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Development of a Real-Time PCR for the Detection of Lupine DNA (*Lupinus* Species) in Foods

ANJA DEMMEL,[†] CHRISTINE HUPFER,[‡] EVELYN ILG HAMPE,[§] ULRICH BUSCH,[‡] AND KARL-HEINZ ENGEL*,[†]

Chair of General Food Technology, Technische Universität München, Am Forum 2, D-85350 Freising-Weihenstephan, Germany; Bavarian Health and Food Safety Authority, Veterinärstrasse 2, D-85764 Oberschleissheim, Germany; and Cantonal Laboratory Basel City, Kannenfeldstrasse 2, P.O. Box, CH-4012 Basel, Switzerland

Lupine flour, protein, and fiber have become common ingredients in food products. The association of lupine-related allergic incidents with peanut allergy is a cause for concern as the latter may bring about severe reactions. In this study, a hybridization probe-based real-time PCR assay for the detection of lupine DNA in foods was developed. Particular attention was paid to the specificity of the method, which was verified by analysis of DNA extracts from more than 50 potential food ingredients such as legumes, cereals, seeds, nuts, spices, fruits, and meat. The limit of detection of the method was determined as 0.1 mg/kg. The successful detection of the presence/absence of lupine DNA in 20 samples proved the suitability of the assay for the analysis of frequently encountered food matrices.

KEYWORDS: Food allergen; PCR; lupine; detection methods

INTRODUCTION

The genus *Lupinus*, belonging to the Leguminosae familiy, 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 (*1*). Varieties with low alkaloid contents were obtained by subsequent breeding. Today, species such as *Lupinus albus*, *Lupinus angustifolius*, *Lupinus luteus*, and *Lupinus mutabilis* are of agricultural importance. The use of lupine-based ingredients in human nutrition is increasing due to beneficial technological and nutritional aspects. An important reason is the fact that ingredients made from lupines can substitute for soy in products that are to be produced without the use of genetically modified organisms. Lupines can be cultivated under a variety of climatic conditions, making them a more easily available and therefore cheaper protein source than other legumes. In addition, the protein content in lupines is higher than that in other crops (*1*). 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 (*1*). Products such as pasta, bread, crackers, or cookies made from wheat flour enriched with about 10% of lupine flour show good consumer acceptance (*1*). A survey of 112 commercial samples from the Norwegian market showed that lupine has become a common ingredient in food products (*2*).

Approximately 4−5% of the adult population in industrialized countries is affected by food allergies (*3, 4*). Because adverse reactions to food change with age and vary between countries, the percentage of allergic individuals can only be estimated. Lupine allergy can occur as a result of cross-reactivity in people allergic to peanuts, yet it can also emerge by primary sensitization (*5*). Subjects with peanut allergy are at high risk to cross-react with lupine (*6*). The first case of lupine allergy was reported in 1994 (*7*). 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 (*8*) and respiratory symptoms (*9*).

In the European Union the presence of lupine materials in foods has to be labeled starting from December 23, 2008, according to Commission Directive 2006/142/EC (*10*). For products investigated in a Norwegian survey (*2*), labeling corresponded in most cases to the actual content of lupine-derived ingredients. The indication of lupine components in bakery products, though, frequently proved to be false. Both positive results for goods without declaration of lupine and negative results for samples with declared lupine content were obtained. Furthermore, two "pure soy flours" turned out to be adulterated with lupine flour. Cross-contamination in the course of food production apparently also occurs, as traces of lupine protein were found in chocolate spread and biscuits. Therefore, sensitive and specific methods for the detection of lupine-based ingredients in food products are needed for the supervision of compliance with labeling directives and for the protection of sensitive consumers.

* Author to whom correspondence should be addressed [telephone +49 (0)8161 714250; fax +49 (0)8161 714259; e-mail k.h.engel@wzw.tum.de].

[†] Technical University Munich.

[‡] Bavarian Health and Food Safety Authority.

[§] Cantonal Laboratory Basel City.

