



Analytical Methods

Development and validation of a duplex real-time PCR assay for the simultaneous detection of three mustard species (*Sinapis alba*, *Brassica nigra* and *Brassica juncea*) in foodMonika Palle-Reisch^{a,b}, 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 Molecular Biology and Microbiology, Spargelfeldstraße 191, 1220 Vienna, Austria

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ABSTRACT

The paper presents a duplex real-time PCR assay for the simultaneous detection of three potentially allergenic mustard species commonly used in food: white mustard (*Sinapis alba*), black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*). White mustard is detected in the “green” and black/brown mustard in the “yellow” channel. The duplex real-time PCR assay does not show cross-reactivity with other *Brassicaceae* species including broccoli, cauliflower, radish and rapeseed. Low cross-reactivities (difference in the Ct value ≥ 11.91 compared with the positive control) were obtained with cumin, fenugreek, ginger, rye and turmeric. When applying 500 ng DNA per PCR tube, the duplex real-time PCR assay allowed the detection of white, black and brown mustard in brewed model sausages down to a concentration of 5 mg/kg in 10 out of 10 replicates. The duplex real-time PCR assay was applied to verify correct labelling of commercial foodstuffs.

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1. Introduction

It is estimated that about 1–7% of all food allergic patients suffer from mustard allergy (EFSA (European Food Safety Authority), 2004). Because of different eating habits the prevalence of mustard allergy varies largely between different geographic regions. In Central Europe, in particular in France where the consumption of mustard is rather high, mustard allergy is more prevalent than in other European countries (Rancé, Kanny, Dutau, & Moneret-Vautrin, 1999).

Three mustard species, all belonging to the plant family *Brassicaceae*, are used as ingredient in foods: white mustard (*Sinapis alba*), black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*). The following proteins have already been identified as allergens in white mustard: a 2S seed storage albumin (Sin a 1) (Menéndez-Arias, Moneo, Domínguez, & Rodríguez, 1988), a seed storage 11S globulin (Sin a 2) (Palomares et al., 2005), a non-specific lipid transfer protein (Sin a 3) and a profilin (Sin a 4) (Sirvent et al., 2009). A 2S seed storage albumin (Bra j 1) with similar structural and immunological characteristics as Sin a 1 is known to be a major allergen in brown mustard (Gonzales de la Peña, Menéndez-Arias, Monsalve, & Rodríguez, 1991). Studies indicate

that Sin a 1 and Sin a 3 are rather resistant to degradation by high temperatures and digestive enzymes and therefore likely withstand food processing. In contrast, Sin a 4 was found to be partly denatured at 85 °C and completely digested by gastric treatment (Sirvent, Palomares, Cuesta-Herranz, Villalba, & Rodríguez, 2012).

At present, the only option for mustard allergic patients is to completely avoid the consumption of mustard. In order to protect these individuals from accidental intake, 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). In 2012, Canada, one of the leading mustard producing and exporting countries, updated her food allergen labelling regulations by making the declaration of mustard mandatory on the ingredient list of pre-packed foodstuffs.

An analytical method should allow the detection of all three mustard species potentially being present in food in order to be applicable for verifying correct labelling of commercial foods. In addition, the method should not show cross-reactivity with other members of the *Brassicaceae* family (e.g., cabbage, cauliflower, brussels sprout, turnip, radish, broccoli and rape) or other species commonly present in mustard containing foodstuffs. Furthermore, the method should detect traces of mustard not only in raw but also in highly processed foods. However, the three mustard ELISAs published so far (Koppelman et al., 2007; Lee, Hefle, & Taylor, 2008; Shim & Wanasundara, 2008) suffer from cross-reactivity, either

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with rapeseed (Lee et al., 2008; Shim & Wanasundara, 2008) or with milk, egg yolk and soy (Koppelman et al., 2007). A real-time PCR method presented by Mustorp et al. allows the detection of white, black and brown mustard but shows cross-reactivity with other *Brassica* species, e.g., radish (Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008). In a previous paper our group presented a real-time PCR assay for white mustard that we have carefully validated with the result that it is specific and does not show cross-reactivity with 67 species tested, including 12 *Brassica* species (Fuchs, Cichna-Markl, & Hohegger, 2010). And very recently, we have published a real-time PCR assay for the simultaneous detection of black and brown mustard. This method was found to show only minor cross-reactivity with white mustard, cinnamon, cumin, fenugreek, ginger, rye and turmeric (Palle-Reisch, Wolny, Cichna-Markl, & Hohegger, 2013). In the present study we aimed at combining the two singleplex real-time PCR assays published previously to a duplex real-time PCR assay allowing the detection of all three mustard species potentially being present in foodstuffs.

2. Materials and methods

2.1. Chemicals and foodstuffs

N-Cetyl-N,N,N-trimethylammoniumbromide (CTAB), ethylenedinitritetetracetic acid disodium salt dihydrate (EDTA), proteinase K, chloroform, iso-amylalcohol, 2-propanol, ethanol, hydrochloric acid, and sodium chloride were delivered from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris) was provided by 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). For DNA extraction and PCR reactions, in house ultrapure water (Millipore, Synergy®, Merck, Darmstadt, Germany) was used. White, black and brown mustard seeds, pork meat, table salt and several foodstuffs were bought at local merchants or supermarkets. Dried spices were provided by Kotányi (Wolkersdorf, Austria).

