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Detectability of Lupine Seeds by ELISA and PCR May Be Strongly Influenced by Potential Differences between Cultivars

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ABSTRACT: Accurate methods for allergen detection are needed for the verification of allergen labeling and the avoidance of hidden allergens. But systematic data on the influence of different cultivars of allergenic crop species on their detectability in enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are lacking. As one example, seeds of 14 different cultivars of lupine (*Lupinus albus*, *Lupinus angustifolius*, *Lupinus luteus*) were investigated for total protein according to a Kjeldahl method, and for their relative quantitative detectability in three commercial lupine-specific ELISA tests and four lupine-specific PCR methods. Total Kjeldahl nitrogen allowed an accurate quantification of total protein. Relative differences in quantitative response between cultivars of 390−5050% and 480−13 600% were observed between ELISA kits and PCR methods, respectively. Hence, quantitative results of selected ELISA and PCR methods may be strongly influenced by the examined lupine cultivar.

KEYWORDS: *lupine (Lupinus spp.), real-time PCR, ELISA, allergen quantification, cultivar, bio variability*

1. INTRODUCTION

Lupine is a member of the *Fabaceae* botanical family. Mainly four out of more than 450 *Lupinus* species are of universal agricultural interest: white lupine (*Lupinus albus*), yellow lupine (*Lupinus luteus*), blue or narrow-leafed lupine (*Lupinus angustifolia*), and pearl lupine (*Lupinus mutabilis*).[1] In central Europe only the first three of these four species, belonging to the "sweet lupines", are grown and used as food and feed. Despite their relationship, the single species and cultivars are quite different in nutrient content. As summarized recently by Kohajdová et al. (2011) and Jansen et al., (2010) the protein content may vary from 20 to 48% depending on the characteristics of growing conditions and soil types.[2,3] Lupine has been reported to be used as an ingredient in different foods such as curd cheese, tofu, sauces, sausage, schnitzel, spread, pasta, coffee substitutes, gluten-free baking mixtures, and all kinds of bakery wares.[4] In the food market, lupine is increasingly used due to its nutritional value, functional properties in food products, and as potential replacement for genetically modified soy bean.[5] The consumption of lupine has also been associated with beneficial health effects, such as the prevention of obesity and cardiovascular disease.[6]

However, beneficial effects do not apply to everyone, since lupines are known to be an allergenic food. Food allergy to lupine has been studied by various authors: Symptoms may range from mild oral allergy syndrome to generalized urticaria, laryngeal edema, and anaphylactic shock. Thus, allergy to lupine is potentially life-threatening.[7−15] To protect lupine-allergic individuals against allergic reactions, the European Union requires mandatory labeling of lupine when used as an ingredient in prepackaged foods.[16] Thus, the food authorities have a demand for sensitive and accurate methods for lupine detection in order to verify the compliance between lupine food labeling and composition. Several protein-[17−19] and DNA-[20−22]based detection methods have been published in the scientific literature, and commercial test kits for lupine detection are available. But systematic data on the influence of different lupine cultivars on their relative quantitative detectability in the most frequently applied methods, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), are lacking.

Lupine, one example of a potentially allergenic crop species, was studied. We aimed at investigating the relative quantitative detectability of various lupine cultivars versus various lupine-specific methods. The relative quantitative detectability of 14 different cultivars of white, yellow, and blue lupine was studied with three selected commercially available ELISA tests, three published real-time PCR methods, and a newly developed real-time PCR.

■ MATERIALS AND METHODS

Materials. Foods for specificity testings were purchased at local retailers. Seeds of 14 different lupine cultivars were obtained from different German seed dealers (Table 1). Each twelve gram of seeds of the individual lupine cultivars were ground under liquid nitrogen using an analytical mill. Finely ground lupine flours were stored at −80 °C until further use in ELISA and PCR analysis. The ELISA tests "nutriLínia Lupine-E (NC-6003/96, Nutricor s.r.o., Slovakia)"[32] "RIDASCREEN®-FAST Lupine (R6102)",[31] and "Veratox Lupine (#8500)"[33] were purchased from Transia (Ober-Mörlen, Germany), R-Biopharm (Darmstadt, Germany), and Neogen (Ayr, Scotland, UK), respectively. All primers and amplicon-specific fluorescent probes were from biomers.net GmbH (Ulm, Germany). Proteinase K (activity, >600 mAU mL^{-1}) was from Qiagen

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Table 1. Investigated Lupine Cultivars and Their Mean Protein Content (Including Standard Deviation STD) as Determined with the Kjeldahl Total Nitrogen Method[a]

species	no.	cultivar	seed dealer	protein content (%) ± STD	% CV
Lupinus angustifolius	Lu01	Idefix	1	22.0 ± 0.7	3.0
	Lu02	Sonate	2	23.1 ± 0.1	0.6
	Lu03	Vitabor	2	26.7 ± 3.4	12.7
	Lu04	Boruta	2	24.3 ± 1.0	4.0
	Lu05	Boregine	2	25.1 ± 0.9	3.4
	Lu06	Bora	2	29.7 ± 1.5	4.9
	Lu07	Borlu	2	29.8 ± 0.9	3.1
	Lu08	Probor	2	28.8 ± 0.4	1.5
Lupinus luteus	Lu09	Juno	3	35.2 ± 1.4	3.9
	Lu10	Mister	3	38.1 ± 0.4	1.1
	Lu11	Bornal	2	35.3 ± 0.2	0.5
Lupinus albus	Lu12	Luxe	1	30.1 ± 1.9	6.3
	Lu13	Feodora	4	30.6 ± 0.2	0.5
	Lu14	Fortuna	4	31.2 ± 0.2	0.5

[a]Abbreviation of seed dealers: Lupina Handels-gesellschaft mbH (1), Saatzucht Steinach GmbH & Co KG (2) Feldsaaten Freudenberger(3), Südwestdeutsche Saatzucht GmbH und Co KG (4).

