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DISSERTATION

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„Development and validation of a multiplex real-time PCR method for the simultaneous detection of white, black and brown mustard and celery in food“

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1. INTRODUCTION

1.1. Food allergy

1.1.1. Definition of food allergy

Any aberrant reaction caused by the ingestion of food or a food additive is called an adverse food reaction. Adverse reactions to food comprise toxic and non-toxic reactions. Toxic adverse reactions occur in any person if the exposure is high enough. In contrast, non-toxic reactions only occur in sensitised individuals. Food allergies and food intolerances are non-toxic reactions [1, 2]. Food allergies and food intolerances are different types of adverse effects to food. Unfortunately, these terms are frequently used indiscriminately. Food allergy is "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food". In contrast, food intolerance is "a non-immune reaction that includes metabolic, pharmacologic and undefined mechanisms" [3].

Immune-mediated reactions can be classified into IgE-mediated (involving the production of immunoglobulin E antibodies), non-IgE-mediated (e.g. cell-mediated) and mixed (IgE- and non-IgE-mediated) reactions [4, 5].

1.1.2. IgE-mediated allergy - immunochemical response

The immunochemical response to food proteins is caused by the interaction of various immune cells (e.g. microfold cells (M-cells), enterocytes and antigen presenting cells). These interactions can lead either to oral tolerance or the loss of tolerance, resulting in the occurrence of food hypersensitivity as described in the following section [6-8].

Most food proteins are enzymatically degraded to amino acids during digestion, however, some proteins remain stable and can come into contact with the mucosal immune system. These proteins are able to pass the epithelial cells that line the gastrointestinal (GI) tract either by active or passive transport (e.g. with the help of M-cells or dendritic cells (DCs)). Once in the lamina propria, the proteins are captured by DCs, internalised and degraded to peptides which are further presented by MHC class II molecules to naive $CD4^+$ T-cells in the mesenteric lymph nodes. In the presence of cytokines (e.g. interleukin (IL)-4, IL-5 and IL-13), naive $CD4^+$ T-cells differentiate to allergen-specific Th2-cells. Th2-cell proliferation and cytokine generation lead to activation of B-cells and induction of antibody class switching from IgA to

antigen-specific IgE. Antigen-specific IgEs, generated by B-cells, bind to surface receptors (e.g. FcεRI) of antigen presenting cells, such as mast cells, basophils or macrophages. After repeated exposure to the offending antigens (allergens), cross-linking of IgE-FcεRI complexes by these allergens activates the release of mediators. These mediators can induce the acute phase of an immune-mediated-type reaction (e.g. by histamine) or are responsible for inflammation, resulting in a late-phase reaction (e.g. by leukotrienes, prostaglandins and cytokines). In young children, the immunologic immaturity and immaturity of the GI barrier (epithelial cells) could be a reason for the increasing prevalence of food allergies [9-12].

The balance between oral tolerance and allergy is affected by a complex web of interrelated factors. Those that have been identified to date include age, sex (male sex in children), race/ethnicity, genetics, microbial milieu (hygiene hypothesis), breast-feeding, dose, timing and route of antigen exposure, vitamin D levels, and other dietary factors. In order to understand the immunopathogenesis of food allergy, it is of crucial importance to elucidate the complex interplay of environmental influences and genetics [13].

1.1.3. Clinical symptoms of food allergies

Food allergens can induce a range of clinical manifestations in sensitised persons, involving the skin, the GI tract, the airways or the cardiovascular system. Most of them are mild, but severe life-threatening anaphylactic reactions are also possible.

IgE-mediated adverse reactions frequently affect the skin and can lead to acute urticaria, with or without angioedema, within minutes due to allergen ingestion. Most commonly, the complex system of the GI tract is involved, resulting in symptoms within minutes to two hours like nausea, vomiting, gastric retention, intestinal hypermotility, abdominal pain due to colonic spasms and/or diarrhea. Respiratory disorders, such as allergic rhinoconjunctivitis or allergic asthma are often associated with skin and gastrointestinal dysfunction [1].

Another frequent manifestation of food allergy is the oral allergy syndrome (OAS), a particular type of IgE-mediated contact urticaria characterised by reactions within one hour, leading to local itching and/or swelling (angioedema) of lips, tongue, palate, throat, ears and/or nose. Allergic patients, especially those suffering from a pollen-food allergy syndrome, mainly exhibit mild local oral symptoms, whereas the more severe systemic symptoms, like anaphylaxis, are rare [2, 14, 15].

Anaphylaxis is defined as “a severe life-threatening generalised or systemic hypersensitivity reaction” regardless of the mechanism [16], or “a serious allergic reaction that is rapid in onset and may cause death”. It is identified by defined clinical criteria involving severe respiratory and cardiovascular disorders [17]. Clinical symptoms are induced by abrupt, massive release of mast cells and/or basophil mediators. These affect the skin or mucous membranes, the GI tract and are life-threatening, if they concern the respiratory and/or cardiovascular system [18]. Even traces of allergenic food can elicit an anaphylactic reaction in seconds in highly sensitised individuals [2].

Food-dependent exercise induced anaphylaxis is also an IgE-mediated food allergy but occurs only if the ingestion of the offending food is followed by physical exercise, as in a case reported by Beak et al., where exercise after celery consumption led to an anaphylactic reaction [19].

In order to help physicians to evaluate the severity of food-induced anaphylaxis, a grading scheme according to the severity of clinical symptoms (grade 1–5) has been developed [20].

1.1.4. Diagnosis of IgE-mediated reactions

Diagnosis require a careful medical history, laboratory studies like skin tests, and *in vitro* allergy diagnosis. In order to confirm both sensitisation and food allergy, oral provocation tests (food challenges), preferentially carried out placebo controlled, have to be applied [11].

The term sensitisation is defined as the formation of allergen specific IgEs caused by the exposure of the immune system to food proteins, whereas the term clinical allergy is defined as the development of symptoms after food consumption [2].

Skin prick tests (SPTs) are frequently used to identify sensitisation to an allergen because of its cost-efficient and easy performing. Standardised food allergen extracts or native, fresh foods (e.g. fresh vegetables or fruits) are used for provocation by physicians after pricking patient’s skin with a lancet. A negative skin response excludes an IgE-mediated food allergy, whereas a positive reaction indicates the presence of food-specific IgE, but does not predict clinical symptoms [2]. Although an increasing mean wheal diameter seems to directly correlate with a greater likelihood of clinical reaction (e.g. to egg, milk and peanut in children), it still cannot be used as a quantitative parameter to assess the severity of outcome [11, 21-24].

Alternatively to skin tests, the level of allergen-specific IgEs present in human serum can be quantitatively detected using commercially available assays of different design. Meanwhile, the well-known RAST (radioallergosorbent test) has been replaced by more reliable quantitative tests, such as ELISAs (enzyme linked immunosorbent assays). The allergen-specific IgE level was also demonstrated to correlate directly with the likelihood of clinical reactions to foods, but not very well with the severity of outcome of allergic symptoms [2, 11, 25-29].

Component-resolved diagnosis (CRD) is a further diagnostic technique, adapted from the traditional skin or *in vitro*-specific IgE testing. On a microarray chip, specific allergenic proteins (purified natural or multiple recombinant) are used to detect the presence of specific IgE antibodies in a small amount of blood, in order to detect the sensitivity to the allergenic food in question. The possible presence of antibodies to different specific proteins within a particular food may provide useful information about the severity of an food allergy, or if a patient is able to pass a food challenge [30, 31].

However, since skin testing and allergen-specific IgE testing in human serum are not sensitive enough to correlate directly with the severity of an allergic outcome, in rare cases oral provocation tests are necessary to confirm a food allergy and allow to design a correct elimination diet. The so-called food challenges (FC) can be performed open (OFC, both the physician and the patient know if the patient is exposed to the allergen or a placebo), single-blind (SB), controlled by placebo (PC), (SBPCFC, only the medical team and the investigators know if the patient is exposed to the allergen or a placebo) or double-blind (DBPCFC, none of the participants knows if the patient is exposed to the allergen or a placebo). However, the double-blind, placebo-controlled food challenge (DBPCFC) remains to be the gold standard in the diagnosis of food allergies, although the procedure is time consuming, labor- and cost intensive, and sometimes of high risk for the patient [32]. To date, several efforts have been made to standardise DBPCFC, but for evaluating food allergies in large-scale epidemiology DBPCFC are not yet a practical way [2, 33-36].

1.1.5. Prevalence of food allergic individuals

Studies estimated that around 2-4% of the adult population and 6-8% of young children are affected by food allergy [37, 38]. Schneider Chafen et al., evaluated literature that met the "Guidelines for the Diagnosis and Management of Food Allergy in the United States" [3] and reported that more than 1-2% but less than 10% of the population are affected by food allergy [39]. The assessment of accurate food allergy prevalence is very challenging because it can be influenced by misclassification, different study populations, differences in study design and methodology, geographic variations, age and other factors as reviewed in detail by Sicherer et al., in 2011 [40]. In the course of the EuroPrevall program, Rona et al., performed a meta-analysis to assess the prevalence of food allergies. The prevalence of cow's milk, hen's egg, peanut, fish and shellfish was found to be 0.6, 0.9, 0.75, 0.2 and 0.6%, respectively [41]. In a meta-analysis based study carried out by Zuidmeer et al., food allergies to vegetables, tree nuts, fruits, soy and wheat were reported to be 2.7, 4.5, 4.2, 0.6 and 1.2% (based on symptoms with sensitisation and SPT) [42]. Burney et al., assessed the prevalence and distribution of IgE sensitisation to food-associated allergens of adults aged 20-54 years from general population in eight different regions of Europe, indicating heterogeneity and a strong association to the prevalence of IgE to pollen allergens [43]. Considering that every food could elicit an allergic reaction, only eight main foods, i.e. milk, egg, peanut, tree nut, soy, wheat, fish and shellfish, turned out to be responsible for more than 90% of food allergies. Among young children peanut, cow's milk and hen's egg allergy are often very severe, but they possibly can outgrow their allergy by developing spontaneous tolerance (80% for egg and milk, 20% for peanut allergy) [44, 45].

The prevalence of food allergies is likely to increase. They are a common health and social issue and will cause a significant impact on quality and cost of life [13, 46, 47].

1.1.6. Management of food allergy

Oral immunotherapy has been found to be a safe and efficient approach to induce desensitisation in most patients suffering from milk, egg and/or peanut allergy [9]. Further strategies for therapy of food allergic individuals, such as the use of herbal medicines derived from traditional Chinese medicine, are under research [48-50]. Several studies indicate that vitamin D seems to play a role in food allergies but the findings are contradictory. According

to Allen et al., vitamin D sufficiency may be an important protective factor for food allergy in the first year of life. However, Weisse et al., reported that high vitamin D levels in pregnancy and at birth may contribute to a higher risk for food allergy [51, 52].

So far, the safest way to cope with a food allergy is to completely avoid the consumption of the offending allergenic food. Rapid emergency treatment has to be initiated in case of an anaphylactic reaction caused by unintended consumption [11, 17]. In order to protect food allergic patients and to make diet planning easier, the European Union (EU) food legislation requires, according to the Commission Directive 2007/68/EC, the labelling of thirteen potentially allergenic foods and products thereof. This list comprises “the big eight”, cereals containing gluten (wheat, rye, barley and related grains), crustaceans, eggs, fish, peanut, soybeans, milk, tree nuts (almonds, hazelnuts, walnuts, cashew nuts, pecan nuts, Brazil nuts, pistachio nuts, macadamia nuts and Queensland nuts), and in addition celery, mustard, sesame seed, lupine and molluscs [53]. Since the 13th of December 2014, the existing labelling directive has been extended by the new regulation 1169/2011 on the provision of food information to consumers. It provides detailed information on how to present allergen information on the respective product labels and extends allergen labelling to non-pre-packed foods [54]. The EU list of food allergens is the most comprehensive one. In other countries like Canada, Australia and New Zealand or the United States a low number of allergenic foods are on the national lists. In Japan, only labelling of wheat, buckwheat, egg, milk, peanut and crustaceans is mandatory. The allergenic foods milk, egg, gluten-containing cereals, crustaceans, peanuts and tree nuts are recovered by almost all labelling regulations [55].

Since food-allergic consumers represent a small, but rather significantly growing portion of the general population, food industry has to control and communicate the presence of allergens, in order to handle allergenic ingredients rather than eliminate them from the food supply chain. Supporting tools like guidelines and allergen management plans are established. Regarding to the inadvertent presence of so-called “cross-contact” allergens, protective measures, such as “may contain …” or “contains trace of …” labelling, have been widely adopted by food processing industry to prevent possible legal actions [56, 57]. Due to overuse of these precautionary labelling, the confidence of allergic consumers in food safety has been negatively affected [58].

1.2. Food allergens

Proteins from both foods of animal and plant origin can trigger food allergic reactions. Since the late 1980s, many hundreds of allergens have been identified and characterised and numerous allergen databases have been established [59]. Due to the similarity of their protein sequences and structures, proteins can be grouped in more than 10,000 protein families. However, proteins with allergenic activity are restricted to only 2% of those known protein families [60].

Food allergens of animal origin belong predominantly to three families, the tropomyosins, parvalbumins and caseins [61]. Tropomyosins are the major allergens of shellfish (crustacea as well as mollusca). Together with the proteins actin and myosin they regulate muscle contraction. They show a typical coiled-coil structure (formed by α-helices). Parvalbumins, classified into α- and β-parvalbumins, are abundant in the white muscle of many fish species, and regulate the calcium levels, which are important for muscle fibre relaxation. Structurally they are characterised by a helix-loop-helix, a calcium-binding motif known as EF-hand. Caseins, α_{s1}-, α_{s2}-, β-, and κ-caseins, constitute the major protein fraction in milk, and bind calcium by forming casein micelles [62].

About 65% of all plant food allergens belong to just four protein families, the prolamins, cupins, Bet v 1 (birch pollen protein) homologues and profilins [63]. The prolamin superfamily includes three major groups of plant food allergens, 2S albumins, nonspecific lipid transfer proteins (nsLTPs) and cereal α-amylase/trypsin inhibitors. They all have similar three-dimensional structures rich in α-helices stabilised by disulphide bonds, and are stable to thermal processing and proteolysis. The cupin superfamily comprises the 7S (vicilin-like) and 11S (legumin-like) seed storage globulins, characterised by a β-barrel (cupin) structure. Bet v 1, the major birch pollen allergen, and its homologues belong to family 10 of the pathogenesis-related proteins (PR-10) and may play a role in plant protection. The profilins are a group of proteins that regulate actin polymerisation, e.g. by binding the monomeric actin and are involved in the pollen-fruit allergy syndrome [62].

Partial homologies in the 3D surface structures are a reason for IgE cross-reactivity between allergens from different food sources, and can lead to allergic response to similar proteins. For example, following sensitisation to the Bet v 1 birch pollen allergen, patients often tend to

develop allergic symptoms after ingestion of fruits, nuts or vegetables (e.g. celery), due to the presence of Bet v 1 related proteins [64, 65].

1.2.1. Food processing - stability of food allergens

In general, the regions of an antigen (allergen) that bind to an antibody are referred to as epitopes. They are classified into continuous or linear epitopes, which can bind to antibodies in their folded or unfolded molecular state, and discontinuous or conformational epitopes showing secondary, tertiary and quaternary structural elements, which may not be recognised by antibodies if the protein is unfolded [62].

Different food processing techniques such as thermal treatment, high pressure treatment, extrusion or ultrasound may have effects on the allergen structure, and thus potentially increase or decrease the allergenicity of a protein [66]. Processing can lead either to unfolding, aggregation or chemical modification of proteins and thus influence their stability to digestion and consecutively the form they are presented to the immune system. However, it is difficult to predict how a certain allergen is affected by food processing.

For example, heating or cooking can unfold or destroy the conformational epitopes of Bet v 1 homologues as found in vegetables and fruits. However, the thermostability differs among the Bet v 1 homologues, e.g. the allergen from apple, Mal d 1, is quite heat labile and unfolds on heating to 90 °C, whereas Api g 1, the allergen from celery, does not start to unfold until heated above 80 °C and can refold to its native structure on cooling. In addition, the type of heating seems to play a role in the loss of allergenicity of proteins, e.g. roasting (dry heating) does not denature Cor a 1, the Bet v 1 homologue in hazelnut, as effective cooking (wet processing) would do [67-69].

In contrast to processing-labile proteins like Bet v 1 homologues, proteins from the prolamin superfamily, e.g. 2S albumin allergens or nsLTPs, are very stable to a variety of chemical and physical denaturants. Their characteristic folded structures constrained by disulphide bonds are not affected by low pH-value, chaotropes, high temperature or pressure. Therefore, they retain their allergenic properties in processed foods.

In addition, there are allergens that comprise, in the presence of well-defined epitopes, regions with ill-defined interchanging conformations that do not show the same transition on heating from a folded to a partial folded or unfolded structure as in more ordered proteins.

Seed storage globulins show another type of process stability. They denature to some extent and form aggregated networks in a highly hydrated structure during heating [67]. Chemical modifications which may affect the allergenicity of the proteins can be induced by the Maillard's reaction occurring at high temperatures between free amino groups of proteins in the presence of sugar, forming structurally diverse compounds, known as Amadori or advanced glycation end products (AGEs).

Further interactions with matrix components, like oxidised lipids, carbohydrates or enzymatic modification with polyphenols, may have a strong effect on the protein structure. However, it is difficult to predict which influence this kind of food processing might have on the allergenicity [67, 70]. Novel processing techniques such as high-pressure treatment, pulsed electrical field or γ-irradiation are used for pasteurisation or sterilisation in order to retain the flavour or texture of the product. However, these techniques do not change the protein conformation and thus are not expected to have an impact on the allergenicity of proteins [71].

Since food processing can lead to changes in the protein structure or reduction of the solubility of the target protein, it can have an influence on the limit of detection of analytical methods, e.g. immunoassays, an important aspect that has to be considered from an analytical point of view [72].

1.2.2. Thresholds for food allergens

Since the exposure to even small amounts of an offending food can elicit adverse reactions in food allergic patients, in the last years great efforts have been undertaken in estimating threshold doses below which allergic reactions are unlikely to occur.

A threshold dose might be defined as the lowest amount of the allergenic food that would elicit mild, objective symptoms, e.g. mild urticaria, in the most sensitised persons [73]. An individual's threshold lies between the highest dose that does not elicit any adverse effect, termed as no-observed-adverse-effect level (NOAEL), and the lowest dose that elicits an adverse reaction, termed as the lowest-observed-adverse-effect level (LOAEL). These parameters are determined experimentally by clinical food challenge studies, e.g. DBPCFC. Clinical data obtained from such studies indicate, that the minimal eliciting dose (MED) can vary in a wide range between persons suffering from the same type of food allergy, e.g. within

the peanut allergic population about 5-6 orders of magnitude [74, 75], and can change within an individual over time, because of factors like age, exercise, stress or medication. In addition, clinical threshold doses differ for various allergenic foods. Thus, it is a difficult task to establish an individual's threshold dose or even a population threshold dose, which would be the largest amount of the allergenic food that will not cause a reaction when tested experimentally in a defined population of allergic individuals [76].

Population threshold dose distributions that can be used to elaborate eliciting doses (EDs), represent a tool to assess the risk of allergen exposure for allergic consumers, and can be used to define action levels for precautionary labelling. The accuracy of these threshold parameters strongly depends on the number of allergic individuals included in the population threshold analysis (60 or more participants are recommended) as well as on the dosing scheme used for oral food challenge, or the employed statistical distribution, as investigated by Klein Entink et al. [77].

Recently, Blom et al., examined threshold dose distributions for five major allergenic foods based on clinical data of children that were routinely tested by DBPCFCs to diagnose food allergy. The protein dose at which 5% of the allergic population is likely to respond (ED_{05}) with objective reactions was found to be 1.6 mg for peanut, 1.1 mg for cow's milk, 1.5 mg for hen's egg, 7.4 mg for cashew nut and 0.29 mg for hazelnut [78].

International expert groups, such as the VITAL (Australian Voluntary Incidental Trace Allergen Labelling) initiative or the ILSI (International Life Sciences Institute) Europe Food Allergy Task Force, reviewed data sets based on all publically available valid oral food challenge data in order to establish food allergen reference doses, which will enable manufacturers to perform rational risk assessment approaches. Reference doses could be determined on the ED_{01} level (only 1% of the population is likely to be affected) for peanut, cow's milk, hen's egg and hazelnut, as well as on the ED_{05} level for wheat, soybean, cashew, shrimp, sesame seed, mustard and lupine, in terms of mg total protein from the allergenic food. Overall, reference doses for 11 potentially allergenic foods were determined, ranging from 0.03 mg for egg to 10 mg for shrimp protein (on the ED_{01} level). However, for fish and celery no reference doses could be established due to insufficient data for modelling. These defined reference doses, which form the basis of the VITAL 2.0 thresholds and are now recommended in Australia, have generated much interest [79, 80].

However, to date, threshold levels which would define tolerable risk levels for allergenic sources in foods are still under discussion. The implementation of such thresholds would lead to benefit for all involved stakeholders. For example, evidence based reference doses which are associated with a certain risk of reaction in the food allergic population would provide information about the safety of a product and lead to an increased choice of products for food allergic persons. In addition, they would restore the credibility of precautionary labelling and improve food safety for allergic consumers. Regulatory authorities would have a more secure basis for product recall if cross-contamination is detected in a product and could assess the overall population health hazard associated with certain allergen exposures in contaminated or recalled products.

From an analytical point of view, the implementation of thresholds would require standardised sensitive allergen detection methods with limits of quantification (LOQ) below the threshold level set. In addition, the development of such accurate allergen detection methods will be dependent on the effort of developing certified allergen reference materials [81].

1.3. Allergenic foods

Since the doctoral thesis deals with the development and validation of analytical methods for the detection of mustard and celery, the following sections focus on these two allergenic foods.

1.3.1. Mustard

Mustard plants belong to the *Brassicaceae* family like many other food crops, for example, various types of cabbage (cauliflower, Brussel sprouts, kohlrabi and Chinese cabbage), turnip, radish, broccoli, watercress, horseradish or rapeseed. Commonly the seeds of three mustard species, in particular white or yellow mustard (*Sinapis alba*), brown or Indian mustard (*Brassica juncea*), but also black mustard (*Brassica nigra*) are used for culinary purposes [82]. All three plant species are yellow flowering annual plants, with various growth heights of about 80 cm for white mustard, 1.2-1.5 m for brown mustard and about 2.5 m for black mustard. The seeds grow within seedpods and are usually about 2.5 mm (white mustard), 1.5-2 mm (brown mustard) and 1 mm (black mustard) in diameter [83].

In the year 2013, Canada and Nepal were the main producers of mustard seeds with 154500 t in Canada and 142920 t in Nepal, respectively. But also in Europe, large amounts of mustard seeds were harvested: Russian Federation 54682 t, Ukraine 30170 t, France 14000 t, the Czech Republic 13378 t, and Germany 10500 t, among other countries with lower production, according to the Food and Agriculture Organisation of the United Nations (FAOSTAT, statistics division). Pakistan and India are also known to produce mustard seeds but these data are not collected by the FAOSTAT [84].

Mustard seeds are a rich source of proteins (ca. 28%), oil (20-36%) and thioglycosides. The oil has a high content of unsaturated fatty acids like 42% erucic acid, 22% oleic acid, 16% linoleic acid, 7% arachidic acid and 7% linolenic acid. Because of its high content of erucic acid, which can lead to fatty degeneration of the heart, mustard oil for consumption should not contain more than 5% of the total fatty acid content [83]. After cell rupture, the thioglycosides are exposed to the activity of the thioglucosidase enzyme myrosinase, resulting in free isothiocyanates. The allyl isothiocyanates obtained from sinigrin in black and brown mustard and the *p*-hydroxybenzoyl isothiocyanates from sinalbin in white mustard are responsible for the pungent burning taste [85]. In China, where mustard seeds are used largely as a spice and in traditional Chinese medicine, it has recently been found that mustard seeds possess anti-oxidant, anti-inflammatory and anticancer bioactivity, due to its multiple components such as isothiocyanate, erucic acid, phenols and phytin [86].

Mustard is mainly consumed as mustard paste or used as a spice for cooking, roasting or pickling, as ingredient to season prepared foods, in sausages or meat, sauces, soups, marinades or salad dressings, applied as whole seeds or ground [82]. The seeds can also be used to produce mustard oil, and the leaves can be eaten as mustard greens. White mustard seeds are less pungent than those of brown and black mustard. They are a main ingredient in American-style mustards, whereas English mustard contains white and brown mustard and European or Chinese mustards mainly contain brown mustard seeds. The seeds are sold whole, ground into powder or processed into prepared mustard [87].

1.3.1.1. Prevalence of mustard allergy

France is one of the largest European producers and also one of the largest European consumers of mustard, before Germany and Great Britain, which could be a reason for the increased prevalence of mustard allergy in France [82].

Andre et al., 1994, followed 580 patients with pathological reactions to foods in France over a period of nine years. They found out, that the frequency of sensitisation to different foods, among them celery and mustard, was definitely increasing, because of increased consumption of food allergens and increasing clinical research efforts [88]. Rancé et al., reported that in France mustard allergy is the fourth common food allergy among young children after allergy to eggs, peanuts and cow's milk, starting early in life [89].

1.3.1.2. Clinical symptoms of mustard allergy

Mustard allergens can induce severe allergic reactions, such as atopic dermatitis (mixed IgE- and non-IgE-mediated reaction), the most clinical manifestation of mustard allergy in children, or OAS, very frequently among adults, with systemic anaphylaxis [87]. In the early 90ties, cases of anaphylactic reactions triggered by mustard after the ingestion of e.g. pizza [90], a small amount of mustard sauce [91, 92], chicken dips (masked mustard) [93], as well as hypersensitivity to mustard seeds [94] were reported.

1.3.1.3. Diagnosis of mustard allergy

Skin prick tests and specific serum IgE determinations [91, 92] seem to be appropriate methods for the diagnosis of mustard hypersensitivity. But positive skin prick tests and the presence of specific IgE only indicate sensitisation to mustard. Since mustard contains irritating substances like isothiocyanates and capsaicin, which may cause non-immune reactions with similar symptoms to allergic reactions, it is required to base the diagnosis of a mustard allergy on a food challenge, preferably a DBPCFC, as already mentioned (Chapter 1.1.4.). Rancé et al., based clinical diagnosis at children on open and single-blind, placebo-controlled food challenges (SBPCFC) [89]. Morisset et al., were the first who performed a DBPCFC in addition to SBPCFC, which still is a challenging task, because masking the strong intense taste of mustard is difficult. Mustard allergy was diagnosed in 42% of children under

3 years (SBPCFC), and in 23.3% of young patients aged 3 to 20 years (SBPCFC or DBPCFC) [89, 95].

Clinical implication of mustard allergy:

The use of recombinant (r, e.g. rSin a 1) well characterised sequences from mustard allergens, like Sin a 1, Sin a 2, Sin a 3 and Sin a 4, instead of crude allergenic extracts opens new perspectives to an easier standardisation for accurate diagnosis, as well as to the development of specific anti-allergic treatments [96-98].

1.3.1.4. Allergens in mustard

To date, in white mustard (*Sinapis alba*) four allergens have been identified. These include Sin a 1, a 2S seed storage albumin with a molecular weight of 14 kDa [99-101], Sin a 2, a 11S seed storage globulin with subunits of 50-60 kDa [97, 102], Sin a 3, a non-specific lipid transfer protein (nsLTP) with 12 kDa, and Sin a 4, a profilin with 14 kDa [98]. A 2S seed storage albumin in brown mustard (*Brassica juncea*), Bra j 1 with 16 kDa, is structurally closely related to Sin a 1 [103, 104]. The allergenic proteins from black mustard have not been identified so far.

1.3.1.5. Stability

The major allergens, Sin a 1 from white mustard and Bra j 1 from brown mustard, are highly thermostable and proteolysis resistant. In addition, it was shown that Sin a 1 is stable against acid and alkaline treatment [96, 105, 106]. The study of Sirvent et al., confirmed the structural stability of Sin a 1 and Sin a 3 to high temperatures and proteolysis digestion. Thus, the allergenicity of these proteins is not affected by processing steps usually involved in mustard derived foods preparation. In contrast to this stable major allergens, Sin a 4 was significantly modified at 85 °C and digested by gastric treatment. Although this allergen does not act as a sensitising mustard allergen, it plays a role as cross-reactive agent involved in pollen and/or other food allergies [107].

Since in the last years the interest in developing genetically modified crops to improve tolerance to pests or herbicides, or nutritional quality has been increased, evaluation of the allergenicity of these plants became essential. However, studies assessing the allergenic risk of transgenic mustard plants indicated no significant differences in protein stability and thus

in allergenic potential compared to native mustard. They confirmed that native as well as genetically modified mustard elicit equal allergic responses in mice [108, 109].

1.3.1.6. Cross-reactivity

Cross-reactions involving clinical manifestations between mustard and other *Brassicaceae* family allergens are rare [82]. However, Caballero et al., reported that data on mustard sensitised patients with a systemic reaction (by OFC) indicate an association to pollen hypersensitivity (pollinosis) or an allergy to other vegetables [110], whereas Rancé and Dutau did not observe any particular association with pollen allergy in children [89]. Figueroa et al., reported significant associations with mugwort pollinosis and several plant-derived food allergies (nuts, legumes, *Rosaceae* fruits and corn) in patients suffering from mustard allergy (by DBPCFC), and proposed a new mustard-mugwort allergy syndrome. In addition, they related this syndrome to food-dependent exercise-induced anaphylaxis in Spain [87].

Sirvent et al., 2009, identified new mustard allergens (Sin a 3 and Sin a 4) and showed IgE cross-reactivity with allergens from fruits like peach or melon [98]. Mustard allergic patients who have IgE to rSin a 3 frequently suffer from allergy to other plant-derived foods such as *Rosaceae* fruits, mainly peach, or *Artemisia vulgaris* pollen. These results are in agreement with the involvement of the lipid transfer protein family in cross-reactivity among cabbage, mugwort pollen and peach as previously reported [111].

1.3.1.7. Threshold dose for mustard

As already mentioned, tolerable risk levels for allergenic sources in foods have not been established so far. In a clinical study based on open or SBPCFC with mustard powder the provoking dose varied between 1 mg (corresponding to 0.3 mg of mustard proteins; the lowest dose tested) and 936 mg (the highest dose given) in children [89]. In a DBPCFC study performed by Morisset et al., the lowest eliciting dose for one patient was 40 mg of mustard seasoning that is equivalent to 13.5 mg of mustard seeds and roughly equivalent to 0.8 mg of mustard proteins (mustard seasoning is considered to contain 6% of proteins) [95]. Recently, Taylor et al., reported the outcome of efforts from an expert panel to establish appropriate

reference doses for allergenic food residues as a part of the VITAL program (as mentioned in Chapter 1.2.2.). The reference dose for mustard was estimated to be 0.05 mg of mustard protein (based on the ED_{05} level), respectively [80].

1.3.2. Celery

Celery (*Apium graveolens*) is a member of the *Apiaceae* family. It has already been cultivated by the Greeks and Romans and used as food and medicine since the Middle Age. Because of their aromatic flavour, three varieties of celery, celery roots (*Apium graveolens var. rapaceum*), celery stalks (*A. g. var. secalinum*) and leaf celery (*A. g. var. dulce*), are commonly used. They can be consumed raw or cooked, e.g. as salad and vegetable juice, or as condiment in soups, sauces, meat products and marinades. Celery powder (from celery roots) and aromatic extracts from celery are often used in processed foods [112]. The typical celery aroma is caused by the occurrence of phthalides in leaves, root, and seeds [113].
However, the consumption of celery can induce allergic reactions in sensitised persons, ranging from oral contact urticaria to life-threatening anaphylaxis.

1.3.2.1. Prevalence of celery allergy

In Europe, particular in Switzerland, France and Germany, celery allergy is one of the most common food allergies. It is frequently associated with birch and/or mugwort pollinosis, also known as birch-mugwort-celery syndrom [114, 115]. A study carried out in Switzerland with 383 food allergic patients (60.3% with additional manifestation to pollen allergy) showed that 36.3% of the tested allergic patients were sensitisised to celery roots [116]. Similar numbers have been reported in a French study. 30% of 580 individuals with food allergy were sensitised to celery, with most of them suffering from severe allergic symptoms [88]. In Germany, a group of 167 pollen-related food allergic persons was examined with skin prick tests and enzyme allergosorbent tests (EASTs). 70% of them were found to be sensitised and 14% (based on anamnestic data) suffered from allergy to celery [117].

1.3.2.2. Clinical symptoms of celery allergy

The ingestion of celery can elicit symptoms from mild OAS (oral itching and swelling of the lips and oral mucosa), gastroenteritis and diarrhea, to severe attacks of urticaria, angioedema, the respiratory system, or even life-threatening systemic anaphylactic reactions [116, 118-120].

1.3.2.3. Diagnosis of celery allergy

In 2000, a DBPCFC study with patients having adverse reactions history to celery was performed for the first time [121]. In a study carried out in 2000, DBPCFC was found to be applicable to confirm celery allergy in 22 of 32 patients with a history of adverse reactions to celery root [122]. However, since DBPCFCs are very cost intensive, time consuming and involve high risk for the patient (as mentioned in Chapter 1.1.4.), researchers try to identify specific IgE binding proteins and peptides in celery to provide suitable diagnostic tools to distinguish between healthy and celery-allergic patients [123].

1.3.2.4. Allergens in celery

Currently, four celery proteins have been identified that have the potential to trigger an allergic reaction in individuals sensitised to celery. Api g 1, with a molecular weight of about 16 kDa, is a Bet v 1 homologue (40% identity, 60% similarity) and belongs to the PR-10 family of pathogenesis-related proteins [124, 125]. Two isoforms, Api g 1.01 and Api g 1.02, have been characterised [126, 127]. The profilin Api g 4, 14 kDa, is homologous to Bet v 2 [128, 129]. Api g 5 has been identified as a glycoprotein of about 60 kDa carrying at least 3 N-glycans attached at different amino acid positions [130, 131]. In previous papers Api g 5 had been described as cross-reactive carbohydrate determinants (CCD) showing allergenic potential [132, 133]. Recently, Api g 2 was identified. It is a non-specific lipid transfer protein (nsLTP), belongs to the PR-14 family and occurs only in celery stalks [134].

Numerous studies have already been carried out to characterise celery allergens in more detail in order to elucidate the molecular mechanisms of celery allergy. One aim is to produce hypoallergenic proteins in order to improve diagnosis of celery allergy and to develop effective immunotherapy [120, 127, 134-138].

1.3.2.5. Stability

Stability tests of the allergens Api g 1, Api g 4 and CCD in celery (now identified as Api g 5) indicated that these allergens are relatively stable under gastrointestinal conditions and the resulting allergenic activity is mainly due to undigested allergens [139].

