



Analytical Methods

Development and validation of a real-time PCR method for the simultaneous detection of black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*) in foodMonika Palle-Reisch^{a,b}, Martina Wolny^a, Margit Cichna-Markl^{a,*}, Rupert Hochegger^b^a Department of Analytical Chemistry, University of Vienna, Währinger Straße 38, 1090 Vienna, Austria^b AGES, Austrian Agency for Health and Food Safety, Institute for Food Safety, Department of Molecularbiology and Microbiology, Spargelfeldstraße 191, 1220 Vienna, Austria

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ABSTRACT

The paper presents a real-time PCR method allowing the simultaneous detection of traces of black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*) in food. The primers and the probe target the *B. nigra* partial RT gene for reverse transcriptase from gypsy-like retroelement 13G42-26. The real-time PCR method does not show any cross-reactivity with other *Brassicaceae* species with the exception of white mustard. Low cross-reactivities with cinnamon, cumin, fenugreek, ginger, rye and turmeric can be ignored because in common mustard containing foodstuffs these biological species are present in very low amounts. By analysing serially diluted DNA extracts from black and brown mustard, the DNA of both mustard species could be detected down to 0.1 pg. With 10 ng DNA per PCR tube the real-time PCR method allows the detection of 5 ppm black and brown mustard in brewed sausages.

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1. Introduction

Mustard is a popular spice added to enhance the flavour and taste of foods. It is frequently present as an ingredient in spice blends, marinades, salad dressings, sausages and convenience products. Food may contain mustard seeds from three plant species of the family *Brassicaceae*: white mustard (*Sinapis alba*), black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*). Black mustard seeds have the most pungent taste, whereas white mustard seeds are the mildest ones. Brown mustard seeds are used to produce Dijon mustard.

In sensitised persons, the ingestion of mustard is known to elicit allergic symptoms. Several cases of severe allergic reactions including anaphylaxis have been reported (Figuerola et al., 2005; Jorro, Morales, Brasó, & Peláez, 1995; Kanny, Fremont, Talhouarne, & Nicolas, 1995; Malet, Valero, Lluh, Bescos, Amat, & Serra, 1993; Monreal, Botey, Pena, Marin, & Eseverri, 1992). An estimated 1–7% of all food allergic patients are allergic to mustard (EFSA (European Food Safety Authority), 2004). In France, where consumption of mustard is generally high, it is the fourth common allergenic food for children, after eggs, peanuts and cow's milk (Rancé, Kanny, Dutau, & Moneret-Vautrin, 1999). The following allergenic proteins have already been identified in white mustard: a 2S seed storage albumin with a molecular weight of 14 kDa (Sin a 1) (Menéndez-

Arias, Moneo, Domínguez, & Rodríguez, 1988), a seed storage 11S globulin (51 kDa, Sin a 2) (Palomares, Cuesta-Herranz, Vereda, Sirvent, Villalba, & Rodríguez, 2005), a non-specific lipid transfer protein (9 kDa, Sin a 3) and a profilin (14 kDa, Sin a 4) (Sirvent, Palomares, Vereda, Villalba, Cuesta-Herranz, & Rodríguez, 2009). In brown mustard, a 2S seed storage albumin (16 kDa, Bra j 1) is known to be allergenic (Gonzales de la Peña, Menéndez-Arias, & Monsalve, 1991).

The only option for allergic patients is to strictly avoid the consumption of mustard. In order to make diet planning easier, mustard and products thereof have to be declared in the European Union according to the Directive 2007/68/EC (Commission of the European Union, 2007). Selective and sensitive analytical methods are necessary to control the implementation of the legal regulations. So far, enzyme linked immunosorbent assays (ELISAs) and methods based on the polymerase chain reaction (PCR) play the most important role in food allergen analysis. Three mustard ELISAs have already been developed (Koppelman et al., 2007; Lee, Hefle, & Taylor, 2008; Shim & Wanasundara, 2008). However, two ELISAs suffer from high cross-reactivity with rapeseed (Lee et al., 2008; Shim & Wanasundara, 2008), whereas the third shows some cross-reactivity with milk, egg yolk and soy (Koppelman et al., 2007). A few companies offer commercial mustard ELISAs, one has recently been validated in an interlaboratory study (Cuhra, Gabrovská, Rysová, Hanák, & Šturm, 2011). Up to now, two real-time PCR methods (Fuchs, Cichna-Markl, & Hochegger, 2010; Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008) and a multiplex quantitative ligation dependent probe

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amplification (MLPA) method (Mustorp, Drømtorp, & Holck, 2011) have been published for the detection of mustard in foods. The MLPA method makes it possible to simultaneously determine mustard and seven allergenic foods, but it also detects other members of the *Brassicaceae* family, e.g. radish, broccoli and cabbage. The real-time PCR method presented by Mustorp et al. (2008) also shows some cross-reactivity with other *Brassica* species whereas the real-time PCR method presented by Fuchs et al. (2010) enables the specific detection of white mustard.

Since food may contain not only white but also brown and/or black mustard, the present study aimed to develop and validate a real-time PCR method that allows the simultaneous detection of black and brown mustard.

