SLOVENSKI STANDARD
SIST-TS CEN/TS 15633-3:2012

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Živila - Odkrivanje prisotnosti alergenov v živilih z imunološkimi metodami - 3. del: Kvantitativno določanje lešnika z encimsko-imunološko metodo z uporabo poliklonalnih protiteles in detekcijo beljakovin po Lowryju

Foodstuffs - Detection of food allergens by immunological methods - Part 3: Quantitative determination of hazelnut with an enzyme immunoassay using polyclonal antibodies and Lowry protein detection

Lebensmittel - Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 3: Quantitative Bestimmung von Haselnuss mit einem Enzym-Immunoassayverfahren unter Verwendung von polyklonalen Antikörpern und Proteindetektion nach Lowry

Produits alimentaires - Détection des allergènes par des méthodes immunologiques - Partie3 : Détermination quantitative de la présence de noisette par un immuno-essai enzymatique à l'aide d'anticorps polyclonaux et détection des protéines par la méthode de Lowry

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Foodstuffs - Detection of food allergens by immunological methods - Part 3: Quantitative determination of hazelnut with an enzyme immunoassay using polyclonal antibodies and Lowry protein detection

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Foreword

This document (CEN/TS 15633-3:2012) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

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

This Technical Specification consists of the following parts:

— CEN/TS 15633-1, *Foodstuffs — Detection of food allergens by immunological methods — Part 1: General considerations*

— FprCEN/TS 15633-2, *Foodstuffs — Detection of food allergens by immunological methods — Part 2: Quantitative determination of hazelnut with an enzyme immunoassay using monoclonal antibodies and bicinchoninic acid-protein detection*

— CEN/TS 15633-3, *Foodstuffs — Detection of food allergens by immunological methods — Part 3: Quantitative determination of hazelnut with an enzyme immunoassay using polyclonal antibodies and Lowry protein detection*

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this Technical Specification: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

The hazelnut (*Corylus avellana)* belongs to a group of foods commonly referred to as tree nuts. Allergic reactions to hazelnut ranging from oral allergy syndrome to severe anaphylaxis have been widely reported. Food labelling legislation requires the presence of hazelnut in food to be declared in numerous nations of the European Union, North America, Asia and Australasia.

IgE binding studies from the sera of sensitized patients have revealed a number of allergens, including both pollen related and non-pollen related allergens [1]. Threshold dose studies have reported provocative doses as low as 1 mg of hazelnut protein [1].

Hazelnut material may occur unintentionally in foods for several reasons. It may be present in contaminated ingredients or cross contamination may occur during food manufacture. Consequently there is a need for sensitive and reliable tests for the detection of hazelnut in food samples.

1 Scope

This Technical Specification specifies an enzyme-linked immunosorbent assay (ELISA)-method for the determination of hazelnut concentration in food samples.

Spiking experiments with diluted ground hazelnut have been used to validate the method's use on food matrices such as mixed grain cereals, dark chocolate (45 % cocoa) and ice cream. The range of the method is 0,5 mg to 5,0 mg hazelnut protein per kg of food sample. As hazelnut kernels typically contain between 12 % to 15 % protein [2], [3], this equates to approximately 3,7 mg to 37 mg hazelnut kernel per kg of food sample. The upper limit of the range of quantitation can be extended, if required, by further dilution of sample extracts.

The method is commercially available[1)] and has been validated in-house by the manufacturer. These data are included in Annex A.2.

The method has been successfully validated by a collaborative study. The study was organized by the Working Group established by the Federal Office of Consumer Protection and Food Safety (BVL) for the execution of § 64 of the German Food and Feed Code (LFGB) for the determination of hazelnut content in dark chocolate. Thirteen German laboratories participated in the collaborative study. These data are included in Annex A.3.

2 Normative references

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

EN 15633-1:2008, *Foodstuffs – Detection of food allergens by immunological methods – Part 1: General considerations*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 15633-1 apply.

4 Principle

A direct sandwich ELISA is used for detection of hazelnut protein.

