



Analytical Methods

Development and validation of a duplex real-time PCR method for the simultaneous detection of celery and white mustard in food

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ABSTRACT

The developed duplex real-time PCR method allows the simultaneous detection of traces of potentially allergenic white mustard (*Sinapis alba*) and celery roots (*Apium graveolens* var. *rapaceum*), celery stalks (*A. g.* var. *dulce*) and leaf celery (*A. g.* var. *secalinum*). The duplex assay does not show any cross-reactivity with 64 different biological species, including various members of the *Brassicaceae* and *Apiaceae* family. In raw model sausages spiked with white mustard and celery roots, the LOD was found to be 0.001% white mustard and 0.005% celery. In model sausages brewed at 75–78 °C for 15 min the LOD was found to be 0.005% white mustard and 0.005% celery. The duplex real-time PCR assay was applied to check if commercial food products are labelled in compliance with the legal regulations.

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1. Introduction

Celery and mustard are known to elicit severe allergic reactions in sensitised persons, including the oral allergy syndrome (OAS), symptoms affecting the skin, the gastrointestinal tract, the respiratory system as well as life-threatening reactions like anaphylaxis. Both allergies are rather common in Central Europe. Allergy to celery is mainly a problem in Switzerland and France where about 30–40% of all food allergic persons suffer from celery allergy (Andre, Andre, Colin, Cacaraci, & Cavagna, 1994; EFSA, 2004). 1–7% of all food allergic patients are estimated to be allergic to mustard (EFSA, 2004). In France, mustard allergy is the fourth common allergy among children, following allergy to eggs, peanuts and cow's milk (Rancé, Kanny, Dutau, & Moneret-Vautrin, 1999).

Several proteins of celery are known to be allergenic: Api g 1, a 16.2 kDa protein homologous to Bet v 1, and its two isoforms Api g 1.01 and Api g 1.02 (Wangorsch, Ballmer-Weber, Rösch, Holzhauser, & Vieths, 2007), Api g 4 (a 14.3 kDa profilin similar to the birch profilin Bet v 2) (Scheurer, Wangorsch, Hausteine, & Vieths, 2000), Api g 5 (a glycoprotein with size variants from 53 to 58 kDa) (Ganglbauer et al., 2000) and CCD (cross-reactive carbohydrate determinants, 32–70 kDa) (Fötisch, Altmann, Hausteine, & Vieths, 1999). Very recently, a novel allergen belonging to the non-specific lipid-transfer protein family 1 has been characterised and desig-

nated as Api g 2 (Gadermaier et al., 2011). The following allergens have been identified in mustard: Sin a 1 (a 14 kDa 2S seed storage albumin) (Menéndez-Arias, Moneo, Domínguez, & Rodríguez, 1988), Sin a 2 (a 51 kDa seed storage 11S globulin) (Palomares et al., 2005), Sin a 3 (a 9 kDa nonspecific lipid transfer protein) and Sin a 4 (a 14 kDa profilin) (Sirvent et al., 2009) in white mustard (*Sinapis alba*) and Bra j 1 (a 16 kDa 2S seed storage albumin) (Gonzales de la Peña, Menéndez-Arias, Monsalve, & Rodríguez, 1991) in brown mustard (*Brassica juncea*).

Celery and mustard are commonly used as ingredients in spices, sausages and convenience products. Celery is eaten raw, cooked or processed whereas mustard is predominantly consumed in form of mustard seeds. Numerous studies indicate that food processing may significantly affect the allergenicity of food allergens (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). The celery allergen Api g 1 has been found to be less heat resistant than Api g 4 and CCD (Ballmer-Weber et al., 2002; Jankiewicz et al., 1997). Non-thermal processes like (ultra) high-pressure treatment, high voltage impulse treatment, drying or γ -irradiation only slightly affect the allergenicity of celery (Houska et al., 2009; Jankiewicz et al., 1997). The major allergens in mustard are known to be highly resistant to heat, acid and alkali treatment and proteolytic degradation (EFSA, 2004).

