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BSI Standards Publication

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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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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Contents

Page

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.

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).

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.

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.

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.

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.

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.

5.8 Flowcharts

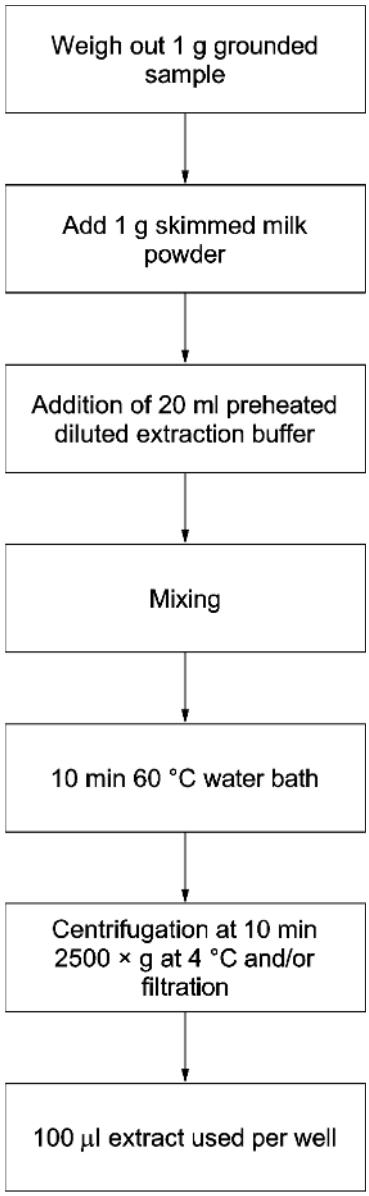


Figure 1 — Extraction flowchart

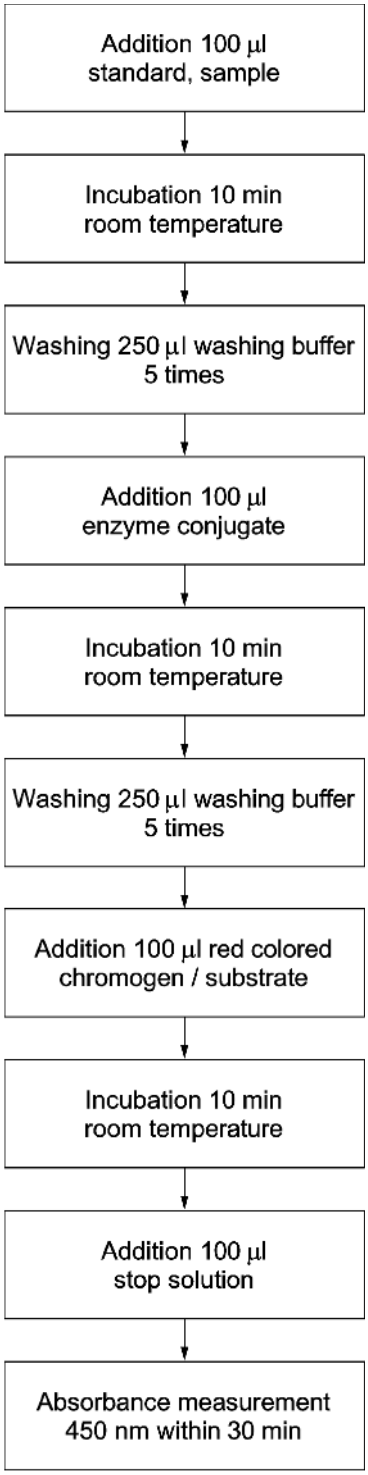


Figure 2 — ELISA procedure flowchart

6 Evaluation

6.1 Summary of validated performance characteristics

The performance characteristics of the presented Hazelnut ELISA meet the following specification:

Time required for completion of the sample preparation was below 20 min and less than 40 min for the test implementation.

Specificity of the test with hazelnut-free blank samples was 100 % (no false positive samples were detected).

Analytical Sensitivity was found at LOD < 1,5 mg/kg hazelnut as measured by 10-fold determination of various hazelnut-free food matrices. LOQ was set at 2,5 mg/kg hazelnut, which was confirmed by multiple measurement of a sample contaminated close to that value. Accuracy was investigated with spiked samples of various matrices at different defined levels between 2,5 mg/kg and 20 mg/kg hazelnut. Mean recovery was found at 98,2 %.

The ELISA is not sensitive to temperature changes between 18 °C and 37 °C.

The ELISA is not sensitive to variation of incubation time between 3 × 9 min and 3 × 11 min.

The ELISA is not sensitive to small changes of reagent volumes between 90 µl and 110 µl.

The test kit components are stable as indicated on the test kit labels (shelf life is usually more than 12 months).

This Hazelnut ELISA was successful evaluated by a collaborative study performed by the working group "Lebensmittel-Allergene" (Food Allergens) of the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (Federal Office of Consumer Protection and Food Safety, BVL), Germany, for implementing LFGB, using dark chocolate as matrix. Four spiked samples at different levels (in the range of 2,5 mg/kg to 20 mg/kg) have been investigated; a mean recovery of 106,3 % has been calculated. The RSD_r was calculated at 9,5 % and the RSD_R at 17,3 %.

6.2 Internal validation (manufacturer's in house study)

To determine the precision three samples (one negative and two hazelnut containing samples) have been included into the experiments. The mean, the standard deviation (SD) and the recovery have been calculated concerning the intra-assay and the inter-assay variance. Over all a mean recovery was found at 99,3 % with a CV (%) of 8,7 %.

The limit of detection (LOD) of the Hazelnut ELISA, evaluated with four clear negative kinds of samples (cookies, cereals, ice cream and chocolate), was measured close to standard 1 (zero mg/kg) at 0,99 mg/kg under consideration of the three times standard deviation and was set at 1,5 mg/kg hazelnut.

