PD CEN/TS 15633-2:2013

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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

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National foreword

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A list of organizations represented on this committee can be obtained on request to its secretary.

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Produits alimentaires - Détection des allergènes alimentaires par des méthodes d'analyse immunologiques - Partie 2: Détermination quantitative de la présence de noisette par un immuno-essai enzymatique à l'aide d'anticorps monoclonaux et détection des protéines avec l'acide bicinchoninique

Lebensmittel - Nachweis von Lebensmittelallergenen mit immunologischen Verfahren - Teil 2: Quantitative Bestimmung von Haselnuss mit einem Enzym-Immunoassayverfahren unter Verwendung von monoklonalen Antikörpern und Proteindetektion mit Bicinchoninsäure

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Foreword

This document (CEN/TS 15633-2:2013) 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 document consists of the following parts:

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

— CEN/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*.

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Introduction

Hazelnuts (*Corylus avellana*) have a wide distribution in food industry, especially in chocolate and nougat production. In these cases, the content of hazelnut determines the quality of a product. Hazelnuts are also frequently used in confectionaries, bakery products, biscuits, breakfast cereals and ice-creams.

Unfortunately, hazelnuts are one of the major causes of food allergy. The amount of hazelnut which causes an allergic reaction depends on the sensitivity of the individuals. Even consumption of a few milligrams of hazelnut can induce allergic reactions in highly sensitive allergic consumers. Amounts ranging from 0,7 mg/kg to 100 mg/kg can induce reactions in sensitised individuals [1]. Symptoms of an allergic reaction include local itching of the mouth and throat to severe life-threatening anaphylaxis. Thus deliberately added non-declared hazelnuts in food products are particularly dangerous. Also trace amounts of hazelnuts or nougat, as a result of cross contamination, pose a health risk.

The allergy is caused among other proteins by glycoproteins like corylin, an 18 kDa storage protein contained in the hazelnut, which is similar to the Cor a1-antigen of hazelnut pollen and homologous to the Bet v1 antigen of birch pollen. Corylin is one of the main allergenic proteins beside Cor a8, Cor a9 and Cor a11 as representatives of seed storage and lipid transfer proteins (LTP-proteins). Corylin is differentiated between pollen associated allergy and non-pollen associated allergy.

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1 Scope

This Technical Specification specifies an enzyme linked immunsorbent assay (ELISA) method for the determination of hazelnut from food samples. In the ELISA the antibodies bind to hazelnut proteins from the food sample. The result of the ELISA is given in mg hazelnut/kg (ppm) because the calibrators consist of an extract of whole hazelnut.

Matrices like cereals, ice cream, cookies, chocolate, sausage, cottage cheese, yogurt and salad dressing were validated by spiking experiments with a carboxymethylcellulose-suspension containing hazelnut paste [2].

The monoclonal antibodies, raised against the whole aqueous extract of hazelnut, detect proteins with approximate molecular weights of 14 kDa, 18 kDa, and 42 kDa. The antibodies detect the major thermostable allergen Cor a9 (11S storage protein). Both antibodies were evaluated by western blots with partially purified hazelnut extracts and purified allergenic proteins.

The ELISA test method is commercially available[1)]. The performance has been validated by an in house validation performed by the manufacturer. All parameters of interest are indicated.

In addition, the ELISA was successfully validated by a collaborative study in order to determine the interlaboratory reproducibility. This ring trial was organised 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. Fourteen German laboratories participated in this collaborative study.