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 delivered 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 purchased from Roche (Mannheim, Germany). Inhouse bidistilled water was used for DNA extraction and for carrying out PCR reactions. Brown mustard and black mustard seeds were bought at a local market. Dried spices were provided by Kotányi (Wolkersdorf, Austria). Pork meat and table salt were bought in local supermarkets.

2.2. Production of model sausages spiked with either black or brown mustard

Model sausages spiked with 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% or 0.0001% (w/w) of each black mustard, white mustard and celery were produced in cooperation with the Department for Foods of Animal Origin (Institute for Food Control, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria). In addition, a second set of model sausages was prepared containing the same percentages of white mustard and celery but brown mustard instead of black mustard. The matrix free of mustard and celery was produced by homogenising 1400 g of minced pork meat with 600 g of ice/ice water and 40 g of salt in a cutter (robot coupe R5 plus, Toperczer, Schwechat–Rannersdorf, Austria) for 5 min. Ten grams of black, brown and white mustard seeds and celery roots were ground separately in a mixer (Thermomix, type 21, Vorwerk, Hard, Austria) for 5 min. The model sausage spiked with 1% (w/w) of each black (or brown) mustard, white mustard and celery was produced by mixing 194 g of the allergen free matrix with 2 g of each homogenised black (or brown) mustard, white mustard and celery roots in a mixer (Type: GVA1, Krups, Brunn am Gebirge, Austria) for 5 min. Model sausages spiked with 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% (w/w) of each black (or brown) mustard, white mustard and celery were prepared by serially “diluting” the model sausage containing 1% (w/w) of each black (or brown) mustard, white mustard and celery with the adequate amount of allergen free matrix.

Control sausages only consisted of allergen free matrix.

DNA extraction was carried out immediately after production. The remaining model and control sausages were filled in beakers and stored at -20°C .

3 g of the sausages was 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 kept constant using a temperature sensing device (alarm thermometer, $^{\circ}\text{C}$, $^{\circ}\text{F}$, Testo GmbH, Vienna, Austria). The brewed sausages were directly used for DNA extraction.

2.3. DNA extraction

DNA was extracted according to the CTAB protocol 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

Sequence data for the design of primers and probes were taken from the NCBI GenBank database. In total, 12 primer pairs and corresponding probes were designed, nine (1–9) at the Department of Analytical Chemistry using the Beacon Designer 7.0 (Premier Biosoft International, Palo Alto, CA, USA) and three (10–12) at the AGES using the software Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) (see Table 1). The primers and the probes (only those corresponding to the primer pairs 10–12) were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.4.2. Cross-reactivity experiments

2.4.2.1. Primer pairs 1–9. Each primer pair was tested under its optimal concentration and the optimal annealing temperature using Sybr Green as fluorescent dye. The optimal conditions were previously determined by investigating the influence of the annealing temperature (in the range from 52.8 to 61.5°C) and the concentration of the primer pair (100 , 200 and 300 nmol L^{-1}) on the Ct value.

The biological species listed in Table 2a were tested for cross-reactivity. All experiments were carried out in 96 well plates using the iCycler thermocycler, equipped with the IQ5 multicolor real-time PCR detection system (BioRad, Vienna, Austria). Each reaction was carried out in a total volume of $25 \mu\text{L}$, consisting of $12.5 \mu\text{L}$ of IQ Sybr Green Supermix (BioRad), the primer forward and primer reverse in the optimised concentration, $5 \mu\text{L}$ of template DNA ($20 \text{ ng}/\mu\text{L}$) and $\text{H}_2\text{O}_{\text{dd}}$. The temperature program was as follows: initial denaturation at 95°C for 3 min followed by 45 cycles of denaturation at 95°C for 30 s and primer annealing and elongation at the optimised annealing temperature for 40 s. After an elongation step at 72°C for 3 min, the amplicons were subjected to melting curve analysis starting at 50°C and increasing the temperature in steps of 0.5°C .

2.4.2.2. Primer/probe sets 10–12. Cross-reactivity tests of the primer/probe sets 10–12 were carried out with the Rotor Gene Q equipped with a 72-well rotor (Qiagen, Hilden, Germany). In preliminary experiments, only *Brassicaceae* species were tested. The experiments were carried out with $12.5 \mu\text{L}$ of TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nmol L^{-1} forward primer, 200 nmol L^{-1} reverse primer, 150 nmol L^{-1} probe, $5 \mu\text{L}$ of DNA extract ($20 \text{ ng}/\mu\text{L}$) and $\text{H}_2\text{O}_{\text{dd}}$. The following temperature program was used: 2 min at 50°C , 10 min at 95°C , 45 cycles of 15 s at 95°C and 60 s at 55°C .

After optimising the primer and probe concentrations and the annealing temperature, further cross-reactivity tests were carried out with primer/probe set 11. DNA extracts from more than 75 biological species were analysed, including all *Brassicaceae* species

Table 1
Primer and probe sequences.

