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BSI Standards Publication

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

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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

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

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

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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].

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

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.

5.3.2 Quantification and normalization of DNA concentration

The concentration of a DNA aliquot can be determined by means of 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.

NOTE 1 The DNA concentration may also be estimated using other suitable procedures.

NOTE 2 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.

5.4 Procedure: Real-time PCR set-up

5.4.1 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 5.4.3 to 5.4.7 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.

A PCR mix should be prepared or set up 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 pipetting reserve of approximately 10 %.

Two replicates of each DNA extract should be analysed by PCR. For each reaction, 5 µl of DNA extract should be used.

NOTE In order to exclude false-negative results occurring due to PCR inhibition or highly degraded DNA, suitability of the isolate DNA can be checked by, e.g., amplification of universal sequences from plants [4].

Table 1 — Reaction mix for real-time PCR

Reagent	Final substance concentration
a) 2x PCR buffer solution (3.4.1)	1x
b) Primer Cel-MDH iF	0,3 µmol/l
c) Primer Cel-MDH iR	0,3 µmol/l
d) Probe Cel-MDH probe	0,2 µmol/l
e) Water	
f) DNA extract (approx. 20 ng/µl, max. 50 ng/µl)	

— Mix the PCR reagents (see Table 1, a) to e)), centrifuge shortly (not longer than 10 s) and pipette 20 µl per PCR reaction into the reaction vials.

— For each of the sample reactions, pipette 5 µl of sample DNA extract into the PCR mix.

- For the **positive DNA target control** (5.4.2), pipette 5 µl of a celery reference DNA into the PCR mix.
- For the **negative DNA target control** (5.4.3), pipette 5 µl of a celery free sample DNA extract into the PCR mix.
- For the **PCR inhibition controls** (5.4.4), pipette one aliquot of celery control DNA (e.g. 100 copies of the 279 bp amplicon described in reference [1]) to an aliquot of (each of) the extracted sample DNA.
- For the **amplification reagent control** (5.4.5), pipette 5 µl of water into the PCR mix.
- For the **extraction blank control** (5.4.6), pipette 5 µl of the corresponding control extract into the PCR mix.
- For the **positive extraction control** (5.4.7), pipette 5 µl of DNA extract of a reference sample containing celery into the PCR mix.
- Place the reaction vials in the PCR device and start the temperature/time programme as described in 5.4.8.

5.4.2 Positive control for DNA targets

In general, there are four types of positive controls for DNA targets:

1. Reference DNA
2. DNA extracted from a certified reference material
3. Known positive sample representative of the sequence
4. Target under study.

NOTE The control is intended to demonstrate what the positive test samples are expected to look like.

5.4.3 Negative control for DNA targets

In general, there are three types of negative controls for DNA targets

1. Reference DNA
2. DNA extracted from a certified negative reference material (blank matrix)
3. Known negative sample not containing the sequence under study.

NOTE The control is intended to demonstrate the result of negative test samples (i. e. samples not containing the sequence under study).

5.4.4 PCR inhibition control

Control DNA which is added to an aliquot of the extracted nucleic acid sample in a specified amount or number of copies. This aliquot is then used in a separate reaction batch in order to check the influence of co-extracted substances from the sample matrix on the amplification.

NOTE In the collaborative trial, a PCR amplicon containing the target sequence of the real-time PCR with a length of 279 bp was used as positive PCR control. This PCR control was prepared in accordance with the information given in reference [1] and purified by means of solid phase extraction through spin filter columns. After measuring the DNA concentration it was adjusted to 100 copies/5 µl by diluting with a suitable DNA solution (commercially available lambda DNA (ρ = 5 ng/µl)). The volume of water in the PCR reaction mix was accordingly reduced.

5.4.5 Amplification reagent control

Control containing all the reagents, except for the extracted test sample template DNA, e,g. instead of the template DNA, a corresponding volume of nucleic acid free water is added to the reaction. The water used should be double distilled or equivalent, free from DNA and nucleases (molecular biology grade).

