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DNA-based analysis of food allergens - development and validation of a real-time PCR method for the detection of DNA from lupine in foods

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Contents

1   Introduction and Objectives

In industrialized countries, approximately 3 to 4% of adults and 5% of young children suffer from food allergies. In these sensitive individuals the ingestion of the respective allergen can cause various symptoms. They differ in severity and may range from rather mild indications such as in the oral allergy syndrome to an anaphylactic reaction in the worst case. The only measure allergic individuals can take to minimize their risk is the strict avoidance of the offending food. Labelling of specific ingredients became mandatory in the European Union with Commission Directive 2003/89/EC. Annex IIIa of the directive contains a list of ingredients that must under all circumstances appear on the label so that consumers who are allergic to a specific ingredient can inform themselves on the presence of this ingredient in a certain food product. In 2006, molluscs and lupine were added to the Annex through Commission Directive 2006/142/EC [1]. Currently, the list contains 14 allergenic ingredients.

For the surveillance of the compliance with recently established allergen labeling regulations by food safety authorities, specific and sensitive methods for the detection of allergens in foods are needed. These can also serve food-producing companies for the analysis of raw materials and for the surveillance of the production process in terms of allergen handling. Requirements focus on specificity, sensitivity, the applicability in a broad range of different foodstuffs and the suitability for the detection of the potential allergen in processed foods. Analytical methods targeting either proteins or specific DNA sequences are used for the detection of food allergens.

The polymerase chain reaction (PCR) is the method of choice for the detection of DNA sequences [2]. Specific primers - oligonucleotides with a nucleotide sequence that is complementary to their target sequence - enable the amplification of specific DNA fragments using a thermostable polymerase. Reporter dyes or fluorescing probes can be used for monitoring the increase in amplification products in real-time. The identity of these products can be verified by the use of oligonucleotide probes. When these probes hybridize to the amplified fragment due to a complementarity of the sequences,

they emit a signal. In TaqMan real-time PCR, a quencher and a reporter are bound to the opposite ends of the probe. When the polymerase hydrolyzes the probe during primer elongation, the fluorescence of the reporter is no longer suppressed by the quencher due to their spatial separation. Therefore, a fluorescence increase only occurs when the targeted specific DNA fragment is amplified. Therefore, this technique enables the development of highly specific assays.

The objective of this study was the development of a hybridization probe-based real-time PCR assay for the detection of lupine DNA in foods. This included the choice of an appropriate target sequence and the validation of the designed method in terms of specificity, sensitivity, its applicability in various matrices and importantly the evaluation of the method's suitability for the analysis of processed foods. Furthermore, the comparison with commercially available protein- and DNA-based detection kits was part of this thesis.

During the design of the real-time PCR method, particular attention was paid to the ability of the assay to detect a variety of lupine species while at the same time discriminating lupine DNA from DNA from closely related species. The specificity was tested by the analysis of DNA extracts from potential food ingredients such as legumes, cereals, seeds, nuts, spices, fruits and meat.

For the determination of the detection limit in complex food matrices, spiked foods were prepared and analyzed using the developed real-time PCR method. This included spiking experiments involving flour from three different lupine species for the determination of individual detection limits and the determination of the copy number of the real-time PCR target sequence in five lupine species and varieties.

Regarding the detectability of lupine DNA in processed foods, the impact of methods of food production, such as yeast fermentation or thermal treatment, was studied. Additionally, the effect of low pH values brought about by acidic ingredients on the integrity of the analyte was investigated. Model foods were prepared and analyzed to achieve this objective.

The comparison of the developed real-time PCR method for the detection of lupine DNA to an ELISA assay and to PCR-based detection kits for lupine regarding the de-

tectability of various lupine species and varieties, the method's specificity, the limit of detection in a food matrix and the applicability to complex and processed foods were also within the scope of this work.

An additional objective of this study was to investigate the suitability of the real-time PCR method for the quantitative determination of *Lupinus angustifolius*-flour in the range of 1 to 10 mg per kg wheat flour. The implementation of upper limits for the content of allergens in food that do not need to be labeled is to be expected. Therefore, quantitative methods for the determination of food allergens are needed. The quantification of *Lupinus angustifolius* flour in wheat flour was achieved employing statice (*Limonium sinuatum*) seeds as an internal standard that enabled the conversion of the detected lupine target sequence copy numbers to mg lupine flour per kg wheat flour.

2 Background

2.1 Food hypersensitivity

Food-related pathological symptoms include both toxic and non-toxic reactions. While toxic reactions are, for example, brought about by spoiled foods, non-toxic reactions are rooted in a food hypersensitivity. Figure 2.1 gives an overview on the categories of hypersensitivity.
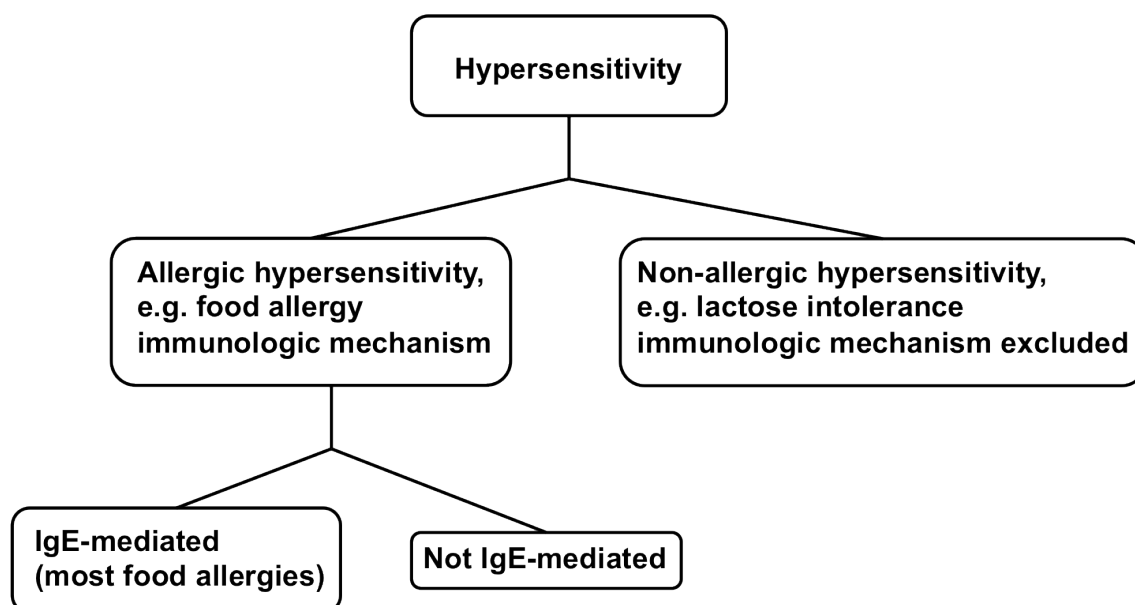
Figure 2.1: Overview on the categories of hypersensitivity (adapted from [3]). Food allergies are based on an immune reaction and are mostly IgE-mediated.

Lactose intolerance is an example for a non-allergic food hypersensitivity, that is, without involvement of the immune system. A deficit in lactase prevents the cleavage of the disaccharide lactose into the two monosaccharides glucose and galactose, which would normally be absorbed by the intestinal mucosa. Instead, the lactose remains in the gut and causes symptoms such as abdominal cramping [4]. Allergic hypersensitivities, in contrast, are based on an immune response to the eliciting antigen. The immune reaction is either cell-mediated or immunoglobulin E (IgE)-mediated [3]. The

majority of food-related immune reactions is IgE-mediated [5].

The molecular and cellular interactions leading to the allergic response include a sensitization phase and a manifestation phase [6]. Both are shown schematically in Figure 2.2.
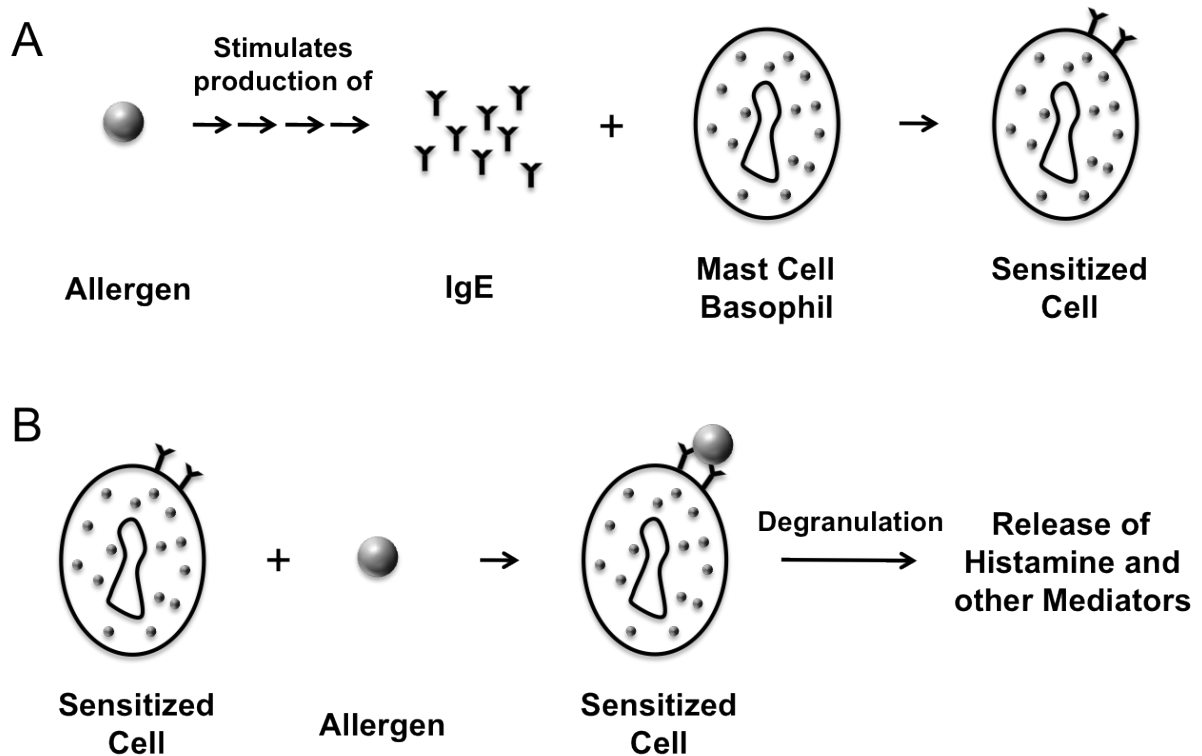
Figure 2.2: Mechanism of IgE-mediated allergic reactions (adapted from [7]). During sensitization (A), the allergen triggers the production of antigen-specific IgE, which is then bound to surface receptors of antigen-presenting cells. At the next encounter with the allergen in the manifestation phase (B), the antigen is linked to the receptor-bound IgEs and causes an allergic reaction.

During sensitization (Figure 2.2, A), antigen-presenting cells (APCs) present peptide fragments of the antigens and MHC (major histocompatibility complex) class II molecules to T cells [8]. Binding of this MHC-complex to T cells bearing the appropriate receptor triggers the proliferation of T cells and cytokine synthesis as well as the activation of TH2-like cells. The TH2-like cells, in turn, bind to B cells carrying receptors specific to the antigen and trigger the generation of antigen-specific IgE through

the production of interleukins (IL-4, IL-9, IL-13) [9]. The immune system is then pre-
pared for an allergic reaction by the binding of these specific IgEs to surface receptors
of mast cells, basophils, macrophages, and other antigen-presenting cells [8]. At this
stage, no allergic reaction is elicited. At the next encounter with the allergen the aller-
gic reaction becomes manifest (Figure 2.2, B). The antigen is linked to receptor-bound
IgEs, resulting in an immediate response caused by the release of mediators such
as histamine, N-acetylhexoamidase, proteases, leukotrienes or proinflammatory cy-
tokines [10]. The delayed response a few hours after the contact with the allergen is
also caused by inflammation-eliciting cytokines [6].

2.2 Food allergy

Two groups of food allergens can be distinguished: Class I food allergens are stable enough to pass the gastrointestinal tract, are absorbed by the intestinal mucosa and therefore cause sensitization after oral ingestion. Examples for class I food allergens are peanut, wheat, fish, cow's milk and soybean [11]. Class II antigens are pollen- or non-pollen-homologous allergens causing secondary sensitization to cross-reactive food allergens. A well-known example for class II food allergy is fruit allergy based on a sensitization to birch pollen [11]. This cross-reactivity can be explained by the high sequence similarity and the similarity of the 3D structures of the allergenic proteins from e.g. apple, hazelnut, cherry, carrot, celery and soybean to the major birch pollen Bet v 1 [11, 12]. In general, a sequence similarity of 50% or more indicates a possible cross reactivity. Cross reactions through similarities of the allergenic proteins can however also be induced by Class I antigens, for example between lupine, peanut and soybeans [11].

Possible manifestations of food allergy include oral, gastrointestinal, respiratory, cardiovascular and skin disorders [13]. The severity of the allergic reaction cannot be determined from previously incurred reactions [14] and may even include life-threatening anaphylaxis. In emergency departments in westernized countries, foods are the single leading cause for the treated anaphylactic reactions [13]. As reported by German pediatricians, 77% of anaphylactic reactions in children were caused by food allergens [15]. Adverse reactions to food change with age and vary between countries. In contrast to some European countries, peanut is the most frequent elicitor of food allergies in the US and the UK [16], hereby reflecting the relevance of the respective food in the different regions. In coastal areas, for example in Hong Kong, fish or crustacea allergies are more frequent than in countries with few or no coastal regions [17], while Israeli children often show allergic reactions to sesame [18]. Hence, the overall percentage of allergic individuals can only be estimated. Additionally, the prevalence value varies depending on the method of data collection used: In a meta-analysis of 51 studies on allergy to milk, egg, peanut and seafood, self-reported allergy ranged from 3% to 35%.

The prevalence estimates from 6 oral food challenge studies, however, were only 1 to 10.8%. In industrialized countries, approximately 3 to 4% of adults and 5% of young children suffer from food allergies, which appear to have increased in prevalence [9]. From 1997 to 2007, childhood food allergy increased by 18% in the United States [9]. The reasons for the rise in prevalence are yet unknown. However, some explanations have been suggested. The argument of the so-called "hygiene hypothesis" is that the observed increase in asthma and allergic diseases is caused by the reduced exposure to microbes through higher overall hygiene standards, which hinders the proper maturation of the immune system [19]. Other explanations are the decreased consumption of micronutrients possibly relevant to immune maturation due to the use of highly refined ingredients in food products [20] and the increase in the number of food ingredients [21].

In principle, any food can elicit an allergic reaction in sensitive individuals. However, the following foods cause the most reactions: milk, egg, peanut, tree nuts, shellfish, fish, wheat and soy [22]. Especially peanut [23, 24, 25], tree nuts [26, 27] and also sesame frequently cause anaphylactic reactions [18, 28, 29, 30].

Major food allergens are water-soluble glycoproteins with a size of 10 to 70 kDa [9]. Most of them belong to a few predominating protein families or superfamilies: 29 protein families were identified which contained at least one allergen [31]. 65% of all plant food allergens are a member of either the prolamin superfamiliy, the cupin superfamily, the Bet v 1 family or the profilins [12]. Three protein families - the tropomyosins, parvalbumins and caseins - predominate for food allergens of animal origin [12]. In the following, examples for allergens contained in the relevant protein families are given.

The *prolamin superfamily* contains gliadins, glutenins, α-amylase inhibitors and protease inhibitors from cereals as well as 2S albumins [12] and non-specific lipid transfer proteins (LTPs) that are major allergens, e.g. in tree nuts. Examples for allergens from the *cupin* superfamily are the vicilins with proteins from peanut (Ara h 1 and Ara h 3), walnut (Jug r 2), sesame (Ses i 3), soybean (glycinin), brazil nut (Ber e 2) and buckwheat (Fag e 1) [32]. *Bet v 1*-related proteins are found in a variety of fruits, such as apple, pear, cherry and kiwi; nuts, e.g. hazelnut; and vegetables (carrot, celery,

parsley). They exhibit a high similarity to pollen proteins from trees of the botanical order *Fagales* (e.g. birch) inspite of their in part rather distant botanical relation. The primary sensitization is often caused by the pollen allergens. Later on, sensitized individuals develop allergic symptoms after ingestion of foods from the groups mentioned above [12]. Sensitization to *profilins* increases the risk to develop multiple pollen-associated food allergies because of the high structural and sequence similarity of these proteins, which accounts for IgE cross-reactivity [32]. While this cross-reactivity does not necessarily cause clinical symptoms, an association between profilin sensitization and clinical allergy to citrus fruits, banana, tomato [33], melon [33, 34] and orange [35, 36] was observed. *Tropomyosins* from invertebrates, e.g. crustaceans and molluscs, are highly cross-reactive food allergens. *Parvalbumin*, a Ca^{2+}-binding protein relevant for muscle relaxation, is the major allergen in fish [12]. *Caseins*, allergenic proteins from mammalian milk, show IgE cross-reactivity between caseins from cow's milk and caseins from goat and sheep milk [37].

The potential of allergenic proteins to elicit adverse reactions can change through modifications occurring due to food processing. Thermal treatment, for example, can both reduce as well as increase the allergenicity of the respective protein: while frying or boiling of peanuts decreases the allergenic potential of Ara h 1, Ara h2 and Ara h 3 as expected, roasting causes an increase in peanut allergenicity [38, 39]. This is in accordance with the fact that the prevalence of peanut allergy is lower in China, where peanuts are mainly consumed boiled or fried rather than roasted like in the United States [39]. Some allergenic proteins are remarkably stable in regard to heat treatment. For lupine protein, a reduction in IgE binding capacity was only observed after autoclaving a raw lupine seed extract for at least 20 min at 138 °C and a pressure of 2.56 atm [40]. After 30 min of autoclaving, however, a new IgE-binding band (70 kD) was observed. While these conditions are unusually harsh, they again illustrate that normal food processing conditions do not necessarily result in reduced allergenicity. In composed foods, however, matrix effects play an important role. When lupine-containing foods instead of raw seed extracts were heated, a decrease in the capacity of the respective lupine proteins to bind IgE was observed [41]. The allergenic potential

of lupine proteins can also be lowered by abrupt pressure changes. When moistened lupine seeds were exposed to steam pressure (up to 6 bar) for a maximum of 3 minutes followed by a sudden pressure drop, modifications of the protein patterns were observed by SDS-PAGE analysis [42]. These changes corresponded to a decrease in IgE-binding capacity. Treating proteins from legumes with enzymatic hydrolysis using pepsin and trypsin also decreased their allergenic potential [43, 44].

Currently, the prevention of allergic reactions can only be accomplished by the strict avoidance of the respective allergenic food or foods [45]. Sensitive individuals therefore need to read labels carefully and be aware of the potential presence of the offending food in the respective establishment when eating out. At home, cross-contact of other foods and the allergy-eliciting food should be avoided by using separate knives, cutting boards etc. [9].

2.3 Allergen labelling

For the above mentioned reasons, allergic consumers rely on the information found on labels of foods [45]. In 2003, Directive 2000/13/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs [46] was amended by Directive 2003/89/EC [47] regarding the indication of the ingredients present in foodstuffs. For better information of all consumers and for the protection of the health of sensitive consumers, it was made obligatory to include potentially allergenic substances present in the foodstuff in the list of ingredients. Annex IIIa of the Directive included the following foods, ingredients and other substances recognized as causing hypersensitivity:

- Cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, kamut or their hybridized strains) and products thereof

- Crustaceans and products thereof,

- Eggs and products thereof,

- Fish and products thereof,

- Peanuts and products thereof,

- Soybeans and products thereof,

- Milk and products thereof (including lactose),

- Nuts i. e. Almond (*Amygdalus communis* L.), Hazelnut (*Corylus avellana*), Walnut (*Juglans regia*), Cashew (*Anacardium occidentale*), Pecan nut (*Carya illinoiesis* (Wangenh.) K. Koch), Brazil nut (*Bertholletia excelsa*), Pistachio nut (*Pistacia vera*), Macadamia nut and Queensland nut (*Macadamia ternifolia*) and products thereof,

- Celery and products thereof,

- Mustard and products thereof,

- Sesame seeds and products thereof,

- Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/L expressed as SO_2.

Referring to the potential severity of allergic reactions to lupine-containing foods and to the possible cross-allergenicity in peanut-allergic sufferers [48], labelling of lupine-containing foods was made mandatory by Directive 2006/142/EC [1], which also included mollusks in the list of allergenic ingredients.

These 14 allergens, however, need only to be mentioned on the label if they are deliberately added to the food. The only measure allergic individuals can take to minimize their risk - the strict avoidance of the offending food - proves difficult, since small amounts of allergens can be adventitiously brought into food products through unintentional cross contact during manufacturing processes [49]. The following examples illustrate the problematic of "hidden allergens" in foods: A consumer allergic to cow's milk reacted to dark chocolate sprinkles, which were not declared to contain milk proteins [45]. Twenty-two percent of allergic reactions in Spanish adults were found to be caused by hidden allergens [50]. When cookies and chocolates without declaration of peanut or hazelnut from different European countries were analyzed, 11% of the cookies and 25% of the chocolates contained peanut proteins. Hazelnut protein was present in 25% of the cookies and 53% of the chocolates [51].

For liability reasons, the precautionary labelling of foods with statements such as "may contain ..." is on the rise [52], irrespective of the actual risk that emanates from the considered food for allergic consumers. For this reason, the allergic consumers' dietary choice is unnecessarily restricted. A measure all stakeholders would benefit from would be the implementation of upper limits for the content of allergens in food that do not need to be labeled. These limits need to be based on clinical threshold data and to take the NOAEL (no observed adverse effect level) for each of the considered allergens into account. Regulatory thresholds of this kind could provide food allergic consumers with important information on the risk emanating from certain products by reducing unnecessary precautionary labelling and could constitute a basis for decisions in the management of food allergens in the manufacturing industry [49]. In

addition, food safety authorities would profit from the increased legal certainty regarding the assessment of food labels in respect to allergens. The "food allergy" working group of the German Society for Allergology and Clinical Immunology and of the Association of German Allergologists proposed upper limits of 10 to 100 mg/kg of the allergenic food or 1 to 10 mg/kg of the protein fraction, depending on its allergenicity, that are thought to protect most allergic consumers from severe allergic reactions [53].

2.4 Lupines

The genus *Lupinus* from the *Leguminosae* family comprises a broad range of species. Several of them have been cultivated in the Andean highlands and around the Mediterranean Sea since ancient times for use in the human diet [54]. Their suitability for crop rotations, which is based on the enrichment of the soil with nitrogen, was already noted by Virgil (70 - 19 BC) (as cited in [55]): "Or, changing the season, you will sow there yellow wheat, whence before you have taken up the joyful pulse, with rustling pods, or the vetch's slender offspring and the bitter lupin's brittle stalks and rustling grove." Since lupines can be cultivated under a variety of climatic conditions, they are a more easily available and therefore a cheaper protein source than other legumes. However, the naturally occurring bitter lupines contain 5 to 40 grams of alkaloids per kilogram [55] and can only be consumed after cooking and subsequent extraction with a hypertonic aqueous salt solution [11]. In the 1920s, after screening 1.5 million lupine plants, von Sengbusch discovered five mutant lupines (yellow and blue) containing only 0.5% alkaloids [56]. Varieties with low alkaloid contents - the so-called "sweet lupines" - were obtained from these mutants by subsequent breeding. They contain less than 200 mg alkaloids per kilogram [57]. Today, species such as *Lupinus albus* (white lupine), *Lupinus angustifolius* (narrow-leaf or blue lupine), *Lupinus luteus* (yellow lupine) and *Lupinus mutabilis* (pearl lupine) are of agricultural importance. While sweet white and yellow lupines were first used as a crop plant in Germany because of a shortness of protein-rich food due to World War I and are today found in the Mediterranean area and Africa (white lupine) and Central Europe (yellow lupine), blue lupines are being cultivated in Australia since the 1950s [11, 55]. In the past ten years, 85% of the world production of lupines originated from Australia [58]. *Lupinus mutabilis* is cultivated in South America [11].

