SLOVENSKI STANDARD
SIST-TS CEN/TS 15634-2:2012

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Živila - Odkrivanje prisotnosti alergenov v živilih z metodami molekularne biologije - 2. del: Zelena (Apium graveolens) - Kvalitativno določanje specifičnega niza DNK v obarjenih klobasah s PCR v realnem času

Foodstuffs - Detection of food allergens by molecular biological methods - Part 2: Celery (Apium graveolens) - Qualitative determination of a specific DNA sequence in cooked sausages by real-time PCR

Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 2: Sellerie (Apium graveolens) - Qualitative Bestimmung einer spezifischen DNA-Sequenz in Brühwürsten mittels Real-time-PCR

Produits alimentaires - Détection des allergènes alimentaires par des méthodes d'analyse de biologie moléculaire - Partie 2: Céleri (Apium graveolens) - Détermination qualitative d'une séquence d'ADN spécifique dans des saucisses cuites par PCR en temps réel

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COMITÉ EUROPÉEN DE NORMALISATION
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Foreword

This document (CEN/TS 15634-2:2012) 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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1 Scope

This Technical Specification specifies a method for the qualitative detection of celery (*Apium graveolens*) in emulsion-type sausages (e.g. Frankfurter, Wiener).

Real-time PCR detection of celery is based on an 101 bp (base pair) sequence from the gene of the mannitol dehydrogenase (GenBank Acc. No. AF067082) of celery (*Apium graveolens*).

The method has been validated on emulsion-type sausages (Bavarian “Leberkäse”) spiked with celery. For this purpose meat batter containing mass fractions of 50 % pork meat, 25 % pork fat, 23 % crushed ice and 1,8 % of a mixture of sodium chloride, nitrite, nitrate, phosphates and ascorbates was prepared according to a standard procedure for emulsion-type sausage. The meat batter was spiked with either ground celery seeds or celery root powder to 1000 mg/kg. Lower spiking levels were obtained by diluting with celery-free meat batter. The batter was stuffed into casings and heated at 65 °C for 60 min [2].

2 Principle

Total DNA from emulsion-type sausages are isolated from the sample matrix. DNA is released from the sample matrix using the cetyltrimethylammonium bromide (CTAB) approach. Potential PCR inhibitors are removed from the isolated DNA by purification with solid phase columns. Real-time PCR is used to detect, amplify and quantify a celery specific sequence. The real time PCR method involves a fluorescence approach with a sequence specific hydrolysis probe [1], [2].

3 Reagents

3.1 General

The following general conditions for analysis shall be followed, unless specified differently. Use only analytical grade reagents suitable for molecular biology. Reagents shall be stored in small aliquots to minimise the risk of contamination. 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.

3.2 Extraction reagents

3.2.1 Chloroform, CAS 66-67-3.

3.2.2 Ethanol, volume fraction φ = 70 %, CAS 64-17-5.

3.2.3 Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), CAS 6381-92-6.

3.2.4 Cetyltrimethylammoniumbromide (CTAB), CAS 57-09-0.

3.2.5 Hydrochloric acid, φ = 37 %, CAS 7647-01-0.

3.2.6 Isoamyl alcohol, CAS 123-51-3.

3.2.7 Isopropanol, CAS 67-63-0.

3.2.8 Proteinase K, EC 3.4.21.64.

3.2.9 Sodium chloride, CAS 7647-14-5.

3.2.10 Sodium hydroxide, CAS 1310-73-2.

3.2.11 Tris(hydroxymethyl)aminomethane (TRIS), CAS 7-86-1.

3.2.12 Chloroform isoamyl alcohol mixture, 24 parts by volume of chloroform (3.2.1) are mixed with one part by volume of isoamyl alcohol (3.2.6).

NOTE Similar mixtures available commercially may be used.

3.2.13 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 shall be adjusted to read 8,0 by adding hydrochloric acid.

3.2.14 Proteinase K solution (ρ = 20 mg/ml)

NOTE Store in the form of aliquots at -20 °C after dissolving. Do not autoclave.