A DBPCFC study demonstrated the high allergenic potential of celery consumed as spice and even after prolonged cooking (76 min at 100 °C). The heat resistance of celery allergens decreased in the order of CCD (Api g 5) > Api g 4 > Api g 1 (results obtained by EAST inhibition), which is in accordance with previous results [68, 140]. Since Api g 1 seems to be the most heat labile allergen in celery, patients suffering from celery allergy were often advised to consume celery only after heating. However, Bohle et al., could show that T-cell cross-reactivity between Bet v 1 and related food allergens such as Api g 1 occurs indepently of IgE cross-reactivity and still may cause T-cell-meditated late phase skin reactions in allergic individuals [141]. In order to investigate the thermodynamic parameters for conformational stability, Bollen et al., carried out CD spectroscopy experiments with natural and recombinant Bet v 1 and the homologous allergens Api g 1 and Dau c 1 (from carrot). They showed that the three allergens were affected differently by various denaturation conditions, pH and temperature [142, 143]. Api g 2 showed remarkable stability to gastrointestinal proteolysis and thermal treatment, and has been found to trigger severe allergic reactions upon consumption of cooked celery stalks [134].

1.3.2.6. Cross-reactivity

Various studies found association between sensitisation to celery and birch and/or mugwort pollinosis [118, 119]. The terms “celery-mugwort-spice syndrom” [144], “celery-carrot-mugwort-condiment syndrom” [144], and associated with birch pollen “celery-birch-mugwort syndrom” [115] have been used. About 70% of patients with birch pollen allergy exhibit an OAS (oral mucosa) after eating certain fruits and vegetables such as celery. As already mentioned above, this association is caused by cross-reactive IgE antibodies directed against birch pollen allergens and the related food proteins, due to structurally similarities of homologous allergens from the PR-10 protein family and profilin [124, 128, 143, 145].

Vallier et al., 1988, studied allergens in celery with cross-reactivity to mugwort and birch pollen with the immunoblotting technique. They found that the detected allergens with

similar molecular masses (14, 15 and 16 kDa) display some structural identity which is maybe responsible for cross-sensitivity to celery, mugwort and birch pollen [146]. Bauer et al., 1996, determined cross-reacting structures in birch pollen, mugwort pollen and celery by means of immunoblotting. They found out that three distinct cross-reacting groups of allergens, the homologues of Bet v 1, the profilins (Bet v 2) as well as proteins with a molecular weight ranging from 46-60 kDa, are shared by birch pollen and celery. Two of them (Bet v 2 and the 46-60 kDa proteins) are also present in mugwort pollen [114]. Hoffman-Sommergruber et al., identified a Bet v 1 homologue from carrot (Dau c 1) and verified the existence of common B-cell epitopes present on Dau c 1, Api g 1 and Bet v 1 [137]. Later on these allergens were purified from natural sources under non-denaturing methods and characterised. These experiments revealed that the allergens naturally occur in mixtures of various isoforms [135].

In addition to the many cross-reactivities amongst the *Apiaceae* (celery and carrot), high IgE prevalence to other plant food allergens belonging to the *Rosaceae* (apple, pear, cherry, peach) or *Solanaceae* (potatoe, tomato) as well as melon and mango have been described [145, 147-154] due to homology of LTPs and CCD (Api g 5) in celery and vegetables or fruits [155].

1.3.2.7. Threshold dose for celery

To date, only a few studies are available which provide information about the minimal eliciting dose of celery. DBPCFCs studies performed by Ballmer-Weber et al., showed minimum provocation doses of 0.7 g of raw celery, as well as 0.9 g of cooked celery that caused an OAS. However, celery spice triggered OAS or systemic symptoms at only 0.16 g, respectively [68, 121]. Due to poor model fits with existing data the VITAL expert panel could not establish an appropriate reference dose for the allergenic celery [80].

1.4. Analytical methods for allergen determination in food

Analytical methods are needed to be able to verify correct food labelling but also to check for allergen contamination during food processing. These methods have to be both selective and sensitive. Since the individual threshold dose or lowest observed adverse effect level (LOAEL)

can vary widely among the food allergic population, in Europe, still no maximal tolerable risk levels for allergenic ingredients in foods have been defined by official authorities (see Chapter 1.2.2.). Thus, an analytical method does not necessarily need to yield quantitative information but should allow the detection of allergenic ingredients as sensitive and reliable as possible [156].

1.4.1. Requirements for an analytical method

There are a number of requirements for an analytical method to be applicable to allergen analysis in food. It needs to be selective for the targeting compound, but also sensitive so that preferably the lowest amount, even traces of allergens that are able to trigger an allergic reaction, can be detected. In order to avoid false positive and false negative results, it must be able to detect the allergenic food of interest in the presence of food matrix components and after food processing. For the application in routine analysis, the method should be rapid, reliable, robust and cost-effective [157, 158].

1.4.1.1. Selectivity

In order to avoid false positive results, highly selective analytical methods are of utmost importance. An analytical method does not necessarily need to target the allergenic protein itself. Any characteristic protein or DNA sequence of the allergenic food can serve as marker and would be suitable to indicate the presence of an allergenic food. In order to select a target that meets these requirements, extensive search in databases followed by practical experiments are necessary to exclude possible cross-reactivity [159].

1.4.1.2. Sensitivity

Highly sensitive analytical methods can be developed by selecting multiple target analytes or markers that do not possess any allergenic potential, but are highly abundant in the allergenic commodity (food) [159]. Due to the lack of defined threshold doses (tolerable risk levels) of allergens in food, the limit of detection should ideally be in the range of threshold doses determined by oral food challenge studies or maybe even a magnitude lower, taking into account the quantity of the food product typically consumed. According to a review article

published by Poms et al., 2004, the scientific community generally agreed with limits of detection of analytical methods between 1 and 100 mg allergenic protein per kg food (ppm) [72]. The “food allergy” working group of the German Society for Allergology and Clinical Immunology and the Association of German Allergologists recommend limits between 10 and 100 mg/kg allergenic food or 1 and 10 mg/kg of protein fraction, to protect most allergic consumers from severe allergic reactions [160].

1.4.1.3. Accuracy

The accuracy of an analytical method can be demonstrated by the use of reference materials, ideally certified, for calibration. Reference materials are defined materials or substances whose property values are sufficiently homogenous and well-established to be used for method development and validation or for proof of method performances (ISO Guide 33) [161]. But to date, available food allergen reference materials are limited and lacking for mustard and celery.

The sensitivity and selectivity of an analytical method are usually evaluated in buffer systems. However, extracts from food products are much more complex than the buffer system. Hence, so-called spike-and-recovery experiments can be applied to investigate the accuracy of the method in one or more food matrices which are representative for the allergenic food. Since these spike-and-recovery experiments do not reflect the effect of food processing, the use of naturally incurred standards for method calibration is recommended. Naturally incurred standards are produced by adding the allergenic food into the food formulation and treating them under “real” food processing conditions. Extracts from these naturally incurred standards are used as calibrants in order to calibrate the method [162]. Recently, Siegel et al., described in detail the production and homogeneity testing of selected food materials such as sausages, cookies and sauce hollandaise powder, containing all allergenic ingredients (with the exception of black and brown mustard) that have to be declared according to EU legislation [163].

1.4.1.4. Application on foodstuffs

Analytical methods for food allergen detection should be applicable to complex and highly processed food products. This can be a challenging task for the development of an analytical

method, since food matrices can mask allergenic proteins, especially if these are present only in trace amounts. In addition, the food matrix can lead to an inhomogeneous distribution of allergenic residues, e.g. in foods having a more particulate nature. Further matrix influences, such as the pH-value, chemical modifications or aggregation phenomena can differently affect the extractability of both proteins and DNA. Currently, no standardised extraction protocols are available in the field of food allergen detection, neither for DNA-based methods nor for ELISAs. In addition, matrix components could inhibit the polymerase activity in PCR based methods. In ELISAs, they could disturb the antigen-antibody binding or interact with the antibody instead of the target protein. Moreover the sensitivity of an analytical method can be decreased by food processing, e.g. heating, fermentation, acidification or irradiation,by affecting the integrity, stability and recovery of DNA and proteins.

Since matrix effects and food processing can lead to false negative as well as false positive results, the developed methods have to be validated ideally for each food product, but at least for one typical product containing the allergenic food of interest, and the effect of food processing needs to be taken into account [157, 162].

1.4.1.5. Validation of an analytical method

There is an urgent need for method validation in order to ensure the accuracy of the obtained results [72]. In single laboratories, important characteristics including selectivity, sensitivity, accuracy, limit of detection, limit of quantification and the applicability have to be determined [161]. Analytical methods should be robust and yield reliable results. Thus, international collaborative trial studies, so-called ring trials, should be performed to address these issues in order to increase confidence. The performance of such ring trial studies is described, e.g. in the international harmonized protocol for the proficiency testing of analytical chemistry laboratories [164]. To date, only a few ELISAs and real-time PCR assays have been validated through inter-laboratory studies according to the AOAC International official protocol (Mermelstein, 2008) [165].

1.4.2. Analytical methods available for food allergen detection

Classical analytical methods for food allergen detection can be divided into protein-based methods and DNA-based methods. Protein-based methods, e.g. enzyme-linked immunosorbent assays (ELISAs) are fequently based on the interaction between specific antibodies and proteins of the allergenic food. DNA-based methods are most commonly based on the real-time polymerase chain reaction (PCR) which amplifies a DNA fragment of a gene coding for the allergenic or other proteins of interest for detection.

ELISAs and PCR methods are currently applied as official methods for routine screening or, if requested, for quantification of food allergens by food safety authorities, whereas certain other promising methods, e.g. LC-MS, are still used mainly in the research field, because they are too costly for routine analysis [166].

1.4.2.1. Protein-based methods - immunoanalytical techniques

Immunoanalytical methods such as ELISAs are based on the formation of an antibody-antigen complex. For the development of a sensitive assay, the selection of antigen-specific antibodies with high affinity is of crucial importance [167]. Polyclonal antisera from rabbit, goat, sheep or chicken consists of a heterogenous mixture of antibodies with various specificity for epitopes of a single protein or a range of proteins. In contrast, monoclonal antibodies from mice are raised against a particular epitope of a protein and thus can be more specific than polyclonal antibodies. They guarantee long time supply and are often used in combination with polyclonal antibodies [162, 168].

There are different formats of ELISAs available, such as the sandwich ELISA, the most common type for the detection of food allergens, and the competitive ELISA.

In the sandwich ELISA the antigen (protein) of interest is captured by an immobilised antibody, forming an antigen-antibody complex. For the detection of this complex, a second protein-specific antibody labelled with an enzyme binds to the antigen and an antibody-antigen-antibody complex (sandwich) is formed. In a next step, a chromogenic substrate solution is added, that, catalysed by the enzyme, is converted to a coloured product. After the enzyme reaction is stopped, the optical density of the solution can be measured photometrically. The obtained signal is directly proportional to the analyte concentration in the sample. The

concentration of the analyte can be determined by a standard curve generated with standard solutions.

In a common competitive ELISA format, immobilised antigens (allergens) are competing with the allergens in the sample, forming an antigen-antibody complex with the protein-specific enzyme-labelled antibody (in case of direct detection; if the protein specific antibody is not labelled, a second enzyme labelled antibody for detection has to be used (indirect detection)). The more antigen is present in the sample, the less antibody will bind to the immobilised antigen and the lower will be the measured absorbance. Thus, the obtained signal is inversely proportional to the concentration of the analyte in the sample [72].

To date, the ELISA is the most commonly used protein-based technique for allergen analysis, due to its high specificity and sensitivity, quick and easy handling, and full automation of the test procedure resulting in high screening capacity.

Since an antibody recognise a particular epitope of a protein, cross-reactivity can appear if the particular epitope is present in closely related proteins, leading to false positive results. If the 3D structure of the epitopes have been altered because of food processing, false negative results could be obtained [72, 168, 169].

1.4.2.2. Protein-based methods – mass spectrometry

A further trend in analysing food allergenic proteins is the application of mass spectrometry (MS) methods, such as liquid chromatography coupled with mass spectrometry (LC-MS, LC-MS/MS), which can overcome the above mentioned drawbacks. In general, the MS technique provides information about the molecular mass and enables the identification and characterisation of more than one specific marker peptides of allergenic proteins. Quantitative MS assays are carried out by measuring the marker peptides in the presence of an internal standard and establishing a calibration function in order to calculate the relative concentration of the allergenic protein. However, a special challenge is to achieve effective sample preparation and to reduce matrix interferences [170].

The advantages of LC-MS/MS over currently available ELISAs are the possibility to detect several target peptides per allergen and the capability of multiplexing. MS methods have been shown to be applicable to processed foods. Because of using marker peptides, the detection is independent of the 3D structure of the allergenic protein. Mass spectrometry can be

employed to determine allergenic foods which contain only low amounts or no DNA at all, such as egg, milk or soy [169]. Recently, a multiple LC-MS/MS method for the simultaneous detection of seven allergens (milk, egg, soy, hazelnut, peanut, walnut and almond) was developed and validated by analysing baked bread [171].

1.4.2.3. DNA-based methods – real-time polymerase chain reaction

DNA-based methods target a DNA sequence of a gene, either coding for the allergenic protein or for other proteins of interest. In most cases, the specific sequence is amplified by the polymerase chain reaction in order to detect it [166]. Since DNA-based methods do not target the (allergenic) protein itself, it is difficult to correlate results that refer to the DNA to actual protein quantities and to use this method for risk assessment studies and management [157].

1.4.2.3.1. Polymerase chain reaction – PCR

In general, the polymerase chain reaction (PCR) is a molecular biological method that amplifies a well defined DNA sequence *in vitro*. For the amplification reaction, target DNA, a thermostable DNA polymerase, two oligonucleotide molecules, so-called primers (forward and reverse), free deoxynucleotide triphosphates (dNTPs) and a reaction buffer containing magnesium, are combined. This reaction mixture is placed in a machine, a so-called thermal cycler, where the polymerase chain reaction is carried out by several repetitions of a series of different temperatures and periods of time. The adjustment of this series of temperatures and time represents one cycle of amplification, involving the steps template denaturation, primer annealing and primer extension (elongation) [172].

In the first step, at a temperature of 94-95 °C hold for a few seconds (initially for 2 minutes), the double stranded DNA (dsDNA) is denatured to separate the complementary single strands (ssDNA).

In the second step, the temperature is rapidly reduced to approximately 40-60 °C (annealing temperature) for a period of 15-60 seconds in order to allow the sequence specific primers to hybridise (anneal) to the complementary bases on the ssDNA. This annealing step is highly dependent on the melting temperature of the primers. Too low temperature can lead to unspecific hybridisation of the primers. If the temperature is too high the primers are unable to bind to the template DNA.

In the final step, the reaction mix is heated to a temperature of 72 °C for approximately 1-2 minutes in order to achieve efficient extention of the hybridised primers. Catalysed by the heat stable DNA polymerase, the complementary DNA strand is synthesised by adding the appropriate dNTPs. Since (at least in theory) the amount of template doubles in each cycle, the DNA sequence is exponentially amplified [173].

1.4.2.3.2. Real-time PCR

Real-time PCR is based on the continuous detection and collection of fluorescent signals from one or more polymerase chain reactions over a range of cycles, which can be visualised on the computer monitor. There are several different types of real-time PCR detection methods available, among them the TaqMan method. Since TaqMan assays were developed in the presented doctoral thesis, the principle of TaqMan probes will be explained (see Figure 1). TaqMan assays are based on the 5'→3' exonuclease activity of the applied Taq DNA polymerase, a heat stable enzyme from the bacterium *Thermus aquaticus* (Taq).

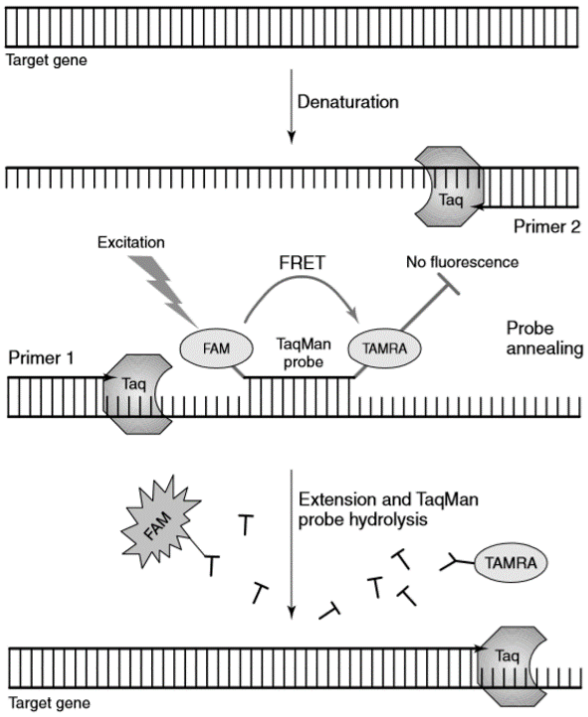

Figure 1: A schematic diagram showing the principle of real-time PCR using a TaqMan probe. Taq: Taq DNA polymerase; FAM: 6-carboxyfluorescein (reporter dye); TAMRA: tetramethylrhodamine (quencher); FRET: fluorescence resonance energy transfer [174].

In addition to the specific forward and reverse primer, a third oligonucleotide, known as TaqMan probe, labelled with a fluorescent dye (reporter) at the 5' end and a quencher molecule (acceptor) at the 3' end, is used. If reporter and quencher are in close proximity, the fluorescence from the reporter dye is absorbed by the quencher, thus, no fluroescence signal can be detected. After the annealing of the primers and the probe, the Taq DNA polymerase extends the primer and synthesises the complementary DNA strand as described before. When reaching the 5' end of the TaqMan probe, the polymerase cleaves the probe base to base from the strand, because of its exonuclease activity as mentioned above. Since the reporter dye is released into solution it seperates from the quencher and the fluorescence signal can be detected at the reporter dye's emission wavelength [174, 175]. For analysis, the kinetics of the PCR reaction is used. The real-time detection system generates a fluorescence curve on the computer monitor, called an amplification plot, that is composed of four distinct phases (see Figure 2): baseline (1), exponential (2), linear (3) and plateau (4). During the first cycles (baseline phase) only background fluorescence is detected. Although the template is amplified exponentially, the fluorescence signal is still below the limit of detection of the real-time instrument.

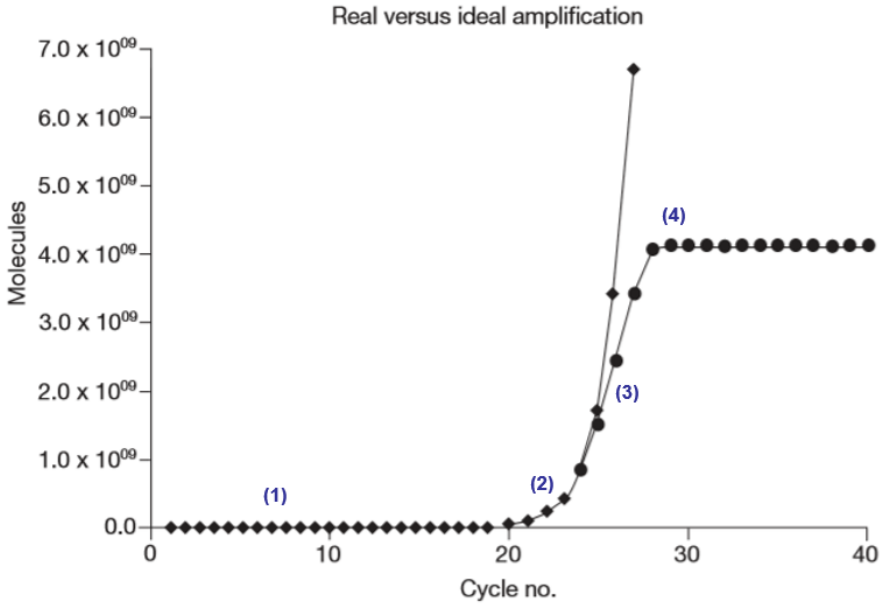

Figure 2: Graphical representation of the fluorescence signals from an ideal vs. an actual real-time PCR reaction. In an ideal PCR, there are two phases (1, 2): a baseline where the signal is below the limit of detection of the instrument followed by an increase in fluorescence that continues over the remaining cycles of the experiment. However, in an actual PCR, there are four phases (1-4). With increasing cycle number the amplification becomes less than ideal leading to a linear phase and finally a plateau where no further increase in signal occurs. ◆ ideal PCR, ● simulated real amplification curve [175].

The exponential phase is comprised of the earliest detectable signal from a polymerase chain reaction where the amplification is proceeding at its maximal exponential rate. The length of this phase depends on the template concentration and the quality of the real-time assay. In an ideal reaction there are two complete molecules synthesised from every one template available in the exponential phase, defined as 100% efficiency. In the linear phase, the amplification efficiency begins to decrease. It is shown as straight line but amplification is no longer two products from every one template molecule in each cycle but gradually declines in template replication efficiency further until the plateau phase is reached where amplification rapidly ceases as a consequence of changes in the relative concentrations of certain components of the reaction [175].

The cycle at which the fluorescence signal significantly exceeds a chosen threshold line above the backround fluorescence is defined as the cycle of threshold (Ct) or crossing point (Cp). This "Ct value" is inversely correlated to the logarithm of the initial DNA copy number [176].

If it is of interest to determine the exact copy number in an unknown sample, real-time PCR provides the possibility for quantification, e.g. with the help of a standard curve established by analysing serially diluted extracts with exactly known DNA copy number or concentration. In addition, the relative standard curve method can be applied, based on the use of two independent standard curves (reference DNA sequence and target DNA sequence). A percentage value for the target relative to the reference gene sequence can be calculated [177].

The quality of a real-time PCR assay is determined by the amplification efficiency (E), which indicates the rate of PCR amplicons generated. The efficiency can be calculated from the slope of the standard curve using following equation: $E\ [\%] = (10^{(-1/slope)} - 1) \times 100$. A slope of a regression line of -3.32 corresponds an amplification efficiency of 100%, indicating that in each cycle the template doubles in quantity. To obtain accurate and reproducible results, reactions should have efficiencies as close to 100% as possible [176].

In order to develop a real-time PCR assay with high amplification efficiency, the design of appropriate primers and a probe for the specific amplification of a DNA sequence can be a challenging task. After selecting a species specific DNA sequence, e.g. from the NCBI database, and exclusion of sequence similarity with other species, e.g. with the basic local alignment search tool (BLAST) algorithm, sequence specific primers and a probe can be designed using a primer and probe design software. In general, to obtain high amplification efficiencies, the

amplicon (target) length spanned by the primers should be kept relatively short (between 70 and 150 base pairs (bp)). Each primer should be between 15 and 30 bp long and show a content of guanidine and cytosine (GC) in a range between 35% and 65%. The melting temperature (T_m) of primer forward and primer reverse should be similar and in the range from 45-65 °C. Self-complementary sequences should be avoided, since the formation of hairpins or primer dimers can drastically reduce the efficiency of the amplification reaction. TaqMan probes (as described above) should consist of 20-30 bp, ideally with a balanced GC ratio and a T_m value of about 8-10 °C higher than the T_m value of the primers [176].

The use of various reporter dyes in TaqMan probes, which emit fluorescence signals at different wavelengths, offers the potential of simultaneous amplification of more than one DNA sequence in a single experiment (multiplexing). The big challenge in developing a mulitplex real-time PCR assay is to design compatible primers and probes that do not form primer and/or probe dimers but achieve high amplification efficiencies for each template under the same conditions, independent of the concentration of the others. However, the advantages of applying a multiplex real-time PCR system are saving time and costs, needing lower amount of sample DNA and reducing the probability of cross-contamination [178].

Under pure conditions (without matrix), PCR makes it possible to detect very low amounts of DNA (5-50 copies). However, the limit of detection is strongly influenced by the integrity and quality of the extracted DNA and the presence of polymerase inhibiting compounds [165, 179].

1.4.2.4. ELISA versus real-time PCR

Careful selection of primers or antibodies allow both techniques (DNA-based as well as antibody-based methods) developing specific assays to determine food allergens. Currently, ELISAs and PCR assays play the most important role in the detection of food allergens. Both methods have strengths but also limitations.

In general, the development of DNA-based assays seems to be easier since databases for sequence information and software for primer and probe design can be used. The development of immunoassays can be more difficult, because the target of interest is often less characterised and the production as well as further characterisation of antibodies can take a lot of time and costs. Another advantage of PCR methods over ELISAs is, that the PCR

chemistry can be purchased anytime in a constant quality, whereas the availability and quality of antibodies can be limited, especially if polyclonal antibodies are applied. Both PCR assays and ELISAs offer the advantage to be very selective. The selectivity of PCR assays critically depends on the quality of primer and probe design, that of ELISAs on the cross-reactivity of the antibodies. In practice, ELISAs frequently show a higher degree of cross-reactivity due to epitope homology of proteins from different species.

In general, ELISAs have been found to be more sensitive than PCR assays. In products that naturally contain low DNA amounts such as egg or milk, ELISAs are more applicable than PCR methods. Sometimes pure, DNA free protein extracts are added to foodstuffs for process related reasons. In such products food allergens can only be detected by the use of protein-based methods like ELISAs. In general, ELISAs are less labour intensive and involve less expensive equipment than PCR assays.

Both analytical techniques can be affected negatively by the presence of matrix compounds or food processing, because the sensitivity of both assays strongly depends on the purity, integrity and stability of the isolated protein or DNA.

If it is necessary, both methods allow to quantify the target of interest. In the field of food allergen analysis quantification is not required, due to the lack of safe threshold doses for allergens in foods, as mentioned before. However, quantitative information may be of great interest, since the occurrence and severity of allergic reactions clearly depend on the allergen content of the offending food and the amount consumed.

In addition, both techniques have the potential for multiple allergen detection in one reaction, but in contrast to multiplex real-time PCR assays, no multiplex ELISA has been published in this field.

Although a lot of DNA-based methods exist as additional or complementary method, they will most probably not replace the protein-based methods which are the official screening methods for allergen detection in foods. Laboratories have to choose a certain analysis format depending on the concerned food, the food matrix, the availability of specific tests, the performance characteristics of the tests or the time required to obtain analysis results [180].

1.4.2.5. Analysis of mustard in food - state of the art

1.4.2.5.1. ELISAs

In 2007, Koppelman et al., developed a competitive ELISA method for the detection of mustard proteins in highly refined mustard seed oil [181]. The polyclonal antibodies from rabbits were raised against a mixture of *Brassica juncea* proteins from mustard seeds, commercially used to produce mustard seed oil. The limit of detection (LOD) of the competitive ELISA was 1.5 mg/kg mustard protein in mustard seed oil. Minor cross-reactivity was obtained with milk (0.28%), egg yolk (0.20%) and soy (0.016%). They demonstrated that steam-distilled mustard oil, which is used in very low quantities as a flavouring with a powerful sensory impact, does not contain substantial amounts of mustard protein and thus will not elicit an allergic reaction in mustard-allergic individuals.

Shim and Wanasundara, 2008, developed a sandwich ELISA using rabbit polyclonal antibodies for the specific detection and quantification of Sin a 1 from white (yellow) mustard (*Sinapis alba* L.). The polyclonal antibodies showed 100% reactivity with Sin a 1 proteins, however, they also cross-reacted with *Brassica napus* napin (NAP, proteins from rapeseed) to 30.3% and *Arabidopsis thaliana* (thale cress) NAP to 2.1%. The sandwich ELISA has a LOD of 0.3 µg/mL for purified Sin a 1 and allows the detection of 20 µg Sin a 1/g yellow mustard meal [182].

Lee et al., 2008, developed two sandwich ELISAs, one based on rabbit antibodies and the other one on sheep antibodies, for the detection of mustard (white and brown) with limits of quantification (LOQs) of 1 and 3 µg of ground, whole mustard seeds/mL, respectively. Both ELISAs showed cross-reactivity with rapeseed, which has to be considered especially when detecting mustard in foods that may come into contact with rapeseed. The mean recovery in model sausages containing mustard flour in a range from 0 to 1000 mg/kg was found to be 95.3% ± 10.7%. The applicability of the assays was shown by analysing 29 foodstuffs [183].

In 2009, Lee et al., investigated the effects of acidity on the detectability of mustard in salad dressings using the two previously developed mustard ELISAs. They found out that acid precipitation of mustard proteins probably renders them insoluble and non-extractable. Thus, the acidic salad dressing matrices lowered the detectability of mustard [184].

In 2011, Cuhra et al., published the results obtained by validating a commercially available ELISA in an inter-laboratory study performed in 12 laboratories. The sandwich ELISA, based on

polyclonal antibodies, has a LOD of 0.5 mg mustard protein/kg food and a LOQ of 1.8 mg mustard protein/kg food [185].

1.4.2.5.2. PCR assays

In 2008, Mustorp et al., presented a real-time PCR method detecting all three mustard species (white, black and brown mustard). However, the determination of mustard is not specific, because the primers and the probe target a sequence of the Sin a 1 gene that is homologous in many other *Brassica* species. Cross-reactivity with broccoli, rapeseed, swede, turnip, Chinese cabbage, cabbage as well as radish was obtained. A standard curve was established by analysing serially diluted DNA from brown mustard. The slope of the curve was -3.41, corresponding to an amplification efficiency of 96%, and the square regression coefficient (R^2) was 0.994. The method allows the detection of mustard down to 0.005% (w/w), corresponding to 50 mg/kg level in spiked barbecue spice and wheat flour [186].
In our working group, Magdalena Fuchs et al., 2010, developed a real-time PCR method for white mustard. The method was carefully validated with regard to important analytical parameters. It was found to be specific and does not cross-react with 67 species tested, including 12 *Brassica* species. In raw model sausages, the limit of detection was found to be 0.001% or 10 mg white mustard/kg food. The applicability of the method was demonstrated by analysing 20 commercial foodstuffs [187].

1.4.2.6. Analysis of celery – state of the art

1.4.2.6.1. ELISAs

To date, no antibody based method that allows the specific detection of celery has been published. Most probably the problem is to produce antibodies that do not show cross-reactivity to other food species especially to other *Apiaceae* members.

1.4.2.6.2. PCR assays

Several PCR methods for the determination of celery have been published. Stephan et al., 2004, presented a real-time PCR method to determine celery root in washing water to monitor industrial cleaning procedures for contamination with celery allergens. The

assay targets the Api g 1 gene from celery and has a LOD between 0.6 and 1.5 mg celery mush (10-30 µg protein)/mL washing water. Since the method showed cross-reactivity with some spices (not specified), the authors do not recommend to use this method for analysis of celery in food products [188]. Mustorp et al., argued that FASTA search for the Api g 1 gene revealed homology to carrot genes and might probably lead to cross-reaction between celery and carrot [186].

Dovičovičová et al., 2004, developed a conventional PCR method based on gel electrophoresis for the detection of celery. The primers targeted a 279 bp sequence of the celery specific mannitol dehydrogenase gene. The assay is selective for four celery varieties and does not show cross-reaction to other plant species. The limit of detection of 0.1% (w/w, or 1000 mg/kg) in meat pâtés is a magnitude above the range recommended by the scientific community [72]. Therefore, the method is not sensitive enough to determine traces of celery in foods [189].

In 2007, Hupfer et al., presented a real-time PCR method for the specific and sensitive detection of celery DNA in food, amplifying a 101 bp fragment within the mannitol dehydrogenase gene. The PCR method was found to be specific for celery by testing more than 50 food relevant species. The limit of detection of 5 to 10 mg/kg ground celery seeds in emulsion-type sausages demonstrates the sensitivity of the method in food matrix [190].

Mustorp et al., 2008, also presented a real-time PCR assay for the detection and quantification of celery in food, targeting a 151 bp fragment within the mannitol dehydrogenase gene. The method was found to be specific and does not cross-react with other tested species. The slope of the standard curve was -3.49 with a R^2 of 0.999, indicating an amplification efficiency of 93%. The authors emphasised, as in their previous paper, that the real-time PCR assay can be used to obtain quantitative results [186].

In 2011, our research group developed and validated a real-time PCR method for the detection of celery in food. The method targets a sequence of the *Apium graveolens* NADPH-dependent mannose-6-phosphate reductase. It allows the detection of three celery varieties (celery roots, celery stalks and leaf celery) without showing cross-reaction with 64 biological species, including ten members of the *Apiaceae*. The LOD, determined by analysing serially diluted celery extracts, was found to be 10 pg celery DNA for all three celery varieties. The method was validated by analysing model sausages containing celery roots. Three different extraction methods were applied, a commercial kit, the CTAB and the Wizard protocol. The DNA

extraction method had an influence on the extraction yield and on the number of false negative results when analysing model sausages spiked with lower levels of celery. The limit of detection determined by analysing celery spiked model sausages was found to be 0.005% (w/w) or 50 mg/kg [191].

1.4.2.7. Multiplex analysis of mustard and celery

In 2011, Mustorp et al., developed a multiplex quantitative ligation dependent probe amplification (MLPA) method to simultaneously determine eight allergenic foods: sesame, soy, hazelnut, peanut, lupine, gluten, mustard and celery. The ligated probes were amplified by PCR, and the amplicons were detected by capillary electrophoresis. The method was validated by analysing different foods spiked with mustard, celery, soy or lupine flour in the range from 1-0.001% (corresponding to 10000-10 mg/kg). However, since the ligation probes for mustard were designed in the vicinity of the PCR primer/probe binding sites that were previously published by Mustorp et al., 2008, cross-reactivity with radish, broccoli, swede, Chinese cabbage and cabbage was obtained [180].

Köppel et al., 2012, presented two hexaplex quantitative real-time PCR systems, which have been developed from earlier published tetraplex systems [192], in order to detect and quantify DNA from twelve allergenic foods. The first system allows determining cashew, peanut, hazelnut, celery, soy and mustard DNA, the second one milk (beef), egg (chicken), almonds, sesame, pistachio and walnut. The two systems showed LOD of 0.1% for each of the analytes. However, since the primers and probe for the detection of mustard were taken from the single PCR assay published by Mustorp et al., 2008, the mustard system also detects other *Brassicaceae*, e.g. broccoli, radish, cabbage and horse radish [193].

In 2013, our group presented a duplex real-time PCR method allowing the simultaneous detection of celery and white mustard [194]. The duplex assay does not show cross-reactivity with 64 different biological species, including members of the *Brassicaceae* and *Apiaceae* family. When analysing brewed model sausages, spiked with white mustard and celery roots, the limit of detection was found to be 0.005% or 50 mg/kg white mustard and celery. The duplex real-time assay was applied to verify correct mustard and celery labelling of commercial food products.

Very recently, Luber et al., published a tetraplex real-time PCR method for the simultaneous determination of soy bean, celery, white mustard and brown mustard. The tetraplex assay is based on two single real-time PCR assays developed in our group, the singleplex assay for white mustard [187] and a singleplex assay for black and brown mustard developed in course of the present doctoral thesis (see Chapter 3.1.). The limit of quantification was found to be 8.5 mg/kg for soy bean, 2.6 mg/kg for brown mustard, 3.7 mg/kg for celery and 36.8 mg/kg for white mustard. The applicability of the tetraplex assay was demonstrated by analysing foods with various labelling concerning the analytes of interest [160].

2. AIM OF THE DOCTORAL THESIS

In central Europe, in particular in France and Switzerland, allergies to mustard and celery are rather prevalent. Since to date therapies of food allergies are still under research, the only option for concerned persons is to strictly avoid the consumption of food they are allergic to. In order to protect these individuals, the declaration of allergenic foods on commercial foodstuffs is mandatory in many countries. Within the European Union, thirteen potentially allergenic foods, including mustard and celery, have to be declared (Directive 2007/68/EC). Analytical methods are needed to verify if food products containing mustard and/or celery comply with the EU labelling directive. In order to be applicable in routine analysis, these analytical methods should be selective, sensitive and suitable for high throughput analysis.

