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SLOVENSKI STANDARD
SIST EN 15634-4:2023

01-maj-2023

Živila - Določevanje alergenov v živilih z molekularno biološkimi metodami - 4. del: Arašidi (Arachis hypogaea) - Kvalitativno določanje specifičnega zaporedja DNK v čokoladi s PCR v realnem času

Foodstuffs - Detection of food allergens by molecular biological methods - Part 4: Peanut (Arachis hypogaea) - Qualitative detection of a specific DNA sequence in chocolate by real-time PCR

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Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 4: Erdnuss (Arachis hypogaea) - Qualitativer Nachweis einer spezifischen DNA-Sequenz in Schokolade mittels Real-time PCR

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Produits alimentaires - Détection des allergènes alimentaires par des méthodes d’analyse de biologie moléculaire - Partie 4 : Arachide (Arachis hypogaea) - Détection qualitative d’une séquence d’ADN spécifique dans du chocolat, par PCR en temps réel

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67.190	Čokolada	Chocolate

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English Version

Foodstuffs - Detection of food allergens by molecular biological methods - Part 4: Peanut (*Arachis hypogaea*) - Qualitative detection of a specific DNA sequence in chocolate by real-time PCR

Produits alimentaires - Détection des allergènes alimentaires par des méthodes d'analyse de biologie moléculaire - Partie 4 : Arachide (*Arachis hypogaea*) - Détection qualitative d'une séquence d'ADN spécifique dans du chocolat, par PCR en temps réel

Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 4: Erdnuss (*Arachis hypogaea*) - Qualitativer Nachweis einer spezifischen DNA-Sequenz in Schokolade mittels Real-time PCR

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European foreword

This document (EN 15634-4:2023) has been prepared by Technical Committee CEN/TC 275 “Food analysis – Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by August 2023, and conflicting national standards shall be withdrawn at the latest by August 2023.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes CEN/TS 15634-4:2016.

In comparison with CEN/TS 15634-4:2016, the following technical modifications have been made:

a) the document was converted from a Technical Specification into a European standard;

b) normative references and terms and definitions clause added;

c) PCR controls moved from Clause 3 “Reagents” to Clause 7 “Procedure”;

d) new subclause 7.4.9 “Accept/Reject criteria” added;

e) restructured clauses in alignment with EN 15634-2:2019.

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Introduction

For the use of this document the term:

— 'shall' indicates a requirement;

— 'should' indicates a recommendation;

— 'may' indicates a permission;

— 'can' indicates a possibility and/or a capability.

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1 Scope

This document specifies a method for the detection of peanut (*Arachis hypogaea*) in chocolate.

Real-time PCR (Polymerase Chain Reaction) detection of peanut is based on an 86 bp (base pair) sequence from the Ara h 2 gene of peanut.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 15634-1:2019, *Foodstuffs - Detection of food allergens by molecular biological methods - Part 1: General considerations*

EN 15842, *Foodstuffs - Detection of food allergens - General considerations and validation of methods*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 15842 and EN 15634-1 apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at https://www.electropedia.org/
- ISO Online browsing platform: available at https://www.iso.org/obp/ui

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4 Principle

Total DNA from chocolate is extracted from the sample using a cetyltrimethylammoniumbromide (CTAB) method. Potential PCR inhibitors are removed from the DNA extracted by purification with solid phase columns and the DNA content is measured by photospectrometry. Real-time PCR is used to detect a peanut specific DNA sequence. The real-time PCR method involves a fluorescence detection with a sequence specific hydrolysis probe [1], [2].

5 Reagents

5.1 General

The following general conditions for analysis should be followed, unless specified differently. Use only analytical grade reagents suitable for molecular biology. All water shall be free from DNA and nucleases, e.g. double distilled or equivalent (molecular grade). Solutions shall be prepared by dissolving the appropriate reagents in water and autoclaving, unless specified differently.

5.2 Extraction reagents

5.2.1 Chloroform.

5.2.2 Ethanol, volume fraction φ = 70 %.

5.2.3 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA).

5.2.4 Cetyltrimethylammoniumbromide (CTAB).

5.2.5 Hydrochloric acid, mass fraction w = 37 %.

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5.2.6 Isoamyl alcohol.

5.2.7 Isopropanol.

5.2.8 Proteinase K.

5.2.9 Sodium chloride.

5.2.10 Sodium hydroxide solution.

5.2.11 Tris(hydroxymethyl)aminomethane (TRIS).

5.2.12 Chloroform isoamyl alcohol mixture, 24 parts by volume of chloroform (5.2.1) are mixed with one part by volume of isoamyl alcohol (5.2.6).

Similar mixtures commercially available may be used.

