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BSI Standards Publication

Foodstuffs — Detection of food allergens by molecular biological methods

Part 3: Hazelnut (*Corylus avellana*) — Qualitative detection of a specific DNA sequence in chocolate by real-time PCR

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National foreword

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Foodstuffs - Detection of food allergens by molecular biological methods - Part 3: Hazelnut (Corylus avellana) - Qualitative detection of a specific DNA sequence in chocolate by real-time PCR

Produits alimentaires - Détection d'allergènes alimentaires par des méthodes de biologie moléculaire - Partie 3: Noisette (Corylus avellana) - Détection qualitative d'une séquence d'ADN spécifique dans du chocolat, par PCR en temps réel

Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 3: Haselnuss (Corylus avellana) - Qualitativer Nachweis einer spezifischen DNA-Sequenz in Schokolade mittels Real-time PCR

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European foreword

This document (CEN/TS 15634-3:2016) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

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EN 15634, Foodstuffs — Detection of food allergens by molecular biological methods, is currently composed with the following parts:

— Part 1: General considerations;

— Part 2: Celery (Apium graveolens) — Qualitative determination of a specific DNA sequence in cooked sausages by real-time PCR [Technical Specification];

— Part 3: Hazelnut (Corylus avellana) — Qualitative detection of a specific DNA sequence in chocolate by real-time PCR [Technical Specification];

— Part 4: Peanut (Arachis hypogaea) — Qualitative detection of a specific DNA sequence in chocolate by real-time PCR [Technical Specification];

— Part 5: Mustard (Sinapis alba) and soya (Glycine max) — Qualitative dectection of a specific DNA sequence in cooked sausages by real-time PCR [Technical Specification].

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1 Scope

This Technical Specification describes a procedure for the qualitative detection of hazelnut *(Corylus avellana)* in chocolate. DNA is extracted from the chocolate and a specific DNA sequence for hazelnut detected from the gene for corA 1 [4], [5].

2 Principle

The total DNA is extracted from the sample and the DNA content estimated. A 152 bp long sequence from the gene for corA 1 is multiplicated using real-time PCR. The amplicon formed in this way is detected by annealing a sequence-specific probe and generating a fluorescence signal [4], [5].

3 Reagents

As a rule, analytical grade chemical reagents suitable for molecular biology shall be used. The water used shall be double distilled or equivalent quality. Solutions should be prepared by dissolving the appropriate reagents in water and autoclaving, unless indicated differently.

3.1 DNA extraction with CTAB

3.1.1 Chloroform.

3.1.2 Ethanol, volume fraction φ = 96 %.

3.1.3 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA).

3.1.4 Cetyltrimethylammoniumbromide (CTAB).

3.1.5 Hydrochloric acid, mass fraction w = 37 %.

3.1.6 Isoamyl alcohol.

3.1.7 Isopropanol.

3.1.8 Proteinase K.

3.1.9 Sodium chloride.

3.1.10 Sodium hydroxide.

3.1.11 Tris(hydroxymethyl)aminomethane (TRIS).

3.1.12 Chloroform isoamyl alcohol mixture.

Mix 24 parts by volume of chloroform (3.1.1) with one part by volume of isoamyl alcohol (3.1.6).

Commercially available and comparable mixtures can be used.

3.1.13 CTAB extraction buffer solution, containing CTAB (mass concentration ρ = 20 g/l), sodium chloride (substance concentration c = 1,4 mol/l), TRIS (c = 0,1 mol/l), Na_2EDTA (c = 0,02 mol/l). Adjust the pH value with hydrochloric acid to pH = 8,0.

3.1.14 Ethanol solution, φ = 70 %.

3.1.15 Proteinase K solution, ρ = 20 mg/ml.

The freshly produced Proteinase K solution should be stored in the form of aliquots at -20 °C.

3.1.16 TE buffer solution, containing TRIS (c = 0,001 mol/l) and Na_2-EDTA (c = 0,000 1 mol/l). Adjust the pH value with hydrochloric acid or sodium hydroxide solution to pH = 8,0.

