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Development and Validation of a Real-Time PCR Method for the Detection of White Mustard (*Sinapis alba*) in Foods

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This paper presents a real-time PCR method allowing the detection of traces of white mustard (*Sinapis alba*) in complex food matrices. The primers and the probe are targeted at the gene coding for *S. alba* MADS D. The real-time PCR method was found to be specific for white mustard and did not show any cross-reactivity with 67 biological species, including 12 members of the Brassicaceae family. The limit of detection, determined by analyzing serially diluted white mustard DNA extracts, was 1 pg of white mustard DNA/μL, corresponding to 5 pg of white mustard DNA. In model sausages, the limit of detection was found to be 0.001% white mustard (corresponding to 10 ppm or 10 mg/kg). The real-time PCR method was applied to verify the correct declaration of 20 foodstuffs purchased from Austrian supermarkets. White mustard DNA was detected in one of three samples labeled with "may contain traces of mustard" and in one of seven samples without any information on the presence of mustard.

KEYWORDS: White mustard; *Sinapis alba*; food allergen; real-time PCR

INTRODUCTION

Food allergies are a rising health problem because the number of persons affected is increasing, particularly in industrialized countries. About 6−8% of the children and 3.5−4% of the adults are affected by food allergies (*1*, *2*). The term "food allergy" is, however, often misused. Whereas food allergies are immune-mediated (either IgE- or non-IgE-mediated), food intolerance refers to an adverse physiological response to food and is not immune-mediated (*2*, *3*). Allergenic food can cause the oral allergy syndrome (OAS) as well as mild symptoms affecting the skin, gastrointestinal tract, and/or respiratory system, but it can also lead to life-threatening reactions such as anaphylaxis (*3*, *4*).

Mustard is one of the foods known to be strongly allergenic. Several cases of allergic reactions, including anaphylaxis, have already been caused by the ingestion of mustard (*5*−*9*). However, there is still not much known about mustard allergy. It is assumed that 1−7% of all food-allergic persons suffer from mustard allergy (*10*), being the fourth most common allergy among children in France, following allergy to eggs, peanuts, and cow's milk (*11*). The major mustard allergens identified so far are Sin a 1 (a 2S seed storage albumin with a molecular weight of 14 kDa) (*12*), Sin a 2 (a seed storage 11S globulin, 51 kDa) (*13*), Sin a 3 (a nonspecific lipid transfer protein, 9 kDa), and Sin a 4 (a profilin, 14 kDa) (*14*) in white mustard (*Sinapis alba*) and Bra j 1 (a 2S seed storage albumin, 16 kDa) (*15*) in brown mustard (*Brassica juncea*). Sin a 1 and Bra j 1 are known to be closely related to other 2S albumins, for example, from rapeseed, castor bean, and Brazil nut (*10*). Because the major mustard allergens are resistant to heat and enzymatic digestion, food processing does not significantly lower the allergenicity of mustard (*10*).

The only way for allergic persons to cope with their allergy is to strictly avoid the allergenic food(s). To facilitate obtaining information on the presence of allergenic ingredients in food, 14 groups of allergenic species have to be declared in the European Union according to Directive 2007/68/EC (*16*), including mustard and products thereof.

Individuals may, however, be exposed to allergenic food despite their attempts to avoid any contact with it. In a retrospective study, 22.4% of the analyzed allergic reactions were considered to be caused by so-called hidden allergens. Hidden allergens are not declared on the list of ingredients and may be introduced to the foodstuff via contamination during food-processing steps (*17*). Many products are therefore labeled with precautionary warnings such as "may contain traces of ...".

Sensitive analytical methods are necessary to control the implementation of the legal regulations and to detect hidden allergens in nondeclared foodstuffs. In general, there are two analytical approaches for the detection of allergens in food: the detection of specific proteins by immunoanalytical methods and the detection of DNA from allergenic species. Among immunoanalytical methods, enzyme-linked immunosorbent assays (ELISAs) play the most important role. DNA from allergenic species is most frequently detected by the polymerase chain reaction (PCR).

ELISAs, based on the specific interactions between antigens and antibodies, are used to detect either allergenic or species-specific proteins (*18*). Several ELISAs have already been presented for the detection of mustard (*19*−*21*); none of the methods is, however, specific for mustard. Two ELISAs suffered from high cross-reactivity with rapeseed (*20*, *21*), whereas the ELISA presented

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by Koppelman et al. was found to show some cross-reactivity with milk, egg yolk, and soy. These authors did, however, not give any information on cross-reactivity with rapeseed (*19*).

In PCR a certain DNA sequence is amplified and detected either by loading the PCR products on an agarose gel or, in real-time PCR, by using probes labeled with fluorescent dyes. So far, only one real-time PCR method has been published for the detection of mustard (*22*). Because the primers were designed to detect a sequence of the Sin a 1 gene in as many allergenic *Brassica* species as possible, the assay was obviously not specific for mustard but showed cross-reactivity with all *Brassica* species tested, including rapeseed and radish.

The present study aimed at the development and validation of a real-time PCR method specific for white mustard (*S. alba*) without showing any cross-reactivity with other Brassicaceae. White mustard was selected because it is the most commonly used mustard species in Europe.