Currently, strict avoidance of the offending food is the only way to cope with a food allergy. In order to make the selection of appropriate foodstuffs easier, declaration of 14 groups of allergenic species, including celery and mustard and products thereof, is

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mandatory within the European Union according to Directive 2007/68/EC (Commission of the European Union., 2007). Several conventional PCR methods and real-time PCR methods have already been developed to verify correct labelling of celery (Dovičová, Olexová, Pangallo, Siekel, & Kuchta, 2004; Fuchs, Cichna-Markl, & Hohegger, 2012; Hupfer, Waiblinger, & Busch, 2007; Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008) or mustard (Fuchs, Cichna-Markl, & Hohegger, 2010; Mustorp et al., 2008) containing foodstuffs. In contrast to singleplex assays, multiplex assays allow the simultaneous detection of more than one template in one tube. Multiplex assays therefore offer the possibility of saving time and costs and lower the probability of cross-contamination. The challenge is, however, to design compatible primers and probes that have similar annealing temperatures and do not form primer/probe dimers. Another challenge in the development of multiplex assays is to achieve high amplification efficiency for each template, independent of the concentration of the other template(s). Several multiplex PCR assays have already been published for the detection of food allergens but most of them do not target at celery and/or mustard (Ehlert, Demmel, Hupfer, Busch, & Engel, 2009; Pafundo, Gulli, & Marmioli, 2010; Rossi et al., 2006; Schöringhumer, Redl, & Cichna-Markl, 2009). The tetraplex real-time PCR system developed by Köppel et al., however, allows the simultaneous determination of celery, hazelnut, peanut and soy (Köppel et al., 2010). The multiplex quantitative ligation dependent probe amplification method published by Mustorp et al. makes it possible to simultaneously determine eight allergenic foods including celery and mustard (Mustorp, Dromtorp, & Holck, 2011). It does, however, not only detect mustard but also other *Brassicaceae*, e.g., radish, broccoli and cabbage.

The aim of the present study was to develop a duplex real-time PCR method allowing the simultaneous detection of celery and white mustard, the most common mustard species in Europe, without showing any cross-reactivity with other common food ingredients, in particular members of the families *Brassicaceae* and *Apiaceae*. The duplex assay should be developed starting from two recently published singleplex assays for white mustard and celery (Fuchs et al., 2010; Fuchs et al., 2012).

2. Materials and methods

2.1. Chemicals and food samples

2-Propanol, chloroform, ethanol, ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA), hydrochloric acid, iso-amylalcohol, N-cetyl-N,N,N-trimethylammoniumbromide (CTAB), proteinase K, and sodium chloride were obtained from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris) was purchased from J.T. Baker (Deventer, Netherlands), phenol/chloroform/iso-amylalcohol 25:24:1 (v/v/v) from Sigma Life Sciences (Buchs, Switzerland). RNase and α -amylase were delivered from Roche (Mannheim, Germany). The SureFoodPREP Allergen Kit was from Congen (Berlin, Germany). Inhouse bidistilled water was used for DNA extraction and PCR reactions. White mustard seeds, celery roots, celery stalks and leaf celery were bought from different local merchants. White mustard varieties were provided by the State Institute of Chemical and Veterinarian Analysis (CVUA) (Freiburg, Germany), dried spices by Kotányi (Wolkersdorf, Austria). Pork meat, table salt and food samples were bought in local supermarkets.

2.2. Production of celery and white mustard spiked sausages

Model sausages spiked with 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% or 0.0001% celery and white mustard as well as

allergen free sausages were produced in cooperation with the Department for Foods of Animal Origin (Institute for Food Control, Austrian Agency for Health and Food Safety, Vienna, Austria). The allergen free matrix was produced by homogenising 700 g of minced pork meat with 300 g of ice/ice water and 20 g of salt in a cutter (robot coupe R5 plus, Toperczer, Schwechat–Rannersdorf, Austria) for 5 min. 10 g of dried celery roots and 10 g of white mustard seeds were ground in a mixer (Thermomix, type 21, Vorwerk, Hard, Austria) for 5 min. The model sausage spiked with 1% celery and 1% white mustard was produced by mixing 196 g of the allergen free matrix with 2 g of homogenised celery roots and 2 g of homogenised white mustard in a mixer (Type: GVA1, Krups, Brunn am Gebirge, Austria) for 5 min. The model sausages spiked with 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% celery and white mustard were prepared by serially “diluting” the model sausage containing 1% of celery and 1% of white mustard with the adequate amount of allergen free matrix. DNA extraction was carried out immediately afterwards. The remaining model sausages were filled in beakers and stored at -20°C .

3 g of the celery and white mustard spiked sausages were filled in 50 mL beakers and brewed for 15 min at $75\text{--}78^{\circ}\text{C}$ in a water bath (GFL, Burgwedel, Germany). The water temperature of the water bath was controlled using a temperature sensing device (alarm thermometer, $^{\circ}\text{C}$, $^{\circ}\text{F}$, Testo GmbH, Vienna, Austria). The obtained brewed model sausages were directly used for DNA extraction.