3.2 DNA purification by means of solid phase extraction

For the DNA purification, different methods may be used.

Various systems are commercially available for DNA purification by means of solid phase extraction, including spin filter columns or plates or also with vacuum operated systems. Commercially available kits can also be used. Observe the manufacturer's data for this (see also 6.3.1).

3.3 Real-time PCR reagents

3.3.1 PCR master mix[1]**,** containing reaction buffer, dNTPs, $MgCl_2$ and Hotstart Taq polymerase.

3.3.2 Oligonucleotides, 5 µmol each.

3.3.2.1 Hazelnut iF, 5´– TAC AgC ATC ATC gAg ggA ggT C – 3´.

3.3.2.2 Hazelnut iR, 5´– CTC CTC ATT gAT TgA AgC gTT g – 3´.

3.3.2.3 Hazelnut probe, 5´– FAM – AgA Tgg Cgg CAg CCC CTC AT – TAMRA – 3´[2]

3.3.3 Negative PCR control, conducted with DNA-free water instead of the DNA extract from the sample and without PCR inhibitors.

3.3.4 Negative extraction control, performing all steps of the DNA extraction procedure, except addition of the test portion, e.g. by substitution of a corresponding amount of water for the test portion.

3.3.5 Negative process control, sample of the food matrix without target sequence, which passes through all steps of the analytical process.

3.3.6 Positive PCR control[3]**,** reaction containing the target DNA in a specified quantity or number of copies.

3.3.7 Positive process control, sample of the food matrix with known quantity of hazelnut, which passes through all steps of the analytical process.

3.3.8 External amplification control (inhibition control), control DNA that is added to an aliquot of the extracted nucleic acid in a specified quantity or number of copies and used in a separate reaction to check the influence of co-extracted substances from the sample matrix on the amplification.

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1) Ready-to-use reagents or single components may be used as a PCR master mix, insofar as they provide comparable or better results.

2) FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine; equivalent reporter dyes and/or quencher dyes may be used if they are shown to give comparable or better results.

3) DNA for the positive PCR control is extracted from phenotypically identified pure hazelnuts as described in 5.3 and 5.4. DNA mass concentration is determined as described in 5.5.

4 Apparatus and equipment

General aspects are described in EN ISO 24276 [3].

Plastic and glass materials shall be sterilized and free of DNA before use. In addition, the use of aerosol protected filter tips is obligatory due to the high sensitivity of the PCR analytics and the resultant risk of DNA contamination. In addition to the usual laboratory facilities, the following equipment is required.

4.1 DNA extraction

4.1.1 Suitable reaction vials, 1,5 ml and 2 ml, DNA-free.

4.1.2 50 ml centrifuge tubes, sterile.

4.1.3 Thermostat or water bath, preferably with shaker function.

4.1.4 Centrifuge, suitable for centrifuging 50 ml centrifuge tubes at 8 000 *g*[4)].

4.1.5 Centrifuge, suitable for centrifuging 1,5 ml and 2 ml reaction vials at 14 500 *g*.

4.1.6 Apparatus and/or material for grinding the sample, e.g. blender or mill.

4.1.7 UV spectrometer or other detection instruments, suitable for estimating the amount of DNA.

4.2 PCR

4.2.1 Suitable PCR tubes.

4.2.2 Microcentrifuge for PCR tubes.

4.2.3 Real-time PCR equipment, suitable for excitation and for emission measurement of fluorescence-marked oligonucleotides.

NOTE Laboratories participating in the interlaboratory study used the following real-time PCR equipment: Rotor Gene 2000™ or 3000™, Stratagene Mx 3005P, ABI PRISM® 7000 or 7500, ABI PRISM® 7700 or 7900HT and Roche LightCycler®.[5)]

5 Procedure

5.1 General

General aspects are described in EN ISO 24276 [3].

5.2 Sample preparation

Ensure, e.g. by milling or homogenizing, that the test sample is representative of the laboratory sample.

