
Živila - Odkrivanje prisotnosti alergenov v živilih z metodami molekularne biologije - 5. del: Gorčica (*Sinapis alba*) in soja (*Glycine max*) - Kvalitativno odkrivanje specifičnega niza DNK v obarjenih klobasah s PCR v realnem času

Foodstuffs - Detection of food allergens by molecular biological methods - Part 5: Mustard (*Sinapis alba*) and soya (*Glycine max*) - Qualitative detection of a specific DNA sequence in cooked sausages by real-time PCR

Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 5: Senf (*Sinapis alba*) sowie Soja (*Glycine max*) - Qualitativer Nachweis einer spezifischen DNA-Sequenz in Brühwürsten mittels Real-time PCR

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This draft Technical Specification is submitted to CEN members for formal vote. It has been drawn up by the Technical Committee CEN/TC 275.

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COMITÉ EUROPÉEN DE NORMALISATION
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Contents	Page
European foreword.....	3
1 Scope	4
2 Principle	4
3 Reagents	4
4 Apparatus and equipment	6
5 Sampling	6
6 Procedure	6
6.1 General	6
6.2 Sample preparation	7
6.3 DNA extraction with CTAB	7
6.4 DNA purification by means of solid phase extraction	7
6.5 Measuring the mass concentration of the extracted DNA and setting to target concentration	8
6.6 Real-time PCR	8
6.7 Temperature/Time program	9
7 Validation status and performance criteria	9
7.1 General information on the interpretation of the real-time PCR	9
7.2 Reliability of the method	10
7.2.1 Setup of the interlaboratory study	10
7.2.2 Results of the interlaboratory study samples	10
7.2.3 Qualitative interpretation	10
8 Test report	12
Bibliography	13

European foreword

This document (FprCEN/TS 15634-5:2015) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This document is currently submitted to the Formal Vote.

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; currently at Formal Vote stage];*
- *Part 4: Peanut (*Arachis hypogaea*) — Qualitative detection of a specific DNA sequence in chocolate by real-time PCR [Technical Specification; currently at Formal Vote stage];*
- *Part 5: Mustard (*Sinapis alba*) and soya (*Glycine max*) — Qualitative detection of a specific DNA sequence in cooked sausages by real-time PCR [Technical Specification; currently at Formal Vote stage].*

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FprCEN/TS 15634-5:2015 (E)

1 Scope

This draft Technical Specification specifies a procedure for the qualitative detection of species specific DNA from white mustard (*Sinapis alba*) and soya (*Glycine max*) in cooked sausages using singleplex real-time PCR based on the genes MADS-D (mustard) and lectin (soya). A mustard content of 10 mg/kg or greater and a soya content of 10 mg/kg or greater can be detected with a probability of > 95 %.

2 Principle

The DNA of the sample is extracted and is set to a definite concentration after photometric measurement. A 74 base pair (bp) long sequence of the DNA for the MADS-D protein of *Sinapis alba* (NCBI accession no. Y08626) or a 81 bp long sequence from the soya lectin gene is multiplied from the sample DNA by real-time PCR. The amplicons formed are detected and verified by annealing a sequence-specific probe and generating a fluorescence signal [4].

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 $\varphi = 96 \%$.

3.1.3 Ethylenediaminetetraacetic acid disodium salt (Na₂EDTA).

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 comparable mixtures can be used.

3.1.13 CTAB extraction buffer solution, containing CTAB (mass concentration $\rho = 20$ g/l), sodium chloride (substance concentration $c = 1,4$ mol/l), TRIS ($c = 0,1$ mol/l), Na₂-EDTA ($c = 0,02$ mol/l). Adjust the pH value with hydrochloric acid to 8,0.

3.1.14 Ethanol solution, $\varphi = 70 \%$.

3.1.15 Proteinase K solution, $\rho = 20 \text{ mg/ml}$.

Store aliquots of the solution at $-20 \text{ }^\circ\text{C}$.

3.1.16 TE buffer solution, containing TRIS ($c = 0,01 \text{ mol/l}$) and $\text{Na}_2\text{-EDTA}$ ($c = 0,001 \text{ mol/l}$). Adjust the pH value with hydrochloric acid or sodium hydroxide solution to $\text{pH} = 8,0$.

3.2 DNA purification by means of solid phase extraction:

Various systems are commercially available, among them spin filter columns or plates or also using vacuum operated systems. Suitable, commercially available kits can be used. Observe the manufacturer's data for this.

3.3 Real-time PCR reagents:

3.3.1 Universal master mix (2 ×) for the real-time PCR, containing thermostable DNA polymerase (for hot-start PCR) and PCR buffer solution¹⁾ (containing reaction buffers, dNTPs, MgCl_2 and Hotstart Taq polymerase), double concentrated.

3.3.2 Oligonucleotides [4]:

Primers and probes for the real-time PCR are shown in Table 1.

Table 1 — Primers and probes for the real-time PCR

Name	DNA sequence of the oligonucleotide
Soya lectin gene	
Lectin-F	5'- TCC ACC CCC ATC CAC ATT T - 3'
Lectin-R	5'- ggC ATA gAA ggT gAA gTT gAA ggA - 3'
Lectin probe	5'- FAM - AAC Cgg TAg CgT TgC CAg CTT Cg - TAMRA-3' ^a
Mustard (<i>Sinapis alba</i>) MADS D protein [4]	
MADS D-F	5'- TGA AAA CTC TCT TCC CCT CTT AGG - 3'
MADS D-R	5'- ACA AAT GCA CAC AAG ACA GAG ATA TAG A - 3';
MADS D probe	5'- FAM - TAC ATG ATG CTT ACC TCG C - TAMRA - 3' ^a
^a FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine; equivalent reporter and/or quencher dyes may be used if they are shown to give comparable or better results.	