2.3. DNA extraction

The SureFoodPREP Allergen Kit was used to extract DNA from white mustard varieties, for all other matrices the CTAB method was applied. DNA extraction with the SureFoodPREP Allergen Kit was carried out according to the producer's instruction sheet. The CTAB method was described previously (Fuchs et al., 2010). The absorbance of the DNA extracts was measured at 260 nm (A_{260}) and 280 nm (A_{280}) with a spectrophotometer (Nano Photometer, Implen, Munich, Germany). The DNA concentration was calculated using the following equation: $c [\text{ng}/\mu\text{L}] = A_{260} \times 50 \times \text{dilution factor}$. The ratio A_{260}/A_{280} provided information about the purity of the isolated DNA.

2.4. Real-time PCR analysis

2.4.1. Primers and probes

The primers and probes targeting at the *S. alba* mRNA for MADS D protein (NCBI accession number: Y08626) and the *Apium graveolens* NADPH-dependent mannose-6-phosphate reductase (M6PR) mRNA (NCBI accession number: U83687) were already used in the singleplex real-time PCR assays for white mustard and celery (Fuchs et al., 2010; Fuchs et al., 2012). The sequences (5' $\rightarrow$ 3') of the primers and probes were as follows: white mustard: MADS D primer forward: TGA AAA CTC TCT TCC CCT CTT AGG, MADS D primer reverse: ACA AAT GCA CAC AAG ACA GAG ATA TAG A, MADS D probe: **FAM**-TAC ATG ATG CTT ACC TCG C-BHQ1, amplicon size: 74 bp; celery: M6PR primer forward: GGC GTA GTG ACC GTA ATG AAA, M6PR primer reverse: AGC ACA GTC AAA GTG ACG ATA ACC, M6PR probe: **Cy5**-CAA GAA TCT CCT CCT TTC CGC GAT TAA CC-BHQ2, amplicon size: 77 bp. The primers and the MADS D probe were synthesized by Eurofins MWG Operon (Ebersberg, Germany), the M6PR probe by Sigma–Aldrich (St. Louis, MO, USA).

2.4.2. PCR conditions

Real-time PCR analysis was carried out on a RotorGene RG-3000 with a 72-well rotor from Corbett Life Sciences (Hilden, Germany), using strip tubes with caps (0.1 mL, purchased from Qiagen, Hilden, Germany) in a total reaction volume of 25 μL . Two different

master mixes were used within the study, the TaqMan Universal PCR Master Mix and the TaqMan Environmental Master Mix (both from Applied Biosystems). All samples were analysed in duplicate. Two non-template controls and two positive controls were implemented in each PCR run.

2.4.3. Optimised duplex real-time PCR assay

Reactions were carried out with 200 nM M6PR forward primer, 300 nM M6PR reverse primer, 50 nM M6PR probe, 200 nM MADS D forward primer, 300 nM MADS D reverse primer, 150 nM MADS D probe, 12.5 µL TaqMan Environmental Master Mix, 5 µL of DNA extract and H₂O_{dd}. The following PCR protocol was applied: 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 30 s at 55 °C.

2.4.4. Specificity

In total, 64 biological species were tested for cross-reactivity, among them many members of the *Apiaceae* and *Brassicaceae* plant families, different meat sorts, spices and seeds. Furthermore, three varieties of celery (celery roots: *A. graveolens* var. *rapaceum*, celery stalks: *A. graveolens* var. *dulce* and leaf celery: *A. graveolens* var. *secalinum*) and 10 varieties of white mustard (*S. alba* var. *Albatros*, *Gaudi*, *Pasodoble*, *Veto*, *Sinus*, *Braco*, *Erica*, *Mirly*, *Tango* and *Slata*) were analysed. In all experiments, 5 µL of DNA extract with a concentration of 20 ng/µL, corresponding to 100 ng DNA absolute, were loaded per tube. All samples were analysed in duplicate.

2.4.5. Limit of detection, amplification efficiency and reliability

The limit of detection (LOD) and the amplification efficiency were determined by analysing serially diluted (20 ng/µL–20 fg/µL) DNA extracts from white mustard, celery roots, celery stalks and leaf celery. In addition, DNA extracts from model sausages spiked in the range from 1.0% to 0.0001% white mustard and celery roots as well as allergen free model sausages were analysed. These DNA extracts were diluted to a concentration of 100 ng/µL. The amplification efficiency was calculated from the slope of the standard curves according to the following equation: $E [\%] = [10^{(-1/(\text{slope}))} - 1] \times 100$.