The limit of quantitation (LOQ) was carried out with a sample, which had a hazelnut content of about 2,5 mg/kg, near standard two. The mean value and the standard deviation of the sample and of standard 2 have been calculated and compared with standard 1 under consideration of the three times standard deviation. It could be shown that the test is able to determine samples near standard point 2 sure, significant different from zero.

Zero samples and spiked samples on a range of 2,5 mg/kg to 20 mg/kg hazelnut were analysed with the test for determination of accuracy. Mean value and recovery have been calculated. A recovery over all of 97,8 % was found. Hazelnut free samples have been validated clearly negative, no false positive results occur.

Cross reactivity of the test was checked with 43 different compounds of interest (grains, vegetables, seeds, nuts and miscellaneous samples). All samples were extracted with the extraction buffer included in the kit. As a result most of the compounds were measured close to standard one without any measurable cross reaction. Some of the substances showed reactivities at a 100 % level. These compounds have been repeated at a level of 100 mg/kg (0,01 %) related to the AOAC Research Institute validation requirements for performance tested methods [3]. All of the tested compounds have been measured well below standard 2 at the level of 100 mg/kg (0,01 %). Matrices which have been checked were:

— cereals like corn, rice, wheat, rye, barley, oats, buckwheat;

— seeds like sesame, linseed and poppy seed;

— nuts like walnut, almond and cashew;

— vegetable like peas and beans varieties;

— processed products like skim milk powder and gelatine.

Ruggedness experiments were designed to elucidate the test performance of the Hazelnut ELISA, when small changes above and below the environmental and operating parameters specified in the leaflet are introduced. Time, temperature and pipetting volume have been varied. No influences of these parameters have been found on the test result.

Data are presented in Annex A.

6.3 Collaborative trial

Within the aims to investigate methods for hazelnut detection in food with an LOD of approximate 2 mg/kg, a ring trial for the determination of hazelnut in dark chocolate was initiated in Germany.

A total of 10 blind coded dark chocolate samples (20 % cocoa content) were sent to 14 German laboratories each. The set of samples contained two blank samples and eight samples spiked with hazelnuts (roasted 20 min at 150 °C). The samples were spiked at 2 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg hazelnut. Each spike level was sent as two individual samples. Each sample was extracted once according to the extraction procedure with pre-heated extraction buffer for 10 min at 60 °C. The samples were measured twice in two independent runs. The raw data were evaluated with commercial available software (RIDA®SOFT Win, Art. No Z9999, version 1.34, R-Biopharm AG). A statistical evaluation was made without elimination of outliers. Mean recovery values of the two series of four spiked samples shown in Table B.1 were calculated at 106,3 %. The precision parameters of the ring trial are presented in Table B.2. Overall a relative repeatability RSD_r was validated at 9,5 % and the relative reproducibility RSD_R at 17,3 %. All blank samples were negative.

Data are presented in Annex B.

Annex A
(informative)

Internal validation (manufacturer's in house study)

A.1 Precision (intra- and inter-assay variance)

A.1.1 Intra-assay variance

The variance within one run was measured as a six fold determination of the standards and a ten-fold determination of one negative and four hazelnut containing samples at two levels of 5 mg/kg and 10 mg/kg. The results are shown in Table A.1 and A.2. The mean intra-assay CV (%) over the standard curve was calculated with 9,9 %, over all samples with 7,1 %.

Table A.1 — Intra-assay variance of standards (n = 6)

Standard concentration mg/kg	**Mean absorbance**	**SD**
0	0,097	0,007
2,5	0,220	0,034
5	0,279	0,046
10	0,624	0,028
20	1,544	0,086
Mean CV (%)		**9,9**

Table A.2 — Intra-assay variance of samples (n = 10)

Matrix	**Spike** mg/kg	**Concentration measured** mg/kg	**SD**	**CV** %
Nut-free chocolate	0	< 2,5		
Chocolate	10	9,9	0,532	5,4
Cereals	5	4,1	0,530	12,9
Ice cream	5	5,9	0,236	4,0
Ice cream	10	9,9	0,582	5,9
Mean			**0,470**	**7,1**

A.1.2 Inter-assay variance

The inter-assay variance was investigated with the five standard solutions in duplicates in 6 independent runs (Table A.3). The mean CV (%) over all standard curves is calculated at 12,8 %.

Three samples have been measured, one of them as a hazelnut free sample and two hazelnut spiked samples at two different levels (5 mg/kg and 10 mg/kg) (Table A.4). The recovery of the spiked samples has been calculated at 99,3 %. The mean CV over the standard curve has been determined at 12,8 % and over the samples at 5 %.

Table A.3 — Inter-assay variance of six independent runs of standards (n = 2 per run)

	Std conc. 0 mg/kg	**Std conc. 2,5** mg/kg	**Std conc. 5** mg/kg	**Std conc. 10** mg/kg	**Std conc. 20** mg/kg
Run 1	0,120	0,282	0,370	0,797	1,885
Run 2	0,138	0,279	0,392	0,908	1,936
Run 3	0,100	0,338	0,469	0,954	1,985
Run 4	0,140	0,300	0,405	0,877	1,897
Run 5	0,174	0,367	0,448	0,925	1,924
Run 6	0,107	0,221	0,361	0,703	1,715
Mean absorbance	0,130	0,298	0,408	0,861	1,890
SD	0,026	0,050	0,043	0,094	0,092
CV (%)	20,7	17,1	10,5	10,9	4,9
Mean SD over all	**0,061 2**		**Mean CV (%) over all**		**12,8**

Table A.4 — Inter-assay variance of spiked samples (six independent runs)