(Hilden, Germany). Mussel glycogen (20 mg mL^{-1}) was from Roche Diagnostics (Mannheim, Germany). "Platinum Taq DNA polymerase" and "Taq DNA Polymerase PCR Buffer" (10× concentrate) were from Invitrogen (Life Technologies, Carlsbad, CA). Uracil-N-glycosylase was from Jena Bioscience (Jena, Germany). "TaqMan® Universal PCR Master Mix" and "Power SYBR® Green PCR MasterMix" was from Applied Biosystems (Life Technologies, FosterCity, CA). "SensiMix II Probe Kit" was purchased from Bioline (Luckenwalde, Germany). "iQ SYBR® Green Supermix" were obtained from BioRad (Hercules, CA). TE-buffer (pH 8.0) consisted of 10 mM Tris/HCl, 1 mM EDTA and 1% Tween 20. "6x Orange DNA Loading Dye" and "O'Gene Ruler Low Range DNA Ladder" were from Fermentas (St. Leon-Rot, Germany). The chemicals cetyltrimethylammonium bromide (CTAB), isopropanol, disodium ethylenediamine-tetraacetic acid (EDTA), sodium chloride (NaCl) and tris(hydroxymethyl)-aminomethane (Tris) were of analytical-reagent grade and obtained from Carl Roth GmbH+Co.KG (Karlsruhe, Germany). Tween 20 was from Sigma–Aldrich (St.Louis, MO). Ultrapure water was used for buffer preparations. Consumables for real-time PCR (optical strips and tubes) were purchased from Stratagene (Agilent Technologies, Cedar Creek, TX). Kjeldahl tablets (Missouri catalyst), sodium hydroxide solution (about 32%), sulfuric acid 96% Suprapur, and boric acid were from Merck (Darmstadt, Germany).

Instrumentation. Lupine seeds and foods for specificity testings were ground with an analytical mill (M20) from IKA (Staufen, Germany) or a knife mill (Grindomix GM200) from Retsch (Han, Germany). The optical densities of ELISA were read in a Spectra Max 340 PC microplate reader (Molecular Devices, Sunnyvale, CA). The "Soft-Max Pro5.2" software was used for controlling the instrument. Enhanced sample homogenization and lysis for DNA extraction was done in a FastPrep 24 instrument using "Lysing Matrix A" tubes (MP Biomedicals, Solon, OH). The "Thermomixer comfort" was from Eppendorf (Hamburg, Germany). Agarose gel electrophoresis was done with the "Rapid Agarose Gel Electrophoresis System" (RAGE system) from Cascade Biologics (Portland, OR). Documentation of agarose gels after electrophoresis was done on a UV transilluminator (Intas Science Imaging Instruments GmbH, Göttingen, Germany). UV spectrometric DNA quantification was performed with a "Nanodrop" absorbance reader from PEQLAB Biotechnologie GmbH (Erlangen, Germany). Real-time PCR experiments were performed on a Stratagene MX3005P cycler from Stratagene (Agilent Technologies, Cedar Creek, TX). After each cycle of amplification, the fluorescence in SYBR Green and Taqman real-time experiments was measured using the carboxyfluorescein (FAM)-channel (excitation and emission 492/517 nm). The cycle threshold C_T was calculated collectively for all samples of one run by the software program MxPro-Mx3005P v4.10 Build 389, Scheme 385, of the Mx3005P real-time cycler using the automatic threshold determination algorithm. Total Kjeldahl nitrogen was determined using the block digestion system "Kjeldatherm KBL40S" and the automated distillation system with titration "Vapodest 50s" from C. Gerhardt GmbH & Co. KG (Königswinter, Germany).

Total Kjeldahl Nitrogen. The quantitative determination of total Kjeldahl nitrogen (TKN) was done in concordance with an established in-house standard operation procedure and considering optimized parameters of digestion and titration with regard to the study samples. Each test series consisted of lupine samples, blank samples and reference samples of known TKN. Each sample was digested twice and each digestion was analyzed twice in a fully automated distillation system with integrated titration. In summary, 0.120 g finely ground lupine flour, 1 g of the catalyst, two boiling stones and 3 mL of 96% sulfuric acid were transferred into a 100 mL Kjeldahl microtube and digested in the preheated Kjeldatherm at 410 °C for 40 min or until the digestion was complete, which was indicated by a clear and transparent reaction solution. After cool-down, the digested samples were adjusted to 20 mL. Subsequently, 4 mL of the digestion were used for the automated determination of TKN in the Vapodest 50s. According to the publication from Doxastakis et al. (2002) and the manual of the TKN apparatus the conversion factor used to calculate the protein content was 5.7.[23] For each cultivar, two samples were analyzed in duplicate.

Lupine-Specific ELISA. The lupine-specific ELISA tests RIDASCREEN FAST Lupine and nutriLínia Lupine-E were performed according to the manufacturers' instructions. For both ELISA tests, the sample size was each one gram of study lupine. The Veratox Lupine Allergen ELISA was performed according to the manufacturer's instruction except for the amount of sample: Instead of five gram, the sample weight was one gram. The sample to buffer ratio including the addition of extraction additive was according to the manufacturer's instruction. In pretestings for each lupine cultivar the optimized dilution factor was determined for each ELISA test to obtain sample ODs within the middle of the calibration curve. For each ELISA experiment, each ground lupine sample was individually extracted as described above, diluted and analyzed in duplicate wells. All commercial ELISA tests are of the so-called sandwich type that makes use of capture and detector antibodies, the latter being provided as an enzyme conjugate. According to the manuals, all studied test kits are intended for the quantitative analysis of lupine proteins in food products. No detailed information is given about the type of antibody, or the antigen used for antibody and standard generation. The RIDASCREEN FAST Lupine ELISA test is described to detect

Table 2. Nucleotide Sequences and Labels of Investigated Primer/Probe Combinations According to This Study and Published Methods

reference	target gene	oligo labeling	sequence (5′-3′)
new lupine PCR (this work)	conglutin beta gene	Lup sense	ccaragaaccaatagacttgagaatct
		Lup antisense	gctctaccattgagtacaacgagg
		Lup probe	6-FAM-actctcattctccctaaacactctgatgctg-BHQ-1
Demmel et al. (2008)	internal transcribed spacer sequences of 18S-26S nuclear rDNA	Lupine F	cctcacaagcagtgcga
		Lupine R	ttgttattaggccaggagga
		Lupine probe	6-FAM-cccctcgtgtcaggaggcgc-Tamra
Galan et al. (2011)	mitochondrial gene for initiator tRNA-MET	forward primer	actaagcagaagcaagacgga
		reverse primer	ctgttccaactctgcgttcat
		Lupine probe	6-FAM-gggcagtttgatggctatgataggcgcg-Tamra
Scarafoni et al. (2009)	CcA32 gene	cγA32−5f	atggtgtacacccttaacc
		cγA32−3r	ggtatgaagatgatgatgatg

lupine proteins, including γ-conglutin, of all relevant lupine species. The results of the three ELISA tests are reported as mg kg^{-1} (ppm) lupine proteins in food products.