Lupine seeds contain 30 - 40% protein [55]. The nutritional value of the protein fraction is higher than in beans or peas, but lower than in soy beans [11]. The biological value of *Lupinus albus* protein was found to be 91% of that of egg protein [59]. As the amino acid composition of lupine protein is complementary to that of cereal protein, blends

with increased biological value of the protein fraction can be produced. Products such as pasta, bread, crackers, or cookies made from wheat flour enriched with about 10% of lupine flour show good consumer acceptance [54].

The oil content of lupine seeds ranges from 4 to 15%, of which about 70 to 80% are unsaturated fats. Accordingly, the main fatty acids are oleic acid (50% in *Lupinus albus* and *Lupinus mutabilis*) and linoleic acid (35-50% in *Lupinus angustifolius* and *Lupinus luteus*). The ratio of omega-6 fatty acids to omega-3 fatty acids is comparable to that of soy oil [55].

Lupines contain a number of anti-nutritional factors. The contents of phytate, tannins, saponins and oligosaccharides are low. An advantage of lupines over other grain legumes, for example soy beans, is their low content in protease inhibitors and lectins [55, 60]. The alkaloids present in higher amounts in the wild bitter lupine species are the elicitors of "lupinism". The symptoms of this alkaloid intoxication are restlessness, spasms, shortness of breath and somnolence. In case of an respiratory arrest, the poisoning can be fatal [61]. The seeds of modern sweet lupine varieties, however, are safely edible due to their low alkaloid content.

Lupines are phytoestrogen-free [62] and do not contain gluten. Lupine flour is therefore used as an ingredient in products for wheat allergic and celiac patients [63]. It is also a valuable protein source for vegetarians and for milk allergic individuals [63]. In addition, ingredients made from lupine can substitute for soy in products that are to be produced without the use of genetically modified organisms. Besides these advantages of lupines, their consumption has been linked to various health benefits. These include triglyceride and plasma cholesterol lowering effects [62, 64, 65]; anti-atherogenic action [66], inhibition of the angiotensin converting enzyme (ACE) [67], anti-hypertensive properties [68, 69]; and a blood glucose lowering effect [70, 71].

In addition to these beneficial nutritional aspects, lupine-based ingredients show valuable technological properties. They have high water binding and emulsifying capacities [11] and improve color, texture and taste of the respective product [72]. For all these reasons, the use of lupine-based ingredients has increased and lupine has become a common ingredient in food products [73]. Lupine flour is for example added to bakery

15

products and pasta, and lupine proteins can be used as a an addition to meat products or as a base for meat substitutes, tofu-like products and for sauces that resemble soy sauces in flavor and texture [55]. Last but not least, lupine seeds are traditionally consumed as snack food ("lupini"). Like soy sprouts, lupine sprouts can be used for salads and other dishes [55].

Lupine-based ingredients, however, can elicit allergic reactions in sensitive individuals. The majority of allergenic proteins of legumes are a member of the following four protein superfamilies and families: cupins, prolamins, profilins and parthogenesis-related proteins, the first two of them containing seed storage proteins [74]. The conglutins found in lupine are seed storage proteins: the 11S or legumin-like α-conglutins and the 7S or vicilin-like β-conglutins [75]. In 2008, the *Lupinus angustifolius* β-conglutin has been named Lup an 1 by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee [76]. Two other groups of conglutins found in lupine are the γ- and δ-conglutins [77]. IgE binding to all lupine conglutins was observed by immunoblotting and ELISA experiments using sera from lupine allergic patients [41]. The results suggested a particularly strong allergenicity of α-conglutin and the presence of cross-reactive allergens in peanut and almond. Peanut sensitive individuals are at high risk to cross-react with lupine [48]. In a recent study, 82% of the peanut-sensitized patients were also sensitized to lupine [78]. Other legumes, such as soybeans, lentils, beans, chickpeas and peas, may also cause cross-reactions to lupine [11].

While lupine allergy can occur as a result of cross-reactivity in, for example, peanut sensitive individuals, it can also emerge by primary sensitization [79, 80]. The first case of lupine allergy was reported in 1994 [81]. A 5-year-old child sensitive to peanut developed urticaria and angioedema after ingesting pasta fortified with lupine flour. Since then, various cases of lupine allergy have been reported, including contact urticaria [82] and respiratory symptoms [83] that mostly occurred under occupational conditions [84, 85, 86]. In 1999, anaphylaxis due to lupine was described the first time [87]. Whether lupine allergy can evolve from inhalant sensitization to lupine pollen is yet unclear [48, 11]. The same holds true for sensitization via the skin, which has been

16

observed for peanuts [11].

No data on the overall prevalence of primary lupine allergy are available [88]. In a double-blind, placebo-controlled food challenge with peanut-sensitized patients the eliciting dose (ED) for subjective allergy symptoms was determined to be 0.5 mg lupine flour. This is very low, since it is only five times the ED of peanut. The no observed adverse effect level (NOAEL) was 0.1 mg.

2.5 Analytical methods for the detection of allergens in foods

For the analysis of raw materials in food companies as well as for the surveillance of regulatory labelling requirements by food safety authorities, specific and sensitive methods for the detection allergens in foods are needed. The most frequently used methods for food allergen analysis can be classified into DNA-based and protein-based techniques.

2.5.1 Protein-based methods

Enzyme-linked immunosorbent assays (ELISAs) detect the allergenic protein or a marker protein for the respective species using an antibody [89]. For the analysis of allergenic proteins in foods, serums derived from animals immunized with the respective allergen are used. These serums contain allergen-specific immunoglobulin G (IgG) [10] in contrast to immunoglobulin E (IgE), which causes the allergic reaction in sensitive humans.

The principle of sandwich ELISA assays is shown in Figure 2.3. The antigens extracted from the samples bind to the first antibody, which is linked to the wells of a microtiter plate (Figure 2.3, A). After the removal of free analyte molecules, the bound antigens are detected using a second, enzyme-linked antibody (Figure 2.3, B). After the addition of a chromogenic substrate, the amount of coloured product is measured photometrically. The colour intensity is directly proportional to the concentration of the analyte in the sample. Quantification can be achieved using a standard curve.

Figure 2.4 illustrates the principle of competitive ELISA assays. The wells of the microtiter plate are coated with antigens (as opposed to the antibody-coated wells used for sandwich ELISA assays). Together with the antigens extracted from the samples, a defined amount of antibodies is added to the plate (Figure 2.4, A). The bound antigens and the antigens originating from the samples therefore compete for the antibody molecules. After the removal of unbound sample antigens, the antigen-antibody-

A

Fixed antibodies   Antigens

B

Substrate   Reporter signal
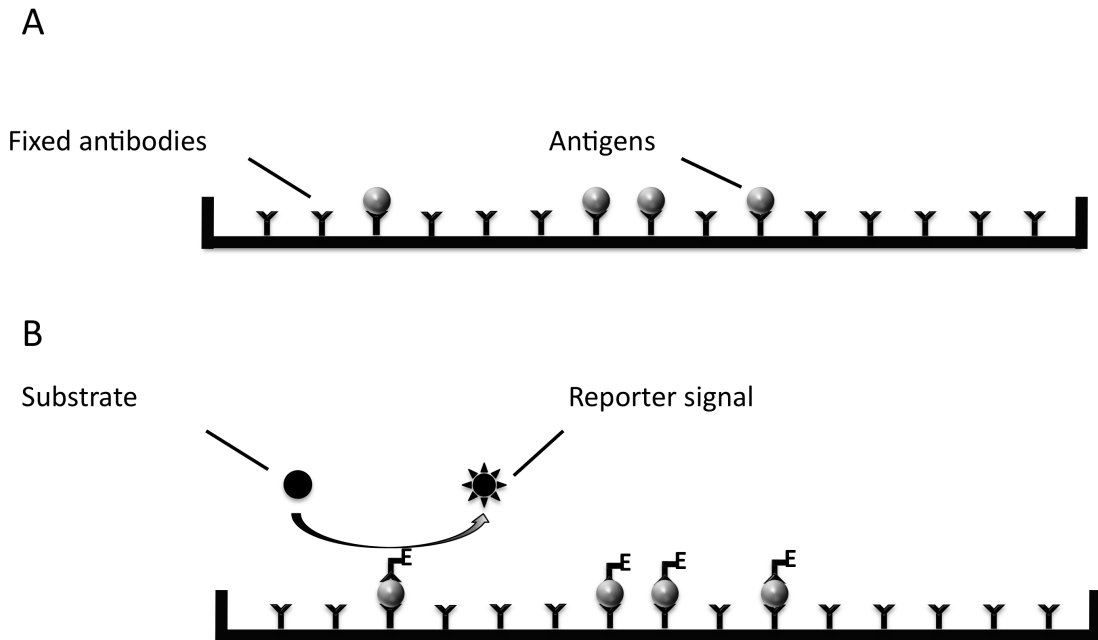
Figure 2.3: Principle of sandwich ELISA assays. Antigens from the sample are bound to the immobilized first antibody (A). After binding of a second, enzyme-linked antibody and the addition of a chromogenic substrate, the amount of coloured product is measured photometrically (B). The colour intensity is directly proportional to the concentration of the analyte in the sample.

complex is detected using a second, enzyme-linked antibody and the subsequent colour reaction (Figure 2.4, B). The concentration of the analyte is indirectly proportional to the colour intensity and can be determined using a standard curve. Competitive ELISA assays are better suited for the detection of small molecules than sandwich ELISA assays, because only one epitope is recognized by the antibody. Sandwich ELISA assays, however, have a higher specificity due to the detection of at least two different epitopes of the analyte [90].

Cross-reactivity, for example to proteins of closely related species, or non-specific bind-
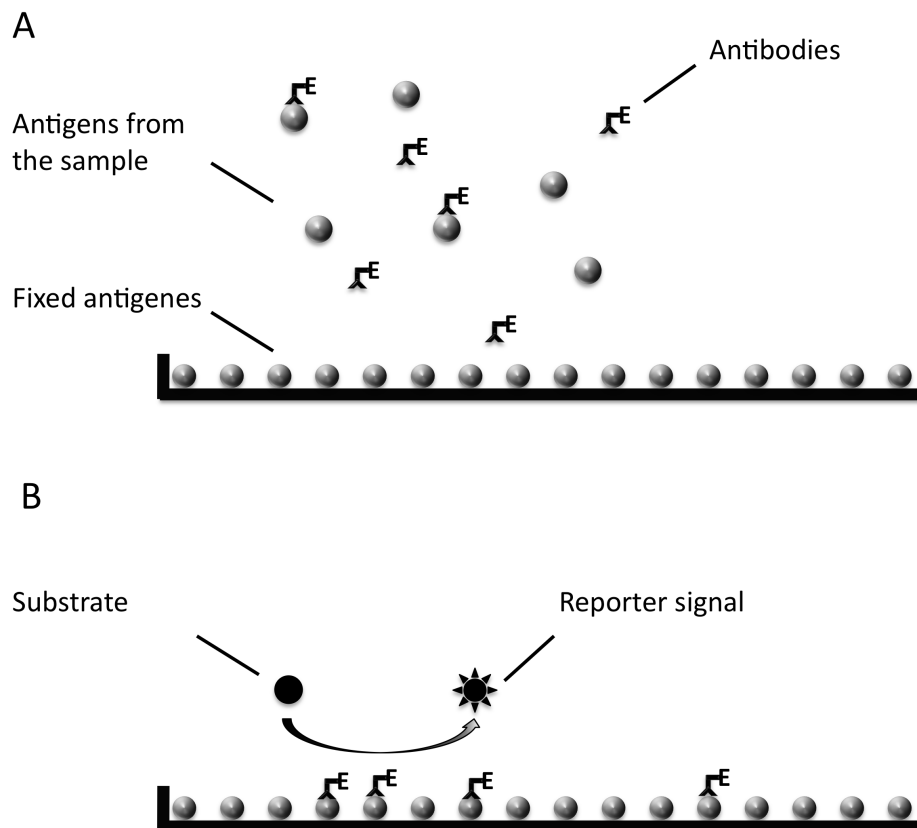
Figure 2.4: Principle of competitive ELISA assays. The fixed antigens and the antigens from the sample compete for enzyme-linked antibodies (A). After the removal of unbound antigens and antigen-antibody complexes, a chromogenic substrate is added (B). The subsequently detected colour intensity is indirectly proportional to the concentration of the analyte.

ing of the antibodies to other food components can result in false positives. In addition, food processing affects the detectability of the analytes because of structural changes. One attempt to overcome these drawbacks is the use of liquid chromatography (LC) in combination with mass spectrometry (MS): The peptides resulting from the enzymatic digestion of the allergens can be detected without an intermediary detection agent using MS/MS. This method enables the direct identification of allergens and is not dependent on the particular three-dimensional structure of the allergenic protein as ELISA assays are [10]. The quantification of allergens can be achieved using standard mate-

rials, which should resemble the analyte as much as possible. The allergen itself can serve as an external standard, with the advantage that in this case no response factor needs to be determined. When an internal standard is preferred, a synthetic analyte tagged with stable isotopes is the ideal choice [10]. The selection of target peptides that are abundant in foods even after processing enables the detection of allergens in processed foods [91]. Another advantage of the availability of MS-based allergen detection methods is the possibility to offer "allergen free" foods [10], provided that the reliability of the assays is high enough and that they are suited for routine analysis. In contrast to the widely used ELISA- and PCR-methods, MS-based detection methods are not yet regularly employed.

2.5.2 Rapid immunochemical detection methods

The costly equipment and the amount of time and training needed for the allergen detection methods currently in use give reasons for the development of rapid and easy-to-handle assays for the detection of food allergens which can, for example, be applied routinely by manufacturers. These methods include rapid ELISA assays, lateral flow assays and dipstick tests [92]. Rapid ELISA assays give semiquantitative results within 30 to 60 min. Lateral flow assays and dipsticks function in the same way as ELISA assays, but are portable because instrumentation such as a microplate reader is not needed since they are read out visually. The results, which are usually obtained within a few minutes, are however qualitative or semiquantitative unless a test strip reader is used [92].

2.5.3 DNA-based methods

Analytical methods applied in the field of food allergen testing should be capable of detecting the ingredient under consideration in the low mg/kg range [2]. Currently, polymerase chain reaction (PCR)-based methods, which target species-specific DNA sequences, offer the best options for the sensitive detection of food allergens [2]. They show detection limits in the required range and the extracted DNA can be used for the detection of more than one species, while protein-based methods require specific

21

sample extractions for different targets. The principle of the polymerase chain reaction, which consists of a succession of three steps that is repeated 25 to 45 times, is shown in Figure 2.5. Higher cycle numbers enable the amplification of small amounts of target DNA, however, the number of unspecific PCR products rises as well [93]. The ideal number of cycles therefore depends on the specific assay.

In the first step of the polymerase chain reaction, the doublestranded DNA is denatured for 30 to 60 s at 94 - 96 °C. At a temperature dependent on the melting temperature of the primers (usually between 45 °C and 65 °C), they anneal to the complementary sequences on the now singlestranded target DNA. The specificity of the PCR depends on the ability of the primers to anneal properly to the target sequence and only to the target sequence. If the primers do however not anneal effectively or not at all, for example due to mismatches or an improperly chosen annealing temperature, unspecific amplificates or no PCR products result [93]. In the third step of the PCR, a heat-resistant polymerase elongates the annealed primers from their 3'-ends with the deoxyribonucleotides (dNTPs) that are complementary to the DNA strand the primer is bound to. The elongation is carried out at the optimal working temperature of the polymerase, which is 72 °C. At the end of each three-step cycle and at an ideal efficiency, the number of target molecules has doubled compared to the starting number. Therefore, repeating the cycle multiple times results in an exponential amplification [93].

In qualitative PCR, the amplification products are separated in an agarose gel and visualized under UV light using DNA staining reagents, for example ethidium bromide. In contrast to this end-point detection, the progress of the PCR can also be monitored in real-time using fluorescent dyes or fluorogenic probes [93]. While fluorescent intercalating dyes interact with doublestranded DNA regardless of its specific sequence, hybridization probes need to bind to their complementary sequence on the target DNA before a fluorescence signal is emitted. The verification of the sequence of the amplification products is therefore part of hybridization-probe based real-time PCR assays. Figure 2.6 illustrates the mechanism of this assay type. After the denaturation of the DNA, the annealing of the primers and the probe, the extension of the primers and the detection of the emitted fluorescence signal are carried out in a single step at 60

22
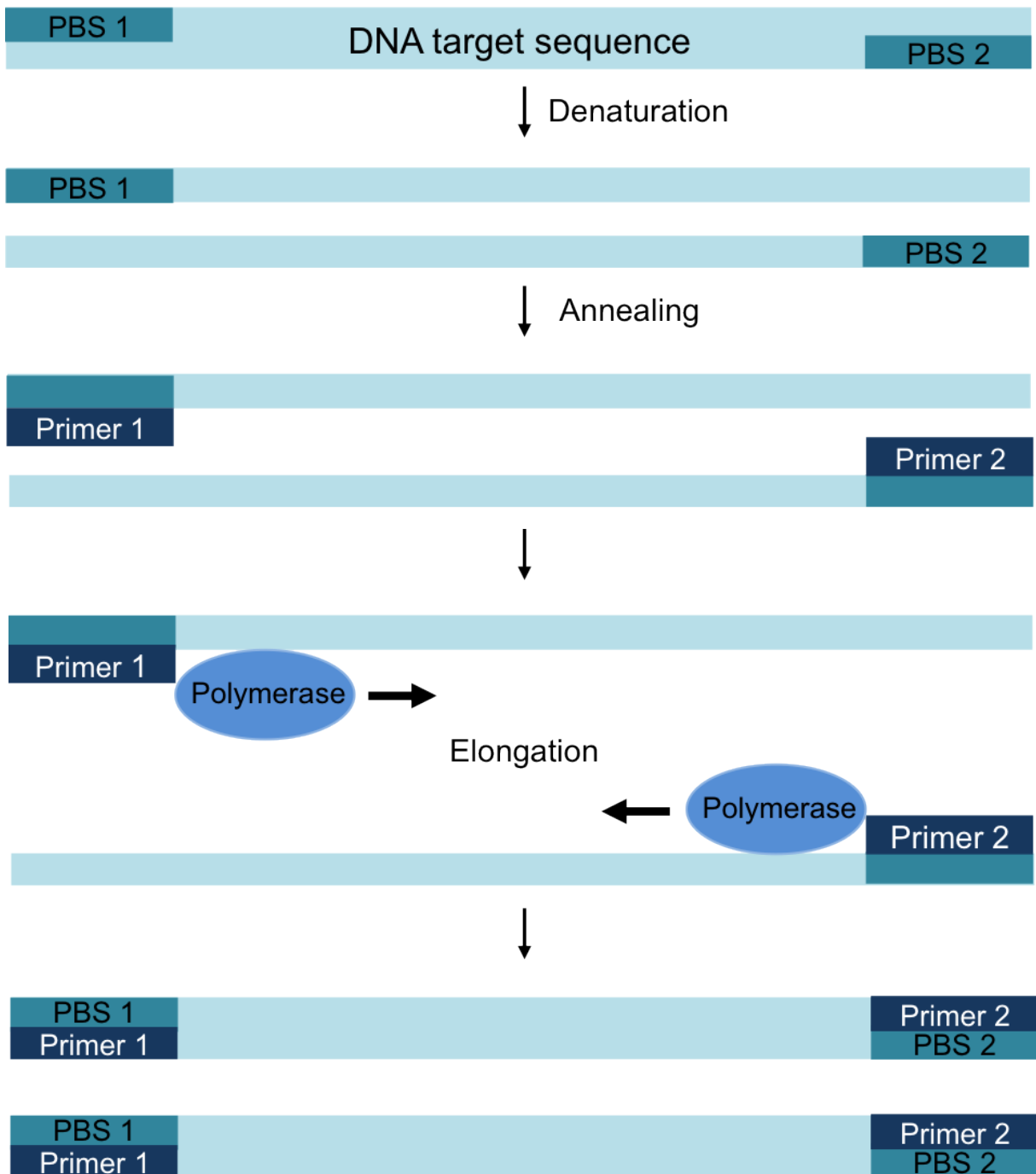
Figure 2.5: Principle of the polymerase chain reaction. Each of the cycles consists of the three steps denaturation, annealing and elongation. After the denaturation of the target sequence, the primers anneal. During the elongation phase, they are extended by the polymerase using free deoxyribonucleotides (dNTPs), resulting in two doublestranded DNA fragments from one template. Repeating the cycle multiple times results in an exponential amplification.
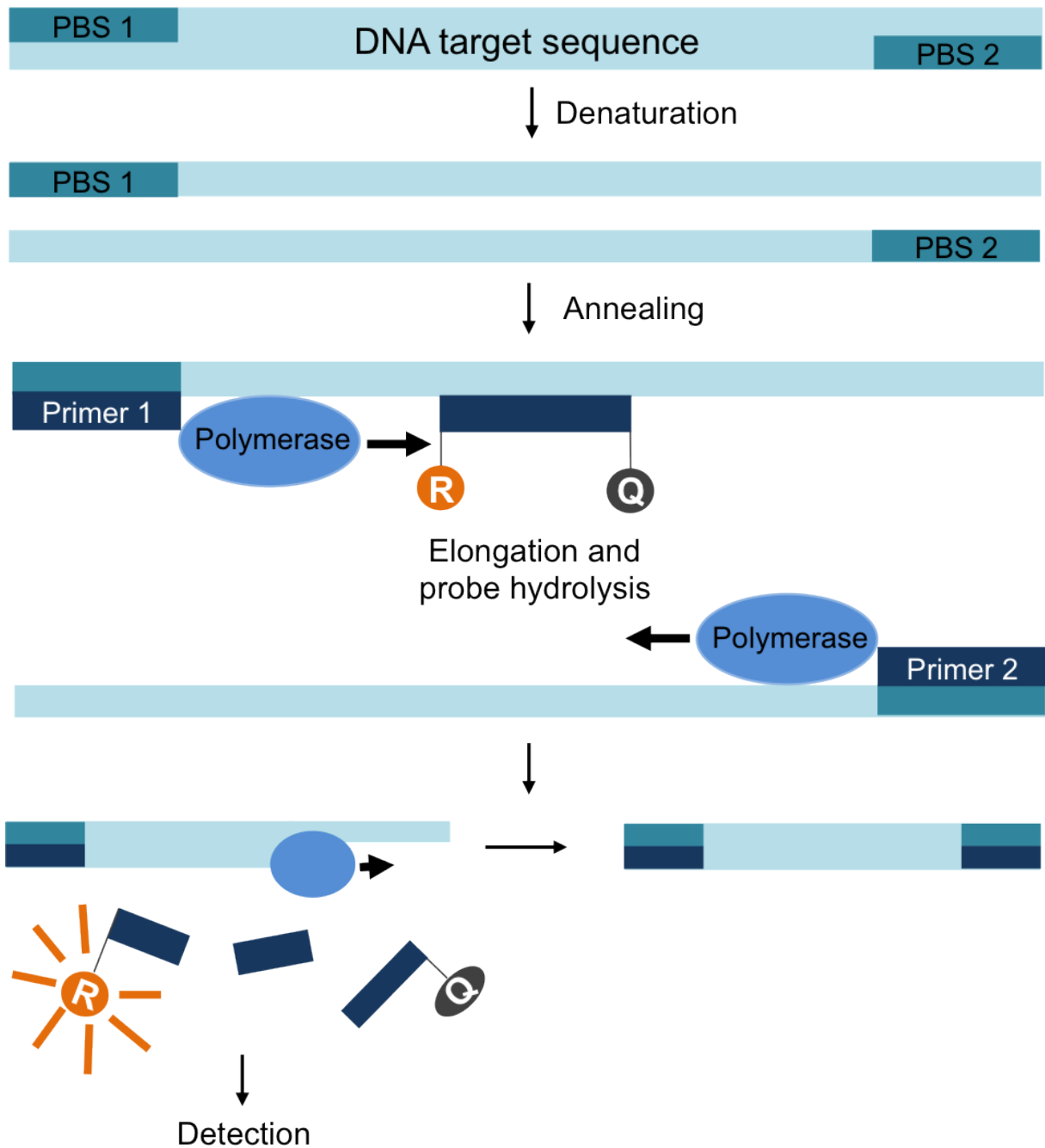
Figure 2.6: Mechanism of the hydrolysis-probe based real-time PCR. After the denaturation of the target DNA, the primers and the probe anneal to their complementary sequences. Due to the close location of the quencher to the reporter dye, no fluorescence is emitted. A fluorescence signal can only be detected after the extension of the primers and the hydrolysis of the probe by the polymerase. The signal increases proportionally to the increase in the number of amplification products.

