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Potraviny – Metody pro detekci geneticky modifikovaných organismů a odvozených produktů – Metody založené na stanovení proteinů

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

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ICS 07.100.30; 67.060

English version

Foodstuffs - Methods for the detection of genetically modified organisms and derived products - Protein based methods (ISO 21572:2004)

Produits alimentaires - Méthodes pour la détection d'organismes génétiquement modifiés et de produits dérivés - Méthodes basées sur les protéines (ISO 21572:2004)

Lebensmittel - Verfahren zum Nachweis von gentechnisch modifizierten Organismen und ihren Produkten - Proteinverfahren (ISO 21572:2004)

This European Standard was approved by CEN on 3 November 2003.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Central Secretariat or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

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Contents

Foreword

This document (EN ISO 21572:2004) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN, in collaboration with Technical Committee ISO/TC 34 “Agricultural food products”.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by September 2004, and conflicting national standards shall be withdrawn at the latest by September 2004.

The demonstration of the presence of genetically modified proteins can either be qualitative or quantitative. These steps are laid down in this draft European Standard.

Other standards dealing with methods of analysis for the detection of genetically modified organisms and derived products in foodstuffs are the following:

prEN ISO 21568 *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Sampling (ISO/DIS 21568:2003).*

prEN ISO 21571 *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products - Nucleic acid extraction (ISO/DIS 21571:2002)*

prEN ISO 21569 *Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods (ISO/DIS 21569:2002)*

prEN ISO 21570 *Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods (ISO/DIS 21570:2003)*

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

prEN ISO 24276 *Foodstuffs – Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products – General requirements and definitions (ISO/DIS 24276:2002)*

Annex A is normative.

This document may touch copyrights and patents: for further information, contact your National Standardisation Institute.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Slovakia, Spain, Sweden, Switzerland and the United Kingdom.

Introduction

Analyses to detect genetically modified organisms (GMOs) and derived products can either be performed to screen, identify or quantify GMOs and their derived products in a given matrix.

For the detection of the transgenic origin of ingredients, the basic principle of a protein-based method is to:

— take a representative sample of the matrix;

— extract the proteins;

— detect and/or quantify the specific protein derived from GMO(s) under study.

As new methods become validated and accepted, they will be annexed to this standard.

1 Scope

This European Standard provides general guidelines and performance criteria for methods for the detection and/or quantitation of specific proteins derived from genetically modified (GM) plant material in a specified matrix.

These general guidelines address existing antibody based methods. Methods other than those described in annex A may also detect the protein. The same criteria as outlined in this standard generally apply.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

prEN ISO 21568 *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Sampling (ISO/DIS 21568:2003).*

3 Terms and definitions

For the purposes of this European Standard, the following terms and definitions apply.

3.1 General terms

3.1.1
sample
One or more sampling units taken from a population and intended to provide information of the population.

[ISO 3534-1:1993]

3.1.2
laboratory sample
Sample intended for laboratory inspection or testing.

3.1.3
test sample (test portion)
Sample, as prepared for testing or analysis, the whole quantity being used for analysis or testing at one time.

[ISO 3534-1]

3.1.4
matrix
All components in the sample with the analyte. Each matrix generally has a common name which permits classification.

3.1.5
denaturation of proteins
Physical and/or (bio)chemical treatment which destroys or modifies the structure of the analyte. The denaturation may modify structural, functional, enzymatic or antigenic properties of the protein.

3.2 Terms relative to antibodies

3.2.1
antibody
Protein (immunoglobulin) produced and secreted by B lymphocytes in response to a molecule recognised as foreign (antigen). The antibody is capable of binding to that specific antigen.

3.2.2
antigen
Substance that is recognised as foreign by the immune system and elicits an immune response.

3.2.3
clone
Population of identical cells derived from a single cell line.

3.2.4
cross-reactivity
Binding of the antibody to substances other than the analyte of primary interest.

3.2.5
monoclonal antibody
Antibody produced from a single hybridoma clone and directed to a single antigen determinant.

3.2.6
polyclonal antibody
Antibody produced by several clones of lymphocytes.

3.2.7
Specificity of an antibody
Ability of an antibody to specifically bind to an antigen determinant and not to other similar structures on that or other antigens.

3.3 Terms relative to techniques

3.3.1
conjugate
Material produced by attaching two or more substances together.

NOTE Conjugates of antibodies with fluorochromes (e.g. coloured particles), radiolabelled substances, or enzymes are often used in immunoassays.

3.3.2
Western blotting
Transfer of an antigen (i.e. the protein of interest), following electrophoretic separation, to a binding surface. The antigen may be visualised with a specific radiolabelled or enzyme-conjugated antibody.

3.3.3
ELISA – enzyme linked immunosorbent assay
In vitro assay that combines enzyme-linked antibodies and a substrate to form a coloured reaction product. Depending on the application, this assay can be used for qualitative or quantitative purposes.

3.3.4
test kit
Set of chemicals, materials and instructions for use, packaged together and intended for in vitro measurement for detection of a specified analyte.

3.3.5
dip stick format
Qualitative and rapid assay formats, including lateral flow strips, where an antibody or an analyte is coated to a solid surface.

