



Effect of food matrix and thermal processing on the performance of a normalised quantitative real-time PCR approach for lupine (*Lupinus albus*) detection as a potential allergenic food



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ABSTRACT

Lupine is widely used as an ingredient in diverse food products, but it is also a source of allergens. This work aimed at proposing a method to detect/quantify lupine as an allergen in processed foods based on a normalised real-time PCR assay targeting the *Lup a 4* allergen-encoding gene of *Lupinus albus*. Sensitivities down to 0.0005%, 0.01% and 0.05% (w/w) of lupine in rice flour, wheat flour and bread, respectively, and 1 pg of *L. albus* DNA were obtained, with adequate real-time PCR performance parameters using the Δ Ct method. Both food matrix and processing affected negatively the quantitative performance of the assay. The method was successfully validated with blind samples and applied to processed foods. Lupine was estimated between 4.12 and 22.9% in foods, with some results suggesting the common practice of precautionary labelling. In this work, useful and effective tools were proposed for the detection/quantification of lupine in food products.

1. Introduction

Lupine is a legume from the Leguminosae family, comprising approximately 200–600 species, from which only four are of agronomic interest and frequently used for food and feed (Prusinski, 2017). Native from the Mediterranean region, the *Lupinus albus* (white lupine) is the most widely cultivated lupine species in this region. The *Lupinus angustifolius* (blue or narrow-leaved lupine) and the *Lupinus luteus* (yellow lupine) are from Australia and Southern Europe respectively, and the less known *Lupinus mutabilis* (pink or Andean lupine) belongs to South America. The first three lupine species are known as “sweet lupines” due to their low levels of potentially toxic alkaloids. Like soybean and milk, lupine is considered as a highly functional food, being commonly used by the industry as technological aids (ingredients) in all kinds of food products (bakery, confectionary, snacks). Due to their nutritional properties, lupine species are also used as feed (Ramanujam, Fiocchi, & Smith, 2016; Sanz, De Las Marinas, Fernández, & Gamboa, 2010). In 2014, Australia was the main producer of lupine with 625.600 tonnes, followed by Poland and Russia (FAOSTAT, 2017). In Europe, lupine is a common substitute for milk and soybean in bakery and dietary products, being commonly used as a functional ingredient in gluten-free foods (Scarafoni, Ronchi, & Duranti, 2009).

Since the first reported case of adverse immunological reactions to lupine in 1994 (Hefle, Lemanske, & Bush, 1994), the allergy to lupine

has been referred with an increasing prevalence (Sanz et al., 2010). Clinical manifestations can vary from urticaria, angioedema, respiratory and abdominal symptoms to fatal reactions (anaphylactic shock). Although lupine allergy can emerge by primary sensitisation, in most of the cases it occurs owing to cross-reactivity in individuals allergic to other legumes, principally peanut and soybean (Sanz et al., 2010). The lowest eliciting dose for allergic reactions to lupine, responsible for inducing mild symptoms in peanut-sensitised patients, was 0.5 mg of lupine flour as reported by Peeters et al. (2009). More recently, the VITAL (Voluntary Incidental Trace Allergen Labelling) program of the Allergen Bureau of Australia and New Zealand (ABA) established 4 mg of protein as the reference allergenic dose for lupine (Taylor et al., 2014).

As consequence of the significant rise of the reported cases of lupine allergy, this legume seed was included in European Union regulations as a food susceptible of inducing allergies and whose presence must be declared and highlighted in the list of labelled ingredients of pre-packaged foods, regardless of its amount (Regulation (EU) No. 1169/2011). The application of the present labelling legislation, while protecting the health of sensitised consumers, it can also restrict the range of available/safe foods if the producers excessively use the precautionary labelling. This demands highly sensitive and quantitative methods in order to identify cross-contamination that justifies the precautionary labelling or the presence of lupine as an ingredient.

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Therefore, analytical methods able to detect and quantify lupine as an allergenic food with high specificity and sensitivity are of great importance to help food industry in allergen management, and to guarantee the life quality of sensitised/allergic individuals.