3.4 Controls:

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

1) Ready-to-use reagents or single components may be used as a PCR master mix. The following was used as part of the interlaboratory study: Quantitect Multiplex Mastermix (2×), QIA- GEN, Hilden.

FprCEN/TS 15634-5:2015 (E)

3.4.2 Extraction blank control, performing all steps of the DNA procedure, except addition of the test portion, e.g. by substitution of a corresponding amount of water for the test portion.

3.4.3 Positive PCR control, sample containing the target sequence, which shall be treated in the same way as the samples to be examined.

4 Apparatus and equipment

General aspects are described in EN ISO 24276 [3]. In addition to the usual laboratory facilities, the following equipment is required.

Due to the high sensitivity of the PCR analytics and the risk of DNA contamination resulting from it, the use of aerosol protected filter tips is obligatory.

4.1 DNA extraction:

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

4.1.2 50 ml centrifuge tubes, Nucleic acid-free.

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^2).

4.1.5 Centrifuge, suitable for centrifuging 1,5 ml and 2 ml reaction vials at 16 000 g .

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

4.1.7 UV spectrometer, suitable for estimating the amount of DNA.

4.2 PCR: <https://standards.iteh.ai/catalog/standards/sist/6ca1e6d1-7510-4380-9420-8659878a4e9e/sist-ts-cen-ts-15634-5-2016>

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.

5 Sampling

Ensure that a representative sample is made available to the laboratory for investigation.

The sample shall be transported and stored so that damage and/or changes are prevented.

6 Procedure

6.1 General

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

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

6.2 Sample preparation

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

6.3 DNA extraction with CTAB

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

An extraction blank control (3.4.2) shall be performed in parallel.

- Weigh 2 g of the homogenized test sample into 50 ml centrifuge tubes;
- Add 10 ml of CTAB buffer (3.1.13);
- Add 30 µl of Proteinase K (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 2 ml reaction vials;
- Add 700 µl of supernatant and mix thoroughly for 30 s;
- Centrifuge for 15 min at approximately 16 000 *g*;
- Place 500 µl of cold isopropanol (3.1.7) in 1,5 ml reaction vials;
- Add 500 µl of supernatant (aqueous phase) and mix carefully;
- Incubate for 30 min at room temperature;
- Centrifuge for 15 min at approximately 16 000 *g*;
- Carefully remove and discard the supernatant;
- Fill the reaction vial with 500 µl ethanol solution, $\varphi = 70 \%$ (3.1.14) and swirl the reaction vial several times;
- Centrifuge for 5 min at about 16 000 *g*;
- Remove supernatant carefully and discard;
- Dry the extracted DNA at 50 °C (alternatively in vacuum);
- Dissolve the dried DNA extract in 100 µl of TE buffer solution (3.1.16).

6.4 DNA purification by means of solid phase extraction

Purification shall be performed by means of solid phase extraction. In the interlaboratory study, the QIAQuick® PCR Purification Kit³⁾ (Qiagen, Hilden) was used corresponding to the manufacturer's

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

FprCEN/TS 15634-5:2015 (E)

information. Other suitable methods for the DNA purification by means of solid phase extraction can be applied.

6.5 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 a wavelength of 260 nm. Calculate the DNA mass concentration as follows:

$$\rho \text{ (DNA) in } \mu\text{g/ml} = 50 \times \text{optical density} \times \text{dilution factor of the measured aliquot}$$

NOTE Other suitable methods for the DNA mass concentration determination can be applied.

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 260 nm and 280 nm should be approximately 1,8. The DNA extracts are brought to a mass concentration of approximately 250 ng/ μ l by dilution, e.g. with sterile water.

If one or more of the DNA samples exhibit a DNA mass concentration below 250 ng/ μ l, repeat the extraction for these sample(s).

If the majority of the DNA solutions exhibit a mass concentration below 250 ng/ μ l and/or the mass concentration 250 ng/ μ l still cannot be attained even after repeated extraction, use the DNA solutions for the real-time PCR analysis as they are.

6.6 Real-time PCR

Prior to use, the gently thawed reagents should be carefully mixed and centrifuged briefly. Store the reagents cooled in the ice bath or cooling block while preparing the PCR batch.

To prevent false-negative results due to co-extracted PCR inhibitors or highly degraded DNA, the amplifiability of the extracted DNA should be assessed prior to the analysis via real-time PCR.

NOTE This can for example be achieved through the amplification of a universal plant DNA sequence [7].

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 pipetting reserve of 10 %. Every DNA extract is examined in two PCR batches (in duplicate). For each reaction, 5 μ l of DNA extract should be used.

Production of the PCR reaction batches:

Table 2 — Reaction batch master mix soya, final volume/concentration in the reaction vial

Reagent	Final concentration	Volume per reaction μ l
PCR master mix (2 \times), s. 3.3.1	1 \times	12,5
Primer Lectin-F (9 μ mol) ^a	0,9 μ mol	2,5
Primer Lectin-R (9 μ mol) ^a	0,9 μ mol	2,5
Lectin probe (2 μ mol) ^a	0,2 μ mol	2,5
Water		–
DNA extract (250 ng/ μ l)		5,0

^a Other working concentrations may be used. The volumes shall then be adjusted correspondingly.