2 Principles

A direct sandwich ELISA is used for detection of hazelnut. The basis of the test is an antigen-antibody reaction. Two hazelnut specific monoclonal antibodies are used to detect the analyte. The antibodies recognise the hazelnut specific protein Cor a9. A microtiter plate is coated with the capture monoclonal antibody mouse anti Cor a9 antibody. Hazelnut standards provided with the kit or sample extracts are incubated for 10 min. After washing, a detector monoclonal antibody mouse anti Cor a9 antibody, labelled with peroxidase, is added as the enzyme conjugate for further 10 min. The conjugate binds to the hazelnut protein antibody complex on the plate. Any unbound enzyme conjugate is then removed by a washing step. Chromogen/substrate is added to the wells and incubated for 10 min. Bound enzyme converts the chromogen into a blue coloured product. The addition of stop reagent inhibits the enzymatic process and causes a shift of the coloured product to yellow. Absorbance measurement is performed at 450 nm against air. The resulting absorbance values are proportional to the concentration of hazelnut of a sample. The result is expressed as hazelnut in mg/kg. The standard stock solution used is an aqueous hazelnut extract of six different varieties of hazelnut (Hallesche Riesen, Levantiner, Kerassunder, Piemonteser, round Römer, Barcelona Giants). These six varieties, raw and roasted, are representative for the hazelnuts used in food products world-wide by food industry. The standard stock solution is further diluted (see 3.1.2). The extract from the different hazelnuts has a protein content of approx. 9 % protein, measured by the photometric protein determination method according to BCA (Pierce).

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1) RIDASCREEN®FAST Hazelnut is the trade name of a product supplied by R-Biopharm AG, Darmstadt, Germany. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by CEN-CENELEC of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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3 Reagents

The assay can be performed in a standard laboratory environment. The test kit shall be stored under cool conditions (4 °C to 8 °C).

3.1 Reagents usually provided with the test kit.

All reagents used for preparing the components and buffers shall be of analytical grade.

3.1.1 Microtiter plate, 48 wells (6 strips with 8 wells each) coated with anti-Cor a9 monoclonal antibody.

3.1.2 Standards, 1,3 ml each, namely 0 mg/kg (zero standard), 2,5 mg/kg , 5 mg/kg , 10 mg/kg and 20 mg/kg hazelnut in aqueous solution; ready to use. (These concentrations correspond to the actual hazelnut amounts of 0 ng/ml, 125 ng/ml, 250 ng/ml, 500 ng/ml and 1000 ng/ml hazelnut in the vials).

3.1.3 Conjugate, 11-fold concentrated aqueous solution of horseradish peroxidase labelled detector anti-Cor a9 monoclonal antibody. The amount, which is necessary, has been determined by titration. The conjugate buffer consists of 10 mmol/l phospate buffer one plus one mixed with Stabilzyme®[2)] containing finally 150 mmol/l saline, 5 % sorbitol, 2 % BSA (bovine serum albumin) 0,1 % Kathon®[2)], Tartrazin/Patent blue as color.

3.1.4 Chromogen/Subtrate, coloured, ready to use Tetramethylbenzidine (TMB)/urea peroxide solution (commercial product provided by e. g. KemEnTec, Denmark).

3.1.5 Stop reagent, 1 mol/l sulphuric acid ready to use solution.

3.1.6 Sample extraction buffer, 20-fold concentrated, PBS-Tween buffer, diluted to 0,01 mol/l phosphate buffer containing 0,9 % saline, 0,05% Tween 20 Ph (8,0 ± 0,2), store at 4 °C to 8 °C over the shelf life of the component (at least 36 months).

3.1.7 Washing buffer, 10-fold concentrate, PBS buffer, finally consisting of 0,01 mol/l phosphate buffer, containing 0,9 % saline, 0,01 % Synperonic®[2)], 0,01 % Thimerosal Ph (7,2 ± 0,2), store at 4 °C to 8 °C over the shelf life of the component (at least 18 months).

3.2 Chemicals not supplied with the test kit

3.2.1 Distilled water

Mono-distilled water or purified by reverse osmosis.

3.2.2 Skim milk powder (food grade like offered in a normal supermarket).

It is necessary to make sure that the skim milk powder is hazelnut free.