°C. A fluorescent reporter dye R is bound to one end of the probe oligonucleotide. As long as the probe is intact, the reporter dye's fluorescence is suppressed by the nearby quencher Q. During primer extension, the hybridized probe is hydrolyzed due to the 5'-exonuclease activity of the polymerase. When the reporter dye and the quencher are separated, a fluorescence signal becomes detectable. This is only the case if the sequence-specific probe has annealed to the target DNA, thus improving the specificity of the method. As shown in Figure 2.7, the fluorescence signal increases proportionally to the increase in the number of amplification products [2] over time. The cycle number needed to exceed a specified fluorescence threshold is called the cycle threshold (Ct) value. The higher the amount of target DNA in a sample, the lower the Ct value, since less cycles are needed to exceed the amount of amplification product that corresponds to the fluorescence threshold.
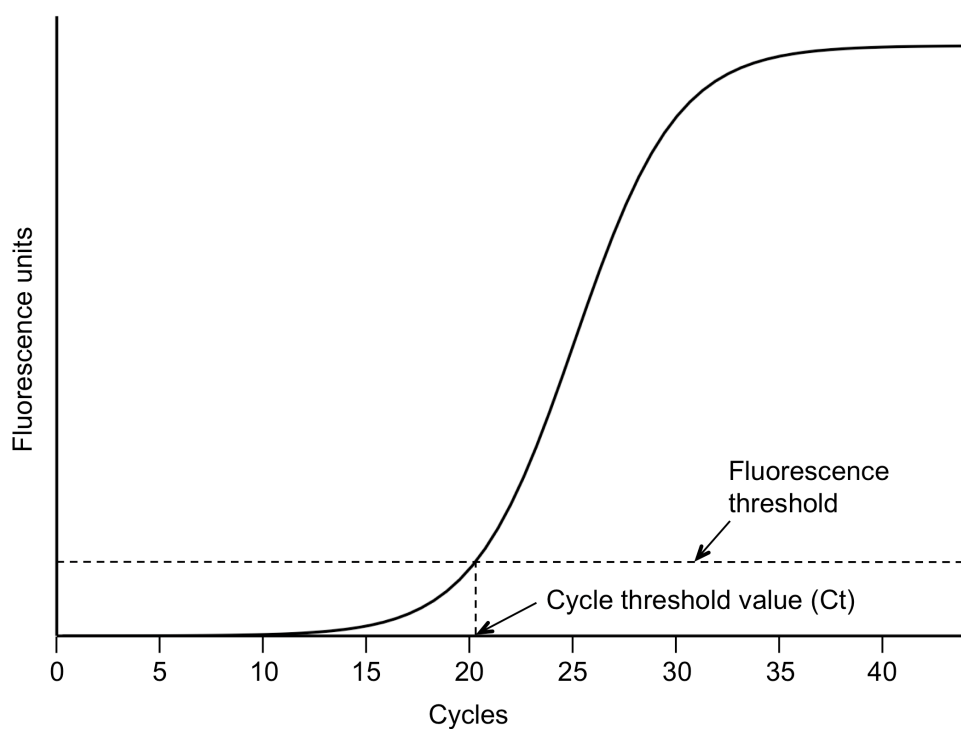
Figure 2.7: Real-time PCR amplification curve obtained by plotting the intensity of the fluorescence signal of the reporter dye against the cycle number. The cycle threshold value (Ct) can be determined after the definition of a fluorescence threshold.

2.5.4 Comparison of ELISA- and real-time PCR methods for the detection of allergens

While the sensitivities of ELISA- and real-time PCR methods are comparable, a higher specificity can often be obtained with PCR assays. A careful selection of the target sequence enables the exclusion of similar DNA sequences, for example from closely related species, from amplification. ELISA assays, in contrast, often exhibit cross-reactivity of the antibodies to structurally similar proteins. False-negative results in processed products caused by structural changes of the analyte are another drawback of the ELISA method. In contrast to proteins, DNA is more heat-resistant, which makes PCR the method of choice for processed food samples. When samples with low pH are to be analyzed, ELISA assays are better suited due to the higher stability of proteins towards acidic ingredients. In addition, milk and egg proteins can only be determined using ELISA [93], since the DNA contained in egg and in chicken meat, for example, is the same. The presence of chicken meat in an egg-free food would therefore cause a false-positive PCR result when testing for the presence of egg. The variability between different ELISA kits is however higher than between different PCR methods, which is caused by the use of different target proteins for the different ELISA kits [94]. In addition, a DNA extract can be used for the detection of a number of analytes, while each ELISA kit has its specific protocol for the extraction of the particular analyte from the sample. This also results in the usage of more sample material when, for example, a food needs to be analyzed for more than one allergen. Special equipment such as a thermocycler is however needed for PCR, while standard laboratory equipment is sufficient for ELISA assays. The decision for one of the both methods is therefore dependent on the type of the sample and on the respective analyte. It should be noted that ELISA kits often detect a marker protein for the allergenic species rather than the allergenic protein itself, as it is also the case for the detection of DNA from an allergenic organism using a PCR method. Therefore both methods do not directly detect the allergen itself. In conclusion, ELISA and PCR are complementary methods and verified results are best obtained by their combination, if feasible.

2.5.5 Influence of food processing conditions on the detectability of allergens using PCR

Most food products pass several manufacturing steps upon production, which may influence the integrity of the analyte [95]. These range from simple mechanical treatments, such as milling, to complex chemical or enzymatic reactions. The degradation rate of DNA is especially dependent on the influence of heat and low pH values. The detectability of a long (1914 bp) DNA fragment is negatively affected by thermal treatment, with the effect being most pronounced under acidic conditions [96]. Baking of salmon fillets for 10 minutes reduced the number of detectable Sal s1 gene copies to about 50% [97]. When the α-conglutin sequence from *Lupinus angustifolius* in spiked cookies was analyzed by real-time PCR, rising cycle threshold (Ct) values were observed that corresponded to increased baking times [98]. Besides heating, fermentation is another important procedure applied in food and feed production. For example, lactic acid fermentation takes place during the ensiling of maize, where it leads to a lowering of the pH. Consequently, a significant decrease in DNA fragment length was observed by agarose gel electrophoresis after 106 days of ensiling of conventional and transgenic Bt (*Bacillus thuringiensis*) maize [99]. This illustrates the importance of using short targets for reliable PCR methods. However, even for real-time PCR systems optimized for the use with processed foods by aiming at short fragments, a significant rise in Ct value has been observed after thermal treatment [100].

2.5.6 Methods for the detection of lupine in foods

So far, four enzyme-linked immunosorbent assays (ELISA) for the detection of lupine protein in foods have been published [73, 101, 102, 103]. The sandwich ELISA developed in 2005 showed lower sensitivity toward unprocessed lupine protein compared to processed lupine protein and slight cross-reactivity with other legumes [101]. A recently described polyclonal-monoclonal-based sandwich ELISA also showed positive results for almond, cashew, pumpkin seed, sunflower seed, and roasted hazelnut [73]. According to the authors, this is probably due to the detection of a protein structure

that occurs with high resemblance in each of the species tested positive. Recently, a sandwich ELISA for the detection of lupine in foods with a limit of quantification of 1 mg/kg has been developed [102] that exhibits a minor cross-reactivity to soybean and black bean. Another sandwich ELISA was developed by Ecker et al. in 2012 [103]. It enables the detection of 1 mg/kg lupine flour in raw noodle or biscuit dough as well as in the noodles and biscuits themselves. The specificity was tested with 34 plant species that could be used as ingredients in lupine-containing foods. No cross-reactivity was observed.

In 2009, a real-time PCR method using SYBR green and targeting the γ-conglutin gene has been published [104]. No cross-reactivity was observed with the tested DNA from other potential food ingredients. The sensitivity of the assay enabled a detection of 7 pg of lupine DNA per reaction.

Two hybridization probe-based real-time PCR methods targeting the α- and δ-conglutine sequences, respectively, were recently developed [98]. The authors compared the two assays regarding their specificity and sensitivity. Using DNA from related legumes or other edible plants as well as from animals, no amplification was observed with any of the methods. A limit of detection of 10 mg/kg in a cookie matrix was determined for both assays, while the sensitivity of the α-conglutin method was 0.5 ng of lupine DNA per reaction as compared to 0.05 ng for the δ-conglutin method.

Of two commercial kits for the detection of lupine DNA using PCR available in 2008, one employed a conventional PCR with subsequent agarose gel electrophoresis [105]. Its main disadvantage is the high limit of detection (1% according to the manual), which is not suitable for the detection of allergens in clinically relevant amounts. In addition, a verification of the amplification products is missing. Details on the specificity of this assay have not been provided.

The second kit is a SYBR-green based real-time PCR assay [106]. It shows a remarkably lower limit of detection (<10 copies), but also lacks a verification of the amplicon sequence because of the employment of an intercalating flourescent dye. According to the specificity data given in the manual, the primer pair from the kit is specific for lupine DNA, but was tested with only three different lupine species.

3 Materials and Methods

3.1 Materials

3.1.1 Lupine seeds and lupine flour

Seeds of all lupine varieties that were available at that time at the Leibniz Institute for Plant Genetics and Crop Plant Research (Gatersleben, Germany) were used as material for analysis. Details on the accessions used are listed in Table 3.1. Additionally, a sample of sweet lupine flour was made available by the Chemical and Veterinarian Research Institute Freiburg (Freiburg, Germany).

3.1.2 Statice seeds

Statice seeds (*Limonium sinuatum*, Himmelblau) were obtained from Ernst Benary Samenzucht GmbH (Hannoverisch Münden, Germany) and ground for 1 min in a food processor (Vorwerk Thermomix, Wuppertal, Germany) to a fine powder.

3.1.3 Materials for specificity tests

Plant materials used for assessing the specificity of the real-time PCR methods are listed in Table 3.2. In addition, DNA samples isolated from cattle, chicken, lamb, pig, turkey, and yeast were included in the specificity survey. These materials were obtained from the Bavarian Health and Food Safety Authority (Oberschleissheim, Germany).

3.1.4 Preparation of lupine-spiked foods

3.1.4.1 Commercial ice cream spiked with lupine flour

A commercial sample of ice cream (showing a negative result in the test for the presence of lupine DNA [107]) was spiked with sweet lupine flour to an initial concentration

Table 3.1: Lupine species investigated and countries of origin.

Accession no.	Name	Country of origin
LUP 232	*Lupinus albus* L. ssp. *albus*	Germany
LUP 521	*Lupinus albus* L. ssp. *graecus*	Italy
LUP 121	*Lupinus angustifolius* L. ssp. *angustifolius*	unknown
LUP 489	*Lupinus angustifolius* L. ssp. *reticulatus*	Spain
LUP 471	*Lupinus hispanicus* Boiss. et Reut.	Portugal
LUP 552	*Lupinus hispanicus* Boiss. et Reut. ssp. *bicolor*	Spain
LUP 384	*Lupinus luteus* L.	Germany
LUP 575	*Lupinus mexicanus* Cerv. ex Lag.	unknown
LUP 514	*Lupinus micranthus* Guss.	Portugal
LUP 580	*Lupinus mutabilis* Sweet	Peru
LUP 55	*Lupinus nanus* Douglas ex Benth.	USA
B 1016	*Lupinus perennis* L.	USA
LUP 90	*Lupinus polyphyllus* Lindl. var. *polyphyllus*	unknown
LUP 84	*Lupinus polyphyllus* Lindl. var. *prunophilus*	Canada
LUP 94	*Lupinus pubescens* Benth.	unknown
LUP 6684	*Lupinus* sp.	Germany
LUP 586	*Lupinus subvexus* C. P. Sm.	USA
LUP 48	*Lupinus succulentus* Dougl. ex K. Koch	USA
LUP 583	*Lupinus variicolor* Steud.	unknown

Table 3.2: Plant materials used to assess the specificity of the method.

Plant	Source	Plant	Source
allspice	local supermarket	marjoram	local supermarket
almond	local supermarket	mung bean	local supermarket
anise	local supermarket	linseed	local supermarket
apple	BFSA[1]	mustard	local supermarket
apricot	BFSA[1]	nectarine	BFSA[1]
banana	BFSA[1]	nutmeg	local supermarket
barley	BFSA[1]	oat	local supermarket
bean	local supermarket	onion	local supermarket
bell pepper	local supermarket	oregano	local supermarket
blackberry	BFSA[1]	parsley	CVRI Freiburg[3]
brazil nut	local supermarket	peach	BFSA[1]
bread wheat	BG Ulm[2]	peanut	local supermarket
buckwheat	local supermarket	pear	BFSA[1]
caraway	local supermarket	pea	local supermarket
cardamom	local supermarket	pecan	local supermarket
cashew nut	local supermarket	pepper (black)	local supermarket
celery	CVRI Freiburg[3]	pepper (white)	local supermarket
chervil	ocal supermarket	pine nut	local supermarket
chickpea	local supermarket	pistachio	local supermarket
chive	BFSA[1]	plum	BFSA[1]
cinnamon	local supermarket	poppy	local supermarket
clove	local supermarket	raspberry	BFSA[1]
cocoa	local supermarket	rice	BFSA[1]
coconut	local supermarket	rosemary	local supermarket
coriander	CVRI Freiburg[3]	rye	BFSA[1]
cumin	local supermarket	sesame	local supermarket
fennel	local supermarket	sour cherry	BFSA[1]
garlic	local supermarket	soybean	BFSA[1]
hazelnut	local supermarket	spelt wheat	BFSA[1]
heart cherry	BFSA	strawberry	BFSA[1]
laurel	local supermarket	sultana	local supermarket
lentil	local supermarket	sunflower	LIPG[4]
linseed	local supermarket	thyme	local supermarket
macadamia nut	local supermarket	walnut	local supermarket
maize	BFSA[1]		

[1] BFSA, Bavarian Health and Food Safety Authority (Oberschleissheim, Germany)

[2] BG Ulm, botanical garden Ulm (Ulm, Germany)

[3] CVRI Freiburg, Chemical and Veterinarian Research Institute Freiburg (Freiburg, Germany)

[4] LIPG, Leibniz Institute for Plant Genetics and Crop Plant Research (Gatersleben, Germany)

of 1000 mg/kg. Serial dilutions (100, 10, 1, and 0.1 mg/ kg) were obtained by consecutively mixing the spiked product with lupine-free ice cream.

3.1.4.2 Wheat flours spiked with lupine flour

The wheat flour used as base material for the spiking experiments was at first tested regarding the absence of lupine DNA; the previously developed real-time PCR [107] gave a negative result. The wheat flour (999 g) was then mixed with sweet lupine flour (1 g) in a stand mixer (Braun, Kronberg, Germany), resulting in a concentration of 1000 mg lupine flour per kg wheat flour. Two further dilution steps (1 part of the spiked material with 9 parts of the pure wheat flour) were carried out to prepare a mixture containing 10 mg of lupine flour per kg wheat flour. This sample was finally diluted 1:2 and 1:10 with pure wheat flour to obtain mixtures containing 5 mg and 1 mg lupine flour per kg, respectively. In addition, wheat flours containing 0.1, 1, 5 and 10 mg of either *Lupinus luteus* or *Lupinus albus* flour per kg were prepared from a starting mixture of 0.1 g of the respective lupine flour with 99.9 g wheat flour.

3.1.4.3 Lupine-spiked pizza

Two grams of yeast and 75 mL water were added to 180 g of of the flour mixtures containing 1000, 100, 10, 1, 0.1, and 0.01 mg of blue lupine flour per kg and the ingredients were stirred in the food processor using a dough hook (Multisystem K3000, Braun, Germany) until a smooth dough was obtained. After blending for 10 min, it was allowed to rise for 30 min at room temperature. Subsequently, the dough was stored at - 20 °C for two weeks. After thawing, each portion was divided into two parts and pizzas were formed. One of them was coated with 40 g tomato puree before baking. Baking of the pizzas was carried out at 190 °C for 25 min. In addition to the spiked samples, blanks were prepared from the lupine-free wheat flour as controls.

3.1.4.4 Ice cream with lupine flour

In case of the commercial ice cream, lupine flour was added after food processing. For this reason, the influence of processing on the detectability of lupine DNA could not

be determined. Therefore, an ice cream base was manufactured including lupine flour as an ingredient. One hundred and fifty grams of sugar were diluted in 250 mL whole milk by mixing in a food processor (Braun, Kronberg, Germany). After adding 200 mL whipping cream (30% fat), 0.1 g sweet blue lupine flour were stirred into 9.9 g of the resulting mixture to obtain an ice cream base containing 10 000 mg lupine flour per kg. Ice cream bases with lupine contents of 1000, 100, 10, 1, 0.1, and 0.01 mg/kg were prepared by subsequently diluting the mixture 1:10 with lupine-free ice cream base. All samples were frozen at -20 °C.

3.1.4.5 Lupine-spiked mayonnaise

Fifteen grams of egg yolk, 0.5 g salt, 20 mL water, 5 g sugar, 7.5 mL lemon juice, and 10 g ground mustard seeds were mixed in a food processor (Braun, Kronberg, Germany). Subsequently, 225 mL vegetable oil were added while stirring. Mayonnaise containing 10 000, 1000, 100, 10, 1, 0.1 and 0.01 mg lupine flour per kg was prepared as described above for spiked ice cream.

3.1.4.6 Double-baked wheat bread ("Zwieback")

Two doughs were each prepared from 250 g wheat flour, 40 g butter, 62.5 g sugar, 125 mL milk, 0.25 g salt and 10 g baking yeast. Lupine flour was added to one of the doughs to obtain a final concentration of 1000 mg. After 1 h of yeast fermentation at room temperature, both breads were baked for 1 h at 180 °C. On the following day, the cooled bread loafs were cut in 0.5 cm thick slices and baked a second time at 100 °C for 30 min. Since the concentration of lupine flour increases because of the loss of water during baking, both the dough containing lupine and the resulting double-baked bread were weighed and the actual lupine flour content of the spiked bread was calculated. After grinding both breads separately in a food processor (Vorwerk Thermomix, Wuppertal, Germany), adequate amounts of the lupine-free bread and the spiked bread were mixed to reconstitute a concentration of 1000 mg lupine flour per kg breadcrumbs. Concentrations of 100, 10, 1, 0.1, and 0.01 mg lupine / kg were obtained by consecutively diluting the spiked bread 1:10 with lupine-free bread.

3.1.5 Food samples

Lupine-containing and lupine-free foods were provided by the Institute for Product Quality (Berlin, Germany) and the Fraunhofer Institute for Process Engineering and Packaging (Freising, Germany). Additional samples were purchased at local stores in Oberschleißheim (Germany).

3.1.6 Primers and probes

A primer set published by Taberlet et al. [108] was used for verification of the amplifiability of the extracted DNA. Noncoding regions of chloroplast DNA were amplified in an endpoint PCR using the primer pair A1/A2 (Table 3.3).

Table 3.3: Primers for the determination of the amplifiability of the extracted DNA.

Primer	Sequence	Amplicon length
A1	5'-CGA AAT CGG TAG ACG CTA CG-3'	variable
A2	5'-GGG GAT AGA GGG ACT TGA AC-3'	

Primers and probe for the real-time PCR detection of a lupine-specific DNA sequence were manually designed as described in sections 3.2.3 and 4.1.1. The sequences of the oligonucleotides and the length of the resulting amplicon are given in Table 3.4.

Table 3.4: Primers and probe for the specific detection of lupine DNA.

Primer/probe	Sequence	Amplicon length (bp)
Lupine F	5'-CCT CAC AAG CAG TGC GA-3'	129 bp
Lupine R	5'-TTG TTA TTA GGC CAG GAG GA-3'	
Lupine probe	5'-FAM-CCC CTC GTG TCA GGA GGC GC-TAMRA-3'	

Primers for the generation of lupine and statice (*Limomium sinuatum*) amplicons with overlapping ends, which were used for the construction of a reference molecule, were developed in this work (Table 3.5; the overlapping regions are shown in bold letters).

Table 3.5: Primers and probe for the generation of amplicons for a reference molecule containing the lupine and statice target sequences.

Primer/probe	Sequence	Amplicon length (bp)
ov-Lupine R	5'-**ACA GTT GAG CTC GAC GCA TT** TTG TTA TTA GGC CAG GAG GA-3'	149 bp (in combination with primer Lupine F)
ov'-Limo-62	5'-**AAT GCG TCG AGC TCA ACT GT** TTG GAC GTG TAT CCC TTG TGG TTC-3'	121 bp (in combination with primer Limo-162)

The real-time PCR system published by Hirao et al. [109] was applied for the detection of statice DNA. Sequences and amplicon lengths are shown in Table 3.6.

Table 3.6: Primers and probe for the detection of statice DNA [109].

Primer/probe	Sequence	Amplicon length (bp)
Limo-62	5'-TTG GAC GTG TAT CCC TTG TGG TTC-3	181 bp
Limo-162	5'-CAC GAA GGT GAA AGT TGC GTT CAT-3'	
Limo-probe	5'-FAM-TGT GCG ACG CGG AAT G-MGB-TAMRA-3'	

All oligonucleotides were obtained from TIB MOLBIOL (Berlin, Germany).

3.1.7 Commercial kits for the detection of lupine DNA

3.1.7.1 Cibus detection kit for plant specific lupine DNA

The Cibus Detection Kit for Plant Specific Lupine DNA in Raw, Processed and Heated Products (CIB-P-Kit LU-EX/20, Cibus Biotech GmbH, Gütersloh, Germany) contains the following reagents: $10\times$ PCR buffer, a $MgCl_2$ solution (25 mM), a dNTP solution, the primer solution LU-EX, DNA polymerase solution, and a positive control (Control DNA lupine).

3.1.7.2 SYBR-Green real-time PCR kit PCR-Fast Lupine

The kit SYBR-Green real-time PCR Kit PCR-Fast Lupine (Institut für Produktqualität (ifp), Berlin, Germany) contains 6 colourless stripes, each with 8 0.2 mL reaction vessels coated with specific primers (for samples, negative controls, and extraction controls) and 6 red stripes, each with eight 0.2 mL reaction vessels coated with specific primers and specific control DNA (for positive controls and inhibition controls). In addition, a SYBR green solution is provided.