Several real-time PCR methods for the detection of mustard or celery have already been published. However, most methods for mustard are not specific and show cross-reactivity with other members of the *Brassicaceae*. Recently, our research group developed and validated a singleplex real-time PCR method for the specific detection of white mustard and another one for the determination of celery.

Since not only white mustard but also two further mustard species, black and brown mustard, are widely present in foods, the first aim of the doctoral thesis was the development and validation of a singleplex real-time PCR method allowing the detection of black and/or brown mustard. The novel real-time PCR assay for black/brown mustard should then be combined with the real-time PCR assay for white mustard in order to establish a duplex real-time PCR assay allowing the determination of all three mustard species simultaneously in one PCR tube. Since the simultaneous detection of all three mustard species and celery would allow saving time and costs, the novel duplex real-time PCR assay for mustard should then be aligned with the singleplex real-time assay for celery to obtain a triplex real-time PCR assay for mustard and celery. The singleplex assay for black/brown mustard, the duplex assay for the three mustard species and the triplex assay for mustard and celery should be validated with regard to important analytical parameters, e.g. selectivity, sensitivity, accuracy and the applicability to raw and processed foods. Another aim of the doctoral thesis was to compare the performance of the mustard real-time PCR methods with that of commercially available mustard ELISA kits. In addition, the multiplex real-time PCR assays for mustard and celery should be applied to verify correct labelling of commercial food products.

3. RESULTS

Paper 1 (Chapter 3.1.)

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

Monika Palle-Reisch, Martina Wolny, Margit Cichna-Markl, Rupert Hochegger

Food Chemistry 138 (2013) 348-355

Paper 2 (Chapter 3.2.)

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 food

Monika Palle-Reisch, Margit Cichna-Markl, Rupert Hochegger

Food Chemistry 153 (2014) 66-73

Paper 3 (Chapter 3.3.)

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 food

Monika Palle-Reisch, Margit Cichna-Markl, Rupert Hochegger

Food Chemistry 153 (2014) 66-73

Paper 4 (Chapter 3.4.)

Validation and comparison of two commercial ELISA kits and three in-house developed real-time PCR assays for the detection of potentially allergenic mustard in food

Monika Palle-Reisch, Rupert Hochegger, Stephan Štumr, Kveta Korycanova, Margit Cichna-Markl

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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 food

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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.

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 (Figueroa et al., 2005; Jorro, Morales, Brasó, & Peláez, 1995; Kanny, Fremont, Talhouarne, & Nicolas, 1995; Malet, Valero, Lluch, 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 le 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, & Štumr, 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 75–78 °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, °C, °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 [ng/μL] = A_{260} *x* 50 *x 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 μL, consisting of 12.5 μL of IQ Sybr Green Supermix (BioRad), the primer forward and primer reverse in the optimised concentration, 5 μL of template DNA (20 ng/μL) and H_2O_{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 μL 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 μL of DNA extract (20 ng/μL) and H_2O_{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	
AJ415649	11f	GTT GAG CCG AGG GTC ATA ATT TC	59.8	76
	11r	TCG ACT TAG GCA TCC TTA CGG	58.0	
	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	*Brassica nigra*	+	+	+	+	+	+	+	+	+
Brown mustard	*Brassica juncea*	+	+	+	+	+	+	+	+	+
White mustard	*Sinapis alba*	+	+[1]	+[1]	+	+[1]	+	+	+	+
Anise	*Pimpinella anisum*	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	–	n.a.
Beetroot	*Beta vulgaris ssp. vulgaris*	n.a.	+	+[1]	+	+[1]	+	+	±	+
Broccoli	*Brassica oleracea var. silvestris*	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.
Caraway	*Carum carvi*	n.a.	n.a.	+[1]	n.a.	+[1]	n.a.	n.a.	–	n.a.
Cauliflower	*Brassica oleracea var. botrytis*	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.
Celery	*Apium graveolens var. secalinum*	+[1]	+	+[1]	+[1]	+	n.a.	n.a.	–	n.a.
Celery root	*Apium graveolens var. rapaceum*	n.a.	n.a.	+[1]	n.a.	+	n.a.	n.a.	±	n.a.
Chinese cabbage	*Brassica rapa ssp. pekinensis*	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	+	n.a.
Chive	*Allium schoenoprasum*	n.a.	n.a.	n.a.	n.a.	+[1]	n.a.	n.a.	–	n.a.
Coriander	*Coriandrum sativum*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.
Cumin	*Cuminum cyminum*	n.a.	n.a.	+[1]	n.a.	+[1]	n.a.	n.a.	±	n.a.
Dill	*Anethum graveolens*	n.a.	n.a.	+[1]	n.a.	+[1]	n.a.	n.a.	–	n.a.
Ginger	*Zingiber officinale*	n.a.	n.a.	+	n.a.	n.a.	n.a.	n.a.	–	n.a.
Kohlrabi	*Brassica oleracea var. gongylodes*	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.
Lovage	*Levisticum officinale*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	±	n.a.
Marjoram	*Origanum majorana*	+	+[1]	+[1]	+	+[1]	n.a.	n.a.	–	n.a.
Nutmeg	*Myristicia fragrans*	n.a.	n.a.	+[1]	n.a.	n.a.	n.a.	n.a.	–	n.a.
Oregano	*Origanum vulgare*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–	n.a.
Pak choi	*Brassica rapa chinensis*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	n.a.
Parsley	*Petroselinum crispum*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	+
Rapeseed	*Brassica napus*	+[1]	+	+[1]	+[1]	+	+	+	–	+
Summer savoury	*Satureja hortensis*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	n.a.
Tarragon	*Artemisia dracunculus*	n.a.	n.a.	n.a.	n.a.	+	n.a.	n.a.	+	n.a.
Thyme	*Thymus vulgaris*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	n.a.
Turmeric	*Curcuma longa/domestica*	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 $\leqslant$40).
± Positive/negative PCR result.
n.a. not analysed.
[1] Ct value $\leqslant$40 but the melting temperature (Tm) differed $\geqslant$1 °C from the Tm 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	11
Black mustard	*Brassica nigra*	+	+	18.10
Brown mustard	*Brassica juncea*	+	+	16.95
White mustard	*Sinapis alba*	+	+	34.47
Celery	*Apium graveolens var. secalinum*	n.a.	+	39.67
Cinnamon	*Cinnamomum zeylanicum*	n.a.	+[2]	37.44
Cumin	*Cuminum cyminum*	n.a.	+	29.98
Fenugreek	*Trigonella foenum-graecum*	n.a.	+	29.92
Flaxseed	*Linum usitatissimum*	n.a.	±	37.78 /–
Ginger	*Zingiber officinale*	n.a.	+	32.27
Rice	*Oryza sativa*	n.a.	±	37.30 /–
Rye	*Secale cereale*	n.a.	+	35.89
Turmeric	*Curcuma longa/domestica*	n.a.	+	37.51
Allspice	*Pimenta dioica*	n.a.	–	–
Almond	*Prunus dulcis*	n.a.	–	–
Anise	*Pimpinella anisum*	n.a.	–	–
Apple	*Malus domesticus*	n.a.	–	–
Barley	*Hordeum vulgare*	n.a.	–	–
Bay leaf	*Laurus nobilis*	n.a.	–	–
Bean	*Phaseolus vulgaris*	n.a.	–	–
Beef	*Bos taurus*	n.a.	–	–
Beet	*Brassica rapa ssp. rapa*	–[1]	–	–
Beetroot	*Beta vulgaris ssp. vulgaris*	n.a.	–	–
Black pepper	*Piper nigrum*	n.a.	–	–
Brazil nut	*Bertholletia excelsa*	n.a.	–	–
Buckwheat	*Fagopyrum esculentum*	n.a.	–	–
Broccoli	*Brassica oleracea var. silvestris*	–[1]	–	–
Caraway	*Carum carvi*	n.a.	–	–
Cardamom	*Ellettaria cardamomum*	n.a.	–	–
Carrot	*Daucus carota*	n.a.	–	–
Cauliflower	*Brassica oleracea var. botrytis*	–[1]	–	–
Celery root	*Apium graveolens var. rapaceum*	n.a.	–	–
Celery stalks	*Apium graveolens var. dulce*	n.a.	–	–
Chicken	*Gallus gallus*	n.a.	–	–
Chilli	*Capsicum sp.*	n.a.	–	–
Chinese cabbage	*Brassica rapa ssp. pekinensis*	–[1]	–	–
Chive	*Allium schoenoprasum*	n.a.	–	–
Coriander	*Coriandrum sativum*	n.a.	–	–
Cucumber	*Cucumis sativus*	n.a.	–	–
Dill	*Anethum graveolens*	n.a.	–	–
Fennel	*Foeniculum vulgare*	n.a.	–	–
Garlic	*Allium sativum*	n.a.	–	–
Horse	*Equus ferus*	n.a.	–	–
Horseradish	*Armoracia rusticana*	n.a.	–	–
Kohlrabi	*Brassica oleracea var. gongylodes*	–[1]	–	–
Leek	*Allium porrum*	n.a.	–	–
Lentil	*Lens culinaris*	n.a.	–	–
Lovage	*Levisticum officinale*	n.a.	–	–
Maize	*Zea mays*	n.a.	–	–
Marjoram	*Origanum majorana*	n.a.	–	–
Nutmeg	*Myristicia fragrans*	n.a.	–	–
Oat	*Avena sativa*	n.a.	–	–
Onion	*Allium cepa*	n.a.	–	–
Oregano	*Origanum vulgare*	n.a.	–	–
Pak choi	*Brassica rapa chinensis*	–[1]	–	–
Paprika	*Capsicum annum*	n.a.	–	–
Parsley	*Petroselinum crispum*	n.a.	–	–
Parsnip	*Pastinaca sativa*	n.a.	–	–
Pea	*Pisum sativum*	n.a.	–	–
Pork	*Sus scrofa*	n.a.	–	–
Porso millet	*Panicum miliaceum*	n.a.	–	–
Potato	*Solanum tuberosum*	n.a.	–	–
Radish	*Raphanus sativus*	n.a.	–	–
Rapeseed	*Brassica napus*	–[1]	–	–
Rosemary	*Rosmarinus officinalis*	n.a.	–	–
Sage	*Salvia officinalis*	n.a.	–	–
Sesame	*Sesamum indicum*	n.a.	–	–
Sheep	*Ovis orientalis aries*	n.a.	–	–
Soy	*Glycine max*	n.a.	–	–
Spelt	*Triticum aestivum ssp. spelta*	n.a.	–	–
Summer savoury	*Satureja hortensis*	n.a.	–	–
Tarragon	*Artemisia dracunculus*	n.a.	–	–
Thyme	*Thymus vulgaris*	n.a.	–	–
Tomato	*Solanum lycopersicum*	n.a.	–	–

(*continued on next page*)

Table 2b (*continued*)

Name	Botanical name	Increase of fluorescence signal		Mean Ct value
		10	11	11
Turkey	*Meleagris gallopavo*	n.a.	–	–
Wheat	*Triticum durum*	n.a.	–	–
White cabbage	*Brassica oleracea var. capitata f. alba*	–[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/μL, 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 μL using the Rotor Gene Q equipped with a 72-well rotor. Reactions were carried out with 12.5 μL TaqMan Universal PCR Master Mix, 300 nmol L^{-1} forward primer, 300 nmol L^{-1} reverse primer, 50 nmol L^{-1} probe, 5 μL of DNA extract and H_2O_{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: $E\,[\%] = [10^{(-1/(slope))} - 1]\ x\ 100$.

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-

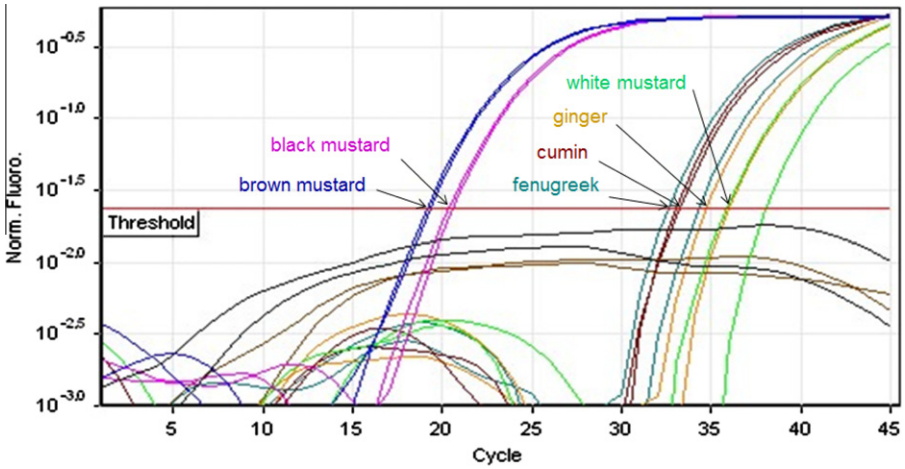

Fig. 1. Amplification curves obtained with cross-reacting species. DNA amount: 10 ng per tube.

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

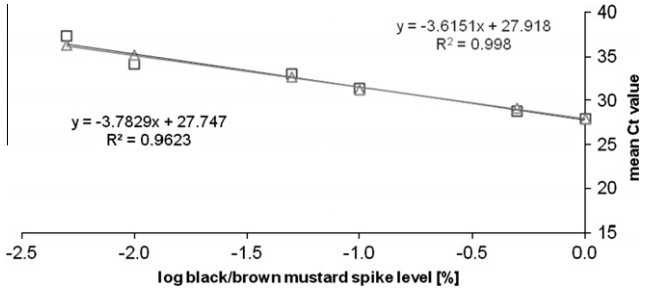

Fig. 2a. Standard curves obtained by amplifying DNA extracted from raw model sausages. DNA amount: 1 ng per tube. □ sausages spiked with black mustard, Δ sausages spiked with brown mustard.

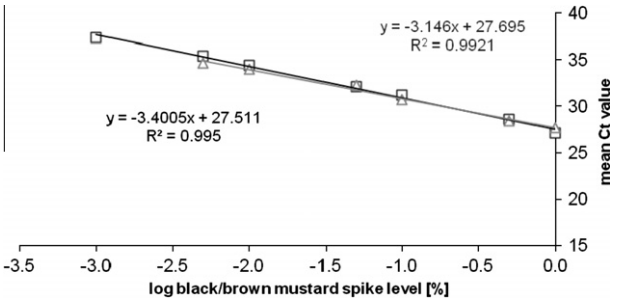

Fig. 2b. Standard curves obtained by amplifying DNA extracted from brewed model sausages. DNA amount: 1 ng per tube. □ sausages spiked with black mustard, Δ sausages spiked with brown mustard.

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	*Brassica nigra*	18.10	20.50	21.55	24.54
Brown mustard	*Brassica juncea*	16.95	19.24	19.68	22.74
White mustard	*Sinapis alba*	34.47	36.97	36.35	39.39
Cinnamon	*Cinnamomum zeylanicum*	n.a.	37.44[1]	–	–
Cumin	*Cuminum cyminum*	29.98	33.10	34.17	–
Fenugreek	*Trigonella foenum-graecum*	29.92	33.44	34.92	38.57
Ginger	*Zingiber officinale*	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			
0.0005	5	35.28	34.21	35.25	1.06	2.99	36.40	35.18	36.02	1.54	4.26
		35.29	34.38				37.38	36.24			
		35.39	36.19				38.07	34.24			
		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			
0.0001	1	–	36.75	37.65	1.14	3.03	–	–	37.16	1.39	3.73
		–	39.07				36.79	–			
		–	–				38.02	–			
		37.17	37.26				35.78	36.17			
		37.62	36.51				39.11	–			
		–	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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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 food

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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 $\geqslant$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.

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 le 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, & Hochegger, 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, & Hochegger, 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), ethylenedinitrilotetraacetic 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 75–78 °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 [ng/μL] = $A_{260} \times 50 \times$ 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 ± 10 nm, detection 510 ± 5 nm), that of YAK (yakima yellow) in the yellow channel (excitation 530 ± 5 nm, detection 557 ± 5 nm). An increase of the fluorescence signal within 40 cycles (Ct 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	*Brassica nigra*	18.37	<LOD
Brown mustard	*Brassica juncea*	16.60	<LOD
White mustard	*Sinapis alba*	31.90	21.20
Cumin	*Cuminum cyminum*	30.28	<LOD
Fenugreek	*Trigonella foenum-graecum*	31.93	<LOD
Ginger	*Zingiber officinale*	35.05	<LOD[a]
Rye	*Secale cereale*	39.14	<LOD
Turmeric	*Curcuma longa/domestica*	37.12	<LOD
Beet	*Brassica rapa ssp. rapa*	<LOD	<LOD
Broccoli	*Brassica oleracea var. silvestris*	<LOD	<LOD
Cauliflower	*Brassica oleracea var. botrytis*	<LOD	<LOD
Celery	*Apium graveolens var. secalinum*	<LOD	<LOD
Chinese cabbage	*Brassica rapa ssp. pekinensis*	<LOD	<LOD
Cinnamon	*Cinnamomum zeylanicum*	<LOD[b]	<LOD[b]
Cod	*Gadus morhua*	<LOD	<LOD
Flaxseed	*Linum usitatissimum*	<LOD	<LOD
Horseradish	*Armoracia rusticana*	<LOD	<LOD
Kohlrabi	*Brassica oleracea var. gongylodes*	<LOD	<LOD
Pak choi	*Brassica rapa chinensis*	<LOD	<LOD
Plaice	*Pleuronectes platessa*	<LOD	<LOD
Radish	*Raphanus sativus*	<LOD	<LOD
Rapeseed	*Brassica napus*	<LOD	<LOD
Rice	*Oryza sativa*	<LOD	<LOD
Salmon	*Salmo salar*	<LOD	<LOD
White cabbage	*Brassica oleracea var. capitata f. alba*	<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

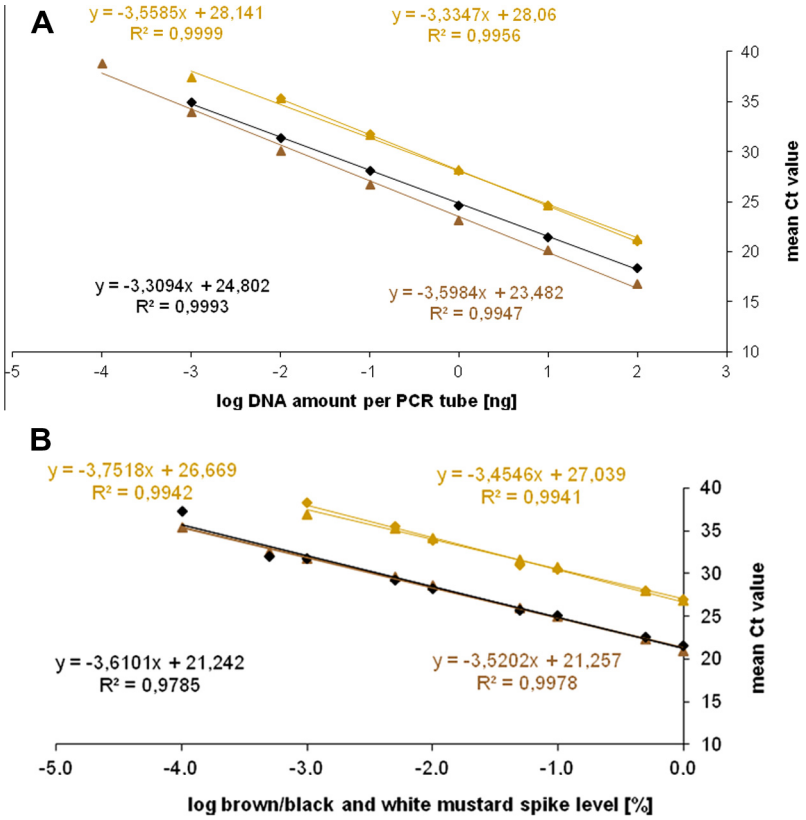

Fig. 1. Standard curves obtained by (A) amplifying serially diluted DNA extracts and (B) amplifying DNA extracted from brewed model sausages. (A) 100 ng DNA amount per mustard species per tube. ◇ 1:1 mixture of DNA extracts from black and white mustard, Δ 1:1 mixture of DNA extracts from brown and white mustard. (B) 100 ng DNA amount per tube. ◇ model sausage set 1, spiking level 1–0.0001% (w/w) per mustard species, Δ 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										
	Black mustard					*White mustard*				
10	31.54	32.08	31.63	0.39	1.23	35.65	37.70	37.36	0.94	2.52
	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			
	31.42	31.16				<LOD	36.93			
5	32.96	32.87	32.94	0.42	1.28	36.85	38.08	37.10	0.85	2.29
	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										
	Brown mustard					*White mustard*				
10	32.11	31.59	31.64	0.33	1.05	38.16	38.96	37.65	1.06	2.81
	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			
	31.44	31.46				37.96	37.97			
5	32.66	32.82	32.80	0.41	1.25	<LOD	37.72	<LOD	-	-
	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 $\geqslant$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											
		Black mustard					*White mustard*				
10	500	29.34	29.17	29.38	0.14	0.49	35.26	34.86	35.06	0.48	1.36
		29.29	29.66				35.11	35.96			
		29.45	29.33				34.67	34.93			
		29.49	29.33				35.47	35.39			
		29.24	29.49				34.43	34.51			
	200	30.58	30.94	30.57	0.27	0.88	36.66	35.52	36.34	0.64	1.75
		30.87	30.56				36.48	37.09			
		30.37	30.48				36.51	35.52			
		30.56	29.98				36.45	35.67			
		30.72	30.59				37.19	<LOD			
5	500	30.72	30.31	30.51	0.18	0.59	35.33	35.70	35.75	0.46	1.28
		30.46	30.65				35.88	36.12			
		30.18	30.58				35.06	36.26			
		30.43	30.47				36.20	36.26			
		30.75	30.55				35.24	35.43			
	200	31.71	31.08	31.74	0.43	1.35	37.45	38.89	<LOD	-	-
		31.52	31.46				<LOD	36.88			
		31.31	32.43				<LOD	36.61			
		31.82	31.81				<LOD	<LOD			
		32.35	31.93				37.65	36.94			
1	500	34.82	32.68	33.06	0.73	2.21	37.02	<LOD	<LOD	-	-
		32.41	32.97				38.14	<LOD			
		32.24	32.74				36.64	<LOD			
		32.90	33.12				37.88	36.81			
		33.63	33.06				<LOD	38.36			
	200	34.64	34.14	34.02	0.65	1.92	<LOD	<LOD	<LOD	-	-
		34.24	35.19				<LOD	<LOD			
		33.38	33.29				<LOD	38.00			
		33.68	33.13				<LOD	<LOD			
		34.44	34.08				<LOD	<LOD			
Model sausage set 2											
		Brown mustard					*White mustard*				
10	500	29.42	29.64	29.43	0.24	0.82	34.90	35.41	34.90	0.81	2.32
		29.09	29.74				34.44	34.79			
		29.15	29.50				33.87	33.83			
		29.21	29.33				35.50	35.02			
		29.42	29.78				34.64	36.56			
	200	30.80	30.62	30.67	0.19	0.62	35.42	37.94	36.67	0.87	2.37
		30.37	30.67				<LOD	35.75			
		30.67	30.89				36.30	<LOD			
		31.02	30.57				36.29	37.33			
		30.54	30.55				37.10	37.26			
5	500	30.40	30.33	30.19	0.26	0.85	36.04	35.25	36.15	0.57	1.57
		30.17	29.73				36.26	35.87			
		29.93	30.38				35.93	36.04			
		30.62	30.23				36.14	36.31			
		30.10	30.05				37.53	36.13			
	200	31.72	31.51	31.56	0.25	0.80	36.57	35.29	36.54	0.85	2.34
		31.40	31.86				37.20	<LOD			
		31.96	31.22				<LOD	35.98			
		31.65	31.36				<LOD	37.95			
		31.63	31.25				36.56	36.25			
1	500	32.11	32.27	32.68	0.44	1.36	37.28	36.68	<LOD	-	-
		32.75	32.66				38.05	<LOD			
		32.07	32.44				<LOD	38.02			
		32.96	33.26				<LOD	36.94			
		33.22	33.06				38.47	<LOD			
	200	33.96	33.69	34.22	0.72	2.12	38.01	36.47	<LOD	-	-
		34.76	35.38				<LOD	<LOD			
		34.12	33.33				<LOD	<LOD			
		33.21	34.68				37.86	38.14			
		35.03	34.00				<LOD	37.04			

<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/μL. 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 $\geqslant$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.

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Development and validation of a triplex real-time PCR assay for the simultaneous detection of three mustard species and three celery varieties in food

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ABSTRACT

The paper presents a triplex real-time PCR assay allowing the simultaneous detection of three mustard species (white, black and brown mustard) and three celery varieties (celery roots, celery stalks and leaf celery) in foodstuffs. The triplex assay does not show cross-reactivity with other *Brassicaceae*. Low cross-reactivities were observed with fenugreek, cumin, ginger, caraway, turmeric, lovage and rye, the ΔCt values were, however, ⩾12 compared to positive controls. The triplex assay allows the detection of traces of DNA of the allergenic components in spite of an excess of the other DNA templates. Analysis of extracts from model sausages containing defined concentrations of mustard and celery showed that the triplex assay is applicable to both raw and processed foods. It was found to allow the detection of 1 ppm black/brown mustard and 50 ppm white mustard and celery in raw and brewed sausages with a probability ⩾95%.

1. Introduction

In spite of an extensive discussion of food allergies in the mass media the general public is not aware of the fact that in Europe mustard and celery allergies are prominent among the spectrum of allergies detected by epidemiologists. Especially in France and Switzerland, where mustard and celery are consumed very frequently, allergies to mustard and celery are rather prevalent. Mustard allergy accounts for about 1–7% of food allergies in France. About 30–40% of food allergic patients living in France or Switzerland are sensitised to celery (EFSA, 2004).

Commercial food products may contain three mustard species belonging to the plant family *Brassicaceae*, white mustard (*Sinapis alba*), black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*). Three varieties of celery, a member of the *Apiaceae* family, play a role in food industry: celery roots (*Apium graveolens var. rapaceum*), celery stalks (*Apium graveolens var. dulce*) and leaf celery (*Apium graveolens var. secalinum*).

It has been shown that allergic reactions to white mustard are mainly triggered by a number of allergenic proteins: a 2S seed storage albumin (Sin a 1) (Menéndez-Arias, Moneo, Domínguez, & Rodríguez, 1988), an 11S seed storage 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). Allergic symptoms to brown mustard are elicited by a 2S seed storage albumin (Bra j 1) structurally similar to Sin a 1 (Gonzales de la Peña, Menéndez-Arias, Monsalve, & Rodríguez, 1991).

To date, the following allergenic proteins have been identified in celery: a protein homologous to Bet v 1 (Api g 1) (Breiteneder et al., 1995) and two isoforms (Api g 1.01 and Api g 1.02) (Wangorsch, Ballmer-Weber, Rosch, Holzhauser, & Vieths, 2007), a non-specific lipid-transfer protein (Api g 2) (Gadermaier et al., 2011), a profilin similar to Bet v 2 (Api g 4) (Scheurer, Wangorsch, Haustein, & Vieths, 2000) and a glycoprotein (Api g 5) (Ganglberger et al., 2000), in previous studies described as cross-reactive carbohydrate determinants (CCD) (Fötisch, Altmann, Haustein, & Vieths, 1999).

Several studies indicate that food processing does not affect allergenicity of mustard and celery proteins or decreases allergenicity only to a minor extent because of their rather high resistance to heat and digestive enzymes (Ballmer-Weber et al., 2002; Jankiewicz et al., 1997; Sirvent, Palomares, Cuesta-Herranz, Villalba, & Rodríguez, 2012).

Both mustard and celery have been reported to potentially cause anaphylaxis, which is, in addition to other symptoms, characterised by a rapid drop of blood pressure that can be life-threatening. Persons sensitised to mustard and/or celery are

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therefore well advised to strictly avoid being exposed. In order to help excluding mustard and celery from the diet, both foods have to be indicated on the ingredient list of prepacked foodstuffs in many countries including the member states of the European Union (Commission of the European Union., 2007).

Analytical methods suitable to verify correct labelling of allergenic ingredients have to be selective to avoid false positive results. To avoid false negative results, the methods must be sensitive and applicable to raw and highly processed foods. Since foodstuffs, e.g. instant noodles, sauces and sausages, may contain both mustard and celery, a method allowing the simultaneous detection of white, black and brown mustard, celery roots, celery stalks and leaf celery would help saving time and costs and lowering the risk of cross-contamination.

Mustorp et al. published a multiplex quantitative ligation dependent probe amplification method for the detection of eight allergenic foods including mustard and celery (Mustorp, Drømtorp, & Holck, 2011). Köppel et al. presented a hexaplex real-time PCR method allowing the simultaneous detection of hazelnut, peanut, celery, soy, mustard and cashew (Köppel, van Velsen-Zimmerli, & Bucher, 2012). However, in both multiplex assays the mustard system is not specific for mustard but also detects other members of the *Brassicaceae* family, e.g. broccoli, radish and cabbage. Recently, our group developed and validated two duplex real-time PCR assays, one for the simultaneous detection of white mustard and celery (Fuchs, Cichna-Markl, & Hochegger, 2013), the other one for the simultaneous detection of white, black and brown mustard (Palle-Reisch, Cichna-Markl, & Hochegger, 2014). The duplex assay for white mustard and celery does not show cross-reactivity with 64 biological species, including various members of the *Brassicaceae* and *Apiaceae* family. The duplex assay for the simultaneous detection of white, black and brown mustard does not detect other *Brassicaceae*, but it shows low cross-reactivities with cinnamon, cumin, fenugreek, ginger, rye and turmeric. Cross-reactivity does, however, not influence the accuracy of the assay since the Ct (threshold cycle) values obtained for cinnamon, cumin, fenugreek, ginger, rye and turmeric were significantly higher than those obtained for black and brown mustard.

Very recently, Luber et al. published a tetraplex real-time PCR method for the simultaneous determination of soy bean, celery, white mustard and brown mustard (Luber, Demmel, Pankofer, Busch, & Engel, 2015). The tetraplex assay is based on two singleplex real-time PCR assays developed in our group, a singleplex assay for white mustard (Fuchs, Cichna-Markl, & Hochegger, 2010) and a singleplex assay for black and brown mustard (Palle-Reisch, Wolny, Cichna-Markl, & Hochegger, 2013).

In the present study we aimed at combining the primers and probes previously used in singleplex (Fuchs, Cichna-Markl, & Hochegger, 2012; Fuchs et al., 2010; Palle-Reisch et al., 2013) and duplex assays (Fuchs et al., 2013; Palle-Reisch et al., 2014) to a triplex real-time PCR assay in order to be able to simultaneously detect white, black and brown mustard, celery roots, celery stalks and leaf celery. The triplex assay should then be validated with regard to selectivity, limit of detection (LOD) and its applicability to detect the three mustard species and celery varieties in raw and processed foods.

2. Materials and methods

2.1. Chemicals and foods

N-Cetyl-N,N,N-trimethylammoniumbromide (CTAB), ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA), phenol/chloroform/iso-amylalcohol 25/24/1 (v/v/v) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Life Sciences (Buchs, Switzerland). Proteinase K, chloroform, isoamyl-alcohol, 2-propanol, ethanol, hydrochloric acid and sodium chloride were delivered from Merck (Darmstadt, Germany). RNase, α-amylase and herring sperm DNA were purchased from Roche (Mannheim, Germany). Ultrapure water (Millipore, Synergy®, Merck, Darmstadt, Germany) was used for extracting DNA and for carrying out PCR reactions. Seeds from white, black and brown mustard, celery roots, celery stalks and leaf celery, pork meat, table salt and foodstuffs were bought at local merchants or supermarkets. Dried spices were provided by Kotányi (Wolkersdorf, Austria).

2.2. Production and brewing of model sausages

For method validation, two sets of model sausages with defined concentrations of mustard and celery as well as a control sausage free of mustard and celery were produced as described previously (Palle-Reisch et al., 2013). Model sausages of set 1 contained 10,000, 5000, 1000, 500, 100, 50, 10, 5 or 1 mg/kg of each white mustard, black mustard and celery roots, sausages of set 2 10,000, 5000, 1000, 500, 100, 50, 10, 5 or 1 mg/kg of each white mustard, brown mustard and celery roots. Aliquots of 3 g were filled in 50 mL beakers and brewed for 15 min at 75–78 °C in a water bath (GFL, Burgwedel, Germany) equipped with an alarm thermometer (Testo GmbH, Vienna, Austria). The brewed sausages were directly used for DNA extraction. Raw sausages were stored at −20 °C.

2.3. DNA extraction

DNA was isolated from plant species, model sausages and commercial food products with the CTAB method according to the protocol described previously (Fuchs et al., 2010). In brief, 3.0 g of the homogenised sample was mixed with 10 mL CTAB extraction buffer, 80 μL α-amylase and 18 μL proteinase K solution and incubated (50 °C, overnight). After centrifugation (4000 rpm, 15 min, model 5810 R, Eppendorf, Vienna, Austria) 1 mL of the supernatant was mixed with 600 μL chloroform/isoamylalcohol (24/1, v/v) and centrifuged (13,200 rpm, 10 min, model 5415 R, Eppendorf). 660 μL of the aqueous supernatant was blended with 1320 μL precipitation solution and incubated (room temperature, 60 min). After a further centrifugation step, the precipitate was dissolved by adding 450 μL 1.2 M NaCl solution, 50 μL 10-fold RNase buffer and 5 μL RNase and incubating (56 °C, 10 min). After cooling, the solution was mixed with 500 μL phenol/chloroform/isoamyl-alcohol (25/24/1, v/v/v) and centrifuged. Next, 400 μL of the aqueous supernatant was mixed with 400 μL isopropanol, incubated (−20 °C, 60 min) and centrifuged. The obtained DNA precipitate was washed with 70% ethanol solution. The dried DNA pellet was dissolved in 100 μL H_2O_{dd} (56 °C, 20 min). The DNA extracts were stored at −20 °C.

After measuring the absorbance of the DNA extracts at 260 nm (A_{260}) and 280 nm (A_{280}) with a nano photometer (Implen, Munich, Germany), the concentration was calculated according to the following equation: c [ng/μL] = $A_{260} \times 50 \times$ dilution factor. The ratio of A_{260}/A_{280} was calculated to assess the purity of the extracts.

2.4. Mixtures of DNA extracts

For method validation, a series of mixtures of DNA extracts from the three mustard species and celery varieties were prepared. The composition of the DNA mixtures is given in Table 1.

2.5. Real-time PCR analysis

Real-time PCR was performed with a Rotor-Gene Q instrument using 0.1 mL strip tubes with caps in a 72-well rotor (Qiagen,

Table 1
Composition of DNA mixtures used for validation [ng/PCR tube].