Matrix	**Spike** mg/kg	**Mean concentration measured** mg/kg	**SD**	**CV** %
White chocolate	0	< 2,5		
Cereals	5	4,8	0,351	7,4
Ice cream	10	10,2	0,252	2,5
Mean SD over all			**0,302**	
Mean CV (%) over all				**5,0**

A.2 Sensitivity

A.2.1 Limit of detection (LOD)

All the tested samples have been extracted with sample extraction buffer. A total of 10 replicates/sample matrices were investigated. LOD was calculated per definition as the concentration equal to the mean value + 3 × standard deviation of the corresponding mg/kg values of the measurements (see Tables A.5 to A.8). Single concentration values were all found well below standard 2 (2,5 mg/kg) of the Hazelnut ELISA. One of the matrices (chocolate) was found to be very close to standard 1, an extrapolation of the corresponding mg/kg data was not possible.

Finally all matrices investigated were calculated at LOD-values close to zero as mg hazelnut/kg sample. The mean limit of detection over the four matrices was calculated at 0,99 mg/kg hazelnut (3s) (Table A.9) with a range of 0,8 mg/kg to 1,25 mg/kg and was set at 1,5 mg/kg hazelnut.

Table A.5 — Limit of detection with hazelnut free butter cookies

Sample ID	Matrix	Absorbance	Hazelnut mg/kg[a]
HN-430-1	Butter cookies	0,244	1,2
HN-430-2		0,225	1,1
HN-430-3		0,237	1,2
HN-430-4		0,211	1,1
HN-430-5		0,232	1,1
HN-430-6		0,233	1,1
HN-430-7		0,233	1,1
HN-430-8		0,220	1,1
HN-430-9		0,231	1,1
HN-430-10		0,216	1,1
Mean		**0,225**	**1,12**
SD		**0,01**	**0,042**
3 · SD		**0,03**	**0,127**
LOD (mean + 3 · SD)		**1,25 mg/kg**	

[a] Zero standard has been measured with absorbance = 0,109 (n=2). Standard 2 with 0,569.

Table A.6 — Limit of detection with hazelnut free ice cream

Sample ID	Matrix	Absorbance	Hazelnut mg/kg[a]
HN-429-1	Ice cream	0,150	0,9
HN-429-2		0,164	0,9
HN-429-3		0,152	0,9
HN-429-4		0,153	0,9
HN-429-5		0,157	0,9
HN-429-6		0,173	1,0
HN-429-7		0,153	0,9
HN-429-8		0,169	0,9
HN-429-9		0,165	0,9
HN-429-10		0,166	0,9
Mean		**0,160**	**0,914**
SD		**0,01**	**0,031**
3 · SD		**0,03**	**0,094**
LOD (mean + 3· SD)		**1,01 mg/kg**	

[a] Zero standard has been measured with absorbance = 0,086 (n=2). Standard 2 with 0,563.

Table A.7 — Limit of detection with hazelnut free cereals

Sample ID	Matrix	Absorbance	Hazelnut mg/kg[a]
HN-544-1	Cereals	0,098	0,9
HN-544-2		0,100	0,9
HN-544-3		0,087	0,9
HN-544-4		0,089	0,9
HN-544-5		0,086	0,9
HN-544-6		0,089	0,9
HN-544-7		0,085	0,9
HN-544-8		0,098	0,9
HN-544-9		0,090	0,9
HN-544-10		0,094	0,9
Mean		**0,092**	**0,9**
SD		**0,01**	**0**
3 · SD		**0,03**	
LOD (mean + 3 · SD)		**0,9 mg/kg**	

[a] Zero standard has been measured with absorbance = 0,097 (n=2). Standard 2 with 0,594.

Table A.8 — Limit of detection with hazelnut free chocolate

Sample ID	Matrix	Absorbance	Hazelnut mg/kg[a]
HN-526-1	Chocolate	0,090	< std 1
HN-526-2		0,103	< std 1
HN-526-3		0,095	< std 1
HN-526-4		0,083	< std 1
HN-526-5		0,090	< std 1
HN-526-6		0,102	< std 1
HN-526-7		0,103	< std 1
HN-526-8		0,099	< std 1
HN-526-9		0,106	< std 1
HN-526-10		0,099	< std 1
Mean		**0,097**	
SD		**0,003**	
3 · SD		**0,009**	
LOD (mean + 3 · SD)		**0,8 mg/kg[b]**	

[a] Zero standard has been measured with absorbance = 0,102 (n=2). Standard 2 with 0,603.

[b] Extrapolated from the mean absorbance + 3 · standard deviation.

Table A.9 — Limit of detection. Summary of four main matrices

Matrix	Mean	SD	LOD (mean + 3 · SD)
Butter cookies	1,120	0,042	1,25
Ice cream	0,914	0,031	1,01
Cereals	0,900	0	0,9
Dark chocolate	< std 1	not calculable	0,8
LOD mean over all			**0,99 mg/kg**

A.2.2 Limit of quantitation (LOQ)

The limit of quantitation (LOQ of 2,5 mg/kg hazelnut) was verified by analysing 8 replicates of a food sample, which contains a hazelnut concentration close to standard 2 (2,5 mg/kg hazelnut). The sample HN-499, a hazelnut-contaminated chocolate (about 2,5 mg/kg hazelnut), has been extracted according to the described sample extraction procedure. In parallel standard 1 (zero) and standard 2 (2,5 mg/kg hazelnut) have been measured eight times to determine the corresponding variance (Table A.10). The variation of standard 1 (0 mg/kg hazelnut) was confirmed with an absorbance of $0{,}082 \pm 0{,}008$ ($3 \cdot$ SD). The hazelnut containing sample HN-499 was measured significantly above zero (standard 1) at 2,31 mg/kg with a small CV of 4,7 % similar to the precision of standard 2 (CV = 3,7 %), highly acceptable for a precise measurement. The measured sample within the range of standard 2 is significantly different from zero considering the three times standard deviation.