DNA Extraction and Purification. The DNA extraction was performed similar to a previously described CTAB method,[24] but further modified. Briefly, 100 mg of the ground lupine powder were transferred to a 2 mL micro reaction tube containing ceramic beads (Lysing matrix A Tube),and 1.4 mL CTAB buffer (55 mM CTAB, 1400 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA)·Na_2·$2H_2O$, 100 mM Tris) and 20 μL of proteinase K were added. The content of the tubes was accelerated for 60 s at 4 m s^{-1} using the FastPrep-24 instrument. Afterward, the tubes were further simultaneously mixed and incubated at 65 °C for 60 min and 1000 rpm in a "Thermomixer comfort". After centrifugation at 15 000*g* for 10 min, 800 μL supernatant was transferred to a 1.5 mL micro reaction tube and mixed with 600 μL chloroform. After centrifugation at 15 800*g* for 5 min, 600 μL supernatant was added to 1 μL of mussel glycogen in another 1.5 mL micro reaction tube, and after short mixing 500 μL ice-cold (−20 °C) isopropanol was added. The tube was carefully inverted 10 times and centrifuged for 90 min at 1000*g* and 4 °C, followed by 15 min centrifugation at 14 000*g*. After supernatant removal, the DNA pellet was washed with 500 μL ice-cold (−20 °C) 70% ethanol and briefly centrifuged. After removal of ethanol, the pellet was dried at 50 °C for 10 min. Afterward, the dried DNA pellet was resuspended and resolved in 100 μL of TE buffer overnight at 4 °C using the "Thermomixer comfort" with interval cycles of 20 s mixing and 20 s chilling. The resuspended DNA pellet was further purified by following the manual of the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). All lupine DNA extracts were diluted 1:100 or as indicated in TE buffer. DNA extracts from foods for specificity testings were diluted 1:10 and 1:100. To exclude the presence of inhibitors that possibly lead to false negative results, the quality of DNA extracts to properly allow PCR amplification was confirmed by amplification with the universal eukaryotic primers TR03/TR04.[25,26] The DNA extracts and respective dilutions were stored at −20 °C until use.

Evaluation of Genes and Oligonucleotides for Lupine-Specific Real-Time PCR. Three different genes were analyzed in silico for sequence stretches suitable for specific amplification of lupine DNA (accession number for a lupine-specific sequence in parentheses): conglutin beta mRNA (EU352876), extensin peroxidase mRNA (AF403735), and glutamate dehydrogenase 1 (GDH1) mRNA (AY681352). The Vector NTI Software (Version 10.3.0) and the BLAST algorithms of NCBI[27] were used for sequence alignments between cDNAs from different lupine species and other legumes as far as available in the NCBI database (default settings). Potential primers were derived with "Beacon Designer Free Edition" from Premier Biosoft International.[28] The characteristics of the derived primers were verified with the web-based software "Oligo Analyzer 3.1" (IDT SciTools, Integrated DNA Technologies, Inc., Coralville, IA).[29] The oligonucleotide sequences specific for the conglutin gene used for final real-time PCR development are displayed in Table 2 for SYBR Green and Taqman PCR, respectively.

Real-Time PCR Methods for Lupine Detection. The real-time PCR developed in this study was performed as follows: For primer pretesting SYBR Green real-time PCRs were run at a final volume of 25 μL, using 5 μL of DNA eluate, "Power SYBR® Green PCR Master Mix", and each 300 nM sense and antisense primers.

The optimized primers and probe for sequence specific Taqman real-time PCR are shown in Table 2, and the optimized composition of the Mastermix for Taqman real-time PCR was 1× PCR buffer, each 200 μM dATP, dUTP, dGTP, and dCTP, 3.5 mM $MgCl_2$, 0.25 mg mL^{-1} BSA, 300 nM sense primer "Lup sense", 300 nM antisense primer "Lup antisense", 100 nM fluorescent probe "Lup probe", 0.125 Units Uracil-N-glycosylase, 0.625 Units Platinum Taq DNA polymerase, 5 μL of sample DNA eluate and reagent-grade water that was added to a final volume of 25 μL. The optimized final real-time PCR program consisted of a first UNG digestion step at 50 °C for 5 min, followed by initial denaturation for 7 min at 95 °C, and followed by 45 cycles of 15 s at 95 °C for denaturation, and 30 s at 64 °C for annealing with polymerization.

Previously published real-time PCR experiments were conducted according to the publications from Demmel et al. (2008),[20] Galan et al. (2011),[22] and Scarafoni et al. (2009)[21] except for the following alterations: The master mix kit "SensiMix Probe Kit" described by Demmel et al. (2008)[20] is no longer available and has been replaced by "SensiMix II Probe Kit". All real-time PCR experiments were performed with a Stratagene Mx 3005P real-time Cycler. Primers and probes used in this study are listed in Table 2.

Each cultivar was extracted twice and each extract was investigated in duplicate PCRs. All experiments of all applied PCR methods were performed with the identical DNA extracts and dilutions within a time period of 24 h. All lupine sample extracts, diluted at 1:100, were quantified using a standard curve obtained from serial dilutions of the DNA extract obtained from cultivar Lu01, DNA extract 1 (Table 1). The selection of cultivar Lu01 was random. Any studied cultivar was considered suitable for the generation of a DNA standard. The following serial 10-fold dilutions were used for generation of the standard curve: 1:10; 1.100; 1:1000; 1:10 000; 1:100 000; 1:1 000 000, and 1:10 000 000. Each standard dilution was recorded in duplicate. The relative quantitative response between the study cultivars was calculated as the ratio of measured DNA dilution that was derived from the standard curve, between the highest and the smallest mean cycle threshold (C_T) value of the investigated lupine cultivars.

Agarose Gel Electrophoresis. Post PCR 25 μL of PCR product were mixed with 6× loading buffer and loaded onto a 3% (w/v) agarose gel. The size of the PCR products was controlled by comparison with the "Low Range DNA Ladder" in gel electrophoresis with 1 X TAE buffer, pH 8.0, at 275 V (50 mL gel) for approximately 25 min. After gel electrophoresis the gel was stained in 0.75 μg mL^{-1} ethidium bromide for 15 min and visualized and documented on a UV (312 nm) transilluminator.