Mixture	White mustard DNA	Black mustard DNA	Brown mustard DNA	Celery roots DNA	Celery stalks DNA	Leaf celery DNA
A	100	100	–	100	–	–
B	100	–	100	100		
C	100	50	50	–	100	–
D	100	50	50	–	–	100
E	95	1.25	1.25	2.5	–	–
F	2.5	47.5	47.5	2.5	–	–
G	2.5	1.25	1.25	95	–	–
H	90	2.5	2.5	5	–	–
I	5	45	45	5	–	–
J	5	2.5	2.5	90	–	–
K	75	6.25	6.25	12.5	–	–
L	12.5	37.5	37.5	12.5	–	–
M	12.5	6.25	6.25	75	–	–
N	45	22.5	22.5	10	–	–
O	10	22.5	22.5	45	–	–
P	45	5	5	45	–	–

Hilden, Germany). White mustard was detected in the green channel (excitation 470 ± 10 nm, detection 510 ± 5 nm), black and brown mustard in the yellow channel (excitation 530 ± 5 nm, detection 557 ± 5 nm) and celery in the red channel (excitation 625 ± 5 nm, detection 660 ± 10 nm). An increase of the fluorescence signal within 40 cycles (Ct value < 40) was considered as a positive result. All DNA extracts, the positive and the no template control were analysed in duplicate.

2.5.1. Primers and probes

The sequences of the primers and probes (white mustard: primer forward$_w$, primer reverse$_w$, probe$_w$; black/brown mustard: primer forward$_{b/b}$, primer reverse$_{b/b}$, probe$_{b/b}$; celery: primer forward$_c$, primer reverse$_c$, probe$_c$) were published recently (Fuchs et al., 2010, 2012; Palle-Reisch et al., 2013). Probe$_w$ was labelled with FAM and TAMRA, probe$_{b/b}$ with YAK and BHQ1 and probe$_c$ with Cy5 and BHQ2. All primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany), with the exception of probe$_c$ which was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.5.2. Optimisation of the triplex real-time PCR assay

The triplex real-time PCR assay was optimised by analysing serially diluted mixtures of DNA extracts from white mustard, black mustard and celery roots (Table 1, mixture A) and white mustard, brown mustard and celery roots (Table 1, mixture B). The DNA concentration of each species ranged from 20 ng/μL to 0.002 pg/μL, which corresponds to 100 ng to 0.01 pg total DNA amount per PCR tube.

Two Master mixes, 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), were tested. 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 temperature program was: 2 min at 50 °C, 10 or 15 min at 95 °C, 45 cycles of 15 s at 95 °C and 45 s or 75 s at 60 °C.

2.5.3. Optimised triplex real-time PCR assay

Each PCR tube was filled with 12.5 μL QTM Mix, 0.2 μM primer forward$_w$, 0.3 μM primer reverse$_w$, 0.15 μM probe$_w$, 0.2 μM primer forward$_{b/b}$, 0.2 μM primer reverse$_{b/b}$, 0.05 μM probe$_{b/b}$, 0.2 μM primer forward$_c$, 0.3 μM primer reverse$_c$, 0.05 μM probe$_c$, H_2O_{dd} and 5 μL DNA extract (20 ng/μL or 40 ng/μL). The total reaction volume was 25 μL. 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 60 °C.

2.5.4. Cross-reactivity experiments

Cross-reactivity experiments were carried out with DNA extracts from members of the *Brassicaceae* and *Apiaceae* family and other plant species commonly used for food production. The DNA extracts were adjusted to 20 ng/μL, corresponding to 100 ng total DNA amount per PCR tube.

2.5.5. Limit of detection, amplification efficiency and reliability

The limit of detection (LOD) of the triplex real-time PCR assay and the amplification efficiency (E) for each allergenic component were determined by analysing serially diluted mixtures of DNA extracts from the three mustard species and celery varieties as well as by analysing DNA extracts from model sausages.

DNA mixtures A–D (Table 1) were serially diluted with water to obtain mixtures containing from 20 ng DNA/μL to 0.002 pg DNA/μL (corresponding to 100 ng–0.01 pg DNA per PCR tube) of white mustard, black and/or brown mustard, and celery roots or celery stalks or leaf celery.

DNA mixtures E–P were serially diluted down to 2 pg/μL with a 20 ng/μL herring sperm DNA solution in order to keep the total DNA amount constant.

DNA extracts (40 ng DNA/μL) were prepared from raw and brewed model sausages containing 10,000–1 mg/kg of each white mustard, black mustard and celery (set 1) or white mustard, brown mustard and celery (set 2).

The amplification efficiencies were calculated from the slopes of the standard curves as follows: $E\,[\%] = [10^{(-1/(\text{slope}))} - 1] \times 100$.

To evaluate the reliability of the triplex assay near the LODs, DNA extracts (40 ng DNA/μL) from raw and brewed model sausages containing 1, 5, 10 or 50 mg/kg of each allergenic component were analysed in 20 replicates.

Inhibition control reactions contained 5 μL DNA extract (40 ng/μL) from the negatively tested food sample, 12.5 μL QTM Mix, 0.2 μM primer forward$_w$, 0.3 μM primer reverse$_w$, 0.15 μM probe$_w$, 0.2 μM primer forward$_{b/b}$, 0.2 μM primer reverse$_{b/b}$, 0.05 μM probe$_{b/b}$, 0.2 μM primer forward$_c$, 0.3 μM primer reverse$_c$, 0.05 μM probe$_c$, 5 μL of the positive control (mixture of DNA extracts containing 20 ng/μL of each white mustard, brown mustard and celery root DNA) and H_2O_{dd}. The reactions were carried out in duplicates.

3. Results and discussion

3.1. Development and optimisation of the triplex real-time PCR assay

The triplex real-time PCR assay was developed by combining primers and probes from recently published singleplex real-time PCR assays for white mustard (Fuchs et al., 2010), black/brown mustard (Palle-Reisch et al., 2013) and celery (Fuchs et al., 2012), which were already successfully applied in a duplex assay for celery and white mustard (Fuchs et al., 2013) and a duplex assay for white mustard and black/brown mustard (Palle-Reisch et al., 2014). Although in primer and probe design we had paid attention to avoid complementarity in order to lower the probability of formation of heterodimers, we had to test the compatibility of the primers and probes in practice.

First the compatibility of primers and probes for black/brown mustard and celery was tested by analysing serially diluted mixtures of DNA extracts from black or brown mustard and celery roots (1:1, 20 ng/μL to 0.002 pg/μL of each species). Amplification was carried out by using 0.3 μM of primer forward (f), f$_{b/b}$, 0.3 μM of primer reverse (r), r$_{b/b}$, 0.05 μM of probe$_{b/b}$ (as in the singleplex assay for black/brown mustard (Palle-Reisch et al., 2013)),

0.2 μM of primer f_c, 0.3 μM of primer r_c, 0.05 μM of $probe_c$ (as in the singleplex assay for celery (Fuchs et al., 2012)) and the TaqMan Univeral PCR Master Mix (TMU Mix). Amplification efficiencies were 100.0% for black mustard, 98.3% for brown mustard and 91.3% for celery. Black mustard and brown mustard DNA could be detected down to a concentration of 0.2 and 0.02 pg/μL (corresponding to 1 and 0.1 pg total DNA amount per PCR tube), respectively, and celery DNA down to 20 pg/μL (100 pg DNA per PCR tube). Since efficiencies and LODs were similar to those obtained with the singleplex assays we assumed that the primers and probes for black/brown mustard and celery are compatible.

In order to test the compatibility of all three primer pairs and probes, serially diluted mixtures (mixtures A and B, Table 1) of DNA extracts from white mustard, black or brown mustard and celery roots were analysed. The DNA concentration of each allergenic component was in the range from 20 ng/μL to 0.002 pg/μL. Experiments were carried out with 0.2 μM primer f_w, 0.3 μM primer r_w, 0.15 μM $probe_w$ (as in the singleplex assay for white mustard (Fuchs et al., 2010)), 0.2 μM primer $f_{b/b}$, 0.2 μM primer $r_{b/b}$, 0.05 μM $probe_{b/b}$ (as in the duplex assay for white mustard and black/brown mustard (Palle-Reisch et al., 2014)), 0.2 μM primer f_c, 0.3 μM primer r_c, 0.05 μM $probe_c$ (as in the singleplex assay for celery (Fuchs et al., 2012)) and 5 μL DNA extract mixture. In order to achieve high amplification efficiencies, a PCR kit particularly recommended for multiplexing, the QuantiTect Multiplex RT-PCR NR Kit (QTM Mix), was used. The following PCR protocol was applied: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s 95 °C and 45 s at 60 °C. Amplification efficiencies were found to be 92.2% for white mustard, 95.7% for black mustard, 101.4% for brown mustard and 97.9% for celery. Applying the QTM Mix instead of the TMU Mix hardly affected the Ct values for black/brown mustard (20 ng DNA/μL, mean Ct: 17.65/16.16 (QTM Mix), 18.30/17.34 (TMU Mix)) but led to significantly lower Ct values in case of celery (20 ng DNA/μL, mean Ct: 21.63 (QTM Mix), 24.20 (TMU Mix)). Similar effects had been observed for white mustard (mean Ct: 21.04, QTM Mix) when developing the duplex assay for white mustard and black/brown mustard (20 ng DNA/μL, mean Ct value: 21.31 (QTM Mix), 23.83 (TMU Mix) (Palle-Reisch et al., 2014)). Modifying the temperature program by extending the annealing time from 45 s to 75 s (as recommended in the manual of the QTM Kit) did not affect the amplification efficiencies for white mustard (93.3%), black mustard (97.7%) and celery roots (98.5%). In contrast, elongating the initial denaturation time from 10 to 15 min (as recommended by the supplier of the QTM Kit) or varying the concentration of the primers or probe for celery (0.2 μM primer f_c, 0.2 μM primer r_c, 0.05 μM $probe_c$; 0.3 μM primer f_c, 0.3 μM primer r_c, 0.05 μM $probe_c$) resulted in higher Ct values and thus lower amplification in case of celery DNA. Thus, all further experiments were carried out with the QTM Mix applying the conditions given in Section 2.5.3.

3.2. Cross-reactivity tests

The triplex real-time PCR assay allows the detection of white mustard (green channel, mean Ct value: 20.91), black and brown mustard (yellow channel, mean Ct values: 17.75 and 16.29, respectively) as well as the three celery varieties (celery roots, celery

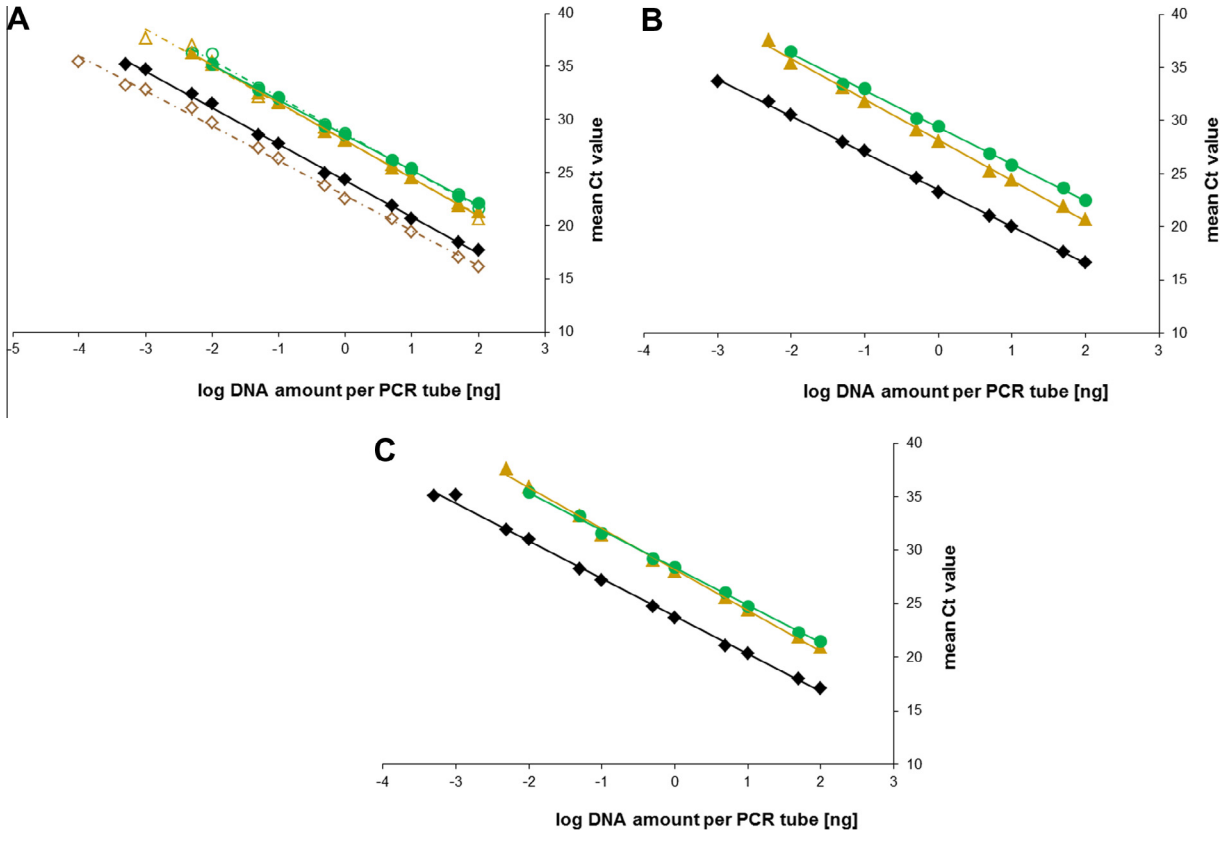

Fig. 1. Standard curves obtained by analysing serially diluted mixtures of DNA extracts. The Ct values are means of two replicates. (A) DNA mixture A: ▲ white mustard: $R^2 = 0.9986$, $y = -3.5488x + 28.039$, $E = 91.3\%$, ♦ black mustard: $R^2 = 0.9985$, $y = -3.4298x + 24.256$, $E = 95.7\%$ and ● celery roots: $R^2 = 0.9989$, $y = -3.3007x + 28.551$, $E = 100.9\%$, DNA mixture B: △ white mustard: $R^2 = 0.9941$, $y = -3.5031x + 28.017$, $E = 93.0\%$, ◇ brown mustard: $R^2 = 0.9973$, $y = -3.2882x + 22.819$, $E = 101.4\%$ and ○ celery roots: $R^2 = 0.9975$, $y = -3.4533x + 28.647$, $E = 94.8\%$, (B) DNA mixture C: ▲ white mustard: $R^2 = 0.9972$, $y = -3.8035x + 28.173$, $E = 83.2\%$, ♦ black/brown mustard: $R^2 = 0.9992$, $y = -3.4736x + 23.5$, $E = 94.0\%$ and ● celery stalks: $R^2 = 0.9980$, $y = -3.4554x + 29.359$, $E = 94.7\%$, (C) DNA mixture D: ▲ white mustard: $R^2 = 0.9963$, $y = -3.8179x + 28.21$, $E = 82.8\%$, ♦ black mustard/brown: $R^2 = 0.9974$, $y = -3.5295x + 23.826$, $E = 92.0\%$ and ● leaf celery: $R^2 = 0.9986$, $y = -3.5044x + 28.362$, $E = 92.9\%$.

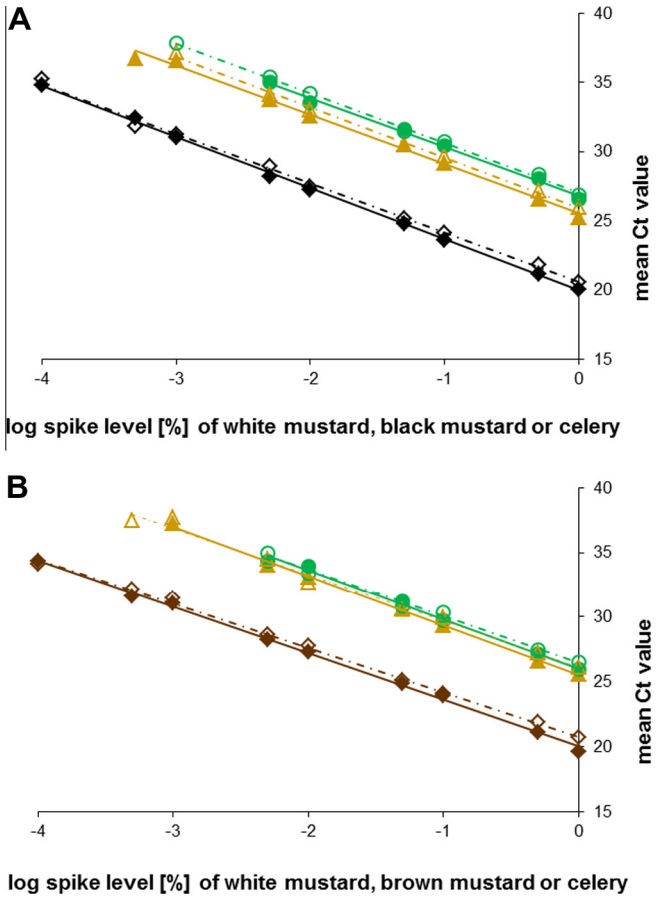

Fig. 2. Standard curves obtained by analysing DNA extracts (40 ng/μL) from raw and brewed model sausages containing from 10,000 to 1 mg/kg of each potentially allergenic component. The Ct values are means of two replicates. (A) Model sausages of set 1: raw: △ white mustard: $R^2 = 0.9976$, $y = -3.6434x + 25.938$, $E = 88.1\%$, ◇ black mustard: $R^2 = 0.9974$, $y = -3.559x + 20.573$, $E = 91.0\%$ and ○ celery roots: $R^2 = 0.9984$, $y = -3.6078x + 27.017$, $E = 89.3\%$. Model sausages of set 1: brewed: ▲ white mustard: $R^2 = 0.9947$, $y = -3.5458x + 25.549$, $E = 91.4\%$, ♦ black mustard: $R^2 = 0.9990$, $y = -3.6987x + 19.965$, $E = 86.4\%$ and ● celery roots: $R^2 = 0.9951$, $y = -3.5327x + 26.821$, $E = 91.9\%$. (B) Model sausages of set 2: raw: △ white mustard: $R^2 = 0.9885$, $y = -3.5912x + 26.113$, $E = 89.9\%$, ◇ brown mustard: $R^2 = 0.9980$, $y = -3.4237x + 20.775$, $E = 95.9\%$ and ○ celery roots: $R^2 = 0.9941$, $y = -3.6077x + 26.433$, $E = 89.3\%$. Model sausages of set 2: brewed: ▲ white mustard: $R^2 = 0.9983$, $y = -3.8277x + 25.515$, $E = 82.5\%$, ♦ brown mustard: $R^2 = 0.9979$, $y = -3.5946x + 20.021$, $E = 89.8\%$ and ● celery roots: $R^2 = 0.9936$, $y = -3.7958x + 26.022$, $E = 83.4\%$.

stalks and leaf celery; red channel, mean Ct values: 22.15, 22.10 and 21.49, respectively).

Although the selectivity of the primers and probes had already been investigated in the singleplex and duplex assays published previously (Fuchs et al., 2010, 2012, 2013; Palle-Reisch et al., 2013, 2014), DNA extracts from ten common *Brassicaceae* species (broccoli, cauliflower, Chinese cabbage, horseradish, kohlrabi, pak choi, radish, rapeseed, turnip, white cabbage), ten members of the *Apiaceae* (anise, caraway, carrot, coriander, cumin, dill, fennel, lovage, parsley, parsnip) and further plant species (apple, black pepper, cardamom, cinnamon, fenugreek, flaxseed, ginger, marjoram, oregano, rice, rye, sage, summer savory, tarragon, turmeric) were tested for cross-reactivity. No cross-reactivity was observed for the ten *Brassicaceae* species tested. Ginger (mean Ct value: 36.10) resulted in an increase of the fluorescence signal in the green channel. In the yellow channel fenugreek (mean Ct value: 29.83), cumin (mean Ct value: 30.36), ginger (mean Ct value: 32.33), caraway (mean Ct value: 35.09), turmeric (mean Ct value: 36.17), lovage (mean Ct value: 37.43) and rye (mean Ct value: 39.45) yielded Ct values <40. Cross-reactivities with these species have already been observed in the singleplex assay for black/brown mustard and the duplex assay for white and black/brown mustard (Palle-Reisch et al., 2013, 2014). Since the Ct values were at least 12 cycles higher than those obtained for the positive controls (white, black and brown mustard) and these plant species commonly occur only in traces in mustard containing foodstuffs, the possibility of causing a false positive result is very low. Thus in our opinion these low cross-reactivities do not limit the applicability of the triplex real-time PCR assay in practice.

3.3. LOD, amplification efficiency and reliability

In order to determine the limits of detection (LODs) and the amplification efficiencies (E) of the triplex real-time PCR assay, serially diluted mixtures of DNA extracts from the three mustard species and celery varieties were analysed. The standard curves obtained by plotting the Ct values against the logarithm of the initial DNA amount are shown in Fig. 1. When analysing serially diluted mixtures A and B (Table 1) LODs were found to be 1 and 0.2 pg/μL (5 and 1 pg DNA per PCR tube) for white mustard DNA, 0.1 pg/μL (0.5 pg DNA per PCR tube) for black mustard DNA, 0.02 pg/μL (0.1 pg DNA per PCR tube) for brown mustard DNA and 2 and 1 pg/μL (10 and 5 pg DNA per PCR tube) for celery roots DNA, respectively (Fig. 1A). The slopes of the standard curves were in the range from −3.2882 to −3.5488, indicating amplification efficiencies of 91.3% and 93.0% for white mustard DNA, 95.7% for black mustard DNA, 101.4% for brown mustard DNA and 100.9% and 94.8% for celery roots DNA. Satisfying LODs and amplification efficiencies were also obtained when mixtures containing DNA from white mustard, black/brown mustard and celery stalks or leaf celery (DNA mixtures C and D, Table 1) were analysed. Celery stalks DNA (Fig. 1B) and leaf celery DNA (Fig. 1C) could be detected down to 2 pg/μL (corresponding to 10 pg DNA per PCR tube). Amplification efficiencies for celery stalks and leaf celery DNA were found to be 94.7% and 92.9%, respectively. The LODs and amplification efficiencies achieved with the triplex real-time PCR assay are close to those obtained with the singleplex real-time PCR assays (white mustard assay: LOD: 1 pg DNA/μL, $E = 91.8\%$ (Fuchs et al., 2010); black/brown mustard assay: LOD: 0.02 pg DNA/μL, $E = 100.6\%/86.2\%$ (Palle-Reisch et al., 2013); celery assay: LOD: 2 pg DNA/μL, E (celery roots) = 93.9%, E (celery stalks) = 92.9%, E (leaf celery) = 87.4% (Fuchs et al., 2012)).

In order to investigate if the triplex real-time PCR assay also allows efficient amplification when the DNA templates are present in different ratios, DNA mixtures E–P (Table 1) were analysed. These mixtures were serially diluted down to 2 pg DNA/μL of each allergenic component with a 20 ng/μL herring sperm DNA solution in order to keep the total DNA amount per PCR tube constant. In general, satisfying amplification efficiencies were obtained for all templates independent if they were present in excess or in deficiency. Analysis of serial dilutions of DNA mixture E, for example, resulted in amplification efficiencies of 94.2% for white mustard, 100.0% for black/brown mustard and 114.5% for celery roots. For serially diluted DNA mixture G amplification efficiencies for white mustard, black/brown mustard and celery roots were found to be 88.8%, 85.0% and 91.6%, respectively.

In another series of experiments the LODs and amplification efficiencies of the triplex assay were investigated by analysing two sets of model sausages containing defined amounts (10,000, 5000, 1000, 500, 100, 50, 10, 5, 1 mg/kg) of either white mustard, black mustard or celery roots (model sausage set 1) or white mustard, brown mustard or celery roots (model sausage set 2). In order to investigate any influence of food processing on the detectability

Table 2
Reliability of the triplex real-time PCR assay near the LOD. DNA extracts from raw and brewed model sausages, set 1 (A) and set 2 (B), were analysed in 20 replicates, applying 200 ng total DNA amount per PCR tube.

(A)

Spike level (mg/kg)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)
Model sausage set 1	White mustard					Black mustard					Celery				
Raw															
50	34.52	34.71	34.09	0.54	1.57	28.97	28.82	28.89	0.19	0.66	34.61	36.08	35.10	0.80	2.29
	33.78	33.76				28.74	28.69				35.78	33.88			
	34.70	33.95				28.79	29.05				–	35.24			
	34.54	34.13				28.90	29.02				34.93	35.33			
	33.42	33.70				28.95	29.43				35.23	35.88			
	33.62	34.20				29.07	28.98				35.70	34.85			
	33.63	33.76				29.02	28.69				34.04	34.86			
	35.53	33.82				28.75	28.97				33.92	34.83			
	33.43	33.95				28.54	28.77				36.19	34.78			
	34.07	34.67				28.91	28.76				36.66	34.13			
10	39.11	37.58	36.83	1.08	2.93	30.54	31.81	31.40	0.42	1.32	–	–	n.c.	n.c.	n.c.
	37.05	38.75				32.50	31.46				–	36.05			
	36.38	35.60				31.39	31.29				36.68	36.88			
	37.04	36.76				31.87	31.02				–	37.14			
	36.92	36.37				31.93	31.20				–	36.73			
	–	–				31.11	31.39				37.83	37.32			
	35.08	36.14				31.12	31.61				–	–			
	35.83	35.57				31.54	31.10				–	–			
	37.40	–				31.05	31.44				36.96	36.92			
	37.69	36.88				31.24	31.37				37.42	37.43			
5	36.89	–	n.c.	n.c.	n.c.	31.98	32.71	32.44	0.34	1.06	36.08	–	n.c.	n.c.	n.c.
	–	37.34				32.06	33.02				36.21	–			
	36.95	–				32.59	32.28				37.10	–			
	37.43	37.50				31.68	32.30				37.22	–			
	–	–				32.65	32.43				–	36.24			
	–	36.42				32.80	32.48				36.13	–			
	37.87	36.53				32.63	31.98				–	–			
	35.41	–				33.02	32.43				36.10	36.31			
	36.14	–				32.26	32.35				–	–			
	–	37.71				32.55	32.53				36.76	36.07			
1	–	–	n.c.	n.c.	n.c.	32.85	35.59	35.04	1.53	4.37	–	–	n.c.	n.c.	n.c.
	38.30	–				34.03	–				–	–			
	–	–				34.97	38.41				–	36.35			
	38.04	36.55				35.06	35.05				–	–			
	–	37.50				36.00	34.24				–	–			
	–	–				34.20	35.04				–	–			
	37.02	–				33.39	35.25				–	–			
	37.01	–				39.23	34.61				–	–			
	–	–				34.96	33.90				36.10	–			
	36.83	–				34.51	34.39				–	–			
Brewed															
50	33.56	32.99	34.11	0.70	2.06	28.17	28.33	28.36	0.14	0.49	35.76	34.44	34.84	0.93	2.67
	33.63	34.86				28.32	28.26				35.12	35.36			
	34.14	34.76				28.26	28.53				34.66	34.27			
	36.20	34.48				28.40	28.53				35.22	34.98			
	33.56	34.64				28.61	28.58				34.42	34.41			
	33.87	33.27				28.27	28.38				33.53	37.45			
	34.53	33.79				28.23	28.28				36.28	34.06			
	33.63	33.75				28.05	28.40				35.39	33.49			
	34.17	34.16				28.39	28.33				34.53	34.49			
	34.29	33.91				28.47	28.31				34.87	34.01			
10	36.64	36.25	36.68	0.90	2.45	31.29	31.35	31.11	0.26	0.84	–	–	n.c.	n.c.	n.c.
	36.01	36.90				31.03	30.82				–	37.23			
	38.00	37.66				31.11	31.50				–	–			
	36.63	37.90				31.01	31.16				35.31	–			
	37.58	35.75				30.96	30.78				–	–			
	–	35.21				31.28	31.04				36.92	–			
	36.83	36.07				31.23	31.05				–	–			
	36.22	36.91				31.26	30.60				36.46	–			
	37.98	35.74				31.30	31.69				–	37.53			
	37.46	35.26				30.81	30.94				–	–			
5	–	–	n.c.	n.c.	n.c.	32.07	31.89	32.16	0.44	1.38	36.71	–	n.c.	n.c.	n.c.
	36.19	36.98				33.23	32.04				–	–			
	37.75	–				32.33	32.04				–	–			
	37.63	–				32.65	32.32				–	–			
	36.47	–				31.81	32.37				37.20	–			
	39.13	37.76				31.72	31.69				–	–			

(*continued on next page*)

Table 2 (*continued*)

(A)

Spike level (mg/kg)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)
Model sausage set 1	White mustard					Black mustard					Celery				
	36.78	–				31.41	32.63				–	–			
	37.77	–				32.50	32.05				–	38.33			
	–	36.76				32.07	31.90				–	–			
	–	38.10				32.78	31.65				–	–			
1	38.15	–	n.c.	n.c.	n.c.	33.52	34.85	34.73	0.79	2.27	–	–	n.c.	n.c.	n.c.
	–	37.78				34.62	35.15				–	–			
	–	–				36.60	33.97				–	–			
	–	–				34.61	34.66				–	–			
	–	37.41				35.18	34.85				–	–			
	–	–				34.14	33.22				–	36.68			
	–	–				34.65	35.05				–	–			
	–	–				34.54	35.08				–	–			
	38.04	–				34.91	34.03				–	–			
	–	–				34.66	36.27				–	–			

(B)

Spike level (mg/kg)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)
Model sausage set 2	White mustard					Brown mustard					Celery				
Raw															
50	34.28	34.17	34.48	0.63	1.84	29.13	29.25	28.98	0.20	0.70	34.98	34.25	34.61	0.68	1.97
	34.71	34.00				29.28	28.52				34.70	33.91			
	35.03	34.47				28.98	29.08				34.35	34.07			
	33.97	34.27				29.11	28.97				33.99	34.95			
	34.01	34.02				28.93	29.09				33.06	35.86			
	35.45	34.36				28.64	28.93				34.37	33.82			
	34.42	33.75				28.88	29.32				35.05	35.34			
	34.05	36.31				28.72	28.93				35.63	34.94			
	35.04	35.12				29.02	28.97				34.29	35.33			
	34.28	33.85				28.95	28.80				34.55	34.78			
10	37.48	37.24	36.82	1.01	2.73	31.91	32.01	31.55	0.39	1.22	–	35.87	36.33	0.83	2.30
	35.83	35.26				31.28	30.98				35.79	35.63			
	36.42	35.82				31.63	32.00				35.35	35.69			
	36.36	–				31.35	30.97				36.07	36.30			
	38.28	36.10				31.27	31.66				–	35.22			
	37.31	36.69				31.08	31.53				37.29	36.96			
	36.66	38.41				32.00	31.56				–	36.09			
	38.64	38.09				31.51	31.90				–	36.05			
	37.11	35.68				31.72	31.00				37.08	38.29			
	36.04	36.14				31.42	32.27				36.36	37.29			
5	37.12	–	37.49	0.81	2.17	32.09	32.39	32.35	0.33	1.02	–	–	n.c.	n.c.	n.c.
	–	–				32.79	31.89				–	–			
	39.55	38.10				32.29	32.33				–	36.89			
	36.47	37.42				32.52	32.75				–	37.49			
	37.62	–				32.67	31.79				35.85	37.34			
	–	37.98				32.14	32.10				37.74	–			
	37.63	37.10				32.21	32.10				–	–			
	37.63	–				32.86	32.37				–	–			
	36.43	36.90				32.21	33.05				36.67	37.11			
	38.14	36.80				32.14	32.37				36.71	–			
1	–	–	n.c.	n.c.	n.c.	34.05	33.82	34.87	0.79	2.28	–	–	n.c.	n.c.	n.c.
	–	–				34.08	34.80				–	–			
	–	–				35.03	36.44				–	37.09			
	–	–				36.22	35.84				–	–			
	–	–				35.43	35.27				36.94	–			
	–	–				33.87	33.95				–	–			
	37.74	–				34.35	34.80				–	–			
	–	–				34.62	35.42				–	–			
	37.98	–				35.71	34.64				–	–			
	–	–				34.96	34.10				–	–			
Brewed															
50	33.90	33.99	34.37	0.56	1.64	28.11	27.89	28.26	0.16	0.58	35.23	33.67	34.39	0.69	2.00
	33.43	35.69				28.26	28.55				35.41	34.54			
	34.10	35.19				28.41	28.14				33.81	34.38			
	34.84	33.98				28.26	28.27				34.85	33.98			
	34.15	33.74				28.26	28.43				33.25	35.87			
	33.96	34.82				28.26	28.08				34.78	34.03			
	33.79	34.18				28.15	28.07				34.28	34.68			
	34.96	34.56				28.31	28.48				34.58	34.50			

Table 2 (*continued*)

(B)															
Spike level (mg/kg)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)	Ct value		Mean Ct value	*S*	RSD (%)
Model sausage set 2	White mustard					Brown mustard					Celery				
	34.12	34.50				28.16	28.47				33.55	33.92			
	34.76	34.75				28.40	28.30				33.48	34.91			
10	35.80	37.43	37.10	1.24	3.34	30.61	30.66	30.85	0.29	0.95	35.80	37.22	36.42	0.73	2.00
	35.38	–				30.95	31.15				36.31	–			
	39.11	39.02				30.78	30.67				–	36.95			
	37.62	36.68				30.71	31.54				37.13	–			
	38.05	–				30.81	30.52				36.34	35.81			
	36.99	39.69				31.01	31.21				37.08	37.05			
	36.16	37.24				30.51	30.99				36.91	–			
	35.81	36.01				31.06	30.87				35.29	–			
	37.27	36.23				30.72	31.24				–	37.08			
	36.09	37.18				30.65	30.36				35.71	35.23			
5	–	38.37	n.c.	n.c.	n.c.	31.89	31.26	31.68	0.62	1.96	–	–	n.c.	n.c.	n.c.
	38.71	–				31.39	32.11				–	39.57			
	–	36.85				31.07	31.71				37.15	36.98			
	–	–				31.17	31.54				–	–			
	36.39	37.20				31.85	31.98				35.13	36.81			
	–	35.72				32.73	31.18				37.06	35.55			
	38.13	37.73				31.73	32.12				–	–			
	36.84	–				31.66	31.60				–	–			
	–	–				31.71	32.99				35.48	–			
	35.94	34.94				31.96	30.03				37.12	–			
1	–	–	n.c.	n.c.	n.c.	34.07	34.53	34.28	0.77	2.24	–	36.73	n.c.	n.c.	n.c.
	–	–				33.40	34.01				–	–			
	–	–				34.47	33.60				–	–			
	–	37.07				33.64	35.48				–	37.29			
	–	–				33.87	33.92				–	–			
	–	–				33.64	34.18				–	36.19			
	–	37.47				36.11	35.41				–	36.78			
	–	–				34.31	34.92				–	–			
	–	–				33.70	34.78				–	–			
	36.93	–				34.46	33.03				–	–			

–: no increase of the fluorescence signal within 40 cycles.
n.c.: not calculated.

of mustard and celery, not only DNA extracts from raw but also from brewed sausages were analysed. The DNA extracts were adjusted to 40 ng/μL (corresponding to 200 ng DNA per PCR tube). The standard curves obtained are shown in Fig. 2A (set 1) and 2B (set 2). In raw sausages of set 1, the LODs were found to be 10 mg/kg for white mustard and celery and 1 mg/kg for black mustard. Amplification efficiencies, calculated from the slopes of the standard curves, were found to be 88.1% for white mustard, 91.0% for black mustard and 89.3% for celery. Similar LODs and amplification efficiencies were obtained in case of the brewed model sausages of set 1. When analysing model sausages of set 2, LODs for white mustard, brown mustard and celery were found to be 5 mg/kg, 1 mg/kg and 50 mg/kg in raw and 10 mg/kg, 1 mg/kg and 50 mg/kg in brewed sausages, respectively.