The reliability of the duplex assay near the LOD was determined by analysing DNA extracts from raw and brewed model sausages spiked with 0.005%, 0.001% or 0.0005% celery roots and white mustard in 20 replicates.

2.4.6. Inhibition control

Food samples which were tested negative were subjected to inhibition control reactions. The tube contained 12.5 µL TaqMan Environmental Master Mix, 200 nM M6PR forward primer, 300 nM M6PR reverse primer, 50 nM M6PR probe, 200 nM MADS D forward primer, 300 nM MADS D reverse primer, 150 nM MADS D probe, 2.5 µL white mustard DNA extract (20 ng/µL), 2.5 µL celery roots DNA extract (20 ng/µL), 5 µL DNA extract from negative tested food samples (100 ng/µL) and H₂O_{dd}. All inhibition control reactions were carried out in duplicate according to the PCR protocol described above.

3. Results and discussion

3.1. Development and optimization of the duplex real-time PCR assay

The duplex real-time PCR assay was developed by combining the recently published singleplex assays for white mustard (Fuchs et al., 2010) and celery (Fuchs et al., 2012). Since the annealing temperatures of the two singleplex assays were quite different (55 and 60 °C, respectively) the first experiments were carried out by applying the primer and probe concentrations of the singleplex assays and varying the annealing temperature in the range

from 55 to 60 °C. The optimization experiments, carried out with serially diluted DNA extracts from white mustard and celery roots (20 ng/µL–20 fg/µL) showed that in the case of celery the Ct value was hardly influenced by the annealing temperature. At an annealing temperature of 55.9 °C the DNA extract with a concentration of 20 ng/µL DNA yielded a mean Ct value of 25.32, at an annealing temperature of 60.3 °C a mean Ct value of 24.86 was obtained. In contrast, in the case of white mustard the annealing temperature strongly affected the Ct value. Lower annealing temperatures resulted in lower Ct values. At 55.9 and 60.3 °C, the DNA extract with a concentration of 20 ng/µL DNA yielded mean Ct values of 26.21 and 29.40, respectively. All following experiments were therefore carried out at an annealing temperature of 55 °C.

Next, the influence of two different master mixes (TaqMan Universal PCR Master Mix and TaqMan Environmental Master Mix) on the performance of the duplex real-time PCR method was investigated. Using the TaqMan Universal Master Mix, the mean Ct values obtained for serially diluted white mustard DNA extracts ranged from 22.89 (20 ng/µL, 100 ng absolute) to 37.82 (2 pg/µL, 0.01 ng absolute). The slope of the standard curve was –3.645, indicating an amplification efficiency of 88.1%. For serially diluted celery roots DNA extracts, the mean Ct values were found to be between 25.32 (20 ng/µL, 100 ng absolute) and 41.90 (2 pg/µL, 0.01 ng absolute). The slope of the standard curve was –4.0435, indicating an efficiency of 76.7%. When the duplex real-time PCR assay was carried out with the TaqMan Environmental Master Mix, the mean Ct values for serially diluted white mustard DNA extracts ranged from 22.36 to 35.92. The slope of the standard curve, –3.4675, indicates a PCR efficiency of 94.3%. For serially diluted celery roots DNA extracts, mean Ct values between 24.50 (20 ng/µL) and 38.18 (2 pg/µL) were obtained. The slope of the standard curve was –3.4301, indicating an efficiency of 95.7%. Since the TaqMan Environmental Master Mix resulted in higher amplification efficiencies and slightly lower mean Ct values, all the following PCR runs were carried out with the TaqMan Environmental Master Mix. The results presented above were obtained by applying an annealing time of 60 s. By reducing the annealing time to 30 s amplification efficiencies of 99.0% (slope of the standard curve –3.347) and 99.4% (slope of the standard curve –3.3375) were obtained for white mustard and celery DNA, respectively (see Fig. 1). In all the following experiments the annealing time was therefore shortened to 30 s.