Different methods have been developed for the detection of lupine allergens in processed foodstuffs, targeting the allergenic proteins by enzyme-linked immunosorbent assay (ELISA) (Ecker & Cichna-Markl, 2012; Ecker et al., 2013; Holden, Faeste, & Egaas, 2005; Holden, Moen, Sletten, & Dooper, 2007; Kaw, Hefle, & Taylor, 2008) or mass spectrometry (MS) platforms (Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017; Mattarozzi, Bignardi, Elviri, & Careri, 2012). As alternative to protein analysis, the indirect identification of DNA encoding sequences of allergens takes advantage of the high stability of the target analytes to processing and the ubiquity of nucleic acids in the cells. Therefore, the number of studies focused on the development of DNA-based methods in allergen detection has rapidly evolved in recent years (Costa, 2013; Costa, Fernandes, Villa, Oliveira, & Mafra, 2017). For the specific detection of lupine, some quantitative real-time PCR approaches with specific hydrolysis probes (Demmel, Hupfer, Busch, & Engel, 2011, 2012; Demmel, Hupfer, Hampe, Busch, & Engel, 2008; Galan, Brohée, de Andrade Silva, van Hengel, & Chassaigne, 2011; Galan, Brohée, Scaravelli, van Hengel, & Chassaigne, 2010; Köppel, van Velsen-Zimmerli, & Bucher, 2012; Röder, Kleiner, Sachs, Keil, & Holzhauser, 2013) or with binding dyes (Scarafoni et al., 2009) have been proposed. Most reports used mitochondrial DNA markers of *Lupinus* spp., except the method described by Röder et al. (2013) that targeted a DNA sequence encoding for the β -conglutinin allergen of *L. angustifolius*. Besides, all of them describe the absolute quantification of lupine DNA, with a rough estimation of lupine content as an ingredient.

In this work, a real-time PCR method targeting a sequence of Lup a 4 encoding gene of *L. albus* and using different model mixtures of lupine as calibrators was developed in order to detect and quantify lupine at trace amounts, and to assess the effect of food matrix and thermal processing on the analytical performance of the assay. A normalised approach was proposed to achieve a reliable and accurate estimation of the relative content of lupine by the use of a reference endogenous gene to minimise the effects associated with food matrix and processing. The successfully validated approaches were further applied to detect and quantify lupine as a potential allergenic ingredient in commercial foods and to verify the compliance with the labelled information.

2. Materials and methods

2.1. Sampling

Seeds of different species of *Lupinus* genus, namely *L. albus* (accessions no. 91-0035D and 91-0023D), *L. luteus* (accessions no. 90-0578D and 90-0579D), *L. mutabilis* (accessions no. 90-0580D and 90-0581D)

and *L. angustifolius* (accession no. PI167938, PI615407, PI606487, PI180708, PI237721 and PI 660698) were gently provided by the Bank of Germplasm of University of Arizona (Boyce Thompson Arboretum, AZ, USA) and by the National Genetic Resources Program (NGRP) (Washington State University, Pullman, WA, USA). Flours of *L. luteus* and *L. albus* were gently provided by the company Germisem (Coimbra, Portugal) or acquired at local markets.

For method specificity testing, a total of 71 different species used as food were assayed: tree nuts (macadamia, pine nut, chestnut, almond, hazelnut, brazil nut, pecan nut, cashew nut, walnut and pistachio nut) and peanut; fruits (tomato, cherry, plum, apricot, blackberry, strawberry, nectarine, peach, mango, pineapple, orange and bitter orange); meats (boar, duck, partridge, hare, quail, pheasant, deer, rabbit, chicken, turkey, lamb, goat, ostrich, cow, horse and pig); fishes (European seabass, whiting pout, Atlantic horse mackerel, tadpole codling, rose fish, rock ling, Pacific mackerel); spices/condiments (onion, garlic, parsley, pepper, bay leaf, sweet chilli, chilli, oregano, basil, coriander and turmeric); cereals and other edible crops (fava bean, colza, sunflower, oat, barley, rye, maize, rice, pumpkin seeds, wheat, soybean, potato and cassava).