5.2.13 CTAB extraction buffer solution, containing:

— CTAB (5.2.4), mass concentration ρ = 20 g/l;

— sodium chloride (5.2.9), substance concentration c = 1,4 mol/l;

— TRIS (5.2.11), substance concentration c = 0,1 mol/l;

— Na_2EDTA (5.2.3), substance concentration c = 0,02 mol/l.

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The pH is adjusted to 8,0 by adding hydrochloric acid (5.2.5).

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

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

5.2.15 TE buffer solution, containing:

— TRIS (5.2.11), c = 0,001 mol/l;

— Na_2EDTA (5.2.3), c = 0,000 1 mol/l.

The pH is adjusted to 8,0 by adding hydrochloric acid (5.2.5) or sodium hydroxide solution (5.2.10).

5.3 DNA purification by means of solid phase extraction

For the DNA purification, different methods may be used.

Several formats are commercially available, among them spin filter columns or plates. Commercially available kits may be used if appropriate. Follow the manufacturer's instructions for this.

5.4 Real-time PCR reagents

5.4.1 PCR master mix, containing reaction buffer, dNTPs, $MgCl_2$ and Hotstart Taq polymerase.

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

5.4.2 Oligonucleotides, 10 µmol each.

5.4.2.1 Peanut (AR-58 F), 5’ – gCA gCA gTg ggA ACT CCA Agg AgA CA – 3´.

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5.4.2.2 Peanut (AR-143 R), 5’ – gCA TgA gAT gTT gCT CgC Ag – 3´.

5.4.2.3 Peanut probe (AR-103 T), 5’ – FAM – CgA gAg ggC gAA CCT gAg gCC – TAMRA – or BHQ1 – 3´[1].

Equivalent reporter dyes and/or quencher dyes may be used if they are shown to give comparable or better results.

6 Apparatus and equipment

6.1 General

In addition to the usual laboratory facilities, the following equipment shall be used.

Due to the high sensitivity of the PCR analytics and the risk of DNA contaminations resulting from it, the use of aerosol protected filter tips in the DNA extraction procedure is obligatory. Plastic and glass materials shall be sterilized and free of DNA before use.

Further general requirements are given in EN ISO 21571.

6.2 DNA extraction

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

6.2.2 50 ml centrifuge tubes, sterile.

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6.2.3 Thermostat or water bath, preferably with shaker function.

6.2.4 Centrifuge, suitable for centrifuging 50 ml centrifuge tubes at 8 000 *g*[2].

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

6.2.6 Equipment and/or material for grinding the sample, e.g. blender or mill.

6.2.7 UV spectrophotometer or other detection instruments, suitable for estimating the amount of DNA.

6.3 PCR

6.3.1 Suitable PCR tubes.

6.3.2 Microcentrifuge for PCR tubes.

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

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[1] FAM: 6-carboxyfluorescein, TAMRA: 6-carboxytetramethylrhodamine, BHQ1: Black Hole Quencher 1

[2] $g = 9{,}81\ \mathrm{m \cdot s^{-2}}$

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7 Procedure

7.1 General

General aspects are described in EN 15634-1 and EN ISO 21571.

7.2 Sample preparation

It should be ensured, that the test sample taken after milling or homogenizing is representative of the laboratory sample.

7.3 Preparation of extracts

7.3.1 DNA extraction with CTAB and DNA purification

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, the controls listed in 7.4.3, 7.4.6 and 7.4.7 should be performed adequately.

The analyses should be carried out **twice** in accordance with the following scheme:

— Weigh 2 g of the homogenized sample into 50 ml centrifuge tubes (tube A).

— Add 10 ml of CTAB extraction buffer solution (5.2.13).

— Add 30 µl of Proteinase K solution (5.2.14) and mix.

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

— Centrifuge for 5 min at 6 000 *g* to 8 000 *g* at room temperature.

— Place 500 µl of chloroform isoamyl alcohol mixture (5.2.12) in a 2 ml reaction vial (tube B).

— Add 700 µl of supernatant from tube A to tube B and mix thoroughly for 30 s.

— Centrifuge for 15 min at about 14 500 *g* at room temperature.

— Place 500 µl of cold isopropanol (5.2.7) in a 1,5 ml reaction vial (tube C).

— Add 500 µl of supernatant (aqueous phase) from tube B to tube C and mix carefully.

— Incubate for 30 min at room temperature.

— Centrifuge for 15 min at about 14 500 *g* at room temperature.

— Carefully remove and discard the supernatant.

— Fill the reaction vial with 500 µl of ethanol (5.2.2) and mix several times.

— Centrifuge for 5 min at about 14 500 *g* at room temperature.

— Carefully remove and discard the supernatant.

— Dry the extracted DNA.

— Dissolve the dried DNA extract in 100 µl of TE buffer solution (5.2.15).