3.2. Specificity of the duplex assay

The specificity of the duplex assay was investigated by analysing DNA extracts from biological species belonging to the *Brassicaceae* family: white mustard (*S. alba*), black mustard (*Brassica nigra*), brown mustard (*B. juncea*), beet (*Brassica rapa* ssp. *Rapa*), broccoli (*Brassica oleracea* var. *Silvestris*), chinese cabbage (*Brassica rapa* ssp. *pekinensis*), cauliflower (*Brassica oleracea* var. *Botrytis*), horseradish (*Armoracia rusticana*), kohlrabi (*Brassica oleracea* var. *Gongylodes*), pak choi (*Brassica rapa chinensis*), radish (*Raphanus sativus*), rapeseed (*Brassica napus*) and white cabbage (*Brassica oleracea* var. *capitata* f. *alba*); species belonging to the *Apiaceae* family: celery roots (*A. graveolens* var. *rapaceum*), celery stalks (*A. graveolens* var. *dulce*), leaf celery (*A. graveolens* var. *secalinum*), anise (*Pimpinella anisum*), caraway (*Carum carvi*), carrot (*Daucus carota*), coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*), dill (*Anethum graveolens*), fennel (*Foeniculum vulgare*), lovage (*Levisticum officinale*), parsley (*Petroselinum crispum*) and parsnip (*Pastinaca sativa*); and further biological species: allspice (*Pimenta dioica*), apple (*Malus domestica*), bay leaf (*Laurus nobilis*), beef (*Bos taurus*), beetroot (*Beta vulgaris* ssp. *Vulgaris*), black pepper (*Piper nigrum*), brazil nut (*Bertholletia excelsa*), cardamom (*Ellettaria cardamomum*), chicken (*Gallus gallus*), chilli (*Capsicum* sp.), chive (*Allium schoenoprasum*), cinnamon (*Cinnamomum zeylanicum*), cucumber (*Cucumis*

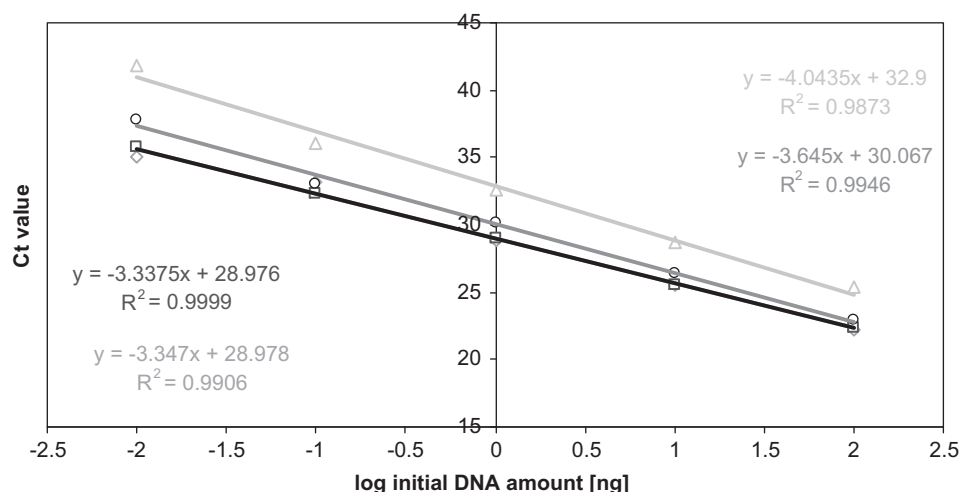


Fig. 1. Standard curves obtained by amplifying serially diluted DNA extracted from white mustard and celery roots with the TaqMan Universal Master Mix (annealing time 60 s) and the TaqMan Environmental Master Mix (annealing time 30 s): (○) white mustard – TaqMan Universal Master Mix; (◇) white mustard – TaqMan Environmental Master Mix; (△) celery roots – TaqMan Universal Master Mix; (□) celery roots – TaqMan Environmental Master Mix.

sativus), fenugreek (*Trigonella foenum-graecum*), flaxseed (*Linum usitatissimum*), garlic (*Allium sativum*), ginger (*Zingiber officinale*), horse (*Equus ferus*), leek (*Allium porrum*), maize (*Zea mays*), majoram (*Origanum majorana*), nutmeg (*Myristicia fragrans*), onion (*Allium cepa*), oregano (*Origanum vulgare*), paprika (*Capsicum annum*), pea (*Pisum sativum*), pork (*Sus scrofa*), potato (*Solanum tuberosum*), rice (*Oryza sativa*), rosemary (*Rosmarinus officinalis*), rye (*Secale cereale*), sage (*Salvia officinalis*), sesame (*Sesamum indicum*), sheep (*Ovis orientalis aries*), soy (*Glycine max*), summer savoury (*Satureja hortensis*), tarragon (*Artemisia dracunculus*), thyme (*Thymus vulgaris*), tomato (*Solanum lycopersicum*), turkey (*Meleagris gallopavo*), turmeric (*Curcuma longa/domestica*) and wheat (*Triticum durum*).