GenBank acc. no.	Primer/probe	Sequence 5' → 3'	T _m (°C)	Amplicon length (bp)
EF470566	1f	ATC GTC GAT ACT AAT GAT GAG TGG	57.0	134
	1r	GCA ACC TCC GCC ATT TCA AG	58.1	
GU222694	2f	GGT CGA ACC ATC GTT TCT AAC AAC	59.4	93
	2r	AAT CAC CCC TTT CTA GCG TAT CTG	59.1	
DQ359129	3f	GCG ACG CCA AGC TGA TTC TC	60.1	135
	3r	CGG CGG TGG ATG ATT TCT GC	60.1	
GU230157	4f	GAT TGC GTT TTC CAA AGA CGA TAG	58.0	150
	4r	TGA GAT AAT CTC TCG GCA AGA CTC	58.7	
DQ359129	5f	GAA TCT AGG TCC GTT TCC CGA AG	59.5	149
	5r	GTT CCA TTG CGA GTT GTG CTT	58.3	
X73032	6f	TCC GAA CGG TAT GGG TTA ACG	58.4	82
	6r	AAT GTG ACC GGG ACT GTC AAC	58.8	
X73032	7f	AAA TTT CCT TTG GCT GTT CTG TTC	57.6	135
	7r	CCA AGT GTA CTG AAG GTA GTG AAC	58.0	
AY714982	8f	GGC CTA ATG ATA AAA GGG TTA CGC	59.0	101
	8r	CAC TTC TTT GCC AGA AAC CTA AGC	59.5	
AY714982	9f	ACT TCA TCT TCC ATC GTA TCA AGC	57.9	149
	9r	CTG GGC CAC ACT ATC TCT AGC	57.9	
X67836	10f	GAG GCT CCG GTT GAG TAT GC	58.4	99
	10r	CTT CTT TGG TGT TGT TGG AGT CTC	58.0	
	10p	CAC CAC CAC GCG AGA CTC CAA CAC	68.7	76
AJ415649	11f	GTT GAG CCG AGG GTC ATA ATT TC	59.8	
	11r	TCG ACT TAG GCA TCC TTA CGG	58.0	82
	11p	CGA GAG TCC GAA TAC TGG GCT GGG GTC	71.5	
AJ621248	12f	TAA CTT TTG CCC CGT GTG G	58.1	82
	12r	GAC CTA CCA CCG ACC TAA ATG G	58.4	
	12p	ATA GGA CGC CCA CAG CCC CAT TTT G	69.9	

f primer forward

r primer reverse

p probe

Table 2a
Results of cross-reactivity tests obtained with 100 ng DNA per well. Primer pairs 1–9.

Name	Botanical name	Increase of fluorescence signal								
		1	2	3	4	5	6	7	8	9
Black mustard	<i>Brassica nigra</i>	+	+	+	+	+	+	+	+	+
Brown mustard	<i>Brassica juncea</i>	+	+	+	+	+	+	+	+	+
White mustard	<i>Sinapis alba</i>	+	¹	¹	+	¹	+	+	+	+
Anise	<i>Pimpinella anisum</i>	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	–	n.a.
Beetroot	<i>Beta vulgaris ssp. vulgaris</i>	n.a.	+	¹	+	¹	+	+	±	+
Broccoli	<i>Brassica oleracea var. silvestris</i>	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.
Caraway	<i>Carum carvi</i>	n.a.	n.a.	¹	n.a.	¹	n.a.	n.a.	–	n.a.
Cauliflower	<i>Brassica oleracea var. botrytis</i>	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.
Celery	<i>Apium graveolens var. secalinum</i>	¹	+	¹	¹	+	n.a.	n.a.	–	n.a.
Celery root	<i>Apium graveolens var. rapaceum</i>	n.a.	n.a.	¹	n.a.	+	n.a.	n.a.	±	n.a.
Chinese cabbage	<i>Brassica rapa ssp. pekinensis</i>	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	±	n.a.
Chive	<i>Allium schoenoprasum</i>	n.a.	n.a.	n.a.	n.a.	¹	n.a.	n.a.	–	n.a.
Coriander	<i>Coriandrum sativum</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.
Cumin	<i>Cuminum cyminum</i>	n.a.	n.a.	¹	n.a.	¹	n.a.	n.a.	±	n.a.
Dill	<i>Anethum graveolens</i>	n.a.	n.a.	¹	n.a.	¹	n.a.	n.a.	–	n.a.
Ginger	<i>Zingiber officinale</i>	n.a.	n.a.	+	n.a.	n.a.	n.a.	n.a.	–	n.a.
Kohlrabi	<i>Brassica oleracea var. gongylodes</i>	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.
Lovage	<i>Levisticum officinale</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	±	n.a.
Marjoram	<i>Origanum majorana</i>	+	¹	¹	+	¹	n.a.	n.a.	–	n.a.
Nutmeg	<i>Myristicia fragrans</i>	n.a.	n.a.	¹	n.a.	n.a.	n.a.	n.a.	–	n.a.
Oregano	<i>Origanum vulgare</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.
Pak choi	<i>Brassica rapa chinensis</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	n.a.
Parsley	<i>Petroselinum crispum</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	+
Rapeseed	<i>Brassica napus</i>	¹	+	¹	¹	+	+	+	–	+
Summer savoury	<i>Satureja hortensis</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.
Tarragon	<i>Artemisia dracuncululus</i>	n.a.	n.a.	n.a.	n.a.	+	n.a.	n.a.	+	n.a.
Thyme	<i>Thymus vulgaris</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	n.a.
Turmeric	<i>Curcuma longa/domestica</i>	n.a.	n.a.	¹	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

– No increase of the fluorescence signal within 40 cycles.