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Table 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 2. Plant Materials Used To Assess the Specificity of the Method[a]

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	mustard	local supermarket
apricot	BFSA	nectarine	BFSA
banana	BFSA	nutmeg	local supermarket
barley	BFSA	oat	local supermarket
bean	local supermarket	onion	local supermarket
bell pepper	local supermarket	oregano	local supermarket
blackberry	BFSA	parsley	CVRI Freiburg
Brazil nut	local supermarket	peach	BFSA
bread wheat	BG Ulm	peanut	local supermarket
buckwheat	local supermarket	pear	BFSA
caraway	local supermarket	pea	local supermarket
cardamom	local supermarket	pecan	local supermarket
cashew nut	local supermarket	pepper (black)	local supermarket
celery	CVRI Freiburg	pepper (white)	local supermarket
chervil	local supermarket	pine nut	local supermarket
chickpea	local supermarket	pistachio	local supermarket
chive	BFSA	plum	BFSA
cinnamon	local supermarket	poppy	local supermarket
clove	local supermarket	raspberry	BFSA
cocoa	local supermarket	rice	BFSA
coconut	local supermarket	rosemary	local supermarket
coriander	CVRI Freiburg	rye	BFSA
cumin	local supermarket	sesame	local supermarket
fennel	local supermarket	sour cherry	BFSA
garlic	local supermarket	soybean	BFSA
hazelnut	local supermarket	spelt wheat	BFSA
heart cherry	BFSA	strawberry	BFSA
laurel	local supermarket	sultana	local supermarket
lens	local supermarket	sunflower	LIPG
linseed	local supermarket	thyme	local supermarket
macadamia nut	local supermarket	walnut	local supermarket
maize	BFSA		

[a] Abbreviations: BFSA, Bavarian Health and Food Safety Authority (Oberschleissheim, Germany); BG Ulm, botanical garden Ulm (Ulm, Germany); CVRI Freiburg, Chemical and Veterinarian Research Institute Freiburg (Freiburg, Germany); LIPG, Leibniz Institute for Plant Genetics and Crop Plant Research (Gatersleben, Germany).

The most frequently used methods for food allergen analysis can be classified into DNA-based and protein-based techniques. So far, two enzyme-linked immunosorbent assays (ELISA) for the detection of lupine protein in foods have been published (*2, 11*). 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 (*11*). A recently described polyclonal−monoclonal-based sandwich ELISA also showed positive results for almond, cashew, pumpkin seed, sunflower seed, and roasted hazelnut (*2*). 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. For this reason, the use of nonimmunological methods, such as the Polymerase Chain Reaction (PCR), has been suggested (*2*).

Table 3. 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
lupine R	5′-TTG TTA TTA GGC CAG GAG GA-3′	
lupine probe	5′-FAM−CCC CTC GTG TCA GGA GGC GC−TAMRA-3′	

Up to now, two commercial kits for the detection of lupine DNA using PCR are available. One of them employs a conventional PCR with subsequent agarose gel electrophoresis (*12*). 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 (*13*). It shows a remarkably lower limit of detection (<10 copies), but also lacks verification of the amplicon sequence. 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.

The aim of this study was the development of a real-time PCR procedure for the detection of lupine DNA in foods. Particular attention was paid to the specificity of the method. Therefore, extensive testing with a variety of food ingredients such as other legumes, cereals, seeds, nuts, spices, fruits, and meat was carried out. Another aspect considered during the design was the ability of the system to detect DNA from a broad range of lupine species. Hence, 20 different species of *Lupinus* were included in the survey.

MATERIALS AND METHODS

Materials and Food Samples. Seeds of different lupine varieties were obtained from the Leibniz Institute for Plant Genetics and Crop Plant Research (Gatersleben, Germany). Details on the accessions used are listed in **Table 1**. Sweet lupine flour was made available from the Chemical and Veterinarian Research Institute Freiburg (Freiburg, Germany).

Plant materials used for assessing the specificity of the method are listed in **Table 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).

Lupine-containing foods either were provided by the Institute for Product Quality (Berlin, Germany) and the Fraunhofer Institute for Process Engineering and Packaging (Freising, Germany) or were purchased at local stores.

Preparation of a Serial Dilution of Genomic DNA. 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/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.

Spiking of Ice Cream with Lupine Flour. A commercial sample of ice cream (showing a negative result in the test for the presence of

Figure 1. Positions of primers and probe in the target region of the internal transcribed spacer 1 of *Lupinus angustifolius* (GenBank accession no. Z72202).