The duplex method was found to detect celery roots, celery stalks, leaf celery and all white mustard varieties tested (*S. alba* var. Albatros, Gaudi, Pasodoble, Veto, Sinus, Braco, Erica, Mirly, Tango and Slata) (see Table 1). Mean Ct values ranged from 21.79

to 24.40 (varieties of white mustard) and from 21.54 to 22.97 (varieties of celery). The duplex assay was found to be specific for white mustard and celery and did not show any cross-reactivity with the other 64 biological species tested.

3.3. LOD, efficiency and reliability

In order to investigate the LOD and the amplification efficiency of the duplex real-time PCR assay, serially diluted DNA extracts from celery roots, celery stalks, leaf celery and white mustard as well as DNA extracts from raw and brewed model sausages spiked with white mustard and celery were analysed.

When analysing serially diluted DNA extracts, the LOD for celery roots, celery stalks, leaf celery and white mustard was found to be 2 pg DNA/μL (corresponding to 0.01 ng DNA absolute). The LOD of the duplex assay is very similar to the LODs of the recently published singleplex assays for white mustard (1 pg/μL) (Fuchs et al., 2010) and celery (2 pg/μL) (Fuchs et al., 2012). Plotting the Ct value against the logarithm of the initial DNA concentration in the range from 20 ng/μL to 2 pg/μL resulted in correlation coefficients (R^2) of 0.9906 for white mustard, 0.9999 for celery roots, 0.9958 for celery stalks and 0.9982 for leaf celery. The slopes of the standard curves were found to be −3.347 (white mustard), −3.3375 (celery roots), −3.1385 (celery stalks) and −3.4085 (leaf celery), indicating amplification efficiencies of 99.0%, 99.4%, 108.3% and 96.5%, respectively. The amplification efficiencies of the duplex assay are slightly higher than those of the recently published singleplex assays (white mustard: 91.8%; celery roots: 93.9%; celery stalks: 92.9%; leaf celery: 87.4%) (Fuchs et al., 2010; Fuchs et al., 2012).

In raw model sausages white mustard could be detected down to a spike level of 0.001% (corresponding to 10 mg/kg or 10 ppm) and celery roots down to 0.005% (50 mg/kg, 50 ppm). The slope of the standard curves was −3.2969 (white mustard) and −3.5860 (celery roots), indicating an amplification efficiency of 101.1% and 90.0%, respectively. The amplification efficiency of the duplex assay for white mustard was higher and that for celery roots lower than those of the singleplex assays (white mustard: 87.1%, celery roots: 106.6%) (Fuchs et al., 2010; Fuchs et al., 2012). In brewed model sausages the LODs were found to be as low as in raw model sausages, the amplification efficiency was, however, slightly reduced (91.2% for white mustard, 85.8% for celery roots).

Table 1
Detectability of different varieties of celery and white mustard.

Variety	Ct value	Mean Ct value
<i>S. a.</i> Albatros	24.07	23.64
	23.20	
<i>S. a.</i> Gaudi	23.10	22.99
	22.88	
<i>S. a.</i> Pasodoble	21.85	21.79
	21.72	
<i>S. a.</i> Veto	23.86	23.36
	22.85	
<i>S. a.</i> Sinus	23.95	23.78
	23.61	
<i>S. a.</i> Braco	24.16	24.40
	24.63	
<i>S. a.</i> Erica	24.20	24.29
	24.37	
<i>S. a.</i> Mirly	23.70	23.83
	23.96	
<i>S. a.</i> Tango	23.98	24.37
	24.75	
<i>S. a.</i> Zlata	24.06	24.11
	24.16	
<i>A. g.</i> rapaceum	22.41	22.37
	22.33	
<i>A. g.</i> dulce	23.19	22.97
	22.74	
<i>A. g.</i> secalinum	21.56	21.54
	21.52	

Table 2

Reliability of the duplex real-time PCR method in raw model sausages near the LOD.

Spike level (%)	White mustard					Celery				
	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
0.005	33.50	33.77	33.71	0.59	1.75	34.00	34.87	34.19	0.51	1.51
	34.38	34.28				34.82	34.54			
	34.09	33.10				33.81	33.95			
	33.33	33.65				34.09	34.65			
	33.49	32.95				34.58	33.58			
	33.46	33.78				34.92	34.44			
	33.07	33.74				33.69	35.02			
	32.81	33.38				34.27	34.03			
	35.30	34.25				33.68	34.08			
	33.57	34.23				33.41	33.40			
0.001	37.01	35.71	36.73	0.86	2.33	–	37.32	36.68	0.88	2.41
	37.16	36.54				–	36.55			
	35.96	36.85				34.67	37.71			
	36.49	38.24				37.22	–			
	35.39	37.76				36.90	–			
	36.43	37.14				36.94	37.05			
	35.71	35.95				36.54	37.17			
	37.32	36.58				35.46	–			
	37.55	38.41				–	–			
	36.70	35.73				–	–			
0.0005	36.90	36.98	36.87	0.89	2.41	36.39	–	36.89	1.02	2.75
	36.78	36.41				36.68	–			
	37.88	36.95				–	37.59			
	36.41	36.66				–	35.94			
	35.72	38.55				36.94	–			
	–	37.76				–	–			
	38.38	36.37				38.48	–			
	35.87	35.38				–	–			
	36.44	–				–	37.70			
	–	37.31				35.36	–			