4 Apparatus and equipment

Usual laboratory equipment should be used and in particular as listed in 4.1 to 4.10:

4.1 Temperature controlled water bath, capable for maintaining (37 ± 4) °C and (60 ± 5) °C.

4.2 Centrifuge, capable for producing a centrifugal acceleration of at least 2 500 *g* at 4 °C.

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2) Stabilzyme®, Kathon®, Synperonic® and Vortex® are examples of suitable products available commercially. This information is given for the convenience of the users of this standard and does not constitute an endorsement by CEN-CENELEC of these products.

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4.3 Microtiter plate reader, capable of reading absorbance at 450 nm.

4.4 Analytical Balance, capable of weighing gram amounts (max. (150 ± 0,01) g).

4.5 Laboratory mill/grinder

4.6 Precision micropipettes, capable of delivering 20 µl to 200 µl and 200 µl to 1 000 µl.

4.7 Mixer, e.g. Vortex®[2)].

4.8 Multi-channel pipette or a repetitive pipette (25 µl to 1 000 µl) (optional).

4.9 Reagent reservoirs for multi-channel dispensing (optional).

4.10 Automated plate washer (optional).

5 Procedure

5.1 Warnings or precautions

WARNING — Wash buffer (thiomersal), harmful by inhalation in contact with skin and if swallowed. Chromogen/substrate (tetramethylbenzidine/urea peroxide), avoid skin and eye contact. Stop solution contains diluted sulfuric acid (1 mol/l), irritating and corrosive, avoid skin and eye contact. Chemicals should be treated with care. Waste shall be disposed according to good laboratory practice.

5.2 Sample collection, transport, preservation and storage

From the whole food aggregated samples shall be collected (according to a sampling protocol). The aggregated sample (combination of different parts of the food) is mixed and the laboratory samples are taken. The sample material is mixed very well to ensure homogeneity of the sample prior to weighing. The food stuff should be transported in sealed bags or closed vials to prevent cross contamination. The samples should be stored in a cool room until use, unless the sample requires freezing (e.g. ice cream) or storage at room temperature (e.g. muesli).

5.3 Sample preparation

Sample preparation shall be carried out according appropriate instructions. An example for an appropriate sample preparation for the test procedure described in this Technical Specification the following:

— collect 5 g of the food sample and ground as fine as possible by high speed for 3 min to 5 min (the temperature of the sample should not be higher than 40 °C); chocolate should be melted at 30 °C to 40 °C and mixed well before weighing;

— weigh 1 g of the milled or melted sample and add 1 g of skim milk powder (3.2.2);

— add 20 ml of pre-heated diluted extraction buffer; mix intensively;

— extract 10 min at 60 °C in a water bath by shaking casually (4.1);

– cool down to room temperature (if possible on ice), centrifuge (10 min at 2 500 *g* at 4 °C (if possible)) and/or filter the extract on a folded paper filter (3 hw grade or similar grade) to avoid particles in the extract;

– use 100 µl of the extract per well.

Sample extracts can be stored at –20 °C for three months.

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5.4 Method Performance

5.4.1 General

All reagents shall be brought to room temperature (20 °C to 25 °C) prior to use. All reagents shall be returned to 2 °C to 8 °C immediately after use. Microwells shall not dry between working steps. Reproducibility in any ELISA is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the ELISA test procedure. Direct exposure to sunlight during all incubations shall be avoided. Covering the microtiter plates is recommended. Chromogen/Substrate reaction should be carried out in the dark.

5.4.2 Physical/environmental conditions

No special laboratory conditions are required. Equipment for standard biochemical working condition is needed.

5.4.3 Instrument calibration

The pipettes shall be calibrated and the laboratory balance shall be checked regularly as written in the laboratories' quality management documents. The microtiter plate reader should be calibrated according to the laboratories' quality management documents.