Table A.10 — Determination of the limit of quantitation with a hazelnut containing chocolate

Replicate (n = 8) measurement of standard 1, standard 2 and sample HN-499

Replicates	Standard 1	Standard 2		Sample HN-499	
	Absorbance	Absorbance	Concentration mg/kg	Absorbance	Concentration mg/kg
1	0,082	0,411	2,54	0,400	2,46
2	0,080	0,423	2,42	0,408	2,36
3	0,080	0,410	2,39	0,412	2,24
4	0,058	0,380	2,61	0,388	2,52
5	0,081	0,434	2,54	0,366	2,20
6	0,088	0,415	2,64	0,389	2,14
7	0,083	0,419	2,30	0,368	2,21
8	0,081	0,392	2,58	0,358	2,37
Mean	**0,082**	**0,406**	**2,5**	**0,381**	**2,31**
SD	**0,003**	**0,015**	**0,119**	**0,018**	**0,136**
3 · SD	**0,008**	**0,045**	**0,358**	**0,053**	**0,407**
CV (%)	**3,2**	**3,7**	**4,8**	**4,7**	**5,9**

A.3 Accuracy/Trueness

Various matrices have been spiked with a hazelnut containing carboxymethylcellulose-suspension according to M. Trucksess. *et al.* [2] at 2,5 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg hazelnut levels. In parallel a series of hazelnut free compounds have been measured (Table A.11). These samples have been calculated well below standard 2 in each case. No false positive results have been found.

The recovery of the spiked samples over a range of 2,5 mg/kg to 20 mg/kg hazelnut is given in Tables A.12 and A.13. The mean recovery over all was calculated at 97,8 %.

Table A.11 — Investigation of hazelnut free compounds

ID	Sample	Target value mg/kg	Value found mg/kg
HN-428	White milk chocolate	0	< 2,5
HN-547	Vanilla ice cream	0	< 2,5
HN-549	Buttermilk ice cream	0	< 2,5
HN-568	Yogurt ice cream	0	< 2,5
HN-570	Butter cookies	0	< 2,5
HN-571	Nut free chocolate	0	< 2,5
HN-572	Cereals	0	< 2,5
HN-574	Home-made chocolate	0	< 2,5
HN-582	Dark chocolate I[a]	0	< 2,5
HN-647	Dark chocolate II[a]	0	< 2,5
PN-218	Instant soup	0	< 2,5
PN-214	Sausage	0	< 2,5
PN-253	Cottage cheese	0	< 2,5
PN-216	Salad dressing	0	< 2,5

[a] Two different kinds of chocolate (cocoa content in the same range 15 % to 20 %).

Table A.12 — Investigation of hazelnut spiked compound (2,5 mg/kg and 5 mg/kg)

ID	sample	Target value mg/kg	Value found mg/kg	Recovery %
HN-491	Vanilla ice cream	2,5	2,4 [a]	96
HN-499	Butter cookies	2,5	2,1 [a]	84
HN-535	Nut free chocolate I	2,5	2,4 [a]	96
HN-562	Cookies	2,5	2,5	100
HN613	Nut free chocolate II	2,5	2,7	108
HN-6791	Cereals	2,5	2,4 [a]	96
HN-484	Home-made chocolate	5	5,3	106
HN-496	Ice cream	5	5,6	112
HN-500	Butter cookies	5	5,1	102
HN-680	Cereals I	5	5,0	100
HN-555	Nut free chocolate I	5	4,7	94
HN-621	Dark chocolate	5	5,4	108
HN-656	Salad dressing I	5	5,1	102
HN-658	Salad dressing II	5	4,6	92
HN-662	Cottage cheese	5	4,8	96
HN-664	Instant soup	5	4,8	96
HN-688	Cookies	5	4,8	96
HN-672	Dark chocolate	5	4,6	92
HN-676	Banana Yoghurt	5	4,1	82
HN-680	Cereals II	5	5,0	100
Sam-17	Sausage	8	10,7	134

[a] The result is lying below the LOQ and above the LOD. This means that the results have only been estimated.

Table A.13 — Investigation of hazelnut spiked compounds (10 mg/kg, 15 mg/kg, 20 mg/kg)

ID	sample	Target value mg/kg	Value found mg/kg	Recovery %
HN-433	Home-made chocolate	10	11,4	114
HN-436	Vanilla ice cream	10	11,7	117
HN-497	Buttermilk ice cream	10	9,5	95
HN-501	Butter cookies	10	10,5	105
HN-509	Cereals I	10	10,0	100
HN-622	Dark chocolate I	10	9,7	97
HN-655	Dark chocolate II	10	10,3	103
HN-657	Salad dressing I	10	7,7	77
HN-659	Salad dressing II	10	8,5	85
HN-661	Cottage cheese	10	10,1	101
HN-665	Instant soup	10	8,5	85
HN-681	Cereals II	10	9,2	92
HN-486	Home-made chocolate	15	14,8	99
HN-498	Ice cream	15	15,0	100
HN-5028	Butter cookies	15	14,0	93
HN-668	Instant soup	15	12,8	85
HN-591	Dark chocolate I	20	28,7	144
HN-623	Dark chocolate II	20	16,8	84
HN-682	Cereals	20	23,6	118

Table A.14 — Summary of the mean values, standard deviation (SD) and recovery of hazelnut spiked compounds (N=41)

	Range 1	Range 2	Range 3	Range 4
Target value (mg/kg)	2,5	5	10	15
Value found (mg/kg)	2,4	4,9	10,2	14,2
Amount of samples	6	14	14	4
SD (mg/kg)	0,19	0,38	1,13	1,22
Uncertainty lower limit	2,02	4,14	7,94	11,76
Uncertainty upper limit	2,78	5,66	12,46	16,64
Recovery (%)	96	98	102	95
Mean recovery (%)	**97,8**			

A.4 Specificity/Selectivity (Interferences)

Test specificity was investigated with a variety of 43 compounds including cereals, nuts, vegetables and fruits. All samples have been milled and prepared according to the instruction extraction procedure and measured in the test without further dilution as a 100 % sample (1 g/20 ml extracted). Most of the samples react negative when the pure substances (100 % level) were analysed (Table A.15 and A.16).