3.4 Terms relative to control

3.4.1
reference material
Material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

[ISO Guide 30]

3.4.2
standard
Measured material, measuring instrument, reference material or measuring system intended to define, realise, conserve or reproduce a unit of one or more values of a quantity to serve as a reference or preparation of known characteristics used to standardise the analysis.

3.5 Terms relative to validation

3.5.1
accuracy
Closeness of agreement between a test result and the accepted reference value.

NOTE The term accuracy, when applied to a set of test results involves a combination of random components and a common systematic error or a bias component.

[ISO 3534-1:1993]

3.5.2
precision
Closeness of agreement between independent test results obtained under stipulated conditions.

NOTE 1 Precision depends only on the distribution of random errors and does not relate to the true value or the specified value.

NOTE 2 The measure of precision usually is expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is reflected by a larger standard deviation.

NOTE 3 "Independent test result" means results obtained in a manner not influenced by any previous result on the same or similar test object. Quantitative measures of precision depend critically on the stipulated conditions. Repeatability and reproducibility conditions are particular sets of extreme stipulated conditions.

[ISO 3534-1:1993]

3.5.3
Bias
Estimate of the systematic (consistent) deviation of the measured result from the true result of a given sample.

3.5.4
sensitivity
Capacity to record a small variation in concentration of a substance in the test material.

In this context, sensitivity usually is meant as the smallest quantity or concentration of the analyte that can be reliably distinguished from background.

3.5.5
specificity
Property of a method to respond exclusively to the characteristic or analyte defined in the Codex standard.

3.5.6
Limit of detection (LOD)
Limit of detection for qualitative methods is the lowest concentration or content of the analyte that can be detected reliably, but not necessarily quantified, as demonstrated by satisfactory collaborative trial or single-laboratory validation [1], [2].

3.5.7
Limit of quantitation (LOQ)
Limit of quantitation of an analytical procedure is the lowest amount or concentration of analyte in a sample which can be quantitatively determined with an acceptable level of precision and accuracy as demonstrated by satisfactory collaborative trial or single-laboratory validation according to ISO 5725, [2], or [3].

3.5.8
Applicability range (range of quantification/linearity/dynamic range)
Upper and lower limits of quantitation as expressed by a set of reference materials (or dilutions).

3.5.9
Repeatability [Reproducibility] limit
Value less than or equal to the absolute difference between two test results, as expected under repeatability [reproducibility] conditions with a probability of 95 %.

NOTE The symbol used is r [R].

[ISO 3534-1]

When examining two single test results obtained under repeatability [reproducibility] conditions, the comparison should be made with the repeatability [reproducibility] limit r [R] = 2,8 s_r [s_R].

3.5.10
Reproducibility
Precision under reproducibility conditions.

[ISO 3534-1:1993]

3.5.11
Reproducibility conditions
Conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

[ISO 3534-1:1993]

3.5.12
Repeatability
Precision under repeatability conditions.

[ISO 3534-1:1993]

3.5.13
Repeatability conditions
Conditions where independent results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.

[ISO 3534-1:1993]

3.5.14
Repeatability [Reproducibility] standard deviation
The standard deviation of test results obtained under repeatability [reproducibility] conditions.

NOTE Repeatability [Reproducibility] standard deviation is a measure of the dispersion of the distribution of test results under repeatability [reproducibility] conditions. Similarly “repeatability [reproducibility] variance” and “repeatability [reproducibility] coefficient of variation” could be defined and used as measures of the dispersion of test results under repeatability [reproducibility] conditions.

[ISO 3534-1]

3.5.15
Recovery
Ability to measure or recover a known amount of analyte from fortified samples over a range of quantitation.

4 Principle

The target protein is extracted according to the procedure described for that specific matrix, and a specific antibody is used to detect or measure the concentration of the protein in the sample.

5 Reagents

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and only de-ionised or distilled water or water that has been purified, or equivalent.

Other reagents, such as antibodies, conjugates, substrate, stop solutions and buffer components are method specific. Please refer to the method for specifics regarding reagents such as protein standards or reference materials, antibodies coated to a solid surface or free, controls and samples.

6 Apparatus and equipment

Apparatus and equipment is specified in A.5.

7 Sampling

Sampling is described in detail in prEN ISO 21568.

8 Procedure

8.1 General

Storage conditions and shelf-life of antibodies, conjugate, substrate, etc shall be clearly specified by the provider.

For the use of this standard, general requirements of quality assurance for laboratories shall be observed (e.g. concerning calibration of apparatus, double determination, blanks, use of reference materials, preparation of calibration curves, etc). Carefully clean all equipment coming into direct contact with the sample to prevent contamination.

Use appropriate laboratory equipment with low protein binding capacity (e. g. polypropylene tubes) to prevent protein adsorption during the whole procedure.

8.2 Preparation of sample solution

Once a representative sample is obtained, specific sample preparation procedures may be found in annex A.

Grind samples as specified in the method before test portions are taken, if necessary. Powders/flour might have swelling properties and need sometimes to be extracted with double volume of extraction solution.

Laboratory samples containing high amounts of fat may be inhomogeneous and a larger test sample should be extracted. If applicable, instructions may be found in annex A.