For assay applicability, a total of 26 commercial foods whose labels stated the presence of lupine (cookies, cupcakes, cakes, wafers and bakery products) were acquired at different Portuguese retail markets.

2.2. Model mixtures and sample preparation

In the absence of certified reference or testing materials for the specific detection of lupine in food products, two independent sets of binary model mixtures containing 50.0%, 10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% (w/w) of lupine flour (*L. albus*, Biosagesse, France) in rice or in wheat flours were prepared. The first spiked reference mixture, containing 50% of lupine, was prepared by the addition of 200 g of lupine flour to 200 g of rice flour or wheat flour. All the following spiking levels of lupine were made by successive additions of each matrix flour until the proportion of 0.0001% of lupine. For method validation, blind samples were prepared similarly to reference mixtures, but independently, adding 25%, 2.5%, 0.8%, 0.25% and 0.025% (w/w) of lupine flour to rice or wheat flours and further analysed as unknown samples.

For the preparation of model bread, starting from binary mixtures of lupine in wheat flour, 180 g of water, 3 g of bread improver, 4.5 g of salt and 6 g of baker's yeast were added to a final amount of 300 g of the respective mixture. Dough was cooked in a bread machine Moulinex OW6101 during 3 h. After cooling, breads were cut in the middle to remove slices, which were ground in the laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). All materials and different blender containers were previously treated with DNA decontamination solution to avoid contaminations. All samples were

Table 1

Key data of primers and probe designed to specifically target DNA sequences of the Lup a 4 encoding gene of lupine and a conserved eukaryotic region (nuclear 18S rRNA).

Primers	Sequence (5' → 3')	Amplicon (pb)	NCBI (Accession no.)	Reference
<i>Lupinus</i> spp.				
La4-F1	GGTGGCCTGGAACCATTAAGA	134	AJ000108.1	This work
La4-R1	GGTAACCCAACTCCCAACTA			
La4-F2	AACCAAAGGAGATGCTAAACCTAA	124	AJ000108.1	This work
La4-R2	GAGTTGAGTTTAGTTGTAATCAGG			
La4-P	FAM-AAGAGGGTAAAGCTGCTAAGGCTAGA-BHQ-1			
La4-F3	TAGTTGGTGGAGTTGGGTTACC	133	AJ000108.1	This work
La4-R3	TTAGGTTTAGCATCTCCTTTGGTT			
Eukaryotes				
EG-F	TCGATGGTAGGATAGTGGCCTACT	109	AF412275	Villa et al. (2017)
EG-R	TGCTGCCTTCCTTGGATGTGGTA			
EG-P	FAM-ACGGGTGACGGAGAATTAGGGTTTCGATTC-BHQ-1			This work

immediately stored at -20°C until further analysis.

Commercial foods were also minced/homogenised separately in a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). Due to their high hardness, seeds of *L. albus* and *L. luteus* were ground in a mill suitable for flours with granulometry of 0.1 mm (MFC-90D MicroHammer Mill, Culatti, Zurique, Switzerland), while seeds and grains of *L. angustifolius* and *L. mutabilis* were soaked in distilled water and homogenised separately in a mortar.

2.3. DNA extraction

DNA was extracted using the Wizard method as described by Mafra, Silva, Moreira, Ferreira da Silva and Oliveira (2008) and/or NucleoSpin food kit (Macherey-Nagel, Düren, Germany), according to manufacturer instructions, using 200 mg of each sample. The extractions were performed in duplicate for each binary mixture and food. The extracts were kept at -20°C until further analysis.

UV spectrophotometric DNA quantification on a SynergyHT multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), using a Take 3 micro-volume plate accessory, was performed to evaluate the yield and purity of DNA extracts. The DNA content was assessed by the nucleic acid quantification protocol in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA) with sample type defined for double-strand DNA.