2.2. Production of model and control sausages

Two sets of model sausages containing different concentrations of mustard (and celery) as well as a matrix free of mustard and celery (control sausage) were used for method validation. Sausages of set 1 contained 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 or 0.0001% (w/w) of each black and white mustard (and celery), sausages of set 2 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 or 0.0001% (w/w) of each brown and white mustard (and celery). Model sausages and the control sausage were produced in cooperation with the Department of Foods, Institute for Food Safety, Austrian Agency for Health and Food Safety (AGES, Vienna, Austria) as previously described (Palle-Reisch et al., 2013). The sausages were filled in beakers and stored at -20°C .

2.3. Brewing of model and control sausages

After defrosting, 3 g of the sausage was weighed in in a beaker (50 mL) and brewed for 15 min at $75\text{--}78^{\circ}\text{C}$ in a water bath (GFL, Burgwedel, Germany) equipped with an alarm thermometer (Testo GmbH, Vienna, Austria).

2.4. DNA extraction

DNA was extracted according to the CTAB method described previously (Fuchs et al., 2010). The concentration of the DNA extracts was determined with a nano photometer (Implen, Munich,

Germany) at 260 nm (A_{260}) and 280 nm (A_{280}) using the following equation: $c [\text{ng}/\mu\text{L}] = A_{260} \times 50 \times \text{dilution factor}$.

2.5. Real-time PCR analysis

Real-time PCR was performed with a Rotor-Gene Q instrument equipped with a 72-well rotor (Qiagen, Hilden, Germany), using 0.1 mL strip tubes with caps (Qiagen). The fluorescence signal of FAM was measured in the green channel (excitation $470 \pm 10 \text{ nm}$, detection $510 \pm 5 \text{ nm}$), that of YAK (yakima yellow) in the yellow channel (excitation $530 \pm 5 \text{ nm}$, detection $557 \pm 5 \text{ nm}$). An increase of the fluorescence signal within 40 cycles (C_t value <40) was considered as a positive result. All experiments were carried out in duplicate. Within each run a positive as well as a no template control were analysed.

2.5.1. Primers and probes

The sequences of primer forward, primer reverse and the probe for white mustard ("primer f_w ", "primer r_w " and "probe $_w$ ", respectively) and black/brown mustard ("primer $f_{b/b}$ ", "primer $r_{b/b}$ " and "probe $_{b/b}$ ", respectively) were published recently (Fuchs et al., 2010; Palle-Reisch et al., 2013). Probe $_w$ was labelled with FAM at the 5' end and TAMRA at 3' end, probe $_{b/b}$ with YAK at the 5' end and BHQ1 at the 3' end. The primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.5.2. Optimisation of the duplex real-time PCR assay

The duplex real-time PCR assay was optimised by analysing serially diluted mixtures (1:1) of DNA extracts from white and black mustard and white and brown mustard. The DNA concentration of each species ranged from 20 ng/ μL to 0.002 pg/ μL , corresponding to a DNA amount (of each species) of 100 ng–0.01 pg per PCR tube.

Two master mixes were tested, the TaqMan Universal PCR Master Mix ("TMU Mix", Applied Biosystems, Foster City, CA, USA) and the QuantiTect Multiplex RT-PCR NR Kit ("QTM Mix", Qiagen). When using the TMU Mix, the PCR protocol was as follows: 2 min at 50°C , 10 min at 95°C , 45 cycles of 15 s at 95°C and 60 s at 55°C . In case of the QTM Mix, the following PCR protocol was applied: 2 min at 50°C , 10 min at 95°C , 45 cycles of 15 s at 95°C and 60 s, 45 s or 30 s at 60°C . The primer and probe concentrations used in the optimisation experiments are given in Table 1.

2.5.3. Optimised duplex real-time PCR assay

The total volume per PCR tube was 25 μL . Analysis was carried out using 12.5 μL QTM Mix, 200 nmol/L primer f_w , 300 nmol/L primer r_w , 150 nmol/L probe $_w$, 200 nmol/L primer $f_{b/b}$, 200 nmol/L primer $r_{b/b}$, 50 nmol/L probe $_{b/b}$, 5 μL DNA extract (100 ng/ μL) and H_2O_{dd} . The following PCR protocol was applied: 2 min at 50°C , 10 min at 95°C , 45 cycles of 15 s at 95°C and 45 s at 55°C .

2.5.4. Cross-reactivity experiments

The species tested for cross-reactivity are given in Table 2. Cross-reactivity experiments were carried out after adjusting the concentration of the extracts to 20 ng/ μL (DNA amount 100 ng per tube).

2.5.5. Limit of detection, amplification efficiency and reliability

The limit of detection (LOD) and the amplification efficiency (E) of the duplex real-time PCR assay were determined by analysing serially diluted mixtures (1:1) of DNA extracts from white and black mustard or white and brown mustard. The DNA concentration of each species was in the range from 20 ng/ μL to 0.002 pg/ μL (corresponding to 100 ng–0.01 pg DNA amount of each species per PCR tube). In addition, DNA extracts (20 ng/ μL) from brewed model sausages (set 1 and set 2) (containing 10000–1 mg/kg of

Table 1

Optimisation of primer and probe concentrations in the duplex real-time PCR assay – investigated combinations.