In order to investigate the reliability of the triplex assay near the LODs, DNA extracts from raw and brewed model sausages of both sets, containing 50, 10, 5 or 1 mg/kg of each allergenic component, were adjusted to 40 ng/μL (corresponding to 200 ng DNA per PCR tube) and analysed in 20 replicates. The Ct values are given in Table 2A (set 1) and 2B (set 2). Fig. 3 shows the percentages of replicates yielding an increase of the fluorescence signal within 40 cycles. The figure indicates that the triplex real-time PCR assay allows the detection of 1 mg/kg black/brown mustard in raw and brewed sausages with a probability $\geqslant$95%. It can be seen that the triplex assay is not as sensitive for white mustard and celery (which is in agreement with the singleplex and duplex assays published previously). Fifty mg/kg white mustard and celery can, however, be detected with a probability of at least 95% in both raw and processed sausages.

3.4. Analysing commercial foodstuffs

Twenty-three commercial foodstuffs were analysed with the triplex real-time PCR assay to verify implementation of existing legislation on food allergen labelling. Some food products were declared to contain or possibly contain mustard and/or celery whereas other products did not have any information regarding the presence of mustard and celery. According to the ingredient list, twelve products potentially contained both mustard and celery.

DNA of high purity (A_{260}/A_{280} ratios between 1.8 and 2.0) could be extracted from 15 out of 23 of the products. In general, DNA concentrations ranged from 100 to 1200 ng/μL. However, extracts with very low DNA concentrations ($\leqslant$13.5 ng/μL) were obtained from products containing high water, salt and/or fat content, such as dressings, spice mixes or sauces. After adjusting the DNA concentration to 40 ng/μL (200 ng DNA per PCR tube) the extracts were analysed with the triplex assay (extracts with concentrations <40 ng/μL were used without further dilution). The results are summarised in Table 3. Mustard was detected in seven out of eight products declared to contain mustard. Five of these products yielded an increase of the fluorescence signal in the green and the yellow channel, indicating that they contained both white and black/brown mustard. In meat spread 1 only white mustard

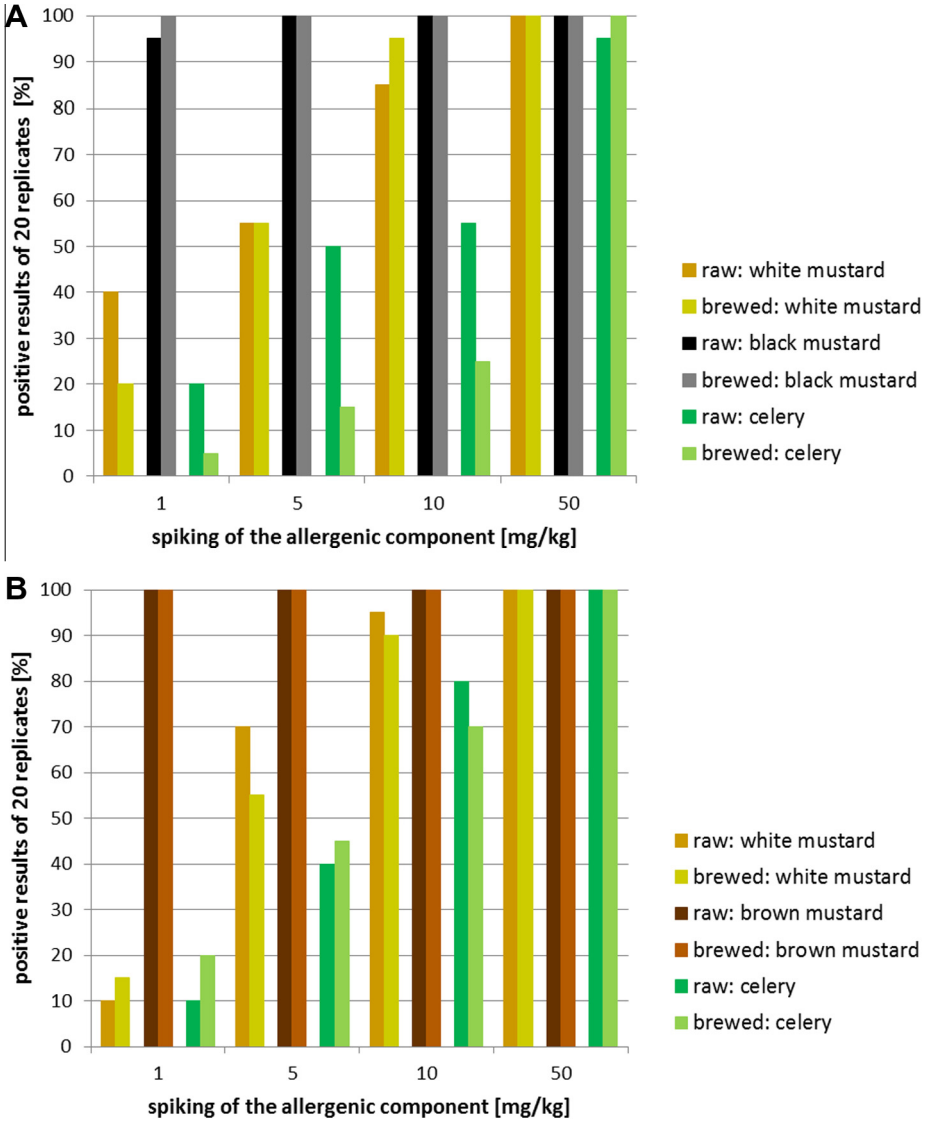

Fig. 3. Reliability of the triplex assay near the LODs by analysing DNA extracts from raw and brewed model sausages: (A) model sausages of set 1, (B) model sausages of set 2.

was detected, whereas the American sauce gave positive signal only for black/brown mustard. In one sample (mayonnaise) declared to contain mustard neither white nor black/brown mustard was detected.

In two out of nine foodstuffs labelled to potentially contain mustard (Mediterranean vegetable pan and spice mix for fish 2), black/brown mustard was detected.

Five of the six products that did not have any hint on the presence of mustard were labelled correctly, since none of the three mustard species was detected. However, one product (spice mix for fish 3), was found to contain white as well as black/brown mustard although mustard was not declared at all.

Only for two out of six products that were declared to contain celery (spaghetti Bolognese and meat spread 2), an increase of the fluorescence signal was observed in the red channel. We assume that in the other four products (fried noodles curry, meat spread 1, meat spread 3 and vegetable aspic) the celery concentration was below 50 ppm and thus below the LOD of the triplex assay. Two out of ten foodstuffs that potentially contain celery yielded positive results. Celery was not detected in the seven products that did not have any hint on the presence of celery.

Inhibition control experiments showed that none of the negative PCR results was caused by polymerase inhibition.

4. Concluding remarks

The triplex real-time PCR assay allows the detection of three mustard species, white mustard (*S. alba*), black mustard (*B. nigra*) and brown mustard (*B. juncea*), and three celery varieties, celery roots (*A. g. var. rapaceum*), celery stalks (*A. g. var. dulce*) and leaf celery (*A. g. var. secalinum*) in food. White mustard is detected in the green channel, black/brown mustard in the yellow channel and the three celery varieties in the red channel.

Cross-reactivity tests with ten *Brassicaceae*, ten *Apiaceae* and further plant species showed that the triplex assay does not detect other *Brassicaceae* species playing a role in food industry, e.g. broccoli, cabbage, cauliflower and rapeseed. Low cross-reactivities were found with fenugreek, cumin, ginger, caraway, turmeric, lovage and rye. These species yielded an increase of the fluorescence signal in the yellow channel, however, at rather high Ct values ($\Delta Ct \geqslant 12$, compared to black/brown mustard DNA).

Table 3
Analysis of commercial foodstuffs. Declaration: + contains mustard/celery, ± may contain mustard/celery, − mustard/celery not declared; total DNA amount: 200 ng per PCR tube.

Foodstuff	Declaration	PCR result (mean Ct value)		Declaration	PCR result (mean Ct value)
	Mustard	Green channel	Yellow channel	Celery	Red channel
Fried noodles curry	+	+ (37.12)	+ (36.08)	+	− (IC: 22.16)
Meat spread 1	+	+ (35.78)	− (IC: 16.56)	+	− (IC: 21.70)
Vegetarian nuggets	+	+ (30.32)	+ (24.61)	±	− (IC: 22.05)
American sauce	+	− (IC: 21.01)[1]	+ (34.9)[1]	−	− (IC: 21.93)[1]
Yoghurt dressing garlic	+	+ (35.54)[2]	+ (32.49)[2]	−	− (IC: 22.00)[2]
Yoghurt dressing Italian herbs	+	+ (35.46)[3]	+ (32.43)[3]	−	− (IC: 22.22)[3]
Sausage (salanettis)	+	+ (32.68)	+ (35.19)	−	− (IC: 21.90)
Mayonnaise	+	− (IC: 20.97)[4]	− (IC: 16.33)[4]	−	− (IC: 21.95)[4]
Spaghetti Bolognese	±	− (IC: 20.83)	− (IC: 16.33)	+	+ (29.18)
Fried noodles	±	− (IC: 21.11)	− (IC: 16.44)	±	− (IC: 22.10)
Fried noodles chicken	±	− (IC: 21.00)	− (IC: 16.47)	±	+ (38.73)
Fried noodles duck 1	±	− (IC: 21.13)	− (IC: 16.28)	±	− (IC: 22.08)
Mediterranean vegetable pan	±	− (IC: 21.02)	+ (37.11)	±	− (IC: 22.15)
Pasta ai funghi	±	− (IC: 21.25)	− (IC: 16.61)	±	− (IC: 21.97)
Sausage (brat maxe)	±	− (IC: 20.86)	− (IC: 16.52)	±	− (IC: 21.84)
Spice mix for fish 1	±	− (IC: 21.03)[5]	− (IC: 16.43)[5]	±	− (IC: 22.10)[5]
Spice mix for fish 2	±	− (IC: 21.13)[6]	+ (37.12)[6]	±	− (IC: 22.05)[6]
Meat spread 2	−	− (IC: 21.17)	− (IC: 16.29)	+	+ (36.54)
Meat spread 3	−	− (IC: 21.18)	− (IC: 16.68)	+	− (IC: 22.23)
Vegetable aspic	−	− (IC: 21.05)	− (IC: 16.41)	+	− (IC: 21.88)
Fried noodles duck 2	−	− (IC: 21.14)	− (IC: 16.43)	±	+ (36.24)
Aspic	−	− (IC: 20.86)	− (IC: 16.62)	−	− (IC: 21.97)
Spice mix for fish 3	−	+ (35.47)[7]	+ (33.25)[7]	−	− (IC: 21.78)[7]

PCR result:
+: increase of the fluorescence signal within 40 cycles.
−: no increase of the fluorescence signal within 40 cycles.
IC: inhibition control.
[1]2.5, [2,4]5.0, [3]10.0, [5,7]22.5, [6]67.5 ng DNA per PCR tube.

Cross-reactivities with the species mentioned above will therefore not affect the accuracy of the triplex assay.

Results obtained by analysing a variety of mixtures of extracts from the three mustard species and celery varieties indicate that the triplex assay allows the detection of traces of DNA of the allergenic components in spite of an excess of the other templates. Analysis of extracts from model sausages containing defined concentrations of mustard and celery showed that the triplex assay is applicable to both raw and processed foods.

In our opinion the triplex real-time PCR assay is highly suitable for being applied in routine analysis since it is selective, sensitive, applicable to raw and processed foods and, due to multiplexing, offers the possibility to save time and costs.

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Analytical Methods

Validation and comparison of two commercial ELISA kits and three in-house developed real-time PCR assays for the detection of potentially allergenic mustard in food

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Validation

ABSTRACT

The study compares the applicability of two commercial mustard ELISA kits (*Mustard ELISA Kit-specific* and *Mustard ELISA Kit-total*) and three in-house developed real-time PCR assays (singleplex assay for white mustard, singleplex assay for black/brown mustard and duplex assay for the detection of white, black and brown mustard). Analyses of raw and brewed model sausages containing white and black/brown mustard in the range from 1 to 50 ppm indicate that both ELISAs and the three real-time PCR assays allow the detection of traces of mustard in raw and in brewed sausages. The ELISAs were found to be more sensitive than the real-time PCR assays. When the ELISAs and real-time PCR assays were applied to the analysis of 15 commercial foodstuffs differing in their labelling concerning mustard, in one sample mustard was detected with both ELISAs and the three real-time PCR assays although mustard was not indicated on the food ingredient list.

1. Introduction

Mustard is able to elicit allergic reactions in sensitised persons. It is estimated that mustard allergy accounts for about 1–7% of all food allergies (EFSA, 2004). The prevalence of mustard allergy strongly depends on the geographic region. In Central Europe and in Canada, where the consumption of mustard is rather high, mustard allergy is more prevalent than in other countries. In children living in France, mustard allergy is the fourth leading cause of food allergy, after milk, eggs and peanuts (Rancé, Kanny, Dutau, & Moneret-Vautrin, 1999). Allergic symptoms caused by the consumption of mustard range from skin manifestations (e.g. itching and swelling) over gastrointestinal manifestations (e.g. vomiting and diarrhoea) to potentially life-threatening anaphylaxis.

Mustard can be contained in a variety of food products, including salads, sauces, spice mixtures and instant foods. Three members of the plant family *Brassicaceae* can be present in foods: white mustard (*Sinapis alba*), black mustard (*Brassica nigra*) and brown mustard (*Brassica juncea*). Studies have shown that some mustard allergens, particularly Sin a 1 and Sin a 3, are rather resistant to heat and enzymatic degradation (Sirvent, Palomares, Cuesta-Herranz, Villalba, & Rodríguez, 2012). Common food processing steps will therefore not drastically reduce the allergenic potential of mustard.

Mustard belongs to the 14 allergenic foods which have to be listed on food labels in the European Union (Commission of the European Union, 2007). Analytical methods are necessary in order to verify if mustard containing foodstuffs are labelled according to allergen legislation. In order to avoid false negative results, the methods should allow the detection of all three mustard species potentially being present in food. The methods should be selective for the mustard species and should not show cross-reactivity with other species commonly present in mustard containing foodstuffs. In addition, the methods should allow the detection of traces of mustard in raw as well as in highly processed foodstuffs.

So far, enzyme linked immunosorbent assays (ELISAs) and real-time polymerase chain reaction (PCR) assays are most frequently applied in food allergen analysis. Several ELISAs and real-time PCR assays for the detection of mustard have already been published (Koppelman et al., 2007; Lee, Hefle, & Taylor, 2008; Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008; Shim & Wanasundara, 2008). In general, both ELISAs and real-time PCR assays are characterised by high selectivity as well as low limits of detection (LODs). Food processing may, however, limit the applicability of both techniques. The detectability of proteins by

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antibodies may be hampered either by conformation changes caused by food processing or by a decreased extractability of proteins from highly processed foods. Although DNA is generally more stable than proteins, food processing may affect its integrity by causing DNA fragmentation.

Since food processing may influence the stability of proteins and DNA in different ways and to different degrees (Diaz-Amigo & Popping, 2010) the present study aimed at comparing the performance of two commercial ELISA kits (*Mustard ELISA Kit-specific* and *Mustard ELISA Kit-total*) and three in-house developed real-time PCR assays (Fuchs, Cichna-Markl, & Hochegger, 2010; Palle-Reisch, Wolny, Cichna-Markl, & Hochegger, 2013; Palle-Reisch, Cichna-Markl, & Hochegger, 2014), for the detection of mustard in food.

According to the information given by the producer, the *Mustard ELISA Kit-specific* is intended for the quantitative determination of white mustard in raw as well as heat treated foodstuffs. Cross-reactivities with white mustard, black mustard, brown mustard and rapeseed are reported to be 100%, 5%, 5% and <0.01%, respectively. The *Mustard ELISA Kit-specific* has recently been validated in an interlaboratory study performed in 12 laboratories (Cuhra, Gabrovská, Rysová, Hanák, & Štumr, 2011). According to the producers manual, the *Mustard ELISA Kit-total* is intended for the quantitative determination of mustard (white, black and brown mustard). The ELISA shows cross-reactivities with white mustard (100%), black mustard (58%), brown mustard (62%) and rapeseed (15%).

The real-time PCR assays developed in-house comprise a singleplex assay for white mustard, a singleplex assay for black/brown mustard and a duplex assay for the simultaneous detection of white, black and brown mustard. The singleplex assay for white mustard was found to be specific for white mustard and does not show cross-reactivity with 67 species tested, including 12 *Brassica* species such as broccoli, cauliflower, radish and rapeseed (Fuchs et al., 2010). The singleplex assay for the simultaneous detection of black and brown mustard shows minor cross-reactivities with cinnamon, cumin, fenugreek, ginger, rye and turmeric. It does, however, not show cross reactivity with other *Brassicaceae* species (Palle-Reisch et al., 2013). Like both singleplex assays the duplex assay for the simultaneous detection of white, black and brown mustard does not show cross-reactivity with other *Brassicaceae* species. Similar to the singleplex assay for black/brown mustard low cross-reactivities were obtained with cumin, fenugreek, ginger, rye and turmeric (Palle-Reisch et al., 2014).

In the present study the two commercial ELISAs and the three in-house developed real-time PCR assays were validated by analysing sets of model sausages produced under well-defined conditions. In order to investigate the influence of food processing on the detectability of mustard, the model sausages were analysed before and after brewing. In addition to validate the mustard ELISAs and PCR assays we investigated their applicability by the analysis of 15 commercial foodstuffs.

2. Materials and methods

2.1. Foods

2.1.1. Model sausages

Two sets of model sausages, spiked with either white mustard, black mustard and celery (“model sausage set 1”) or white mustard, brown mustard and celery (“model sausage set 2”), as well as an allergen free model sausage (“control sausage”) were produced in the course of a previous study (Palle-Reisch et al., 2013). The sausages of set 1 contained 0.005%, 0.001%, 0.0005% or 0.0001% (w/w) of each white mustard and black mustard (and celery), the sausages of set 2 0.005%, 0.001%, 0.0005% or 0.0001% (w/w) of each white mustard and brown mustard (and celery). The sausages were stored in beakers at −20 °C. Aliquots of 15 g were filled in 50 mL beakers and brewed at 75–78 °C for 15 min in a water bath (GFL, Burgwedel, Germany) equipped with an alarm thermometer (Testo GmbH, Vienna, Austria).

2.1.2. Commercial foodstuffs

Fifteen commercial foodstuffs, three of them declared to contain mustard, six labelled with “may contain traces of mustard” and six products without any declaration concerning mustard, were bought in Austrian supermarkets. The whole content of each foodstuff (ranging from 24 to 250 g) was carefully homogenised in a cutter (speedy pro, Krups, Brunn am Gebirge, Austria).

2.2. Determination of the protein concentration of mustard seeds

The protein concentration of seeds from white, black and brown mustard (used for producing the model sausages) was determined by the Kjeldahl method. 50 g aliquots of the seeds were ground in a mixer (IKA®A11 basic, IKA-Werke, Staufen, Germany) for 1–2 min. Then 0.5 g of the homogenised seed sample was weighed in and transferred into a digestion glass tube. After adding two Kjeldahl tablets (Missouri, Büchi Labortechnik AG, Flawil, Switzerland) and 20 mL of sulphuric acid (98%, VWR International, Vienna, Austria) the sample was digested at 420 °C for 90 min (digestion system K-438, Büchi). After cooling, the clear solution was diluted with distilled water, neutralised with 32% sodium hydroxide solution (VWR) and distilled under 100% steam pressure for 4 min into a 4% boric acid solution (Sigma–Aldrich, Vienna, Austria) (destillation unit K-355, Büchi). The nitrogen content was determined by titration with 0.25 M sulphuric acid (Sigma–Aldrich) using bromcresolgreen/methylred (Sigma–Aldrich) as indicator. The protein content was calculated from the nitrogen content by applying a conversion factor of 5.4 (Tkachuk, 1969).

2.3. ELISA

The *Mustard ELISA Kit-specific* and the *Mustard ELISA Kit-total* were provided by SEDIUM RD (Pardubice-Nemosice, Czech Republic).

2.3.1. Protein extraction

The protein fraction of the model sausages and the commercial foodstuffs was extracted following the protocol given in the producer’s manual. An 1.00 g aliquot of the homogenised food was mixed with 10 mL of the extraction buffer (delivered with the ELISAs), continuously shaken for 30 min and centrifuged (at 2250 rpm for 10 min, model 5810 R, Eppendorf, Vienna, Austria). The supernatant was collected and stored at −20 °C. All samples were extracted twice.

2.3.2. ELISA analysis

The ELISA kits were applied according to the manuals supplied by the producer. Both ELISAs are based on a sandwich format. All extracts were analysed with both the *Mustard ELISA Kit-specific* and the *Mustard ELISA Kit-total* in duplicates. The extracts were analysed without dilution, except extracts from model sausages containing 50 ppm of white and black or brown mustard that were diluted fourfold prior to analysis.

100 μL of the assay buffer and 100 μL of the extract or 100 μL of the calibrator solution (mustard protein concentration: 0, 0.5, 1.5, 5.0 or 15.0 mg/kg) were transferred into the well and incubated at room temperature for 60 min without shaking. After washing the well with the washing solution (300 μL for four times, multichannel pipette, Eppendorf), 200 μL of the conjugate solution was

added and incubated as described above. After a further washing step, 200 μL of TMB substrate solution was added and incubated at room temperature in the dark for 10 min. The enzymatic reaction was stopped by adding 50 μL of stop solution. The optical density (OD) was measured at 450 nm with a microtitre plate reader (Multiskan FC, Thermo Scientific, Vienna, Austria).

In oder to determine the mustard protein concentration in model sausages and commercial foodstuffs, calibration curves were established by plotting the optical density obtained for calibrator solutions against the logarithm of their mustard protein concentration. A sigmoidal four-parameter logistic function was used for non-linear regression:

$$Y = \frac{A - D}{1 + \left(\frac{X}{C}\right)^{b}} + D \quad (1)$$

where Y is the optical density, X the analyte concentration, C the transition point where the curve changes inflection, b the slope of the calibration curve in linear range and A and D are the asymptotic ends. Non-linear regression was carried out with a commercially available fitting software (Immunotech, LIANA software v. 4.7, Prague, Czech Republic).

Recovery of mustard (%) was calculated by dividing the mustard protein concentration determined by the ELISAs by the mustard protein concentration in the model sausages and multiplying it by 100.

2.4. Real-time PCR

2.4.1. DNA extraction

Genomic DNA was isolated with the CTAB (*N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide) method as described previously (Fuchs et al., 2010). All samples were extracted twice. In brief, 3.0 g of the homogenised sample was mixed with 10 mL of the CTAB (Merck, Darmstadt, Germany) extraction buffer, 80 μL of α-amylase (Roche, Mannheim, Germany) and 18 μL of proteinase K solution (Merck) and incubated at 50 °C overnight. After centrifugation (at 4000 rpm for 15 min, model 5810 R, Eppendorf) 1 mL of the supernatant was mixed with 600 μL chloroform:isoamylalcohol (24:1, v/v) (Merck). After centrifugation (at 13200 rpm for 10 min, model 5415 R, Eppendorf), 660 μL of the aqueous supernatant was mixed with 1320 μL of precipitation solution and incubated at room temperature. After a further centrifugation step, the supernatant was removed and the precipitate re-dissolved by adding 450 μL 1.2 M NaCl solution, 50 μL of 10-fold RNAse (Roche) buffer and 5 μL RNAse (Roche) and incubating at 56 °C. After cooling, 500 μL of phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) (Sigma Life Sciences, Buchs, Switzerland) was added. After centrifugation, 400 μL of the aqueous supernatant was transferred to 400 μL isopropanol (Merck), incubated in the freezer and centrifuged. The obtained DNA precipitate was washed with 70% ethanol solution (Merck). The dried DNA pellet was re-dissolved in 100 μL of distilled water at 56 °C. The DNA extracts were stored at −20 °C.

After measuring the absorbance of the DNA extracts at 260 nm (A_{260}) and 280 nm (A_{280}) (nano photometer, Implen, Munich, Germany) the concentration was calculated according to following equation: c [ng/μL] = $A_{260} \times 50 \times$ dilution factor. The ratio of A_{260}/A_{280} between 1.8 and 2.0 indicated pure DNA extracts.

2.4.2. Real-time PCR assays

Three in house developed real-time PCR methods were applied: a singleplex assay for the detection of white mustard (Fuchs et al., 2010), a singleplex assay for the detection of black/brown mustard (Palle-Reisch et al., 2013) and a duplex assay for the simultaneous detection of white, black and brown mustard (Palle-Reisch et al., 2014). Each DNA extract was analysed in duplicate. All experiments were carried out in strip tubes with caps in a 72-well rotor, using a Rotor-Gene Q thermo cycler (Qiagen AG, Hilden, Germany). A positive as well as a no template control were analysed in each PCR run. The experimental details are summarised in Table 1. An increase of the fluorescence signal within 40 cycles (*Ct* value <40) was regarded as positive result. If not all of the replicates yielded an increase of the fluorescence signal within 40 cycles, the result was regarded as negative.

3. Results and discussion

3.1. Analysis of model sausages

Two sets of model sausages, spiked with 50, 10, 5 or 1 ppm white mustard, black mustard and celery (set 1) or spiked with 50, 10, 5 or 1 ppm white mustard, brown mustard and celery (set 2), and an allergen free sausage serving as a control were produced (Palle-Reisch et al., 2013) and analysed with the two commercial ELISAs and the three real-time PCR assays. The model sausages were analysed before and after brewing (75–78 °C, 15 min) in order to obtain information on the influence of heat treatment on the applicability of the methods.

3.1.1. ELISAs

Table 2 shows the results obtained by extracting the protein fraction twice and by analysing each extract in duplicates. In order to be able to calculate the recovery, the protein concentration of white, black and brown mustard seeds used for preparing the model sausages was determined by the Kjeldahl method. The protein concentration of white, black and brown mustard seeds was found to be 27%, 21% and 23%, respectively.

The *Mustard ELISA Kit-total*, intended for the quantitative determination of white, black and brown mustard, allowed the detection of mustard in raw and brewed model sausages down to a mustard concentration of 2 ppm (the sausages contained 1 ppm of each white and black mustard (set 1) or 1 ppm of each white and brown mustard (set 2)). By analysing raw and brewed model sausages of set 1, the LOQ was determined to be 10 ppm mustard, corresponding to a mustard protein concentration of 2.4 ppm. In raw and brewed model sausages of set 2, the LOQ was found to be 2 ppm mustard, corresponding to 0.5 ppm mustard protein. In raw sausages, the recovery ranged from 93.2% to 113.1% (set 1) and from 104.3% to 129.0% (set 2). In brewed sausages, recovery was in the range from 71.7% to 83.3% (set 1) and 113.0% to 170.8% (set 2).

The *Mustard ELISA Kit-specific*, intended for the quantitative determination of white mustard, allowed the detection of white mustard down to a concentration of 1 ppm (in the presence of 1 ppm black or brown mustard) in both raw and brewed sausages. At this spiking level, which corresponds to a white mustard protein concentration of 0.27 ppm, white mustard could not only be detected but also quantified. In general, the *Mustard ELISA Kit-specific* resulted in recoveries between 46.1% and 70.4% in raw and between 43.1% and 84.3% in brewed sausages.

These data indicate that the commercially available ELISAs are very sensitive and applicable to detect and quantify traces of mustard not only in raw but also in brewed sausages. With both ELISAs satisfying recoveries were obtained. The *Mustard ELISA Kit-total* generally yielded higher recoveries than the *Mustard ELISA Kit-specific*. In case of model sausage set 2, recoveries obtained with the *Mustard ELISA Kit-total* were, however, higher than 100%.

3.1.2. Real-time PCR assays

Table 2 also summarises the Ct values obtained by analysing DNA extracts from the model sausages by the in-house developed real-time PCR assays. In raw model sausages, the singleplex assay

Table 1
Experimental conditions of the in-house developed real-time PCR assays.

	Singleplex PCR assay for white mustard	Singleplex PCR assay for black/brown mustard	Duplex PCR assay for white mustard and black/brown mustard	
GenBank acc. No./ reference	Y08626/Fuchs et al. (2010)	AJ415649/Palle-Reisch et al. (2013)	Palle-Reisch et al. (2014)	
Sequences (5′ → 3′)				
Primer forward	TGA AAA CTC TCT TCC CCT CTT AGG	GTT GAG CCG AGG GTC ATA ATT TC	TGA AAA CTC TCT TCC CCT CTT AGG	GTT GAG CCG AGG GTC ATA ATT TC
Primer reverse	ACA AAT GCA CAC AAG ACA GAG ATA TAG A	TCG ACT TAG GCA TCC TTA CGG	ACA AAT GCA CAC AAG ACA GAG ATA TAG A	TCG ACT TAG GCA TCC TTA CGG
Probe	*FAM*-TAC ATG ATG CTT ACC TCG C-*TAMRA*	*FAM*-CGA GAG TCC GAA TAC TGG GCT GGG GTC-*TAMRA*	*FAM*-TAC ATG ATG CTT ACC TCG C-*TAMRA*	*YAK*-CGA GAG TCC GAA TAC TGG GCT GGG GTC-*BHQ1*
Optimised concentrations [nmol L^{-1}]				
Primer forward	200	300	200	200
Primer reverse	300	300	300	200
Probe	150	50	150	50
Initial DNA amount [ng] per PCR tube	200	10	100 (model sausages), 500 (commercial foodstuffs)	
Reaction mix	TaqMan Universal Master Mix	TaqMan Universal Master Mix	QuantiTect Multiplex RT-PCR NR Kit	
PCR protocol				
Initial step	2 min at 50 °C	2 min at 50 °C	2 min at 50 °C	
Initial denaturation	10 min at 95 °C	10 min at 95 °C	10 min at 95 °C	
45 cycles: denaturation, annealing	15 s at 95 °C, 60 s at 55 °C	15 s at 95 °C, 60 s at 55 °C	15 s at 95 °C, 45 s at 55 °C	

FAM (6-carboxyfluorescein).
YAK (yakima yellow).
TAMRA (tetramethylrhodamine).
BHQ1 (black hole quencher).

for white mustard allowed the detection of white mustard down to the 50 ppm (set 1) or the 10 ppm (set 2) spiking level. In brewed sausages, mustard could be detected down to a mustard concentration of 10 ppm (sets 1 and 2). Although the singleplex assay for black/brown mustard was carried out with a lower initial DNA amount (10 ng) than the singleplex assay for white mustard (200 ng), the LODs were found to be lower. In raw and in brewed sausages the LOD for black and brown mustard was found to be 5 ppm. The duplex assay for the simultaneous detection of white, black and brown mustard was carried out with an initial DNA amount of 100 ng. In contrast to the singleplex assays which were used to analyse raw and brewed sausages the duplex assay was only applied to analyse the brewed model sausages. It allowed the detection of white mustard down to a spiking level of 10 ppm (set 1) or 5 ppm (set 2) whereas black and brown mustard could be detected down to 1 ppm (set 1 and set 2, respectively).

These results indicate that the singleplex assay for black/brown mustard is more sensitive than the singleplex assay for white mustard, in spite of applying a significantly lower initial DNA amount. Ct values obtained with the singleplex assay for black/brown mustard were generally lower than those obtained with the singleplex assay for white mustard. The duplex assay turned out to be more sensitive for black and brown mustard than for white mustard. For the singleplex assay for black/brown mustard, the same LODs were determined in raw and in brewed sausages. By analysing raw and brewed sausages with the singleplex assay for white mustard, in raw sausages the LODs were found to be 50 ppm (set 1) and 10 ppm (set 2), in brewed sausages the LOD was 10 ppm (sets 1 and 2). In general, similar Ct values were obtained for sausages of set 1 and those of set 2.

3.1.3. Comparison of the ELISAs and real-time PCR methods

Both commercial ELISAs and the three in-house developed real-time PCR assays proved to be able to detect mustard in raw as well as in brewed sausages. The ELISAs were found to be more sensitive than the real-time PCR assays. In contrast to the real-time PCR methods the ELISAs do not only allow to detect but also to determine mustard quantitatively. In general, with both ELISAs high recoveries were obtained.

In a previous study in which we compared the performance of three in-house developed ELISAs (one sandwich ELISA and two competitive ELISAs) and an in-house developed real-time PCR assay for the detection of lupine the ELISAs were also found to be more sensitive than the real-time PCR assay (Ecker et al., 2013).

3.2. Analysis of commercial foodstuffs

In addition to the model sausages, fifteen commercial food products were analysed with the ELISAs and the real-time PCR assays. Three products were labelled to contain mustard, six to potentially contain mustard. The ingredient lists of six products did not have any information on the presence of mustard. The results are summarised in Table 3.

3.2.1. ELISAs

In two products declared to contain mustard, mustard was detected with both ELISAs. For one product (meat spread 1) only the *Mustard ELISA Kit-specific* yielded a positive result. In three out of six products labelled with "may contain mustard" mustard was detected with both ELISAs. In two products the mustard concentration was below the LODs of the ELISAs. In one product (spice mix for fish 2) mustard was detected with the *Mustard ELISA Kit-specific* but not with the *Mustard ELISA Kit-total*. In two out of six samples that should not contain mustard, mustard was detected with both ELISAs. In the other samples the mustard concentration was below the LODs of both ELISAs.

In none of the samples the mustard concentration was above the LOQ of the *Mustard ELISA Kit-specific*. In two samples mustard could be quantified with the *Mustard ELISA Kit-total*, the mayonnaise that actually was declared to contain mustard but also in

Table 2
Analysis of raw and brewed model sausages.