2.4. Oligonucleotide primers and probes

DNA sequences available at NCBI database (<http://www.ncbi.nlm.nih.gov/>) corresponding to the gene encoding the allergenic PR-10 protein of *L. albus* (Lup a 4) (accession no. AJ000108.1 and AB070618.1) and *L. luteus* (accession no. AF170091.1, AF322226.1, AY288355.1, AF322225.1 and AF170092.2) were aligned using the software BioEdit V7.2.5. (Ibis Biosciences, Carlsbad, CA, USA). Common regions between sequences were selected for the design of three sets of new primers (La4-F1/La4-R1; La4-F2/La4-R2 and La4-F3/La4-R3) for the specific detection of *Lupinus* spp. (Table 1). The design of primers was performed with the software Primer3 Output designing tool (<http://frodo.wi.mit.edu/primer3/>). The hydrolysis probe (La4-P) was also designed based on a specific region for *L. albus* in the same gene (accession no. AJ000108.1), labelled with FAM as fluorescent reporter and BHQ-1 as quencher (Table 1 and Fig. S1A, supplementary material). For specificity purposes, *in silico* analysis of the nucleotide and primer sequences was performed using the basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), respectively. OligoCalc software (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) was also used to check primer properties and to ensure the absence of primer hairpins and self-hybridisation.

Supplementary Figs. S1–S3 associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.04.079>.

The amplification capacity of extracts was performed with universal eukaryotic primers (EG-F/EG-R) targeting a highly conserved 18S rRNA nuclear region (NCBI accession no. AF412275), acting also as an endogenous control gene (Villa, Costa, Oliveira, & Mafra, 2017). For the development of the normalised real-time PCR systems, a probe for the same region (EG-P) was also designed. The primers and probes listed in Table 1 were synthesised by STABVIDA (Lisbon, Portugal).

2.5. Qualitative PCR

In order to optimise PCR conditions, different concentrations of MgCl_2 and temperatures of annealing were tested for each primer pair (Table 1). Using the optimised PCR conditions, the reactions were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 25 μL containing 2 μL of

template DNA (100 ng), 1 $\times$ buffer (67 mM of Tris-HCl (pH 8.8), 16 mM of $(\text{NH}_4)_2\text{SO}_4$, 0.1% of Tween 20), 200 mM of each dNTP (Grisp, Porto, Portugal), 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM (for primers La4-F1/La4-R1, La4-F2/La4-R2 and EG-F/EG-R) or 3.5 mM (for primers La4-F3/La4-R3) of MgCl_2 and 200 nM of each primer (except for primers EG-F/EG-R, for which 280 nM were used) (Table 1). The amplification programs were defined as following: initial denaturation at 95°C for 5 min; 35 (for primers EG-F/EG-R) or 40 cycles (for all the other primers) at 95°C for 30 s, 59°C (for primers La4-F2/R2), 62°C (for primers La4-F1/R1 and La4-F3/R3) or 63°C (for primers EG-F/EG-R) for 30 s and 72°C for 30 s; and a final extension at 72°C for 5 min.

PCR products were verified by electrophoresis in a 1.5% agarose gel stained with GelRed 1 $\times$ (Biotium, Inc., Hayward, CA, USA) and carried out in 1 $\times$ SGTB (Grisp, Porto, Portugal) for 20–25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was recorded using Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each extract was amplified at least in two independent runs.

2.6. Real-time PCR

For the real-time PCR amplifications, the reaction mixture of 20 μL included 1 $\times$ of SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 300 nM of each primer (EG-F/EG-R or La4-F2/La4-R2), 200 nM of each probe (EG-P or La4-P) for eukaryotic or lupine genes (Table 1), respectively, and 2 μL of DNA extract (100 ng). Each target sequence (eukaryotic and lupine genes) was amplified simultaneously in parallel reactions, using a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: 95°C for 5 min, 50 cycles at 95°C for 15 s and 60°C for 45 s, with collection of fluorescence signal at the end of each cycle. The software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) was used to evaluate the data from each real-time PCR run. Cycle of quantification (Cq), also known as cycle threshold (Ct), values were calculated using the software at user defined settings. Real-time PCR trials were repeated in two independent runs using $n = 4$ replicates in each one.