MATERIALS AND METHODS

Chemicals and Food Products. Ethylenedinitrilotetraacetic acid disodium salt dehydrate (EDTA), *N*-cetyl-*N*,*N*,*N*-trimethylammonium-bromide (CTAB), sodium chloride, hydrochloric acid, proteinase K, chloroform, isoamylalcohol, ethanol, and 2-propanol were purchased from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris) was obtained from J. T. Baker (Deventer, The Netherlands). Phenol/chloroform/isoamylalcohol 25:24:1 (v/v/v) was purchased from Sigma Life Sciences (Buchs, Switzerland). RNase and α-amylase were obtained from Roche (Mannheim, Germany), and the SureFoodPREP Allergen Kit was from Congen (Berlin, Germany). TaqMan Universal PCR Master Mix was purchased from Applied Biosystems (manufactured by Roche, Branchburg, NJ). In-house bidistilled water was used for DNA extraction and PCR reactions. White mustard was bought from five different local merchants. Dried spices were provided by Kotányi (Wolkersdorf, Austria). Pork meat, table salt, and food samples were bought in local supermarkets.

Production of White Mustard Spiked Sausages. The sausages spiked with 0, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, or 10% white mustard (w/w) were produced at the Department for Foods of Animal Origin (Institute for Food Control, Austrian Agency for Health and Food Safety, Vienna, Austria). Two hundred grams of white mustard seeds was ground in a mixer (Thermomix, type 21, Vorwerk, Hard, Austria) for 5 min. Twelve kilograms of pork meat was minced with a meat chopper (type FW-N 22/2, Bizerba, Balingen, Germany), using a 3 mm insert. Five grams of table salt and an adequate amount of ground white mustard were carefully mixed in a crystallization plate for 5 min. After an adequate amount of minced meat had been weighed, the meat was filled into a cutter (robot coupe R5 plus, Toperczer, Schwechat-Rannersdorf, Austria). After the salt/mustard mixture had been sprinkled over the meat, 300 g of ice/ice water was added. A small aliquot of the ice water was not directly added to the meat but used for rinsing the crystallization plate to ensure complete removal of the salt/mustard mixture. The mixture was then homogenized in the cutter for 5 min. The model sausages were filled in beakers and stored at −20 °C until use.

DNA Extraction. Genomic DNA was isolated according to a CTAB protocol. About 10−50 g of the samples was ground in a mixer (type A11 basic, IKA-Werke, Staufen, Germany; Speedy Pro, type 720, Krups, Brunn am Gebirge, Austria) for 2−5 min. In the case of convenience foods, the whole package was ground and homogenized in a Thermomix (type 21, Vorwerk, Hard, Austria) for 5 min. The samples were extracted immediately after grinding. An aliquot of 3 g of the ground sample was weighed, mixed with 10 mL of the CTAB extraction solution (20 mg/mL CTAB, 0.02 M EDTA, 0.1 M Tris, 1.4 M NaCl, adjusted to pH 8.0 with 4 M HCl, autoclaved) and 80 μL of α-amylase (10 mg/mL), and incubated at 50 °C in an oven (type 8863423, B. Braun Biotech Int., Melsungen, Germany) under shaking for at least 1 h. Eighteen microliters of proteinase K (600 mAnson-U/mL) was added to the solution, and the mixture was incubated at 50 °C in an oven under shaking overnight. After centrifugation for 15 min at 4000 rpm (model 5810 R, Eppendorf, Vienna, Austria), 1 mL of the supernatant was transferred into a 2 mL microreaction tube, where 600 μL of chloroform/isoamylalcohol (24:1, v/v) had already been provided. This and all following steps were carried out in duplicate. After 30 s of vortexing, the mixture was centrifuged for 10 min at 13000 rpm (model 5415R, Eppendorf). Thirteen hundred and twenty microliters of CTAB precipitation solution (5 g/L CTAB, 0.04 M NaCl) was prepared in a new 2 mL microreaction tube, mixed with 660 μL of the aqueous phase, and incubated at room temperature for at least 1 h. After centrifugation for 10 min at 13000 rpm, the supernatant was decanted and the residue resolved under shaking for 10 min at 56 °C in the thermomixer (model comfort, Eppendorf) in 450 μL of 1.2 M NaCl, 50 μL of 10× RNase buffer (3 M NaCl, 100 mM Tris, 50 mM EDTA, adjusted to pH 7.4 with 4 M HCl), and 5 μL of RNase (500 μg/L). Five hundred microliters of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) was added after cooling to room temperature, and then the mixture was vortexed for 30 s and afterward centrifuged for 10 min at 13000 rpm. Four hundred microliters of isopropanol was prepared in a new 1.5 mL microreaction tube, and 400 μL of the aqueous phase was added, mixed, and incubated for at least 1 h at −20 °C in the freezer. After centrifugation for 10 min at 13000 rpm, the supernatant was decanted, and the precipitate was washed with 500 μL of ethanol 70% (v/v) by vortexing for 30 s. After a further centrifugation step (10 min, 13000 rpm), the supernatant was decanted carefully. The pellet was dried in a vacuum centrifuge (model V R 1, Heraeus, Hanau, Germany) for 15−30 min. Afterward, 100 μL of H_2O_{dd} was added, and the DNA was dissolved under shaking at 56 °C for 20 min in the thermomixer. The DNA extract was stored at −20 °C until analysis.