Model sausages	Spiked mustard concentration (mg/kg)			Mustard protein concentration (mg/kg)			Mustard ELISA Kit-total				Mustard ELISA Kit-specific				Singleplex PCR assay[b]			Singleplex PCR assay[c]			Duplex PCR assay[d]	
							Recovery (%)		Mean recovery (%)[a]	SD (%)[a]	Recovery (%)		Mean recovery (%)[a]	SD (%)[a]	White mustard Mean *Ct* value			Black/brown mustard Mean *Ct* value			White mustard Mean *Ct* value	Black/brown mustard
							Extract a	Extract b			Extract a	Extract b			Extract a	Extract b	SD (%)	Extract a	Extract b	SD (%)	Extract a	
Set 1	White mustard	Black mustard	Total mustard	White mustard	Black mustard	Total mustard																
Raw																						
	0	0	0	0	0	0	n.a.	<LOD	–	–	<LOD	n.a.	n.a.	–	<LOD	<LOD	–	<LOD	<LOD	–	n.a.	n.a.
	1	1	2	0.27	0.21	0.48	<LOQ	<LOQ	–	–	<LOQ	<LOQ	<LOQ	–	38.71/<LOD	39.28/<LOD	–	37.92	<LOD	–	n.a.	n.a.
	5	5	10	1.34	1.05	2.38	92.0	97.5	94.8	3.9	50.2	41.9	46.1	5.9	<LOD	39.63/<LOD	–	36.13	35.08	0.7	n.a.	n.a.
	10	10	20	2.67	2.09	4.76	112.4	113.7	113.1	0.9	70.8	70.0	70.4	0.6	36.84/<LOD	39.89/<LOD	–	34.58	34.38	0.1	n.a.	n.a.
	50	50	100	13.35	10.45	23.80	92.3	94.1	93.2	1.3	62.9	64.1	63.5	0.8	35.95	36.16	0.1	32.09	32.34	0.2	n.a.	n.a.
Brewed																						
	0	0	0	0	0	0	<LOD	n.a.	–	–	<LOD	n.a.	n.a.	–	<LOD	<LOD	–	<LOD	<LOD	–	<LOD	<LOD
	1	1	2	0.27	0.21	0.48	<LOQ	<LOQ	–	–	<LOQ	<LOQ	<LOQ	–	<LOD	<LOD	–	36.42/<LOD	38.89/<LOD	–	<LOD	37.24
	5	5	10	1.34	1.05	2.38	86.6	56.7	71.7	21.1	48.7	37.5	43.1	7.9	38.13/<LOD	38.45	–	35.26	36.35	0.8	<LOD	32.02
	10	10	20	2.67	2.09	4.76	78.8	69.7	74.3	6.4	54.7	51.7	53.2	2.1	38.83	38.40	0.3	34.97	34.72	0.2	38.23	31.69
	50	50	100	13.35	10.45	23.80	56.0	110.6	83.3	38.6	40.4	76.7	58.6	25.7	36.59	36.59	0.0	32.02	32.11	0.1	35.46	29.19
Set 2	White mustard	Brown mustard	Total mustard	White mustard	Brown mustard	Total mustard																
Raw																						
	0	0	0	0	0	0	n.a.	<LOD	–	–	<LOD	n.a.	n.a.	–	<LOD	<LOD	–	<LOD	<LOD	–	n.a.	n.a.
	1	1	2	0.27	0.23	0.49	107.5	107.5	107.5	0.0	<LOQ	<LOQ	<LOQ		<LOD	<LOD	–	36.14/<LOD	<LOD	–	n.a.	n.a.
	5	5	10	1.34	1.13	2.47	121.7	120.1	120.9	1.1	55.4	55.4	55.4	0.0	39.14	<LOD	–	34.18	34.88	0.5	n.a.	n.a.
	10	10	20	2.67	2.26	4.93	117.4	140.6	129.0	16.4	65.2	59.6	62.4	4.0	37.68	36.81	0.6	34.86	34.17	0.5	n.a.	n.a.
	50	50	100	13.35	11.30	24.65	87.8	120.7	104.3	23.3	68.9	71.0	70.0	1.5	35.36	35.93	0.4	32.15	31.65	0.4	n.a.	n.a.
Brewed																						
	0	0	0	0	0	0	<LOD	n.a.	–	–	<LOD	n.a.	n.a.		<LOD	<LOD	–	<LOD	<LOD	–	<LOD	<LOD
	1	1	2	0.27	0.23	0.49	148.1	121.7	134.9	18.7	<LOQ	<LOQ	<LOQ		<LOD	38.73/<LOD	–	36.10/<LOD	37.85	–	<LOD	35.40
	5	5	10	1.34	1.13	2.47	155.4	162.7	159.1	5.2	72.7	66.7	69.7	4.2	<LOD	<LOD	–	35.66	34.66	0.7	36.94	32.49
	10	10	20	2.67	2.26	4.93	175.5	166.1	170.8	6.6	92.5	76.0	84.3	11.7	37.54	38.52	0.7	33.49	34.29	0.6	35.30	31.74
	50	50	100	13.35	11.30	24.65	93.8	132.1	113.0	27.1	77.0	80.6	78.8	2.5	36.21	35.94	0.2	31.68	31.36	0.2	34.11	29.56

n.a. not analysed.
[a] Calculated from results obtained by analysing extract a and extract b in duplicate on one and the same day ($n = 4$).
[b] 200 ng DNA per PCR tube.
[c] 10 ng DNA per PCR tube.
[d] 100 ng DNA per PCR tube.

Table 3
Analysis of 15 commercial foodstuffs.

Commercial foodstuffs	Declaration of mustard	Mustard protein concentration [mg/kg]				Mean Ct value					
		Mustard ELISA Kit-total All mustard species		*Mustard ELISA Kit-specific* White mustard		Singleplex PCR assay[a] White mustard		Singleplex PCR assay[b] Black/brown mustard		Duplex PCR assay[c]	
										White mustard	Black/brown mustard
		Extract a	Extract b	Extract a	Extract b	Extract a	Extract b	Extract a	Extract b	Extract a	
American sauce	+	<LOQ	<LOQ	<LOQ	<LOQ	37.84	39.53	33.76	33.01	36.06	34.13
Mayonnaise	+	0.85	0.72	<LOQ	<LOQ	<LOD	38.80/<LOD	39.31/<LOD	36.85/<LOD	37.54	37.24/<LOD
Meat spread 1	+	<LOD	<LOD	<LOQ	<LOQ	<LOD	39.43/<LOD	39.66/<LOD	<LOD	35.93	36.68
Sausage (brat maxe)	+/−	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	36.41/<LOD
Spice mix for fish 1	+/−	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	38.65/<LOD	<LOD
Spice mix for fish 2	+/−	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Fried noodles duck 1	+/−	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	37.22/<LOD
Fried noodles chicken	+/−	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	37.60
Spaghetti bolognese	+/−	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Aspic	–	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Vegetable aspic	–	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Meat spread 2	–	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Meat spread 3	–	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Fried noodles duck 2	–	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	37.55/<LOD
Spice mix for fish 3	–	1.43	1.54	<LOQ	<LOQ	37.99	36.99	34.86	33.75	36.22	33.98

LOD = 0.05 mg/kg mustard protein, *Mustard ELISA Kit-total*.
LOQ = 0.5 mg/kg mustard protein, *Mustard ELISA Kit-total*.
LOD = 0.06 mg/kg mustard protein, *Mustard ELISA Kit-specific*.
LOQ = 0.5 mg/kg mustard protein, *Mustard ELISA Kit-specific*.
n.a. not analysed.
[a] 200 ng DNA per PCR tube.
[b] 10 ng DNA per PCR tube.
[c] 500 ng DNA per PCR tube.

the spice mix for fish 3 that did not have any hint on the presence of mustard.

3.2.2. Real-time PCR assays

Isolation of DNA from highly processed foodstuffs can be a difficult task. The CTAB protocol applied in the present study generally resulted in extracts with DNA concentrations ranging from 70 to 1200 ng/μL. Lower extraction yields were, however, obtained for products with high salt and/or water content, for example for spice mixes, sauces or marinades. For most DNA extracts A_{260}/A_{280} ratios between 1.8 and 2.0 were determined, indicating that the extracts were almost free of proteins.

Only for one out of three products declared to contain mustard (American sauce) an increase of the fluorescence signal was observed with both singleplex assays and the duplex assay. With the duplex assay mustard was also detected in the other two samples declared to contain mustard. In case of meat spread 1, an increase of the fluorescence signal was observed in both channels, indicating that white and black or brown mustard were present. In case of the mayonnaise sample, an increase of the fluorescence signal was only observed with the primer/probe system targeting white mustard. In none of the foodstuffs that potentially contained mustard, mustard was detected with the singleplex assays. In only one of these samples (fried noodles chicken) black/brown mustard was detected with the duplex assay. For five out of six foods that should not contain mustard a negative result was obtained with all three real-time PCR assays. For the spice mix for fish 3, however, white and black/brown mustard were detected with the respective singleplex assays as well as with the duplex assay.

Inhibition control experiments were carried out for all samples in which mustard could not be detected. These experiments indicated that none of the negative results was caused by the presence of polymerase inhibitors.

In general, the results obtained with the duplex assay and the respective singleplex assays were in good agreement. Due to the higher initial DNA amount applied in the duplex assay in three cases the mustard concentration was below the LOD of the singleplex assays but above the LOD of the duplex assay.

3.2.3. ELISA versus PCR results

For only one out of three samples declared to contain mustard (American sauce) both ELISAs and the three real-time PCR assays yielded a positive signal. In the mayonnaise sample, mustard was detected with both ELISAs. In addition, an increase of the fluorescence signal was observed with the duplex assay, however, only in the channel detecting white mustard. In meat spread 1, mustard was detected with the *Mustard ELISA Kit-specific* and the duplex assay. For two out of six foodstuffs that potentially contained mustard neither the ELISAs nor the PCR assays yielded a positive result. In two of these foodstuffs both ELISAs but none of the real-time PCR assays led to a positive result. In case of fried noodles chicken, mustard was detected with both ELISAs and black/brown mustard with the duplex assay. In spice mix for fish 2, white mustard was detected with the *Mustard ELISA Kit-specific*. In four foodstuffs that should not contain mustard the mustard concentration was below the LODs of the ELISAs and the real-time PCR assays. In case of fried noodles duck 2 both ELISAs but none of the real-time PCR assays yielded a positive result. In spice mix for fish 3, mustard was detected with both ELISAs as well as the three real-time PCR assays. We therefore assume that this sample contained both white and black/brown mustard although the ingredient list did not contain any hint on the presence of mustard.

4. Conclusion

The study aimed at comparing the applicability of two commercial ELISAs and three in-house developed real-time PCR assays for

the detection of mustard in raw and processed foodstuffs. Results obtained by analysing raw and brewed (at 75–78 °C for 15 min) model sausages containing white and black/brown mustard in the range from 1 to 50 ppm indicate that both ELISAs and the three real-time PCR assays allow the detection of traces of mustard in raw as well as in brewed sausages. The ELISAs turned out to be more sensitive than the real-time PCR assays. Recoveries achieved with the ELISAs were quite satisfying.

In addition to the analysis of model sausages the ELISAs and real-time PCR assays were used to detect mustard in commercial foodstuffs. Fifteen foodstuffs differing in their declaration concerning mustard were analysed. Fourteen out of 15 samples were found to be labelled in accordance with legal regulations. In one sample mustard was detected with both ELISAs and the three real-time PCR assays although mustard was not indicated on the food ingredient list.

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4. DISCUSSION

The aim of the doctoral thesis was to develop and validate a multiplex real-time PCR method allowing the simultaneous detection of mustard and celery in food. The method should allow the detection of three mustard species (white, black and brown mustard) and three celery varieties (celery roots, celery stalks and leaf celery) that are potentially present in commercial foodstuffs. The method should be selective, sensitive and be applicable to both raw and processed food products.

Our strategy in developing the triplex assay is shown in Figure 3. Since it is rather difficult to find primers and a probe for the selective determination of all three mustard species, my colleague Magdalena Fuchs developed a method for white mustard targeting the *Sinapis alba* mRNA for MADS D protein. The method was found to allow the specific detection of white mustard, without showing any cross-reactivity to other 67 tested biological species, including 12 *Brassica* members [187]. In the course of her doctoral thesis she also developed a singleplex assay for celery [191].

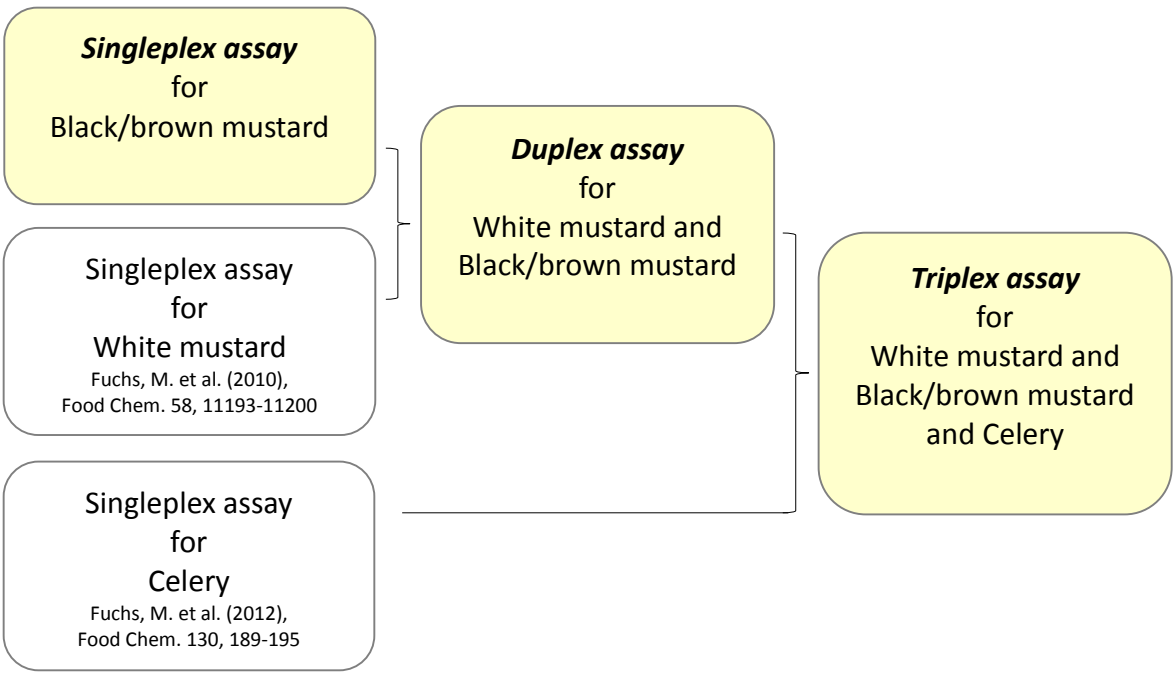

Figure 3: Our strategy for developing a triplex real-time PCR assay for the simultaneous detection of three mustard species and celery.

We started with the development and validation of a singleplex assay allowing the detection of both black and brown mustard. In a further step, we tried to combine the singleplex assays for white mustard and black/brown mustard to a duplex assay allowing the detection of all three mustard species.

Finally, we tried to combine the novel duplex assay with the singleplex assay for celery (celery roots, celery stalks and leaf celery) to a triplex assay for the detection of all three mustard species and celery varieties.

4.1. Development and optimisation of real-time PCR methods

White, black and brown mustard belong to the *Brassicaceae*, a huge plant family including other species that are frequently present in commercial foodstuffs, e.g. broccoli, Brussels sprout, cabbage, cauliflower, radish, rape seed or turnip. Our aim was to design two primers and a probe allowing the simultaneous detection of black and brown mustard. However, the method should not show cross-reactivity with other members of the *Brassicaceae* or other species that are commonly found in commercial foodstuffs.

4.1.1. Primer and probe design

For the primer/probe design, the NCBI GenBank database was searched for appropriate DNA sequences. Sequences were chosen that did not reveal any sequence homology with other *Brassicaceae* members by intensive search using the BLAST algorithm. With regard to multiplexing, great attention was paid to design compatible primers and probes without forming primer/probe dimers (self-dimer and cross-dimer) or hairpins. Finally, 12 primer pairs and corresponding probes, targeting nine different DNA/mRNA sequences from *Brassica nigra* or *Brassica juncea*, were designed (for details see Paper 1, Chapter 3.1., Table 1 as well as Section 2.4.1. and 3.1.). Three of the primer pairs and corresponding probes (primer/probe set 10-12) were designed in the course of the present doctoral thesis, nine (primer/probe set 1-9) were designed by my colleague Martina Wolny [195]. These sequences were chosen because they did not form primer/probe dimers. Compatibility was checked by performing *in silico* tests applying the software Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA). Preliminary cross-reactivity experiments were carried out under non-optimised conditions (without optimising the annealing temperature and the primer/probe

concentrations), analysing DNA extracts from black mustard, brown mustard, white mustard, broccoli, cauliflower, Chinese cabbage, kohlrabi, pak choi, rapeseed, turnip and white cabbage, all belonging to the *Brassicaceae* family. With the primer/probe set 11, DNA from black mustard as well as from brown mustard was amplified, resulting in rather similar Ct values (Ct: 18.10 and 16.95) whereas DNA from other members of the *Brassica* species (mentioned above), with the exception of white mustard, did not result in an increase of the fluorescence signal within 45 cycles (see Paper 1, Chapter 3.1., Table 2b). All other primer pairs tested were not selective for black and brown mustard DNA and showed cross-reactivity with other *Brassica* members, e.g. often with rapeseed, beetroot or cabbage, and other plant species like celery or marjoram.

Due to these results, the singleplex real-time PCR assay for black and brown mustard was developed with the primer/probe set "11" that target the sequence of the *Brassica nigra* partial RT gene encoding reverse transcriptase from gypsy-like retroelement 13G42-26 (see Figure 4).

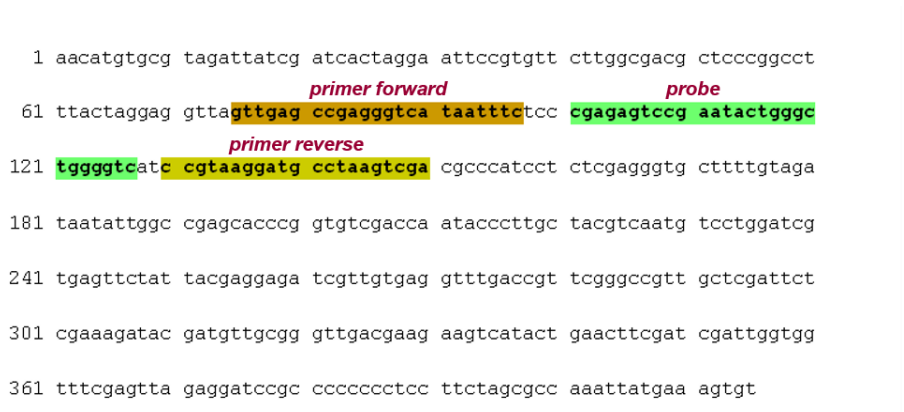

Figure 4: Localisation of the primers and the probe sequences in the Brassica nigra partial RT gene encoding reverse transcriptase from gypsy-like retroelement 13G42-26.

4.1.2. Probe labelling

Since we aimed to use the primers and the probe later on in a duplex and a triplex assay, the probe had to be labelled with fluorescence dyes differing in the excitation and emission wavelength from those used for white mustard and celery. Thus, in the duplex and triplex assay, the probe for black/brown mustard was labelled with YAK (yakima yellow), allowing the detection of black and brown mustard DNA in the yellow channel (excitation at 530 ± 5 nm

and emission at 557 ± 5 nm) of the real-time PCR instrument. White mustard DNA (FAM, 6-carboxyfluorescein) could be detected in the green channel (excitation at 470 ± 10 nm and emission at 510 ± 5 nm) and celery DNA (Cy5, cyanine) in the red channel (excitation at 625 ± 5 nm and emission at 660 ± 10 nm).

4.1.3. Optimisation of the real-time PCR assays

The singleplex assay was developed by optimising the concentrations of the primers and the probe, and the annealing temperature of the primer/probe set for black and brown mustard as described in detail in Paper 1, Chapter 3.1., Section 2.4.3. and 2.4.4.. The influence of the primer concentration, the probe concentration and the annealing temperature on the Ct value is shown in the Figures 5-8.

The lowest Ct values (mean Ct: black mustard 18.10, brown mustard 16.95) were obtained with 300 nmol/L primer forward, 300 nmol/L primer reverse, 50 nmol/L probe and an annealing temperature of 55 °C.

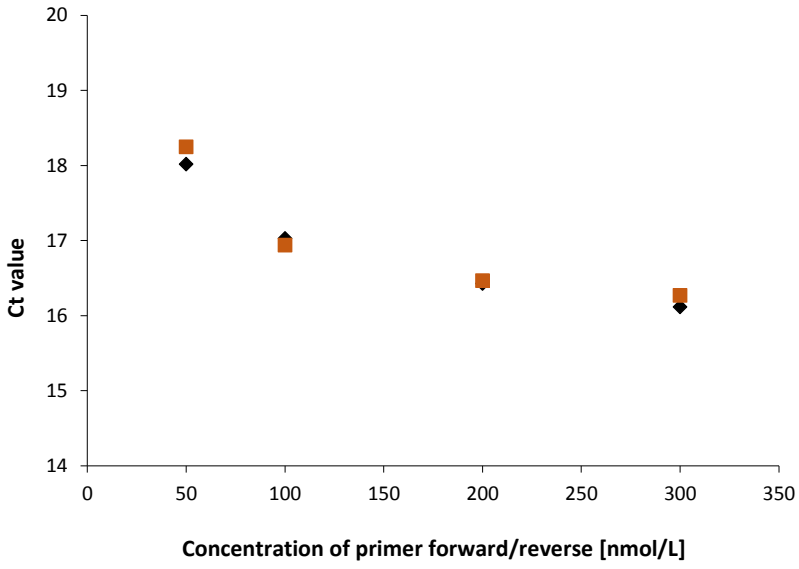

Figure 5: Mean Ct values obtained by varying the concentration of the primers from 50 to 300 nmol/L at constant probe concentration of 150 nmol/L when analysing DNA extracts from ◆ black mustard and ▪ brown mustard by applying a total DNA amount of 100 ng per PCR tube in duplicates.

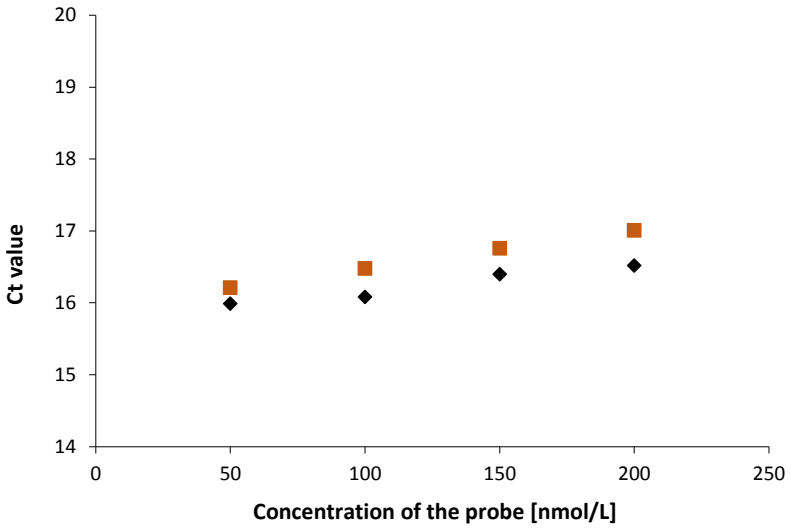

Figure 6: Mean Ct values obtained by varying the concentration of the probe from 50 to 200 nmol/L at optimised primer concentrations of 300 nmol/L when analysing DNA extracts from ◆ black mustard and ■ brown mustard by applying a total DNA amount of 100 ng per PCR tube in duplicates.

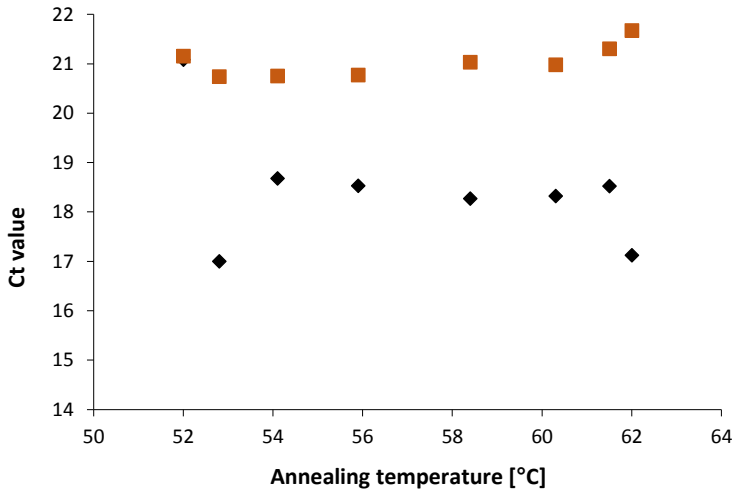

Figure 7: Mean Ct values obtained by varying the annealing temperature from 52-62 °C with optimised primer and probe concentrations (300 nmol/L primer forward/reverse, 50 nmol/L probe) when analysing DNA extracts from ◆ black mustard and ■ brown mustard by applying a total DNA amount of 100 ng per PCR tube in duplicates. (Experiment was carried out with the iCycler thermocycler (BioRad, Vienna, Austria), equipped with the IQ5 multicolor real-time PCR detection system, using a 96-well plate in a total volume of 25 µL).

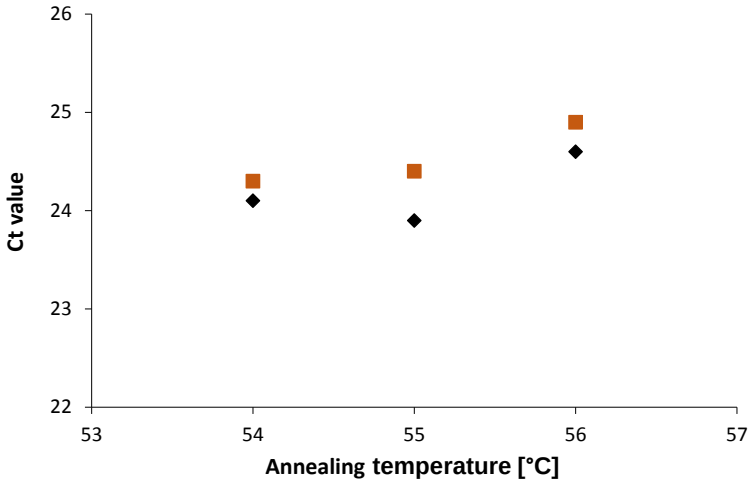

Figure 8: Mean Ct values obtained by varying the annealing temperature from 54-56 °C with optimised primer and probe concentrations (300 nmol/L primer forward/reverse, 50 nmol/L probe) when analysing DNA extracts from ◆ black mustard and ■ brown mustard by applying a total DNA amount of 0.1 ng per PCR tube in duplicates.

In further studies we tried to combine the primers and probe for black/brown mustard with those from the singleplex assay for white mustard, and in addition with those from the singleplex assay for celery, to develop a duplex real-time PCR method to simultaneously determine all three mustard species (Paper 2, Chapter 3.2.), and a triplex assay to detect all three mustard species and celery (Paper 3, Chapter 3.3.) in one PCR tube, respectively.

Table 1: Sequences of primers and probes used in the singleplex real-time PCR assays for black/brown mustard, white mustard and celery.

Primer/ Probe	Sequence [5´ --> 3´]	Probe labelling [dye-quencher]	Amplicon length [bp]	GenBank acc. no. [paper]
Black/brown mustard (b/b)				
Forward$_{b/b}$	GTT GAG CCG AGG GTC ATA ATT TC			
Reverse$_{b/b}$	TCG ACT TAG GCA TCC TTA CGG			AJ415649
Probe$_{b/b}$	CGA GAG TCC GAA TAC TGG GCT GGG GTC	FAM-TAMRA	76	[Palle-Reisch et al., 2013]
White mustard (w)				
Forward$_{w}$	TGA AAA CTC TCT TCC CCT CTT AGG			
Reverse$_{w}$	ACA AAT GCA CAC AAG ACA GAG ATA TAG A			Y08626
Probe$_{w}$	TAC ATG ATG CTT ACC TCG C	FAM-TAMRA	74	[Fuchs et al., 2010]
Celery (c)				
Forward$_{c}$	GGC GTA TGG ACC GTA ATG AAA			
Reverse$_{c}$	AGC ACA GTC AAA GTG ACG ATA ACC			U83687
Probe$_{c}$	CAA GAA TCT CCT CCT TTC CGC GAT TAA CC	FAM-TAMRA	77	[Fuchs et al., 2011]

The sequences of the primers and probes of the three singleplex assays are shown in Table 1. Although the primers and the probe for amplifying DNA from black/brown mustard had been designed carefully to avoid any complementarities between them (as described above) and to have similar annealing temperatures with the primers and probe for white mustard and celery, we had to test the compatibility of all primers and probes and their suitability for multiplexing in practice. Thus, preliminary investigation and optimisation experiments were carried out by analysing serially diluted mixtures of DNA extracts from either black mustard and white mustard, brown mustard and white mustard, black mustard and celery, or brown mustard and celery (1:1, weight/weight (w/w)). The DNA concentrations ranged from 20 ng/µL to 0.002 pg/µL of each species, corresponding to a total DNA amount of 100 ng down to 0.01 pg per PCR tube.

Amplification plots of analysed DNA mixtures from brown mustard/white mustard and brown mustard/celery are shown in Figures 9 and 10.

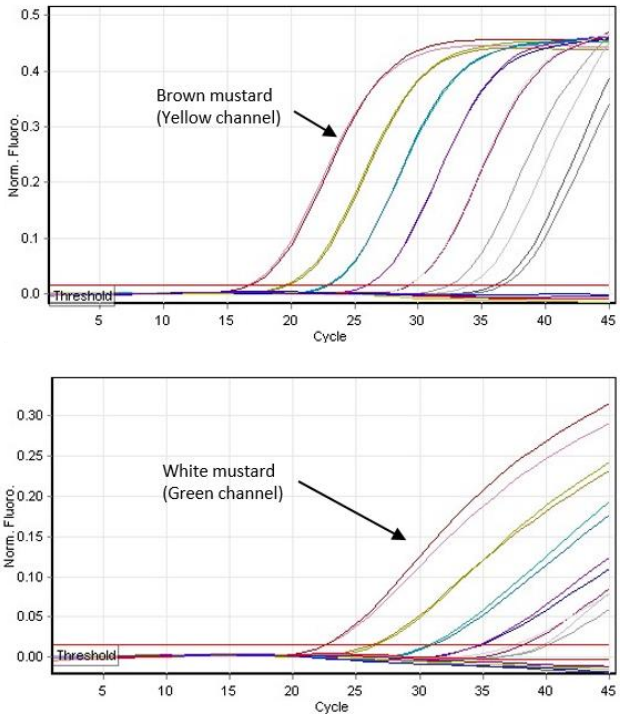

Figure 9: Amplification plots of serially diluted DNA extract mixtures (1:1, w/w) from brown mustard (detected in the yellow channel) and white mustard (detected in the green channel) by applying a total DNA amount ranging from 100 ng down to 0.01 pg of each species per PCR tube analysed in duplicates.

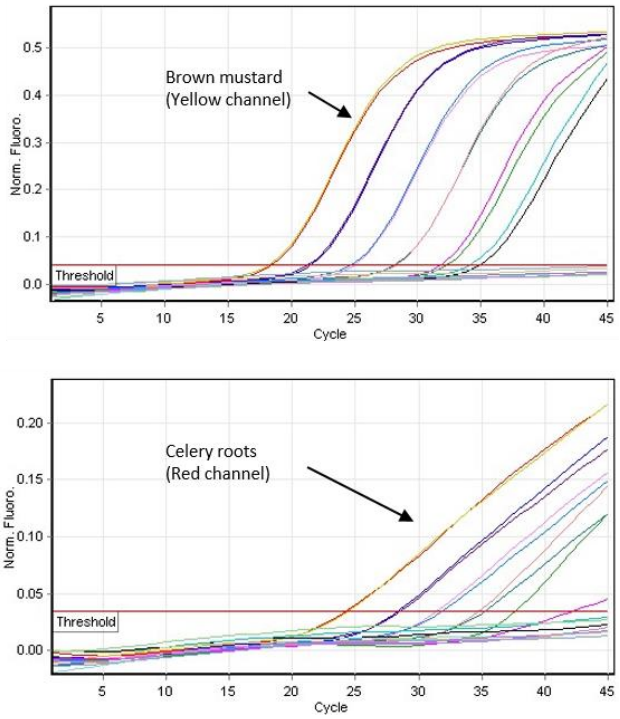

Figure 10: Amplification plots of serially diluted DNA extract mixtures (1:1, w/w) from brown mustard (detected in the yellow channel) and celery (detected in the red channel) by applying a total DNA amount ranging from 100 ng down to 0.01 pg of each species per PCR tube analysed in duplicates.

Since the primers and the probe for black/brown mustard seemed to be compatible with those from the singleplex assays for white mustard and celery we further had to optimise the methods in order to achieve high amplification efficiencies and sufficient limits of detection for all species analysed with the duplex or triplex assay.

When developing the duplex assay, we used two commercial master mixes, the TaqMan Universal PCR Master Mix ("TMU Mix", used in both singleplex assays) and the QuantiTect Multiplex RT-PCR NR Kit ("QTM Mix"). In addition, the annealing temperature, annealing time and concentration of the primers and probes were optimised as described in detail in Paper 2, Chapter 3.2., Section 2.5.1., 2.5.2., 2.5.3, 3.1. and Table 1. High amplification efficiencies of ≥ 84.7% for all three mustard species were achieved when the duplex assay was carried out using the QTM Mix with 200 nmol/L primer forward$_w$, 300 nmol/L primer reverse$_w$, 150 nmol/L probe$_w$ for white mustard DNA, 200 nmol/L primer forward$_{b/b}$, 200 nmol/L primer reverse$_{b/b}$, 50 nmol/L probe$_{b/b}$ for black/brown mustard DNA and 5 µL DNA extract (20 ng/µL down to 0.002 pg/µL of each species) with the following PCR protocol: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 45 s at 55 °C.

In the course of the development of the triplex assay, additional compatibility tests of all primer pairs and probes, and further optimisation experiments had to be carried out. Since the singleplex assay for white mustard has recently been tested at an annealing temperature of 60 °C in an international ring-trial [196] and the singleplex assay for black/brown mustard was tested in our lab at this temperature without lowering the performance of the method, we decided to increase the annealing temperature from 55 °C to 60 °C as in the singleplex assay for celery. For all four species high amplification efficiencies were obtained, for celery 100.9%/94.8%, for white mustard 91.3%/93.0%, for black mustard 95.7% and for brown mustard 101.4%, respectively. Using the QTM Mix instead of the TMU Mix yielded lower Ct values for celery (mean Ct: 21.63 (QTM Mix), 24.20 (TMU Mix), by applying 100 ng DNA amount per species per PCR tube, analysed in duplicates) (for details refer to Paper 3, Chapter 3.3., Section 3.1.). A summary of all optimised real-time PCR methods is given in Table 2.

Table 2: Overview of the optimised real-time PCR assays.

