SLOVENSKI STANDARD
SIST EN 15634-2:2019

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Nadomešča:
SIST-TS CEN/TS 15634-2:2012

Živila - Odkrivanje prisotnosti alergenov v živilih z molekularno biološkimi metodami - 2. del: Zelena (Apium graveolens) - Odkrivanje 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) - Detection of a specific DNA sequence in cooked sausages by real-time PCR

Lebensmittel - Nachweis von Lebensmittelallergenen mit molekularbiologischen Verfahren - Teil 2: Sellerie (Apium graveolens) - Nachweis 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) - Détection d'une séquence d'ADN spécifique dans des saucisses cuites par PCR en temps réel

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ICS:

07.100.30	Mikrobiologija živil	Food microbiology
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English Version

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

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

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European foreword

This document (EN 15634-2:2019) 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of an identical text or by endorsement, at the latest by April 2020, and conflicting national standards shall be withdrawn at the latest by April 2020.

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 15634-2:2012.

Significant technical changes between this standard and CEN/TS 15634-2:2012 are as follows:

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

b) normative references added (2);

c) expression of results (8) updated;

d) requirements regarding the test report added (10)

e) updated bibliography.

According to the CEN-CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

For the use of this document the term:

— ‘shall’ indicates a requirement;

— ‘should’ indicates a recommendation;

— ‘may’ indicates a permission; and

— ‘can’ indicates a possibility and/or a capability.

1 Scope

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

Real-time PCR (polymerase chain reaction) detection of celery is based on an 101 bp (base pair) sequence from the gene of the mannitol dehydrogenase (GenBank Acc. No. AF067082[1)] 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 [1].

This document is intended to be used in addition to EN 15842 and EN 15634-1.

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

4 Principle

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

1) NCBI-GeneBank® is an example of a suitable search tool for free use. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN.

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. Reagents should be stored in small aliquots to minimize 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.

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, φ = 37 %.

5.2.6 Isoamyl alcohol.

5.2.7 Isopropanol.

5.2.8 Proteinase K.

5.2.9 Sodium chloride.

5.2.10 Sodium hydroxide.

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), c = 0,1 mol/l,

— Na_2EDTA (5.2.3), c = 0,02 mol/l.

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

5.2.14 Proteinase K solution, ρ = 20 mg/ml

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

5.2.15 TE buffer solution, containing:

— TRIS (5.2.11), c = 0,001 mol/l, and

— 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.

5.4 Real-time PCR reagents

5.4.1 Concentrated PCR buffer solution [2] (containing reaction buffers, dNTPs, $MgCl_2$ and Hotstart *Taq* polymerase).

5.4.2 Oligonucleotides, c = 20 µmol/l each.

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

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

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

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.

6.2 DNA extraction

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

6.2.2 Thermostat or water bath, preferably with shaker function.

6.2.3 Centrifuge suitable for centrifuging 50 ml centrifuge tubes at 8 000 *g* [4].

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

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

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[2] 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.

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

6.2.6 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.

7 Procedure

7.1 General

General aspects are described in EN 15634-1.

In general, factors compromising DNA detection methods, include PCR inhibitors, acidic pH and/or extensive heat treatment of the food commodity and reduction or elimination of the DNA during the production process. The detection of celery root powder is reduced compared to equivalent amounts of celery seed as demonstrated by the inter-laboratory study.

7.2 Sample preparation

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

In order to minimize 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.

7.3 Preparation of extracts

7.3.1 DNA extraction with CTAB and DNA purification

In parallel to the test samples, the controls listed in 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 buffer (5.2.13).

— Add 30 µl of Proteinase K solution (5.2.14) and mix by inversion, pipetting or vortexing.

— 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 approximately 14 500 *g* at room temperature.

— Place 500 µl of 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 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 (5.2.2) and mix 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 (5.2.15).

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 considered.

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

7.3.2 Quantification and normalization of DNA concentration

The concentration of a DNA aliquot can be determined by UV spectrophotometers at a wavelength of 260 nm (concentration in ng/µl = 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 260 nm and 280 nm should be approximately 1,8.

The DNA concentration may also be estimated using other suitable procedures.

NOTE In the interlaboratory trial the DNA extracts have been adjusted to a mass concentration of approximately 20 ng/µl, e.g. by diluting with water.

7.4 Real-time PCR set-up

7.4.1 Reaction mix for real-time PCR

As an example, the procedure is described in the following for a total reaction volume of 25 µl (20 µl PCR mix and 5 µl DNA extract) with the reagents indicated in Table 1. The final concentrations of the reagents given in Table 1 have been proved to be suitable. The PCR may also be carried out with larger volumes, if the solutions are adapted correspondingly.

In parallel to the test samples, the controls listed in 7.4.3 to 7.4.7 should be carried out adequately.

Prior to use, the reagents should be gently thawed e.g. on an ice/cooling block and centrifuged briefly. If needed, during preparation of the PCR mix the reagents should be stored in an ice bath or cooling block. Care shall be taken to carefully mix any reagents immediately before pipetting.