DNA from white mustard spiked sausages was also extracted with the SureFoodPREP Allergen Kit (Congen) according to the producer's instruction sheet. In brief, the homogenized sample was mixed with lysis buffer and proteinase K and incubated at 65 °C for 60 min. After centrifugation, the supernatant was filtered, mixed with binding buffer, and applied to a spin filter. After washing the spin filter, DNA was eluted with elution buffer. The DNA extracts were stored at −20 °C until analysis.

Determination of the Concentration and Quality of the Isolated DNA. The absorbance of aqueous DNA solutions 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 (ng/μL) = $A_{260} \times 50 \times$ dilution factor. The ratio A_{260}/A_{280} gave information on the purity of the extracted DNA.

Real-Time PCR Analysis. *Primer and TaqMan Probe Design*. To detect white mustard, seven primer pairs and probes for the following four genes were designed with the primer design software Primer Express 3.0 (Applied Biosystems, Foster City, CA) as well as Beacon Designer 6.0 software (Premier Biosoft International, Palo Alto, CA): *S. alba* chloroplast mRNA for sigma factor 2 (SIG2 gene, NCBI accession no. AJ276656), *S. alba* flowering promoting factor 1 (FPF1) mRNA (NCBI accession no. Y11987), *S. alba* mRNA for Gbox binding factor 2A (NCBI accession no. Y16953), and *S. alba* mRNA for MADS D protein (NCBI accession no. Y08626). The following criteria were applied: production of an amplicon with a length from 50−150 bp, a primer length between 9 and 40 bp (20 bp set as optimum), a probe length from 13 to 25 bp, a GC content between 30 and 80%, and a melting temperature from 58 to 60 °C for the primers and from 68 to 70 °C for the probes. All probes were labeled with the reporter dye FAM at the 5′ end and the quencher TAMRA at the 3′ end. The sequences of the primers and probes (**Table 1**) were checked for similarities with sequences from other biological species by using the BLAST (Basic Local Alignment Search Tool) algorithm. The primers and probes were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

PCR Conditions. Real-time PCR reactions were carried out on a RotorGene RG-3000 from Corbett Life Sciences (Hilden, Germany), equipped with a 72-well rotor. Strip tubes with caps (0.1 mL, purchased from Qiagen, Hilden, Germany) were used in a total PCR reaction volume of 25 μL. All experiments were accomplished with the TaqMan Universal PCR Master Mix from Applied Biosystems. All samples were analyzed in duplicate. Two positive controls and two nontemplate controls were applied in each PCR run.

Optimization of the Real-Time PCR Assay. To determine the optimal primer concentrations, the probe concentration was fixed at 150 nM and the primer concentration was set at 100, 200, or 300 nM in a primer matrix. The following temperature program was used: 2 min at 50 °C, 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 60 s at 55 °C. The probe

Table 1. Sequences of the Primers and FAM/TAMRA-Labeled Probes

primer/probe	sequence 5′→3′	amplicon size (bp)
primer SIG2_1 forward	CCT TCT TCC AAC CGC AGT GT	
primer SIG2_1 reverse	TCT GAA TCG GTA TCG AAA GAA GGT	103
probe SIG2_1	FAM-CCA CTT CAC CGC CGT TAT TAA CTT CTA CGC-TAMRA	
primer SIG2_2 forward	TGC TGC TGG AGT CGA TAA AAA A	
primer SIG2_2 reverse	GCA ATT GAA ATG ACG AGA CGA A	105
probe SIG2_2	FAM-TAA CTC ACG GCA CAC AAT GCA AGG ACA-TAMRA	
primer SIG2_3 forward	TGG GAG GAT GAA GAC GTT GC	
primer SIG2_3 reverse	GAA GTA TCA ATC TGC CTC ACT CTC	78
probe SIG2_3	FAM-TGG TGA GAC GAT GGG TGT GAG CCG-TAMRA	
primer FPF1_1 forward	CAA ATA GCC GAA GGA AAG TGA TGG	
primer FPF1_1 reverse	GAG CGT TTG TGG AAT TGG AGA AG	148
probe FPF1_1	FAM-GGG AGA GGT ACT TCG GTG GCG GCG-TAMRA	
primer FPF1_2 forward	TGG TCT ATT TAC CGA CAG GAG AAG T	
primer FPF1_2 reverse	CCG AAG TAC CTC TCC CAT CCA	88
probe FPF1_2	FAM-CTT ACT CCA CGC TCG AGC AGA TCC TCC-TAMRA	
primer Gbox binding factor 2A forward	GAT TCA GTG TGG CCT CTG ATC TG	
primer Gbox binding factor 2A reverse	CCT TAT CTC GAA TCC ATT TGC TTT	96
probe Gbox binding factor 2A	FAM-CCA CTT GAT TGG TTA TGT TT-TAMRA	
primer MADS D forward	TGA AAA CTC TCT TCC CCT CTT AGG	
primer MADS D reverse	ACA AAT GCA CAC AAG ACA GAG ATA TAG A	74
probe MADS D	FAM-TAC ATG ATG CTT ACC TCG C-TAMRA	

concentrations were optimized by applying the optimal primer concentrations and adjusting the probe concentration to 50, 100, 150, or 200 nM. The annealing temperatures were optimized by applying the optimal concentrations for primers and probes and using a temperature gradient. These experiments were carried out with the Bio-Rad iCycler thermocycler, equipped with the IQ5 multicolor real time PCR detection system (Vienna, Austria), using 96 -well plates from Bio-Rad in a total volume of 25 μL (12.5 μL of TaqMan Universal PCR Master Mix, 7.5 μL of a primers/probe/H_2O_{dd} mixture, and 5 μL of DNA extract (20 ng/μL)). The temperature optimization was done according to the following PCR program: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C, and 60 s at a temperature gradient from 52 to 62 °C.