Primer/probe combination	Concentration (nmol/L)								
	1	2	3	4	5	6	7	8	9
Primer f_w	200	200	200	300	200	200	200	200	100
Primer r_w	300	300	300	300	300	300	300	300	150
Probe $_w$	150	150	150	150	250	50	50	50	50
Primer $f_{b/b}$	300	200	100	300	300	300	200	100	100
Primer $r_{b/b}$	300	200	100	300	300	300	200	100	100
Probe $_{b/b}$	50	50	50	50	50	50	50	50	50

Table 2

Results of cross-reactivity tests. DNA amount: 100 ng per PCR tube.

Name	Botanical name	Yellow channel	Green channel
		Mean Ct value	
Black mustard	<i>Brassica nigra</i>	18.37	<LOD
Brown mustard	<i>Brassica juncea</i>	16.60	<LOD
White mustard	<i>Sinapis alba</i>	31.90	21.20
Cumin	<i>Cuminum cyminum</i>	30.28	<LOD
Fenugreek	<i>Trigonella foenum-graecum</i>	31.93	<LOD
Ginger	<i>Zingiber officinale</i>	35.05	<LOD ^a
Rye	<i>Secale cereale</i>	39.14	<LOD
Turmeric	<i>Curcuma longa/domestica</i>	37.12	<LOD
Beet	<i>Brassica rapa ssp. rapa</i>	<LOD	<LOD
Broccoli	<i>Brassica oleracea var. silvestris</i>	<LOD	<LOD
Cauliflower	<i>Brassica oleracea var. botrytis</i>	<LOD	<LOD
Celery	<i>Apium graveolens var. secalinum</i>	<LOD	<LOD
Chinese cabbage	<i>Brassica rapa ssp. pekinensis</i>	<LOD	<LOD
Cinnamon	<i>Cinnamomum zeylanicum</i>	<LOD ^b	<LOD ^b
Cod	<i>Gadus morhua</i>	<LOD	<LOD
Flaxseed	<i>Linum usitatissimum</i>	<LOD	<LOD
Horseradish	<i>Armoracia rusticana</i>	<LOD	<LOD
Kohlrabi	<i>Brassica oleracea var. gongylodes</i>	<LOD	<LOD
Pak choi	<i>Brassica rapa chinensis</i>	<LOD	<LOD
Plaice	<i>Pleuronectes platessa</i>	<LOD	<LOD
Radish	<i>Raphanus sativus</i>	<LOD	<LOD
Rapeseed	<i>Brassica napus</i>	<LOD	<LOD
Rice	<i>Oryza sativa</i>	<LOD	<LOD
Salmon	<i>Salmo salar</i>	<LOD	<LOD
White cabbage	<i>Brassica oleracea var. capitata f. alba</i>	<LOD	<LOD

<LOD: no increase of the fluorescence signal within 40 cycles.

^a Duplicate: Ct value 38.02/<LOD.^b Since the concentration of the isolated DNA extract was only 8.0 ng/μL, the total DNA amount per tube was 40.0 ng.

each species) were analysed. The amplification efficiency was calculated from the slope of the calibration curves according to the following equation: $E [\%] = [10^{(-1/(slope))} - 1] \times 100$.

The reliability of the duplex real-time PCR assay near the LOD was investigated by analysing DNA extracts from brewed model sausages (set 1 and set 2, containing 1, 5 or 10 mg/kg of each species) in 10 or 20 replicates.

3. Results and discussion

3.1. Optimisation of the duplex real-time PCR assay

The primers and probes used for developing the duplex real-time PCR assay had already been used in singleplex assays for the detection of white mustard (primer f_w , primer r_w , probe $_w$) (Fuchs et al., 2010) or black and brown mustard (primer $f_{b/b}$, primer $r_{b/b}$, probe $_{b/b}$) (Palle-Reisch et al., 2013). The concentration of primer f_w , primer r_w and probe $_w$ (in the singleplex assay for white mustard) was 200 nmol/L, 300 nmol/L and 150 nmol/L, the concentration of primer $f_{b/b}$, primer $r_{b/b}$ and probe $_{b/b}$ (in the singleplex assay for black/brown mustard) 300 nmol/L, 300 nmol/L and

50 nmol/L, respectively. In both singleplex assays the following PCR protocol had been applied: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 60 s at 55 °C.

Although primer $f_{b/b}$, primer $r_{b/b}$ and probe $_{b/b}$ had been designed to be not complementary to and to have similar annealing temperatures with primer f_w , primer r_w and probe $_w$, the compatibility of the primers and probes and their suitability for being combined in a duplex real-time PCR assay had to be tested in practice. In addition, the concentration of the primers and probes had to be optimised in the duplex assay in order to achieve high amplification efficiency for all three mustard species.

First experiments were carried out with serially diluted mixtures (1:1) of DNA extracts from white and brown mustard in the range from 20 ng/μL to 0.002 pg/μL by applying the primer and probe concentrations and the temperature protocol used in the singleplex assays. Two master mixes were tested, the TaqMan Universal PCR Master Mix ("TMU Mix"), which had been used in both singleplex assays, and the QuantiTect Multiplex RT-PCR NR Kit ("QTM Mix"). In case of brown mustard, the type of the master mix did not have an influence on the amplification efficiency (TMU Mix: 106.1%, QTM Mix: 96.8%). In case of white mustard, the QTM Mix resulted in lower mean Ct values (20 ng/μL: 22.28; 0.002 ng/

μL : 37.49) than the TMU Mix (20 ng/ μL : 23.83; 0.002 ng/ μL : 41.33). The amplification efficiencies were found to be 85.3% (QTM Mix) and 70.5% (TMU Mix). Due to the higher amplification efficiency, all further experiments were carried out with the QTM Mix. Since the supplier of the mix recommends applying an annealing time of 45 s and an annealing temperature of 60 °C, we investigated if these conditions affect the amplification efficiency. Reducing the annealing time from 60 to 45 s had only minor effects on the amplification of brown mustard DNA. However, Ct values obtained for white mustard DNA ranged from 21.31 (20 ng/ μL) to 35.03 (0.002 ng/ μL) and were thus lower than the Ct values obtained with an annealing time of 60 s. The amplification efficiency was 94.6% and thus higher than that achieved with an annealing time of 60 s. Further reducing the annealing time from 45 to 30 s led to slightly lower amplification efficiency for white mustard (90.2%). Further experiments were therefore carried out by applying an annealing time of 45 s.