5.4.4 Cleanliness of work area

The sample preparation should be performed in a preparation room, separate from the ELISA room to avoid cross contamination to the kit components. Laboratory equipment shall be clean and free from hazelnut residues. After each sample is weighed, the equipment (e. g. spatula, mills) shall be cleaned to avoid cross contamination.

5.5 Preparation of reagents

5.5.1 Antibody Enzyme Conjugate

The conjugate is a 11fold concentrate solution. Since the conjugate has a limited stability only the amount that is required should be diluted. Before diluting the concentrated conjugate should be shaken carefully. The conjugate shall be diluted 1 + 10 with distilled water (3.2.1).

5.5.2 Washing Buffer

The washing buffer is a 10-fold concentrate solution. Before use the buffer shall be diluted 1:10 (1 + 9) with water (i.e. 100 ml buffer concentrate solution + 900 ml distilled water). The diluted ready-to-use buffer is stable at 2 °C to 8 °C for four weeks. Before dilution, dissolve any crystals of the concentrated buffer in a water bath at 37 °C completely.

5.5.3 Sample Extraction Buffer

The sample extraction buffer is a 20-fold concentrated solution. Before dilution of the buffer concentrate solution, dissolve any crystals in a water bath at 37 °C completely and mix well. Then dilute the heated buffer concentrate 1:20 (1 + 19) with dist. water (3.2.1) before use (i. e. 100 ml buffer concentrate + 1 900 ml water) or alternatively follow the kit manufacturer's instruction. The diluted buffer is stable at 2 °C to 8 °C for max. four weeks.

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5.6 Test performance

Insert a sufficient number of wells into the microwell holder. Not more than 3 strips shall be done per run for all standards and samples. Standard and sample positions should be recorded. Standards and samples should be run at least in duplicates. Incubation steps shall occur without shaking unless otherwise stated.

Add 100 µl of each standard solution or prepared sample to separate wells and incubate for 10 min at room temperature (20 °C to 25 °C).

Pour the liquid out of the wells and tap the microwell holder upside down vigorously (consecutively three times) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with approximately 250 µl washing buffer (3.1.7) and pour out the liquid again. Repeat four more times.

Add 100 µl of the diluted enzyme conjugate to each well. Mix gently by rocking the plate. Incubate for 10 min.

Pour the liquid out of the wells and tap the microwell holder upside down vigorously (consecutively three times) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with approx. 250 µl washing buffer and repeat four more times as mentioned above.

Add 100 µl of red colored chromogen/substrate solution to each well.

Mix gently by rocking the plate manually and incubate for 10 min at room temperature (20 °C to 25 °C) in the dark.

Add 100 µl stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 30 min after addition of stop solution.

5.7 Reading/interpretation and test result report

Sample concentrations are calculated on the basis of the standard curve. The curve is generated with an aqueous hazelnut extract corresponding to 0 mg/kg, 2.5 mg/kg, 5 mg/kg, 10 mg/kg, and 20 mg/kg hazelnut (real concentrations see 3.1.2), whereas the extraction dilution factor is already included. Absorbances obtained for hazelnut extract are plotted into a system of coordinates onto semi-logarithmic graph paper versus hazelnut concentration (mg/kg) manually or by suitable software applying cubic spline fitting or 4-parameter curve logistic. Sample results are expressed in mg/kg hazelnut as indicated on the standard vials. The dilution factor of 20 is already included in the standard concentration. If a dilution factor other than 20 is used, this shall be taken into account when calculating the result. All results of the internal study were obtained with commercial available software (RIDA®SOFT Win, Version 1.34, R-Biopharm AG).

A positive internal control sample or a reference material is recommended with each test run. The result shall be expressed as mg/kg hazelnut. The hazelnut content can be converted into approximately hazelnut protein content by multiplying by the factor 0,09. (The protein content of the standard is approximately 9 %. This was measured by the BCA method.) For those who will prepare their own standards, the protein content shall be determined e.g. using BCA assay or equivalent.

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