Optimized Real-Time PCR Method. Reactions were carried out with 12.5 μL of TaqMan Universal PCR Master Mix, 200 nM MADS D forward primer, 300 nM MADS D reverse primer, 150 nM MADS D probe, 5 μL of DNA extract, and H_2O_{dd}. The following PCR protocol 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.

Specificity of the Real-Time PCR Method. The specificity was tested with 67 different biological species, among them many members of the Brassicaceae family such as black mustard, brown mustard, rapeseed, broccoli, and cauliflower, seeds, spices, and different meats. In all cases, 5 μL of DNA extract with a concentration of 20 ng/μL, corresponding to 100 ng of DNA, was loaded per well.

Amplification Efficiency, Limit of Detection (LOD), Reliability, and Repeatability. The amplification efficiency and the LOD were examined with both serially diluted white mustard extracts (1:1 to 1:1000000, corresponding to a DNA concentration of 20 ng/μL to 20 fg/μL and an absolute amount of 100 ng to 100 fg) and DNA extracts (100 ng of DNA/μL, corresponding to 500 ng of DNA absolute) from white mustard spiked sausages obtained either via CTAB extraction or via isolation with the SureFoodPREP Allergen Kit. The amplification efficiency was calculated using the following equation: $E\ (\%) = [10^{(-1/\text{slope})} - 1] \times 100$. The reliability of the real-time PCR method was tested by repeatedly analyzing DNA extracts from model sausages spiked with white mustard at a spike level of 5, 10, or 50 ppm. Each extract was analyzed in 20 replicates. The repeatability was tested with serially diluted white mustard extracts (20 ng/μL, diluted 1:100, 1:200, 1:1000, 1:2000, 1:10000, or 1:20000) on two consecutive days in fourfold determination.

Inhibition Control. Each inhibition control reaction consisted of 12.5 μL of TaqMan Universal PCR Master Mix, 200 nM MADS D forward primer, 300 nM MADS D reverse primer, 150 nM MADS D probe, 5 μL of white mustard DNA extract (20 ng/μL), and 5 μL of template DNA (100 ng/μL) from negative tested food samples and H_2O_{dd}. The reactions were carried out in duplicate according to the PCR protocol described above.

Table 2. Optimized PCR Conditions

primer/probe set	forward primer concn (nM)	reverse primer concn (nM)	probe concn (nM)	annealing temp (°C)
SIG2_1	300	300	100	55
SIG2_2	300	200	50	60
SIG2_3	300	300	50	55
FPF1_1	300	200	50	60
FPF1_2	100	300	50	55
Gbox binding factor 2A	200	200	150	55
MADS D	200	300	150	55

RESULTS AND DISCUSSION

Primer Design. At the beginning of the study five primer pairs were designed, three of them targeted at the *S. alba* chloroplast mRNA for sigma factor 2 (SIG2) and two targeted at the *S. alba* flowering promoting factor 1 (FPF1) mRNA. These sequences were selected because sequence homology search by BLAST had not revealed any cross-reactivity with other Brassicaceae.

Optimization of the Real-Time PCR Assays. The concentrations of the forward and reverse primers and the concentration of the probe as well as the annealing temperature were optimized. The optimal conditions, defined as those yielding the lowest Ct values, are summarized in **Table 2**.

Specificity of the Primers. To investigate the specificity of the five assays for white mustard, the DNA of more than 60 different biological species (e.g., Brassicaceae, spices, different meat sorts, oilseeds) was extracted and analyzed. Despite the negative result obtained by the BLAST search, each of the five primer/probe sets showed numerous cross-reactions with various spices and most of

Table 3. Cross-Reactivity of the Primer/Probe Sets[a]