Weigh an appropriate amount (as specified in the annex) of a representative analytical sample for analysis to create a test portion for extraction. Add extraction solution and homogenise or mix.

8.3 Extraction

Use an extraction procedure suitable for the matrix. Appropriate conditions for the extraction/dilution of the test portions, controls and reference materials, are detailed in annex A.

Care should be taken to use extraction procedures validated for the matrix and, if necessary, to add binding agents or special buffers to aid in extraction of the protein (e.g. tannin binder to be added for analysis of dark chocolate).

8.4 Preparation of calibration curves

For preparation of calibration curves, it is recommended to use matrix matched reference materials or reference materials that have been validated for the matrix.

8.5 Assay procedure

According to the procedure chosen from annex A of this standard, select the required number of strips/tests/etc for the set of sample test solution to be analysed including blanks, references, standards and controls, and add the sample test solution, standards etc., at minimum in duplicate, properly diluted to the assay.

According to the method chosen, mix gently and allow reaction to occur at a given time and temperature range. If necessary, terminate the reaction according to the method described in the annex.

The stability of the final signal may vary. Read the results in a timely manner as specified in the annex.

9 Interpretation and expression of results

9.1 General

The parameters to interpret vary depending on whether the assay is qualitative, semi-quantitative or quantitative.

For quantitative methods, the coefficient of variation of optical density values resulting from replicate measurements of a sample test solution, in general, shall not exceed 15 %. The coefficient of variation of calculated concentrations resulting from replicate measurements of a sample test solution, in general, shall not exceed 20 %.

If the % coefficient of variation limit is exceeded, the analyses should be repeated on freshly prepared sample test solution. To establish a coefficient of variation, in this case, at least three determinations shall be carried out (e.g., values from 3 microtiter wells).

No affirmation shall be made stating that there is no GMO present in the sample analysed. Negative results shall be reported as “negative at the limit of detection”, or “less than the limit of detection”.

Positive results below the limit of quantitation shall be reported as “positive above limit of detection, but below limit of quantification”.

9.2 Quantitative and semi-quantitative analysis

The following parameters are evaluated: raw data (continuous numerical values) of sample test solution, of blank, of reference material or analytical standard, and of negative control, % CV between replicates, % coefficient of variation of standard and % coefficient of variation of control samples.

All final results shall be reported including the measurement uncertainty previously established.

Quantitative results may not be reported by extrapolating above the highest or below the lowest calibration standard measure.

9.3 Qualitative analysis

For qualitative tests, including all applications thereof, the corresponding parameters are described in the annex.

Results should be reported as detected or not detected and include the limit of detection.

10 Specific parameters which may influence results

10.1 General

The performance criteria listed in the method of annex A are a set of performance specifications established for each method during the development, validation and routine use of the method. These parameters were developed for each method and are intended to ensure that data generated by the method are reliable and of consistently high quality. Each time a method is performed, evaluate and compare the data generated with the established method performance criteria.

When a value (e. g. % coefficient of variation of replicate determinations) does not agree with the assay specifications, it signals that the result is atypical and warrants closer evaluation of the data. The list of specifications shall be taken as a whole, individual parameters may in certain instances not meet the specifications, but the data may still be perfectly acceptable. If any of the criteria are not met, it should, however, be acknowledged in writing and the data evaluated to determine if the analysis of results should be adjusted, or if a particular sample or a set of samples should be repeated. These decisions should be based on the judgement of the researcher interpreting the entire set of criteria.

10.2 Special considerations

10.2.1 Specificity

Adequate specificity of the assay for a particular analyte needs to be demonstrated for each analyte (protein) to be measured and in each matrix to be tested. Where appropriate, cross reactivity should be evaluated for analogues (proteins present with a similar sequence). To test for the absence of the analyte in non-GMO, assay the non-GMO containing ingredient and GM-containing ingredient at the appropriate dilutions and compare.

This is generally done during the development and validation of the method and is not necessary during routine analysis of samples for which the method has previously been validated. The method in annex A lists all of the specific substances and matrices for which cross reactivity has been determined.

10.2.2 Extraction efficiency

Special care has to be taken to assess the influence of process parameters applied for the production of a given laboratory sample.

In order to provide for the greatest sensitivity of the immunoassay, extraction efficiency shall be as high as possible. The assay performance is matrix dependent. Extraction efficiency should be determined and documented for each matrix.

The extraction procedure shall be demonstrated to be reproducible and the method of calibration account for incomplete extraction.

10.2.3 Matrix effects

The scope of application clearly and exactly defines the matrices for which the given immunoassay is applicable. The use of matrix matched reference materials allows for direct comparison between reference materials and samples. However, if samples are to be analysed against reference materials which are not the same matrix, then matrix effects will have to be evaluated.

For example, prepare a negative extract for each sample type (matrix) to be analysed by the method and an extract of a positive control of known concentration. Prepare a series of dilutions of the positive control in the negative

extract and compare the resulting dose response curve with the calibration curve from the method. If the two curves are different, then there is a matrix effect. Use a matrix that most closely represents the true samples that will be tested. A dilution curve with a positive control of known concentration should also be included as a reference. The shape of the calibration curve should not change due to a matrix effect.