Table 4. Results Obtained by the Analysis of Ice Cream Spiked with Lupine Flour

lupine flour content (mg/kg)	mean Ct value[a]	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	30.5	10/10

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

lupine DNA) was spiked with sweet lupine flour to an initial concentration of 1000 mg/kg. Serial dilutions (100, 10, 1, and 0.1 mg/kg) were obtained by consecutively mixing the spiked product with ice cream.

DNA Extraction: CTAB Method. *DNA Extraction from Lupine Flour.* DNA from lupine flour was isolated following a CTAB protocol. Two hundred milligrams of ground lupine seeds was 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 (20 mg/mL) in a 2 mL tube, followed by overnight incubation at 65 °C. After 10 min of centrifugation at 14000*g*, 1000 μL of supernatant was transferred into a new 2 mL tube and centrifuged a second time at 14000*g*. 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 14000*g*, 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 14000*g*. 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 14000*g*, 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×). Subsequently, the DNA extracts were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

DNA Extraction from Food Samples. The DNA extraction from food samples followed a modified CTAB protocol. Ten milliliters of CTAB extraction buffer and 30 μL of proteinase K (20 mg/mL) were added to 2 g of homogenized sample material in a 50 mL falcon tube. After mixing and overnight incubation at 65 °C, the samples were centrifuged for 5 min at 5000*g*. One thousand microliters of the supernatant was transferred into a new 1.5 mL tube and centrifuged for 5 min at 14000*g*. Seven hundred microliters of supernatant was mixed with 500 μL of chloroform/isoamyl alcohol (ReadyRed), followed by 15 min of centrifugation at 16000*g*. Five hundred microliters of supernatant was added to 500 μL of cold isopropanol (stored at −20 °C). Thirty minutes of incubation at room temperature was followed by 15 min of centrifugation at 16000*g*. After removal of the supernatant, the pellet was washed with 500 μL of ethanol (70% v/v; stored at −20 °C) and centrifuged 5 min at 16000*g*. The ethanol was discarded and the pellet diluted in 100 μL of TE buffer (1×). Subsequently, the DNA extracts were purified using the QIAquick PCR purification kit (Qiagen).

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 double-stranded DNA was achieved using a calibration curve derived from λ-DNA. Recorded data were evaluated using the Tecan Magellan software package.

Amplifiability. The amplifiability of the extracted DNA was verified by PCR with primers targeting noncoding regions of chloroplast DNA (*14*).

Primers and Probe. 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.). Primers and probe were manually designed on the basis of this alignment and checked using Beacon Designer 7.01 software. Oligonucleotides were obtained from TIB MOLBIOL (Berlin, Germany). Sequences and amplicon length are shown in **Table 3**.

Real-Time PCR. Sample extracts (5 μL) were added to 20 μL of reaction mix containing 2× SensiMix (Quantace, London, U.K.), 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). The threshold was manually set to 0.1. Unless otherwise noted, reactions were performed in duplicate.

Enzyme-Linked Immunosorbent Assay (ELISA). Food samples were analyzed using a commercially available ELISA kit (*15*). Extraction and detection of lupine protein were carried out as described in the manual. Each sample was extracted once, and the extracts were analyzed in duplicate.

RESULTS AND DISCUSSION

Design of a Real-Time PCR Method 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 (*16*). 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.