A 13 kDa to 14 kDa protein band common to both raw and roasted hazelnuts was identified using polyacrylamide gel electrophoresis. This protein marker was purified by high performance liquid chromatography and used to raise rabbit anti-hazelnut polyclonal anti-sera. The IgG fraction of this antiserum was purified by affinity chromatography then used to develop a direct sandwich ELISA as outlined below:

1) Soluble hazelnut protein is extracted from a food sample.

2) The extract is then added to micro-titre wells coated with the anti-hazelnut capture antibody. The sample is allowed to react before thorough washing.

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1) ELISA Systems Hazelnut Residue ELISA is the trade name of a product supplied by ELISA Systems Pty Ltd, Brisbane, Australia. This information is given for the convenience of users of this Technical Specification 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.

3) The conjugate (horseradish peroxidase labelled anti-hazelnut antibodies) is then added and allowed to react before thorough washing. Hazelnut protein captured in step 2 will bind the conjugate to form a sandwich.

4) Tetramethylbenzidine (TMB), the chromogenic substrate, is added and allowed to react. Hazelnut protein-antibody sandwiches will cause development of a blue colour.

5) The reaction is stopped by the addition of 1 mol/l phosphoric acid which causes a colour change to yellow.

A spectrophotometer (450 nm primary wavelength and 620 nm to 650 nm reference wavelength) is used to measure the optical density (*OD*) of each well.

The *OD* produced is determined by the hazelnut protein concentration. The hazelnut concentration of a sample can be calculated by comparison of the *OD* it generates with the *OD* generated by standards. The standards are calibrated in hazelnut protein mg/kg. They were prepared from an aqueous extract of commercial hazelnuts (*Corylus avellana).* The protein concentration was determined by the Lowry method.

5 Reagents

5.1 Reagents usually supplied with the kit, as standard

5.1.1 Extraction Solution

The routine extraction solution is phosphate buffered saline with Tween 20 (PBST). The solution is supplied as a 20-fold concentrate and requires dilution to working strength prior to use (see 5.2.1).

Table 1 — Composition of ELISA Systems Working Strength PBST

Compound	Working Concentration
KH_2PO_4	0,2 g/l
NaH_2PO_4	1,2 g/l
Tween 20	2 ml/l
NaCl	8 g/l
KCl	0,2 g/l
Ethyl(2-mercaptobenzoato-(2-)-O,S)mercurate(1-) sodium (Thiomersal (INN))	0,2 g/l

5.1.2 ESADDSOL (Enhanced Extraction Solution for Samples containing Polyphenols)

A specialized extraction solution should be used for foods containing polyphenols. The solution is supplied as a concentrate and requires dilution prior to use (see 5.2.2). The working strength solution contains PBST (see Table 1) plus 1,5 % Polyvinylpyrrolidone (PVP10 average molecular weight 10 000).

5.1.3 Antibody Coated Strips

48 Micro-titre wells coated with anti-hazelnut protein capture antibodies, with one strip holder.

5.1.4 Standards

Each vial contains 1,7 ml of hazelnut protein in aqueous solution to provide standards of the following values: 0 mg/kg (negative control); 0,5 mg/kg; 1,0 mg/kg; 2,5 mg/kg and 5,0 mg/kg. Coloured pale purple with food dye and ready to use.

NOTE The true concentration of each vial is one tenth of that listed on the label. This compensates for the dilution effect of the extraction procedure.

5.1.5 Enzyme Conjugate, 7 ml rabbit anti-hazelnut polyclonal antibody conjugated to horseradish peroxidase (HRP) coloured with green food dye, ready to use.

5.1.6 Substrate Enhanced KBlue[2], 7 ml of chromogenic substrate, ready to use.

Contains 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide.

5.1.7 Stop Solution, 7 ml of 1 mol/l phosphoric acid, ready to use.

5.1.8 Wash Buffer 20×Concentrate

This PBST solution is supplied as a 20-fold concentrate and requires dilution to working strength prior to use (see 5.2.3). The components are listed in Table 1.