Statistical Analysis. Data were evaluated by means of a mixed linear model with fixed factor *lupine group* (angustifolius, luteus, albus) and random factor *cultivar*. For Kjeldahl, an additional random factor *sample* (up to two samples were tested in duplicates) was included in the model. *P*-values (95% confidence interval) were adjusted for multiple comparisons according to Bonferroni. The statistical analysis was performed with SAS/STAT software, version 9.3, SAS System for Windows.

RESULTS AND DISCUSSION

All of the investigated lupine cultivars are approved field crops in Germany. Except for the cultivars Idefix, Bornal and Fortuna all lupine cultivars are listed in the 29th complete edition of the "common catalogue of varieties of agricultural plant species"[30] and are thus approved field crops in the European Union. The selection of cultivars depended on availability from the seed dealers.

Total Kjeldahl Nitrogen. The Kjeldahl method is the most often used methods for determining the total protein content in foods. In contrast to current food allergen tests like ELISA and PCR, the Kjeldahl method cannot be used to determine protein of a food ingredient in compound foods. However, for the pure food it is the method of choice to obtain reliable data about the total protein content, independent from soluble or insoluble protein fractions. The protein content of the 14 study lupine samples (Table 1) ranged between 22.0 and 38.1% (blue lupine cultivars: 22.0−29.8%, yellow lupine cultivars 35.2−38.1% and white lupine cultivars 30.1−31.2%) and was in concordance with the results published in literature (20−48%).[2,3] This indicated our cultivar selection to be reasonably representative. The individual results are displayed in Figure 1. Remarkable differences in the protein content were observed. The largest difference in relative quantification between cultivars, as calculated by the protein ratio of the study lupine cultivars having the highest and the lowest protein content, respectively, was 1.8 (Table 3).

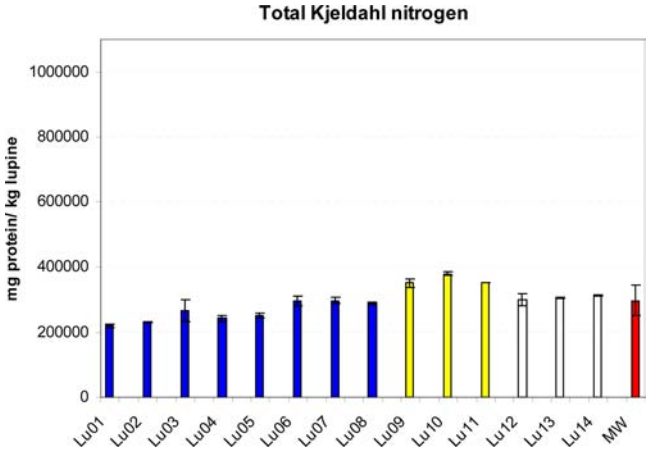

Figure 1. Total protein calculated from the total Kjeldahl nitrogen analysis of the lupine cultivars studied. The bars represent the mean of two extracts and two replicates per extract. Error bars indicate the standard deviation of duplicate measurement. The red bar is the mean of all investigated samples.

Table 3. Relative Quantitative Response of Various Lupine-Specific Methods between All Investigated Lupine Cultivars[a]

method	reference/ ELISA manufacturer	cultivar specific response ratio (mean single extract)	cultivar specific response ratio (mean duplicate extract)
Kjeldahl	in house SOP	1.8	1.7
Sandwich ELISA	manufacturer A	3.9	not done
	manufacturer B	5.1	not done
	manufacturer C	50.5	not done
real-time PCR	Lupine PCR (this work)	9.8	9.4
	Demmel et al. (2008)	135.8	82.9
	Galan et al. (2011)	4.8	4.0
	Scarafoni et al. (2009)	not analyzable	not analyzable

[a]The response ratio was calculated from the minimum and maximum result of the mean single extract measurements (Kjeldahl, ELISA, PCR) and duplicate extracts measurements (Kjeldahl, PCR).

Relative Quantitative Response of Commercially Available Lupine ELISA Tests to Different Lupine Cultivars. According to the suppliers' manuals,[31−33] the different ELISA tests are capable of detecting protein of lupine cultivars in general, but the manuals do not provide information about potential differences in quantitative results between cultivars. Figure 2 displays the lupine protein quantification (mg lupine protein kg^{-1} food) of 14 study lupine cultivars with the three commercial ELISA kits. All of the ELISA gave positive signals for the investigated lupine samples; however, the response was remarkably different for single cultivars and depended on the studied ELISA test. Kit A quantified lupine protein of the investigated cultivars between 9.0 and 35.1% (blue lupine cultivars: 18.0−29.1%, yellow lupine cultivars 9.0−12.8% and white lupine cultivars 13.8−35.1%). The ratio between the highest and lowest protein quantification of the investigated lupine cultivars resulted in a relative quantitative response of 3.9 (Table 3).

Kit B quantified the amount of protein in investigated lupine cultivars between 20.7 and 106.6% (blue lupine cultivars 20.7−