10.2.4 Parallelism/Linearity

For quantitative analyses, the expected linear range of the immunoassay shall be explicitly stated in the scope of applications for all the matrices covered by it.

The number of calibration points supplied should reflect the linear portion of the curve (i.e. if the calibration curve is non – linear more calibration points will need to be generated).

If the calibration curves do not appear “parallel” or of the same slope when graphed side by side, the data should be statistically analysed for a test of dilution comparability.

10.2.5 Limits of detection

Results should not be interpreted below the limit of detection. In this case, reporting of results should be stated to be at or less than the limit of detection.

10.2.6 Limits of quantitation

The limits of quantitation for each set of calibrants (or dilution) need to be stated explicitly.

The estimated concentration of unknown sample test solutions should be interpolated and not extrapolated.

Results should not be interpreted below the limit of quantitation or above the highest or below the lowest calibration points.

11 Confirming method

To establish the credibility of assays, another method such as western blot, HPLC or functional assay can be used to measure split analytical samples of known concentration. The results of both methods are then qualitatively/quantitatively compared. This is especially important for immunoassays, since antibodies could cross react with other analytes present in a matrix.

12 Test report

The test report shall contain at least the following information:

— all information needed to identify the laboratory sample;

— reference to this standard and to the method used, and an indication of whether it was a qualitative, quantitative or semi-quantitative method;

— limit of detection;

— upper and lower limits of quantification;

— date and the type of sampling procedure used (if known);

— date of receipt;

— analysis start date or other appropriate documentation;

— amount of the test portion;

— amount of the sample test solution;

— results and the units used to report them;

— any special points observed during testing;

— any operation not specified in the method or considered to be optional but that can have an effect on the results.

Annex A
(normative)

Detection of genetically modified soybeans (Roundup Ready ®1) tolerant)

A.1 Introduction

Soybeans (*Glycine max*), are an important food and feed source[2]. Recently, a gene, *cp4 epsps*, was introduced into certain soybean varieties through use of modern biotechnology. The gene for glyphosate tolerance comes from *Agrobacterium* sp. strain CP4. The expressed CP4 EPSPS protein confers tolerance to the herbicide glyphosate (Roundup®[1]). The CP4 EPSPS protein produced may be detected by using specific antibodies.

A.2 Scope

This annex specifies an ELISA-method for the determination of CP4 EPSPS protein present in Roundup Ready®[1] soybeans GTS 40-3-2 and derived ingredients.

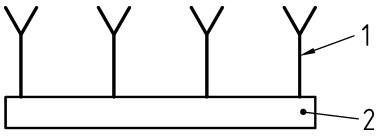
The method is applicable to samples where little or no treatment or processing has been carried out, and, thus, the CP4 EPSPS protein is not denatured. For example, the temperature at which food ingredients are processed may impact the ability of protein detection.

In the case for quantitative measurement, the method can only be applied to food samples consisting entirely of soybeans and derived products in which protein can be detected.

The method has been validated for the detection of CP4 EPSPS protein in ground soya powder (IRMM reference material) and can be used for the determination over the range of 0,5 % to 2 % (w/w) using specific reference material[3]. The corresponding test kit is commercially available[4] and applicable for the analysis in other protein containing matrices as identified by the kit manufacturer's method validation data. For summary of collaborative study and User's Guide, see [3], [4].

A.3 Principle

A direct sandwich enzyme linked immunosorbent assay (ELISA) is used for detection of CP4 EPSPS protein as shown in the figures A.1 to A.4:



Key

1 Monoclonal antibody
2 Coated surface

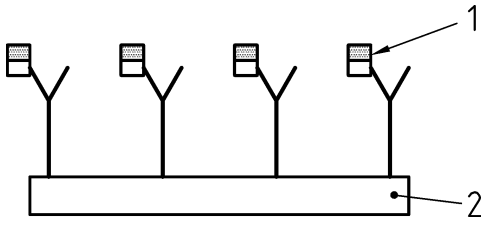
Figure A.1 — The surface of a microtiter plate is coated with a specific monoclonal capture antibody.

1) RoundupReady® and Roundup® are registered trademark of Monsanto company. This information is only given for the convenience of users and does not constitute an endorsement of CEN.

2) Common soybean derived ingredients which are high in protein include: flour, concentrate, isolate and texturized products.

3) Suitable reference material is available from European Commission's Joint Research Centre, Institute for Reference Materials and Measurements (IRMM). This information is only given for the convenience of users of this standard and does not constitute an endorsement of this product.

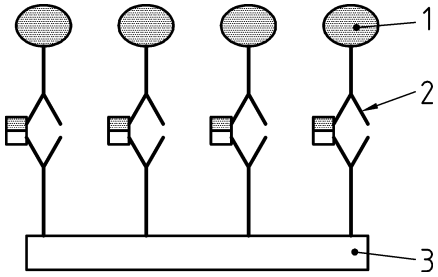
4) Strategic Diagnostics, Inc. Hampshire, England. This information is only given for the convenience of users of this standard and does not constitute an endorsement of this product.