Real-time PCR	Singleplex assay			Duplex assay	Triplex assay
Allergenic food	Black/brown mustard	White mustard*	Celery*	Black/brown mustard and	Black/brown mustard and White mustard and
Condition	(b/b)	(w)	(c)	White mustard	Celery
Primers and probes					
$Forward_{b/b}$ (µmol/L)	300	-	-	200	200
$Reverse_{b/b}$ (µmol/L)	300	-	-	200	200
$Probe_{b/b}$ (µmol/L)	50	-	-	50	50
Probe labelling	FAM-TAMRA	-	-	YAK-BHQ1	YAK-BHQ1
$Forward_{w}$ (µmol/L)	-	200	-	200	200
$Reverse_{w}$ (µmol/L)	-	300	-	300	300
$Probe_{w}$ (µmol/L)	-	150	-	150	150
Probe labelling	-	FAM-TAMRA	-	FAM-TAMRA	FAM-TAMRA
$Forward_{c}$ (µmol/L)	-	-	200	-	200
$Reverse_{c}$ (µmol/L)	-	-	300	-	300
$Probe_{c}$ (µmol/L)	-	-	50	-	50
Probe labelling	-	FAM-TAMRA	Cy5-BHQ2	-	Cy5-BHQ2
Master mix	TMU Mix	TMU Mix	TMU Mix	QTM Mix	QTM Mix
PCR protocol					
Initial step	2 min, 50 °C	2 min, 50 °C	2 min, 50 °C	2 min, 50 °C	2 min, 50 °C
Initial denaturation	10 min, 95 °C	10 min, 95 °C	10 min, 95 °C	10 min, 95 °C	10 min, 95 °C
45 cycles					
Denaturation	15 s, 95 °C	15 s, 95 °C	15 s, 95 °C	15 s, 95 °C	15 s, 95 °C
Annealing	60 s, 55 °C	60 s, 55 °C	60 s, 60 °C	45 s, 55 °C	45 s, 60 °C

*Published by M. Fuchs et al.

4.1.4. Selectivity of the real-time PCR assays

After optimisation of the concentration of the primers and the probe and the annealing temperature for black/brown mustard DNA, the selectivity of the singleplex assay was tested by analysing more than 75 biological species of plant or animal origin. The method showed cross-reactivity with white mustard but not with 10 other members of the *Brassicaceae* family tested. Low cross-reactivity was observed with cinnamon, cumin, fenugreek, ginger, rye and turmeric (listed in Paper 1, Chapter 3.1., Table 2b). Since the Ct values obtained were significantly higher (Δ Ct > 11.9) than those obtained for the positive controls (black/brown mustard DNA), and due to the fact that these plants are usually present only in traces in mustard containing foods, the possibility of obtaining false positives is unlikely. Thus, these low cross-reactivities do not limit the applicability of the singleplex real-time PCR method for the selective detection of black and/or brown mustard.

The previously published singleplex assays for white mustard [187] and celery [191] are specific and do not show cross-reactivity to 67 biological species including 12 members of the *Brassicaceae* (singleplex assay for white mustard) or 64 species, among them ten *Apiaceae* (singleplex assay for celery).

The results of the selectivity experiments carried out with the multiplex assays were in accordance with those obtained for the singleplex assay for black/brown mustard. The multiplex real-time PCR methods do not cross-react with other *Brassica* species. Low cross-reactivity with cumin, fenugreek, ginger, rye and turmeric was obtained with the duplex assay. In the triplex assay, caraway, cumin, fenugreek, ginger, lovage, rye and turmeric led to an increase of the fluorescence signal in the yellow channel and ginger in the green channel. In the red channel, only celery resulted in an increase of the fluorescence signal. Representative amplification plots obtained in cross-reactivity experiments with the triplex assay are shown in Figure 11.

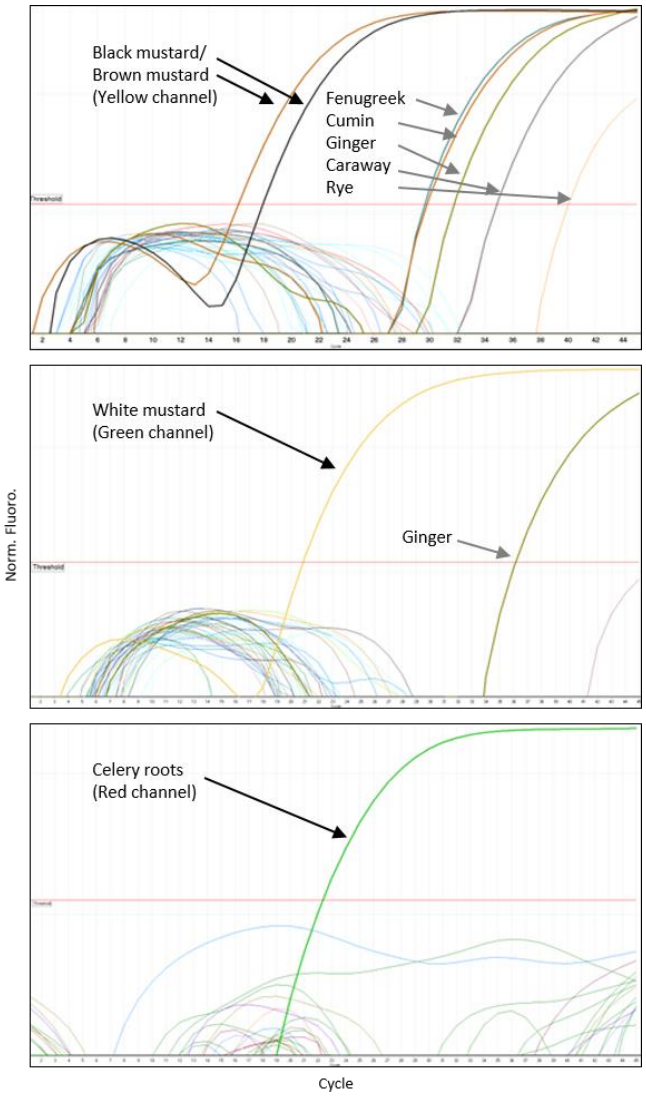

Figure 11: Representative amplification plots obtained when testing the triplex real-time PCR assay for selectivity. Black/brown mustard, white mustard, and celery DNA were simultaneously detected in the yellow, green and red channel.

These results demonstrate that the novel real-time PCR methods for mustard developed in our research group do not cross-react with other *Brassica* species and are therefore more applicable to foodstuffs than the real-time PCR method published by Mustorp et al., 2008, which showed cross-reactivity with broccoli, rapeseed, swede, turnip, Chinese cabbage, cabbage and radish, all members of the *Brassicaceae* family [186]. The selectivity for celery of the novel triplex assay was found to be as high as reported in the previously published singleplex real-time PCR methods for celery [186, 189-191].

4.2. Validation of the developed real-time PCR methods

4.2.1. Amplification efficiency and LOD

In order to determine the amplification efficiencies (E) and the limits of detection (LODs) of the developed real-time PCR assays, serially diluted DNA extracts and DNA extract mixtures were analysed. DNA extracts from black mustard and brown mustard in the range from 0.2 ng/µL to 0.002 pg/µL were applied in the singleplex assay for black/brown mustard. Mixtures of DNA extracts from white and black mustard, and white and brown mustard (1:1, w/w) in the range from 20 ng/µL to 0.002 pg/µL of each species were used in the duplex assay allowing the determination of all three mustard species. Mixtures of DNA extracts from white, black or brown mustard and celery roots (1:1:1, w/w/w) and white, black, brown mustard and celery roots or celery stalks or leaf celery (1:0.5:0.5:1, w/w/w/w) in the range from 20 ng/µL to 0.002 pg/µL of each species were analysed in the triplex assay. For more details refer to Paper 1, Chapter 3.1., Section 2.4.5. and 3.3., Paper 2, Chapter 3.2., Section 2.5.5. and 3.3. and Paper 3, Chapter 3.3., Section 2.5.5. and 3.3..

Table 3 summarises the obtained E [%] and LODs [pg DNA amount per PCR tube] of the real-time PCR assays for mustard and celery developed by our research group. As can be seen in Table 3, the amplification efficiencies and LODs of the multiplex real-time PCR assays are very close to those achieved in the singleplex assays. Amplification efficiencies were in the range from 86.2-101.4% and LODs from 10-0.1 pg DNA amount per PCR tube, indicating the high sensitivity of the developed real-time PCR methods, the singleplex assay for black/brown mustard, the duplex assay for white and black/brown mustard and the triplex assay for all three mustard species and three celery varieties.

Table 3: Efficiencies (E) and limits of detection (LODs) of the developed real-time PCR methods determined by analysing serially diluted DNA extracts or DNA extract mixtures in duplicate.

Real-time PCR	Singleplex assay		Duplex assay			Triplex assay				Singleplex assay*	
Allergenic food	Black mustard	Brown mustard	Black mustard	Brown mustard	White mustard	Black mustard	Brown mustard	White mustard	Celery roots	White mustard	Celery roots
E [%]	100.6	86.2	100.0	89.6	95.3	95.7	101.4	92.2	97.9	91.8	93.9
LOD [pg DNA/PCR tube]	0.1	0.1	1.0	0.1	10.0	0.5	0.1	5.0	10.0	5.0	10.0

*Published by M. Fuchs et al.

A challenge in the development of multiplex real-time PCR assays is to achieve high amplification efficiency for all target DNA sequences, independent of their proportion in the food sample. Thus, we investigated if the triplex assay allows efficient amplification in case the DNA of the three mustard species and celery roots is present in different amounts in a constant background matrix, as simulated with a herring sperm DNA solution (20 ng/µL corresponding to 100 ng DNA per PCR tube). Ratios of 95:2.5:2.5, 90:5:5, 75:12.5:12.5 and 45:45:10 (w/w/w) of each, white, black/brown mustard, and celery roots were analysed (see also Chapter 3.3., Table 1: DNA mixtures E-P). An overview of all Ct values obtained by analysing the DNA mixtures E-P with the triplex assay is given in Table 4.

Ct values and amplification efficiencies obtained for the most interesting mixtures E, F, G (95:2.5:2.5, w/w/w) and H, I, J (90:5:5, w/w/w) are presented in Figures 12 and 13. Satisfying amplification efficiencies were obtained for each template being in excess (E: 84.9-94.2%) but also for those being in deficiency (E: 71.9-129.6%), in presence of a constant background matrix DNA (herring sperm DNA), indicating that the triplex assay allows the sensitive detection of traces of DNA of the allergenic components in spite of an excess of the other templates.

Table 4: Overview of all Ct values obtained by analysing DNA mixtures E-P (Paper 3, Chapter 3.3.) containing various proportions of DNA from the allergenic foods with the triplex real-time PCR assay. The background matrix of 100 ng DNA per PCR tube was kept constant by diluting with a herring sperm DNA solution of 20 ng/µl.

Allergenic food		Black/brown mustard				White mustard				Celery roots			
Mixture	Dilution	DNA [ng/PCR]	Ct value	Ct value	Mean Ct value	DNA [ng/PCR]	Ct value	Ct value	Mean Ct value	DNA [ng/PCR]	Ct value	Ct value	Mean Ct value
E	-	95.0	16.54	16.94	16.74	2.5	30.20	29.80	29.80	2.5	28.85	29.15	29.00
	1:10	9.50	19.68	20.27	19.98	0.25	35.82	32.89	32.89	0.25	32.42	31.59	32.01
	1:100	0.950	22.94	24.08	23.51	0.025	-	-	-	0.025	35.78	33.30	34.54
	1:1000	0.0950	27.53	27.27	27.40	0.0025	36.35	-	-	0.0025	-	-	-
	1:10000	0.00950	30.94	30.51	30.73	0.00025	37.74	-	-	0.00025	-	-	-
F	-	2.5	22.81	22.64	22.73	95.0	20.89	20.92	20.91	2.5	27.32	27.82	27.57
	1:10	0.25	26.54	26.44	26.49	9.50	24.84	24.65	24.75	0.25	31.32	30.86	31.09
	1:100	0.025	29.54	28.21	28.88	0.950	27.82	28.04	27.93	0.025	36.00	34.57	35.29
	1:1000	0.0025	32.96	33.04	33.00	0.0950	31.53	31.80	31.67	0.0025	36.22	36.24	36.23
	1:10000	0.00025	-	39.01	-	0.00950	35.32	34.27	34.80	0.00025	-	-	-
G	-	2.5	21.76	22.16	21.96	2.5	26.54	26.57	26.56	95.0	22.48	22.44	22.46
	1:10	0.25	26.44	25.12	25.78	0.25	30.20	30.37	30.29	9.50	25.81	25.91	25.86
	1:100	0.025	30.32	30.00	30.16	0.025	33.41	34.19	33.80	0.950	29.55	29.93	29.74
	1:1000	0.0025	33.07	32.40	32.74	0.0025	-	36.39	-	0.0950	33.34	32.61	32.98
	1:10000	0.00025	38.70	35.68	37.19	0.00025	-	-	-	0.00950	37.82	-	-
H	-	90	16.88	16.64	16.76	5	26.49	26.96	26.73	5	26.66	27.12	26.89
	1:10	9.0	19.58	19.84	19.71	0.5	29.76	31.07	30.42	0.5	29.29	30.81	30.05
	1:100	0.90	23.01	23.60	23.31	0.05	32.79	33.04	32.92	0.05	34.11	34.31	34.21
	1:1000	0.090	27.27	25.49	26.38	0.005	37.87	-	-	0.005	37.52	-	-
	1:10000	0.0090	31.52	31.22	31.37	0.0005	-	-	-	0.0005	-	-	-
I	-	5	18.57	20.59	19.58	90	20.76	20.72	20.74	5	27.35	26.64	27.00
	1:10	0.5	25.16	24.91	25.04	9.0	24.75	24.38	24.57	0.5	29.89	29.86	29.88
	1:100	0.05	28.22	28.19	28.21	0.90	28.50	28.09	28.30	0.05	33.35	34.46	33.91
	1:1000	0.005	31.54	33.11	32.33	0.090	31.97	31.78	31.88	0.005	-	36.08	
	1:10000	0.0005	34.93	-	-	0.0090	36.14	35.48	35.81	0.0005	-	-	-
J	-	5	20.07	20.55	20.31	5	25.12	25.45	25.29	90	22.04	22.32	22.18
	1:10	0.5	22.10	23.48	22.79	0.5	29.58	29.03	29.31	9.0	25.85	26.03	25.94
	1:100	0.05	28.48	28.23	28.36	0.05	32.21	32.43	32.32	0.90	29.79	29.84	29.82
	1:1000	0.005	32.20	31.93	32.07	0.005	36.09	35.18	35.64	0.090	33.72	33.39	33.56
	1:10000	0.0005	37.58	36.26	36.92	0.0005	37.93	-	-	0.0090	36.22	35.72	35.97
K	-	75.0	16.42	16.67	16.55	12.5	24.55	24.23	24.39	12.5	25.26	24.67	24.97
	1:10	7.50	20.50	20.56	20.53	1.25	27.91	27.59	27.75	1.25	28.52	28.74	28.63
	1:100	0.750	24.41	24.15	24.28	0.125	31.48	31.26	31.37	0.125	32.23	32.02	32.13
	1:1000	0.0750	28.25	28.14	28.20	0.0125	34.89	35.98	35.44	0.0125	36.69	35.80	36.25
	1:10000	0.00750	30.87	31.83	31.35	0.00125	37.17	37.35	37.26	0.00125	36.27	35.72	36.00
L	-	12.5	19.29	19.81	19.55	75.0	21.03	21.21	21.12	12.5	25.23	25.17	25.20
	1:10	1.25	23.09	22.44	22.77	7.50	24.82	25.08	24.95	1.25	28.38	28.71	28.55
	1:100	0.125	26.11	26.54	26.33	0.750	28.18	28.55	28.37	0.125	32.29	32.98	32.64
	1:1000	0.0125	30.36	30.67	30.52	0.0750	31.72	31.61	31.67	0.0125	36.31	35.64	35.98
	1:10000	0.00125	33.18	33.30	33.24	0.00750	35.37	36.03	35.70	0.00125	-	-	-
M	-	12.5	18.32	18.92	18.62	12.5	23.83	23.79	23.81	75.0	22.37	22.59	22.48
	1:10	1.25	22.86	23.04	22.95	1.25	27.60	27.40	27.50	7.50	26.13	26.35	26.24
	1:100	0.125	26.86	25.09	25.98	0.125	31.09	31.16	31.13	0.750	29.35	29.68	29.52
	1:1000	0.0125	30.70	30.75	30.73	0.0125	34.01	35.35	34.68	0.0750	33.46	32.89	33.18
	1:10000	0.00125	34.18	32.82	33.50	0.00125	-	37.12	-	0.00750	37.08	38.56	37.82
N	-	45	17.83	17.95	17.89	45	21.74	21.69	21.72	10	25.84	25.50	25.67
	1:10	4.5	21.03	21.68	21.36	4.5	25.22	25.63	25.43	1.0	29.08	29.13	29.11
	1:100	0.45	24.42	24.86	24.64	0.45	28.92	29.34	29.13	0.10	32.86	32.87	32.87
	1:1000	0.045	27.09	28.57	27.83	0.045	32.75	32.92	32.84	0.010	35.98	35.82	35.90
	1:10000	0.0045	32.21	32.07	32.14	0.0045	38.72	36.99	37.86	0.0010	-	-	-
O	-	10	20.67	20.29	20.48	45	22.03	21.84	21.94	45	23.23	22.88	23.06
	1:10	1.0	24.57	24.17	24.37	4.5	25.89	25.78	25.84	4.5	26.99	26.41	26.70
	1:100	0.10	27.75	25.98	26.87	0.45	29.18	29.12	29.15	0.45	30.27	29.94	30.11
	1:1000	0.010	31.54	31.88	31.71	0.045	32.92	33.27	33.10	0.045	33.60	34.02	33.81
	1:10000	0.0010	34.76	35.04	34.90	0.0045	37.83	36.93	37.38	0.0045	-	37.65	-
P	-	45	17.77	17.76	17.77	10	24.49	24.48	24.49	45	23.32	23.41	23.37
	1:10	4.5	20.64	21.30	20.97	1.0	28.25	28.34	28.30	4.5	26.79	26.72	26.76
	1:100	0.45	23.96	24.33	24.15	0.10	32.12	32.24	32.18	0.45	30.20	30.40	30.30
	1:1000	0.045	27.93	28.42	28.18	0.010	34.45	34.97	34.71	0.045	33.95	35.19	34.57
	1:10000	0.0045	33.01	32.37	32.69	0.0010	-	37.65	-	0.0045	37.54	-	-

- Dilution: Undiluted
- Ct value: No fluorescence signal within 40 cycles

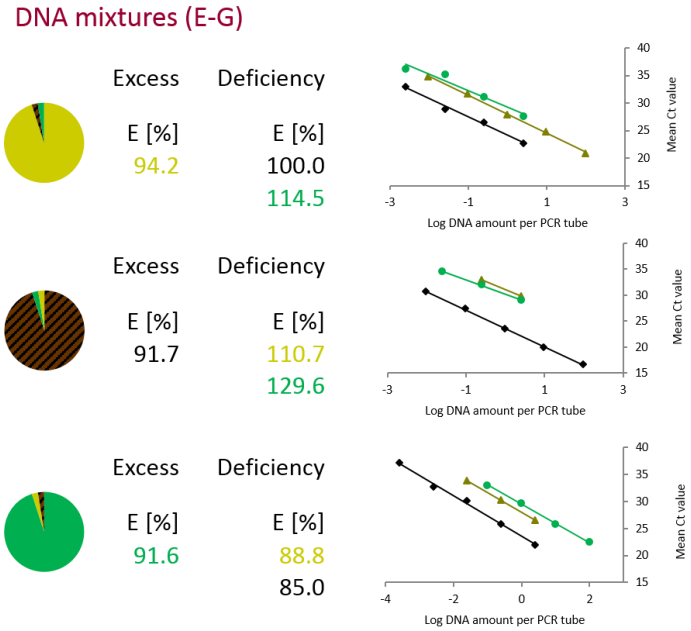

Figure 12: Regression lines and amplification efficiencies obtained for DNA mixtures E, F and G (Paper 3, Chapter 3.3.) with DNA ratios of 95:2.5:2.5 (w/w/w). ● White mustard, ●/● black/brown mustard and ● celery roots DNA.

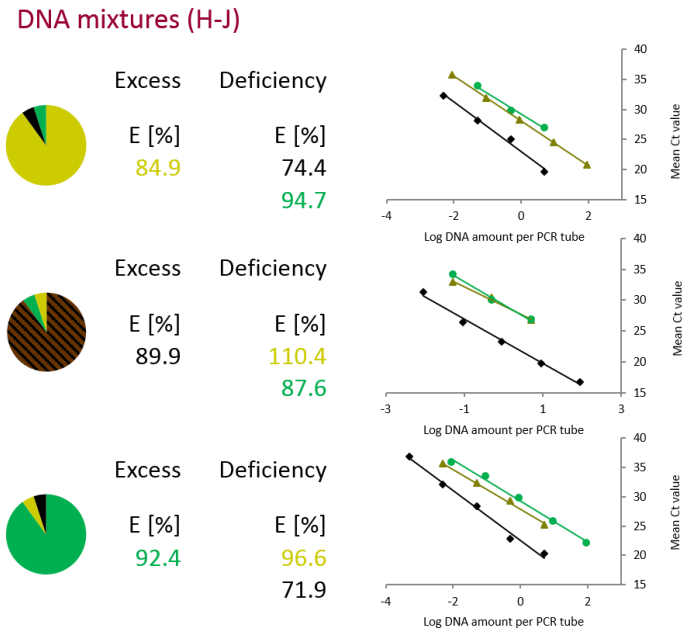

Figure 13: Regression lines and amplification efficiencies obtained for DNA mixtures H, I and J (Paper 3, Chapter 3.3.) with DNA ratios of 90:5:5 (w/w/w). ● White mustard, ●/● black/brown mustard and ● celery roots DNA.

Further investigations to determine the sensitivity and amplification efficiencies of the real-time PCR assays were carried out by analysing in-house produced sets of model sausages (for production see Chapter 4.2.1.1.) containing defined amounts (10000, 5000, 1000, 500, 100, 50, 10, 5, 1 mg/kg) of either ground white mustard, black mustard and celery roots (set 1) or white mustard, brown mustard and celery roots (set 2). To find out, if food processing has any influence on the detectability of the allergenic ingredients, not only DNA extracts from raw, but also from brewed sausages were analysed. An overview of the LODs [mg/kg] and efficiencies E [%] is given in Table 5.

Table 5: LOD [mg/kg] and E [%] achieved with the developed real-time PCR methods by analysing raw and brewed model sausages of set 1 (containing white, black mustard and celery roots) and set 2 (containing white, brown mustard and celery roots).

Real-time PCR		Singleplex assay [1 ng DNA/PCR tube]		Duplex assay [100 ng DNA/PCR tube]			Triplex assay [200 ng DNA/PCR tube]			
Allergenic food		Black mustard	Brown mustard	Black mustard	Brown mustard	White mustard	Black mustard	Brown mustard	White mustard	Celery roots
Model sausages										
Set 1	LOD									
raw	[mg/kg]	50	n.a.	n.a.	n.a.	n.a.	1	n.a.	10	10
	E [%]	83.8	n.a.	n.a.	n.a.	n.a.	91	n.a.	88.1	89.3
Set 1	LOD									
brewed	[mg/kg]	10	n.a.	1	n.a.	10	1	n.a.	10	50
	E [%]	96.8	n.a.	89.2	n.a.	84.7	86.4	n.a.	91.4	91.9
Set 2	LOD									
raw	[mg/kg]	n.a.	50	n.a.	n.a.	n.a.	n.a.	1	5	50
	E [%]	n.a.	89.1	n.a.	n.a.	n.a.	n.a.	95.9	89.9	89.3
Set 2	LOD									
brewed	[mg/kg]	n.a.	50	n.a.	1	10	n.a.	1	10	50
	E [%]	n.a.	107.9	n.a.	92.3	94.8	n.a.	82.5	83.4	89.8

n.a.: not analysed

It shows satisfying amplification efficiencies and LODs in the recommended range from 10 to 100 mg/kg of the allergenic food for all developed methods, in both raw and brewed model sausages. However, it should be stressed that different amounts of DNA per PCR tube were used in the singleplex, the duplex and the triplex real-time PCR method. The DNA amount was increased from 1 ng in the singleplex assay to 100 ng in the duplex assay and to 200 ng in the triplex assay because of the higher LODs of the previous developed singleplex assays for white

mustard (10 mg/kg by applying 500 ng DNA) and for celery (50 mg/kg by applying 500 ng DNA) compared with the singleplex assay for black and brown mustard (5 mg/kg by applying 10 ng DNA).

4.2.1.1. Production of model sausages

Since in 2011 no reference materials for mustard or celery were available, we had to produce model foods in-house. We decided to prepare model sausages, which, in our opinion, is a representative matrix, containing different amounts of the three mustard species and celery. The production of model foods is a quite challenging task, since the allergenic ingredients, which are present in trace amounts, must be homogenously distributed in the food material. First of all it is important to carefully prepare a blank material, in our case a sausage meat, which is free of the allergenic foods. In order to achieve good homogeneity of the spiked material, our model sausages were carefully produced in a cutter.

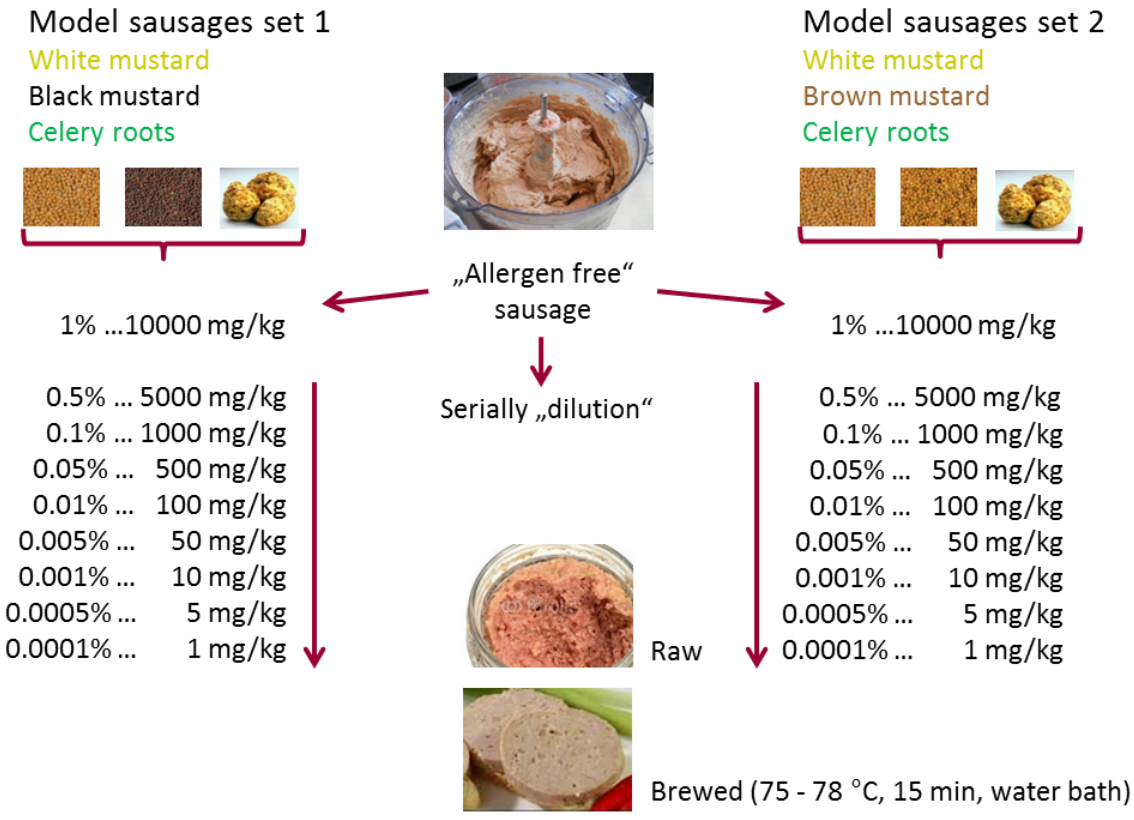

Figure 14: In-house preparation of model sausages: sausages of set 1 (containing black mustard, white mustard and celery roots), sausages of set 2 (containing brown mustard, white mustard and celery roots). The sausages were analysed raw and after brewing.

Since DNA from black mustard and brown mustard is detected with the same primer/probe system, we prepared two sets of sausages, as shown in Figure 14. 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 mustard, white mustard and celery roots, 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 mustard, white mustard and celery roots. After preparation of the "allergen free" matrix, 194 g of the sausage meat was homogenised with 2 g of each ground black (or brown) mustard, white mustard and dried ground celery roots. These sausages with a concentration of 10000 mg/kg (1%, w/w) of each allergenic food were further serially diluted with the "allergen free" matrix down to a concentration level of 1 mg/kg (0.0001%, w/w). In order to simulate food processing, aliquots of the sausages (e.g. 3 g or 15 g) were filled in beakers and brewed at 75–78 °C for 15 min in a water bath. The raw and brewed sausages were directly used for DNA extraction (for detailed information refer to Paper 1, Chapter 3.1., Section 2.2.).

4.2.2. Reliability

We also determined the reliability of each real-time PCR assay near the LODs by analysing raw and brewed model sausages of set 1 and set 2, spiked with 50, 10, 5 or 1 mg/kg of each allergenic component in 10 or 20 replicates.

The singleplex assay for the detection of black/brown mustard proved to be highly sensitive. It allows the detection of 5 ppm black and also brown mustard in brewed sausages without yielding false negative results, in spite of applying only 10 ng DNA per PCR tube, respectively (for more details see Paper 1, Chapter 3.1., Section 3.3.).

In the case of the duplex assay, which allows the simultaneous detection of white mustard and black/brown mustard, in first experiments the reliability of the method near the LOD was tested by applying a DNA amount of 100 ng per PCR tube in 20 replicates. At the spiking level of 10 mg/kg only 80% positive results were obtained for white mustard, whereas all replicates yielded a positive result for black mustard (set 1) and brown mustard (set 2), even at the spiking level of 1 mg/kg. Since a DNA amount of 500 ng per PCR tube had been used in the singleplex assay for white mustard, we carried out further experiments applying 200 or 500 ng DNA in total in 10 replicates, in order to reveal any effect on the detectability. When applying 500 ng DNA per PCR tube, all three mustard species could be detected in brewed model

sausages down to a spiking level of 5 mg/kg without false negative results (10 out of 10 replicates were positive). These results indicate the high sensitivity of the duplex real-time PCR method. For more details refer to Paper 2, Chapter 3.2., Section 3.3.. An overview of the obtained results is given in Figures 15 and 16.

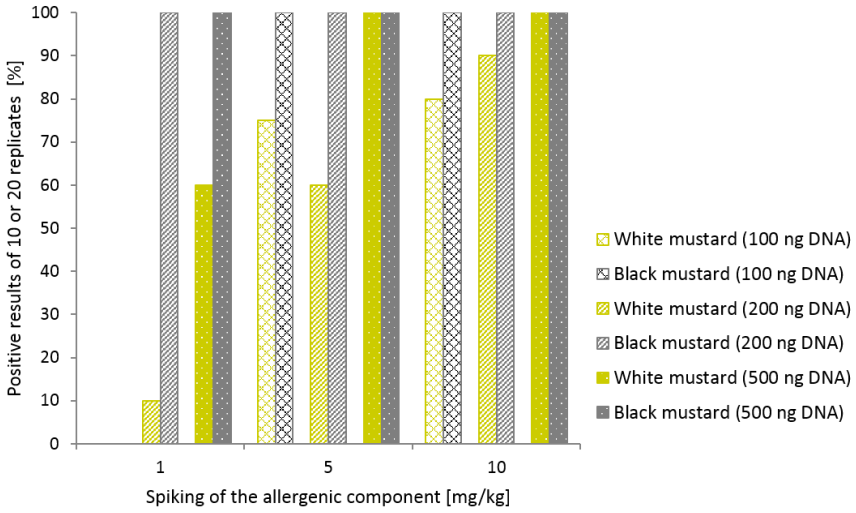

Figure 15: Reliability of the duplex real-time PCR assay by analysing brewed model sausages of set 1 (containing black and white mustard) in 20 (100 ng DNA per PCR tube) or 10 (200, 500 ng DNA per PCR tube) replicates.

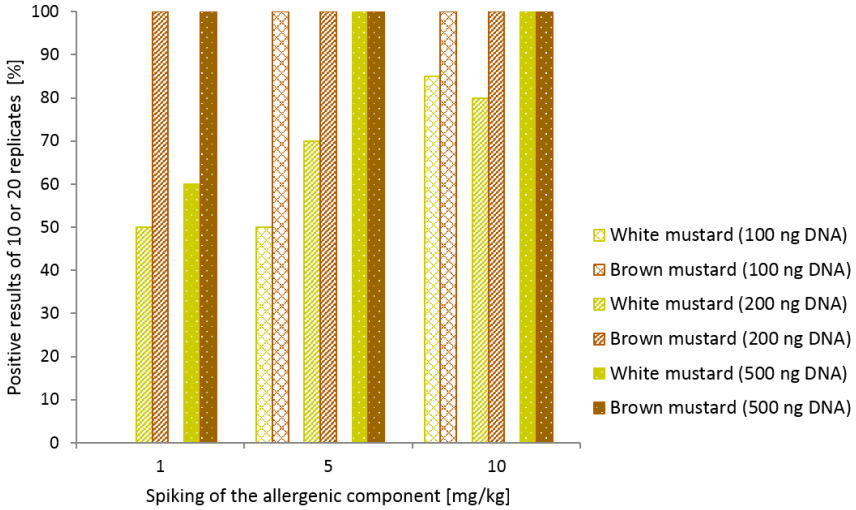

Figure 16: Reliability of the duplex real-time PCR assay by analysing brewed model sausages of set 2 (containing brown and white mustard) in 20 (100 ng DNA per PCR tube) or 10 (200, 500 ng DNA per PCR tube) replicates.

In case of the triplex assay, we applied 200 ng DNA per PCR tube, because this is the DNA amount commonly used in routine analysis in the AGES. The triplex assay was found to allow

the detection of 1 mg/kg black/brown mustard and 50 mg/kg white mustard as well as celery in raw and brewed sausages with a probability of ≥ 95% (19 or 20 out of 20 replicates were positive), respectively, indicating that the triplex real-time PCR method is very sensitive. The results are shown in Figures 17 and 18. Detailed information can be found in Paper 3, Chapter 3.3., Section 3.3..

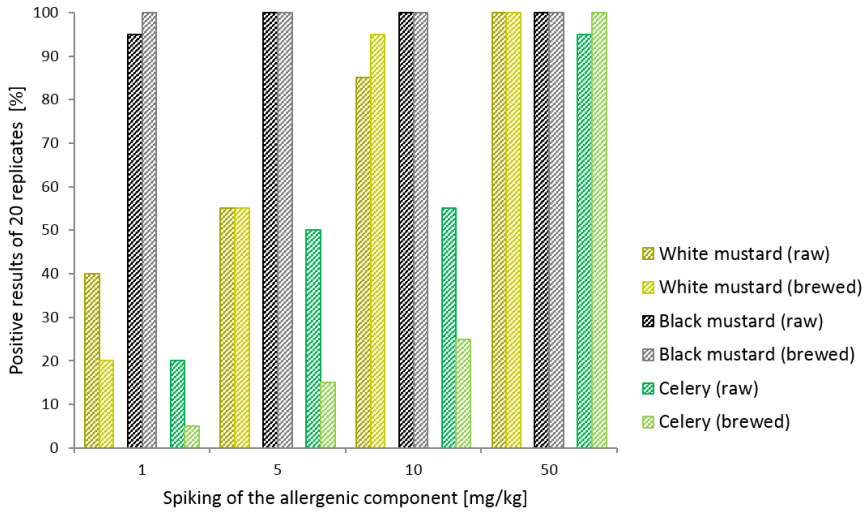

Figure 17: Reliability of the triplex real-time PCR assay by analysing raw and brewed model sausages of set 1 (containing black mustard, white mustard and celery roots) in 20 replicates (200 ng DNA per PCR tube).