4) $g = 9{,}81\ \text{m} \cdot \text{s}^{-2}$

5) Rotor Gene 2000™ and 3000™, Stratagene Mx 3005P, ABI PRISM® 7000 and 7500, ABI PRISM® 7700 and 7900HT and Roche LightCycler® are examples of suitable products available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by CEN of these products. Equivalent products may be used if they can be shown to lead to the same results.

5.3 DNA extraction with CTAB

Measures and work steps to be considered for the DNA extraction are described in EN ISO 21571 [2].

It is acceptable to use a commercially available kit instead of the DNA extraction procedure described below, if it is ensured that comparable or better results are obtained.

In parallel to the test samples, carry out the controls listed in 3.3.4, 3.3.5 and 3.3.7 adequately.

Prepare every sample **twice** in accordance with the following scheme:

— Weigh 2 g of the sample into 50 ml centrifuge tubes;

— Add 10 ml of CTAB extraction buffer solution (3.1.13);

— Add 30 µl of Proteinase K solution (3.1.15) and mix;

— Incubate and shake for 90 min at 65 °C;

— Centrifuge for 5 min at 6 000 *g* to 8 000 *g*;

— Place 500 µl of chloroform isoamyl alcohol mixture (3.1.12) in a 2 ml reaction vial;

— Add 700 µl of supernatant and mix thoroughly for 30 s;

— Centrifuge for 15 min at about 14 500 *g*;

— Place 500 µl of cold isopropanol (3.1.7) in a 1,5 ml reaction vial;

— Add 500 µl of supernatant (aqueous phase) and mix carefully;

— Incubate for 30 min at room temperature;

— Centrifuge for 15 min at about 14 500 *g*;

— Carefully remove and discard the supernatant;

— Fill the reaction vial with 500 µl of ethanol (3.1.2) and swirl the reaction vial several times;

— Centrifuge for 5 min at about 14 500 *g*;

— Carefully remove and discard the supernatant;

— Dry the extracted DNA;

— Dissolve the dried DNA extract in 100 µl of TE buffer solution (3.1.16).

5.4 DNA purification by means of solid phase extraction

Purify DNA extract according to the instructions given by the respective kit manufacturer.

The DNA extract can be stored at 4 °C for a short period. If storage times exceed more than one week, the DNA extracts should be stored at temperatures of −18 °C.

5.5 Measuring the mass concentration of the extracted DNA and setting to target concentration

The mass concentration of a DNA aliquot can be determined by means of a UV spectrometer at 260 nm. Calculate the DNA mass concentration as follows:

ρ (DNA) in ng/µl = 50 × optical density × dilution factor of the measured aliquot

In order to check its purity, the sample can in addition be measured at 280 nm. The ratio of the values for optical density at wavelengths of 260 nm and 280 nm should be approximately 1,8.

The DNA mass concentration may also be estimated using other suitable procedures.

Set the DNA extract to a mass concentration of approximately 20 ng/µl by diluting with sterile water.

5.6 Real-time

— PCR

NOTE 1 In order to exclude false negative results occurring due to PCR inhibition or highly degraded DNA, the PCR suitability of the isolated DNA can be checked by, e.g. an amplification of universal sequences from plants [6]. Alternatively, a possible inhibition of the PCR can be detected by spiking the sample DNA with a positive control in a separate reaction (see 3.3.8).

NOTE 2 The method description for hazelnut detection applies for a total volume per PCR of 25 µl with the reagents indicated in Table 1. The PCR can also be carried out in a larger volume, if the solutions are adapted correspondingly.

The final concentrations of the reagents given in Table 1 have proven to be suitable.

In parallel to the test samples, the controls listed in 3.3.3 to 3.3.8 shall be carried out adequately.

Prior to use, the gently thawed reagents should be centrifuged briefly. In addition, every reagent shall be mixed carefully immediately before pipetting. Keep the reagents cooled while preparing the PCR (e.g. cooling block).

A PCR mix should be prepared containing all the components except for the DNA extract. The required amount of PCR mix is determined by the number of reactions to be carried out plus a safety reserve of 10 %.

Every DNA extract is examined in at least two PCR (duplicates).

For each reaction, 5 µl of DNA extract should be used.