+ Positive PCR result (Ct value ≤40).

± Positive/negative PCR result.

n.a. not analysed.

¹ Ct value ≤40 but the melting temperature (T_m) differed ≥1 °C from the T_m of the positive controls (black and brown mustard DNA).

Table 2b

Results of cross-reactivity tests obtained with 100 ng DNA per tube. Primer pairs 10 and 11.

Name	Botanical name	Increase of fluorescence signal		Mean Ct value
		10	11	
Black mustard	<i>Brassica nigra</i>	+	+	18.10
Brown mustard	<i>Brassica juncea</i>	+	+	16.95
White mustard	<i>Sinapis alba</i>	+	+	34.47
Celery	<i>Apium graveolens</i> var. <i>secalinum</i>	n.a.	+	39.67
Cinnamon	<i>Cinnamomum zeylanicum</i>	n.a.	+ ²	37.44
Cumin	<i>Cuminum cyminum</i>	n.a.	+	29.98
Fenugreek	<i>Trigonella foenum-graecum</i>	n.a.	+	29.92
Flaxseed	<i>Linum usitatissimum</i>	n.a.	±	37.78 /–
Ginger	<i>Zingiber officinale</i>	n.a.	+	32.27
Rice	<i>Oryza sativa</i>	n.a.	±	37.30 /–
Rye	<i>Secale cereale</i>	n.a.	+	35.89
Turmeric	<i>Curcuma longa</i> /domestica	n.a.	+	37.51
Allspice	<i>Pimenta dioica</i>	n.a.	–	–
Almond	<i>Prunus dulcis</i>	n.a.	–	–
Anise	<i>Pimpinella anisum</i>	n.a.	–	–
Apple	<i>Malus domestica</i>	n.a.	–	–
Barley	<i>Hordeum vulgare</i>	n.a.	–	–
Bay leaf	<i>Laurus nobilis</i>	n.a.	–	–
Bean	<i>Phaseolus vulgaris</i>	n.a.	–	–
Beef	<i>Bos taurus</i>	n.a.	–	–
Beet	<i>Brassica rapa</i> ssp. <i>rapa</i>	– ¹	–	–
Beetroot	<i>Beta vulgaris</i> ssp. <i>vulgaris</i>	n.a.	–	–
Black pepper	<i>Piper nigrum</i>	n.a.	–	–
Brazil nut	<i>Bertholletia excelsa</i>	n.a.	–	–
Buckwheat	<i>Fagopyrum esculentum</i>	n.a.	–	–
Broccoli	<i>Brassica oleracea</i> var. <i>silvestris</i>	– ¹	–	–
Caraway	<i>Carum carvi</i>	n.a.	–	–
Cardamom	<i>Ellettaria cardamomum</i>	n.a.	–	–
Carrot	<i>Daucus carota</i>	n.a.	–	–
Cauliflower	<i>Brassica oleracea</i> var. <i>botrytis</i>	– ¹	–	–
Celery root	<i>Apium graveolens</i> var. <i>rapaceum</i>	n.a.	–	–
Celery stalks	<i>Apium graveolens</i> var. <i>dulce</i>	n.a.	–	–
Chicken	<i>Gallus gallus</i>	n.a.	–	–
Chilli	<i>Capsicum</i> sp.	n.a.	–	–
Chinese cabbage	<i>Brassica rapa</i> ssp. <i>pekinensis</i>	– ¹	–	–
Chive	<i>Allium schoenoprasum</i>	n.a.	–	–
Coriander	<i>Coriandrum sativum</i>	n.a.	–	–
Cucumber	<i>Cucumis sativus</i>	n.a.	–	–
Dill	<i>Anethum graveolens</i>	n.a.	–	–
Fennel	<i>Foeniculum vulgare</i>	n.a.	–	–
Garlic	<i>Allium sativum</i>	n.a.	–	–
Horse	<i>Equus ferus</i>	n.a.	–	–
Horseradish	<i>Armoracia rusticana</i>	n.a.	–	–
Kohlrabi	<i>Brassica oleracea</i> var. <i>gongylodes</i>	– ¹	–	–
Leek	<i>Allium porrum</i>	n.a.	–	–
Lentil	<i>Lens culinaris</i>	n.a.	–	–
Lovage	<i>Levisticum officinale</i>	n.a.	–	–
Maize	<i>Zea mays</i>	n.a.	–	–
Marjoram	<i>Origanum majorana</i>	n.a.	–	–
Nutmeg	<i>Myristicia fragrans</i>	n.a.	–	–
Oat	<i>Avena sativa</i>	n.a.	–	–
Onion	<i>Allium cepa</i>	n.a.	–	–
Oregano	<i>Origanum vulgare</i>	n.a.	–	–
Pak choi	<i>Brassica rapa chinensis</i>	– ¹	–	–
Paprika	<i>Capsicum annum</i>	n.a.	–	–
Parsley	<i>Petroselinum crispum</i>	n.a.	–	–
Parsnip	<i>Pastinaca sativa</i>	n.a.	–	–
Pea	<i>Pisum sativum</i>	n.a.	–	–
Pork	<i>Sus scrofa</i>	n.a.	–	–
Porso millet	<i>Panicum miliaceum</i>	n.a.	–	–
Potato	<i>Solanum tuberosum</i>	n.a.	–	–
Radish	<i>Raphanus sativus</i>	n.a.	–	–
Rapeseed	<i>Brassica napus</i>	– ¹	–	–
Rosemary	<i>Rosmarinus officinalis</i>	n.a.	–	–
Sage	<i>Salvia officinalis</i>	n.a.	–	–
Sesame	<i>Sesamum indicum</i>	n.a.	–	–
Sheep	<i>Ovis orientalis aries</i>	n.a.	–	–
Soy	<i>Glycine max</i>	n.a.	–	–
Spelt	<i>Triticum aestivum</i> ssp. <i>spelta</i>	n.a.	–	–
Summer savoury	<i>Satureja hortensis</i>	n.a.	–	–
Tarragon	<i>Artemisia dracunculus</i>	n.a.	–	–
Thyme	<i>Thymus vulgaris</i>	n.a.	–	–
Tomato	<i>Solanum lycopersicum</i>	n.a.	–	–