An elevated cross-reactivity at the 100 % level was observed for sunflower kernels, raw and roasted almond and apricot kernel. These samples have been re-analysed at a level of 100 mg/kg (0,01%) of each compound. As can be seen in Tables A.15 and A.16 no further cross-reactivity was observed at this concentration level.

The cross reactivity of sunflower kernel, raw and roasted almond and of the apricot kernel have been calculated well below 0,02 % (Table A.17) This was commonly calculated by comparing the cross-reactive substance dilution row and the standard curve at 50 % inhibition as mg/kg.

NOTE Cross reaction to almond and apricot kernel was observed in the internal validation with version 06-03-21 of the test kit. Following improvement without changing the test system this cross reactivity could be suppressed in the later version 10-01-19k of the test kit.

Table A.15 — Investigation of cross reactivity with various compounds

Sample ID	Compound	Absorbance	found at 100 % level mg/kg	found at 100 mg/kg mg/kg
PN-93	Barley	0,084	< 2,5	
PN-94	Wheat	0,101	< 2,5	
PN-95	Oats	0,097	< 2,5	
PN-96	Maize	0,100	< 2,5	
PN-114	Rye	0,110	< 2,5	
PN-129	Buckwheat	0,063	< 2,5	
PN-131	Rice	0,064	< 2,5	
PN-132	Millet	0,338	< 2,5	
PN-99	Soy Beans	0,082	< 2,5	
PN-107	Sesame seeds	0,187	< 2,5	
Ma-51	Poppy seed	0,097	< 2,5	
PN-102	Sunflower kernel	1,134	11,5	< 2,5
HN-704	Linseed	0,059	< 2,5	
PN-103	Pumpkin kernel	0,112	< 2,5	
PN-108	Pine kernel	0,080	< 2,5	
PN-104	Cashew raw	0,362	< 2,5	
PN-105	Cashew roasted	0,171	< 2,5	
PN-111	Pistachio	0,119	< 2,5	
PN-109	Almond raw[3)]	2,177	> 20	< 2,5
PN-110	Almond roasted[3)]	1,570	17,0	< 2,5
PN-137	Walnut raw	0,084	< 2,5	
Ma-42	Walnut roasted	0,127	< 2,5	
Ma-50	Coconut	0,073	< 2,5	
Ma-37	Brazil nut	0,079	< 2,5	
Ma-38	Pecanut	0,144	< 2,5	
Ma-39	Chestnut	0,082	< 2,5	
Ma-41	Macadamia	0,156	< 2,5	
Pa-3	Peanut mix raw + roasted	0,133	< 2,5	

3) Cross reaction to almond and apricot kernel was observed in the internal validation with version 06-03-21 of the test kit. Following improvement without changing the test system this cross reactivity could be suppressed in the later version 10-01-19k of the test kit.

Table A.16 — Investigation of cross reactivity with various compounds

Sample ID	Compound	Absorbance	found at 100 % level mg/kg	found at 100 mg/kg mg/kg
Ma-175	White Beans	0,066	< 2,5	
Ma-176	Kidney Beans	0,065	< 2,5	
Ma-177	Quail Beans	0,064	< 2,5	
Ma-178	Lentils	0,069	< 2,5	
PN-138	Green Beans	0,114	< 2,5	
PN-140	Lima Beans	0,250	< 2,5	
PN-142	Chicken Peas	0,270	< 2,5	
PN-141	Green Peas	0,072	< 2,5	
PN-133	Cocoa	0,083	< 2,5	
PN-144	Kiwi	0,080	< 2,5	
PN-39	Lecithin	0,068	< 2,5	
PN-134	Bovine Gelatin	0,074	< 2,5	
PN-136	Porcine Geliatin	0,071	< 2,5	
PN-130	Skim milk powder	0,076	< 2,5	
Ma-164	Apricot kernel [4)]	0,878	6,3	< 2,5

Table A.17 — Summary of cross reactivities (%) at 50 % inhibition of the standard curve

Food matrix	Cross reactivity %
Almond. raw[4)]	0.001
Almond. roasted[4)]	0,006
Sunflower kernel	0,019
Apricot kernel[4)]	0,001

A.5 Robustness of the method (Ruggedness)

Ruggedness of the test was investigated with small modifications to the regular test performance. Temperature, volumes and incubation time of the regular procedure of the test kits were varied. All tests were performed including one negative control sample and two positive control samples (cereals and artificial home-made chocolate spiked with 5 mg/kg and 10 mg/kg hazelnut, using a hazelnut containing Carboxymethylcellulose-suspension for spiking).

4) Cross reaction to almond and apricot kernel was observed in the internal validation with version 06-03-21 of the test kit. Following improvement without changing the test system this cross reactivity could be suppressed in the later version 10-01-19k of the test kit.

Three ELISA runs have been performed at each variation step. Mean values of each variation as obtained by the three runs are shown in the following tables. In addition the mean values over all variations and the corresponding standard deviations are indicated (Tables A.18 to A.20).