For the determination of absolute limit of detection (LOD) and limit of quantification (LOQ) a series of 10-fold serially diluted lupine DNA extracts were prepared (10 ng–1 pg), while the binary mixtures (0.0001–50%) allowed the determination of the relative LOD and LOQ of lupine flour in food matrix. The LOD was considered as the lowest amplified level for 95% of the replicates and the LOQ was established as the lowest amplified level within the linear or dynamic range of the calibration curve (ENGL, 2015).

2.7. Sequencing

DNA from *L. albus*, *L. luteus*, *L. angustifolius* and *L. mutabilis* were amplified with primers La4-F3/La4-R2 to produce fragments of 233 bp for sequencing. The following amplification conditions were used: initial denaturation at 95°C for 5 min; 40 cycles at 95°C for 30 s, 60°C for 45 s and 72°C for 60 s; and a final extension at 72°C for 5 min. PCR products were purified with GRS PCR & gel band purification kit (GRISP, Porto, Portugal) to remove contaminants such as proteins, divalent cations, unincorporated nucleotides and enzyme inhibitors. The purified products were sent to a specialised research facility (GATC Biotech AG, Constance, Germany) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality. Data were analysed using the available BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and FinchTV (Geospiza, Seattle, WA, USA) softwares and further compared with consensus sequence of *L. albus* (NCBI accession no. AJ000108.1).

3. Results and discussion

3.1. DNA extracts

DNA extracts used to test the specificity of the method were obtained with the Wizard method described by Mafra et al. (2008), presenting a wide range of yields (6.0–1608.4 ng/μL) and respective purities (1.6–2.2). *Lupinus* spp. specimens, reference mixtures and commercial samples were extracted with NucleoSpin food kit (Macherey-Nagel, Düren, Germany), providing DNA yields that varied between 9.1 and 1705.1 ng/μL with purities of 1.6–2.3. Prior to any amplification with specific primers and in order to ensure the absence of any possible false-negative results, the amplification capacity of all DNA extracts was tested with universal primers targeting the eukaryotic nuclear 18S rRNA gene (Table 1), revealing positive results for all samples.

3.2. Selection of primers

To evaluate the suitability of the designed new primers (La4-F1/La4-R1, La4-F2/La4-R2 and La4-F3/La4-R3) (Table 1) for lupine

detection by PCR, sensitivity and specificity tests were performed. The sensitivity was assayed by PCR amplification of 10-fold serially diluted lupine (*L. albus*) DNA extracts starting from 100 ng, while specificity was tested using a wide range of plant and animal species commonly used as food (as described in 2.1 section) to assure the absence of any possible cross-reactivity. The PCR assay with primers La4-F1/La4-R1 revealed strong cross-reactivity with soybean from the 71 investigated species (Fig. S2A and S2B, supplementary material), which invalidated their further use. In addition, the obtained PCR fragments show a size much higher than the expected (134 bp), suggesting that the region contains an intron that was probably spliced during the transcription process and, therefore, not reported in the target sequence of *Lupinus albus* mRNA available at NCBI database (accession no. AJ000108.1). La4-F3/La4-R3 primers were able to amplify the expected fragment of 133 bp of lupine DNA (Fig. S2E and S2F, supplementary material), but, again they reacted with soybean, thus invalidating their use. The best results were obtained with La4-F2/La4-R2 primers, whose sensitivity was down to 10 pg of lupine DNA, with an expected fragment of 124 bp and no observable cross-reactivity with any other non-target species (Fig. S2C and S2D, supplementary material). Specificity of the PCR was further assessed with the other *Lupinus* species besides *L. albus*, namely