5.2 Preparation of Working Strength Solutions from Concentrates

5.2.1 Extraction Solution

Use distilled or deionised water to dilute 25 ml of the extraction solution concentrate supplied to a final volume of 500 ml. The working strength extraction solution can be stored at room temperature (20 °C to 25 °C) for up to 12 months.

5.2.2 ESADDSOL (Enhanced Extraction Solution for Samples containing Polyphenols)

A specialized extraction solution should be used for foods containing polyphenols. Use distilled or deionised water to dilute 30 ml of the enhanced extraction solution concentrate supplied to a final volume of 500 ml. The working strength enhanced extraction solution can be stored at room temperature (20 °C to 25 °C) for up to 12 months.

5.2.3 Wash Buffer

Use distilled or deionised water to dilute 25 ml of the wash buffer concentrate supplied to a final volume of 500 ml. The working strength wash buffer can be stored at room temperature (20 °C to 25 °C) for up to 12 months.

5.3 Reagents not supplied with the test kit

5.3.1 pH indicator strips

5.3.2 Distilled or deionised water

5.3.3 Reagents for pH adjustment (optional)

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2) Enhanced KBlue Substrat is the trade name of a product supplied by Neogen, Michigan, U.S.A. This information is given for the convenience of users of this Technical Specification 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.

5.3.3.1 General

The reagents described in 5.3.3.2 and 5.3.3.3 may be required for pH adjustment of sample-extraction solution mixtures (see 7.2.3). These solutions should be prepared using analytical grade reagents and distilled or deionised water.

5.3.3.2 Sodium hydroxide solution, c = 1 mol/l.

5.3.3.3 Hydrochloric acid solution, c = 1 mol/l.

6 Apparatus and equipment

Standard laboratory equipment and in particular the following:

6.1 Temperature-controlled water bath, capable of maintaining temperatures of 60 °C.

6.2 Laboratory balance, capable of weighing to the nearest 0,01 g.

6.3 Plastic containers, of suitable size for the extraction procedure, low protein binding disposable containers are recommended.

6.4 Device to allow sample homogenization, such as blender, grinder, stomacher or tissue homogenizer.

6.5 Laboratory mixer, as a Vortex®[3] style mixer.

6.6 Precision pipette, capable of measuring 100 µl.

6.7 Laboratory wash bottle

6.8 Spectrophotometer, plate reader or strip reader capable of reading at 450 nm (primary) and 620 nm to 650 nm (reference).

6.9 pH meter, (optional).

6.10 Incubator, if room temperatures falls outside the range of 20 °C to 25 °C.

6.11 Centrifuge or **micro-centrifuge,** (optional).

6.12 Equipment required for larger batches (> 16 wells)

6.12.1 Multichannel pipette, capable of measuring 100 µl.

6.12.2 Automated plate washer, (recommended).

7 General instructions/recommendations/preparation

7.1 General

Kits should be stored at 2 °C to 8 °C.

Do not substitute reagents and wells from kits of different lot numbers.

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3) Vortex is an example of suitable products available commercially. This information is given for the convenience of the users of this Technical Specification and does not constitute an endorsement by CEN of these products.

If fewer than 48 wells are required then mix the reagents thoroughly and remove the required wells and aliquots of reagents. Reseal the remaining reagents and wells and return the remainder of the kit to refrigeration.

Reagents should be allowed to reach assay temperature (20 °C to 25 °C) before the assay is commenced. The incubation times listed in 7.3.2 should be adhered to.

7.2 Preparation of sample

7.2.1 Sample type and amounts, including sample identification

Solid and liquid samples can be analysed. Samples should be clearly and unambiguously labelled.

7.2.2 Sample collection, transport, preservation and storage

Representative samples should be collected in accordance with a HACCP (Hazard Analysis and Critical Control Points) plan and sampling standards.

Samples should be transported in sealed bags or containers to prevent leakage and contamination.

Ideally samples should be extracted and analysed promptly. Proteases from microbes and other sources can digest food allergens present in samples and decrease the concentrations detected. If a delay is expected samples should be refrigerated or frozen.