The variation of temperature from 18 °C to 37 °C as well as a variation of ± 10 % of standard/sample volume and a variation of the incubation time from 3 × 9 min to 3 × 11 min demonstrated that small changes above and below the environmental and operating parameters of the ELISA do not affect the test results.

The results obtained with three control samples have been found in good agreement disregarding the variation of the regular procedure; the overall variance of the test results obtained for three control samples was extremely small. The mean CV over all variation of the two samples has been found as 7,1 %.

Table A.18 — Ruggedness versus temperature (18 °C, room temperature (23 °C to 25 °C) and 37 °C)

Mean values and SD values of three test series are indicated.

Standard/sample	**Concentration**[a] mg/kg	**Mean absorbance of three test series**			**Mean over all tests**	**SD over all tests**
		37 °C	Room temperature (23 °C to 25 °C)	18 °C		
Standard 1	0	0, 076	0,147	0,055	0,093	0,048
Standard 2	2,5	0,241	0,338	0,327	0,302	0,053
Standard 3	5	0,393	0,451	0,632	0,492	0,125
Standard 4	10	0,926	0,890	1,050	0,955	0,084
Standard 5	20	2,180	1,964	1,875	2,006	0,157
	Concentration (mg/kg hazelnut)					
Negative control	White chocolate	< 2,5	< 2,5	< 2,5	negative	
Spike 5 mg/kg	Cereals	5,4	5,4	4,1	4,97	0,762
Spike 10 mg/kg	artif. chocolate	9,1	10,2	10,5	9,94	0,709

[a] Concentration of standard solutions: the dilution factor of sample preparation is included.

Table A.19 — Ruggedness versus incubation time (9 min, 10 min (recommended) and 11 min)

Mean values and SD values of three test series are indicated.

Standard/sample	**Concentration**[a] mg/kg	**Mean absorbance of three test series**			**Mean over all tests**	**SD over all tests**
		3 × 9 min	3 × 10 min	3 × 11 min		
Standard 1	0	0,096	0,147	0,126	0,123	0,026
Standard 2	2,5	0,210	0,338	0,297	0,282	0,065
Standard 3	5	0,304	0,451	0,445	0,400	0,083
Standard 4	10	0,616	0,890	0,945	0,817	0,176
Standard 5	20	2,428	1,964	2,144	2,179	0,234
	Concentration (mg/kg hazelnut)					
Negative control	White chocolate	< 2,5	< 2,5	< 2,5	negative	
Spike 5 mg/kg	Cereals	5,1	5,4	4,4	4,97	0,527
Spike 10 mg/kg	artif. chocolate	10,4	10,2	9,7	10,1	0,361

[a] Concentration of standard solutions: the dilution factor of sample preparation is included.

Table A.20 — Ruggedness versus incubation volume (90 µl, 100 µl (recommended) and 110 µl)

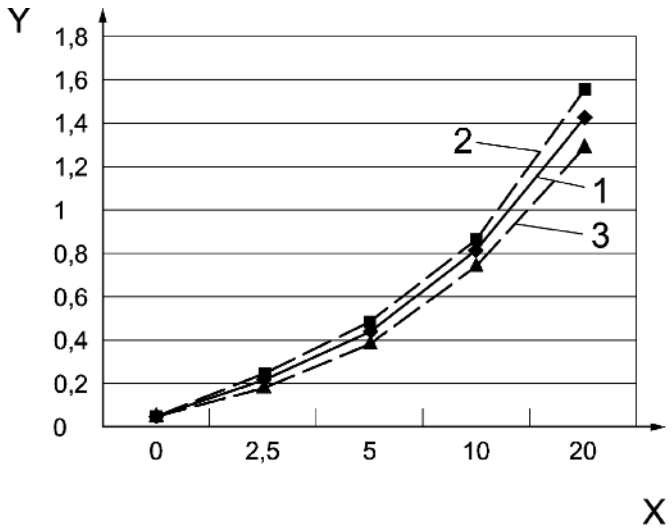
Mean values and SD values of three test series are indicated.

Standard/sample	**Concentration**[a] mg/kg	**Mean absorbance of three test series**			**Mean over all tests**	**SD over all tests**
		3 × 90 µl	3 × 100 µl	3 × 110 µl		
Standard 1	0	0,071	0,092	0,081	0,081	0,011
Standard 2	2,5	0,377	0,383	0,336	0,365	0,025
Standard 3	5	0,738	0,742	0,653	0,711	0,050
Standard 4	10	1,183	1,247	1,102	1,177	0,073
Standard 5	20	2,069	2,041	1,988	2,033	0,041
	Concentration (mg/kg hazelnut)					
Negative control	White chocolate	< 2,5	< 2,5	< 2,5	negative	
Spike 5 mg/kg	Cereals	4,4	4,5	4,4	4,4	0,075
Spike 10 mg/kg	Artif. chocolate	10,6	10,2	9,7	10,2	0,412

[a] Concentration of standard solutions: the dilution factor of sample preparation is included.

A.6 Calibration curve

The calibration curve of the test covers a range of 2,5 mg/kg to 20 mg/kg hazelnut (see Figure A.1). Results of samples read of the standard curve have included the dilution factor of 20 corresponding to the sample preparation.



Key
X concentration (mass fraction) of hazelnut (mg/kg)
Y absorbance
1 mean
2 mean + SD
3 mean - SD

Figure A.1 — Typical Standard calibration curve of the Hazelnut ELISA

Mean of 8 stability tests of lot # 04156 (week 0 to week 24) additionally mean +SD and mean – SD curves are shown.