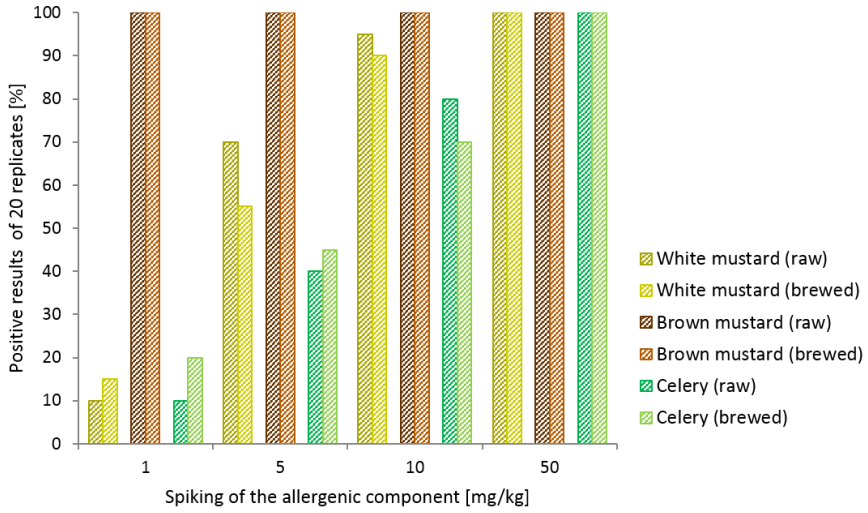

Figure 18: Reliability of the triplex real-time PCR assay by analysing raw and brewed model sausages of set 2 (containing brown mustard, white mustard and celery roots) in 20 replicates (200 ng DNA per PCR tube).

In a recent paper, Köppel et al., showed that the hexaplex method for cashew, peanut, hazelnut, celery, soy and mustard allows the detection of each of the allergenic foods at the lowest spiking level of 32 mg/kg in boiled sausages (however, they only mentioned that the sausages were produced in a professional production plant but did not give further details) [193].

Recently, Luber et al., combined our in house-developed singleplex real-time PCR methods for the detection of black/brown mustard and white mustard with singleplex assays for soy bean and celery to establish a tetraplex real-time PCR method [160]. The authors showed that the tetraplex assay can be used to quantify the allergenic species in foodstuffs. Quantification of the analytes was performed by applying the standard addition method according to Eugster et al., 2010 [197]. In contrast to our studies, the method was validated by analysing standard lysate mixtures prepared by mixing lysates of boiled sausage with lysates of unprocessed (not boiled) allergenic material. Analysis of DNA extracts from lysate mixtures resulted in low LOQs (soy bean: 8.5 mg/kg; brown mustard: 2.6 mg/kg; celery: 3.7 mg/kg; white mustard: 36.8 mg/kg). These results underlines the applicability of the primer/probe system for black/brown mustard designed within the doctoral thesis. However, since the allergenic ingredients were not spiked to the matrix before cooking, the influence of food processing on the performance of the tetraplex assay was not evaluated, as it was the case in our validation studies.

To sum up, we successfully developed three real-time PCR methods, a singleplex assay for black/brown mustard, a duplex assay for all three mustard species and a triplex assay for mustard and celery. Our methods are selective for mustard and celery without showing cross-reactivity with other members of the *Brassicaceae* and *Apiaceae* family. All developed real-time PCR methods were found to be highly sensitive, reaching high amplification efficiencies and low limits of detection within 10 to 100 mg/kg of the allergenic food, as recommended by the scientific community [72, 160]. The applicability of all three developed real-time PCR assays on raw and processed model sausages was shown, achieving reliable results without false negatives in more than 95%.

4.2.3. Real-time PCR versus ELISAs

In a further study we compared the performance of our three in-house developed real-time PCR assays (singleplex assay for white mustard [187], singleplex assay for black/brown mustard and duplex assay for the detection of white, black/brown mustard) with that of two commercial mustard ELISA kits (Mustard ELISA Kit-specific for white mustard and Mustard ELISA Kit-total for all three mustard species). The methods were validated by analysing our in-house developed model sausages containing white mustard and black mustard (set 1) and white mustard and brown mustard (set 2) in concentrations of 50, 10, 5 or 1 mg/kg of each allergenic species. To investigate the influence of food processing on the detectability of mustard, the model sausages were analysed raw and brewed (at 75-78 °C for 15 min).

The Mustard ELISA Kit-total allowed the detection of mustard in raw and brewed model sausages down to a mustard concentration of 2 mg/kg (1 mg/kg of each white and black mustard (set 1) or white and brown mustard (set2)). The LOQ was found to be 10 mg/kg in set 1 and 2 mg/kg in set 2. No difference was found between raw and brewed sausages. The Mustard ELISA Kit-specific allowed the quantification of white mustard down to a concentration of 1 mg/kg in both raw and brewed sausages. With both ELISAs satisfying recoveries were obtained, e.g. the Mustard ELISA Kit-total achieved recovery rates between 93-129% in raw and 72-171% in brewed sausages in a concentration range from about 0.5 to 25 mg/kg total mustard protein. With the Mustard ELISA Kit-specific we obtained recoveries between 46-70% in raw and 43-84% in brewed sausages in a concentration range from about 0.3-13 mg/kg (for detailed results see Paper 4, Chapter 3.4., Table 2).

The singleplex real-time PCR assay for white mustard detected white mustard down to a concentration of 50 mg/kg (set 1) or 10 mg/kg (set 2) in raw sausages and 10 mg/kg in brewed sausages of both sets, using an initial DNA amount of 200 ng. The singleplex real-time PCR assay for black and brown mustard reached an LOD of 5 mg/kg for black and brown mustard in both raw and brewed sausages. The duplex real-time PCR assay for white and black/brown mustard was only applied to analyse brewed sausages and allowed the detection of white mustard down to a concentration of 10 mg/kg (set 1) or 5 mg/kg (set 2) whereas black and brown mustard could be detected down to 1 mg/kg in both sets, respectively. Thus, the duplex real-time PCR assay turned out to be more sensitive for black and brown mustard than for white mustard (for more detailed results refer to Paper 4, Chapter 3.4.).

The results indicate, that the three in-house developed real-time PCR assays and both commercial ELISAs are able to detect traces of mustard not only in raw but also in brewed sausages. The ELISAs were found to be more sensitive than the real-time PCR assays. In addition, in contrast to the real-time PCR methods, the ELISAs allow the quantification of mustard.

These findings are in accordance with a previously published comparative study performed in our group. Four in-house developed methods for the detection of lupine, a sandwich ELISA, two competitive ELISAs and a real-time PCR method, were applied to analyse four different food matrices, comprising bread, biscuits, rice patties and noodles. To investigate the influence of food processing on the detectability, the heat treated model foods and the corresponding doughs were analysed. In general, the ELISAs turned out to be more sensitive than the real-time PCR assay. The sandwich ELISA proved to be the most sensitive method and reached a LOD of 10 mg/kg lupine, independent of the food matrix and independent if the dough or the heat treated food was analysed [198].

4.3. Applicability to commercial foodstuffs

The applicability of our real-time PCR assays (the singleplex assay for white mustard [187], that for black/brown mustard, the duplex assay for all three mustard species and the triplex assay for all three mustard species and three celery varieties) and the two commercial mustard ELISA kits (Mustard ELISA Kit-total for all mustard species and Mustard ELISA Kit-specific for white mustard) was tested by analysing 23 commercial foodstuffs differing in their labelling concerning mustard and/or celery. Eight products were declared to contain mustard, nine to potentially contain mustard and six did not have any information on the presence of mustard on the ingredient list. Concerning celery, on six products celery was listed, ten were declared to possibly contain celery and seven products did not provide any indication on celery. The results are shown in Paper 2, Chapter 3.2., Table 5, Paper 3, Chapter 3.3., Table 3, Paper 4, Chapter 3.4., Table 3 and a summary is given in this Chapter in Table 6.

Table 6: Summary of the results obtained by testing the applicability of the real-time PCR assays for mustard and celery and two commercial ELISA kits for mustard on 23 commercial foodstuffs.

Analytical method		Triplex Real-time PCR [200 ng DNA/PCR tube]		Duplex Real-time PCR [500 ng DNA/PCR tube]		Singleplex Real-time PCR* [500 ng DNA/PCR tube]	Singleplex Real-time PCR [10 ng DNA/PCR tube]	ELISA Kit-total	ELISA Kit-specific		Triplex Real-time PCR [200 ng DNA/PCR tube]	Singleplex Real-time PCR* [500 ng DNA/PCR tube]
Allergenic food (Detection channel)	*Declaration of Mustard*	White mustard Mean Ct value (Green channel)	Black/brown mustard Mean Ct value (Yellow channel)	White mustard Mean Ct value (Green channel)	Black/brown mustard Mean Ct value (Yellow channel)	White mustard Mean Ct value (Green channel)	Black/brown mustard Mean Ct value (Green channel)	White mustard/ Black mustard/ Brown mustard protein conc. [mg/kg]	White mustard protein conc. [mg/kg]	*Declaration of Celery*	Celery Mean Ct value (Red channel)	Celery Mean Ct value (Green channel)
Foodstuffs												
Fried noodles curry	+	+ (37.12)	+ (36.08)	+ (34.32)	+ (35.98)	+ (33.71)*	n.a	n.a	n.a	+	-	-
Meat spread 1	+	+ (35.78)	-	+ (35.93)	+ (36.68)	-	-	<LOD	<LOQ	+	-	-
Vegetarian nuggets	+	+ (30.32)	+ (24.61)	+ (29.30)	+ (23.36)	+ (29.30)*	n.a.	n.a.	n.a.	±	-	-
American sauce	+	- [1]	+ (34.9) [1]	+ (36.06) [1]	+ (34.13) [1]	+ (37.84) [1]	+ (33.76)	<LOQ	<LOQ	-	- [1]	- [1]
Yoghurt dressing garlic	+	+ (35.54) [2]	+ (32.49) [2]	+ (35.62)	+ (31.96)	+ (37.95)*	n.a.	n.a.	n.a.	-	-	-
Yoghurt dressing Italian herbs	+	+ (35.46) [3]	+ (32.43) [3]	+ (34.83) [3]	+ (32.17) [3]	+ (36.71)* [3]	n.a.	n.a.	n.a.	-	- [3]	- [3]
Sausage (salanettis)	+	+ (32.68)	+ (35.19)	+ (32.02)	+(34.82)	+ (34.08)*	n.a.	n.a.	n.a.	-	-	-
Mayonnaise	+	- [2]	- [2]	+ (37.54) [2]	- [2]	- [2]	-	0.85	<LOQ	-	- [2]	- [2]
Spaghetti Bolognese	±	-	-	-	-	-	-	<LOD	<LOD	+	+ (29.18)	-
Fried noodles	±	-	-	-	+ (36.72)	-	n.a.	n.a.	n.a.	±	-	-
Fried noodles chicken	±	-	-	-	+ (37.60)	-	-	<LOQ	<LOQ	±	+ (38.73)	-
Fried noodles duck 1	±	-	-	-	-	-	-	<LOQ	<LOQ	±	-	-
Mediterranean vegetable pan	±	-	+ (37.11)	-	-	-	n.a.	n.a.	n.a.	±	-	-
Pasta ai funghi	±	-	-	-	-	-	n.a.	n.a.	n.a.	±	-	-
Sausage (brat maxe)	±	-	-	-	-	-	-	<LOD	<LOD	±	-	-
Spice mix for fish 1	±	- [4]	- [4]	- [4]	- [4]	- [4]	-	<LOQ	<LOQ	±	- [4]	- [4]
Spice mix for fish 2	±	-	+ (37.12)	- [5]	- [5]	- [5]	-	<LOD	<LOQ	±	-	- [5]
Meat spread 2	-	-	-	-	-	-	-	<LOD	<LOD	+	+ (36.54)	-
Meat spread 3	-	-	-	-	-	-	-	<LOD	<LOD	+	-	-
Vegetable aspic	-	-	-	-	-	-	-	<LOD	<LOD	+	-	-
Fried noodles duck 2	-	-	-	-	-	-	-	<LOQ	<LOQ	±	+ (36.24)	-
Aspic	-	-	-	-	-	-	-	<LOD	<LOD	-	-	-
Spice mix for fish 3	-	+ (35.47) [4]	+ (33.25) [4]	+ (36.22) [4]	+ (33.98) [4]	+ (37.99) [4]	+ (33.75)	1.43	<LOQ	-	- [4]	- [4]

+ Declaration: Contains mustard/celery
± Declaration: Potentially contains mustard/celery
- Declaration: No hint concerning mustard/celery

Less DNA amount per PCR tube [ng]: [1] 2.5, [2] 5.0, [3] 10.0, [4] 22.5, [5] 67.5
+: Increase of the fluorescence signal within 40 cycles
-: No increase of the fluorescence signal within 40 cycles
LOD: 0.05 mg/kg mustard protein, Mustard ELISA Kit-total; 0.06 mg/kg mustard protein, Mustard ELISA Kit-specific
LOQ: 0.5 mg/kg mustard protein, Mustard ELISA Kit-total, Mustard ELISA Kit-specific
n.a.: not analysed *Published by M. Fuchs et al.

By analysing products declared to contain mustard with the triplex real-time PCR assay (initial DNA amount 200 ng; commonly used in routine analysis in the AGES), mustard was detected in seven out of eight foodstuffs. In five of them, both white and black/brown mustard were determined, in meat spread 1 only white mustard and in the American sauce only black/brown mustard was found. In mayonnaise, neither white nor black/brown mustard could be detected.

Analysis with the duplex real-time PCR assay for white and black/brown mustard (initial DNA amount of 500 ng DNA) indicated that mustard is present in all products that were declared to contain mustard. In mayonnaise, only traces of white mustard were detected.

In the course of the comparative study on the applicability of our real-time PCR methods and commercial ELISA kits for mustard, only three out of eight products that should contain mustard (meat spread 1, American sauce and mayonnaise) were analysed with the singleplex real-time PCR assays for white mustard and black/brown mustard. Only in one product, (American sauce) both white mustard as well as black/brown mustard were found. (Further five products were tested positive by Fuchs et al., 2010). By applying the mustard ELISA kits, in two out of three products mustard was detected with both ELISAs, whereas in case of meat spread 1 only the Mustard ELISA Kit-specific for white mustard yielded a positive result.

With regard to foodstuffs that were declared to potentially contain mustard, analyses with the real-time PCR methods indicate that in four out of nine foods mustard was present. Traces of black/brown mustard were detected with the triplex real-time PCR assay in the Mediterranean vegetable pan and spice mix for fish 2, and with the duplex real-time PCR assay in fried noodles and fried noodles chicken. In three out of six products mustard was detected with both ELISAs, but in spice mix for fish 2, only with the Mustard ELISA Kit-specific.

For four out of six samples that did not have any hint on the presence of mustard, negative results were obtained with all real-time PCR assays and with both ELISAs. In the case of the product fried noodles duck 2 none of the real-time PCR methods but both ELISAs yielded a positive result. However, in the product spice mix for fish 3, mustard was detected with all four real-time PCR assays as well as both ELISAs, although mustard was not declared at all.

The results obtained with the triplex, the duplex and the singleplex real-time PCR assays were in general in good agreement. Due to the difference in the initial DNA amounts applied in the real-time PCR assays, in three cases the mustard concentration was found to be below the LOD of the triplex assay and in two cases below the LOD of the singleplex assays but above

the LOD of the duplex real-time PCR method. In three cases (*fried noodles duck 1, spice mix for fish 1* and *fried noodles duck 2*) both ELISAs detected traces of mustard (<LOQ) whereas none of the real-time PCR methods yielded a positive result. Comparison of our real-time PCR methods and the commercial ELISAs for the detection of mustard by analysing model sausages revealed that the ELISA kits for mustard are more sensitive than the developed real-time PCR methods and therefore more applicable to determine traces of mustard in foods. The concentration of mustard in these foodstuffs is most probably above the LOD of the ELISA kits but below the LOD of the real-time PCR methods. The use of pure protein extracts in the production of these foodstuffs can be a further reason for positive results with an ELISA but negatives with a PCR method. In this case, only mustard proteins but no or only a low amount of DNA is present in such products.

5. CONCLUSION

In Europe, especially in France and Switzerland, mustard and celery allergies are prominent among the spectrum of allergies. Since both allergenic foods have been reported to potentially cause anaphylaxis, persons sensitised to mustard and/or celery are well advised to strictly avoid being exposed. In order to simplify diet planning, both allergenic foods have to be indicated on the label of prepacked products according to the Directive 2007/68/EC [53]. In order to manage and verify correct food labelling, the availability of selective and sensitive analytical methods applicable to raw and highly processed foods is of utmost importance.

At the beginning of the doctoral thesis, a singleplex real-time PCR method for the simultaneous detection of black and brown mustard was developed. The primers and the TaqMan probe target the *Brassica nigra* gene encoding reverse transcriptase from gypsy-like retroelement 13G42-26. The method was optimised and carefully validated with regard to selectivity, sensitivity and applicability to processed foods. The singleplex assay showed some cross-reactivity with white mustard, but did not react with other *Brassicaceae* species. Low cross reactivity was also found with cinnamon, cumin, fenugreek and ginger. However, since these biological species are generally present in trace amounts in mustard containing foods, these low cross reactivities will not affect the accuracy of the singleplex assay. The real-time PCR method allows the detection of 5 ppm black and/or brown mustard in brewed (75 °C to 78 °C, 15 min) model sausages. Like the in-house developed singleplex real-time PCR assay for white mustard [187], the singleplex assay for black/brown mustard will be validated in a ringtrial study. The interlaboratory study will be performed by the AGES. If the singleplex real-time PCR assay for black/brown mustard performs well, it will be included in the Official Collection of Methods of Analysis, like the singleplex assay for white mustard.

Based on the singleplex assay for black/brown mustard, a duplex real-time PCR assay for the simultaneous detection of all three mustard species, and a triplex real-time PCR assay for the simultaneous detection of mustard and celery were developed. The big challenge was to investigate if the primers and probes for white mustard, black/brown mustard and celery were compatible, and to optimise the PCR conditions in order to yield high amplification efficiencies for all templates**.** With the duplex real-time PCR assay, high amplification efficiencies (≥ 84.7%) and low limits of detection (LODs) in raw and brewed model sausages were obtained (1 mg/kg black mustard, 1 mg/kg brown mustard and 5 mg/kg white mustard). The triplex real-time PCR

assay allows the detection of 1 mg/kg black and brown mustard, and 50 mg/kg white mustard and celery in raw and brewed model sausages with a probability of ≥ 95%, respectively.

The applicability of the singleplex, duplex and triplex real-time PCR assay for mustard and celery was demonstrated by analysing commercially available foodstuffs.

In a further study, we compared the performance of our three in-house developed real-time PCR assays for mustard (singleplex for black/brown mustard, singleplex assay for white mustard [187] and duplex assay for all three mustard species) and two commercial mustard ELISAs kits, for white mustard and for all three mustard species. All the real-time PCR methods as well as the ELISAs were found to detect traces of mustard in raw and brewed model sausages. However, the ELISAs turned out to be more sensitive than the real-time PCR assays.

In addition, 15 commercial food products were analysed. According to the ingredient list, some of them contained „mustard“, part of them potentially contained mustard and some of them should not contain mustard. In case of one product that should not contain mustard, positive results were obtained with all real-time PCR assays and both ELISA kits.

The results indicate that due to their high selectivity and sensitivity, the real-time PCR methods developed in course of the doctoral thesis can be applied to verify correct labelling of foodstuffs in routine analysis.

6. INDICES

6.1. List of Abbreviations

°C	Degree centigrade
µg	Microgram
µL	Microliter
b/b	Black/brown mustard
B-cells	B-lymphocytes
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pair
c	Celery
CCD	Cross-reactive carbohydrate determinants
CRD	Component-resolved diagnosis
Ct	Cycle of threshold
Da	Dalton
DBPCFC	Double-blind, placebo-controlled food challenge
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
E	Amplification efficiency
e.g.	Exempli gratia (for example)
EAST	Enzymeallergosorbent test
ED	Eliciting dose
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
et al.	Et alii (and others)
EU	European Union
FAM	6-Carboxyfluorescein
FAOSTAT	Food and Agriculture Organisation of the United Nations – Statistics
FC	Food challenge
FRET	Fluorescence resonance energy transfer
g	Gram
GI	Gastrointestinal
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL	Interleukin
ILSI	International Life Sciences Institute
kDa	Kilodalton
kg	Kilogram
L	Liter

LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOAEL	Lowest-observed-adverse-effect level
LOD	Limit of detection
LOQ	Limit of quantification
M-cells	Microfold cells
MED	Minimal eliciting dose
mg	Milligram
MHC	major histocompatibility complex
min	Minute
mL	Milliliter
MLPA	Multiplex ligation-dependent probe amplification
mm	Millimeter
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS	Mass spectrometry
n.a.	not analysed
ng	Nanogram
nm	Nanometer
nmol	Nanomole
NOAEL	No-observed-adverse-effect level
nsLTP	Nonspecific lipid transfer protein
OAS	Oral allergy syndrome
OD	Optical density
PCR	Polymerase chain reaction
ppm	Parts per million
PR-10	pathogenesis-related proteins 10
QTM	QuantiTect Multiplex RT-PCR NR Kit
RAST	Radioallergosorbent test
s	Second
SBPCFC	Single-blind, placebo-controlled food challenge
SPT	Skin prick test
TAMRA	Tetramethylrhodamine
Taq	*Thermus aquaticus*
T-cells	T-lymphocytes
Th2	Type 2 thymus helper cells
T_m	Melting temperature
TMU	TaqMan Universal Master Mix
VITAL	Australian Voluntary Incidental Trace Allergen Labelling
w	white mustard
w/w	Weight per weight

6.2. List of Figures

6.3. List of Tables

6.4. List of references

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7. APPENDIX

7.1. Abstract

Allergies to mustard and celery are rather prevalent in Europe. In France, about 1-7% of all food allergic persons suffer from mustard allergy and in Switzerland, 30-40% of the patients are sensitised to celery. Since therapies of food allergies are still under research, the only option for concerned individuals is the strict avoidance of the responsible food. In order to protect food allergic patients, in Europe thirteen potentially allergenic foods, including mustard and celery, have to be declared on commercial food products (Directive 2007/68/EC). Selective and sensitive analytical methods are necessary to be able to verify if foodstuffs are labelled according to legal regulations. Currently, protein-based and DNA-based methods play the most important role in food allergen analysis. Several real-time PCR methods for the detection of mustard or celery have already been published. However, all real-time PCR methods for mustard show some cross-reactivity, mainly with other *Brassicaceae* species.

Food products may contain white mustard, black mustard and/or brown mustard and three different celery varieties. Since in many commercial foodstuffs, such as meat products, sauces, marinades or convenience products, both mustard and celery can be present, in cooperation with Mag. Rupert Hochegger, Department of Molecular Biology and Microbiology, Institute for Food Safety, Austrian Agency for Health- and Food Safety (AGES), we aimed to develop a multiplex assay allowing the simultaneous detection of all three mustard species and celery.

We started with the development of a real-time PCR method for the simultaneous detection of black and brown mustard. The primers and the probe were designed to target a sequence of the "*Brassica nigra* gene encoding reverse transcriptase from gypsy-like retroelement 13G42-26". This method was then combined with a real-time PCR assay for white mustard, which has been developed previously in our research group. Finally, the novel duplex real-time PCR assay for white, black and brown mustard was extended with a singleplex real-time PCR assay for celery (previously developed in our research group) to a triplex real-time PCR assay for the simultaneous detection of all three mustard species and three celery varieties.

Each real-time PCR assay was validated by analysing serially diluted mixtures of DNA extracts from white and black or brown mustard and celery. In addition, model sausages containing defined amounts of mustard and celery were produced in-house. In order to investigate the applicability of the real-time PCR assays to processed foods, raw and brewed model sausages were analysed.

The real-time PCR assays for black/brown mustard do not show cross-reactivity with other *Brassicaceae* species. Low cross-reactivity was observed with caraway, cumin, fenugreek and ginger, but compared to the positive control, the difference in the Ct value was ≥ 12. All real-time PCR methods developed in course of the doctoral thesis were found to be highly sensitive, with limits of detection (LODs) being in the range from 10 to 100 mg/kg of the allergenic food. The real-time PCR assays were applied to verify correct labelling of commercially available foodstuffs differing in their declaration concerning mustard and celery. In addition, we compared the applicability of the in-house developed singleplex real-time PCR assay for black/brown mustard, the singleplex real-time PCR assay for white mustard and the duplex real-time PCR assay for all three mustard species with two commercial mustard ELISA kits (one for the detection of all three mustard species, the other one being specific for white mustard). Validation of the methods was carried out by analysing raw and brewed model sausages containing 1 to 50 mg/kg white mustard as well as black/or brown mustard. In general, the commercial ELISAs were found to be more sensitive than the real-time PCR methods. However, our results indicate that the ELISAs as well as the three real-time PCR assays can be applied to detect traces of mustard not only in raw but also in brewed sausages.

7.2. Zusammenfassung

Senf- und Sellerieallergien sind in Europa relativ weit verbreitet. In Frankreich leiden ca. 1-7% aller Lebensmittelallergiker an einer Senfallergie und in der Schweiz sind ca. 30-40% aller Patienten gegenüber Sellerie sensibilisiert. Da nach wie vor an Therapien für Lebensmittelallergien geforscht wird, bleibt den betroffenen Personen nur die Option das allergieauslösende Lebensmittel strikt zu vermeiden. Um Lebensmittelallergiker zu schützen, unterliegen in Europa dreizehn potentiell allergene Lebensmittel, darunter auch Senf und Sellerie, einer Kennzeichnungspflicht (Directive 2007/68/EC).

Für die Überprüfung der Einhaltung der Kennzeichnungspflicht von Allergenen in Lebensmitteln werden selektive und sensitive Analysenmethoden benötigt. Gegenwärtig spielen protein-basierende und DNA-basierende Methoden die wichtigste Rolle in der Lebensmittelanalytik. Es wurden bereits einige real-time PCR Methoden für die Detektion von Senf und Sellerie publiziert. Jene für Senf weisen jedoch Kreuzreaktivität vorwiegend mit anderen *Brassicaceae* Spezies auf. Lebensmittel können weißen Senf, schwarzen Senf und/oder braunen Senf und drei unterschiedliche Selleriearten enthalten. Da viele kommerzielle Lebensmittel, wie Fleischprodukte, Soßen, Marinaden und Fertiggerichte, sowohl Senf als auch Sellerie beinhalten können, setzten wir uns, in Zusammenarbeit mit Mag. Rupert Hochegger, Abteilung Molekular- und Mikrobiologie, Institut für Lebensmittelsicherheit Wien, Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), die Entwicklung einer Mulitplex Methode für die simultane Detektion aller drei Senfspezies und Sellerie zum Ziel.

Wir starteten mit der Entwicklung einer real-time PCR Methode für die gleichzeitige Detektion von schwarzem und braunem Senf. Die entworfenen Primer und die Sonde erfassen eine Sequenz des Gens „*Brassica nigra* encoding reverse transcriptase from gypsy-like retroelement 13G42-26“. Diese Methode wurde mit einer real-time PCR Methode für weißen Senf, welche in unserer Arbeitsgruppe entwickelt wurde, kombiniert. Zum Schluss wurde die neue Duplex real-time PCR Methode für weißen, schwarzen und braunen Senf mit einer Singleplex real-time PCR Methode für Sellerie (in unserer Arbeitsgruppe entwickelt) zu einer Triplex real-time PCR Methode für die simultane Bestimmung aller drei Senfspezies und drei Selleriearten erweitert.

Jede real-time PCR Methode wurde durch Analyse von seriell verdünnten Mischungen von DNA Extrakten aus weißem, schwarzem oder braunem Senf, und Sellerie validiert. Zusätzlich

wurden Modellwürste mit definierten Mengen an Senf und Sellerie produziert. Um die Anwendbarkeit der Methoden auf stark verarbeitete Lebensmittel zu prüfen, wurden rohe und gebrühte Modellwürste analysiert.

Die real-time PCR Methoden für schwarzen/braunen Senf zeigen keine Kreuzreaktivität mit anderen *Brassicaceae* Spezies. Geringe Kreuzreaktion wurde mit Kümmel, Kreuzkümmel, Bockshornklee und Ingwer erhalten, doch verglichen zur Positivkontrolle war die Differenz des Ct Wertes ≥ 12. Alle im Rahmen dieser Doktorarbeit entwickelten real-time PCR Methoden sind hoch sensitiv, mit Nachweisgrenzen im Bereich von 10 bis 100 mg/kg allergenes Lebensmittel. Die real-time PCR Methoden wurden zur Überprüfung der korrekten Kennzeichnung von kommerziellen Lebensmitteln mit unterschiedlicher Deklaration hinsichtlich Senf und Sellerie eingesetzt.

Zusätzlich verglichen wir die Anwendbarkeit der „in-house“ entwickelten real-time PCR Methoden, Singleplex für schwarzen/braunen Senf, Singleplex für weißen Senf und Duplex für alle drei Senfspezies, mit zwei kommerziellen Senf ELISA Kits (einen für die Detektion aller drei Senfspezies, ein anderer spezifisch für weißen Senf). Die Validierung der Methoden wurde durch die Analyse von rohen und gebrühten Modellwürsten, welche 1 bis 50 mg/kg weißen Senf sowie braunen oder schwarzen Senf beinhalteten, durchgeführt. Generell waren die kommerziellen ELISAs empfindlicher als die real-time PCR Methoden. Dennoch zeigen unsere Ergebnisse, dass sowohl die ELISAs als auch die drei real-time PCR Methoden für die Detektion von Spuren von Senf nicht nur in rohen sondern auch in gebrühten Würsten eingesetzt werden können.

7.3. CURRICULUM VITAE

MAG. MONIKA PALLE-REISCH

Date of birth	18th November 1976
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EDUCATION

Since 03.2011	Doctoral studies of Chemistry Title of the doctoral thesis: *"Development and validation of a multiplex real-time PCR method for the simultaneous detection of white, black and brown mustard and celery in food"* AGES, Austrian Agency for Health and Food Safety and Department of Analytical Chemistry, University of Vienna
1997 – 2007	Master studies of Chemistry Faculty of Chemistry, University of Vienna Title of the diploma thesis: *„Immunfiltration als Probenaufreinigungsschritt bei der Bestimmung von Zearalenon in Futtermitteln"* Department of Analytical Chemistry, University of Vienna Graduation 15th November 2007
1994 – 1997	Advanced course, HBLW, Linz, Upper Austria
1991 - 1994	College for economic professions, FW, Vöcklabruck, Upper Austria
1987 - 1991	Secondary school, Vöcklabruck, Upper Austria
1983 - 1987	Primary school, Weyregg, Upper Austria

ADDITIONAL QUALIFICATION

07.2011 - 02.2012	Quality assurance in the chemical laboratory, University course, Montanuniversität Leoben

STAY ABROAD

03.2012	Faculty of Science, Charles University, Prague, Czech Republic

WORK EXPERIENCE

Since 09/2014	Project assistant, AGES, Austrian Agency for Health and Food Safety, Vienna
03.2011 - 02.2014	PhD student and freelancer, AGES, Austrian Agency for Health and Food Safety, Vienna
03.2011 – 02.2014	Lecturer, Department of Analytical Chemistry, University of Vienna
03.2006 – 08.2008	Tutor, Department of Analytical Chemistry, University of Vienna
10.2002 – 01.2003	Tutor, Department of Organic Chemistry, University of Vienna
03.2008 – 08.2008	Freelancer, Project EuroFIR BASIS, Institut of Food Research, Norwich Research Park and Department of Nutritional Sciences, University of Vienna
10.2001 – 12.2002	Freelancer, ECHEM, Kompetenzzentrum für Angewandte Elektrochemie GmbH, Wr. Neustadt
07.2001 – 09.2001	Intern, AUSTRIAN RESEARCH CENTERS, Seibersdorf

LANGUAGE SKILLS

German	Mother tongue
English	Fluent
French	Basic

PUBLICATIONS

Development and validation of a triplex real-time PCR assay for the simultaneous detection of three mustard species and three celery varieties in food
Monika Palle-Reisch, Rupert Hochegger, Margit Cichna-Markl
Food Chemistry 184 (2015) 46-56

Validation and comparison of two commercial ELISA kits and three in-house developed real-time PCR assays for the detection of potentially allergenic mustard in food
Monika Palle-Reisch, Rupert Hochegger, Stephan Štumr, Kveta Korycanova,
Margit Cichna-Markl
Food Chemistry 174 (2015) 75-81

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 food
Monika Palle-Reisch, Margit Cichna-Markl, Rupert Hochegger
Food Chemistry 153 (2014) 66-73

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
Monika Palle-Reisch, Martina Wolny, Margit Cichna-Markl, Rupert Hochegger
Food Chemistry 138 (2013) 348-355

ORAL PRESENTATIONS

Detektion von potentiell allergenem Senf und Sellerie mittels eines Triplex real-time PCR Assays
Österreichische Lebensmittelchemikertage 2014, GÖCH, Wien

Development and validation of a triplex real-time PCR method for the simultaneous detection of celery and three mustard species in food
RAFA 2013, 6th International Symposium on Recent Advances in Food, Praque, Czech Republic

Comparison of real-time PCR assays with commercial ELISA kits for the detection of potentially allergenic mustard
CEEPUS (Central European Exchange Program for University Studies), 13th Symposium on Bioanalysis and Summer School 2013, Debrecen, Hungary

Development and validation of a duplex real-time PCR method for the simultaneous detection of black, brown and white mustard
CEEPUS, 12th Symposium on Bioanalysis and Summer School 2012, Cluj-Napoca, Romania

POSTER PRESENTATIONS

Entwicklung einer hochempfindlichen LC-MS/MS-Methode für den Nachweis des Bakterientoxins Cereulid in Lebensmitteln und deren Anwendung
ANAKON 2015, GDCH, Graz

Simultane Bestimmung von braunem und schwarzem Senf in Lebensmitteln mittels real-time PCR
Österreichische Lebensmittelchemikertage 2012, GÖCH, Linz

Development and validation of a real-time PCR method for the simultaneous detection of black mustard (Brassica nigra) and brown mustard (Brassica juncea)
RAFA 2011, 5th International Symposium on Recent Advances in Food, Prag, Tschechien

Development and validation of a real-time PCR method for the simultaneous detection of black mustard (Brassica nigra) and brown mustard (Brassica juncea)
CEEPUS 11th Symposium on Bioanalysis and Summer School 2011, Graz

Sample clean-up by immuno-ultrafiltration to determine Zearalenone in food
Österreichische Chemietage 2009, GÖCH, Wien

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