5.4.6 Extraction blank control

Control performing all steps of the extraction procedure, except the addition of the test sample, e.g. by substitution of water for the test portion. The water used should be double distilled or equivalent, free from DNA and nucleases (molecular biology grade).

NOTE It is used to demonstrate the absence of contaminating nucleic acid during extraction.

5.4.7 Positive extraction control

Control sample meant to demonstrate that the nucleic acid extraction procedure has been performed in a way that will allow for extraction and subsequent amplification of the target nucleic acid, i.e. by using a sample material known to contain the target nucleic acid.

NOTE Information about controls can be found in EN ISO 24276 [5].

5.4.8 Temperature/time programme (real- time PCR)

The temperature/time programme described in Table 2 has been shown to be suitable for reaction vials made of synthetic material and the reagents used.

Table 2 — Temperature/time programme

<table>
<tr><th>Step</th><th colspan="2">Parameter</th><th>Temperature</th><th>Time</th><th>Fluorescence measurement</th><th>Cycles</th></tr>
<tr><td>1</td><td colspan="2">Uracil-N-glycosylase (UNG) activation (optional)</td><td>50 °C</td><td>2 min</td><td>No</td><td>1</td></tr>
<tr><td>2</td><td colspan="2">Initial denaturation</td><td>95 °C</td><td>10 min</td><td>No</td><td>1</td></tr>
<tr><td rowspan="2">3</td><td rowspan="2">Amplification</td><td>Denaturation</td><td>95 °C</td><td>15 s</td><td>No</td><td rowspan="2">45</td></tr>
<tr><td>Annealing and Extension</td><td>60 °C</td><td>60 s</td><td>Yes</td></tr>
</table>

5.4.9 Accept/Reject criteria

The results obtained including the controls, should be unambiguous and yield the expected results. Otherwise, the whole procedure, starting from the extraction of DNA, should be repeated.

Results from two test samples should be consistent. If one test sample gives a positive result and the other gives a negative result, then the analysis shall be repeated. If possible, the quantity of template nucleic acid in the reaction should be increased, so as to get consistent results for both test samples.

Moreover, as a minimum, the purity of the template nucleic acid should be checked by including a PCR inhibition control. Other controls to check the length and integrity of the template nucleic acid may be useful.

The evaluation shall be carried out with the appropriate data analyser, specific for the device. Indication of the amplification may differ depending on the real-time PCR device used. When no detectable PCR products are obtained (negative result) the indication may read, e. g., “undetermined” or “no amp”, or else the maximum number of cycles set may be given in the test report. In case of amplification of the DNA target sequence in a

sample (positive result), the cycle count at which a predefined fluorescence threshold value (the Ct or Cp value) was exceeded should be calculated.

NOTE 1 If the automatic evaluation does not give any sensible result due to atypical fluorescence measurement data, manual adjustment of the baseline and the threshold value may be required prior to evaluating the data. When doing so, follow the device specific instructions given in the respective manual for using the evaluation software.

The PCR result will be either

— Positive, if a specific PCR product has been detected and all the controls give the expected results, or

— Negative, if a specific PCR product has not been detected and all the controls give the expected results.

The target sequence is considered to be detected, if:

— an increase in the measured fluorescence can be observed that is caused by the amplification, by means of celery specific primers and the probe;

— no increase in fluorescence caused by amplification can be observed in the PCR control reactions without added celery DNA (negative DNA target control, extraction blank control or amplification reagent control); and if:

— the expected Ct values are obtained in the positive control reactions and, if appropriate, in the inhibition control.

NOTE 2 In the collaborative trial, the Ct values obtained were $35,6 \pm 1,2$ with $n = 23$ for the positive DNA target control corresponding to 100 copies of target DNA and $36,3 \pm 2,3$ with $n = 19$ for the positive extraction control spiked with 100 mg/kg celery seed.