Next we investigated the influence of increasing the annealing temperature from 55 to 60 °C. The performance of the singleplex assay for white mustard has recently been investigated at an annealing temperature of 60 °C in an international ring-trial (Siegel, Schnur, Boernsen, Pietsch, & Waiblinger, 2012), that of the singleplex assay for black/brown mustard was tested in our lab (data not shown). The increase of the annealing temperature did not lower the performance of the singleplex assays. However, since in the duplex real-time PCR assay an annealing temperature of 60 °C resulted in slightly higher Ct values and lower amplification efficiencies for white mustard, all following experiments were carried out at an annealing temperature of 55 °C.

The primer and probe concentrations were optimised by analysing serially diluted mixtures (1:1) of DNA extracts from white

and black mustard and white and brown mustard in the range from 0.02 ng/ μL to 0.2 pg/ μL (corresponding to 0.1 ng–0.001 ng DNA per PCR tube). By testing nine combinations of primer and probe concentrations (Table 1), including the concentrations used in the singleplex assays, the lowest Ct values were obtained by applying 200 nmol/L primer f_w , 300 nmol/L primer r_w , 150 nmol/L probe $_w$, 200 nmol/L primer $f_{b/b}$, 200 nmol/L primer $r_{b/b}$ and 50 nmol/L probe $_{b/b}$. These primer and probe concentrations were used in all further experiments.

3.2. Cross-reactivity tests

The singleplex assay for white mustard had been found to be specific and had not shown cross-reactivity with 67 species tested, including 12 *Brassica* species (Fuchs et al., 2010). The singleplex assay published very recently (Palle-Reisch et al., 2013) allows the simultaneous detection of black and brown mustard and shows minor cross-reactivity with white mustard, cinnamon, cumin, fenugreek, ginger, rye and turmeric. In order to assess the selectivity of the optimised duplex real-time PCR assay, DNA extracts from ten common *Brassicaceae* species and nine plant species that were found to show low cross-reactivity in the singleplex assay for black/brown mustard, were analysed. Since mustard is a common ingredient in fish dishes, cod, plaice and salmon were tested too. The duplex assay did not show cross-reactivity with the ten *Brassicaceae* species and the three fish species tested (Table 2). In the yellow channel, an increase of the fluorescence signal was observed for cumin, fenugreek, ginger, rye and turmeric. In agreement with the singleplex assay for black/brown mustard, Ct values were >11 higher than those obtained for black and brown mustard (Table 2). Since only traces of these biological species

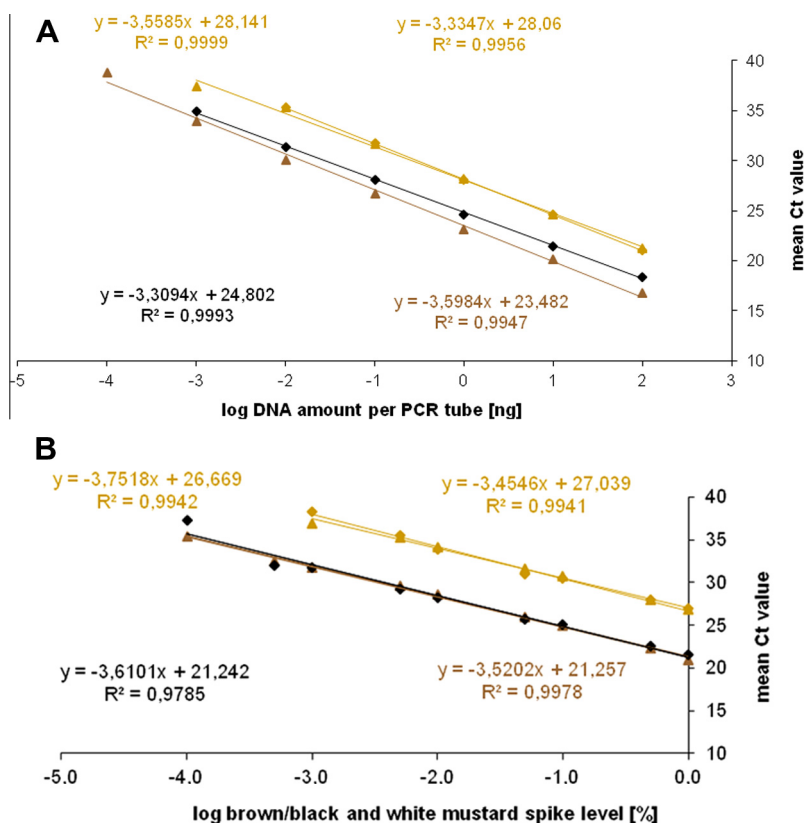


Fig. 1. Standard curves obtained by (A) amplifying serially diluted DNA extracts and (B) amplifying DNA extracted from brewed model sausages. (A) 100 ng DNA amount per mustard species per tube. $\diamond$ 1:1 mixture of DNA extracts from black and white mustard, $\triangle$ 1:1 mixture of DNA extracts from brown and white mustard. (B) 100 ng DNA amount per tube. $\diamond$ model sausage set 1, spiking level 1–0.0001% (w/w) per mustard species, $\triangle$ model sausage set 2, spiking level 1–0.0001% (w/w) per mustard species.