Key

1 Antigen
2 Coated surface

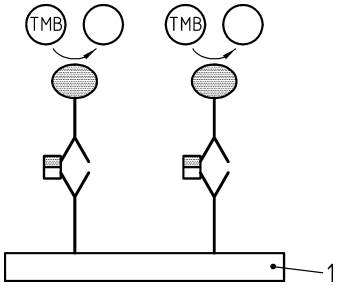
Figure A.2 — When the sample of interest is added, the capture antibody binds the antigen. Unbound components of the sample are removed by washing.



Key

1 HRP
2 Detector antibody
3 Coated surface

Figure A.3 — After washing, a polyclonal antibody, covalently linked to horseradish peroxidas (HRP) is added, which is specific for a second antigenic site on the bound CP4 EPSPS protein.



Key

1 Coated surface

Figure A.4 — After washing, a tetramethylbenzidine (TMB) chromogenic substrate for horseradish peroxidase is added. The horseradish peroxidase generates a colour signal which is proportional to the concentration of antigen in a linear range. To stop the colour development, a stop solution is added. The degree of colour produced is measured at a wavelength of 450 nm.

A.4 Reagents

A.4.1 General

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and de-ionised or distilled water.

Any deviation from the defined performance criteria may indicate a lack of reagent stability. If the substrate components have already changed colour from clear to blue, this reagent should be discarded. Turbid buffer or turbid conjugate solutions should not be used.

All kit components should be stored at approximately 2 °C to 8 °C. The shelf life of the kit components is indicated by expiry date. Based on accelerated stability testing, the expiry date for the test kit has been set for 9 months at approximately 2 °C to 8 °C.

The antibody conjugate stock solution (A.6.6.1) and the antibody conjugate working solution (A.6.6.2) should be stored at 2 °C to 8 °C until the expiry date of the kit. The diluted wash buffer should be stored at approximately 2 °C to 8 °C and not longer than expiry date of the kit.

A.4.2 Reagents usually provided with the test kit

A.4.2.1 Soya extraction buffer, sodium borate buffer, pH 7,5

A.4.2.2 Soya assay buffer, PBS, Tween 20, bovine serum albumin, pH 7,4

A.4.2.3 Coated strip wells, 12 strips each containing 8 wells coated with the monoclonal capture antibodies, one strip holder.

A.4.2.4 Rabbit anti-CP4 EPSPS protein conjugated to horseradish peroxidase (HRP), lyophilised

A.4.2.5 Conjugate diluent buffer, 10 % heat inactivated mouse serum

A.4.2.6 Chromogenic substrate, K-Blue^{TM}[5)] (Tetramethylbenzidine (TMB), hydrogen peroxide, 5 % dimethylformamide as base solvent)

A.4.2.7 Stop solution, 0,5 % sulfuric acid

A.4.2.8 10 fold wash buffer concentrate, PBS, Tween 20, pH 7,1

A.4.2.9 Matrix matched negative and positive reference standards, e.g. of 0,1 %, 0,5 %, 1 %, 2 %, and 5 % w/w

A.4.3 Chemicals not supplied with the test kit

A.4.3.1 Alcohol, such as methanol, volume concentration ϕ of 70 %, or ethanol, ϕ of 95 %

A.4.3.2 Detergent, for ultrasonic bath, optional

5) K-Blue is the trade name of a product supplied by Neogen Corp, Lansing, Michigan, USA. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

A.5 Apparatus and equipment

A.5.1 General

Usual laboratory equipment and, in particular:

A.5.2 Refrigerator, working at approximately 4 °C

A.5.3 Polypropylene conical centrifuge tubes, sealable, e.g. 15 ml

A.5.4 Plastic wrap or aluminium foil

A.5.5 Plastic tape to prevent strips movement during plate washing

A.5.6 Wash bottle, e.g. of 500 ml

A.5.7 Precision micropipettes capable of delivering e.g. 20 µl to 500 µl

A.5.8 Mixer, e.g. Vortex ®[6)]

A.5.9 Balance capable of weighing to the nearest 0,01 g

A.5.10 Centrifuge capable for producing a centrifugal acceleration of at least 5 000 x *g* at the outer end of the centrifuge tubes

A.5.11 Microtiter plate reader capable of reading absorbance at 450 nm

A.5.12 Incubator oven or water bath capable of maintaining 37 °C

A.5.13 Sieve of aperture size of 450 µm, or equivalent

A.5.14 Sieve of aperture size of 150 µm (100mesh), or equivalent

A.5.15 Multi-channel pipette, e.g. of 50 µl to 300 µl (optional)

A.5.16 Reagent reservoirs for multi-channel dispensing (optional)

A.5.17 Automated plate washer (optional)

A.5.18 Test tube rack for 15 ml centrifuge tubes (optional)

A.5.19 Ultrasonic bath (optional)

A.6 Procedure

A.6.1 Warning or precautions

Precautions should be taken when using acid solutions or solutions containing TMB.

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6) Vortex is an example of a suitable product available commercially. This information is given for the convenience of the users of this standard and does not constitute an endorsement by CEN of this product.

Natural ventilation is sufficient.