5.4.10 Identification

Verification of a positive detection is obtained by the sequence-specific hybridization of the real-time hydrolysis probe.

6 Validation status and performance criteria

6.1 Robustness of the method

Participants of the inter-laboratory ring-trial used different types of real-time cycler devices (2× capillary based system, 10× 96-well block-based instrument and 2× rotary design cycler). The DNA extraction method and the PCR reaction mix were defined. All reagents except for primers and probe had to be provided by the participants. Therefore, the results of the ring trial reflect a broad spectrum of variation in reaction conditions.

The higher false-negative rate observed for the detection of celery root powder is indicative of the reduced detectability of this part of the celery plant. This reduced detectability may be due to lower DNA-content and/or DNA extraction efficiency of celery root powder compared to celery seed.

6.2 Intra-laboratory data

The intra-laboratory validation of the relative repeatability standard deviation (RSDr) was determined on the basis of a celery-containing concentrated beef broth sample. This apparently homogeneous matrix was selected in order to minimize errors due to inhomogeneity of the sample material itself.

The overall variation of both, the DNA extraction procedure and the amplification reaction, was estimated by DNA extraction of 15 test samples (500 mg each) and subsequent PCR analysis. Copy numbers were calculated on the basis of a calibration curve obtained by analyzing serial dilutions of the target sequence

ranging from 1 to 10^5 copies. All reactions were carried out in triplicate. Measurements yielded RSD_r values of ± 5 copies or relative standard deviations (CV) of ± 41 %. Since the calculated mean copy number of target DNA in the PCR (= 13 copies per reaction) was very low, i.e. close to the detection limit, the measured RSD_r is acceptable considering the fact that it reflects the reproducibility of the whole analysis procedure including the variability of the DNA extraction step [2].

Kappa values cannot be provided, due to the lack of alternative detection methods.

6.3 Selectivity

6.3.1 General

Real-time PCR detection of celery is based on a 101 bp (base pair) sequence from the gene of the mannitol dehydrogenase (GenBank Acc. No. AF067082) of celery (*Apium graveolens*) [2].