Table 3
Reliability of the duplex real-time PCR assay near the LOD. DNA extracts from brewed model sausages (set 1 and set 2) were carried out in 20 replicates. DNA amount: 100 ng per PCR tube.

Spiking level (mg/kg)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)
Model sausage set 1										
10	Black mustard		31.63	0.39	1.23	White mustard		37.36	0.94	2.52
	31.54	32.08				35.65	37.70			
	31.60	32.26				37.29	36.89			
	31.65	31.32				36.50	38.94			
	32.36	31.31				<LOD	36.98			
	31.41	31.51				<LOD	37.51			
	31.48	31.56				37.06	37.75			
	31.05	31.50				38.32	37.72			
	31.30	32.11				36.10	39.24			
	32.33	31.66				<LOD	37.13			
5	31.42	31.16	<LOD	36.93	37.10	0.85	2.29			
	32.96	32.87	36.85	38.08						
	33.05	32.62	36.10	37.97						
	33.03	32.77	<LOD	37.77						
	33.25	32.86	<LOD	37.85						
	33.50	32.63	38.15	36.56						
	32.58	33.57	<LOD	35.95						
	32.47	32.67	36.53	38.13						
	32.63	33.34	37.61	<LOD						
	32.18	33.88	36.26	36.43						
33.26	32.65	<LOD	36.23							
Model sausage set 2										
10	Brown mustard		31.64	0.33	1.05	White mustard		37.65	1.06	2.81
	32.11	31.59				38.16	38.96			
	31.80	32.31				36.93	37.55			
	31.41	32.21				39.55	38.17			
	31.19	31.55				37.85	<LOD			
	31.26	31.88				35.32	36.79			
	31.77	31.53				37.29	38.71			
	31.10	31.45				<LOD	37.53			
	31.80	31.93				36.87	38.40			
	31.45	31.50				<LOD	36.00			
5	31.44	31.46	37.96	37.97	<LOD	-	-			
	32.66	32.82	<LOD	37.72						
	32.87	32.62	38.25	37.50						
	33.02	32.63	<LOD	<LOD						
	32.20	32.64	37.82	<LOD						
	32.13	32.58	<LOD	37.56						
	32.60	32.63	37.89	36.85						
	33.13	32.46	<LOD	<LOD						
	33.78	32.77	<LOD	38.88						
	33.22	32.53	38.08	<LOD						
33.54	33.18	36.44	<LOD							

<LOD: no increase of the fluorescence signal within 40 cycles.

are commonly used in commercial mustard containing foodstuffs, these cross-reactivities of the duplex real-time PCR assay can be neglected and therefore do not limit its applicability in practice.

3.3. LOD, amplification efficiency and reliability

The limit of detection (LOD) of the duplex assay was determined by analysing serially diluted mixtures (1:1) of DNA extracts from white and brown mustard as well as white and black mustard in the range from 20 ng/μL to 0.002 pg/μL. Analysis of serially diluted mixtures of white and black mustard DNA resulted in LODs of 0.2 pg/μL (1 pg DNA per PCR tube) for black mustard DNA and 2 pg/μL (10 pg DNA per PCR tube) for white mustard DNA. When analysing mixtures of white and brown mustard DNA, LODs were found to be 0.2 pg/μL (1 pg DNA per PCR tube) and 0.02 pg/μL (0.1 pg DNA per PCR tube) for white and brown mustard DNA, respectively. This data indicates that the duplex assay is very sensitive for all three mustard species. The correlation coefficients (R^2) of the calibration curves obtained by plotting the Ct values against the logarithm of the initial DNA amounts were found to be ≥ 0.9947 (Fig. 1A). The slopes of the calibration curves, ranging from -3.3094 to -3.5984 , indicate amplification efficiencies of

100.5% for black, 89.6% for brown and 91.0%/99.5% for white mustard.

The sensitivity and amplification efficiency of the duplex assay were also determined by analysing DNA extracts (20 ng/μL, corresponding to 100 ng DNA per PCR tube) from two sets of brewed model sausages, spiked with defined amounts of either black and white mustard (set 1) or brown and white mustard (set 2) (Fig. 1B). In model sausage set 1, the LODs for black and white mustard were found to be 1 mg/kg (spiking level of 0.0001%, w/w) and 10 mg/kg (spiking level of 0.001%, w/w), respectively. The slopes of the standard curves were -3.6101 and -3.7518 , which indicate amplification efficiencies of 89.2% for black and 84.7% for white mustard. The LODs determined by analysing model sausage set 2 comply with those obtained in model sausage set 1 (brown mustard: 1 mg/kg, white mustard: 10 mg/kg). The amplification efficiencies, calculated from the slopes of the standard curves, were 92.3% (brown mustard) and 94.8% (white mustard).