(continued on next page)

Table 2b (continued)

Name	Botanical name	Increase of fluorescence signal		Mean Ct value
		10	11	11
Turkey	<i>Meleagris gallopavo</i>	n.a.	–	–
Wheat	<i>Triticum durum</i>	n.a.	–	–
White cabbage	<i>Brassica oleracea</i> var. <i>capitata</i> f. <i>alba</i>	– ¹	–	–

– No increase of the fluorescence signal within 40 cycles.

+ Positive PCR result (Ct value ≤ 40).

± Positive/negative PCR result.

n.a. not analysed.

¹ Increase of the fluorescence signal after 40 cycles.

² Since the concentration of the DNA extract was only 1.5 ng/ μ L, the DNA amount was 7.5 ng per tube.

that had been tested under non-optimised conditions (see Table 2b).

2.4.3. Optimisation of the real-time PCR assay

The real-time PCR assay was developed and optimised with primer/probe set 11. In order to optimise the primer concentration, the probe concentration was kept constant at 150 nmol L⁻¹ and the primer concentrations varied from 50 to 300 nmol L⁻¹. The optimal probe concentration was determined at the optimised primer concentrations by varying the probe concentration between 50 and 200 nmol L⁻¹. The optimal annealing temperature was determined by varying the temperature from 54 to 56 °C.

2.4.4. Optimised real-time PCR assay

The optimised real-time PCR assay was carried out with primer/probe set 11. Real-time PCR reactions were carried out in strip tubes with caps (0.1 mL, Qiagen, Hilden, Germany) in a total reaction volume of 25 μ L using the Rotor Gene Q equipped with a 72-well rotor. Reactions were carried out with 12.5 μ L TaqMan Universal PCR Master Mix, 300 nmol L⁻¹ forward primer, 300 nmol L⁻¹ reverse primer, 50 nmol L⁻¹ probe, 5 μ L of DNA extract and H₂O_{dd} with the following PCR protocol: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 60 s at 55 °C. Analysis of DNA extracts was carried out in duplicate. Two positive and two non-template controls were analysed within each run.

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 DNA extracts from black mustard and brown mustard (total DNA amount 1 ng–0.01 pg). In addition, DNA extracts from raw and brewed model sausages containing either black mustard or brown mustard (1.0–0.0001% (w/w)) were analysed. An increase of the fluorescence signal within 40 cycles (Ct value ≤ 40) was considered as positive result. A Ct value of 40 was set as threshold, since at the LOD of the PCR method Ct values <40 were obtained. The amplification efficiency was calculated from the slope of the standard curves according to the following equation: $E [\%] = [10^{(-1/(slope))} - 1] \times 100$.

The reliability of the real-time PCR assay near the LOD was determined by analysing DNA extracts from raw and brewed model sausages (containing 0.0001%, 0.0005%, 0.001% or 0.005% black mustard or brown mustard) in 20 replicates.