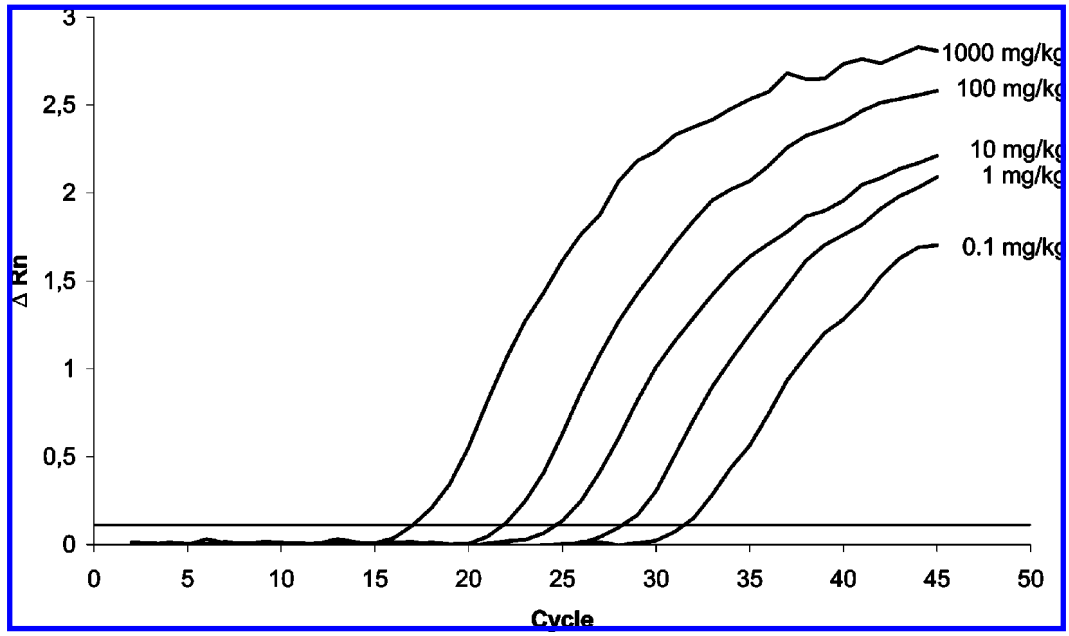

Figure 2. Amplification curves obtained by analysis of ice cream spiked with 1000, 100, 10, 1, and 0.1 mg/kg lupine flour, respectively (line at Δ Rn 0.1 corresponds to cycle threshold).

Table 5. Results Obtained by Analysis of Food Samples, Using the Developed Real-Time PCR and a Commercially Available ELISA Kit, Respectively

product	labeling/ingredients	detection of lupine DNA[a]	detection of lupine protein[b]
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		−	−

[a] Using the real-time PCR method described in this paper. [b] Using a commercially available ELISA kit (*15*).

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 11 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. 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. 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. As an example, the positions of primers and probe in the ITS-1 sequence corresponding to *L. angustifolius* (GenBank accession no. Z72202) are shown in **Figure 1**.

Specificity. DNA from the materials listed in **Table 1** was used to assess the suitability of the method for the detection of lupine DNA in foods in which different lupine species 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 **Table 2**, comprising other legumes, cereals, seeds, nuts, spices, meat, and fruit. No nonspecific amplification was observed.

Sensitivity. 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 (ENGL) (*17*). 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.

Limit of Detection in Matrices. *Analysis of Spiked Ice Cream.* An important aspect of method validation is the applicability of a method to actual food matrices.

Lupine-based ingredients can be used for the production of nondairy ice cream. This product, resembling conventional ice cream in texture, is suitable for people suffering from lactose

intolerance. Because no certified lupine-containing reference materials are available, lupine-free commercial ice cream was spiked with lupine flour to determine whether lupine DNA could still be detected after extraction from a matrix containing fat, proteins, and carbohydrates. The concentration levels were 1000, 100, 10, 1, and 0.1 mg/kg. To obtain representative data, five portions of each level were extracted and the extracts were subjected to real-time PCR in duplicate. The results are shown in **Table 4**. Examples of the resulting amplification curves are given in **Figure 2**.

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 (*18*). 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 265 mg of lupine flour, but no threshold dose for lupine has yet been established (*19*). For peanut, the lowest absolute dose eliciting allergic reactions has been determined to 1 mg (*18*). 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 (*20*). With a limit of detection determined to 0.1 mg/kg lupine flour in ice cream, the developed method meets these requirements.

Using spiked sausages (added amounts of lupine flour ranging from 10 to 1000 mg/kg) as an example, it could be demonstrated that the method also allows the detection of lupine DNA in moist, heated foods.

Analysis of Retail Samples. A spectrum of foods, comprising various bakery products, fruit-flavored gums, and ice cream, was analyzed by PCR as well as by ELISA. The results are shown in **Table 5**. 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.

The newly developed real-time PCR method for the detection of lupine DNA in foods was shown to be specific and sensitive. Although the instrumentation needed is a major investment, the assay can be applied by food producers and food safety authorities to analyze the absence or presence of lupine DNA in food products, thus providing a tool for the surveillance of regulatory labeling requirements and therefore improving the protection of allergic consumers.

Supporting Information Available: Genbank accession numbers of the sequences used for the alignment of ITS-1 and standard curve obtained by real-time PCR analysis of genomic DNA from *L. angustifolius*. This material is available free of charge via the Internet at http://pubs.acs.org.

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