The reliability of the duplex real-time PCR assay near the LOD was investigated by repeatedly analysing DNA extracts from brewed model sausages (set 1 and set 2), containing 10 or 5 mg/kg of each mustard species. In one series of experiments, DNA extracts were adjusted to a concentration of 20 ng/μL and analysed in

Table 4

Reliability of the duplex real-time PCR assay near the LOD. DNA extracts from brewed model sausages (set 1 and set 2) were carried out in 10 replicates. DNA amount: 500 or 200 ng per PCR tube.

Spiking level (mg/kg)	DNA amount per tube (ng)	Ct value		Mean Ct value	S	RSD (%)	Ct value		Mean Ct value	S	RSD (%)										
Model sausage set 1																					
10	500	Black mustard			29.38	0.14	0.49	White mustard			35.06	0.48	1.36								
		29.34	29.17	29.29				29.66	35.26	34.86				35.11	35.96						
		29.45	29.33	29.49				29.33	34.67	34.93				35.47	35.39						
		29.24	29.49	30.58				30.94	34.43	34.51				36.66	35.52						
		30.87	30.56	30.37				30.48	36.48	37.09				36.51	35.52						
	200	30.56	29.98	30.72	30.59	36.45	35.67	37.19	<LOD	36.34	0.64	1.75									
		30.72	30.31	30.46	30.65	35.33	35.70	35.88	36.12	35.75	0.46	1.28									
		30.18	30.58	30.43	30.47	35.06	36.26	36.20	36.26												
		30.75	30.55	31.71	31.08	35.24	35.43	37.45	38.89												
		31.52	31.46	<LOD	<LOD	<LOD	36.88	<LOD	36.61												
31.82	31.81	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD														
5	500	32.35	31.93	34.82	32.68	33.06	0.73	2.21	37.02	<LOD	<LOD	-	-								
		32.41	32.97	32.24	32.74	32.90	33.12	37.88	36.81	37.02	<LOD	<LOD	-	-							
		33.63	33.06	34.64	34.14	34.02	0.65	1.92	<LOD	<LOD	<LOD	-	-								
		34.24	35.19	33.38	33.29	33.68	33.13	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD							
		34.44	34.08	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD								
	Model sausage set 2																				
	10	500	Brown mustard			29.43	0.24	0.82	White mustard			34.90	0.81	2.32							
			29.42	29.64	29.09				29.74	34.90	35.41				34.44	34.79					
			29.15	29.50	29.21				29.33	33.87	33.83				35.50	35.02					
			29.42	29.78	30.80				30.62	34.64	36.56				35.42	37.94					
30.37			30.67	30.67	30.89				<LOD	35.75	<LOD				35.75						
200		30.67	30.89	31.02	30.57	30.54	30.55	36.30	<LOD	36.67	0.87	2.37									
		36.29	37.33	37.10	37.26	36.04	35.25	36.26	35.87	36.15	0.57	1.57									
		36.57	35.29	36.57	35.29	35.93	36.04	36.14	36.31												
		37.10	37.26	37.53	36.13	37.20	<LOD	37.53	36.13												
		37.10	37.26	37.53	36.13	37.20	<LOD	37.53	36.13												
5	500	30.40	30.33	30.17	29.73	30.19	0.26	0.85	36.04				35.25	36.15	0.57	1.57					
		29.93	30.38	30.62	30.23	30.10	30.05	35.93	36.04	36.15	0.57	1.57									
		30.62	30.23	31.72	31.51	31.56	0.25	0.80	36.57				35.29								
		30.10	30.05	31.40	31.86	31.96	31.22	37.20	<LOD												
		31.65	31.36	31.63	31.25	31.96	31.22	<LOD	35.98												
	200	31.65	31.36	31.63	31.25	31.72	31.51	31.56	0.25				0.80	36.57	35.29	36.54	0.85	2.34			
		31.40	31.86	31.96	31.22	31.65	31.36	31.63	31.25	31.72	31.51	31.56	0.25	0.80	36.57	35.29					
		31.40	31.86	31.96	31.22	31.65	31.36	31.63	31.25	31.72	31.51	31.56	0.25	0.80	36.57	35.29					
		31.40	31.86	31.96	31.22	31.65	31.36	31.63	31.25	31.72	31.51	31.56	0.25	0.80	36.57	35.29					
		31.40	31.86	31.96	31.22	31.65	31.36	31.63	31.25	31.72	31.51	31.56	0.25	0.80	36.57	35.29					
1	500	32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
	200	32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00
		32.11	32.27	32.75	32.66	32.07	32.44	32.96	33.26	33.22	33.06	33.96	33.69	34.76	35.38	34.12	33.33	33.21	34.68	35.03	34.00

<LOD: no increase of the fluorescence signal within 40 cycles.

20 replicates. The results are summarised in Table 3. In case of black and brown mustard, all replicates yielded a positive result, even at the 5 mg/kg spiking level. In contrast, for white mustard, at the 10 mg/kg spiking level 80% (set 1) and 85% (set 2) and at the 5 mg/kg spiking level 75% (set 1) and only 50% (set 2) of the replicates led to an increase of the fluorescence within 40 cycles.

Since a DNA amount of 500 ng per tube had been applied in the singleplex assay for white mustard, further experiments were carried out by increasing the DNA amount to 200 ng or 500 ng DNA per PCR tube. In this series of experiments, extracts from brewed model sausages containing 10, 5 or 1 mg/kg of each mustard species were analysed in 10 replicates. Table 4 indicates, that in case

Table 5
Analysis of commercial foodstuffs. Declaration: + mustard listed, ± may contain mustard, – mustard not listed. Mean Ct values before the bracket were obtained with the duplex assay, values in the bracket with the respective singleplex assay. DNA amounts were as follows (unless noted otherwise): duplex assay and singleplex assay for white mustard: 500 ng per PCR tube; singleplex assay for black/brown mustard: 10 ng per PCR tube.