A.7 Stability testing/data

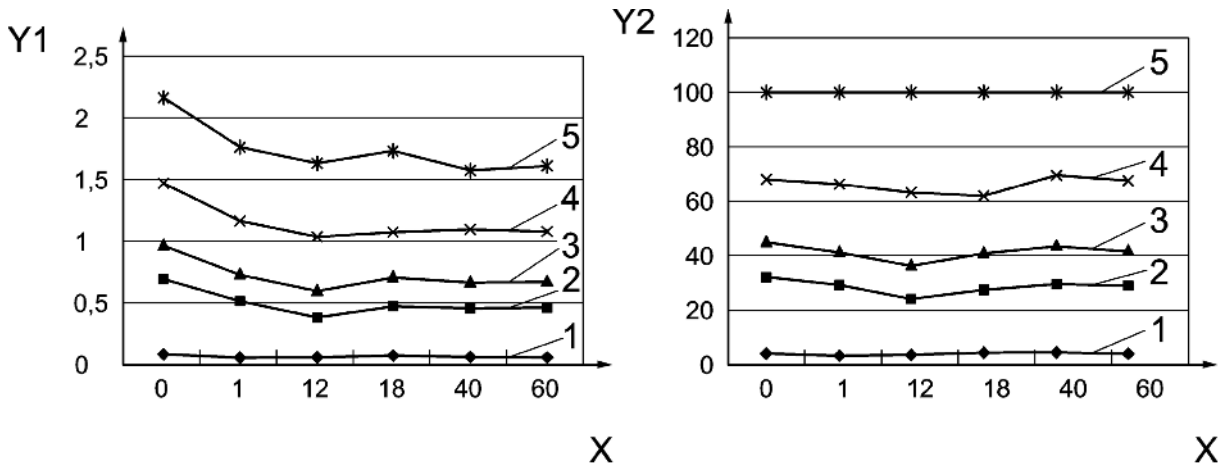
The stability of the test (batch to batch variability) is routinely checked by the manufacturer's quality assurance laboratory after defined storage intervals. Test kits are stored in a cool room at temperatures of 2 °C to 8 °C according to the recommended storage condition. The kit components are temperature equilibrated to room temperature (20 °C to 25 °C) for at least one hour before starting an assay. The B/Bmax (%) (the optical density of the sample divided by the optical density of the highest point of the standard curve multiplied by 100) values of the standards were always found on relative constant levels during the observation time over a period of more than a year.

Table A.21 and A.22 represent the mean values of absorbances and relative absorbance values (B/Bmax (%)).

Real time data was collected for kits stored at 5 °C over a period of 60 weeks. An hour before starting the assay the kits were equilibrated to 20 °C – 25 °C. B/Bmax-values in percent were calculated from the absorbance values as can be seen in Table A.21.

Table A.21 — Stability control data of lot # 02305 (exp date March 2006)

Storage	Absorbance values					B/Bmax %				
Week	**std 1**	**std 2**	**std 3**	**std 4**	**std 5**	**std 1**	**std 2**	**std 3**	**std 4**	**std 5**
0	0,082	0,694	0,970	1,470	2,163	3,8	32,1	44,8	68,0	100
1	0,054	0,511	0,724	1,168	1,764	3,1	29,0	41,0	66,2	100
12	0,052	0,392	0,578	1,030	1,629	3,2	24,1	35,5	63,2	100
18	0,070	0,467	0,709	1,075	1,739	4,0	26,9	40,8	61,8	100
40	0,066	0,458	0,671	1,089	1,578	4,2	29,0	42,5	69,0	100
60	0,056	0,454	0,683	1,078	1,611	3,5	28,2	42,4	66,9	100
					Mean	**3,6**	**28,2**	**41,2**	**65,9**	**100,0**
					SD	**0,5**	**2,7**	**3,1**	**2,8**	**0,0**
#02305		exp	2006-03		**CV (%)**	**12,5**	**9,4**	**7,6**	**4,2**	**0,0**



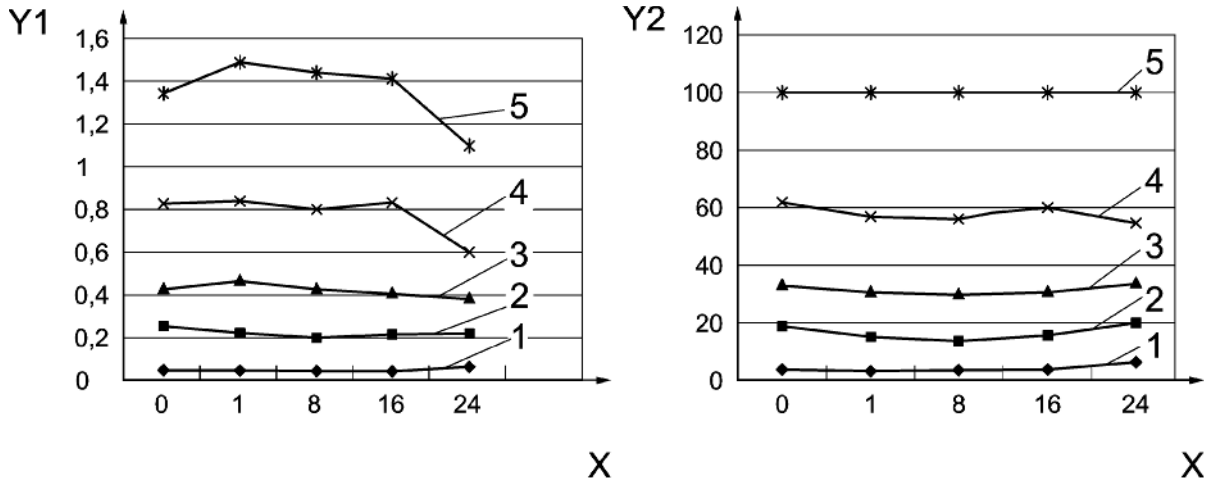
Key
X storage weeks
Y1 absorbance
Y2 B/Bmax (%); calculation: 100 × (Absorbance of std_n/Absorbance of std_5)
1 std 1
2 std 2
3 std 3
4 std 4
5 std 5

Figure A.2 — Stability control of lot # 02305 (exp date March 2006)

Real time data with kits stored at 5 °C over a period of 24 weeks Absorbance values and % B/Bmax values are indicated in Table A.22.

