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SLOVENSKI STANDARD
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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

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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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ICS:

07.100.30	Mikrobiologija živil	Food microbiology
67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products
67.120.10	Meso in mesni proizvodi	Meat and meat products

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TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

CEN/TS 15634-5

June 2016

ICS 07.100.30; 67.120.10

English Version

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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CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

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European foreword

This document (CEN/TS 15634-5:2016) has been prepared by Technical Committee CEN/TC 275 "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

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 detection of a specific DNA sequence in cooked sausages by real-time PCR* [Technical Specification].

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1 Scope

This 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) [1]. 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 multiplicated 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 [2].

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.

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3.1.2 Ethanol, volume fraction φ = 96 %.

3.1.3 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA).

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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_2-EDTA (c = 0,02 mol/l). Adjust the pH value with hydrochloric acid to 8,0.

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

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

Store aliquots of the solution at −20 °C.

3.1.16 TE buffer solution, containing TRIS ($c = 0{,}01$ mol/l) and Na_2-EDTA ($c = 0{,}001$ 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:

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 solution[1)] (containing reaction buffers, dNTPs, $MgCl_2$ and Hotstart Taq polymerase), double concentrated.

3.3.2 Oligonucleotides [2]:

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

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

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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 (Sinapis alba) MADS D protein [2]	
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.

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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. Quantitect Multiplex Mastermix (2×), available from QIA- GEN, Hilden; was used within the interlaboratory study.

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3.4.2 Extraction blank 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.

3.4.3 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*.

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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.

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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.

5 Procedure

5.1 General

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

5.2 Sample preparation

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.

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

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

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5.3 DNA extraction with CTAB

Measures and work steps to be considered for the DNA extraction are described in EN ISO 21571 [4].

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;

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— Incubate for 30 min at room temperature;

— Centrifuge for 15 min at approximately 16 000 *g*;

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— 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).

5.4 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 Kit[3] (Qiagen, Hilden) was used corresponding to the manufacturer's information. Other suitable methods for the DNA purification by means of solid phase extraction can be applied.

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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.