Foodstuff	Declaration	Black/brown mustard Mean Ct value	White mustard Mean Ct value
Sausage (brat maxe)	±	<LOD ^a (<LOD)	<LOD
Sausage (salanettis)	+	34.82	32.02 (34.08)
Meat spread 1	–	<LOD (<LOD)	<LOD
Meat spread 2	+	36.68 (<LOD ^f)	35.93
Meat spread 3	–	<LOD (<LOD)	<LOD
Aspic	–	<LOD (<LOD)	<LOD
Vegetable aspic	–	<LOD ¹ (<LOD)	<LOD
American sauce	+	34.13 ² (33.76 ²)	36.06 ²
Yoghurt dressing Italian herbs	+	32.17 ³	34.83 ³ (37.95 ³)
Yoghurt dressing garlic	+	31.96 ⁴	35.62 ⁴ (36.71 ⁴)
Mayonnaise	+	<LOD ^{5,b} (<LOD ^{5,g})	37.54 ⁵
Spice mix for fish 1	±	<LOD ⁶ (<LOD)	<LOD ^{6,c}
Spice mix for fish 2	±	<LOD ⁷ (<LOD)	<LOD ⁷
Spice mix for fish 3	–	33.98 ⁸ (33.75)	36.22 ⁸
Fried noodles	±	36.72	<LOD (<LOD)
Fried noodles duck 1	±	<LOD ^d (<LOD)	<LOD
Fried noodles duck 2	–	<LOD ^e (<LOD)	<LOD
Fried noodles chicken	±	37.60 (<LOD)	<LOD
Fried noodles curry	+	35.98 ⁹	34.32 ⁹ (33.71 ⁹)
Spaghetti bolognese	±	<LOD (<LOD)	<LOD
Pasta ai funghi	±	<LOD	<LOD (<LOD ^h)
Mediterranean vegetable pan	±	<LOD	<LOD (<LOD)
Vegetarian nuggets	+	23.36	29.30 (29.30)

¹ 435 ng DNA per PCR tube.

² 2.5 ng DNA per PCR tube.

³ 10 ng DNA per PCR tube.

⁴ 5 ng DNA per PCR tube.

⁵ 5 ng DNA per PCR tube.

⁶ 22.5 ng DNA per PCR tube.

⁷ 67.5 ng DNA per PCR tube.

⁸ 22.5 ng DNA per PCR tube.

⁹ 10 ng DNA per PCR tube.

^a Duplicate: Ct value 36.41/<LOD.

^b Duplicate: Ct value 37.24/<LOD.

^c Duplicate: Ct value 38.65/<LOD.

^d Duplicate: Ct value 37.22/<LOD.

^e Duplicate: Ct value 37.55/<LOD.

^f Duplicate: Ct value 39.66/<LOD.

^g Duplicate: Ct value 39.31/<LOD.

^h Duplicate: Ct value 38.58/<LOD.

of black and brown mustard at each spiking level and independent of the initial DNA amount, all replicates yielded a positive result. An initial DNA amount of 500 ng per PCR tube made it possible to detect white mustard in brewed sausages containing 10 or 5 mg/kg of each mustard species in 10 out of 10 replicates. At the 1 mg/kg spiking level, in both sets of model sausages an increase of the fluorescence signal was only observed in 60% of the replicates.

With an initial DNA amount of 200 ng per PCR tube, white mustard was detected in 90% (spiking level 10 mg/kg), 60% (spiking level 5 mg/kg) and only 10% (spiking level 1 mg/kg) of the replicates carried out with DNA extracts from model sausage set 1 and 80% (10 mg/kg), 70% (5 mg/kg) and 50% (1 mg/kg) of the replicates carried out with DNA extracts from model sausage set 2.

These results indicate that the duplex real-time PCR assay should be carried out with 500 ng DNA per PCR tube in order to allow the detection of black, brown and white mustard in brewed sausages down to a concentration of 5 mg/kg without obtaining false negative results.

3.4. Determination of mustard in commercial foodstuffs

Twenty-three commercial food products, labelled with either “contains mustard” or “may contain mustard” and similar products without any information concerning mustard, were analysed with the duplex real-time PCR assay. DNA could be isolated

with high purity (A_{260}/A_{280} ratios between 1.8 and 2.0) in concentrations between 100 and 1200 ng/ μ L. However, some products containing high amounts of water and/or salt, like sauces or spice mixes, yielded lower DNA concentrations. The results obtained by analysing the DNA extracts with the duplex assay are shown in Table 5. Mustard was detected in all samples declared to contain mustard. For seven out of the eight foodstuffs, an increase of the fluorescence signal was observed in both channels, indicating that black/brown and white mustard are present. In two out of nine foodstuffs that were declared to potentially contain traces of mustard, black/brown mustard was detected. For one out of six foodstuffs that did not have any information on mustard (spice mix for fish 3), an increase of the fluorescence signal was obtained in both channels, indicating that black/brown and white mustard were contained although mustard was not declared.

Part of the foodstuffs were also analysed with the singleplex assays for white or black/brown mustard. Table 5 indicates that the results obtained with the duplex assays were in excellent agreement with those obtained with the singleplex assays.