In order to determine the reliability of the duplex real-time PCR method near the LOD, DNA extracts of raw and brewed model sausages (spike level 0.005%, 0.001% and 0.0005% white

mustard and celery roots) were analysed in 20 replicates. The results are given in Table 2 and Table 3. At the 0.005% spike level, all replicates (20/20) yielded positive results. In raw model

Table 3

Reliability of the duplex real-time PCR method in brewed model sausages near the LOD.

Spike level (%)	White mustard					Celery				
	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
0.005	34.44	34.73	34.12	0.43	1.27	34.47	33.79	34.01	0.92	2.72
	34.54	33.90				35.88	32.95			
	33.58	33.24				34.08	32.12			
	34.84	34.30				34.93	33.77			
	33.76	34.31				33.24	34.15			
	33.76	33.58				34.01	35.92			
	34.28	34.04				34.11	33.55			
	34.45	33.95				34.20	33.71			
	34.49	33.60				34.96	33.41			
	34.44	34.09				33.98	33.00			
0.001	–	36.49	37.30	0.54	1.45	–	–	35.93	0.63	1.74
	–	38.67				36.92	–			
	–	37.64				–	–			
	37.26	37.83				35.86	–			
	37.05	37.48				36.76	34.48			
	37.26	–				35.98	36.01			
	37.29	36.75				35.63	35.84			
	37.72	36.78				–	35.96			
	36.76	37.10				35.77	36.06			
	36.94	37.70				–	–			
0.0005	–	–	37.90	0.81	2.13	–	–	35.69	0.48	1.34
	37.22	36.75				–	–			
	–	38.12				–	36.15			
	37.39	39.52				–	35.43			
	37.59	–				–	–			
	38.28	38.24				35.86	–			
	–	37.94				–	36.03			
	36.29	–				–	34.99			
	38.31	37.99				–	–			
	38.35	38.58				–	–			

Table 4

Real-time PCR results obtained by analysing food products from Austrian supermarkets.

Sample	White mustard				Celery			
	Declaration ^a	PCR result ^b	Mean Ct value	Mean Ct value inhibition control ^c	Declaration ^a	PCR result ^b	Mean Ct value	Mean Ct value inhibition control ^d
Fried noodles with curry	+	+	31.82		+	–		23.18
Herbs garlic sauce	+	+	38.64		+	–		23.20
Landjaeger (sausage)	+	+	32.49		+	–		23.50
Salanettis (sausage)	+	+	31.39		–	–		23.42
Salt with seven herbs	+	+	28.36		+	+	30.68	
Spice mix for fish	+	+	25.78		–	+	37.32	
Spice mix for game	+	+	20.96		+	–		23.07
Spice mix for herb butter	+	+	26.09		+	+	32.25	
Vegetarian nuggets	+	+	29.25		±	±	36.38/–	
Yoghurt dressing mild	+	+	31.77		+	–		23.44
Pasta ai funghi	±	+	38.12		±	–		23.33
Bratwurst 1	–	+	31.98		–	–		23.53
Spice mix for pasta asciutta 1	–	+	36.39		–	+	29.94	
Potato cream soup	–	±	38.01/–		+	–		23.60
Fried noodles	±	–		26.42	±	–		23.51
Mediterranean vegetables pan	±	–		26.28	±	–		23.51
Pasta al formaggio	±	–		26.52	+	–		23.53
Spice mix for goulash	±	–		26.23	±	–		23.15
Spice mix for pasta asciutta 2	±	–		26.32	±	–		23.07
Bratwurst 2	–	–		26.96	+	–		23.86
Noodles with spring onions sauce	–	–		26.07	+	+	27.32	
Pasta alla bolognese	–	–		26.12	+	+	28.07	
Spice mix for steak	–	–		26.56	–	–		23.39
Vegetables cream soup	–	–		26.64	–	+	31.97	
Vegetarian burger	–	–		26.27	–	–		23.07