3.1.8 ELISA kit for the detection of lupine protein in foods

The HAVen Lupin ELISA Kit 96 wells (HA-011048) was used for the determination of lupine protein in food samples. For the extraction buffer, 9.17 g glycine and 2.96 g tris(hydroxymethyl)-aminomethane were diluted in 220 mL distilled water. After adjusting the pH to 8.65 - 8.75, distilled water was added to obtain a final volume of 245 mL. The washing buffer was prepared by dilution of the concentrate provided in the kit: to 25 mL of the washing buffer concentrate, distilled water was added to a final volume of 500 mL. The dilution buffer was obtained by diluting the concentrate provided in the kit 1:4 with distilled water.

For sample extraction, 10 mL extraction buffer were added to 1 g of homogenized sample material in a 50 mL falcon tube and thoroughly shaken for 1 min. After 15 min of incubation at 45 °C, the samples were again shaken for 1 min and centrifuged at

1000g at room temperature. The resulting supernatants were then diluted as follows using the dilution buffer:

- buckwheat bread with sesame, bread roll from pretzel dough, crunchy bread with rice and maize, cake slices with whole milk chocolate, ice cream (vanilla), ice cream (soy protein), cherry cake, almond curd cheese stollen, control (low), control (medium): 1:5

- fruit-flavored gums (lupine protein, 100 mg/kg), ice cream (lupine protein, 100 mg/kg): 1:10

- toast, muffin (lupine protein, 2%): 1:4000

- ice cream (lupine protein, 3%): 1:6000

- whole-grain bread with sunflower seeds, rice bread, fruit-flavored gums (lupine fiber, 10%), bread roll (lupine protein, 15 %), wheat brown bread with flax seeds 1 (lupine flour), wheat brown bread with flax seeds 2 (lupine flour), wheat brown bread with pumpkin seeds (lupine flour): 1:10000.

3.2 Methods

3.2.1 DNA extraction - CTAB method

3.2.1.1 Standard CTAB method

The isolation of DNA from lupine flour was performed using a cetyltrimethylammonium bromide (CTAB)-based method. Two hundred milligrams of ground lupine seeds were mixed with 1500 μL of CTAB extraction buffer [2% (w/v) cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-OH/HCl] and 10 μL of proteinase K solution [20 mg proteinase K per mL of storage buffer (50 mM Tris, pH 8, 3 mM $CaCl_2$, 50% glycerol), Invitrogen, Karlsruhe, Germany] in a 2 mL tube, followed by overnight incubation at 65 °C. After 10 min of centrifugation at 14000g (all centrifugation performed in this study were carried out at room temperature), 1000 μL of supernatant was transferred into a new 2 mL tube and centrifuged a second time at 14000g. In a fresh tube, 1300 μL of precipitation buffer was added to 650 μL of supernatant. After 1 h of incubation at room temperature and 5 min of centrifugation at 14000g, the supernatant was removed and the pellet dissolved in 350 μL of 1.2 M NaCl. Addition of 350 μL of chloroform was followed by 10 min of centrifugation. The aqueous phase was then transferred into a 1.5 mL tube. After the addition of 2 μL of glycogen and 350 μL of isopropyl alcohol (100%), the samples were centrifuged for 15 min at 14000g. The supernatant was discarded, and the pellet was washed by the addition of 500 μL of ethanol (70% v/v). After 5 min of centrifugation at 14000g, the supernatant was again removed and the residuals were dried at 50 °C. The pellet was then dissolved in 100 μL of TE buffer (1$\times$). Subsequently, the DNA extracts were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

3.2.1.2 Modified CTAB method

A modified cetyltrimethylammonium bromide (CTAB)-based method was used for the DNA extraction from food samples, which were homogenized in a food processor (Braun, Germany). To 2 g of homogenized sample material in a 50 mL falcon tube,

10 mL of CTAB extraction buffer [2% (w/v) cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-OH/HCl] and 30 μL of proteinase K solution [(20 mg proteinase K per mL of storage buffer (50 mM Tris, pH 8, 3 mM CaCl$_2$, 50% glycerol), Invitrogen, Karlsruhe, Germany] were added and mixed, followed by overnight incubation at 65 °C. One thousand microliters of the supernatant obtained by 5 min of centrifugation at 5000 g (all centrifugation performed in this study were carried out at room temperature) were transferred into a new 1.5 mL tube and centrifuged 5 min at 14000 g. Seven hundred microliters of supernatant were mixed with 500 μL of chloroform/isoamyl alcohol (Ready Red, MP Biomedicals, Heidelberg, Germany), followed by 15 min of centrifugation at 16000 g. Preloading of 1.5 mL sample tubes with 500 μL of cold isopropanol (stored at -20 °C) was followed by addition of 500 μL supernatant and 30 min of incubation at room temperature. After 15 min of centrifugation at 16000 g, the supernatant was discarded and the pellet washed with 500 μL of ethanol (70% v/v; stored at -20 °C) and centrifuged 5 min at 16000 g. The ethanol was removed and the pellet diluted in 100 μL of Tris-EDTA buffer [1$\times$, 100-fold dilution of 100$\times$ TE buffer solution (1.0 M Tris-HCl, pH 8.0, containing 0.1 M EDTA), Sigma Aldrich, Hamburg, Germany]. Subsequently, the DNA extracts were purified with the QIAquick PCR purification kit (Qiagen, Hiden, Germany) according to the manufacturer's instructions.

3.2.2 Fluorimetric determination of DNA concentration

DNA concentrations of the extracts were determined fluorometrically at 520 nm using PicoGreen dsDNA quantification reagent (Invitrogen, Karlsruhe, Germany) and a Tecan GENios plus reader (Männedorf, Switzerland) at an excitation wavelength of 480 nm. The samples were diluted 1:10 with a 1:400 dilution of the PicoGreen stock solution. Quantification of doublestranded DNA was achieved using a calibration curve derived from λ -DNA. Recorded data were evaluated using the Tecan Magellan software package.

3.2.3 Design of the primers and the probe for the detection of lupine DNA

The database entries from NCBI GenBank containing sequences of the internal transcribed spacer 1 of different lupine species were aligned using the SeqMan 5.08 software (DNASTAR, Inc., Madison, WI, USA). Primers and probe were manually designed on the basis of this alignment and checked using the Beacon Designer 4.0 (Premier Biosoft Int., Palo Alto, CA, USA) software.

3.2.4 Polymerase Chain Reaction (PCR)

3.2.4.1 Determination of amplifiability

The amplifiability of the extracted DNA was verified by endpoint PCR with primers targeting noncoding regions of chloroplast DNA [108]: 12.5 μL HotStarTaq Master Mix (HotStarTaq DNA Polymerase in PCR buffer with 3 mM MgCl$_2$ and 400 μM of each dNTP; Qiagen, Hilden, Germany), 25 pmol of each of the primers A1 and A2, 10.5 μL PCR grade water and 1 μL of the DNA extracts were mixed in 0.5 mL reaction tubes. After 15 min of incubation at 95 °C, 40 cycles of the following thermal profile were carried out: 94 °C (30 s), 55 °C (30 s), 72 °C (1 min). Final elongation was 2 min at 72 °C. The PCR products were visualized on an agarose gel as described in section 3.2.6.

3.2.4.2 Real-time PCR for the detection of lupine DNA

Sample extracts (5 μL) were added to 20 μL of reaction mix containing 2$\times$ SensiMix (Quantace, London, UK), 7.5 pmol of each primer, and 5.0 pmol of probe per reaction. PCR reactions were carried out on an ABI 7900HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA) according to the following thermal cycling program: uracil-N-glycosylase (UNG) decontamination (2 min at 50 °C), initial denaturation (10 min at 95 °C), cycle denaturation (15 s at 95 °C), primer annealing, and elongation and data collection (60 s at 60 °C).
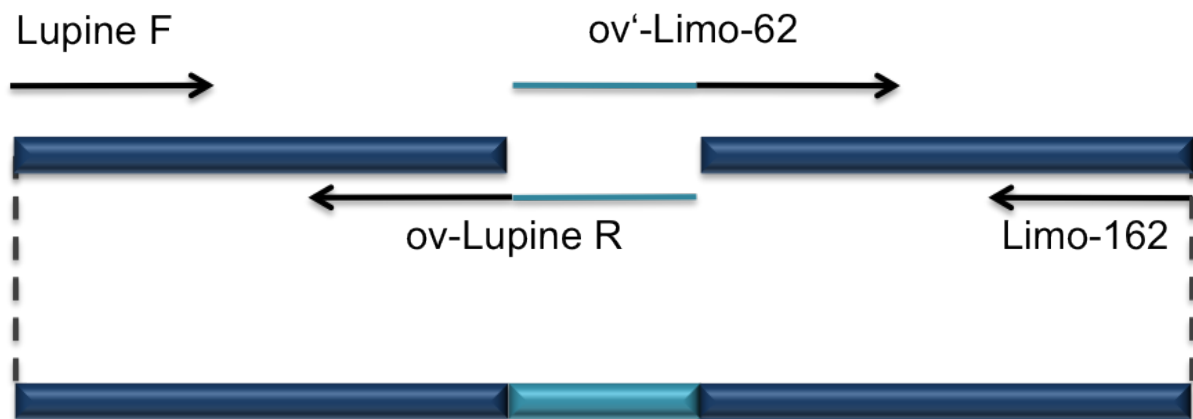
Figure 3.1: Generation of lupine and statice amplicons with overlapping ends and subsequent construction of a reference molecule using the primers Lupine F and Limo-162.

3.2.4.3 Construction of a reference molecule

Prior to the construction of a reference molecule containing the target sequences of both the lupine and the statice real-time PCR systems, the target sequences were each amplified separately using the respective specific primer pair. Two primers - the statice forward- and the lupine reverse primer - were, however, modified at their 5'-ends: the reverse primer ov-Lupine R contains the sequence of the reverse primer Lupine R and an additional 20 base pairs. This extension in its reverse-complemented form is also part of the primer ov'-Limo-62, which in addition contains the sequence of the forward primer Limo-62. This enables the amplification of the aforementioned target sequences and the addition of an extra sequence of 20 bp, which is complementary in the two amplicons. Figure 3.1 illustrates the construction of the reference molecule.

Qualitative PCR on lupine DNA as preparation for the construction of a reference molecule The target sequence of the real-time PCR for the detection of lupine DNA was amplified in a final volume of 25 μL in 0.2 mL reaction vials containing 12.5 μL 2x HotStarTaq Master Mix (HotStarTaq DNA Polymerase in PCR buffer with 3 mM MgCl$_2$ and 400 μM of each dNTP; Qiagen, Hilden, Germany), 10 pmol of each of the

primers Lupine F and ov-Lupine R and 100 ng template DNA. The following temperature program was applied in a Primus 96 plus thermocycler (MWG-Biotech, Ebersberg, Germany): activation of the hotstart polymerase at 95 °C for 8 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing of the primers at 55 °C for 30 s, and elongation at 72 °C for 30 s. The final elongation step was 2 min at 72 °C.

Qualitative PCR on statice DNA as preparation for the construction of a reference molecule Statice DNA was amplified in 0.2 mL reaction vials in a final reaction volume of 25 μL containing 12.5 μL HotStarTaq Master Mix, 20 pmol of each of the primers ov'-Limo-62 and Limo-162 and 100 ng template DNA. The temperature program consisted of a polymerase activation step (8 min at 95 °C) and 45 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30s, and elongation at 72 °C for 30 s, followed by a final elongation step for 2 min at 72 °C.

Qualitative PCR for the preparation of a reference molecule from the lupine and statice amplicons The reference molecule containing one copy of the target sequence of the lupine real-time PCR linked to one copy of the target sequence of the statice real-time PCR was constructed as follows: The PCR product obtained from lupine DNA using the primers Lupine F and ov-Lupine R was linked to the statice amplicon produced with the primer pair Limo-162/ov'-Limo-62 by PCR using the outer primers Lupine F and Limo-162 (Figure 3.1) and the same temperature program as for the qualitative PCR for statice. After the determination of the DNA concentration, the reference molecule was serially diluted in 1$\times$ TE buffer containing 5 ng λ-DNA per μL to increase the stability of the solutions.

3.2.4.4   Real-time PCR for the detection of statice DNA

A final reaction volume of 25 μL containing 12.5 μL QuantiTect Probe PCR Master Mix (HotStarTaq DNA polymerase in QuantiTect Probe PCR buffer, Qiagen, Hilden, Germany), 7.5 pmol of each of the oligonucleotides Limo-62, Limo-162 and Limo-probe, and 100 ng template DNA was set up in 0.2 mL reaction tubes. 2 min of UNG decontamination at 50 °C were followed by 15 min of preincubation at 95 °C and 45 cycles

of denaturation (1 min at 95 °C), annealing (2 min at 66 °C), and elongation and data collection (1 min at 72 °C).

3.2.5 PCR-based detection kits for lupine DNA in food

3.2.5.1 Cibus detection kit for plant specific lupine DNA in raw, processed and heated products

Following the description in the manual of the Cibus Detection Kit for Plant Specific Lupine DNA in Raw, Processed and Heated Products (CIB-P-Kit LU-EX/20, Cibus Biotech GmbH, Gütersloh, Germany) [105], 5 μL of the sample extracts, 12.3 μL PCR-grade water and the following reagents provided in the kit were mixed in 0.2 mL tubes: 2.5 μL 10× PCR buffer, 1.5 μL of a solution containing 25 mM MgCl$_2$, 1.0 μL of the dNTP solution, 2.5 μL of the primer solution LU-EX, and 0.2 μL DNA polymerase solution, resulting in a total volume of 25 μL. The amplification was carried out in a Primus 96 plus thermocycler (MWG-Biotech, Ebersberg, Germany) according to the thermal cycling profile given in the manual: initial denaturation at 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 64 °C, and 30 s at 72 °C. The final elongation step was 5 min at 72 °C. A positive control (Control DNA lupine, provided with the kit) and a negative control (PCR-grade water) were treated in the same way as the samples.

3.2.5.2 SYBR-Green real-time PCR Kit PCR-Fast Lupine

Following the manual [106], 5 μL of each sample extract, 12.5 μL SensiMix (Quantace, London, UK), 0.5 μL SYBR green solution and 7 μL PCR-grade water were pipetted into one of the colourless and one of the red tubes, respectively. Thermal cycling was carried out on an ABI 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the following profile: uracil-N-glycosylase (UNG) decontamination (2 min at 50 °C), initial denaturation (10 min at 95 °C), and 45 cycles of denaturation (15 s at 95 °C), primer annealing, and elongation and data collection (60 s at 60 °C). The samples were prepared for melting curve analysis by heating to 95 °C (15 s) and 60 °C (15 s). Melting curves were subsequently recorded while

increasing the temperature from 60 °C to 95 °C with a ramp rate of 2% and holding at 95 °C for 15 s.

3.2.6 Electrophoresis

The amplification products were mixed with loading buffer [TBE buffer (45 mM Tris/boric acid, 1mM Na-EDTA, pH 8.0), 40% glycerine, 2.5 g/L bromphenol blue sodium salt] and loaded onto 2% agarose gels (E-Gel 2%, Invitrogen, Karlsruhe, Germany) containing ethidium bromide. The gels were run at 65 V for 30 min in the E-Gel station. For visualization, they were digitized using the CCD camera-based documentation system GelDoc 1000 and the corresponding Multi Analyst 1.0.2 software (Bio Rad, München, Germany).

3.2.7 Enzyme-linked immunosorbent assay (ELISA)

Food samples were analyzed using a commercially available ELISA kit [110]. Each sample was extracted once, and the extracts were analyzed in duplicate. 100 μL of the diluted extracts, the standards and the controls were pipetted into the plate provided with the kit, which was then cautiously shaken horizontally. After 1 hour of incubation at room temperature, the wells were emptied by pipetting and by subsequently patting the plate on paper towels. The wells were then filled to the top with washing buffer. After 30 seconds of incubation, the washing buffer was removed. The washing procedure was repeated 6 times using an ELISA washer (BioTek ELx50, Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany). After patting the plate on paper towels to remove potentially remaining liquid, 100 μL anti-lupin-biotin-reagent were pipetted into the wells. Shaking of the plate was followed by 60 min of incubation at room temperature. The liquid was then pipetted off and the remaining liquid was removed as described before. After washing 6 times with washing buffer, 100 μL avidin-HRP-reagent were pipetted into the wells and the plate was cautiously shaken horizontally. 15 min of incubation at room temperature were then followed by revmoval of the liquid and 6 repetitions of the washing procedure. 100 μL of the TMB-substrate solution were immediately pipetted into the wells, followed by cautious mixing by shaking of the plate. After 15 min of

incubation in the dark at room temperature, 100 μL of stop solution were pipetted into the wells of the microtiter plate, which was then shaken cautiously. The optical density in each well was measured in a plate reader (Tecan GENios plus reader (Männedorf, Switzerland)) at 450 nm and converted into ng/μL lupine protein based on the standard curve and using the Tecan Magellan software package.

3.2.8 Calculation of the coefficient value of *Lupinus angustifolius*

DNA from a mixture of 1 g of lupine flour with 200 mg ground statice seeds was extracted and 100 ng were subjected to both the real-time PCR for lupine and the real-time PCR for statice. The starting copy numbers for lupine and statice were determined via the calibration curve obtained by the analysis of the serial dilutions of the reference molecule; in total, 24 extracts were analyzed in triplicate. The coefficient value for lupine was calculated using the following formula (I):

$$cv_{Lupine} = Limonium_0/Lupine_0 \text{ (I)}$$

$Limonium_0$: statice starting copy number of DNA extract from 1 g lupine flour and 200 mg ground statice seeds

$Lupine_0$: lupine starting copy number of DNA extract from 1 g lupine flour and 200 mg ground statice seeds

3.2.9 Calculation of the lupine content

100 ng DNA extracted from a mixture of 1 g of the spiked wheat flours and 200 mg statice seeds were analyzed by both lupine and statice real-time PCR in three replicates per extract. The calibration curves obtained by the analysis of subsequent dilutions of the reference molecule were used for the determination of the starting copy numbers. The lupine content of the spiked wheat flours was calculated using formula (II):

Lupine flour content in the food sample [mg/kg] =

$$(Lupine_1/Limonium_1) * cv_{Lupine} * 10^6 \text{ (II)}$$

$Lupine_1$: lupine starting copy number of the food sample

$Limonium_1$: statice starting copy number of the food sample

cv_{Lupine}: coefficient value (Formula I)

4 Results and discussion

4.1 Development of a real-time PCR for the detection of lupine DNA (*Lupinus* species) in food

4.1.1 Design of primers and a probe for the specific detection of lupine DNA

The internal transcribed spacer sequences of 18S-26S nuclear rDNA provide a useful basis for the phylogenetic comparison of species and closely related genera. Despite underlying rapid evolution, this region also contains conserved segments. These characteristics can be utilized in the design of PCR methods for detection at specific taxonomic levels [111]. Nevertheless, it depends on the group of plants considered whether the detection at the desired taxonomic stage is possible or not. Sequences corresponding to the same taxonomic range may show significant interspecies or even intraspecies variability in one case while matching exactly in another case. The main challenge in the PCR design was the need to develop a method enabling the detection of DNA from as many different lupine species as possible while at the same time omitting false-positive results caused by closely related species that might be present in food. Therefore, a thorough comparison of the relevant sequence data was carried out as the first step. An alignment of all sequences available from NCBI GenBank and corresponding to the internal transcribed spacer 1 (ITS-1) of 12 different lupine species was produced to assess identical sequence regions. In a second step, ITS-1 sequences from other legumes such as chickpea, bean, field bean, lentil, soy and pea were added to the alignment to see if there were any similarities between the ITS-1 sequences of lupines and those of closely related species that are used as food ingredients. The alignment is shown in Figures 4.1 (part 1), 4.2 (part 2) and 4.3 (part 3). The numbers at the top of each alignment are the nucleotide positions of the topmost sequence (GeneBank accession number AH007412.1).

Figure 4.1: Alignment of internal transcribed spacer 1-sequences from 12 different lupine species, chickpea, bean, field bean, lentil, soy and pea and position of the primer "Lupine F".