A.6.2 Limitation of the procedure

This ELISA is limited to samples where the amount of the CP4 EPSPS protein can be correlated with the level of GM material present in the reference material used. For thermally processed food samples and composite food samples, this correlation may not be applicable.

The ELISA test kit is designed to give optimum performance at ambient temperatures of between 15 °C to 30 °C. The absorbance of the highest reference material should be greater than 0,8 optical density (OD) and should not fall outside the linear range of the spectrometer (the upper limit varies from spectrometer to spectrometer). At temperatures greater than 30 °C, OD values will rise more rapidly, a reduced substrate incubation time may be necessary. At low temperatures (less than 15 °C) the substrate incubation time should be increased.

A.6.3 Specimen collection

Sampling shall be carried out according to prEN ISO 21568.

A.6.4 Sample preparation

Take a homogeneous test sample from the laboratory sample in duplicate, see prEN ISO 21568.

For raw soybeans, it is recommended that 2 000 g should be blended and ground until fine enough to be sieved. For quantitative analysis, a particle size of less than 150 µm should be obtained and less than 450 µm for qualitative analysis. To avoid contamination, care should be taken during the sieving step. Furthermore, care should be taken to avoid excessive heating. The action of the blender will both mix and grind the sample. From the ground material approximately 100 g should be taken and passed through a sieve of 450 µm pore size (A.5.13). At least 90 % of this sample should pass through the 450 µm sieve. For a qualitative assay, this material can be used directly, for a quantitative assay the sieved material should be further sieved using a sieve of 150 µm pore size (A.5.14). The material passing through the 450 µm sieve has been shown to be homogeneous. Therefore, it is necessary to sieve only enough material to provide an analytical sample through the 150 µm sieve.

Other types of samples should be treated equivalently although smaller samples sizes may be used.

A.6.5 Measures to avoid contamination during sample preparation

A.6.5.1 General

The ELISA test system is a sensitive technique capable of detecting very small quantities of CP4 EPSPS protein. For this reason, it is imperative that all equipment used to process soya samples be thoroughly cleaned between sample batches. The following procedures involve a first step of physical removal of as much particulate material as possible. The second step, a wash with alcohol, is to denature and render non-reactive any protein that remains on the equipment.

A.6.5.2 Grinder or blender cleaning

Brush clean with a soft bristle brush.

Rinse with alcohol (this may be stored and dispensed from a spray or squirt bottle). Two rinses or sprays are recommended. Then rinse thoroughly with water.

Air dry or, if rapid reuse is required, use e. g. commercial hair dryer.

Periodically wash brush and soak in an alcohol solution (A.4.3.1). Dry brush before subsequent use. Wipe with a soft cloth or laboratory towel.

A.6.5.3 Sieve cleaning

Sieve tends to become caked with soya powder. Sharply tap sieve on hard surface to dislodge caked material.

Brush the sieve with clean soft bristle brush. Soak the sieve in alcohol for at least 5 min and rinse thoroughly with water. Air dry or, if rapid reuse is required, use e. g. commercial hair dryer.

An alternative method would be to use an ultrasonic bath followed by air drying.

Periodically wash brush and soak in a alcohol (A.4.3.1) for at least 1 min. Dry brush before subsequent use. Wipe the brush with soft cloth.

A.6.5.4 Cleanliness of work area

Avoid soy dust contamination in the work area. Do not allow soya dust from one processing to contaminate equipment to be used in a subsequent processing. For optimum performance, run assay in a room separated from the facility where sample grinding and preparation is conducted to avoid potential dust contamination.

A.6.6 Preparation of antibody conjugate

A.6.6.1 Antibody conjugate stock solution

Reconstitute the lyophilised antibody conjugate (A.4.2.4) in conjugate diluent buffer (A.4.2.5), according to the user guide.

Store the antibody conjugate stock solution at 2 °C to 8 °C no longer than the expiry date of the kit.

A.6.6.2 Antibody conjugate working solution

Add 240 µl of the conjugate stock solution (A.6.6.1) to 21 ml of conjugate diluent buffer (A.4.2.5).

Store the antibody conjugate working solution at 2 °C to 8 °C no longer than expiry date of the kit.

A.6.7 Preparation of wash buffer

Dilute the 10 fold wash buffer concentrate (A.4.2.8) 1 : 10 (i.e. 1+9) in water.

A.6.8 Assay procedure

Allow all reagents to reach room temperature.

Remove the coated strips (A.4.2.3) and strip holder from the foil bag. Always seal the foil bag each time after removing the appropriate number of strips. Ten wells are required for reference standards and assay blanks. Each plate shall have its own standards and controls. If using manual washing, tape the edges of all strips (A.5.5) needed for a run to the strip holder to prevent strips from accidentally falling out of the strip holder during the washing steps.

The procedure is summarised in A.7.

A.6.9 Test performance

A.6.9.1 Extraction of test portion and reference standard

The test portions and negative and positive reference materials[7)] are extracted under the same conditions in duplicate, described as follows.

7) Currently commercially available certified reference materials can be obtained from Institute of Reference Materials and Measurements, JRC, Geel, Belgium. This information is only given for the convenience of users of this standard and does not constitute an endorsement by CEN.

