for the evaluation. No outliers were eliminated for the calculations of the coefficient of variation of reproduciblity, $C_{V,R}$. Precision data for sample 5 cannot be given because of the low amount of lectin copies below the LOQ of the method. It is noted, that the precision data reflect the coefficient of variation of reproducibility of the copy numbers extracted from the lecithin samples under reproducibility conditions in the collaborative trial.

1) Product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of this product by ISO. Equivalent products may be used if they can be shown to lead to the same results.

Table A.7 — Summary of the validation data

Lecithin sample No.	Mean lectin copy No.	Coefficient of variation of reproducibility, $C_{V,R}$ %	Mean practical limit of detection, LOD_{prac} %	No. laboratories with LOD_{prac} < 0,9 %
1	17 044	67	0,06	12/12
2	3 630	63	0,28	11/12
3	2 318	57	0,43	10/12
4	8 325	52	0,12	12/12
5	<80	not determined	>10	0/12

A.5.2.4 Principle and summary

Viscous sample material is homogenized after heating and extracted with hexane after addition of a guanidine thiocyanate buffer. Interfering accompanying substances are separated by extraction with chloroform, and the RNA present in the solution is digested with RNase A. The DNA is precipitated with isopropanol in the presence of glycogen, then washed with ethanol and dissolved in water. For purification of the DNA, a subsequent gel filtration step (using a cross-linked dextran gel for size exclusion chromatography) is carried out.

A.5.2.5 Terms and definitions

For the purposes of this subclause, the terms and definitions given in ISO 5725-1[43] and ISO 24276 apply.

A.5.2.6 Sample type and amounts

Ensure that the test sample is representative of the laboratory sample. Measures and operational steps to be taken into consideration are described in 5.1. Sample material should be as homogeneous as possible.

A.5.2.7 Estimation of measurement uncertainty

The measurement uncertainty of the lectin real-time PCR data assessed in the collaborative trial is given as coefficent of variation of reproducibility. The results are given in Table A.7.

A.5.2.8 Interferences

The degree of refinement of the lecithin can interfere with the ability to extract DNA from lecithin samples due to the DNA degradation accompanied by this processing.

A.5.2.9 Physical or environmental conditions

No specific conditions required. See ISO 24276 for details.

A.5.2.10 Apparatus

Usual laboratory equipment and in particular the following.

A.5.2.10.1 Table-top centrifuge for 1,5 ml or 2 ml centrifugation vessels, at least 12 000 × *g*.

A.5.2.10.2 Centrifuge for 50 ml centrifuge tube, at least 4 000 × *g*.

A.5.2.10.3 Polypropylene centrifugation vessels of capacities 1,5 ml, 2,0 ml, and 50 ml, for use in centrifuges at 12 000 × *g* and 4 000 × *g*.

A.5.2.10.4 Heating block with shaking device.[2)]

A.5.2.10.5 Vacuum drier, optional.

A.5.2.10.6 Multi mixer, e.g. Vortex.

A.5.2.10.7 Real-time PCR thermal cycler equipped with an energy source suitable for the excitation of fluorescent molecules and an optical detection system suitable for the detection of the fluorescence signals generated during PCR.

A.5.2.10.8 Reaction vessels and **caps** or **closures** which can be repeatedly heated to 100 °C and cooled to 4 °C without damage and which do not influence the fluorescence signal generated during the amplification.

A.5.2.10.9 UV-spectrophotometer or **fluorometer** to determine the concentration of DNA.

A.5.2.11 Reagents and materials

For the quality of the reagents used, see ISO 24276.

A.5.2.11.1 Guanidine thiocyanate.

A.5.2.11.2 *Tris*(hydroxymethyl)aminomethane (tris) or ***tris*(hydroxymethyl)aminomethane hydrochloride** (tris·HCl).

A.5.2.11.3 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA).

A.5.2.11.4 Pancreatic RNase A.

A.5.2.11.5 Glycogen.

A.5.2.11.6 Polyethyleneglycol tert-octylphenyl ether [4-(1,1,3,3-tetramethylbutyl)phenylpolyethyleneglycol] [Triton X 100[3)]].

A.5.2.11.7 *n*-Hexane.

A.5.2.11.8 Chloroform.

A.5.2.11.9 Sodium chloride.

A.5.2.11.10 Sodium hydroxide.

A.5.2.11.11 Hydrochloric acid, $c(HCl) = 0{,}1$ mol/l.

A.5.2.11.12 Sodium hydroxide solution, $c(NaOH) = 0{,}1$ mol/l. Weigh 10 g of sodium hydroxide into a 250 ml volumetric flask, and fill the flask with water to the calibration mark.

A.5.2.11.13 Isopropanol (2-propanol).

A.5.2.11.14 Ethanol, $\varphi(C_2H_5OH) = 70$ % volume fraction. Mix 70 ml of ethanol with 30 ml of water.

2) The Eppendorf Thermomixer is an example of a suitable 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.

A.5.2.11.15 Tris·HCl buffer, pH = 6,4, c(tris·HCl) = 0,1 mol/l. Dissolve 1,57 g of tris·HCl in approximately 70 ml of water, adjust to pH 6,4 using hydrochloric acid, transfer to a 100 ml volumetric flask, and make up to volume with water.

A.5.2.11.16 Tris·HCl + NaCl buffer, pH 7,5, c(tris·HCl) = 10 mmol/l, c(NaCl) = 15 mmol/l. Dissolve 0,166 g of tris·HCl and 88 mg of sodium chloride in approximately 70 ml of water, adjust to pH 7,5 using hydrochloric acid, transfer to a 100 ml volumetric flask, and make up to volume with water.