Table A.22 — Stability control data of lot # 04156 (exp data April 2007)

Storage	**Absorbance values**					**B/Bmax** %				
Weeks	**std 1**	**std 2**	**std 3**	**std 4**	**std 5**	**std 1**	**std 2**	**std 3**	**std 4**	**std 5**
0	0,051	0,253	0,444	0,830	1,340	3,8	18,9	33,1	62	100
1	0,046	0,220	0,457	0,840	1,484	3,1	14,8	30,8	57	100
8	0,042	0,196	0,426	0,803	1,438	2,9	13,6	29,6	56	100
16	0,043	0,212	0,411	0,833	1,408	3,1	15,1	29,2	59	100
24	0,057	0,215	0,361	0,596	1,097	5,2	19,6	32,9	54	100
					Mean	**3,6**	**16,4**	**31,1**	**57,6**	**100,0**
					SD	**0,9**	**2,7**	**1,8**	**3,0**	**0**
#04156		exp.	2007-04		**CV (%)**	**26,2**	**16,2**	**5,9**	**5,2**	**0**



Key
X storage weeks
Y1 absorbance
Y2 B/Bmax (%); calculation: 100 × (Absorbance of std_n/Absorbance of std_5)
1 std 1
2 std 2
3 std 3
4 std 4
5 std 5

Figure A.3 — Stability control of lot # 04156 (exp date April 2007)

The test kit components provide a good long-term stability as demonstrated with six Hazelnut ELISA batches Two examples are given. One batch with an expiry of March 2006, evaluated over 60 weeks and one batch with an expiry date of April 2007, controlled now over 24 weeks.

Annex B
(informative)

Collaborative trial

Within the aims to investigate methods for hazelnut detection in food with a LOD of approximate 2 mg/kg, a collaborative study for the determination of hazelnut in dark chocolate was initiated in Germany.

A total of 10 blind coded dark chocolate samples (20 % cocoa content) were sent to 14 German laboratories each. The set of samples contained two blank samples and eight samples spiked with hazelnuts (roasted 20 min at 150 °C). The samples were spiked at 2 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg hazelnut. Each spike level was sent as two individual samples. Each sample was extracted once according to the extraction procedure with pre-heated extraction buffer for 10 min at 60 °C. The samples were measured twice in two independent runs. The raw data were evaluated with commercial available software (RIDA®SOFT Win. Art. No Z9999. Version 1.34. R-Biopharm AG). A statistical evaluation was made without elimination of outliers. Mean recovery values of the two series of four spiked samples shown in Table B.1 were calculated at 106,3 %. The precision parameters of the ring trial are presented in Table B.2. Overall a relative repeatability RSD_r was validated at 9,5 % and the relative reproducibility RSD_R at 17,3 %. All blank samples were negative.

Table B.1 — Mean recovery (%) of the five blind coded hazelnut spiked samples measured twice at 14 laboratories

	Target value mg/kg	**Mean value** mg/kg	**Mean recovery** %
Sample 1	0	< 2,5	
Sample 2	2	2,37[a]	118,5
Sample 3	5	4,69	93,8
Sample 4	10	10,66	106,6
Sample 5	20	21,29	106,5
Mean recovery (%)			**106,3**

[a] The result is lying below the LOQ and above the LOD. This means that the results have only been estimated.

Table B.2 — Repeatability and reproducibility of the four blind coded hazelnut spiked samples measured twice at 14 laboratories

Parameter	**Dark chocolate** **Content of hazelnut** mg/kg			
	Sample 1 2 mg/kg	**Sample 2** 5 mg/kg	**Sample 3** 10 mg/kg	**Sample 4** 20 mg/kg
Number of laboratories	14	14	14	14
Laboratories after outlier elimination	14	14	14	14
Number of outliers	0	0	0	0
Mean (mg/kg)	2,37	4,69	10,66	21,29
Repeatability r (mg/kg)	0,62	1,50	3,24	3,97
Repeatability standard deviation s_r (mg/kg)	0,22	0,53	1,16	1,42
Repeatability relative standard deviation RSD_r (%)	9,3	11,3	10,8	6,7
Reproducibility R (mg/kg)	1,14	2,24	5,63	9,48
Reproducibility standard deviation s_R (mg/kg)	0,41	0,80	2,01	3,38
Reproducibility relative standard deviation RSD_R (%)	**17,3**	**17,1**	**18,9**	**15,9**

Bibliography

[1] M. Wensing, A. H. Penninks, S. L. Hefle, J. H. Akkerdaas, R. van Ree, S. J. Koppelman, C. A. F. M. Bruijnzeel-Koomen and A. C. Knulst: *The range of minimum provoking doses in hazelnut-allergic patients as determined by double-blind, placebo-controlled food challenges*. Clin Exp Allergy 32 (12), 1757-1762 (2002)

[2] M. Trucksess, T. B. Whitaker, A. B. Slate, K. M. Williams, V. A. Brewer, P. Whittaker, and J. T. Heeres: *Preparation of peanut butter suspension for determination of peanuts using enzyme-linked immunoassay kits*. J AOAC Int 87 (2), 424-428 (2004)

[3] AOAC Research Institute, *Performance Tested Methods Program* (for evaluation of peanut ELISAs, 2002)

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