Weigh out 0,5 g ± 0,01 g of each reference material and the test portion into individual 15 ml polypropylene centrifuge tubes. When weighing, weigh out each reference material in increasing order of concentration. Subsequently, weigh out the test portions. To avoid contamination, clean the spatula by wiping it with an alcohol soaked tissue (A.4.3.1) followed by drying or use disposable spatula between each reference material and test portion.

Add 4,5 ml of extraction buffer (A.4.2.1) into each centrifuge tube.

Mix the test portion or reference material with extraction buffer by shaking vigorously and agitating (vortex) until they become a homogeneous mixture.

NOTE Defatted flour and protein isolate need a prolonged time of mixing, sometimes more than 15 min. Full fat flour becomes more easily a homogenous mixture (less than 5 min).

Centrifuge the mixtures at approximately 5 000 *g* for 15 min preferably at 4 °C.

Carefully remove approximately 1 ml supernatant of each sample solution and standard reference extract and place each into an individual clean polypropylene centrifuge tube.

Sample solutions can be stored at 2 °C to 8 °C, but not longer than one working day

Prior to starting the assay, dilute the sample solutions and the reference standard solutions with soya assay buffer (A.4.2.2) according to Table A.1.

Table A.1 — Dilution ranges according to the matrix

Matrix	Dilution
Soybean	1 : 300
Soya flour	1 : 300
Defatted soy flour [a]	1 : 300
Protein isolate [a]	1 : 10
a These matrices have not been validated by collaborative trial. Dilution ranges are based on manufacturer's experience.	

A summary of the extraction steps can be found in Table A.2.

A.6.9.2 ELISA immunoassay procedure

A.6.9.2.1 General

The ELISA assay kit can be run in different formats using any number of the 8-well strips. It is recommended to follow a randomised loading scheme, i.e. test samples and controls not always added to the same wells of each assay run to avoid position effects in the plate, if any.

All reactions should be run at minimum in duplicate and the mean absorbance value calculated. Each run consists of the assay blank, the sample blank and the positive reference standard solutions.

When an assay has been started, all steps should be completed without interruption.

A summary of the ELISA procedure can be found in Table A.3.

A.6.9.2.2 Incubation

Using a micropipette, add 100 µl of diluted sample and reference material solutions and the assay blank to the appropriate wells. Use separate disposable tips for each pipetting step to avoid carry-over contamination. Cover plate with plastic wrap or aluminium foil (A.5.4) to prevent contamination and evaporation.

Before starting incubation, it is recommended to gently mix the microtiter plate by gripping the short sides between thumb and forefinger and moving the plate from side to side.

Incubate microtiter plate at 37 °C for 1 h.

A.6.9.2.3 Washing

Wash 3 times with 300 µl wash buffer (A.6.7) by using a mixer (A.5.8).

Manual washing: Empty the wells by inverting over a sink or suitable waste container. Using a 500 ml wash bottle containing working wash solution, fill each well to the top, allow to stand for 60 s, then empty the plate as described above. Repeat the washing step for a total of 3 times. Remove residual liquid and bubbles by tapping upside down on several layers of paper towels.

Prevent the strips from falling out of the frame by securing with adhesive tape.

Automatic washing: At the end of the incubation period aspirate the contents of all wells using a microtiter plate washer, then fill wells with working wash buffer. Repeat the aspiration/fill step for a total of 3 times. Finally, use the washer to aspirate all wells then tap the inverted plate onto a stack of paper towels to remove residual droplets of wash buffer and bubbles.

NOTE 1 Do not let wells dry out, as it may affect assay performance.

NOTE 2 Inadequate washing will cause erroneous results. Whether using manual or automated washer it is important to ascertain that each assay well is washed with identical volumes to all other wells.

A.6.9.2.4 Addition of antibody conjugate

Add 100 µl of antibody conjugate working solution (A.6.6.2) to each well using a micropipette. Cover the plate to prevent contamination and evaporation.

Before starting incubation, gently mix the microtiter plate by gripping the short sides between thumb and forefinger and moving the plate from side to side.

Incubate the microtiter plate at 37 °C for 1 h.

A.6.9.2.5 Washing

At the end of the incubation period, repeat the washing step as described above (A.6.9.2.3).

A.6.9.2.6 Substrate addition

Add 100 µl of the chromogenic substrate (A.4.2.6) to each well using a micropipette. Gently mix the plate and incubate for 10 min at room temperature.

The addition of chromogenic substrate should be completed without interruption. Maintain the same sequence and time interval during the pipetting.

A.6.9.2.7 Stop solution addition

At the end of the incubation period, add 100 µl of stop solution (A.4.2.7) to each well, pipette the stop solution in the same sequence as the colour reagent was added. Gently mix the plate for 10 s to stop colour development and uniformly distribute the stop solution.

The addition of stop solution should be completed without interruption. Protect the microtiter plate from sunlight, otherwise colour intensity is influenced.

A.6.9.2.8 Absorbance reading

Using a microtiter plate reader fitted with a filter appropriate for reading at 450 nm, measure the absorbance of each assay well. All readings should be completed within 30 min of adding the stop solution.