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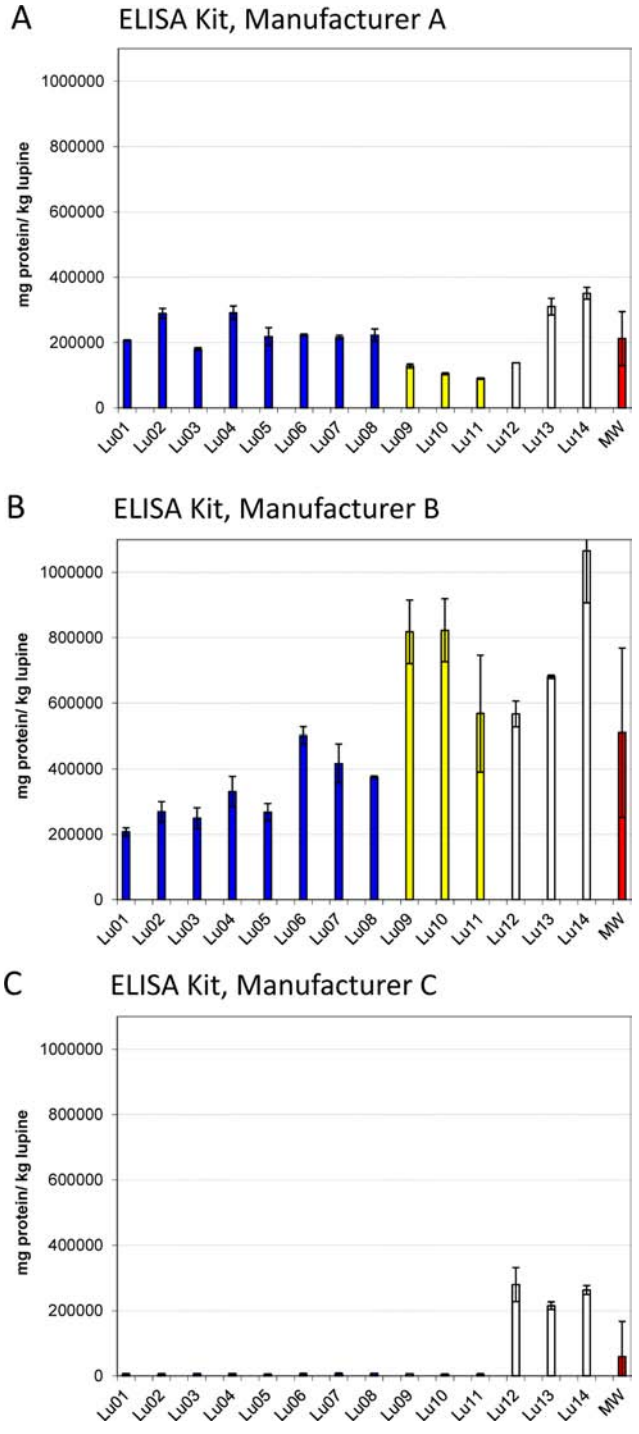

Figure 2. Lupine protein content and response of selected commercially available lupine-specific ELISA to study cultivars of blue, yellow and white lupine. Error bars indicate the standard deviation of duplicate measurement. The red bar is the mean of all investigated samples.

50.1%, yellow lupine cultivars 56.9−82.3% and white lupine cultivars 56.7−106.6%). The relative quantitative lupine response was determined as 5.1 (Table 3).

The read-out of the standard curve of kit C is mg lupine kg^{-1} food. When analyzing 100% of lupine seeds the detected amount of the individual lupine cultivar ranged between 1.3 and 68.2%. According to a personal communication with the European division of the kit manufacturer, this kit quantifies lupine on the basis of a 41% protein proportion. Thus the calculated protein content of the studied cultivars ranged between 0.6 and 28.0% (blue lupine cultivars 0.6−0.8%, yellow lupine species 0.6% and white lupine species 21.5−28.0%). The calculated quantitative response ratio between all cultivars studied was 50.5 (Table 3). This large difference was merely due to a high recovery of *L. albus* cultivars versus a low recovery of *L. angustifolius* and *L. luteus* cultivars, respectively.

In addition, ELISA and Kjeldahl data were analyzed statistically. Because of only duplicate analysis of each cultivar in each ELISA test, statistical analysis for identifying differences between individual cultivars was not viable. Thus, the species were compared with each other as groups. Significant differences ($p < 0.05$) were found between *Lupinus angustifolius* and *Lupinus luteus* for Kjeldahl (p=0.0003) and ELISA kit of manufacturer B (p=0.0026). For ELISA kit of manufacturer C, *Lupinus angustifolius* versus *albus* and *albus* versus *luteus* were different ($p < 0.0001$).

Taking the method of Kjeldahl as a reference, the results obtained with the commercially available ELISA tests were used to calculate the percental recovery (Table 4). The ELISA A data (Figure 2A, Table 4) were quite in tune with the Kjeldahl data (Figure 1, Table 1). However, some underestimation below 50% recovery was observed for the yellow lupine cultivars (Lu09, Lu10 and Lu11) and one white lupine cultivar (Lu12) (Table 4). A comparison of results between the ELISA B (Figure 2B, Table 4) and the Kjeldahl method (Table 1) revealed an overestimation of nearly all studied lupine cultivars. The lupine protein of one-half of all studied cultivars was overestimated by more than 50%. Especially cultivars from yellow and white lupine species were overestimated. The ELISA C was capable of recovering between 70 and 92% protein of white lupine cultivars in contrast to only 1.5−3.0% for blue and yellow lupine cultivars (Figure 2C, Table 4). The large differences in the detection of lupine cultivars between the investigated ELISA kits may be due to the selection of cultivars used as antigen to generate specific antibodies and protein standards. However, no detailed information is available from the kit manuals. The validity of the various ELISA tests for

Table 4. Recovery of Lupine Protein of Commercially Available Lupine-Specific ELISA[a]

Lupine cultivar	manufacturer A	manufacturer B	manufacturer C
Lu01	93.94	94.36	3.01
Lu02	125.27	116.65	2.68
Lu03	67.52	93.38	2.74
Lu04	120.19	136.57	2.90
Lu05	87.03	106.70	2.23
Lu06	75.31	168.94	2.48
Lu07	72.81	139.97	2.66
Lu08	77.35	130.34	2.68
Lu09	36.54	232.63	1.81
Lu10	27.51	215.89	1.48
Lu11	25.43	161.06	1.57
Lu12	45.86	188.06	92.77
Lu13	101.29	222.12	70.20
Lu14	112.57	341.61	84.23

[a]The protein content of investigated lupine cultivars, as determined with the total Kjeldahl nitrogen method (Table 1), was set to 100%. The results obtained with the ELISA were expressed as %recovery of the TKN value.