name	botanical name	FPF1_1	FPF1_2	SIG2_1	SIG2_2	SIG2_3	Gbox	MADS D
white mustard	*Sinapis alba*	+	+	+	+	+	+	+
allspice	*Pimenta dioica*	−	−	−	−	−	na	−
anise	*Pimpinella anisum*	−	−	−	−	−	na	−
apple	*Malus domesticus*	na	na	na	na	na	na	−
bay leaf	*Laurus nobilis*	−	−	−	−	−	na	−
beef	*Bos taurus*	−	−	−	−	−	na	−
beet	*Brassica rapa* ssp. *rapa*	+	na	na	+	na	−	−
beetroot	*Beta vulgaris* ssp. *vulgaris*	na	na	na	na	na	−	−
black mustard	*Brassica nigra*	+	+	+	+	+	−	−
black pepper	*Piper nigrum*	−	+	−	+	+	na	−
brazil nut	*Bertholletia excelsa*	na	na	na	na	na	na	−
broccoli	*Brassica oleracea* var. *silvestris*	+	na	na	+	na	−	−
brown mustard	*Brassica juncea*	na	na	na	na	na	na	−
caraway	*Carum carvi*	+	+	+	+	+	na	−
cardamom	*Ellettaria cardamomum*	−	−	−	−	−	na	−
carrot	*Daucus carota*	na	na	na	na	na	na	−
cauliflower	*Brassica oleracea* var. *botrytis*	+	na	na	+	na	−	−
celery	*Apium graveolens* var. *secalinum*	na	na	na	na	na	na	−
celery root	*Apium graveolens* var. *rapaceum*	+	+	+	+	+	na	−
celery stalks	*Apium graveolens* var. *dulce*	na	na	na	na	na	na	−
chicken	*Gallus gallus*	−	−	−	−	−	na	−
chilli	*Capsicum* sp.	−	−	−	−	−	na	−
chinese cabbage	*Brassica rapa ssp.pekinensis*	+	na	na	+	na	−	−
chive	*Allium schoenoprasum*	−	−	−	−	−	na	−
cinnamon	*Cinnamomum zeylanicum*	−	+	−	−	−	na	−
coriander	*Coriandrum sativum*	−	−	−	−	−	na	−
cucumber	*Cucumis sativus*	na	na	na	na	na	na	−
cumin	*Cuminum cyminum*	+	+	+	+	+	na	−
dill	*Anethum graveolens*	+	+	+	+	+	na	−
fennel	*Foeniculum vulgare*	na	na	na	na	na	na	−
fenugreek	*Trigonella foenum-graecum*	+	−	−	−	+	na	−
flaxseed	*Linum usitatissimum*	na	na	na	na	na	na	−
garlic	*Allium sativum*	na	na	na	na	na	na	−
ginger	*Zingiber officinale*	+	+	+	+	+	na	−
horse	*Equus ferus*	na	na	na	na	na	na	−
horseradish	*Armoracia rusticana*	+	na	na	−	na	−	−
kohlrabi	*Brassica oleracea* var. *gongylodes*	+	na	na	+	na	−	−
leek	*Allium porrum*	na	na	na	na	na	na	−
lovage	*Levisticum officinale*	−	+	−	−	−	na	−
maize	*Zea mays*	−	−	−	−	−	na	−
marjoram	*Origanum majorana*	+	+	+	+	+	na	−
nutmeg	*Myristicia fragrans*	+	+	+	+	+	na	−
onion	*Allium cepa*	na	na	na	na	na	na	−
oregano	*Origanum vulgare*	−	+	+	−	+	na	−
pak choi	*Brassica rapa chinensis*	+	na	na	+	na	−	−
paprika	*Capsicum annum*	−	−	−	−	−	na	−
parsley	*Petroselinum crispum*	−	−	−	−	−	na	−
parsnip	*Pastinaca sativa*	na	na	na	na	na	na	−
pea	*Pisum sativum*	−	−	−	−	−	na	−
pork	*Sus scrofa*	na	na	na	na	na	na	−
potato	*Solanum tuberosum*	na	na	na	na	na	na	−
radish	*Raphanus sativus*	+	na	na	+	na	−	−
rapeseed	*Brassica napus*	+	+	+	+	+	−	−
rice	*Oryza sativa*	−	−	−	−	−	na	−
rosemary	*Rosmarinus officinalis*	−	−	−	−	−	na	−
rye	*Secale cereale*	na	na	na	na	na	na	−
sage	*Salvia officinalis*	−	+	−	−	+	na	−
sesame	*Sesamum indicum*	na	na	na	na	na	na	−
sheep	*Ovis orientalis aries*	na	na	na	na	na	na	−
soy	*Glycine max*	−	na	na	−	na	na	−
summer savory	*Satureja hortensis*	−	−	−	−	−	na	−
tarragon	*Artemisia dracunculus*	−	−	−	−	−	na	−
thyme	*Thymus vulgaris*	−	−	−	−	−	na	−
tomato	*Solanum lycopersicum*	na	na	na	na	na	na	−
turkey	*Meleagris gallopavo*	na	na	na	na	na	na	−
turmeric	*Curcuma longa/domestica*	+	+	+	+	+	na	−
wheat	*Triticum durum*	na	na	na	na	na	na	−
white cabbage	*Brassica oleracea* var. *capitata* f.*alba*	+	na	na	+	na	−	−

[a] +, positive PCR result (Ct < 39); −, negative PCR result; na, not analyzed.

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   1 tgagtatttc ttccagggta aaaagcaaaa gaattccgga gtgagagagg agagaaggaa
  61 agaagaggag aaatgggaag agggagagta gaattgaaga ggatagagaa caagatcaat
 121 aggcaagtga cgtttgcaaa gagaaggaat ggtcttttga agaaagcata cgagctttcg
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 241 ttttgcagta gttccagcat gatccggaca ctggagagat accaaaagtg caactatgga
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 421 ggagaagatc taggacctct tagtacaaaa gagcttgagt tacttgagag acagcttgac
 481 tcttctttga agcagatcag agctctccgg acacagttca tgctcgacca gctcaatgat
 541 ctccaaagta aggaacgcat gttgaatgag acaaataaaa ctctcagact aaggttagct
 601 gatggatacc agatgccact ccaacttaac ccgaaccaag aagatcatca tgttgactac
 661 ggccgtcatg atcaacaaca acaacaaaac tctcatcatg ctttcttcca gcctttggaa
 721 tgcgaaccca ttcttcaaat ggggtatcag gggcagcaag atcatggaat ggaagcagga
 781 ccaagtgaga ataattacat gttgggttgg ttaccttatg acaccaactc tatttgaatc
                                    primer forward              probe
 841 tttcttcaat ttttttgaca ttttatatga aaactctctt cccctcttag gctacatgat
       probe                  primer reverse
 901 gcttacctcg ctctctatat ctctgtcttg tgtgcatttg tgtgtgtgta atgtttatta
 961 tgcccttcta caattaaata atttttgaa cacattaaaa aaaaaa
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Figure 1. Localization of the primers and the probe in the gene coding for *Sinapis alba* MADS D.