Record the results obtained and calculate the mean absorbance values or use a computer programme.

A.7 Flowcharts

Table A.2 — Extraction flowchart

Procedure	Description
Weigh out 0,5 g	Weigh out analytical samples, blank, reference standards.
Addition of 4,5 ml	Addition of extraction buffer (A.4.2.1).
Mixing	Mix the test portion with extraction buffer until it becomes homogeneous, full fat flour less than 5 min, defatted flour, protein isolate more than 15 min.
Centrifugation at 5 000 x *g*	Centrifuge the sample at 5 000 x *g* for 15 min, preferably at 4 °C. Remove supernatant and place it into a clean tube.
Dilution: 1 : 300 or 1 : 10 according to the material investigated	Dilute the resulting sample test solution, assay blank and reference standards.

Table A.3 — ELISA procedure flowchart

Procedure	Volume	Description
Addition	100 µl	Pipette diluted sample test solutions, blank and reference standards into appropriate assay well and mix.
Incubation		Incubate 1 h at 37 °C.
Washing		Wash 3 times with wash buffer (A.6.7).
Addition	100 µl	Dispense antibody conjugate (A.4.2.4) into each assay well and mix.
Incubation		Incubate 1 h at 37 °C.
Washing		Wash 3 times with wash buffer (A.6.7).
Addition	100 µl	Dispense chromogenic substrate (A.4.2.6) into each well and mix.
Incubation		Incubate for 10 min at ambient temperature.
Addition	100 µl	Dispense stop solution (A.4.2.7) into each assay well and mix.
Absorbance measurement		Measure absorbance value of each assay well in plate reader at 450 nm.

A.8 Evaluation

Data should be recorded.

Standard values should be used to develop a standard curve. The value from the assay blank should be subtracted from all values for diluted test sample solutions and reference standards. The average corrected values from each duplicate reference point should be used to create a standard curve. The average data from each duplicate sample test solution should then be used to interpolate a concentration from this curve.

A.9 Accept/reject criteria

Each run shall meet the accept/reject criteria in the procedure to be valid as listed in Table A.4. The run consists of the following, assay blank, positive reference materials, negative reference materials and unknown samples. All sample test solutions, reference material solutions and the assay blank will be run in duplicates. If a run does not meet the assay acceptance criteria, the entire run shall be repeated. Sample test solutions that do not pass the acceptance criteria in any run shall be re-run a second time.

Table A.4 — Criteria for accepting or rejecting results

Assay Buffer Blank	A 450 nm < 0,30
0 % GMO Standard	A 450 nm < 0,30
2,5 % Reference standard	A 450 nm ≥ 0,8
All positive reference standards, OD	CV (OD) of replicates $\underline{\leq}$ 15 %
Unknown samples, solution	CV (OD) of replicates ≤ 20 %

A.10 Status

This method has been tested in an interlaboratory trial on certified reference material CRM 410 according to ISO 5725 as carried out by the Joint Research Centre of the European Commission Ispra, Italy, see [3].

The protocol for the interlaboratory test differed from the description below concerning the following aspects:

— no sieving was applied,

— the shelf life of the reagents was not specified,

— no sample preparation was described,

— the method was only applied with standard reference material containing 0,1 %, 0,5 % and 2 % of CRM 410,

— the reference standards were not analysed in duplicate,

— the amount of sample was not sufficient to repeat the analysis and to apply the acceptance/rejection criteria.

The results of the interlaboratory trial are listed in Table A.5

Table A.5 — Precision data

Sample in percent GMO in soy bean flour	0,5 %	1 %	2 %
Year of inter-laboratory test	1999	1999	1999
Number of laboratories	37	37	37
Number of laboratories retained after eliminating outliers	37	33	35
Number of outliers (laboratories)	0	4	2
Number of accepted results	37	33	35
Mean value $\bar{x}$, % GMO	0,440	0,952	1,902
% of true value	88,1	95,2	95,1
Repeatability standard deviation s_r [a]	0,062	0,092	0,146
Repeatability relative standard deviation RSD_r, % [b]	12,4	9,2	7,3
Repeatability limit r [$r = 2{,}8 \cdot s_r$] [a]	0,176	0,260	0,414
Reproducibility standard deviation s_R [a]	0,083	0,123	0,186
Reproducibility relative standard deviation RSD_R, % [b]	16,6	12,3	9,3
Reproducibility limit [$R = 2{,}8 \cdot s_R$] [a]	0,238	0,349	0,527

a s_r, s_R, r, and R are expressed in units of % GMO.

b RSD_r and RSD_R are expressed as percent of true value.

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TECHNICAL CORRIGENDUM 1

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Foodstuffs — Methods for the detection of genetically modified organisms and derived products — Protein based methods

TECHNICAL CORRIGENDUM 1

Produits alimentaires — Méthodes pour la détection d'organismes génétiquement modifiés et de produits dérivés — Méthodes basées sur les protéines

RECTIFICATIF TECHNIQUE 1

Technical Corrigendum 1 to ISO 21572:2004 was prepared by the European Committee for Standardization (CEN), in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Page 10, Annex A

The status of Annex A shall be “(informative)” and not “(normative)”.

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