4. Conclusion

A duplex real-time PCR assay for the detection of mustard was developed by combining a singleplex assay for the detection of white mustard with a singleplex assay for the simultaneous

detection of black and brown mustard. The duplex assay allows the detection of white mustard in the “green” and black/brown mustard in the “yellow” channel. Cross-reactivity tests were carried out with 12 *Brassicaceae* species, three fish species and food ingredients that had yielded an increase of the fluorescence signal in the singleplex assay for black/brown mustard. The duplex assay neither showed cross-reactivity with the *Brassicaceae* species nor the fish species tested. We obtained low cross-reactivity with cumin, fenugreek, ginger, rye and turmeric, as in the singleplex assay for black/brown mustard. Since in mustard containing foodstuffs these ingredients are commonly present only in traces and, compared with the positive control, the differences in the Ct value were ≥ 11.91 , the probability for obtaining false positive results is very low.

The duplex real-time PCR assay proved to be very sensitive. By applying a DNA amount of 500 ng per PCR tube, the three mustard species could be detected in brewed model sausages down to a concentration of 5 mg/kg in 10 out of 10 replicates.

Twenty-three commercial food products differing in their declaration concerning the presence of mustard were analysed with the duplex assay. Eight products were declared to contain mustard as ingredient, nine were labelled with “potentially contains traces of mustard” and six products were without any information with regard to the presence of mustard. In seven out of the eight foodstuffs that were declared to contain mustard, black/brown and white mustard were detected. In addition, black/brown and white mustard were detected in one out of six foodstuffs (spice mix for fish 3) that did not have any information on the presence of mustard.

References

- Commission of the European Union (2007). Commission directive 2007/68/EC. Official Journal of the European Union, L310/311–L310/314.
- EFSA (European Food Safety Authority). (2004). Opinion of the scientific panel on dietetic products, nutrition and allergies on a request from the commission relating to the evaluation of allergenic foods for labelling purposes. *The EFSA Journal*, 32, 113–128.
- Fuchs, M., Cichna-Markl, M., & Hohegger, R. (2010). Development and validation of a real-time PCR method for the detection of white mustard (*Sinapis alba*) in foods. *Journal of Agricultural and Food Chemistry*, 58, 11193–11200.
- Gonzales de la Peña, M. A., Menéndez-Arias, L., Monsalve, R. I., & Rodríguez, R. (1991). Isolation and characterization of a major allergen from oriental mustard seeds, Bra j 1. *International Archives of Allergy and Immunology*, 96, 263–270.
- Koppelman, S. J., Vlooswijk, R., Bottger, G., Van Duijn, G., Van der Schaft, P., Dekker, J., & Van Bergen, H. (2007). Development of an enzyme-linked immunosorbent assay method to detect mustard protein in mustard seed oil. *Journal of Food Protection*, 70, 179–183.
- Lee, P.-W., Hefle, S. L., & Taylor, S. L. (2008). Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of mustard in foods. *Journal of Food Science*, 73, T62–T68.
- Menéndez-Arias, L., Moneo, I., Domínguez, J., & Rodríguez, R. (1988). Primary structure of the major allergen of yellow mustard (*Sinapis alba* L.) seed, Sin a 1. *European Journal of Biochemistry*, 177, 159–166.
- Mustorp, S., Engdahl-Axelsson, C., Svensson, U., & Holck, A. (2008). Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. *European Food Research and Technology*, 226, 771–778.
- Palle-Reisch, M., Wolny, M., Cichna-Markl, M., & Hohegger, R. (2013). Development and validation of a real-time PCR method for the simultaneous detection of black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*) in food. *Food Chemistry*, 138, 348–355.
- Palomares, O., Cuesta-Herranz, J., Vereda, A., Sirvent, S., Villalba, M., & Rodríguez, R. (2005). Isolation and identification of an 11S globulin as a major allergen in mustard seeds. *Annals of Allergy, Asthma & Immunology*, 94, 586–592.
- Rancé, G., Kanny, G., Dutau, G., & Moneret-Vautrin, D.-A. (1999). Food hypersensitivity in children: clinical aspects and distribution of allergens. *Pediatric Allergy and Immunology*, 10, 33–38.
- Shim, Y.-Y., & Wanasundara, J. P. D. (2008). Quantitative detection of allergenic protein Sin a 1 from yellow mustard (*Sinapis alba* L.) seeds using enzyme-linked immunosorbent assay. *Journal of Agricultural and Food Chemistry*, 56, 1184–1192.
- Siegel, M., Schnur, K., Boersen, B., Pietsch, K., & Waiblinger, H.-U. (2012). First ring-trial validation of real-time PCR methods for the quantification of allergenic food ingredients. *European Food Research and Technology*, 235, 619–630.
- Sirvent, S., Palomares, O., Cuesta-Herranz, J., Villalba, M., & Rodríguez, R. (2012). Analysis of the structural and immunological stability of 2S albumin, nonspecific lipid transfer protein, and profilin allergens from mustard seeds. *Journal of Agricultural and Food Chemistry*, 60, 6011–6018.
- Sirvent, S., Palomares, O., Vereda, A., Villalba, M., Cuesta-Herranz, J., & Rodríguez, R. (2009). NsLTP and profilin are allergens in mustard seeds: Cloning, sequencing and recombinant production of Sin a 3 and Sin a 4. *Clinical & Experimental Allergy*, 39, 1929–1936.