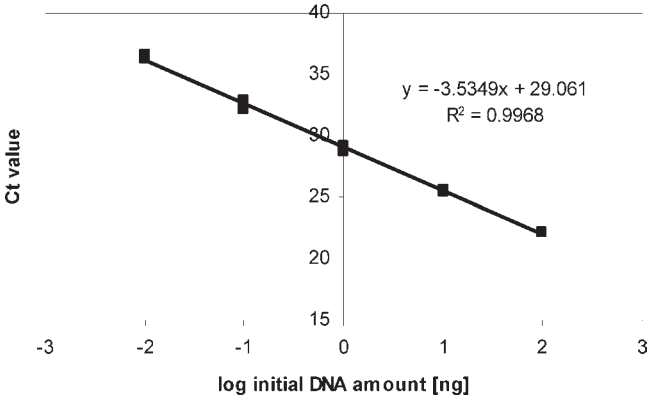

Figure 2. Standard curve obtained by analyzing serially diluted white mustard extracts.

the tested members of the Brassicaceae family, particularly with rapeseed (**Table 3**). For rapeseed even lower Ct values were obtained than for white mustard. We therefore designed two further primer/probe sets pursuing a different strategy. We particularly looked for *S. alba* genes for which the BLAST search revealed cross-reactivity with other Brassicaceae genes and designed the primers and the probes in regions where, according to BLAST, no homology between the sequences occurred. One primer/probe set was targeted at the *S. alba* mRNA for MADS D protein (**Figure 1**), which according to BLAST shows sequence homology to a gene of cauliflower. The other primer/probe set was targeted at the *S. alba* mRNA for Gbox binding factor 2A, which according to BLAST shows sequence homology to genes of rapeseed and radish. The sequences of the primers and the probes are shown in **Table 1**. After the primer and probe concentrations and the annealing temperature had been optimized (optimized conditions see **Table 2**), the assays were tested for their specificity for white mustard. Cross-reactivity tests with members of the Brassicaceae family indicated that none of the two assays suffered from any cross-reactivity with other Brassicaceae. Because with the MADS D primers a lower Ct value (mean Ct value = 22.11) was obtained for white mustard than with the Gbox binding factor 2A primers (mean Ct value = 25.09), further experiments were carried on with the MADS D primers. In total, 67 biological species were tested for cross-reactivity (**Table 3**). The assay amplifying a sequence of the MADS D gene proved to be specific for the detection of white mustard.

Table 4. Purity and Amount of DNA Isolated from Model Sausages by Two Extraction Methods

	SureFood PREP Allergen Kit		CTAB method	
spike level (%)	extracted DNA amount (mg/g)	purity A_{260}/A_{280}	extracted DNA amount (mg/g)	purity A_{260}/A_{280}
0	3.22	2.04	0.84	1.96
	2.11	2.07	1.07	1.99
0.0001	2.28	2.07	0.74	1.98
	2.16	2.05	0.82	2.01
0.0005	3.08	2.03	0.81	1.97
	2.52	2.06	0.72	1.98
0.001	1.87	2.09	0.74	1.95
	2.35	2.08	0.68	1.91
0.005	2.14	2.06	0.56	1.95
	2.22	2.04	0.80	1.94
0.01	2.91	2.04	0.68	1.94
	2.65	2.04	0.65	1.96
0.05	2.07	2.06	0.67	1.92
	2.66	2.05	0.02[a]	2.00
0.1	2.32	2.03	0.99	1.93
	2.21	2.04	0.65	1.92
0.5	2.63	2.05	0.83	1.96
	2.22	2.08	0.82	2.00
1	3.29	2.03	0.67	1.97
	2.67	2.04	0.72	1.98
5	3.72	2.07	0.85	1.95
	2.69	2.05	0.91	1.97
10	1.95	2.03	1.07	1.97
	2.74	2.04	0.97	2.00

[a] The pellet was lost at the decanting step after washing the DNA with 70% ethanol.

To investigate the applicability of the PCR method to different cultivars of white mustard, white mustards from five different suppliers were analyzed. For all samples similar Ct values were obtained.