^a Declaration: + mustard/celery listed as ingredient, – mustard/celery not listed, ± may contain mustard/celery.^b PCR result: – no amplification within 39 cycles.^c Mean Ct value inhibition control: white mustard without matrix: 26.22.^d Mean Ct value inhibition control: celery without matrix: 23.36.

sausages, white mustard was detected in 20 of 20 replicates at the 0.001% spike level and 17 of 20 replicates at the 0.0005% spike level, corresponding to a reliability of 100% and 85%, respectively. In brewed sausages, white mustard was detected in 16 of 20 replicates at the 0.001% spike level and 14 of 20 replicates at the 0.0005% spike level, corresponding to a reliability of 80% and 70%, respectively. Celery was detected in raw sausages in 11 of 20 replicates at the 0.001% spike level and eight of 20 replicates at the 0.0005% spike level, corresponding to a reliability of 55% and 40%, respectively. In brewed sausages, celery was detected in 11 of 20 replicates at the 0.001% spike level and five of 20 replicates at the 0.0005% spike level, corresponding to a reliability of 55% and 25%, respectively.

The results indicate that the duplex real-time PCR assay allows the detection of 0.005% celery in raw and brewed sausages, 0.001% white mustard in raw and 0.005% white mustard in brewed sausages without yielding false negative results.

3.4. Determination of white mustard and celery in food samples

In order to check the correct labelling of commercial food products the duplex assay was applied to the analysis of several food products (e.g., spice mixes, sausages, convenience products, and sauces). Some of the products were declared to contain or possibly contain celery and/or mustard, the ingredient list of the other food-stuffs did not give any information about the presence of celery and/or mustard. Most DNA extracts resulted in A_{260}/A_{280} ratios between 1.8 and 2.0 and DNA concentrations between 100 and 900 ng/μL. For some spice mixes, sauces and soups, the A_{260}/A_{280}

ratios were lower (<1.5) and the DNA concentration was <5 ng/μL. The poor DNA quality and the low extraction yield were probably caused by high amounts of salt and/or water in the food samples.

The results obtained by analysing the DNA extracts with the duplex assay are summarized in Table 4. Since DNA extracts from model sausages spiked with 0.001% white mustard and 0.005% celery led to Ct values <39, PCR runs yielding Ct values >39 were regarded as negative. For all samples declared to contain mustard as ingredient, a positive result was obtained. However, only four out of 12 samples declared to contain celery as ingredient yielded a positive result. We assume that in those food samples the amount of celery was below <0.005%. In only one out of six samples (pasta ai funghi) that possibly contained mustard, white mustard was detected. Celery was not detected in five out of six samples possibly containing celery. In the case of vegetarian nuggets, for one of two replicates a Ct value <39 was obtained. In two (bratwurst 1 and spice mix for pasta asciutta) out of nine samples giving no information about mustard as ingredient, white mustard was detected. In the case of potato cream soup, a Ct value <39 was obtained in one of two replicates. Positive results were obtained for three (spice mix for fish, spice mix for pasta asciutta 1 and vegetables cream soup) out of seven samples giving no information about the presence of celery.

In order to be sure that negative real-time PCR results were not caused by polymerase inhibiting compounds, inhibition control experiments were carried out. Table 4 shows, that the DNA from all food samples was amplified successfully, indicating that the negative results were not caused by PCR inhibition.

4. Conclusion

The duplex real-time PCR method presented in this study allows the specific detection of three different celery varieties (celery roots: *A. graveolens* var. *rapaceum*, celery stalks: *A. graveolens* var. *dulce*, leaf celery: *A. graveolens* var. *secalinum*) and ten different white mustard varieties (*S. alba* var. *Albatros*, *Gaudi*, *Pasodoble*, *Veto*, *Sinus*, *Braco*, *Erica*, *Mirly*, *Tango* and *Slata*). The duplex assay does not show any cross reactivity with 64 biological species, including several members of the *Apiaceae* as well as the *Brassicaceae* family, various spices, and different meat sorts. The LOD in serially diluted celery and white mustard extracts was found to be 2 pg DNA/ μ L, corresponding to 10 pg celery or white mustard DNA. The LOD is similar to those of the recently published singleplex assays for celery and white mustard. In raw model sausages spiked with celery and white mustard, the LOD was found to be 0.001% white mustard and 0.005% celery. In model sausages brewed at 75–78 °C for 15 min, the LOD was found to be 0.005% for both white mustard and celery.

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