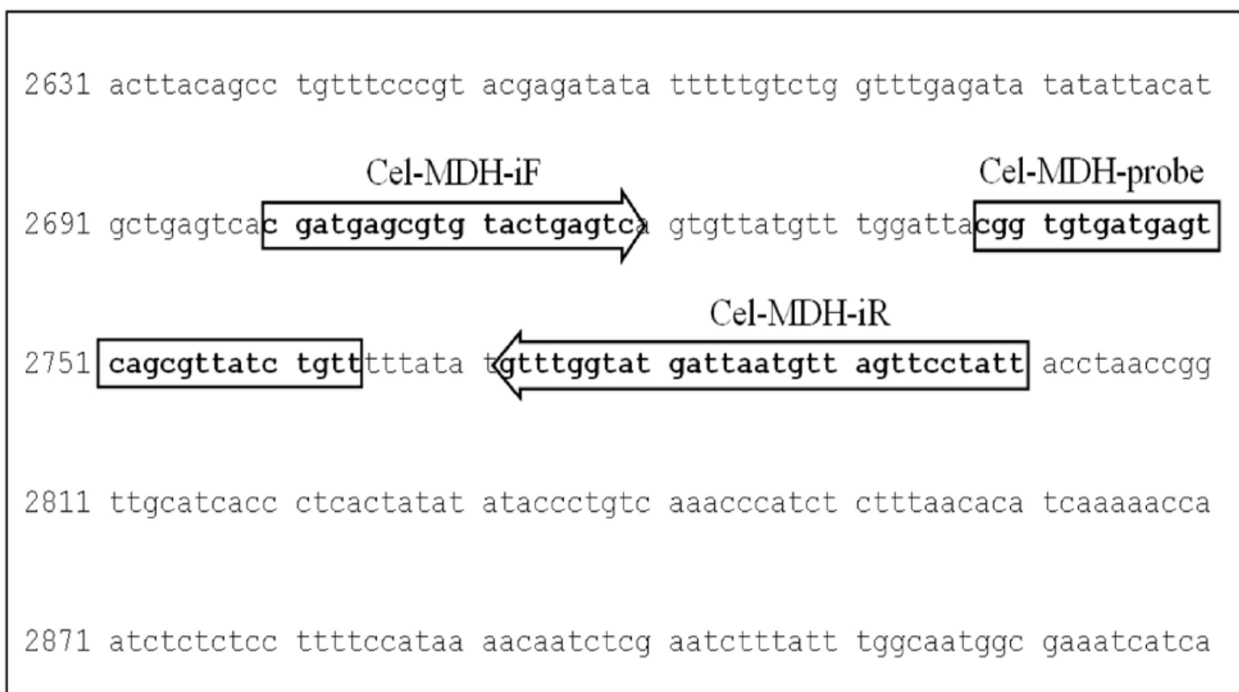

Figure 1 — Sequence from gene of mannitol dehydrogenase of celery (*Apium graveolens*)

6.3.2 Experimental

Intra-laboratory tests for selectivity included the following species of the Apiaceae family:

Anise (*Pimpinella anisum*), burnet (*Pimpinella major*), caraway (*Carum carvi*), carrot (*Daucus carota*), chervil (*Anthriscus cerefolium*), coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*), dill (*Anethum graveolens*), fennel (*Foeniculum vulgare*), lovage (*Levisticum officinale*), parsley (*Petroselinum crispum*) and parsnip (*Pastinaca sativa*).

No unspecific signals were obtained either by real-time PCR or on ethidium bromide stained agarose gels. Furthermore, DNA of the following organisms frequently used in food products yielded negative results in real-time PCR:

Basil (*Ocimum basilicum*), broccoli (*Brassica oleracea var. italica Plenck*), beef (*Bos primigenius taurus*), beetroot (*Beta vulgaris subsp. Vulgaris*), black pepper (*Piper nigrum*), cardamom (*Amomum subulatum),* cauliflower (*Brassica oleracea var. botrytis L.*), chicken (*Gallus gallus domesticus*), chive (*Allium schoenoprasum*), clove (*Syzygium aromaticum)*, corn (*Zea mays*), cucumber (*Cucumis sativus*), curcuma

(*Xanthorhiza simplicissima*), flax (Linum) seed, garlic (*Allium sativum*), ginger (*Zingiber officinale*), hot paprika (*Capsicum annuum*), horseradish (*Armoracia rusticana*), kohlrabi (*Brassica oleracea var. gongylodes L.*), laurel (*Laurus nobilis*), leek (*Allium porrum)*, lucerne (*Medicago sativa*), marjoram (*Origanum majorana*), mustard (*Sinapis spec.)*, nutmeg (*Myristica fragrans*), onion (*Allium cepa*), oregano (*Origanum vulgare*), pimento (*Pimenta officinalis*), pork (*Sus scrofa domestica*), potato (*Solanum tuberosum*), radish (*Raphanus spec*.), rice (*Oryza sativa*), rosemary (*Rosmarinus officinalis*), rye (*Secale cereale*), sage (*Salvia officinalis)*, savory (*Satureja hortensis*), sesame (*Sesamum indicum*), soy (*Glycine max)*, stone pine (Pinus pinea), tarragon (*Artemisia dracunculus*), thyme *(Thymus)*, tomato (*Solanum lycopersicum*), turkey (*Meleagris gallopavo)*, white pepper (*Piper nigrum*), yeast (*Saccharomyces spec.)*, and wheat (*Triticum).*

Prior to selectivity testing the amplificability of the isolated DNA was confirmed by either plant specific or eukaryote specific PCR-detection system.