3. Results and discussion

3.1. Primer design

At the beginning of the study 12 primer/probe sets (see Table 1) were designed to target the following nine DNA/mRNA sequences: *B. juncea* (*B. j.*) 3-ketoacyl ACP synthase III gene (primer/probe set 1), *B. j.* expansion (expa1) gene (primer/probe set 2), *B. j.* NPR1 mRNA (primer/probe set 3 and 5), *B. j.* var. *napiformis* chalcone–

flavanone isomerase mRNA (primer/probe set 4), *B. j.* ribosomal intergenic spacer (primer/probe set 6 and 7), *B. j.* chitinase gene (primer/probe set 8 and 9), *Brassica nigra* (*B. n.*) pBNMbo5 repetitive DNA (primer/probe set 10), *B. n.* partial RT gene for reverse transcriptase from gypsy-like retroelement 13G42-26 (primer/probe set 11), and *B. n.* 5S rRNA gene (partial) and NTS (primer/probe set 12). These sequences were selected because a search for sequence homology using BLAST did not reveal any cross-reactivity with other *Brassicaceae*.

3.2. Cross-reactivity

The selectivity of the primer pairs 1–9 for black and brown mustard was tested under optimised conditions using Sybr Green as fluorescent dye. The results are summarised in Table 2a. None of the nine primer pairs was found to be selective for black and brown mustard. Most of them showed some cross-reactivity with beetroot, celery, cabbage, marjoram, rapeseed and white mustard.

The first experiments with the primer/probe sets 10–12 were carried out under non-optimised conditions. In those preliminary experiments, DNA extracts from species belonging to the *Brassicaceae* family were analysed. With primer/probe set 12 no increase of the fluorescence signal was observed with either DNA from brown and black mustard seeds or with DNA from other *Brassicaceae* species. With the primer/probe sets 10 and 11, DNA from black and brown mustard was amplified whereas DNA from other members of the *Brassicaceae* family did not result in an increase of the fluorescence signal within 40 cycles, with the exception of white mustard. However, in the case of primer/probe set 10, DNA extracts from beet, broccoli, cauliflower, Chinese cabbage, kohlrabi, pak choi, rapeseed and white cabbage yielded Ct values between 40 and 45. Due to these results, primer/probe set 11 was used in all further experiments.

When optimising the concentration of the primers and the probe and the annealing temperature the lowest Ct values were obtained with 300 nmol L⁻¹ primer forward, 300 nmol L⁻¹ primer reverse, 50 nmol L⁻¹ probe and an annealing temperature of 55 °C.

Under the optimised conditions the selectivity of primer/probe set 11 for black and brown mustard was investigated by analysing DNA extracts from more than 75 different biological species. All experiments were carried out with 100 ng DNA per tube. The results are summarised in Table 2b. DNA extracts from black mustard and brown mustard resulted in mean Ct values of 18.10 and 16.95, respectively. The primer/probe set did not show any cross-reactivity with 64 biological species. Low cross-reactivity (mean Ct value ranging from 29.92 to 39.67) was obtained for white mustard, celery, cinnamon, cumin, fenugreek, ginger, rye and turmeric. However, those cross-reactivities do not limit the application of the real-time PCR method for the selective detection of black and brown mustard because compared with the positive controls (mean Ct 18.10 and 16.95, respectively) differences in the Ct value >11 were obtained. In addition, in common foodstuffs white mus-

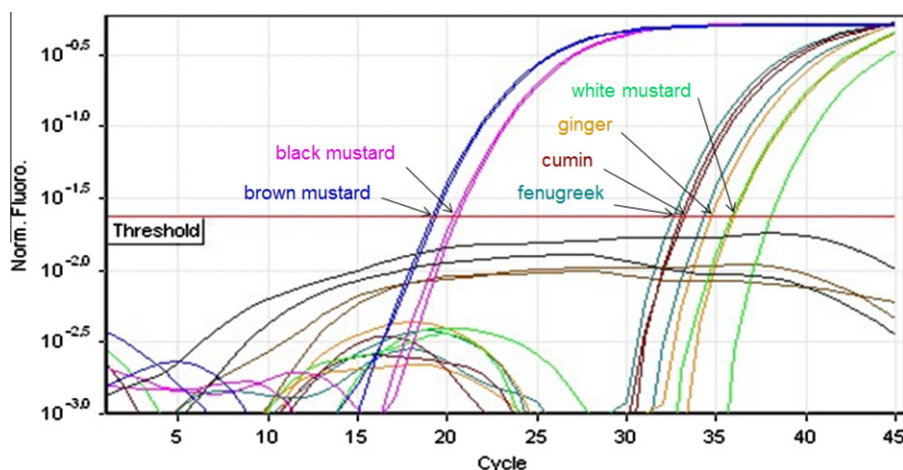


Fig. 1. Amplification curves obtained with cross-reacting species. DNA amount: 10 ng per tube.

tard, cinnamon, cumin, fenugreek, ginger and turmeric are used in trace amounts and not in high concentrations.

Nevertheless, cross-reactivity studies were performed by reducing the DNA amount per tube to 10, 5 or 1 ng. Fig. 1 shows amplification curves obtained with 10 ng DNA per tube. Table 3 indicates that at lower DNA amounts the positive controls still yielded low Ct values (10 ng DNA per tube: Ct 20.50 (black mustard) and 19.24 (brown mustard); 1 ng DNA per tube: Ct 24.54 (black mustard) and 22.74 (brown mustard)) whereas the Ct values of cross-reacting species were >33 (10 ng DNA per tube) or even >38 (1 ng DNA per tube). Next, the LOD and the amplification efficiency of the real-time PCR method were investigated.