A.5.2.11.17 EDTA solution, pH 8,0, c = 0,5 mol/l. Dissolve 18,6 g of EDTA disodium salt in approximately 70 ml of water, adjust to pH 8,0 using first sodium hydroxide pellets and then sodium hydroxide solution, transfer to a 100 ml volumetric flask, and make up to volume with water.

A.5.2.11.18 TE buffer solution, c(tris) = 0,010 mol/l and c(Na_2EDTA) = 0,001 mol/l.

A.5.2.11.19 Guanidine thiocyanate buffer, c(tris·HCl) ≈ 4,6 mol/l, c($Na_2EDTA{\cdot}2H_2O$) ≈ 0,02 mol/l, ρ[Triton X 100[3)]] ≈ 11,8 g/l. Mix 2,6 g of Triton X 100[3)] (A.5.2.11.6) and 120 g of guanidine thiocyanate (A.5.2.11.1) and dissolve in 100 ml of tris·HCl buffer (A.5.2.11.15), 8,8 ml of EDTA solution (A.5.2.11.17) and 13,2 ml of water. The buffer has a final volume of approximately 220 ml at ~pH 7,2. The solution can be kept for at least 12 months at room temperature.

A.5.2.11.20 RNase A solution, ρ(RNase A) ≈ 10 mg/ml, in accordance with the manufacturer's instructions or Reference [3], B.17.

Dissolve 10 mg of RNase A (A.5.2.11.4) in 1,0 ml of tris·HCl + NaCl buffer (A.5.2.11.16). Temper the solution for 15 min to 20 min at 95 °C, allow to cool slowly to room temperature, and then divide into 50 µl aliquots. Store the solution frozen, avoiding repeated freezing and thawing.

A.5.2.11.21 Glycogen solution, ρ(glycogen) ≈ 20 mg/ml. Weigh 200 mg of glycogen into a 15 ml plastics centrifuge tube and dissolve in 10 ml of deionized water. The aliquoted solution can be kept for 24 months at 5 °C.

A.5.2.11.22 Elution buffer, (0,2 TE), c(tris·HCl) = 2 mmol/l, c(EDTA) = 0,2 mmol/l, pH 8,0. Dissolve 158 mg of tris·HCl and 37 mg of EDTA disodium salt in approximately 400 ml of water; adjust to pH 8,0. Transfer the solution to a 500 ml volumetric flask and make up to volume with water. Autoclave the solution. The aliquoted solution can be kept for at least 12 months at room temperature.

A.5.2.11.23 MicroSpin S-300 HR column for additional DNA purification (Amersham Pharmacia Biotech)[3)]

A.5.2.11.24 Quant-iT PicoGreen dsDNA Quantitation Kit for fluorometric quantitation of DNA (Invitrogen), optional.[5)]

A.5.2.12 Sample collection, transport, preservation and storage

No specific requirements.

A.5.2.13 Test sample preparation

Apply the following DNA extraction and purification protocol. Scale adaptation of masses and buffer volumes is required as a function of the selected size of the test portion.

3) The column and test kit indicated 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 these products. Equivalent products may be used if they can be shown to lead to the same results.

Usually, lecithin or plant oils which have not been refined can be weighed in directly. Viscous lecithin samples are heated to 60 °C first and shaken up well prior to being weighed in.

A.5.2.14 Instrument calibration

Instruments should be calibrated as per ISO/IEC 17025.[39]

A.5.2.15 Method steps

A.5.2.15.1 General

For solvent extraction, the viscous sample material is treated with hexane and guanidine thiocyanate buffer. Interfering material is removed by extraction with chloroform and the remaining RNA is digested with RNase A. The DNA is precipitated in the presence of glycogen with isopropanol, washed with ethanol, and dissolved in an aqueous solution. For removal of PCR-inhibitors, the DNA is purified by means of gel filtration.

A.5.2.15.2 DNA extraction procedure

Ensure that the sample material is as homogeneous as possible and transfer 2,5 g to a 50 ml polypropylene centrifugation vessel.

An extraction blank control shall be processed in parallel.

Add 15 ml of *n*-hexane and dissolve lecithin.

Add 2 ml of the guanidine thiocyanate buffer and mix thoroughly (e.g. for 10 s to 20 s using a mixer).

Centrifuge for 10 min at approximately 4 000 × *g*.

Decant the major part of the upper hexane phase.

Transfer the lower, aqueous phase (without sediment) to a sterile 2 ml polypropylene reaction vessel.

Centrifuge for 10 min at 10 000 × *g*.

Transfer 1 ml of the lower, aqueous phase to a sterile 2 ml polypropylene reaction vessel.

Add 0,5 ml of chloroform and mix thoroughly for approximately 2 min.

Centrifuge for 10 min at 10 000 × *g*.

Transfer 500 µl to 750 µl of the aqueous supernatant to a new 1,5 ml polypropylene reaction vessel and add 5 µl of RNase A solution. (The RNase A digestion was performed in a collaborative trial and may be omitted, if applicable, in which case, proceed directly to the addition of glycogen.)

Incubate the mixture for 10 min at room temperature in order to hydrolyse any co-isolated RNA.

Add 4 µl of glycogen solution and 0,8 volumes of isopropanol (relative to the supernatant volume used), mix gently, and incubate for 30 min at room temperature.

Centrifuge for 10 min at no less than 12 000 × *g*.

Discard the supernatant; wash the DNA precipitate with 500 µl of ethanol.

Centrifuge for 5 min at no less than 12 000 × *g*.

Carefully draw off the supernatant, dry the DNA precipitate at room temperature or under vacuum.

Dissolve the DNA precipitate in 50 µl of elution buffer (e.g. in the refrigerator over night; heat slightly if not fully dissolved).