Figure 4.2: Alignment of internal transcribed spacer 1-sequences from 12 different lupine species, chickpea, bean, field bean, lentil, soy and pea and position of the probe "Lupine probe".
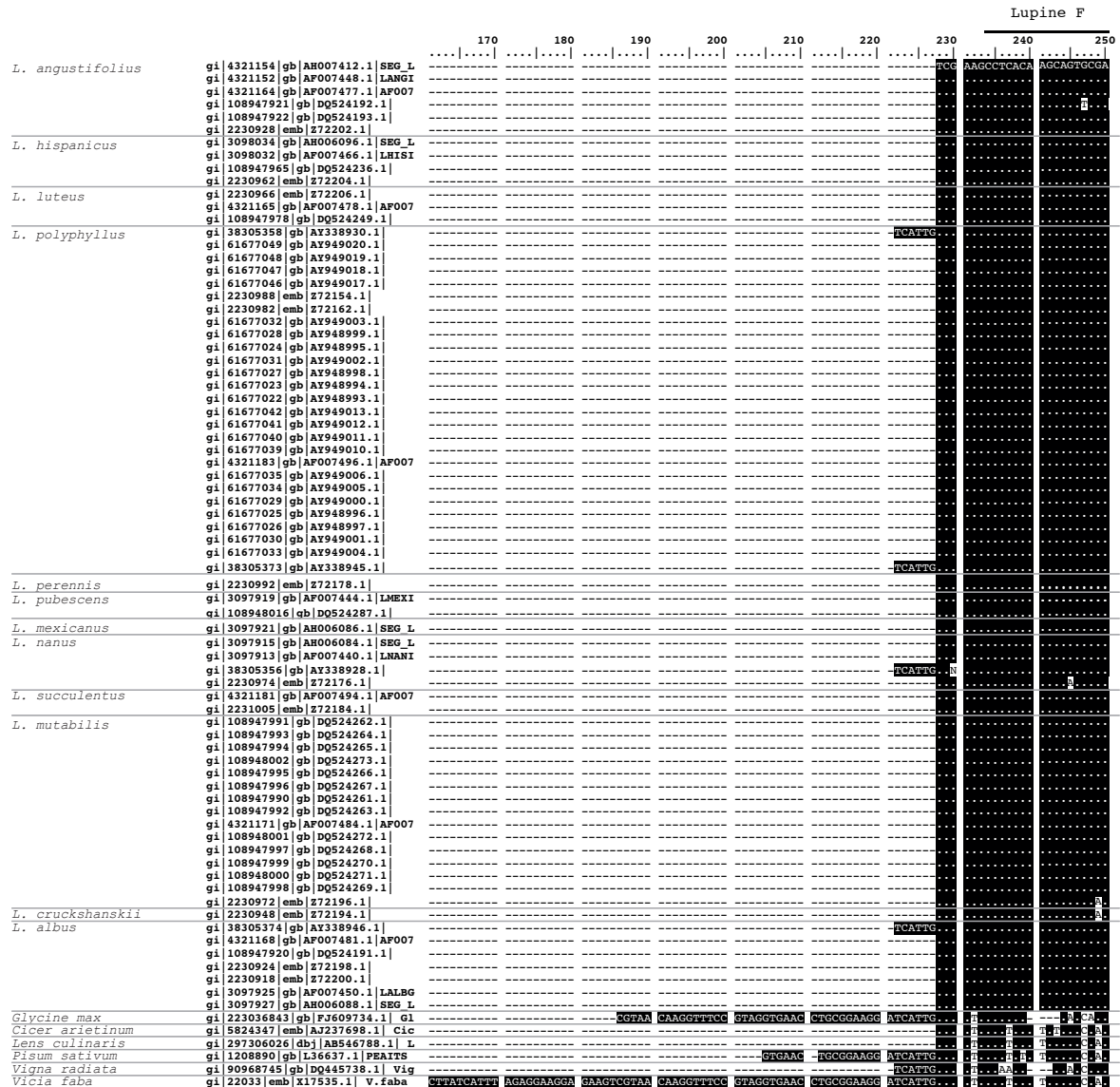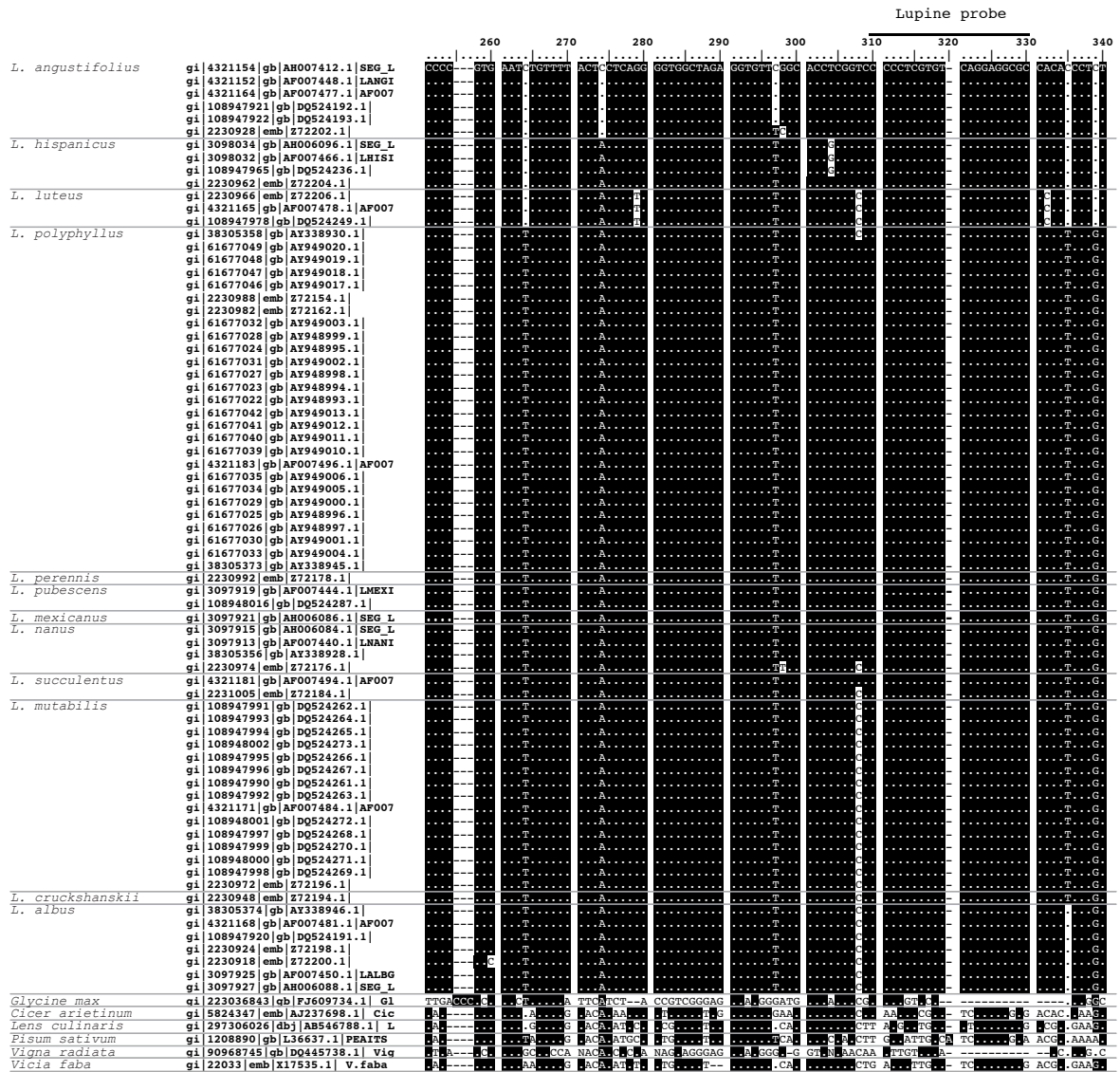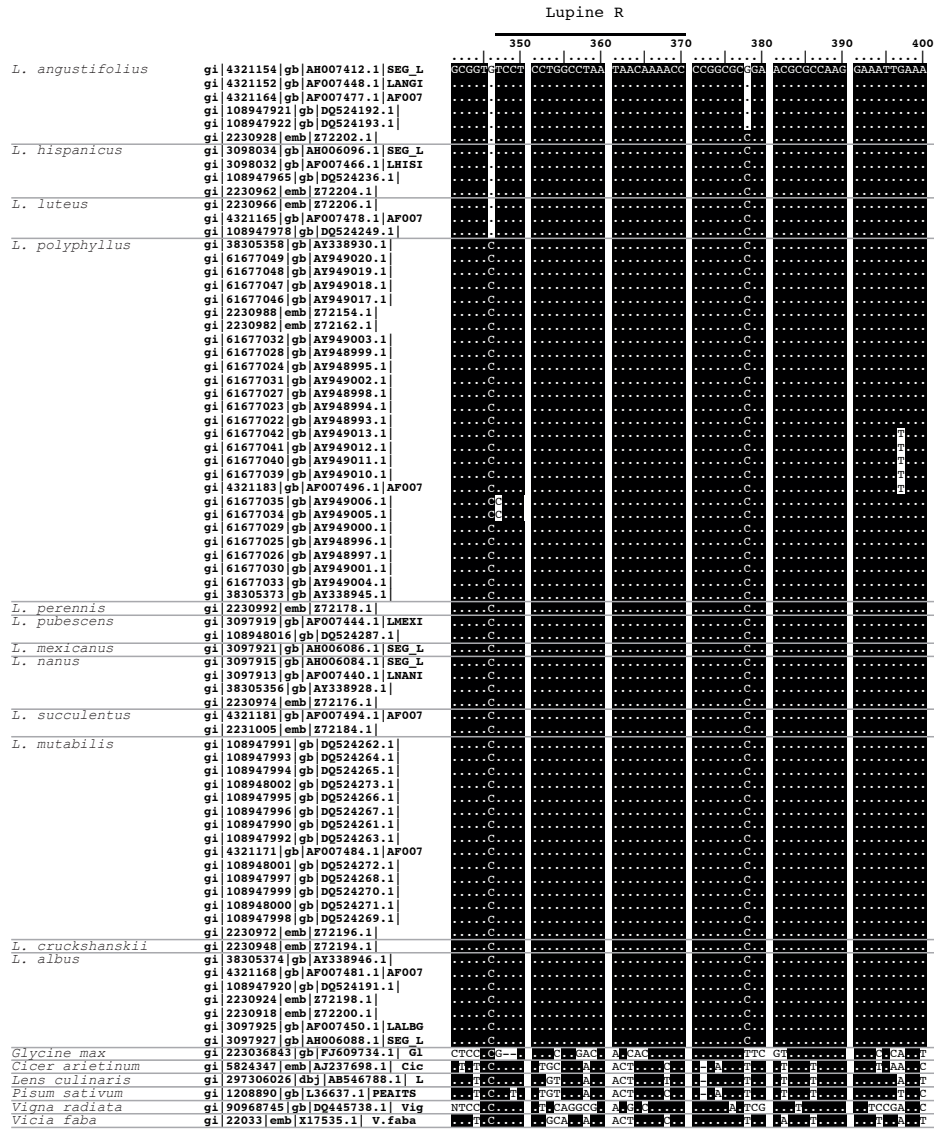
Figure 4.3: Alignment of internal transcribed spacer 1-sequences from 12 different lupine species, chickpea, bean, field bean, lentil, soy and pea and position of the primer "Lupine R".

Finally, the fragments that seemed to be suitable for the design of a real-time PCR which would be specific for lupine DNA, but also capable of detecting DNA from a variety of different lupine species, were analyzed by BLAST search. The results were searched for genera that include species which are used in human nutrition. No significant similarities relevant to food products were found. Primers and probe were manually designed to the fragments considered above and checked using primer design software. A BLAST-analysis of the primer "Lupine F" revealed 100% homologies with species from the genera *Crotalaria* and *Genista*. As described above, the results were searched for species relevant to human nutrition. Again, no significant similarities relevant to potential food ingredients could be found. The same was done for the reverse primer "Lupine R" which showed 100% homology with species from the genera *Lebeckia*, *Lotononis*, *Cytisus*, *Lembotropis*, *Genista*, *Argyrolobium* and *Chamaespartium* and the probe "Lupine probe", that is fully homologous to sequences pertaining to the genera *Echinospartum*, *Cytisus*, *Agrocytisus* and *Genista*. Since the results from the BLAST searches did not include significant similarities with species that are used as food ingredients, the primers and the probe were considered to be suitable for the detection of *Lupinus* species in food according to the *in silicio* analysis. The positions of the primers and the probe in the region of the internal transcribed spacer 1 are shown in Figure 4.1 (position of the primer "Lupine F"), Figure 4.2 (position of the probe "Lupine probe") and Figure 4.3 (position of the primer "Lupine R").

4.1.2 Evaluation of the specificity

DNA extracts from 19 lupine species and varieties (listed in Table 3.1) and from lupine flour provided by the Chemical and Veterinarian Research Institute Freiburg were used to assess the suitability of the method for the detection of lupine DNA in foods in which different lupines could be used. All extracts gave positive signals.

The specificity of the real-time PCR method was tested using DNA extracts of the foods listed in section 3.1.3, comprising other legumes, cereals, seeds, nuts, spices, meat and fruit. No nonspecific amplification was observed.

4.1.3 Evaluation of the sensitivity

Genomic DNA extracted from Lupinus angustifolius ssp. angustifolius (LUP 121) was diluted to concentrations of 0.01, 0.1, 1.0, 10, 100, and 1000 pg per 5 μL. Subsequently, each concentration level was analyzed in five replicates, and the average cycle threshold (Ct) values were plotted against the log of the respective DNA amount. The resulting graph is shown in Figure 4.4.
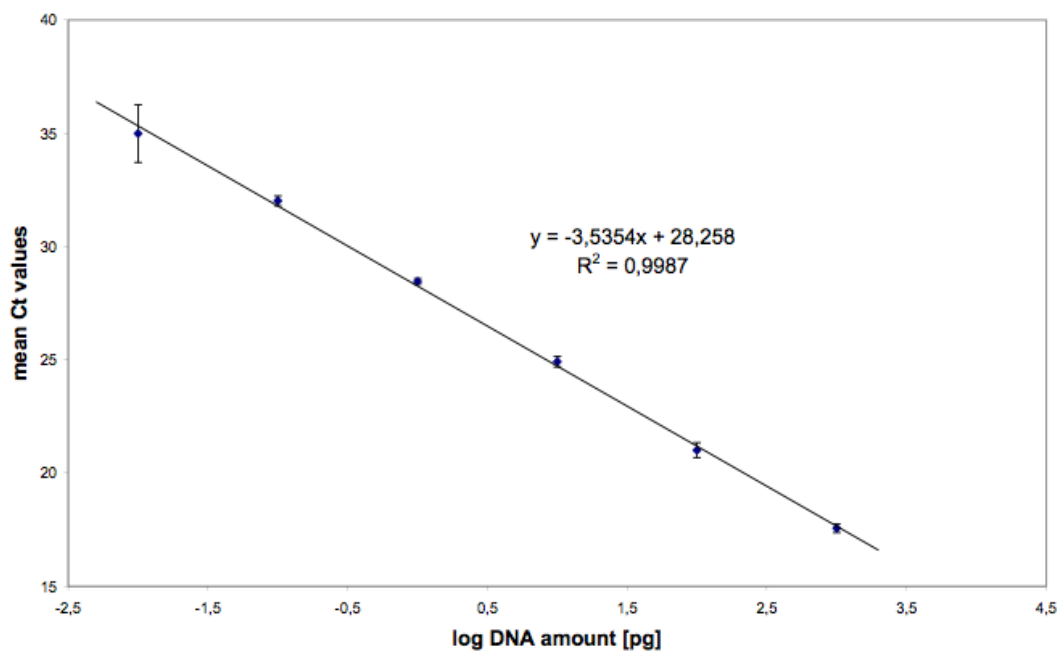
Figure 4.4: Standard curve obtained by real-time PCR analysis of serial dilutions of genomic DNA from *Lupinus angustifolius* in the range of 0.01 to 1000 pg (data points correspond to results from 5 replicates; bars indicate standard deviations).

The method reliably detected 0.01 pg of lupine DNA, whereas 0.001 pg did not give any positive signals. The exceptionally low value for the amount of lupine DNA that can still be amplified is due to the detection of a multicopy target. At present, no general performance criteria for PCR assays are available. The only existing guideline in the field of food analytics is the definition of the minimum performance requirements for analytical methods of GMO testing as set up by the European Network of GMO

Laboratories [112]. With a slope of -3.5 and a correlation coefficient of 0.999, the standard curve obtained from the real-time PCR procedure meets these acceptance criteria. The acceptable values specified by the ENGL are -3.1 to - 3.6 for the slope and a correlation coefficient greater than 0.98.

4.1.4 Detection of lupine DNA in spiked commercial ice cream

The lowest amount of allergen provoking a reaction in sensitive persons is referred to as the threshold dose. Even for a single allergen, the threshold dose may vary significantly between individuals as well as with the food matrix considered [113]. For this reason, the determination of the threshold dose for a specific allergen is challenging. The lowest dose reported to trigger clinical reactions has been 0.5 mg of lupine flour [114], but no threshold dose for lupine has yet been established. A method for allergen analysis should ideally be able to reliably detect the allergen at the threshold dose level. In general, detection limits in the low milligrams per kilogram range are considered to be appropriate [2]. Table 4.1 contains the results obtained by the analysis of ice cream spiked with 1000 mg/kg, 100 mg/kg, 10 mg/kg, 1 mg/kg and 0.1 mg/kg lupine flour, respectively. All samples gave positive results. Therefore, the developed method meets the aforementioned requirements.

Table 4.1: Results obtained by the analysis of ice cream spiked with lupine flour.

Lupine flour content (mg/kg)	mean Ct value[1]	positive results/ no. of reactions
1000	17.3	10/10
100	21.0	10/10
10	23.3	10/10
1	27.0	10/10
0.1	20.5	10/10

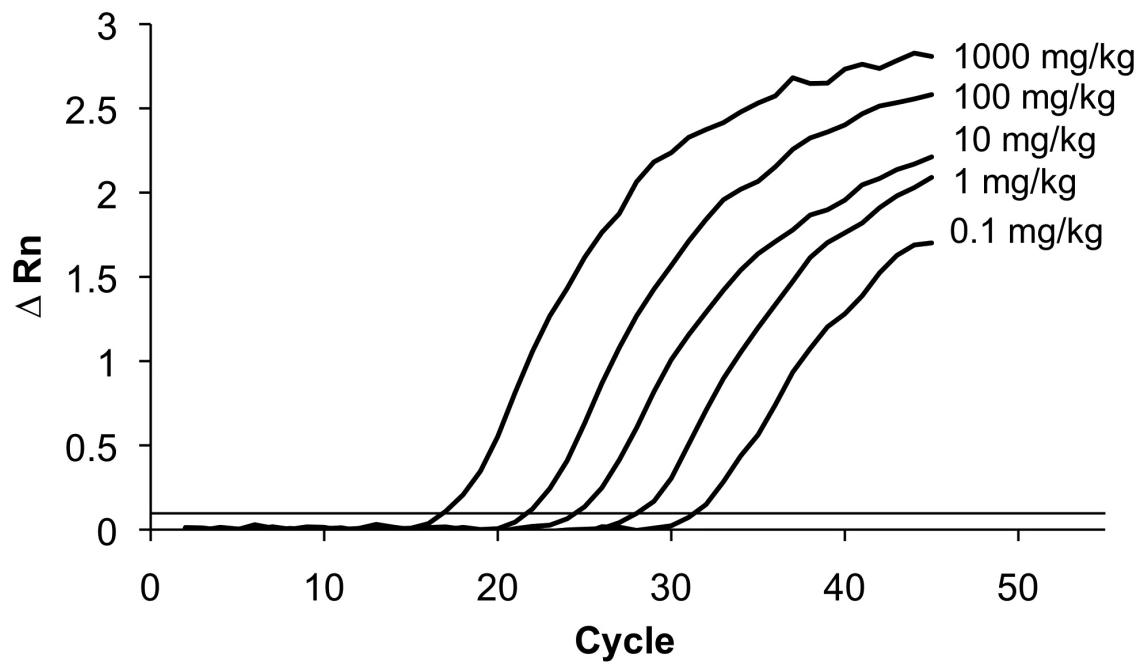[1] Means from five extractions per level, analyzed in duplicate.

Figure 4.5: Amplification curves obtained by analysis of ice cream spiked with 1000 mg/kg, 100 mg/kg, 10 mg/kg, 1 mg/kg and 0.1 mg/kg lupine flour, respectively (line at $\triangle$Rn 0.1 corresponds to cycle threshold).

The corresponding amplification curves are shown in Figure 4.5.

4.1.5 Influence of the lupine species on the limit of detection in wheat flour

4.1.5.1 Determination of the copy number of the real-time PCR target sequence in five lupine species

The number of repetitions of the segments of the ribosomal DNA in plants is highly variable [115]. Therefore, the copy number of the target sequence of the hybridization probe-based real-time PCR for the detection of lupine DNA, the internal transcribed spacer 1 sequence, was analyzed in *Lupinus angustifolius*, *Lupinus angustifolius* ssp. *angustifolius*, *Lupinus albus* ssp. *graecus*, *Lupinus luteus* and *Lupinus mutabilis*. For each species, two different extracts of lupine DNA were analyzed in two replicates each. The amount of DNA per reaction was calculated from the DNA concentration of the extracts (measured as described in section 3.2.2) and the reaction volume of 5 μl. Subsequently, the DNA amount per reaction was divided by the weight of a haploid genome of the respective lupine species as provided by the Plant DNA C-values database of the Kew Royal Botanic Gardens [116] (column "C value" in Table 4.2) to calculate the number of genomes per reaction (Table 4.2, "genome equivalents in 5 μl"). The copy number of the target sequence was then determined using a standard curve obtained by the analysis of a dilution series of the amplification product of the real-time PCR (10 to 10^9 copies per reaction). The copy numbers per reaction were subsequently divided by the number of genomes per reaction (calculated as described above) to obtain the copy number per genome equivalent, referred to as CGE in the following. Gao et al. [117] found 422 rDNA repeats in BAC-end sequences that covered 0.96% of the *Lupinus angustifolius* genome. This corresponds to 43,958 repeats per genome. This number is in the same order of magnitude as the copy numbers of the internal transcribed spacer 1 determined in this work (35,700 for *Lupinus angustifolius* (Table 4.2)). The results in Table 4.2 illustrate the high inter- and intra-species variability of the copy number of the internal transcribed spacer 1 sequence.

Table 4.2: Determination of the copy number of the target sequence of the real-time PCR in five lupine species.

Species/ variety	DNA conc. [ng/μL]	C value (pg per genome)[116]	genome equivalents in 5 μL	Ct value	log copy no.	copy no. in 5 μL	copies per genome equivalent	mean copy no. per gen.	std. dev. copy no. [%]
L. angustifolius	14.45	0.93	77,670	11.77	9.51	$3.21*10^9$	41,276	35,700	14.8
				12.27	9.37	$2.34*10^9$	30,101		
	1.33		7,124	15.93	8.36	$2.31*10^8$	32,456		
				15.64	8.44	$2.78*10^8$	38,966		
L. angustifolius ssp. *angust.*	41.06	0.93	220,731	11.94	9.57	$3.74*10^9$	16,946	21,915	31.7
				12.08	9.53	$3.41*10^9$	15,455		
	2.57		13,820	15.37	8.62	$4.17*10^8$	30,168		
				15.66	8.54	$3.47*10^8$	25,090		
L. albus ssp. *graecus*	42.55	0.6	354,591	15.48	8.59	$3.88*10^8$	1,094	1,199	8.8
				15.16	8.68	$4.76*10^8$	1,343		
	2.49		20,738	19.77	7.40	$2.50*10^7$	1,205		
				19.83	7.38	$2.40*10^7$	1,156		
L. luteus	2.58	1.0	12,918	15.48	8.59	$3.88*10^8$	30,011	19,365	47.4
				15.84	8.49	$3.08*10^8$	23,858		
	0.46		2,279	19.46	7.48	$3.05*10^7$	13,388		
				19.88	7.37	$2.33*10^7$	10,203		
L. mutabilis	28.58	0.95	150,429	11.24	9.77	$5.83*10^9$	38,745	34,675	11.3
				11.37	9.73	$5.38*10^9$	35,797		
	2.02		10,651	15.82	8.50	$3.13*10^8$	29,370		
				15.55	8.57	$3.71*10^8$	34,787		

While the lowest CGE was determined for the white lupine *Lupinus albus* ssp. *graecus*, *Lupinus angustifolius* (blue lupine) shows an approximately 30-fold copy number and therefore the highest of all CGEs determined. Comparison of *Lupinus angustifolius* with another subspecies of blue lupine, *Lupinus angustifolius* ssp. *angustifolius*, reveals additional intra-species variation of the copy number of the target sequence per genome equivalent, with the CGE of the subspecies angustifolius being lower by more than a third.

4.1.5.2 Determination of the limit of detection for *Lupinus angustifolius*, *L. luteus* and *L. albus* in wheat flour

The high variability of the target sequence's copy number per genome equivalent implies different limits of detection (LODs) for different lupine species when analyzed using the developed real-time PCR method. Three extracts per spiked wheat flour (containing 0.1, 1, 5, and 10 mg lupine flour from *Lupinus angustifolius*, *Lupinus luteus*, and *Lupinus albus*, respectively) were analyzed in two PCR replicates each. The limit of detection was defined as the lowest concentration where all performed reactions were rated positive. The results confirmed the presumed variability of the LOD: 0.1 mg *Lupinus angustifolius* per kg wheat flour, 5 mg *Lupinus luteus* per kg wheat flour, and 10 mg *Lupinus albus* per kg wheat flour were the lowest amounts detectable in six of six reactions. This is in accordance with the results for the copy numbers per genome equivalent: the lowest copy number and therefore the highest LOD were found for *Lupinus albus*, while *Lupinus angustifolius* shows the highest copy number and the lowest LOD.

4.2 Comparison of the developed real-time PCR method with two commercial kits for the detection of lupine DNA

4.2.1 Detectability of 20 lupine species and varieties

Different lupine species could be used in foods, therefore DNA extracts from lupine seeds from the different species and varieties listed in Table 3.1 were used to compare the suitability of the the hybridzation probe-based real-time PCR method, the CIBUS kit and the PCR-FAST kit for the detection of lupine DNA. A third kit for the detection of lupine DNA provided by InCura (Cremona, Italy), the LupiKit, only became available later and was therefore not included in the analysis. The kits were applied as described in their respective manuals. For the CIBUS kit, this included an endpoint PCR and subsequent detection of the amplification products using agarose gel electrophoresis. In the case of the PCR-FAST kit, the SYBR-green based real-time PCR was combined with a melting curve analysis. For the developed real-time PCR, the verification of the amplified sequence was accomplished through the use of the hybridization probe. A negative control (5 μl PCR grade water) was analyzed in addition to the samples with each of the three methods. No amplification was observed in any of the control reactions. The results from the analysis of the different lupine species are shown in Table 4.3. Column A contains the results obtained with the developed real-time PCR method. Column B shows the results obtained using the CIBUS kit with subsequent agarose gel electrophoresis, while Column C contains the results obtained with the PCR-Fast kit (column C-1: Ct values of the SYBR green real-time detection, column C-2: results of the additional agarose gel electrophoresis).