6.3.3 Theoretical

All oligonucleotide sequences were successfully checked for the absence of homologies with other known sequences of plant DNA by BLAST search within All GenBank, EMBL, DDBJ, PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences). Last update: March 2010 (BLASTN 2.2.23). No relevant homologies have been found.

Table 3 — Summary of validation parameters of the intralaboratory studies

Validation parameter	Value	Comments
Specificity		No false positive signals with DNA from more than 50 organisms, see 6.3.2.
PCR efficiency	Slope: –3,3 to –3,4 E: 97 % to 101 %	Genomic celery DNA was analyzed by two labs, using a 7-point calibration curve; five PCR replicates per concentration.
False-negative rate	0 %	Detection of 10 copies of target DNA spiked into background DNA extracted from a celery-free seasoning sample, in all 20 replicates.
RSDr	± 5 copies; CV = 41 %	15 test samples of a concentrated beef broth sample containing 13 copies of celery target DNA were analyzed in triplicate. Since the calculated mean copy number of target DNA in the PCR is very low, i.e. close to the detection limit, the measured RSDr is acceptable considering the fact that it reflects the repeatability of the whole analysis procedure including the variability of the DNA extraction step.
LOD	10 mg of celery seed per kg of sausage	Celery in spiked samples was detected by 2 labs in all 18 reactions; (20 of 21 positive detection reactions at the 5 mg/kg level)

7 Sample type and amounts

Intra-laboratory validation of the method was achieved by using emulsion-type sausages spiked with celery seeds at mass fractions ranging from 1 mg/kg to 1 000 mg/kg [2]. The inter-laboratory study comprised mass fractions of 0 mg, 10 mg, 20 mg and 50 mg of celery seed per kg of sausage and an additional sample type spiked with 50 mg celery root powder per kg of sausage. In the interlaboratory study DNA extraction started with a test sample size of 2 g.

Within the scope of validating the applicability of the method in the intra-laboratory study a variety of commercial food products, mainly seasonings and bouillon products with unknown celery concentrations, were analyzed. Using suitable extraction methods including subsequent purification of the extracts and appropriate dilution factors of the DNA extracts no inhibition of the PCR amplification was observed [2].

8 Limit of detection

For the matrix of emulsion-type sausages the limit of detection (LOD) expressed in terms of the lowest concentration reliably detectable was determined by inter-laboratory studies to be 10 mg (0,001 %) of celery seed per kg of sausage [2]. Concerning the reduced detectability of celery root powder, see 7 and 11.3.

9 Interferences

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 has been reduced compared to equivalent amounts of celery seed as demonstrated by the collaborative trial.

10 Test report

10.1 General

According to EN 15634-1 the test report should contain at least the information given in 10.2 to 10.5.

NOTE The statements in 10.2.1 and 10.2.2 are given as examples.

10.2 Expression of results

10.2.1 Expression of a positive result

The following text could appear in the test report:

‘For laboratory sample X, the celery specific 101 bp target sequence of mannitol dehydrogenase was detected.’ ‘For the matrix of emulsion-type sausages the limit of detection (LOD) was determined by inter-laboratory studies to be 10 mg (0,001 %) of celery seed per kg of sausage. [2]’

10.2.2 Expression of a negative result

The following text could appear in the test report:

‘For laboratory sample X, the celery specific 101 bp target sequence of mannitol dehydrogenase was not detected.’ ‘For the matrix of emulsion-type sausages the limit of detection (LOD) was determined by inter-laboratory studies to be 10 mg (0,001 %) of celery seed per kg of sausage. [2]’

A negative result shall never be expressed as “specific target sequence y is not present”.

10.2.3 Ambiguous results

The results shall be expressed unambiguously, i.e. not as “+/-“.

10.2.4 Quality assurance requirements

Results from two test portions should be consistent. If one test portion gives a positive result and the other gives a negative result, then the analysis should be repeated, if possible, by increasing the quantity of template nucleic acid in the reaction, so as to get consistent results for both test portions.