3.2.15 TE buffer solution containing TRIS (c = 0,001 mol/l) and Na_2EDTA (c = 0,000 1 mol/l). The pH shall be adjusted to read 8,0 by adding hydrochloric acid or sodium hydroxide solution.

3.3 DNA purification by means of solid phase extraction

For the DNA purification different methods may be used.

NOTE Several formats are commercially available, among them spin filter columns or plates. Commercially available kits may be used as appropriate.

3.4 Real-time PCR reagents

3.4.1 Concentrated PCR buffer solution [1] (containing reaction buffers, dNTPs, $MgCl_2$ and Hotstart *Taq* polymerase).

3.4.2 Oligonucleotides, c = 20 µmol/l each.

3.4.2.1 General

For information on the DNA target sequence and validation of selectivity, see 6.3.

NOTE In the interlaboratory study, the participants received their primers and the probe from the same production lot.

3.4.2.2 Forward primer (iF), Cel-MDH iF 5'-CgA TgA gCg TgT ACT gAg TC – 3'.

3.4.2.3 Reverse primer (iR), Cel-MDH iR 5'-AAT Agg AAC TAA CAT TAA TCA TAC CAA AC – 3'.

3.4.2.4 Cel-MDH probe 5'-FAM-AAC AgA TAA CgC TgA CTC ATC ACA CCg-TAMRA – 3' [2].

1) Ready-to-use reagent mixtures or single components may be used for the PCR buffer solution as long as they give results comparable to or better than the ones stated for the collaborative trial.

2) 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.

4 Apparatus and equipment

4.1 General

General aspects are described in EN 15634-1 [3]. In addition to the usual laboratory facilities, the following equipment is required.

NOTE 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.

4.2 DNA extraction

4.2.1 Suitable reaction vials with a capacity of 1,5 ml and 2 ml, sterile; 50 ml centrifuge tube, sterile.

4.2.2 Thermostat or water bath, preferably with shaker function.

4.2.3 Centrifuge suitable for centrifuging 50 ml centrifuge tubes at 8 000 g [3)].

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

4.2.5 Equipment and/or material for grinding the sample, e.g., a kitchen blender.

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

4.3 PCR

4.3.1 Suitable PCR tubes

4.3.2 Microcentrifuge for PCR tubes

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

5 Analysis steps

5.1 General

General aspects are described in EN 15634-1 [3].

5.2 Sample preparation

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

In order to minimise the risk of carry-over contaminations, all equipment should be cleaned extensively prior to proceeding with the next sample. Examples of cleaning products or techniques include: DNA-degrading agents, hypochlorite solution, hot water and detergents.

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

5.3 Preparation of extracts

5.3.1 DNA extraction with CTAB and DNA purification

In parallel to the test samples, the controls listed in 5.4.6 and 5.4.7 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 buffer (3.2.13).

— Add 30 µl of Proteinase K solution (3.2.14) and mix by inversion, pipetting or vortexing..

— Incubate and shake for 90 min at a temperature of 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 (3.2.12) 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 approximately 14 500 *g* at room temperature.

— Place 500 µl of isopropanol (3.2.7) 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 by inversion, pipetting or vortexing.

— Incubate tube C for 30 min at room temperature.

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

— Carefully remove and discard the supernatant using a pipette or by gently pouring out.

— Fill the reaction vial with 500 µl ethanol (3.2.2) and swirl several times.

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

— Carefully remove and discard the supernatant using a pipette or by gently pouring out.

— Dry the extracted DNA in order to remove the remaining traces of ethanol, e.g. by inverting tube C and allowing to blot dry on paper towels.

— Dissolve the dried DNA extract in 100 µl of TE buffer solution (3.2.15).

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

— Purify the DNA extract using e. g. solid phase extraction. For commercial kits the instructions given by the respective kit manufacturer are available.

The purified DNA extract may be stored for a short period of time (approx. 1 week) at 4 °C. For long-term storage of several months a temperature of -18 °C should be maintained.