3.3. LOD, amplification efficiency and reliability

Serially diluted DNA extracts from black mustard and brown mustard (concentration from 0.2 ng/μL to 0.002 pg/μL) were analysed. Both black and brown mustard DNA could be detected down to 0.1 pg. The slopes of the standard curves indicated amplification efficiencies of 100.6% and 86.2% for black and brown mustard DNA, respectively.

In addition, the LOD and the amplification efficiency were determined by analysing DNA extracts from raw and brewed model sausages that had been spiked with various amounts of black or brown mustard. First experiments were carried out with 1 ng DNA per PCR tube with the aim to eliminate the influence of cross-reacting species. In raw model sausages spiked with black mustard, the LOD was found to be 50 ppm (spike level of 0.005%, w/w), the amplification efficiency 83.8%. In brewed sausages, a LOD of

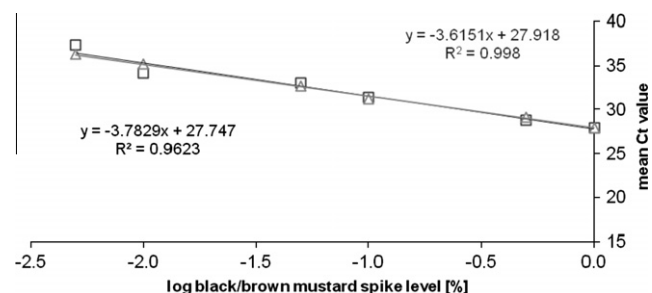


Fig. 2a. Standard curves obtained by amplifying DNA extracted from raw model sausages. DNA amount: 1 ng per tube. □ sausages spiked with black mustard, Δ sausages spiked with brown mustard.

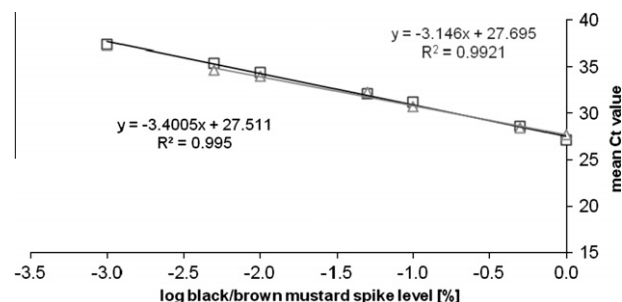


Fig. 2b. Standard curves obtained by amplifying DNA extracted from brewed model sausages. DNA amount: 1 ng per tube. □ sausages spiked with black mustard, Δ sausages spiked with brown mustard.

Table 3
Influence of the DNA amount on the Ct value.

Name	Botanical name	Mean Ct value			
		DNA amount per tube (ng)			
		100	10	5	1
Black mustard	<i>Brassica nigra</i>	18.10	20.50	21.55	24.54
Brown mustard	<i>Brassica juncea</i>	16.95	19.24	19.68	22.74
White mustard	<i>Sinapis alba</i>	34.47	36.97	36.35	39.39
Cinnamon	<i>Cinnamomum zeylanicum</i>	n.a.	37.44 ¹	–	–
Cumin	<i>Cuminum cyminum</i>	29.98	33.10	34.17	–
Fenugreek	<i>Trigonella foenum-graecum</i>	29.92	33.44	34.92	38.57
Ginger	<i>Zingiber officinale</i>	32.27	35.38	37.07/–	38.22/–

– No increase of the fluorescence signal within 40 cycles.

n.a. not analysed.

¹ Since the concentration of the DNA extract was only 1.5 ng/μL, the DNA amount was 7.5 ng per tube.

Table 4a

Reliability of the real-time PCR assay near the LOD investigated by analysing DNA extracts from raw model sausages in 20 replicates. DNA amount per tube: 1 ng.

Spike level (%)	ppm	Black mustard					Brown mustard				
		Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
0.005	50	–	37.47	35.73	1.41	3.94	35.27	36.76	35.25	0.76	2.16
		34.50	35.20				35.34	35.62			
		34.98	36.35				34.03	p.e.			
		35.44	35.81				36.27	34.26			
		35.33	34.53				36.22	33.90			
		36.96	36.09				36.02	34.80			
		36.18	39.83				35.29	35.35			
		36.16	33.98				35.19	35.68			
		36.32	34.81				35.35	35.03			
		35.54	33.46				34.45	34.96			

– No increase of the fluorescence signal within 40 cycles.

p.e. Pipetting error (no reaction mix was added).

Table 4b

Reliability of the real-time PCR assay near the LOD investigated by analysing DNA extracts from brewed model sausages in 20 replicates. DNA amount per tube: 1 ng.