Moreover, as a minimum, the purity of the template nucleic acid should be checked by including a PCR inhibition control. Other controls to check the length and integrity of the template nucleic acid may be useful.

11 Method performance studies

11.1 General

This Technical Specification has been prepared by the working group “Lebensmittel-Allergene“ (Food Allergens) of the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (Federal Office of Consumer Protection and Food Safety, BVL), Germany, for implementing LFGB § 64.

The performance of this method was studied in a collaborative trial with a total of 15 participants.

11.2 Setup of the collaborative trial

Each of the participants received 10 coded samples of emulsified sausage. The sausages were spiked in duplicate with 0 mg, 10 mg, 20 mg and 50 mg of celery seed or 50 mg celery root powder per kg of sausage. Each spiking level was examined in 20 PCR determinations (10 extractions in repeat determination). The homogeneity of each material was confirmed by means of a variance analysis method (ANOVA).

The following DNA solutions were sent to the participants to be used as positive controls.

— A 279 bp PCR amplicon containing the celery specific target sequence of the real-time PCR was used as positive PCR control [1] and purified by means of solid phase extraction through spin filter columns. The DNA concentration was adjusted to 100 copies/5 µl by dilution with a suitable commercially available lambda DNA (5 ng/µl).

— The positive DNA extraction control consisted of a DNA solution obtained from sausage spiked with 100 mg celery seed per kg.

For each of the samples, two DNA extracts had to be examined in two PCR replicates; so that every participant had to report 40 results. Participants were provided with the primer and probe solutions. Other reagents to be used for the DNA extraction and the PCR master mix as well as the real-time PCR device were chosen by the participants on their own.

11.3 Results

All 15 participating laboratories delivered results. The values from one lab had difficulties extracting the sample DNA were excluded from the evaluation (see Table 4, row 2).

NOTE Laboratories taking part in the collaborative trial used the following real-time PCR equipment: Rotor Gene 2000 or 6000, respectively, Stratagene Mx 3005P, ABI 7000, 7500, 7700 or 7900HT, respectively, Roche Light Cycler[4)].

Table 4 — Overview of data regarding the collaborative trial

Number of laboratories	15
Number of laboratories after elimination of outliers	14
Total number of samples per laboratory	10
Number of negative samples per laboratory	2
Number of PCR results per sample	4
Number of PCR results accepted	558 [a]
False-positive results	3 of 112 [b]
False-negative results	26 of 446

[a] One participant reported in two cases no second PCR replicate.

[b] Three participants detected one of four PCR of the same sample as false-positive.

In the collaborative trial samples were tested which covered a mass fraction range of 10 mg to 50 mg of celery per kg of emulsified sausage. For the 50 mg/kg spiking level, samples were presented in the forms of both celery seed and celery root powder. The results are summarized in Table 5.

Table 5 — Number of PCR reactions showing false negative or false positive results

Celery mg/kg	PCR results total	Number of false-negative PCR results	Number of false-positive PCR results	Negative rate %	Positive rate %
0	112	0	3[c]	97	3
10 [a]	112	7	0	6	94
20 [a]	111	4	0	4	96
50 [a]	111	5	0	5	95
50 [b]	112	10	0	9	91

a Celery seed

b Celery root powder

c Three participants detected one of four PCR of the same sample as false-positive.

A content of 10 mg of celery seed per kg of emulsified sausage could be detected in 94 % of all reactions, higher concentrations in celery seed showed even better ratios as was to be expected. In comparison of the detectability of celery root powder was lightly less pronounced. Thus, 50 mg of root powder per kg of emulsified sausage were still detected positively in 91 % of the batches.

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4) Rotor Gene 2000 or 6000, Stratagene Mx 3005P, ABI 7000 or 7500 or 7700 or 7900HT and Roche Light Cycler are examples of suitable products available commercially. This information is given for the convenience of users of this European Technical Specification and does not constitute an endorsement by CEN of these products.

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