— Mix the PCR master mix (see Table 1), centrifuge shortly and pipette 20 µl per PCR into each reaction vial.

Table 1 — Reaction batch for the real-time PCR

Reagent	Final concentration	Volume per reaction µl
Water		7,2
PCR buffer solution 10x		2,5
dNTP solution (2,5 mmol)		1,8
$MgCl_2$ solution (25 mmol)		4,4
Primer hazelnut iF (5 µmol[a])	0,34 µmol	1,7
Primer hazelnut iR (5 µmol[a])	0,34 µmol	1,7
Probe hazelnut (5 µmol[a])	0,12 µmol	0,6
Hotstart DNA polymerase	0,5 U	0,1
Total master mix		20
DNA extract		5

[a] Other working concentrations can be used. The volumes shall then be adjusted correspondingly.

— For the negative PCR control (3.3.3), pipette 5 µl of water into the PCR master mix provided.

— For the sample PCR, pipette 5 µl of DNA extract respectively into the PCR master mix.

— For the negative extraction control (3.3.4), pipette 5 µl of extract from the negative extraction control assay into the PCR master mix provided.

— For the negative process control (3.3.5), pipette 5 µl of the hazelnut-free sample DNA extract into the PCR master mix provided.

— For the positive PCR control (3.3.6), pipette 5 µl of the target containing DNA into the PCR master mix provided.

— For the positive process control (3.3.7), pipette 5 µl of the hazelnut-containing sample DNA into the PCR master mix provided.

— For the external amplification control (inhibition control (3.3.8)), pipette an aliquot of hazelnut control DNA to an aliquot of the extracted sample DNA into the master mix provided.

— Place the reaction vials in the PCR device and start the temperature/time program.

The temperature/time programs given in Table 2 and Table 3 have been proven for the PCR described here when using reaction vessels made from plastic.

Table 2 — Temperature/time program when using a light cycler

Light cycler						
Step	Parameter		Temperature	Time	Fluorescence measurement	Cycles
1	Initial denaturation		95 °C	1 min	No	1
2	Amplification	Denaturation	95 °C	5 s	No	45
		Annealing	60 °C	10 s	No	
		Elongation	65 °C	15 s	Yes	

Table 3 — Temperature/time program when using a block cycler

Block cycler						
Step	**Parameter**		**Temperature**	**Time**	**Fluorescence measurement**	**Cycles**
1	Initial denaturation		95 °C	5 min	No	1
2	Amplification	Denaturation	95 °C	10 s	No	45
		Annealing	60 °C	15 s	No	
		Elongation	65 °C	30 s	Yes	

6 Validation status and performance criteria

6.1 General

The method described in this Technical Specification was elaborated by the working group “Lebensmittel-Allergene“ (Food Allergens) of the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (Federal Office of Consumer Protection and Food Safety, BVL) for implementation of section 64 of the German Food and Feed Code (LFGB) and validated in the interlaboratory study with a total of 14 participants [7].

The measurement results are analysed using the relevant device-specific data analysis program. The amplification result is sometimes indicated differently, depending on the real-time PCR device used. When no detectable PCR products are obtained (negative result) the indication may read, e.g. “undetermined” or “no amp”, or also the maximum number of cycles set may be given in the results report. In case of amplification of the DNA target sequence in a sample (positive result), the cycle count at which a predefined fluorescence threshold value was exceeded (C_t or C_p value) is calculated.

If the automatic evaluation does not give any effective result due to atypical fluorescence measurement data, manual adjustment of the baseline and the threshold value may be required prior to evaluating the data. When doing so, follow the device specific instructions given in the respective manual for using the evaluation software.

In this interlaboratory study, hazelnut mass fractions of 20 mg/kg or greater could be detected with a probability of > 88 %.

6.2 Detection

The target sequence is considered to be detected, if

— an increase in the measured fluorescence can be observed that is caused by the amplification by means of hazelnut-specific primers and the specific probe. The qualitative results of two test samples shall not be contradictory, otherwise the analysis shall be repeated;

— no increase in fluorescence caused by amplification can be observed in the PCR controls without added hazelnut DNA (negative DNA control, negative process control);

— the expected C_t values are obtained in the positive controls and, if appropriate, in the inhibition control.