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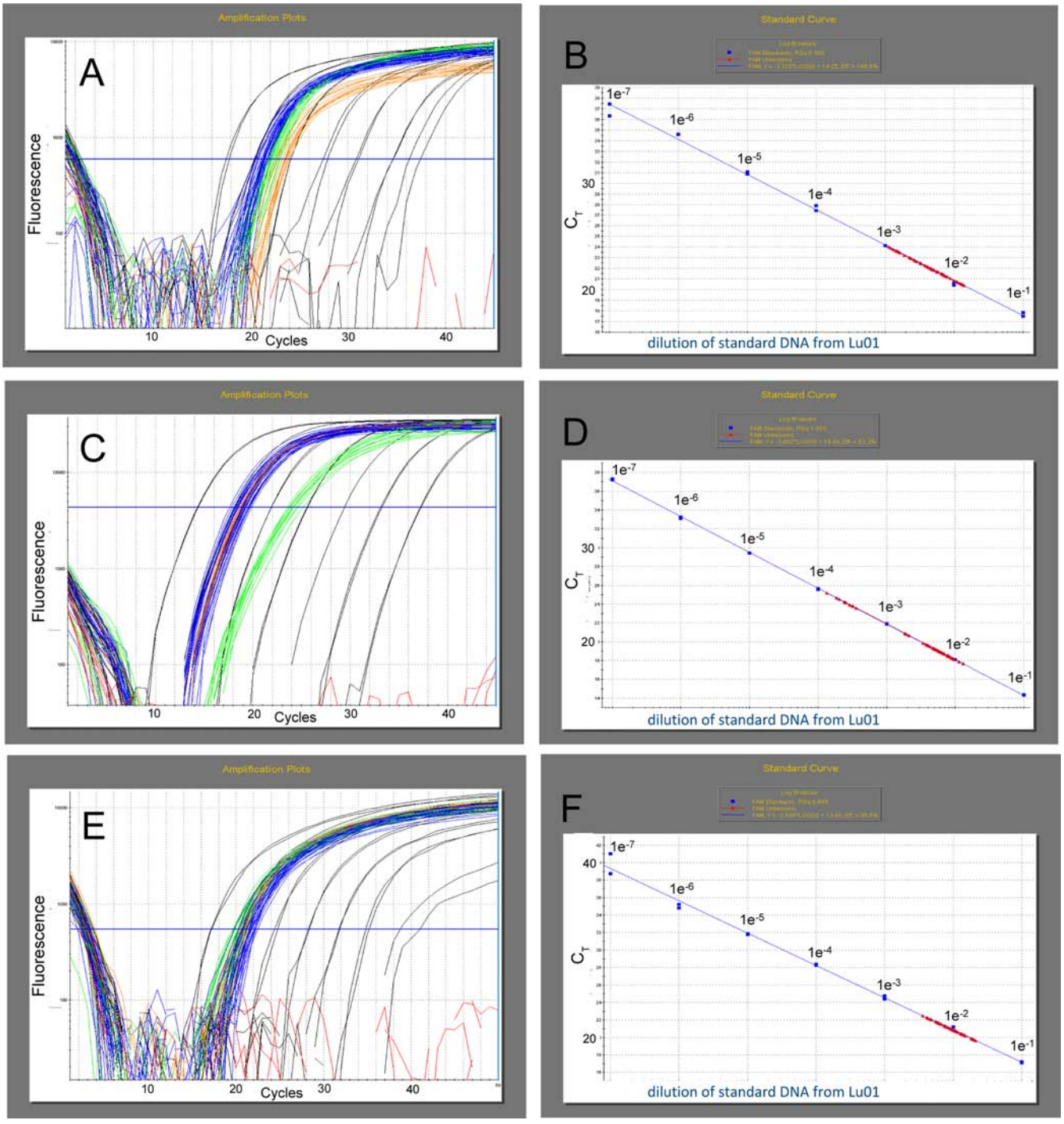

Figure 3. Response of lupine-specific real-time PCR methods to different cultivars of blue (blue curves), yellow (orange curves) and white lupine (green curves). One curve represents one PCR with diluted lupine DNA extract (1:100). Panels A, C, and E depict the recorded real-time PCR fluorescence curves. Panels B, D, and F display the position of quantified lupine samples relative to the standard curve made of serial dilutions of the DNA extract from lupine Lu01, respectively. Newly developed PCR method (A and B), Demmel et al., 2008 (C and D), Galan et al., 2011 (E and F).

measuring total lupine protein or total lupine content based on the particular antibodies that are utilized in each kit very much depends on the specificity of the antibodies. Ideally, the antibodies would quantify individual proteins or a mixture of proteins that are found at comparable and constant quantities in each cultivar. Such quantitative protein data would allow correlating to the lupine content in a food product. In this study, the experimental differences in total lupine protein content according to the Kjeldahl method already indicate that some variability would need to be accepted even for the most ideal molecular marker for quantification. Between the studied ELISA kits, the variability was even larger than the one observed by the Kjeldahl method. Thus, an accurate quantification of the total lupine content by ELISA is limited unless the cultivar is available as a reference.

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Table 5. Species Specific C_T Values (± Standard Deviation) and Response Ratio within One Species (r.w.s) Deduced from the Ratio of the Max/Min Result of Cultivars from One Species[a]

	Lupinus angustifolius		*Lupinus luteus*		*Lupinus albus*	
reference	mean C_T	r.w.s.	mean C_T	r.w.s.	mean C_T	r.w.s.
Lupine PCR (this work)	21.33 ± 0.44	2.3	23.40 ± 0.49	1.9	22.01 ± 1.00	1.8
Demmel et al. (2008)	18.87 ± 0.59	3.2	18.96 ± 0.27	1.3	24.15 ± 0.36	1.5
Galan et al. (2011)	21.43 ± 0.57	2.7	20.34 ± 0.36	1.4	21.02 ± 0.68	1.4
Scarafoni et al. (2009)	not analyzable		not analyzable		not analyzable	

[a]Each cultivar was extracted twice and two replicates were measured per extract.

Development of a Novel Lupine-Specific Real-Time PCR. Three different lupine genes were evaluated for the development of a specific lupine PCR. In-silico analysis suggested the gene (Accession numbers HQ670415.1, EF455724.1, DQ142920.1) of conglutin beta, a major lupine allergen (Lup an 1) as one promising target gene, (data not shown). Two different pairs of primer were designed and investigated experimentally. One primer combination achieved a sensitive and specific detection of different lupine cultivars. A Taqman real-time PCR was designed and optimized with special regard to the $MgCl_2$ concentration of the Mastermix, and the temperature profile. Both parameters were crucial for the balance between sensitivity and specificity.

This lupine-specific real-time PCR was developed for the purpose of investigating the detectability of various cultivars. Primary experiments with serially diluted DNA indicated sufficient sensitivity: the successful amplification of a $1e^{-6}$ dilution indicated a potential detection of 1 mg kg^{-1} whole lupine in food. The specificity of the final primer probe combination was investigated with different cereals and legume foods. The following plant foods tested negative in lupine-specific real-time PCR: barley, soy bean, rye, kidney bean, sunflower seed, celery seed, red lentil, buckwheat, pumpkin seed, sesame seed, soft wheat, navy bean, peanut, linseed, licorice, and chickpea. Forage and catch crops, grown between successive plantings of main crops, that might contaminate subsequent lupine harvesting, were also included in specificity testings but none of the following species tested positive: common broom, fenugreek, alfalfa, red clover, and seradella. If the method, as developed at this stage, should be used for lupine detection in compound food, further validation would need to be done with regard to matrix effects. However, the initial experiments in evaluating specificity and sensitivity were considered sufficient for the purpose of this study.