7.2.3 Test sample preparation

A minimum of two portions of each submitted sample should be extracted. Samples should be allowed to reach assay temperature (20 °C to 25 °C) before processing.

ESADDSOL (5.2.2) is required for samples containing polyphenols such as: dark chocolate, berries, coffee, tea etc. Other samples can be extracted with the routine Extraction Solution.

After the sample is added to the extraction solution the pH of the mixture should be in the range of 6,8 to 7,4. If it is below this range then add 1 mol/l NaOH drop by drop until the correct pH is achieved. If it is above the 6,8 to 7,4 range then add 1 mol/l HCl drop by drop until the pH is in range.

Solid Samples: The sample should be ground to a fine consistency to provide a homogenous mixture. Routinely 5 g of sample is extracted in 50 ml of the extraction solution. If necessary this can be altered as long as the ratio of 1 g of sample to 10 ml of the extraction solution is maintained. The sample/extraction solution mixture should be blended or mixed until homogenous and only minimal clumps are present.

Liquid Samples: Liquid samples should be thoroughly mixed. Routinely 5 ml of sample is added to 45 ml of the extraction solution. If necessary this can be altered as long as the ratio of 1 ml of sample added to 9 ml of the extraction solution is maintained. The sample/extraction solution mixture should be blended or mixed until homogenous.

Extraction: All samples are extracted at 60 °C. Incubate for 5 min then shake/mix for 1 min. Repeat two more times (total incubation time is 15 min). Allow the extract to cool to room temperature (20 °C to 25 °C) and settle. Samples may be centrifuged speeds up to 500 *g* for 5 min. The aqueous phase is used for the ELISA procedure.

Sample extracts should be analysed promptly, or they may be stored refrigerated for up to 24 h. If longer delays before analysis are expected, then aliquots of the aqueous phase should be stored frozen at – 20 °C.

7.3 Immunoassay procedure/Operational Scheme

7.3.1 General

WARNING — Avoid skin and eye contact with all reagents. Dispose of reagents in accordance with local best practice. Stop Solution contains 1 mol/l phosphoric acid. TMB Substrate contains tetramethylbenzidine and hydrogen peroxide. Concentrates of the extraction solutions and wash buffer contain 0,4 % Thiomersal as a preservative. Standards and negative control contain 0,2 % Thiomersal as a preservative. Natural ventilation is sufficient.

The assay should be performed in the temperature range of 20 °C to 25 °C. Reagents should be allowed to reach this temperature range before the assay is commenced. If room temperature falls outside this range then an incubator should be used.

Avoid exposure to direct sunlight.

For quantitative results the full range of standards should be included with each run. Each standard and sample extract should be run in duplicate. It is also recommended to include a positive internal control sample or reference material if available.

Each wash step comprises of:

— Invert the plate and flick the contents out into a sink or discard container.

— Using a wash bottle fill each well to the brim with the working strength wash solution, then invert the plate and flick out the contents.

— Repeat four times (a total of 5 washes).

— Then tap thoroughly onto absorbent paper towels to remove excess wash solution.

An automated plate washer with a 5 wash cycle may be used as an alternative. Washing with multi-channel pipettes is not recommended. Do not allow the plate to totally dry out during the assay.

7.3.2 Assay Steps

— Insert sufficient antibody-coated wells for each standard and sample into a test strip holder. If more than 2 strips are required then the use of a multichannel pipette is recommended to minimize timing errors.

— Record the position of each standard, control and sample.

— Add 100 µl of each standard solution, control and sample extract to their allocated well. Mix by moving the strip holder sideways for 10 s.

— Incubate at room temperature for 10 min then wash.

— Add 100 µl of the enzyme conjugate to each well. Mix by moving the strip holder sideways for 10 s.

— Incubate at room temperature for 10 min then repeat the wash step.

— Add 100 µl of substrate to each well. Mix by moving the strip holder sideways for 10 s.

— Incubate at room temperature for 10 min. Do not wash.

— Add 100 µl of Stop Solution to each well. Mix thoroughly by moving the strip holder sideways for 10 s.