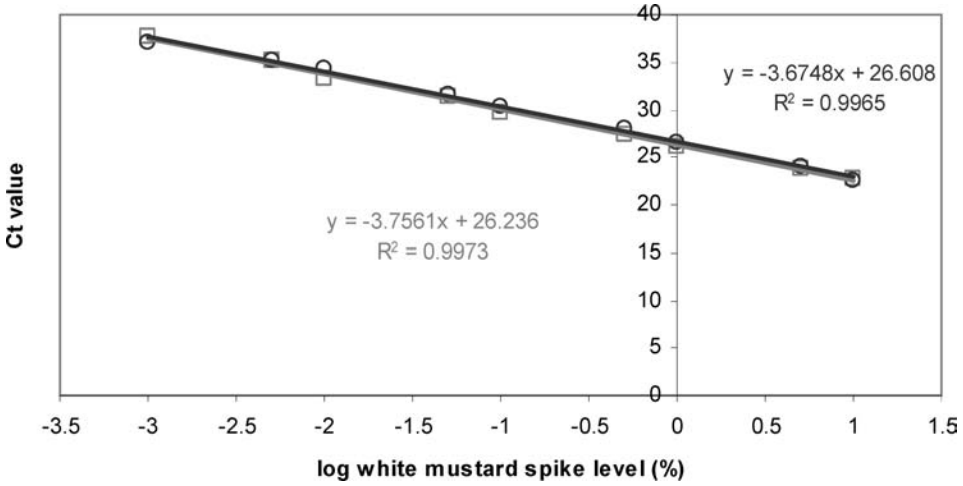

Figure 3. Standard curves obtained by amplifying DNA extracted from model sausages: (□) DNA extraction with kit; (○) DNA extraction with CTAB method.

Table 5. Reliability of the Real-Time PCR Method at DNA Concentrations Near the LOD

	SureFood PREP Allergen Kit					CTAB method				
spike level (ppm)	Ct value		mean Ct value	*S*	RSD (%)	Ct value		mean Ct value	*S*	RSD (%)
50	34.89	33.63	34.07	0.48	1.42	33.83	33.83	33.92	0.43	1.28
	34.01	33.98				33.56	34.24			
	34.25	35.05				34.07	33.64			
	34.24	33.71				33.91	33.79			
	34.01	33.53				33.99	33.81			
	34.35	33.25				33.30	33.54			
	34.15	34.03				33.76	34.86			
	33.37	34.28				34.32	33.54			
	34.06	33.61				33.30	34.03			
	34.86	34.18				34.92	34.12			
10	35.78	–[a]	36.93	1.16	3.15	38.40	36.89	36.77	0.67	1.82
	36.68	35.99				36.27	37.59			
	35.12	38.67				37.34	35.88			
	37.59	37.27				36.09	35.65			
	38.80	37.09				36.82	–			
	35.99	37.55				36.73	36.11			
	36.71	38.33				37.18	36.02			
	35.41	37.48				37.02	37.00			
	35.19	37.41				37.37	36.74			
	38.33	36.19				36.76	36.84			
5	–	37.29	37.51	0.81	2.17	–	–	37.52	0.73	1.96
	38.14	36.93				38.70	–			
	–	36.03				38.93	37.67			
	–	37.24				37.50	36.80			
	36.76	38.93				37.81	36.99			
	37.64	37.59				37.25	–			
	–	38.03				36.18	37.85			
	38.22	37.52				–	37.62			
	36.08	38.67				–	37.20			
	37.75	37.35				–	37.21			

[a] –, no amplification within 39 cycles.

Amplification Efficiency, Limit of Detection (LOD), Reliability, and Repeatability of the Assay. The amplification efficiency and the LOD were investigated with both serially diluted white mustard extracts obtained by the CTAB method and DNA extracts from white mustard spiked sausages obtained by the CTAB method as well as by extraction with the SureFoodPREP Allergen Kit.

The analysis of serially diluted white mustard DNA extracts (DNA concentration from 20 ng/μL to 20 fg/μL) showed linearity down to a concentration of 2 pg/μL (corresponding to the dilution factor 1:10000 or 10 pg of DNA) (**Figure 2**). The correlation coefficient (R^2) of the standard curve was 0.9968, and the slope was −3.5349, indicating an amplification efficiency of 91.8%. The LOD was found to be 1 pg of white mustard DNA/μL, corresponding to 5 pg of white mustard DNA.

To examine the LOD in white mustard spiked samples, model sausages containing 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, or 0.0001% white mustard and white mustard free model sausages were produced. The procedure of preparing mustard

Table 6. Repeatability of the Real-Time PCR Assay[a]

dilution factor	DNA concn (pg/μL)	Ct day 1	Ct day 2	mean Ct	*S*	RSD (%)
1:100	200	29.11	28.78	28.91	0.22	0.78
		28.84	28.66			
		28.96	28.87			
		28.73	29.35			
1:200	100	30.08	30.29	30.08	0.13	0.43
		29.93	30.10			
		29.95	30.21			
		29.96	30.08			
1:1000	20	32.16	33.29	32.60	0.42	1.29
		32.36	31.99			
		32.55	32.76			
		32.88	32.80			
1:2000	10	33.97	33.82	33.72	0.32	0.95
		33.44	34.23			
		33.49	33.53			
		33.97	33.32			
1:10000	2	36.64	38.85	36.51	1.08	2.96
		36.25	36.59			
		36.01	36.19			
		35.00	36.55			
1:20000	1	38.18	38.57	37.48	1.17	3.12
		36.67	38.77			
		36.31	37.34			
		35.63	38.35			

[a] The experiments were performed with serially diluted white mustard extracts in fourfold determination on two consecutive days.

spiked sausages did, however, not include technological treatments commonly used in the industrial production of sausages. **Table 4** compares the amount and the quality of the DNA obtained from the model sausages with both extraction methods. The table indicates that, in contrast to the quality, the amount of the extracted DNA strongly depended on the extraction method. The commercial kit yielded about 2−3 times higher amounts of DNA than the CTAB protocol.