Relative Quantitative Response of Real-Time PCR Methods to Different Lupine Cultivars. Performance characteristics of the newly developed real-time PCR are shown in Figures 3A and 3B: The recorded fluorescence curves, and the position of quantified lupine samples relative to the standard curve made of serial dilutions of the DNA extract from study lupine Lu01, are displayed, respectively. $1e^{-1}$ to $1e^{-6}$ dilutions of extracted DNA of lupine cultivar Lu01 were repeatedly detected positive, indicating a sensitivity to detect 1 mg kg^{-1} whole lupine in foods. This sensitivity is comparable to commercial ELISA tests (0.2 mg kg^{-1} (ELISA B) lupine protein and 0.6 mg kg^{-1} (ELISA A) lupine protein). Amplification of DNA from lupine cultivars of different species succeeded comparably but curves relating to individual species (blue, white, yellow) were visually separated from each other (Figure 3A and 3B). The C_T values were smallest in PCR tests performed with DNA extracts from blue lupine, followed by C_T values from white lupine and C_T-values from yellow lupine (Table 5). The curve shapes appeared to be similar for all three species (Figure 3A). The response ratio within one species was quite low.

As described above for the ELISA analysis, for PCR analysis the relative quantitative response between the studied cultivars was also calculated. Two approaches were considered: First, the response ratio based on mean values of single extracts for one lupine cultivar was calculated to be 9.8 and second, the response ratio based on mean values of duplicate extracts for one lupine cultivar, was 9.4 (Table 3). The results demonstrated that the single extract approach was very much comparable to the duplicate extract approach and a quantitative intercultivar response ratio of approximately 1 order of magnitude would need to be accepted for this method. For reasons of identical sample treatment in ELISA and PCR, the data from the single extract analysis should be considered. The response ratio of this real-time PCR method was twice as high as seen for the best performing ELISA but was still considered acceptable.

The real-time PCR according to Demmel et al. (2008) makes use of a high copy number DNA target, the internal transcribed spacer sequences of 18S-26S nuclear rDNA, for an increase in sensitivity, compared to single copy DNA targets.[20] Figure 3C depicts the recorded fluorescence curves of all real-time PCR runs for the various lupine cultivars and in comparison to a serially diluted lupine DNA. Figure 3D displays the obtained C_T data plotted versus the standard curve made of serially diluted lupine DNA Lu01. The very high sensitivity of this method is reflected by a reproducible duplicate amplification of even the highest dilution of $1e^{-7}$ of genomic DNA. The original work of Demmel et al. (2008) included the investigation of the three lupine species that are used in food production. Two white lupine cultivars were reported with a positive detection. However no details were given. In our study, all investigated lupine cultivars were detected positive. However, fluorescence curves obtained from real-time PCR with DNA extracts of white lupine cultivars appeared shifted to higher C_T values (mean 24.15) in comparison to results obtained with DNA extracts from blue (mean 18.87) and yellow (mean 18.96) lupine cultivars (Table 5). Again the response ratio within one species was very low. Further, a different slope was observed for the amplification curves of white lupine cultivars (Figure 3C). According to the data obtained with studied lupine cultivars it appeared that the method from Demmel et al. (2008) detects blue and yellow lupine cultivars equally. However, the C_T shift of studied white lupine cultivars may suggest that this method underestimates white lupine cultivars. According to the data obtained with study lupine cultivars we conclude that the chosen sequence does not seem to be optimal for the detection of white lupine cultivars. In summary, between all study cultivars the relative quantitative response was calculated to be 135.8 and 82.9 on the basis of single and duplicate extracts,

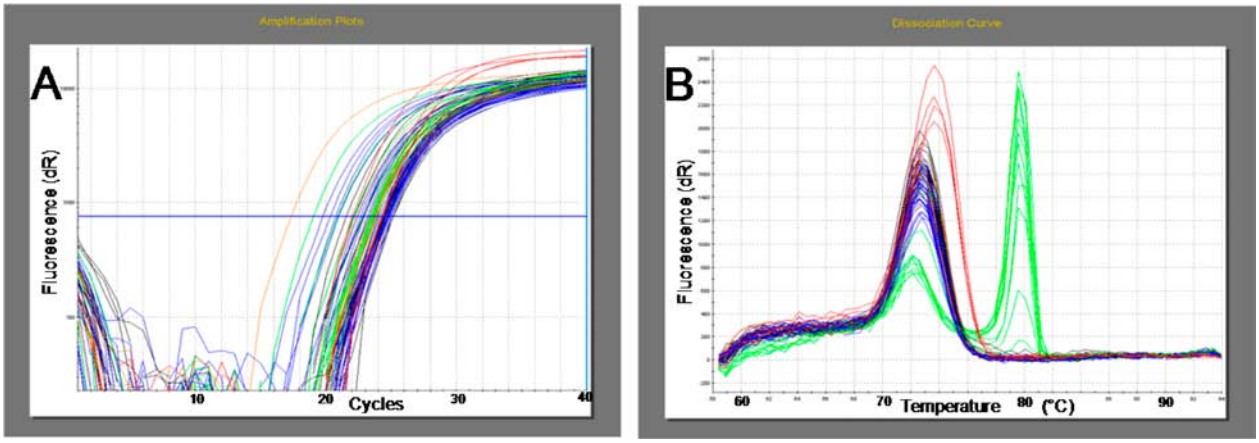

Figure 4. Sybr Green PCR according to Scarafoni et al. 2010. Response of lupine-specific real-time PCR methods to different cultivars of blue (blue curves), yellow (orange curves) white lupine (green curves). One curve represents one PCR with diluted lupine DNA extract (1:100). Fluorescence curves (A) and melting curve analysis (B).

respectively (Table 3). The response ratio of this method was the highest of all methods investigated in this study and the response ratio of the single extract approach was approximately 35 times higher than the one of the best performing ELISA test.