All reactions gave positive signals. In addition to the specific amplicon, a second amplification product from *Lupinus perennis* DNA was detected using the PCR-FAST kit and melting curve analysis. Therefore, the amplification products were additionally analyzed using agarose gel electrophoresis. The result from the melting curve analy-

sis was confirmed: while all other samples yielded only one amplification product that matched the positive control DNA amplicon in length, two bands were visible for *Lupinus perennis* DNA. The primers therefore also anneal to another part of the DNA than the target sequence, hereby reducing the amount of primers available for the amplification of the target sequence. Since the PCR-FAST kit is a commercially available kit, the unspecific amplification product was not sequenced.

All considered methods detected lupine DNA from 20 different species and varieties and therefore comply with a major requirement in allergen detection, since various lupines could be used in foods.

Table 4.3: Comparison of the three PCR methods in terms of the detectability of DNA from different lupine species and varieties. **A**: Hybridization probe-based real-time PCR; **B**: CIBUS kit; **C**: PCR-FAST kit

Name	DNA concentration [ng/µL]	A Ct values	A Gel electrophoresis result	B Gel electrophoresis result	C-1 Ct values	C-2 Gel electrophoresis result
Lupinus albus L. ssp. *albus*	3.0	17.5	+	+	20.3	+
Lupinus albus L. ssp. *graecus*	1.3	17.2	+	+	20.7	+
Lupinus angustifolius L. ssp. *angustifolius*	3.6	14.5	+	+	20.3	+
Lupinus angustifolius L. ssp. *reticulatus*	3.7	14.2	+	+	20.5	+
Lupinus angustifolius	0.2	16.7	+	+	23.5	+
Lupinus hispanicus Boiss. et Reut.	1.3	15.5	+	+	21.6	+
Lupinus hispanicus Boiss. et Reut. ssp. *bicolor*	4.1	13.7	+	+	19.5	+
Lupinus luteus L.	1.3	15.4	+	+	21.2	+
Lupinus mexicanus Cerv. ex Lag.	4.2	13.5	+	+	19.1	+
Lupinus micranthus Guss.	20.0	22.6	+	+	21.2	+
Lupinus mutabilis Sweet	1.4	14.8	+	+	20.8	+
Lupinus nanus Douglas ex Benth.	44.7	18.0	+	+	25.3	+
Lupinus perennis L.	40.0	11.0	+	+	26.2	+, additional unspecific band
Lupinus polyphyllus Lindl. var. *polyphyllus*	2.2	14.2	+	+	29.7	+, additional unspecific band
Lupinus polyphyllus Lindl. var. *prunophilus*	2.4	14.1	+	+	24.6	+
Lupinus pubescens Benth.	4.9	12.9	+	+	19.1	+
Lupinus sp.	3.1	14.0	+	+	19.4	+
Lupinus subvexus C. P. Sm.	5.7	13.1	+	+	24.4	+
Lupinus succulentus Dougl. ex K. Koch	1.5	15.0	+	+	20.8	+
Lupinus variicolor Steud.	3.8	13.5	+	+	28.6	+, additional unspecific band

4.2.2 Specificity

As described in section 4.1.2, no nonspecific amplification was observed for the developed real-time PCR when DNA extracts from legumes, cereals, seeds, nuts, spices, meat and fruit were analyzed. According to the validation report, no cross-reactivity with DNA from peanut, soya, white beans, peas, lentils and chickpeas was observed for the CIBUS kit [118]. The additionally tested mung bean DNA also gave a negative result. The manual of the PCR-FAST kit only gives specificity data on pea, peanut and soya DNA, without any cross-reactivity reported. When the kit was applied to DNA from white beans, chickpeas, and mung beans, Ct values between 31.7 and 33.9 were observed. SYBR green, however, interacts with double-stranded DNA regardless of its particular nucleotide sequence. Therefore, the obtained amplification products were additionally analyzed using melting curve analysis. The melting curves of the chickpea and the mung bean DNA amplicon were not identical to the melting curve of the control DNA, whereas the curve of white bean DNA overlapped with the melting curve of the lupine DNA from the control. The additionally performed agarose gel electrophoresis did also only show the unspecific amplicons for chickpea and mung bean DNA, while the lane corresponding to DNA from white beans contained an amplicon with the same length as the positive control in addition to the unspecific one. Therefore, a cross reactivity with DNA from white beans was detected for the CIBUS kit, which is in contrast to the specificity of the developed real-time PCR method and the PCR-FAST kit.

4.2.3 Detectability of lupine flour in spiked ice cream

When ice cream containing 1000, 100, 10, 1, and 0.1 mg lupine flour per kg was analyzed using the developed real-time PCR method, lupine DNA was detected in all samples. The use of a multicopy target sequence accounts for the exceptionally low detectable amount of lupine flour in a food. The CIBUS kit reliably detected 1000 mg lupine flour per kg ice cream, while only a weak band corresponding to the ice cream containing 100 mg/kg was visible on the agarose gel. Concentrations of 10, 1, and 0.1 mg lupine flour per kg ice cream were not detectable using the CIBUS kit. When the

ice creams were analyzed using SYBR green real-time PCR (PCR-FAST kit), amplification occurred in all spiked samples. Visualization of the amplification products on an agarose gel only allowed the detection of 1000 to 10 mg lupine flour per kg ice cream, while no bands pertaining to the samples containing 1 and 0.1 mg lupine flour were visible. The results are summarized in Table 4.4. In conclusion, the hybridization-probe based real-time PCR and the PCR-Fast kit with SYBR green detection enabled the detection of 0.1 mg lupine flour per kg ice cream. 10 mg/kg were the lowest concentration of lupine flour in ice cream that could be detected using the PCR-Fast kit with subsequent amplicon visualization by agarose gel electrophoresis, while 100 mg/kg was the lowest detectable concentration using the CIBUS kit. According to these results, the hybridization probe-based real-time PCR method and the PCR-Fast kit fulfill the requirement to detect allergens in the low mg/kg range, while the CIBUS kit does not enable the detection of a few mg lupine material per kg sample.

Table 4.4: Detectability of lupine flour in a food matrix: comparison of the three methods.

A: Hybridization probe-based real-time PCR; **B**: CIBUS kit; **C**: PCR-FAST kit.

Lupine flour	**A**	**B**	**C**	**C**
	Ct values	Gel electro-phoresis result	Ct values	Gel electro-phoresis result
1000	18.7	+	26.5	+
100	23.8	+, band weakly visible	31.1	+
10	26.2	-	33.6	+
1	29.9	-	41.2	-
0.1	33.4	-	42.0	-

4.2.4   Detectability of lupine DNA in commercial food products

Foods with declared lupine content as well as foods without a declaration of lupine as ingredient were analyzed to compare the applicability of the three methods. Table 4.5 lists the foods that were tested in this study. Three products that had lupine flour declared in their ingredients list were included as positive controls. Only one of them tested positive with all three methods: false-negative results were obtained for the remaining two products when analyzed using the CIBUS kit. This is in line with the kit's low sensitivity observed for the detection of lupine flour in ice cream. Using the PCR-FAST kit, all three lupine-containing products were correctly identified. Of three products without an indication of the presence of lupine-based ingredients on the label, two came from manufacturers that use lupine flour in some of their products. Lupine DNA was detected in both products using the developed real-time PCR method and the PCR-FAST kit, illustrating that cross contamination is likely to occur when lupine flour is present at a food production site. Due to its low sensitivity, the CIBUS kit did not give any positive signals for these products.

Table 4.5: Comparison of the applicability of the three methods: Analysis of commercial food products. **A**: hybridization probe-based real-time PCR; **B**: CIBUS kit; **C**: PCR-Fast kit.

Name	Labelling	DNA concentration [ng/μL]	A Ct values	B Gel electro-phoresis result	C Ct values	C Gel electro-phoresis result
Mini rolls, toasted	Lupine flour	5.3	21.7	+	22.9	+
		4.1	21.9	+	22.4	+
Whole meal bread from oat	Sweet lupine flour	6.3	31.3	-	23.3	+
		5.7	31.5	-	22.4	+
Cocos cookies with chocolate icing	Sweet lupine flour	29.3	15.7	-	22.5	+
		32.7	16.2	-	22.1	+
Whole meal bread		6.5	32.3	-	21.8	+
		5.6	34.0	-	24.1	+
Cookie with chocolate icing		16.2	37.7	-	27.6	+
		13.3	38.1	-	22.9	+
Crackers		28.2	32.3	-	21.7	+
		24.6	32.3	-	18.6	+

4.3 Comparison of the developed real-time PCR method with an ELISA kit for the detection of lupine

ELISA assays are widely used in the field of food allergen testing. Therefore, a spectrum of foods, comprising various bakery products, fruit-flavored gums and ice cream, was analyzed by hybridization probe-based real-time PCR as well as by ELISA to evaluate the comparability of the two methods. The results are shown in Table 4.6. Thirteen products that had lupine flour, protein, or fiber declared in their ingredients list were included as positive controls. Twelve of them tested positive with the developed real-time PCR method and the ELISA test. This shows that even in commercially employed lupine protein and fiber the content of amplifiable DNA is sufficient to yield positive results with the PCR method. In the remaining sample, traces of lupine DNA were found, whereas no lupine protein was detectable. For seven products no presence of lupine-based ingredients was indicated on the label. Nevertheless, lupine DNA and lupine protein were detected in one of them. Traces of lupine DNA were found in another three products, whereas lupine protein was detectable in only one of those products. For the remaining three samples, the absence of lupine DNA and lupine protein above the detection limits of the respective methods was verified.

Table 4.6: Results obtained by analysis of food samples, using the developed real-time PCR and a commercially available ELISA kit, respectively.

Product	Labelling/ ingredients	Detection of lupine DNA [1]	Detection of lupine protein [2]
bread roll	lupine protein, 15 %	+	+
ice cream	lupine protein, 3 %	+	+
muffin	lupine protein, 2 %	+	+
fruit-flavored gums	lupine protein, 100 mg/kg	+	+
ice cream	lupine protein, 100 mg/kg	+	+
fruit-flavored gums	lupine fiber, 10 %	+	+
toast	sweet lupine flour, 4 %	+	+
whole-grain bread with sunflower seeds	lupine flour	+	+
rice bread	sweet lupine flour	+	+
wheat brown bread with flax seeds, 1	lupine flour	+	+
wheat brown bread with flax seeds, 2	lupine flour	+	+
wheat brown bread with pumpkin seeds	lupine flour	+	+
almond curd cheese stollen	lupine flour	traces	-
ice cream with soy protein		+	+
buckwheat bread with sesame		traces	+
bread roll from pretzel dough		traces	-
cake slices with whole milk chocolate		traces	-
cherry cake		-	-
crunchy bread from rice and maize		-	-
ice cream, vanilla		-	-

[1] Using the real-time PCR method described in this thesis.
[2] Using a commercially available ELISA kit [110].

4.4 Effects of processing and of the food matrix on the detection limit of the developed real-time PCR method

The outcome of the real-time PCR is especially dependent on the integrity of the analyte, which is negatively affected by food processing. Even for real-time PCR systems optimized for the use with processed foods by aiming at short fragments, a significant rise in Ct value has been observed after thermal treatment [100]. Therefore, the objective of this work was to study the effects of processing on the LOD of the real-time PCR method established for the detection of lupine DNA. The preparation of the model product chosen should comprise different processing steps. As this applies to pizza, and due to the fact that a case of lupine allergy after consumption of a pizza has been reported [119], this food was chosen as the model for the study of processing effects on the detectability of lupine DNA. When baked pizza dough prepared without an acidic ingredient was compared to pizza coated with tomato puree, the limit of detection was higher for the latter [120]. In this case, however, the effects of both heat and low pH were regarded in combination. Therefore, they were also considered separately: The influence of acidic ingredients was determined analyzing mayonnaise with different amounts of lupine flour, while the impact of heat was assessed using lupine-spiked, double-baked bread made according to a traditional recipe ("Zwieback"). The previously observed negative effect of freezing and thawing on lupine DNA detectability was investigated using ice creams prepared with varying amounts of lupine flour, which were analyzed after different periods of storage at -20 °C.

4.4.1 Changes in the detectability of lupine DNA using real-time PCR in the course of the preparation of pizza

4.4.1.1 Limit of detection in spiked commercial wheat flour

Wheat flour containing 1000 mg, 100 mg, 10 mg, 1 mg, 0.1 mg, and 0.01 mg of lupine flour per kg, respectively, was each extracted twice and four PCRs per extract were conducted. The employed PCR-method allowed a consistent detection of lupine DNA down to a content of 0.1 mg lupine flour per kg of wheat flour. For the wheat flour containing 0.01 mg lupine flour per kg, amplification was only observed in one of the eight performed reactions, with a high Ct value of 41.6. Due to the high inherent sensitivity of the method, even very low lupine contents result in a signal. Therefore, the limit of detection was defined as the lowest concentration level where all performed reactions were rated positive. The limit of detection determined for wheat flour according to this definition is 0.1 mg/kg. This is in agreement with the value determined in ice cream [107], a matrix in which the lupine flour did also not undergo further processing. The standard curve derived from the analysis of the spiked wheat flour is shown in Figure 4.6. The Ct values were plotted against the logarithm of the lupine flour content of the respective samples. The slope of the resulting regression line indicates the amplification efficiency. As mentioned in section 4.1.3, the slope of the standard curve should be in the range of - 3.1 to - 3.6 and the correlation coefficient should be $= 0.98$ according to the ENGL guideline [112]. With a slope of -3.6 and a correlation coefficient (R^2) of 1.00, the standard curve in Figure 4.6 meets these acceptance criteria. The efficiency of 90% for the analysis of spiked wheat flour is in the required range from 90 to 110%. Figure 4.6 illustrates the high linearity of the real-time PCR method, which enables the detection of lupine materials over five orders of magnitude.
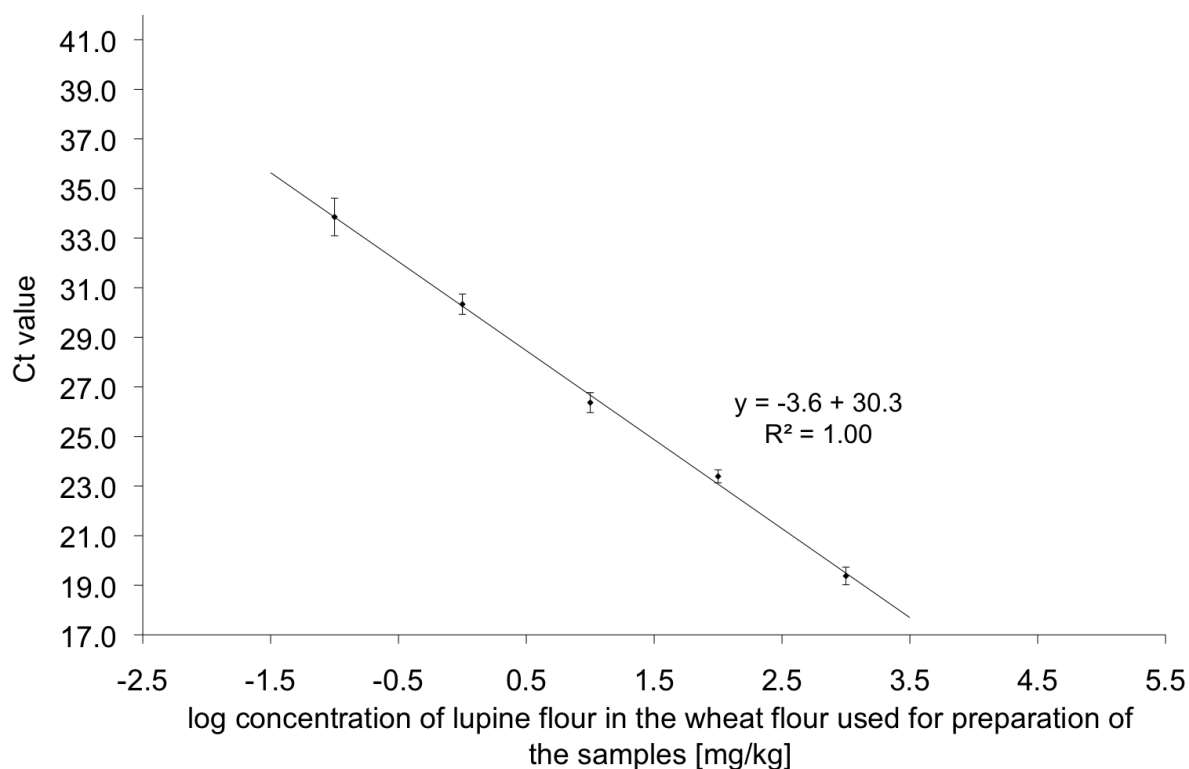
Figure 4.6: Standard curve derived from the analysis of wheat flour containing 0.1 - 1000 mg/kg lupine flour, respectively. Data are means of results obtained by real-time PCR analysis of two extracts (4 PCRs per extract) for each spiking level; bars represent standard deviations.

4.4.1.2 Analysis of spiked pizza dough

During the preparation of pizza dough, mechanical stress is exerted on the ingredients by kneading of the dough. Subsequently, enzymatic reactions originating from the yeast fermentation process take place, which may also influence the integrity of the DNA. Doughs were prepared from the lupine-spiked wheat flours and two samples were extracted per dough; four real-time PCR reactions were performed per extract. The Ct values recorded for the pizza dough after 30 min of rising at room temperature are given in (Table 4.7, column a). From 1000 to 0.1 mg/kg, all performed reactions gave positive results. For 0.01 mg/kg, amplification was observed in four out of eight reactions (extract 1: Ct values 38.1, 38.0, 38.4, no amplification observed; extract 2:

Ct value 42.6, no amplification observed in the remaining three reactions). The limit of detection determined for the dough according to the aforementioned definition is therefore 0.1 mg/kg. The observed amplification efficiency of 97% (Figure 4.7) determined as described above for spiked wheat flour, which is in the range from 90% to 110% as required by the ENGL guideline, indicates that the yeast fermentation process did not influence the integrity of the DNA. No amplification was observed in the reactions performed on the extract from the dough made from the lupine-free wheat flour. In industrial bakeries, raw dough pieces are commonly frozen, distributed in frozen condition and subjected to further processing at a different location. This was simulated by two weeks of storage of the dough at -20 °C. A change of the mean Ct value from 32.8 to 36.2 was observed for the dough prepared from the 0.1 mg/kg flour mixture (Table 4.7, column b). As can be seen in Figure 4.7, these conditions also led to an increase in the slope of the corresponding standard curve from -3.4 for the dough that had not been frozen to -4.1, resulting in a decrease of the amplification efficiency from 97% to 75%. It has been shown that DNA from meat samples is damaged after 15 days of storage at -20 °C [121]. The observed effect can therefore be explained by DNA degradation caused by freezing and subsequent thawing. Detection of lupine DNA was still possible at 0.1 mg/kg with a mean Ct value of 38.4, whereas no amplification was observed in the reactions performed on the 0.01 mg/kg sample and the blank sample.

Table 4.7: Analysis of (a) pizza dough after rising, (b) pizza dough after two weeks of storage at -20 °C, (c) baked pizza without tomato puree, and (d) baked pizza with tomato puree.

Concentration of	Ct values [a]			
lupine flour [mg/kg]	a	b	c	d
1000	19.7 ± 0.2	20.0 ± 0.3	20.2 ± 0.2	19.6 ± 0.3
	19.7 ± 0.3	20.2 ± 0.2	19.8 ± 0.3	19.5 ± 0.2
100	23.6 ± 0.2	21.7 ± 0.4	22.8 ± 0.2	22.4 ± 0.4
	23.7 ± 0.4	23.5 ± 0.5	$23.3. \pm 0.4$	22.8 ± 0.2
10	27.2 ± 0.3	27.4 ± 0.4	26.5 ± 0.7	27.6 ± 0.3
	27.4 ± 0.1	27.5 ± 0.1	27.2 ± 0.3	26.6 ± 0.5
1	31.7 ± 0.3	32.4 ± 0.1	30.9 ± 0.1	30.7 ± 0.4
	31.1 ± 0.4	31.0 ± 0.8	31.4 ± 0.4	29.3 ± 0.1
0.1	32.9 ± 0.1	37.7 ± 0.9	34.6 ± 0.9	37.0 ± 1.4
	32.7 ± 0.5	34.7 ± 0.6	31.8 ± 0.5	34.9 ± 0.4
0.01	[b]	[d]	[c]	[d]
	[b]	[d]	[c]	[d]

[a] two extracts per concentration level were each analyzed four times using real-time PCR.