Spike level (%)	ppm	Black mustard					Brown mustard				
		Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
0.005	50	36.54	36.57	35.12	0.89	2.53	34.58	36.71	35.91	1.51	4.19
		36.48	34.73				36.19	38.80			
		33.96	34.96				36.15	35.55			
		35.63	35.35				35.32	37.47			
		35.52	33.69				36.59	34.00			
		34.84	34.83				34.04	34.86			
		35.78	34.28				35.32	35.04			
		–	35.27				39.76	34.19			
		33.81	35.32				36.52	35.64			
		34.54	–				36.54	35.00			

– No increase of the fluorescence signal within 40 cycles.

Table 5

Reliability of the real-time PCR assay near the LOD investigated by analysing DNA extracts from brewed model sausages in 20 replicates. DNA amount per tube: 10 ng.

Spike level (%)	ppm	Black mustard					Brown mustard				
		Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
0.001	10	33.87	33.85	34.39	0.73	2.13	33.66	33.25	34.53	1.00	2.90
		34.28	35.64				33.64	33.28			
		33.73	33.86				35.25	34.75			
		34.12	34.87				35.91	34.44			
		34.61	35.05				33.68	34.79			
		33.11	34.65				34.52	34.96			
		35.30	34.61				36.30	33.30			
		35.64	35.23				36.51	34.83			
		33.61	33.52				34.01	34.02			
		33.82	34.40				35.59	33.81			
		35.28	34.21				36.40	35.18			
		35.29	34.38				37.38	36.24			
0.0005	5	35.39	36.19	35.25	1.06	2.99	38.07	34.24	36.02	1.54	4.26
		37.80	35.27				38.35	35.89			
		35.59	35.28				33.58	36.32			
		34.21	34.92				36.09	36.43			
		34.27	34.64				35.53	33.54			
		33.68	35.51				39.34	36.03			
		37.61	35.91				37.13	34.89			
		34.41	35.11				35.21	34.46			
		–	36.75				–	–			
		–	39.07				36.79	–			
		–	–				38.02	–			
		37.17	37.26				35.78	36.17			
0.0001	1	37.62	36.51	37.65	1.14	3.03	39.11	–	37.16	1.39	3.73
		–	38.16				35.67	38.56			
		–	–				39.56	36.73			
		36.24	39.86				37.20	36.98			
		–	37.90				–	35.31			
		–	–				–	–			

– No increase of the fluorescence signal within 40 cycles.

10 ppm (spike level of 0.001%, w/w) and an amplification efficiency of 96.8% were observed. In both raw and brewed model sausages spiked with brown mustard, the LOD was 50 ppm (spike level of 0.005%, w/w). The amplification efficiencies in raw and brewed sausages were found to be 89.1% and 107.9%, respectively. (See Fig. 2a and 2b)

The reliability of the real-time PCR method near the LOD was determined by repeatedly ($n = 20$) analysing DNA extracts from raw and brewed model sausages that had been spiked with 50 ppm black mustard or brown mustard. Again 1 ng DNA was applied per tube. Table 4a and Table 4b indicate that, in the case of brown mustard, all replicates yielded a positive result. However, in the case of black mustard only 19 (raw sausage) and 18 (brewed sausage) of 20 replicates yielded a positive result. In further experiments, DNA extracts from brewed model sausages spiked with 10, 5 or 1 ppm black or brown mustard were analysed in 20 replicates by applying 10 ng DNA per tube (Table 5). At the 10 ppm and the 5 ppm spike level, for all replicates a positive result was obtained (20/20). At the 1 ppm spike level, black mustard was only detected in 10 of 20 replicates (50%) and brown mustard in 12 of 20 replicates (60%). These results indicate that with 10 ng DNA per tube the real-time PCR method allows the detection of 5 ppm black and brown mustard in brewed sausages without yielding false negative results.

4. Conclusion

The real-time PCR assay developed with primer/probe set 11 targeting the *B. nigra* partial RT gene for reverse transcriptase from gypsy-like retroelement 13G42–26 allows the simultaneous detection of black mustard (*B. nigra*) and brown mustard (*B. juncea*) in food. As with the previously published real-time PCR method for white mustard (Fuchs et al., 2010), this real-time PCR method does not show any cross-reactivity with other *Brassica* species, with the exception of white mustard. The real-time PCR presented is therefore more applicable than the method published by Mustorp et al. (2008) that shows cross-reactivity with some *Brassica* species. The real-time PCR assay described in the present paper does, however, show some cross-reactivity with cinnamon, cumin, fenugreek, ginger, rye and turmeric. In our opinion, these low cross-reactivities can be ignored because in common mustard containing foodstuffs these biological species are present in very low amounts. By analysing serially diluted DNA extracts from black and brown mustard, the DNA of both mustard species could be detected down to 0.1 pg. In the analysis of DNA extracts from raw and brewed model sausages, which had been spiked with 50 ppm black mustard, with 1 ng DNA per tube, only 19 (raw sausage) and 18 (brewed sausage) of 20 replicates led to a positive result. However, with 10 ng DNA per PCR tube, the real-time PCR method allows the detection of 5 ppm black and brown mustard in brewed sausages without yielding false negative results. We, therefore, recommend using 10 ng DNA per tube for this PCR method.

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