Real-time PCR analysis of the DNA extracts yielded the standard curves shown in **Figure 3**. In both cases, the LOD was found to be 0.001% (corresponding to a concentration of 10 mg of white mustard/kg sausage or 10 ppm). The PCR assay is thus sensitive enough to allow the detection of traces of white mustard in complex foods (*18*). Both standard curves showed high linearity down to a spike level of 0.001%. The slopes of the calibration curves were −3.7561 (kit) and −3.6748 (CTAB protocol), indicating amplification efficiencies of 84.6 and 87.1%, respectively.

To assess the reliability of the PCR method at DNA concentrations near the LOD, DNA extracts from model sausages spiked with 5, 10, or 50 ppm white mustard were analyzed in 20 replicates. DNA was isolated with both the CTAB method and the commercial DNA extraction kit to reveal any influence of the extraction method on the detectability. **Table 5** shows that at the 50 ppm spike level all replicates yielded positive results. At the 10 ppm spike level, positive results were obtained for 19 of 20 replicates, independent of the DNA extraction method. This result indicates that at a concentration of 10 ppm white mustard is detected by the PCR method with a probability of 95%. At the 5 ppm spike level, 16 of 20 replicates were positive when DNA was extracted with the commercial kit and only 13 of 20 replicates when DNA was isolated with the CTAB method.

Table 7. Real-Time PCR Results Obtained by Analyzing Food Samples from Austrian Supermarkets

sample	declaration[a]	PCR result[b]	mean Ct value	mean Ct value inhibition control[c]
spice mix for minced meat	+	+	21.39	
spice mix for fish	+	+	26.49	
spice mix for herb butter	+	+	26.57	
vegetarian nuggets	+	+	29.60	
fried noodels with curry	+	+	33.71	
salanettis (sausages)	+	+	34.08	
curry sauce	+	+	34.12	
bratwurst 1	−	+	34.92	
yogurt dressing with garlic	+	+	36.71	
yogurt dressing with Italian herbs	+	+	37.95	
pasta ai funghi	±	±	38.58/−	
Asian fried noodels	±	−		21.22
Mediterranean vegetables pan	±	−		21.16
pasta al formaggio	±	−		21.02
arlberger (sausage)	−	−		25.19
bratwurst 2	−	−		22.50
bratwurst 3	−	−		23.88
salsa sauce	−	−		21.48
spice mix for barbecue	−	−		21.16
vegetarian burger	−	−		21.48

[a] Declaration: +, mustard listed as ingredient; −, mustard not listed; ±, may contain mustard. [b] PCR result: −, no amplification within 39 cycles. [c] Mean Ct value of the positive control: 21.59.

The repeatability of the PCR assay was investigated by analyzing serially diluted white mustard extracts (20 ng/μL, diluted 1:100, 1:200, 1:1000, 1:2000, 1:10000, and 1:20000). PCR runs were carried out in 4-fold determination on two consecutive days. The data given in **Table 6** demonstrate the high repeatability of the assay.

Determination of White Mustard in Food Samples. To verify the applicability of the real-time PCR method for the detection of white mustard in food, various food products (e.g., sausages, noodles, spice mixes, sauces, and dressings) were bought in different local supermarkets. Some of the food samples listed mustard as an ingredient, some were declared to possibly contain mustard, and some did not have any information about mustard as an ingredient. All samples were analyzed in duplicate.

DNA was extracted with the CTAB protocol. For most of the samples the A_{260}/A_{280} ratio was found to be between 1.8 and 2.0, indicating high purity of the DNA. In most cases, the DNA concentration was in the range from 100 to 900 ng/μL. A_{260}/A_{280} ratios lower than 1.5 as well as very low DNA concentrations (< 5 ng/μL) were obtained for some spice mixes, dressings, and sauces, probably caused by high amounts of salt and/or water in the food products.

For all samples declaring mustard as an ingredient, Ct values of < 39 were obtained, indicating the presence of white mustard (**Table 7**). Because at the LOD (1 pg of white mustard DNA/μL, corresponding to 5 pg of white mustard DNA absolute) Ct values of < 39 were obtained, a Ct value of 39 was set as threshold. In one of four food products declared as "may contain mustard", white mustard was detected in traces (one Ct value was 38.58, the other one > 39). For one of six samples for which mustard was not listed as ingredient, a positive real-time PCR result was obtained. To verify the positive result, DNA of the food sample (bratwurst 1) was extracted with the commercial kit and analyzed by real-time PCR. Again, a Ct value of < 39 was obtained, verifying the previous positive result.

All negative tested food samples were subjected to an inhibition control to ensure that the negative real-time PCR result was not

caused by amplification-inhibiting compounds. The DNA from all negative tested samples was amplified successfully, indicating that the negative results were not caused by PCR inhibition. In most cases, the Ct values were very similar to that obtained with the positive control. Ct values obtained for sausage samples were, however, slightly higher. From these results it can be concluded that sausage matrices will slightly lower the sensitivity of the PCR method.

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