The original publication of Galan and co-workers (2011)[22] aimed at the simultaneous detection of soy and lupine. PCR methods validated for duplex use may be also used for single detection. The detection of lupine is based on primers flanking "DNA sequences coding for mitochondrial gene for initiator tRNA-MET". Using Vector NTI Software to identify primer binding sites, we recognized that the published primer sequences of both the reverse and the forward primer were misleadingly situated on the same DNA strand. Thus, we performed real-time PCR with the reverse complement sequence of the originally published reverse primer. As observed for our newly introduced PCR, the highest serial dilution ($1e^{-7}$) of lupine DNA extract Lu01 was out of linearity and displayed low reproducibility of duplicates (Figure 3F). Nevertheless the second highest serial dilution ($1e^{-6}$) of standard DNA performed well in duplicate PCR analysis. Thus, the sensitivity of the method appears comparable to commercial ELISA tests and the detection limit may be estimated around 1 mg kg^{-1} whole lupine in food. One important observation was the similarity of fluorescence curves between investigated cultivars in both C_T position and maximum fluorescence (Figure 3E): The mean Ct value for blue, yellow and white lupine was 21.43, 20.34, and 21.02, respectively (Table 5). Between all study cultivars the response ratio of 4.8 and 4.0 was calculated from C_T values of single and duplicate extracts, respectively (Table 3). This is almost identical to the performance of ELISA kits A and B.

In contrast to the already described PCR methods, the real-time PCR from Scarafoni et al. (2009) is based on fluorogenic signals generated by a DNA intercalating but sequence unspecific Sybr Green dye.[21] The authors investigated the specificity of the method with DNA from three cultivars of white lupine, one cultivar of blue lupine, and one cultivar of yellow lupine. The five lupine samples had been detected positive. According to the publication from Scarafoni et al. (2009) the size of the generated amplicons should have a length of 159 bp and the melting temperature of amplicons was determined at 81 °C. In our hands, fluorescence signals were obtained for all lupine study samples, but also nontemplate control (NTC) replicates. Figure 4A displays the fluorescence curves of recorded PCR runs. The subsequent melting curve analysis (Figure 4B) revealed that only PCR replicates performed with DNA extracts from white lupine generated lupine-specific amplicons. Their measured melting points of approximately 79 °C were similar to the originally published 81 °C. By contrast, amplified DNA from blue and yellow lupine cultivars as well as NTC replicates generated amplicons having melting temperatures around 72 °C. This signal is believed to be related to artifacts because it most prominently appeared in NTC replicates. These findings were verified by agarose gel electrophoresis (data not shown): Artifacts of 45 bp length were only visible in PCR replicates of NTC and blue and yellow cultivars, whereas amplification of DNA from white lupine cultivars resulted in amplicons of approximately 140 bp size in addition to 45 bp artifacts. Hence, the experimental amplicon length differed from the information given in the original work. Our additional in-silico verification of primer binding sites revealed that the primer sequence of the forward primer published by Scarafoni and co-workers was not identical to the denoted EMBL accession number CAC16394 (DNA sequence AJ297490) which was chosen as template for their PCR development. Our in-silico analysis further revealed that the published forward primer may hybridize to a close-by sequence having three base pair mismatches. In this case, the subsequent product of both primers would generate an amplicon of 136 bp, as seen in our gel electrophoresis experiments. In summary, the method from Galan and co-workers displayed the lowest intercultivar variability of all investigated functional real-time PCR tests, whereas the method from Demmel et al. (2008) exhibited the highest relative quantitative response in this study. The latter recently became an official method (Method BVL L 08.0058(V):2011−06) of the "Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB", which is the official collection of test procedures pursuant to article 64 of the German Food and Feed Code.

Effect of Intercultivar Response Differences in ELISA and PCR Analysis. According to European regulation,[16] certain allergenic food ingredients require mandatory labeling. In the case of potential future regulatory thresholds for allergenic foods, a quantification of the food itself or protein thereof would be necessary. Accordingly, the quantitative

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response of a method to detect different species and cultivars thereof should be reasonably consistent with special regard to the generation of quantitative data to assess, for example, the risk resulting from the amount of an unlabeled potential allergenic food component. The main focus of this study was to investigate the relative quantitative response of both protein based ELISA and DNA based real-time PCR methods to different cultivars of lupine as an example. Because the lupine species and cultivar is likely unknown in a retail sample the worst known case of intercultivar response ratio should be taken into consideration. In the lifelike scenario of an unknown lupine cultivar, the true lupine (protein) content may thus vary between "*x*" times higher or lower the determined amount of lupine (protein) with "*x*" being the known maximum intercultivar response of the test method.

ELISA tests are currently state of the art in allergen detection and quantification. Thus, real-time PCR methods should feature at least similar intercultivar responses if used for allergen quantification. Three real-time PCR methods were also capable of detecting all lupine samples and one method did not work as published. The sensitivity appeared sufficient in the three functional real-time PCR methods. The maximum ratio of intercultivar response ranged in our study from 3.9 to 50.5 and 4.8 to 135.8 in ELISA and PCR analysis, respectively. These data demonstrate that both methodologies appear likely limited in generating quantitative data if prone to a high quantitative intercultivar response and if the detected cultivar is unknown or unavailable as a reference material. Overreliance on the mere analytical read-out without knowing the range of cultivar response could lead to false allergen risk assessment which may result, in the worst case, to undesired severe or fatal allergic episodes. The study results demonstrate that both protein (ELISA) and DNA (PCR) based methods are in general available for a sensitive detection of the numerous lupine cultivars. According to our data, differences below and up to 1 order of magnitude in relative quantification of lupine cultivars would need to be accepted even for the best performing ELISA and PCR methods studied. To date only few data about the relative quantitative response of methods to different cultivars of other allergenic foods are available. In the future there is a necessity to generate such data, also for other plant food species known as allergenic source, in order to elucidate the quantitative features of allergen detection methods. It was demonstrated that, depending on the individual method, large differences in quantitative response to lupine cultivars may occur and thus may circumvent accurate quantification. Thus, optimization of detection methods for allergenic plant foods toward low intercultivar variability should be considered a very critical issue.

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Notes

The authors declare the following competing financial interest(s): Thomas Holzhauser had consultant arrangements with Institut für Produktqualität and Monsanto Company; he is inventor of a method for quantitative detection of an organic substance in a sample (PCT/EP2009/061584).

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