[b] amplification observed in four out of eight reactions (extract 1: Ct values 38.1, 38.0, 38.4, 1x no amplification observed; extract 2: Ct value 42.6, no amplification observed in the remaining three reactions)

[c] amplification observed in one of eight reactions (Ct value 38.6)

[d] no amplification observed

4.4.1.3 Analysis of pizza made from lupine-containing dough

As heat-induced DNA degradation is increased by acidic conditions, which can be brought about by ingredients low in pH, two pizzas were prepared per concentration level. One was baked without topping, while the other one was coated with tomato puree (Table 4.7, columns c and d). The Ct values were equal or lower for the pizza

without tomato puree (Table 4.7, column c) compared to the dough after freezing (Table 4.7, column b). The DNA degradation induced by the heating process is obviously compensated for by the concentration effect owing to the loss of water. The contribution of each of these two effects cannot be experimentally determined, because baking inevitably results in loss of water and an increase in relative lupine content. In contrast to the analysis of the dough after rising and after freezing and due to the increase in relative lupine content, amplification was observed in one of the eight reactions performed on the extracts from the pizza made from the 0.01 mg/kg flour mixture (Ct value 38.6). No amplification was observed in the blank pizza sample. Data obtained for the pizzas coated with tomato puree (Table 4.7, column d) differ from those recorded for the pizzas that were prepared without tomato puree. No amplification was detectable in the pizza samples based on the lupine-free wheat flour and on the 0.01 mg/kg flour mixture. The mean Ct value for the next higher concentration level of 0.1 mg/kg was 36.0 compared to 33.2 for the pizza not coated with tomato puree. The amplification efficiency of 77% calculated from the slope of the corresponding standard curve shown in Figure 4.7 indicates degradation of the DNA. The pH value of the tomato serum derived from the puree through centrifugation was determined to be 4.0. Under acidic conditions, non-enzymatic hydrolysis of DNA takes place. The first step of DNA degradation at low pH conditions is depurination, leading to further hydrolysis and a decrease in average fragment size [122], which can hamper the PCR-based detection of specific sequences. The results obtained for the comparison of pizza with and without an acidic ingredient are in accordance with these findings. Detection of lupine DNA was possible in one of the reactions performed on the pizza based on the 0.01 mg/kg flour mixture and baked without tomato puree, while it failed in all reactions performed on the DNA extract from the respective sample prepared with tomato puree. Figure 4.8 exemplarily illustrates the results obtained from the 0.1 mg/kg-samples. As expected, the Ct values rise rise after freezing of the dough. After baking, an even higher Ct value would be expected. In contrast, the Ct value drops as a result of the loss of water and the corresponding concentration of lupine flour in the samples. The pizza baked with tomato puree, however, shows the expected rise in Ct value compared to the pizza
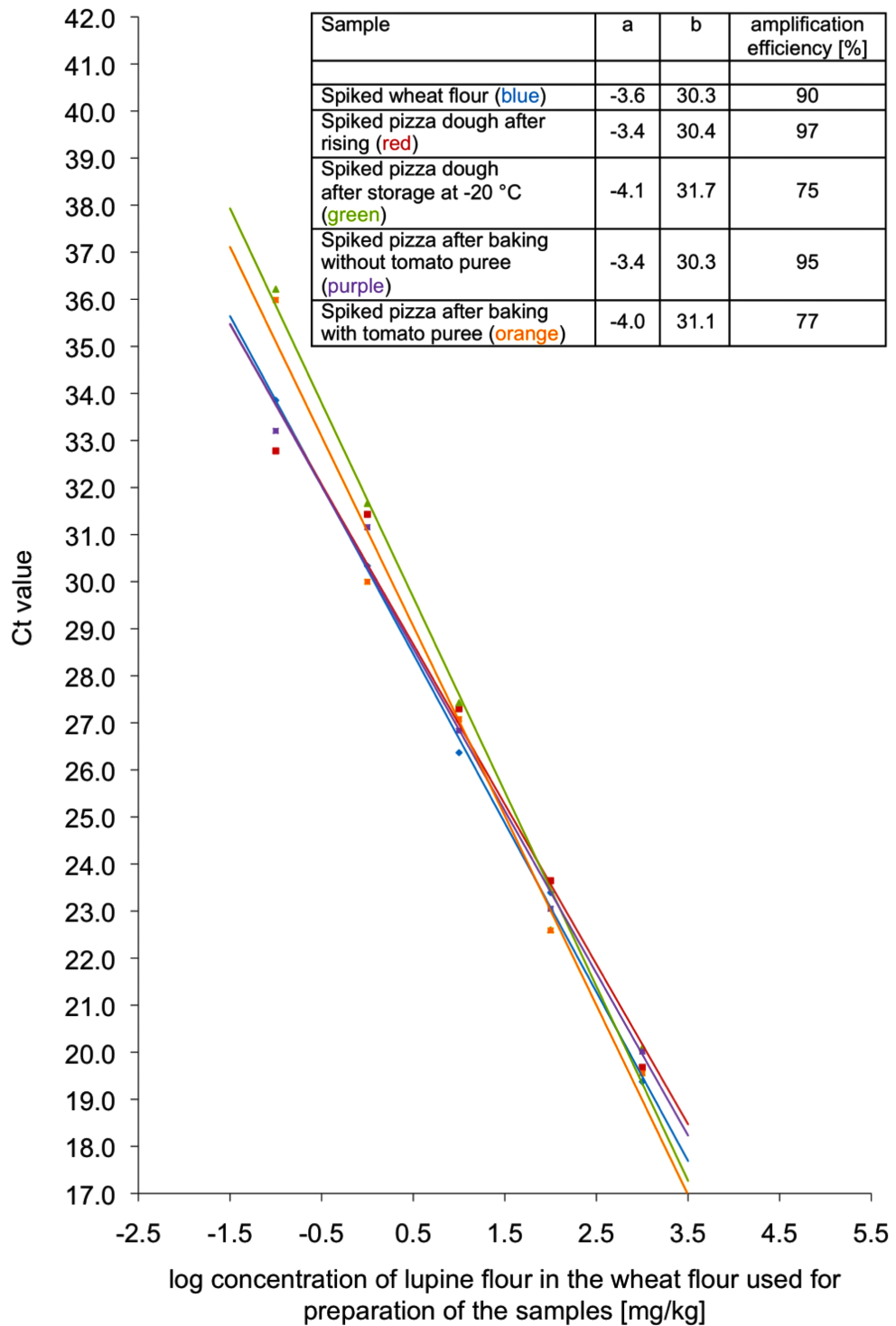
Figure 4.7: Standard curves obtained by the analysis of spiked wheat flour (blue), spiked pizza dough after rising (red) and after storage at -20 °C (green); and of spiked pizza after baking with (orange) and without (purple) tomato puree. y: Ct value; a: slope; x: log lupine flour concentration; b: y axis intercept.
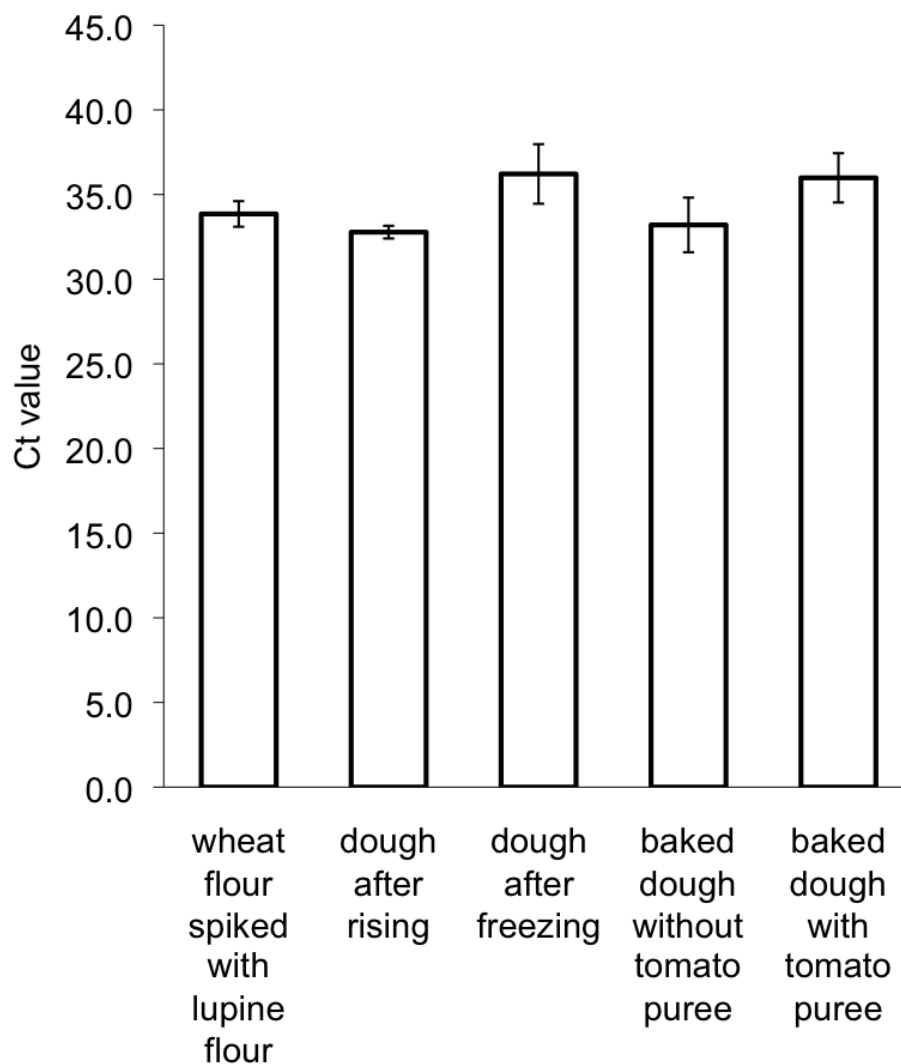
Figure 4.8: Histogram illustrating the mean Ct values obtained from the analysis of the 0.1 mg/kg samples at different processing stages. Bars represent standard deviations.

made without an acidic ingredient. As can be seen from Figure 4.8, the processing steps leading to a rise in Ct value and therefore inducing a degrading effect on the DNA are freezing and thawing as well as the influence of heat under acidic conditions. It has to be kept in mind that a comparison of the Ct values is only valid for the whole products resulting from the different processing steps. A true comparison of the Ct values of the wheat flour contained in these products is not possible, because the relative content of the constituents changes with the processing levels. Therefore, the

actual amount of wheat flour contained in each of these products cannot be determined and the obtained Ct values solely reflect the detectability of lupine DNA in respect to the whole particular product.

The high sensitivity of the hybridization probe-based real-time PCR might result in the detection of amounts that are not relevant to allergic sufferers. In a double-blind, placebo-controlled food challenge the eliciting dose for subjective allergy symptoms was determined to vary from 1 mg or less to 3 mg lupine flour [114]. In order to ingest 1 mg of lupine flour via wheat flour containing 0.1 mg/kg lupine flour, one would have to consume 10 kg of the respective flour. Objective symptoms such as hoarseness and rhinoconjunctivitis only occurred after ingestion of 300 mg lupine flour or more, which could not be achieved by consumption of products made from wheat flour containing 0.1 mg/kg lupine flour. Therefore, a criterion was needed to distinguish possible traces at the detection limit from amounts that might affect sensitive individuals. A lupine flour content of 0.1 mg per kg of wheat flour results in a mean Ct value of 33.9. Accordingly, only samples with Ct values lower than or equal to 33.9 may be considered as containing amounts of lupine materials that are relevant to allergic sufferers. For the dough made from spiked wheat flour, the lowest concentration rated positive according to this Ct-based exclusion criterion is 0.1 mg/kg (Table 4.7). After cold storage and thawing, the practical limit of detection is 1 mg/kg with a mean Ct value of 31.7. Regarding the pizzas baked with and without tomato puree, respectively, the samples containing 1 mg lupine flour per kg pizza with tomato puree and the sample containing 0.1 mg lupine flour per kg pizza without tomato puree are considered as containing relevant amounts of lupine in respect to the allergy eliciting dose. Identification of samples which contain amounts of lupine material that might affect allergic individuals is thus possible even in highly processed foods.

4.4.2 Impact of acidic ingredients, the application of heat and of storage at low temperatures on the limit of detection

4.4.2.1 Effect of storage at low temperatures

For the determination of the influence of the food matrix, food processing and of storage conditions on the detectability of lupine DNA using real-time PCR, ice cream containing lupine flour (1000, 100, 10, 1, 0.1 and 0.01 mg/kg) was manufactured and analyzed after one day, two weeks, and four weeks of storage at -20 °C. Real-time PCR was performed twice on each of two extracts per concentration level. 0.01 mg lupine flour per kg was detectable after all examined storage periods. However, the Ct values observed for this low lupine concentration varied: for example, the highest and the lowest Ct value observed after one day of freezing differed by 1.5 cycles. Therefore, the results for the 0.01-mg/kg samples were not included in the standard curves obtained by plotting the mean Ct values against the logarithm of the respective lupine flour concentration (Figure 4.9). The amplification efficiency E was then calculated from the slopes of the curves using the aforementioned formula $10^{(-1/slope)}$-1 [112].

The slopes of the curves corresponding to one day and two weeks of storage at -20 °C indicate amplification efficiencies of 104 and 98%. Stirring of the lupine-containing ice cream base does therefore not notably influence the detectability of lupine DNA, while a small decrease in amplification efficiency can be observed after two weeks of frozen storage. After four weeks, the efficiency of the PCR decreases to 89%, which can be explained by a decline of amplifiable DNA in the ice cream due to fragmentation [121].

4.4.2.2 Influence of acidic ingredients

In the case of mayonnaise as a food containing an acidic ingredient, lupine DNA was detectable in all samples with Ct values ranging between 21.2 (1000 mg/kg) and 40.5 (0.01 mg/kg). However, the amplification efficiency calculated from the slope of the regression line was only 75%. At low pH, DNA degradation through non-enzymatic hydrolysis following depurination takes place [122]. The resulting decrease in average fragment size [122] influences the detectability of target DNA sequences.
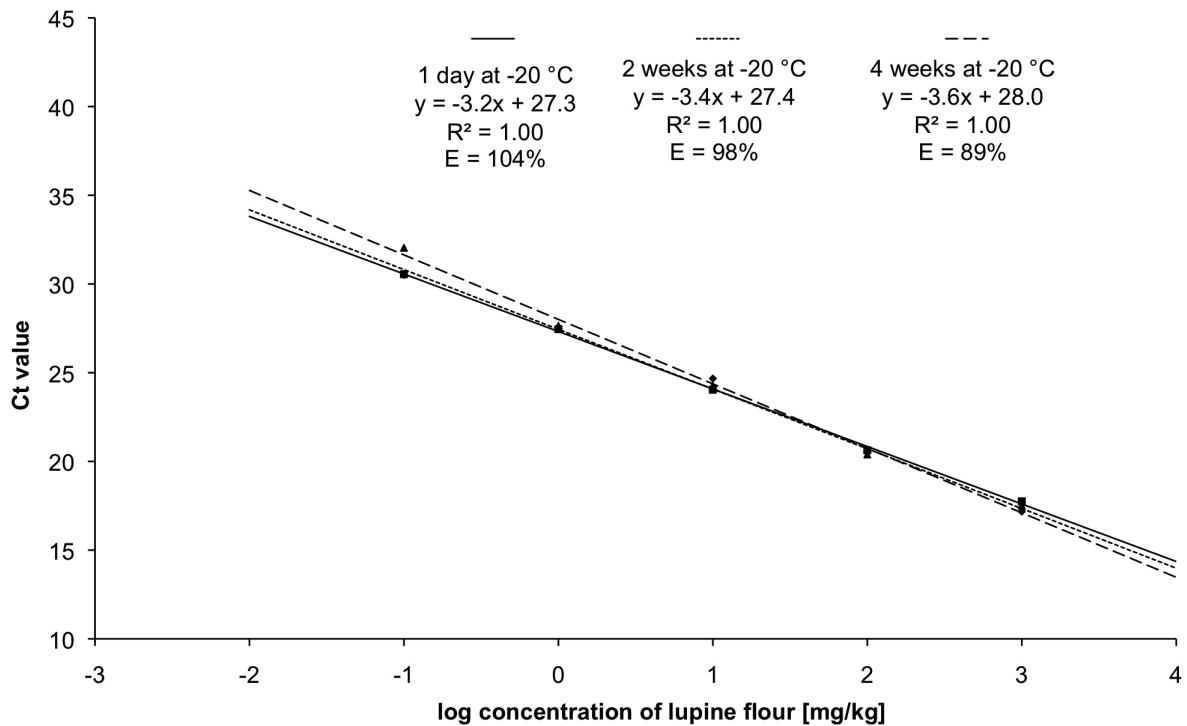
Figure 4.9: Standard curves obtained by the analysis of lupine-containing ice cream (1000, 100, 10, 1, and 0.1 mg/kg) using the developed real-time PCR after storage at -20 °C for 1 day (full line), 2 weeks (dotted line) and 4 weeks (dashed line).

4.4.2.3 Impact of heat treatment

The effect of heat on the detectability of lupine DNA using hybridization-probe based real-time PCR was examined by the analysis of "Zwieback", a wheat bread made following a traditional recipe. The preparation includes a second heat treatment after baking: the bread is dried for preservation at a lower temperature the following day. Lupine DNA was detected in the double-baked breads containing 1000 - 0.1 mg/kg using real-time PCR. Of a total of four reactions performed on extracts from the bread containing 0.01 mg lupine flour per kg, amplification was only observed once and gave a high Ct value of 44.2. The limit of detection in double-baked bread, as opposed to ice cream and mayonnaise with a LOD of 0.01 mg/kg, is therefore 0.1 mg/kg. This result is in accordance with the negative influence of thermal treatment on the detectability of a DNA target sequence described in [96]. Plotting the Ct values obtained for the breads

containing 1000 - 0.1 mg/kg against the logarithm of the lupine flour concentration resulted in a curve with a slope of -3.6. This corresponds to an amplification efficiency of 89%, which also illustrates the impact of the intense heat treatment on the integrity of the DNA.

4.4.2.4 Comparison of the effects of storage at low temperatures, acidic ingredients and of heat treatment on the limit of detection

When comparing the Ct values obtained for the ice cream (after 4 weeks of frozen storage), the mayonnaise, and the bread samples containing 0.1 mg lupine flour per kg, the storage of the ice cream at -20 $^\circ$C had the least impact on the detectability of lupine DNA. The mean Ct value was 32.0 in spite of a low DNA concentration in the extract (0.4 ng/μL). The DNA concentration of the mayonnaise and bread samples was adjusted to 5 ng/μL. Both still gave higher Ct values than the ice cream, indicating that acidic conditions and heat have a higher impact on the integrity of the DNA than frozen storage. The mean Ct value obtained for the mayonnaise containing 0.1 mg lupine flour per kg was 37.9 as opposed to 41.2 for the double-baked bread. The overall higher Ct values observed for the bread samples account for the LOD of 0.1 mg/kg. These results indicate a higher impact of thermal treatment than of acidic ingredients on the detectability of specific DNA sequences. The most pronounced effect has been observed for the combination of both low pH and heat [96, 120].

4.5 Quantification of *Lupinus angustifolius* in wheat flour

In the European Union, labelling of the 14 food allergens listed in annex IIIa of Directive 2000/13/EC is mandatory. For the reasons described in section 2.3, the implementation of upper limits for these allergens is under discussion. Therefore, quantitative analytical methods will be needed to verify compliance with regulatory requirements. The quantification of genetically modified organisms (GMO) in food using real-time PCR is based on the ratio of the copy number of the transgene and the copy number of a species specific reference gene. Since food products are complex mixtures of ingredients that originate from a variety of species, an ubiquitous reference sequence representative for all these species would be needed in order to transfer this principle to the quantification of food allergens using real-time PCR. No such sequence is available, however, a DNA sequence specific to a DNA-containing material that does not naturally occur in food can be used as reference sequence when a defined amount of this standard material is added to the food sample prior to the extraction of the DNA. This method was first described by Hirao et al. [109] for the quantification of buckwheat, a potentially allergenic food ingredient for which labelling is mandatory in Japan.

The aim of this study was to transfer this method to the quantification of an allergen which is relevant in the European Union. As described in [109], statice (*Limonium sinuatum*) seeds were used as the internal standard, since statice is an ornamental plant and is therefore not likely to be present in food. In addition, the statice seeds can be bought at seed stores, making the internal standard material easily available. The feasibility of this method for the determination of lupine contents in the range of 1 to 10 mg/kg was investigated. The following sections describe the assessment of the specificity of the Limo-62/Limo-162 primer pair used for the amplification of the DNA from the internal standard (statice seed) towards lupine DNA and the construction of a reference molecule. Following the description of this preparatory work, details on the establishment of standard curves for the determination of lupine and statice starting

copy numbers using the reference molecule and on the determination of the coefficient value of *Lupinus angustifolius* are given. Finally, the results obtained from the analysis of spiked wheat flours are presented.

4.5.1 Specificity of the Limo-62/Limo-162 primer pair towards *Lupinus angustifolius* DNA

In order to rule out cross-reactivity of the statice PCR primers towards *Lupinus angustifolius* DNA, *Lupinus angustifolius* DNA was subjected to PCR using the primer pair Limo-62/Limo-162. While the control reaction yielded the expected product of 101 bp from statice DNA, no amplification was observed for *Lupinus angustifolius* DNA. Therefore, combining the analyte *Lupinus angustifolius* with statice as internal standard material is feasible.

4.5.2 Construction of a reference molecule containing the target sequences of the lupine and statice real-time PCR systems

Figures 4.10 and 4.11 show the amplicons obtained using (i) the primer pairs Lupine F/ov-Lupine R (Figure 4.10, lane B) and ov'- Limo-62/Limo-162 (Figure 4.10, lane C) and (ii) the reference molecule obtained using the primer pair Lupine F/Limo-162 on a mixture of the two amplicons shown in Figure 4.10 (Figure 4.11, lane B), respectively. Due to the overlapping ends of these amplicons, the resulting hybrid molecule's length is: length (lupine amplicon) + length (statice amplicon) - length (overlapping region) = 149 bp + 121 bp - 20 bp = 250 bp.

Figure 4.10: Amplicons obtained using the primer pairs Lupine F/ov-Lupine R (lane B) and ov'-Limo-62/Limo-162 (lane C), respectively. Lane A: puC8 DNA size standard.
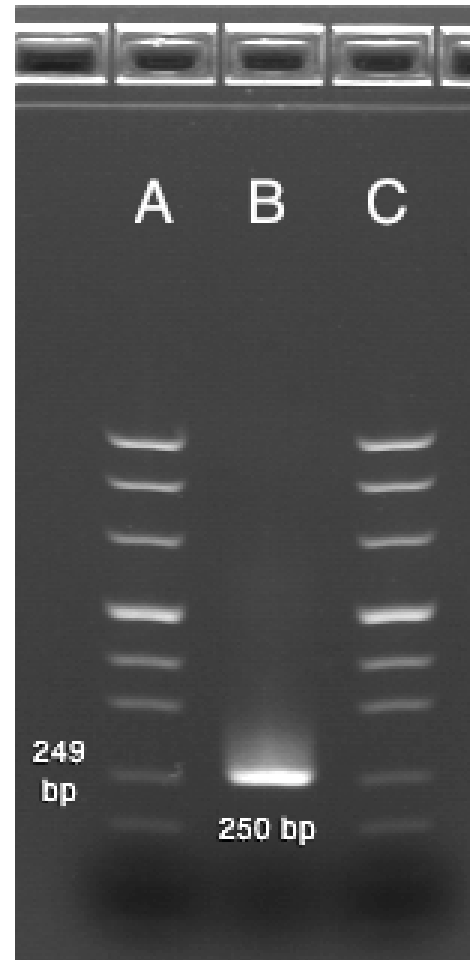

Figure 4.11: Reference molecule obtained using the primer pair Lupine F/Limo-162 on a mixture of the two amplicons shown in Figure 4.10 (lane B). Lanes A and C: puC8 DNA size standard.

