SLOVENSKI STANDARD
kSIST-TS FprCEN/TS 15634-4:2015

01-november-2015

Živila - Odkrivanje prisotnosti alergenov v živilih z metodami molekularne biologije - 4. del: Arašidi (Arachis hypogaea) - Kvalitativno odkrivanje specifičnega niza 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

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

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

Ta slovenski standard je istoveten z: **FprCEN/TS 15634-4**

ICS:

07.100.30	Mikrobiologija živil	Food microbiology
67.190	Čokolada	Chocolate

kSIST-TS FprCEN/TS 15634-4:2015 **en,fr,de**

TECHNICAL SPECIFICATION

SPÉCIFICATION TECHNIQUE

TECHNISCHE SPEZIFIKATION

FINAL DRAFT FprCEN/TS 15634-4

September 2015

ICS 07.100.30; 67.190

English Version

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (FprCEN/TS 15634-4:2015) has been prepared by Technical Committee CEN/TC 275 “Lebensmittelanalytik - Horizontale Verfahren”, the secretariat of which is held by DIN.

This document is currently submitted to the Formal Vote.

1 Scope

This Technical Specification describes a procedure for the qualitative detection of peanut (*Arachis hypogaea*) in chocolate using real-time PCR based on the gene for the peanut allergen Ara h 2 [4], [5].

2 Principle

The total DNA is extracted from the sample and the DNA content estimated. A sequence specific to peanut from the gene for Ara h 2 is multiplicated using real-time PCR. The amplicon with a length of 86 base pairs (bp) formed in this way is detected 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 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). The pH value is set 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). The pH value is set 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 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 buffers, dNTPs, $MgCl_2$ and Hotstart Taq polymerase.

3.3.2 Oligonucleotides, 10 µmol each.

3.3.2.1 Peanut (AR-58 F), gCA gCA gTg ggA ACT CCA Agg AgA CA.

3.3.2.2 Peanut (AR-143 R), gCA TgA gAT gTT gCT CgC Ag.

3.3.2.3 Peanut probe (AR-103 T), FAM – CgA gAg ggC gAA CCT gAg gCC – TAMRA or – BHQ1.

3.3.3 Negative PCR control, conducted with DNA-free water instead of the DNA extract from the sample.

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 (blank sample).

3.3.6 Positive PCR control[2] 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 peanut, which passes through all steps of the analytical process.

3.3.8 External amplification control (inhibition 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 batch to check the influence of co-extracted substances from the sample matrix on the amplification.

4 Apparatus and equipment

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

Plastic and glass materials shall be sterilised 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.

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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) DNA for the positive PCR control is extracted from phenotypical identified pure peanuts as described in 5.3 and 5.4. DNA content is determined as described in 5.5.

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*[3)].

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 trial used the following real-time PCR equipment: Rotor Gene 6000™, Stratagene Mx 3005P, ABI PRISM® 7500, ABI PRISM® 7900HT and Roche LightCycler® 1.5. [4)]

5 Procedure

5.1 General

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

5.2 Sample preparation

It shall be ensured, e.g. by milling or homogenizing, that the test sample is representative of the laboratory sample.

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, the controls listed in 3.3.4, 3.3.5 and 3.3.7 shall be carried out adequately.

Every sample shall be prepared **twice** in accordance with the following scheme:

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

4) Rotor Gene 6000™, Stratagene Mx 3005P, ABI PRISM® 7500, ABI PRISM® 7900HT and Roche LightCycler® 1.5 are examples of suitable products available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products.

— 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 a temperature of 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*;

— Remove supernatant carefully and discard;

— 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*;

— Remove supernatant carefully and discard;

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

The DNA extract can be stored cooled (approximately 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 concentration and purity of the extracted DNA

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

ρ (DNA) in µg/ml = 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.

5.6 Real-time PCR

— PCR batch

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 peanut detection applies for a total volume per PCR batch 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 batch (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 **undiluted** in at least two PCR batches (repeat determination). If inhibitory effects result (see also 3.3.8 external amplification control), the DNA extract is to be diluted 1:4 with sterile water in a repeat process or used diluted to a concentration of 20 ng/µl.

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

— Mix the PCR master mix (Table 1, a) to e)), centrifuge shortly and pipette 20 µl per PCR batch into the reaction vials.

Table 1 — Reaction batch for real-time PCR

Reagent	Final concentration	Volume per reaction µl
a) Water		5,5
b) PCR master mix (see 3.3.1)	1x	12,5
c) Primer AR-58 F, (10 µmol[a])	0,3 µmol	0,75
d) Primer AR-143 R,(10 µmol[a])	0,3 µmol	0,75
e) Probe AR-103 T, (10 µmol[a])	0,2 µmol	0,5
Total master mix		20
i) 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.