The manufacturer's instructions for the relevant analysis software shall be observed for the analysis.

6.3 Reliability of the method

6.3.1 Setup of the interlaboratory study

The reliability of the method was validated in an interlaboratory study with a total of 14 participants.

Every participant received 10 coded samples of dark chocolate that have been fortified with mass fractions w = (0, 2, 5, 10 and 20) mg/kg hazelnut. The homogeneity of the samples was checked for every fortification level via ELISA.

Chocolate containing hazelnut was used as positive controls.

For each of the samples, two DNA extracts had to be examined in two PCR replicates; so that every participant had to report 40 results.

Components of the commercial Sure Prep extraction kit were used for DNA extraction and DNA purification in the interlaboratory study. Several of the participating laboratories revealed in further tests that the extraction procedure described here as well as the commercial kit QIAQuick® Purification Kit[6)] (Qiagen, Hilden) for purification lead to comparable results. The choice of PCR device was free. The primer and probe solutions were ordered centrally and made available to the interlaboratory study participants.

6.3.2 Results

14 laboratories took part in the interlaboratory study, the measurement results of 13 laboratories were considered in the statistical interpretation. The results are shown in Tables 4 and 5. The results of one laboratory were excluded from the study due to completely incongruent data.

A mass fraction of hazelnut of 10 mg/kg could be detected in 87 % of all reactions as expected, higher mass fractions for hazelnut revealed even better detection rates.

Table 4 — Overview data for the interlaboratory study

Number of laboratories	14
Number of laboratories after outlier elimination	13
Total number of samples per laboratory	10
Number of negative samples per laboratory	2
Number of PCR results per sample	4
Number of PCR results accepted (considered)	520
False-positive results	2 of 104[a]
False-negative results	111 of 416[b]

[a] One of the four PCR reactions of a sample respectively was detected as false-positive by two interlaboratory study participants.

[b] It is to be assumed that the reason for the false-negative results also lies in the setting of the DNA to 20 ng/µl amongst other factors. To avoid false-negative results, an undiluted DNA extract should also be used.

6) QIAQuick® Purification Kit is an example of a suitable product available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by CEN of this product.

Table 5 — Number of PCR reactions showing false-negative or false-positive results

Hazelnut mg/kg	PCR results total	Number of false-negative PCR results	Number of false-positive PCR results	Negative rate %	Positive rate %
0	104	0	2	98	2
2	104	54	0	52	48
5	104	32	0	31	69
10	104	13	0	13	87
20	104	12	0	12	88

6.3.3 Specificity

The specificity was tested for relevant species by a laboratory beforehand. The DNA was extracted from the phenotypically identified species as described in 5.3 and 5.4. No cross reactions with hazelnut were ascertained for the species indicated in the list below.

Cashew nut	Pumpkin seed	Oat
Peanut	Sunflower seed	Barley Rye
Macadamia nut	Pine seed	Rice
Almond	Linseed	Maize
Pecan nut	Yellow linseed	Whole egg (hen)
Brazil nut	White bean	Whole milk powder
Walnut	Green peas	Apricots
Coconut	Chickpea Kidney bean	Ground coffee
Parsley	Lentil	
Celery	Soya bean	
Carrot		

7 Test report

The test report should contain the data according to EN ISO/IEC 17025 [1] and at least the following information:

a) all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) a reference to this Technical Specification;

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

d) the date of receipt;

e) the date of test;

f) the test results and the units in which they have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional, which might have affected the results.

Bibliography

[1] EN ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025)*

[2] EN ISO 21571, *Foodstuffs — Methods of analysis for the detection of genetically modified organisms and derived products — Nucleic acid extraction (ISO 21571)*

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[6] Taberlet, P., Gielly, L., Pautou, G., Buovet. Universal primers for amplification of three non-coding regions of chloroplast DNA. *J Plant Mol Biol*. 1991, **17** pp. 1105–1109

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