4.5.3 Standard curves for the determination of lupine and statice starting copy numbers

In order to determine starting copy numbers for lupine and statice DNA in the sample extracts, standard curves for both PCR systems were established. They were obtained by performing real-time PCR on dilutions of the reference molecule and by subsequently plotting the cycle threshold (Ct) values against the logarithm of the respective starting copy numbers per reaction. The resulting graphs that were used to evaluate the linearity of the two PCR systems are shown in Figure 4.12 (lupine) and Figure 4.13 (statice). For starting copy numbers from 10^3 to 10^8 per reaction for lupine and 10^4 to 10^9 per reaction for statice, the correlation coefficient was > 0.99. The amplification efficiencies calculated from the slopes of the standard curves were 87.6% (lupine) and 87.2% (statice).
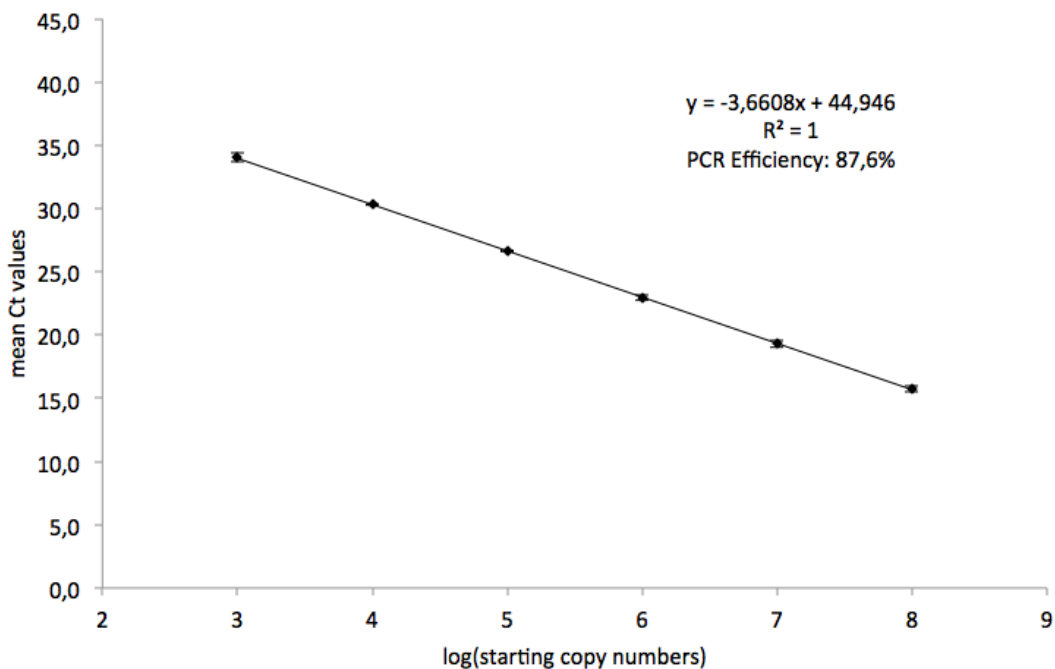
Figure 4.12: Standard curve derived from the analysis of dilutions of the reference molecule with the real-time PCR for the detection of lupine DNA. Data are means of three replicate PCR analyses per dilution; bars represent standard deviations.
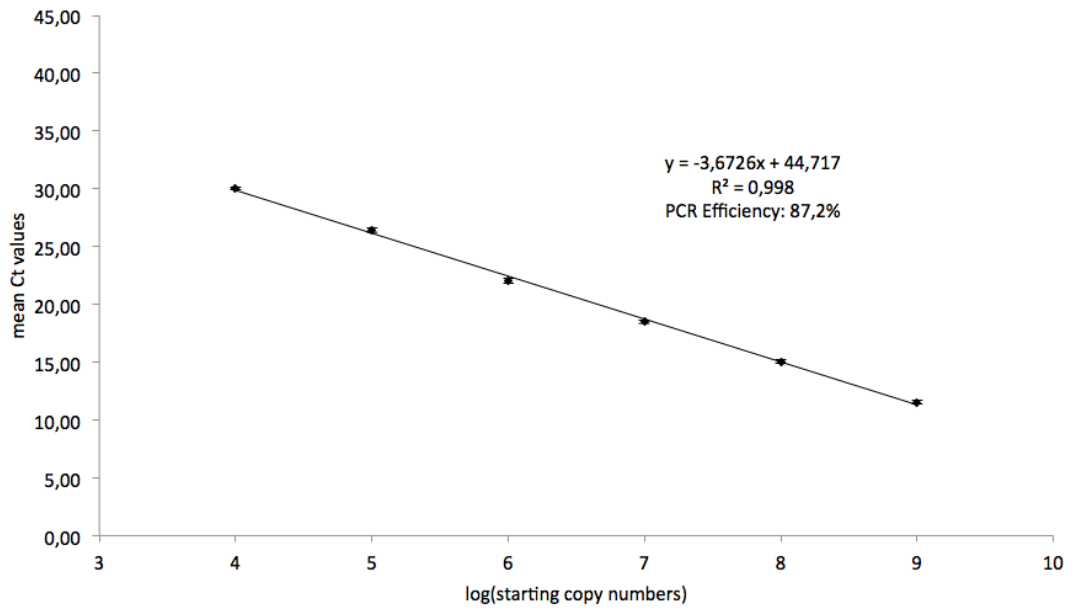
Figure 4.13: Standard curve derived from the analysis of dilutions of the reference molecule with the real-time PCR for the detection of statice DNA. Data are means of three replicate PCR analyses per dilution; bars represent standard deviations.

4.5.4 Determination of the coefficient value for lupine

For calculating the lupine content of the samples from the copy numbers determined for lupine and statice, the coefficient values were determined according to formula (I):

$$cv_{Lupine} = Limonium_0 / Lupine_0 \text{ (I)}$$

$Limonium_0$ = statice starting copy number of DNA extract from 1 g lupine flour and 200 mg ground statice seeds

$Lupine_0$ = lupine starting copy number of DNA extract from 1 g lupine flour and 200 mg ground statice seeds

A mean coefficient value of 0.1017 $\pm$ 0.0150 was determined from a total of 24 extracts. The individual coefficient values obtained for each of the twenty-four extracts of the lupine flour are given in Table 4.8. The mean coefficient values obtained for each of the five independent analysis series illustrate that the coefficient value can be reproducibly determined. Therefore, the number of extracts of the 100% analyte material per extraction series may be reduced for routine analysis purposes after the coefficient value has been reliably established.

Table 4.8: Coefficient values for *Lupinus angustifolius* from 5 temporally separated series of analysis.

Series	Coefficient value	Mean coefficient value
A	0.1020	0.1097
	0.1060	
	0.1117	
	0.1189	
B	0.0869	0.0879
	0.0871	
	0.0774	
	0.0912	
	0.0967	
C	0.1241	0.1071
	0.0982	
	0.0994	
	0.1067	
	0.1070	
D	0.1211	0.1087
	0.1114	
	0.1044	
	0.1040	
	0.1027	
E	0.0740	0.0968
	0.0712	
	0.1009	
	0.1306	
	0.1072	
Overall coefficient value		0.1017 ± 0.0150

4.5.5   Lupine flour contents determined in wheat flour

The lupine flour concentrations determined in the spiked wheat flour samples using real-time PCR and statice seeds as internal standard material are given in Table 4.9. The comparison with the actual lupine flour contents of the investigated samples shows that the measured values are in the respective ranges. The average relative standard deviation is 42%; for the sample containing 10 mg lupine flour per kg wheat flour the relative standard deviation is comparable to that reported by Hirao et al. [109] for the sample containing 10 mg buckwheat per kg wheat flour (49% vs. 40%).

Table 4.9: Quantification of lupine flour in wheat flour using real-time PCR.

Actual lupine flour content $[mg/kg]$	Experimentally determined lupine flour content $[mg/kg]$	Relative standard deviation $[\%]$
1	1.7 ± 0.6[a]	35.3
5	9.3 ± 3.9[b]	41.9
10	14.6 ± 7.2[c]	49.3

[a] Mean of 11 DNA replicate extracts.

[b] Mean of 8 DNA replicate extracts.

[c] Mean of 10 DNA replicate extracts.

Plotting the measured lupine flour content against the actual lupine flour content results in the graph shown in Figure 4.14. The horizontal lines visualize the ranges of the standard deviations of the 1 mg/kg sample (dotted lines) and the 10 mg/kg sample (dashed lines), respectively. Taking these standard deviations into account, a discrimination of samples containing about 1 mg per kg and samples containing about 10 mg/kg is possible.

Methods for the analysis of allergens in foods should be capable of reliably detecting the analyte in the low mg per kg range [2]. Figure 4.14 demonstrates that lupine flour concentrations of a few mg per kg, as relevant to allergen analysis, are within the
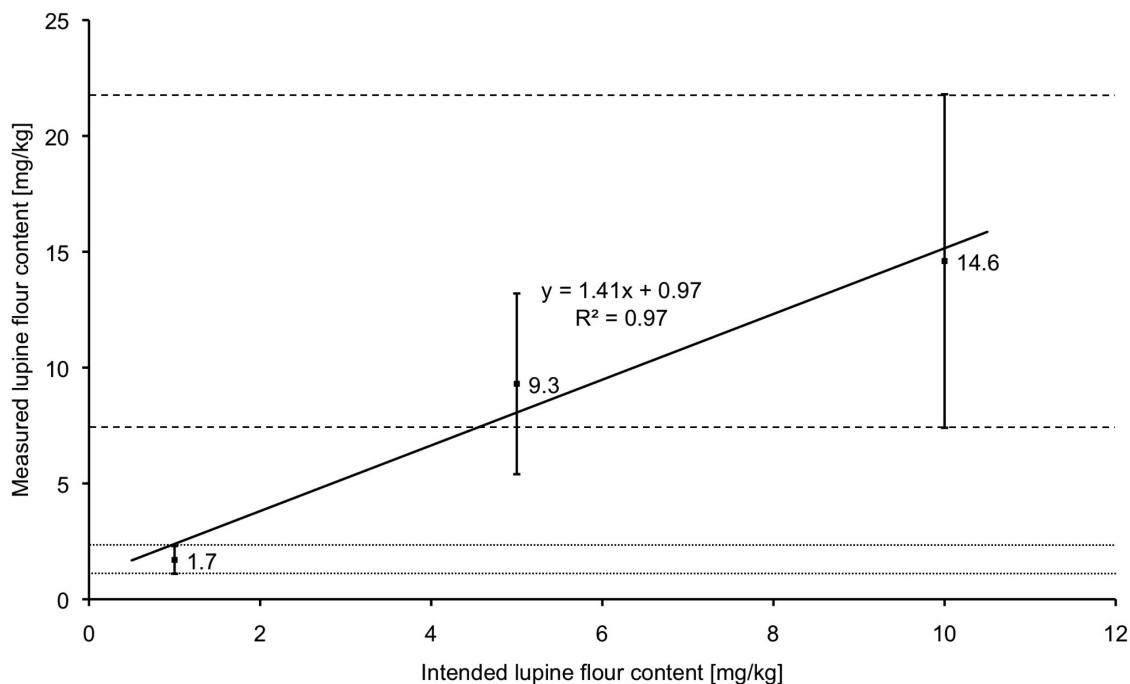
Figure 4.14: Linearity of the quantification of lupine flour content using real-time PCR. The horizontal lines mark the ranges of the standard deviations of the 1 mg/kg sample (dotted lines) and the 10 mg/kg sample, respectively (dashed lines).

linear range of the developed method. The lowest eliciting dose for allergic reactions to lupine, which induced mild symptoms in peanut-sensitized patients, was determined in a recent study [78] to be 0.5 mg. Of a food containing 10 mg lupine per kg, 50 grams suffice for the ingestion of this amount of lupine. At a concentration of 1 mg lupine per kg food, however, a portion of 500 g is needed to reach the same amount. Since the standard material is added to the sample prior to the extraction of DNA, the influence of constituents of the sample on the extraction efficiency and on the performance of the real-time PCRs are accounted for. Another approach to the quantification of allergens in foods is the use of adequate calibration samples, which resemble the samples to be analyzed as much as possible [123]. This method also addresses potential matrix effects on the quantitative results. However, it is very laborious to prepare standard materials for all sample types that might need to be analyzed in a laboratory. So far, no reference materials containing allergens in a variety of food matrices are available. The optimal method to quantify food allergens would be the use a reference gene in

the same way as for the quantification of genetically modified organisms. However, it is hard to find a gene that meets the requirements, as it would need to be present in all species used as food and would also need to occur with a constant and known copy number. Therefore, the use of a unique internal standard material as employed in this study can be considered a practicable alternative to the methods mentioned above. It should however be noticed that since the coefficient value influences the quantification results, the material used for the determination of this value needs to be chosen carefully. The copy numbers of the target sequence of the real-time PCR method vary significantly between different lupine species. Therefore, the quantitative results will only be valid if the allergen contained in the food and the material used for the determination of the coefficient value are identical. Since food ingredients are usually not characterized to this extent, a method to discriminate the commercially most relevant lupine species in advance to the quantification would be beneficial. However, no such method is yet available. Despite these shortcomings, the principle developed by Hirao et al. [109] could be applied successfully to the quantification of an allergen of relevance in the European Union, thus proving the suitability of this approach for the control of a potential future threshold for allergens in food.

4.6 Conclusion

A real-time PCR for the detection of lupine DNA in foods was developed. The method is capable of detecting DNA from a variety of different lupine species and of discriminating lupine DNA against DNA from closely related species that are used as food ingredients such as legumes, cereals, seeds, nuts, spices, fruits and meats. Therefore, the assay can be used for the specific detection of lupine DNA in food.

The developed assay enables the amplification of 0.01 pg of lupine DNA per reaction. Methods for food allergen analysis should be able to detect the respective allergen in the low mg per kg range. In unprocessed foods, such as spiked commercial ice cream or wheat flour, 0.1 mg *Lupinus angustifolius* flour per kg were detectable. The detectability of *Lupinus luteus* and *Lupinus albus* differed in accordance with the observed high variability in copy number of the target sequence in lupine species. The lowest detectable amount for *Lupinus luteus* was 5 mg/kg wheat flour, whereas it was 10 mg lupine flour per kg wheat flour for *Lupinus albus*. In conclusion, the sensitivity of the developed real-time PCR method is in the required range for food allergen analysis.

Two commercial kits for the detection of lupine DNA were compared with the developed real-time PCR method. All tested lupine species and varieties were detectable with the three methods. A cross-reactivity with DNA from white beans was detected for the CIBUS kit, which is in contrast to the specificity of the developed real-time PCR method and the PCR-Fast kit. The lowest amount of lupine flour detectable in spiked ice cream differed for the three methods: while 0.1 mg lupine flour per kg ice cream were detectable using the hybridization-probe based real-time PCR or the PCR-Fast kit with SYBR-green detection, the CIBUS kit only gave positive results from ice cream spiked with 100 mg lupine flour per kg or more. Of three commercial food products with declared lupine content, only one tested positive with all three methods. False-negative results were obtained for the remaining two products when analyzed using the CIBUS kit. In two products that came from manufacturers that use lupine flour in some of their products, lupine DNA was detected using the developed real-time PCR method and the PCR-Fast kit, while the CIBUS kit did not give any positive results, which is

in line with the lower sensitivity of this kit. In summary, the developed hybridization probe-based real-time PCR method proved to be equally sensitive as the SYBR-green real-time PCR kit and more sensitive than the endpoint PCR assay.

Comparable results concerning the presence/absence of lupine in food samples were obtained using the developed real-time PCR assay and a commercially available ELISA kit for the detection of lupine protein in foods. Lupine DNA was detected in all products that had lupine protein, lupine fiber or lupine flour declared in their ingredients list, showing that the content of amplifiable DNA even in commercially employed lupine protein and lupine fiber is sufficient to yield positive results with the PCR method. Lupine protein was detected in twelve of these products, while no lupine protein was detected in a product containing lupine flour according to the labelling. For three products without any declaration of lupine-based ingredients, the absence of lupine DNA and lupine protein above the detection limits of the respective methods was verified. The results for the remaining four products without lupine-based ingredients according to the declaration differed for the two methods: while lupine DNA as well as lupine protein were detected in one of them, lupine protein was only detected in one more product, while lupine DNA was found in all three remaining products.

The influence of processing on the sensitivity of the developed real-time PCR method was analyzed using pizza as a model food. In baked pizza dough coated with the acidic ingredient tomato puree, 1 mg lupine flour per kg was the lowest detectable amount. The method is therefore applicable to the analysis of food allergens in processed products, since lupine contents in the low mg per kg range can be detected. The separate determination of the influence of high and low temperatures and of acidic ingredients showed that frozen storage hast the least influence on DNA integrity, followed by low pH values and heat. The highest impact on the integrity of the analyte was observed for a combination of heat and and the presence of an acidic ingredient.

Lupinus angustifolius flour contents of 1 to 10 mg per kg wheat flour were successfully quantified using real-time PCR and seeds of *Limonium sinuatum* as an internal standard material. Since food products are complex mixtures of ingredients that originate from a variety of species, an ubiquitous reference sequence representative for all

these species would be needed in order to transfer the principle used for the quantification of genetically modified organisms to the quantification of food allergens using real-time PCR. No such sequence is available, however, a DNA sequence specific to a DNA-containing material that does not naturally occur in food can be used as reference sequence when a defined amount of this standard material is added to the food sample prior to the extraction of the DNA. Statice (*Limonium sinuatum*) seeds were used as the internal standard, since statice is an ornamental plant and is therefore not likely to be present in food. In addition, the availability of statice seeds is good, since they can be bought at seed stores. The method proved to be linear in the low mg per kg range that is relevant for food allergic consumers. It allows for the discrimination between samples with an actual lupine content of 1 mg per kg and samples with an actual content of about 10 mg/kg. The relative standard deviation of the result obtained for the sample containing 10 mg lupine per kg was 49%, which is comparable to the relative standard deviation determined by Hirao et al. (40%) [109]. The respective quantification principle described in the literature was successfully adapted to the quantification of an allergen for which labelling is mandatory in the European Union. The approach is therefore suitable for the control of a potential future threshold for allergens in food.

In conclusion, the developed real-time PCR system for the detection of lupine DNA enables the detection of potentially allergenic lupine in unprocessed and processed foods in a range that is relevant for food allergic consumers. Therefore, it can be considered as a valuable tool for the surveillance of the compliance with labelling rules, for the protection of allergic consumers and for the quality assurance in the food industry.

5   Summary

A need for specific and sensitive detection methods arises from the recent implementation of labelling rules for allergenic ingredients in foods. In this study, a real-time PCR for the detection of lupine DNA in foods was developed. The method is capable of detecting DNA from a variety of different lupine species and of discriminating lupine DNA against DNA from closely related species.

The specificity was tested using DNA from 20 lupine species and varieties as positive controls and DNA from more than 70 species potentially used as food ingredients, including other legumes, cereals, seeds, nuts, spices, fruits and meats.

The assay enables the amplification of 0.01 pg of lupine DNA per reaction. 0.1 mg lupine flour in commercial ice cream or wheat flour were detectable. The detection limits for *Lupinus luteus* and *Lupinus albus* differed in accordance with the observed high variability in copy number of the target sequence in lupine species. The limit of detection for *Lupinus luteus* was 5 mg/kg wheat flour, whereas 10 mg/kg wheat flour was the lowest detectable amount for *Lupinus albus*.

When pizza was analyzed as an example for processed foods, a sufficiently low detection limit of 1 mg/kg was observed. This result was obtained in baked pizza dough coated with the acidic ingredient tomato puree. The separate determination of the influence of high and low temperatures and of acidic ingredients showed that heat has a higher impact on DNA integrity than low pH values. Frozen storage had the least influence, while the highest impact on the integrity of the analyte was observed for a combination of heat and and the presence of an acidic ingredient.

Concerning the presence/absence of lupine in food samples, comparable results were obtained using the developed real-time PCR assay and a commercially available ELISA kit for the detection of lupine protein in foods. When compared to two commercial PCR kits for the detection of lupine DNA, the developed method proved to be equally sensitive as a SYBR green real-time PCR method and more sensitive than an endpoint PCR assay.

Lupinus angustifolius flour contents of 1 to 10 mg per kg wheat flour were successfully quantified using real-time PCR and seeds of *Limonium sinuatum* as an internal standard material. The method proved to be linear in the low mg per kg range that is relevant for food allergic consumers. It allows for the discrimination between samples with an actual lupine content of 1 mg per kg and samples with an actual content of about 10 mg/kg. The relative standard deviation of the result obtained for the sample containing 10 mg lupine per kg was 49%. This is comparable to the relative standard deviation determined by Hirao et al. (40%) [109]. The respective quantification principle described in the literature was therefore successfully adapted to the quantification of an allergen for which labelling is mandatory in the European Union. This proves the suitability of this approach for the control of a potential future threshold for allergens in food.

The real-time PCR system established for the detection of lupine DNA in foods can be considered as a valuable tool for the surveillance of the compliance with labelling rules, for the protection of allergic consumers and for the quality assurance in the food industry.

6 Zusammenfassung

Durch die Einführung von Richtlinien zur verbindlichen Kennzeichnung von Lebensmittelallergenen auf Fertigpackungen entstand ein Bedarf an spezifischen und sensitiven Nachweismethoden. In dieser Arbeit wurde eine Echtzeit-Polymerase-Kettenreaktion (real-time polymerase chain reaction, real-time PCR) entwickelt, mit der bei gleichzeitiger Unterscheidung von nahe verwandten Spezies DNA verschiedener Lupinenarten und -sorten nachgewiesen werden kann.

Die Spezifität der Methode wurde mit DNA-Extrakten aus 20 Lupinenarten und -sorten als Positivkontrollen sowie aus über 70 möglichen Lebensmittelbestandteilen getestet, darunter andere Leguminosen, Getreide, Samen, Nüsse, Gewürze, Früchte und Fleisch.

Die Nachweismethode ermöglicht die Amplifikation von 0,01 pg Lupinen-DNA pro Reaktionsansatz. In unverarbeiteten Lebensmitteln (dotierter Eiscreme und dotiertem Weizenmehl) konnte 0,1 mg Lupinenmehl pro Kilogramm Lebensmittel nachgewiesen werden. Die Nachweisgrenzen für *Lupinus luteus* und *Lupinus albus* unterschieden sich hiervon in Übereinstimmung mit den in verschiedenen Lupinenarten detektierten Unterschieden in den Kopienzahlen der Zielsequenz. Für *Lupinus luteus* wurde eine Nachweisgrenze von 5 mg/kg Weizenmehl bestimmt, für *Lupinus albus* betrug diese 10 mg/kg.

In Pizza als einem Modell für verarbeitete Lebensmittel wurde eine ausreichend niedrige Nachweisgrenze von 1 mg/kg ermittelt. Dieses Ergebnis bezieht sich auf mit saurem Tomatenmark bestrichenen und gebackenen Pizzateig. Wurden die Einflüsse von hohen bzw. niedrigen Temperaturen und sauren Zutaten getrennt betrachtet, so ergab sich ein höhere Beeinflussung der DNA-Integrität durch Hitze als durch niedrigen pH-Wert. Der am schwächsten ausgeprägte Effekt ergab sich für die Gefrierlagerung des untersuchten Lebensmittels, während der gleichzeitige Einfluss von Hitze und einer sauren Zutat die Integrität der DNA am stärksten beeinträchtigte.

Vergleichbare Ergebnisse wurden bei der Untersuchung von Lebensmittelproben mit

der entwickelten real-time PCR - Methode und mit einem kommerziell verfügbaren ELISA-Kit erhalten. Beim Vergleich mit zwei kommerziellen Kits zum Nachweis von Lupinen-DNA erwies sich die entwickelte Methode als ebenso sensitiv wie eine SYBR-Green real-time PCR - Methode und als sensitiver als eine Endpunkt-PCR - Methode. Gehalte von 1 bis 10 mg *Lupinus angustifolius*-Mehl in Weizenmehl konnten unter Verwendung der entwickelten real-time PCR - Methode sowie von Samen von *Limonium sinuatum* als internem Standard erfolgreich quantifiziert werden. Die relative Standardabweichung für die 10 mg Lupinenmehl pro kg Weizenmehl enthaltende Probe betrug 49%. Dies ist vergleichbar mit der relativen Standardabweichung, die von Hirao et al. [109] für eine Probe ermittelt wurde, welche 10 mg Buchweizen pro kg Weizenmehl enthielt (40%). Die Methode ist im untersuchten Konzentrationsbereich linear und ermöglichte die Unterscheidung von Weizenmehlen, die mit 1 bzw. 10 mg Lupinenmehl pro kg dotiert waren. Das in der Literatur beschriebene Prinzip zur Quantifizierung mittels PCR und internem Standardmaterial konnte somit erfolgreich zur Bestimmung eines in der Europäischen Union relevanten Allergens angewendet werden. Hiermit wurde die Anwendbarkeit der Methodik zur Kontrolle möglicher zukünftiger Schwellenwerte für Lebensmittelallergene demonstriert.

Das entwickelte real-time PCR - System zum Nachweis von Lupinen-DNA in Lebensmitteln kann damit als in der Praxis anwendbare Methode zur Überwachung der Einhaltung von Kennzeichnungsvorschriften und zum Schutz allergischer Verbraucher sowie zur Qualitätskontrolle in der Lebensmittelindustrie betrachtet werden.

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List of Figures

List of Tables

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