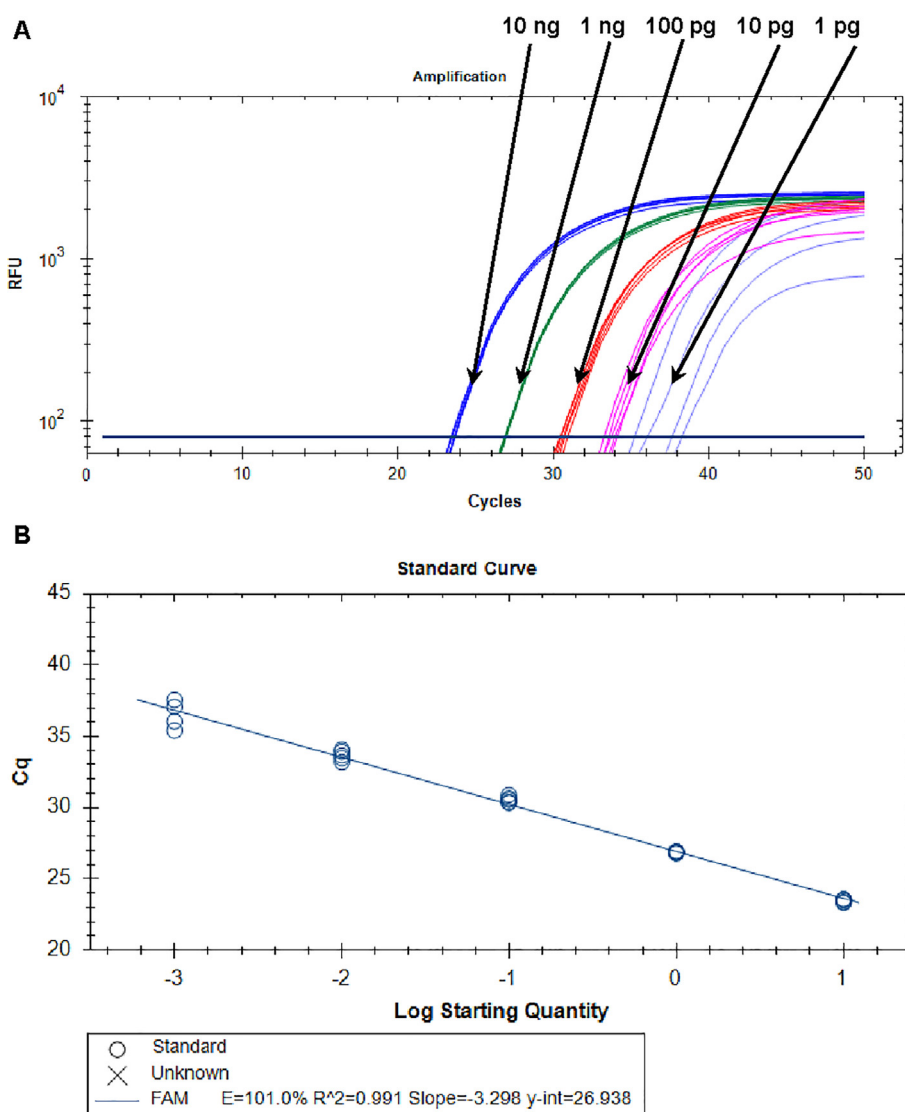


Fig. 1. Amplification curves (A) and respective calibration curve (B) of a real-time PCR assay with a hydrolysis probe targeting the *Lup a 4* gene. The amplified extracts were obtained from 10-fold serially diluted lupine DNA from 10 ng to 1 pg ($n = 4$ replicates). Cq (cycle of quantification) is also known as Ct (cycle threshold).

L. luteus, *L. angustifolius* and *L. mutabilis* (Fig. S3A, supplementary material), which all produced the expected PCR fragment, showing that the assay is *Lupinus* genus specific. Lupine and soybean are species from the same legume family (Leguminosae), sharing structural similarities between their respective allergens. Serologic cross-reactivity between other members of the Leguminosae family and lupine is often common, although it does not always translate in clinically relevant symptomatic allergies (Berkner et al., 2009; Peeters et al., 2007; Ramanujam et al., 2016). The soybean allergen Gly m 4 is a member of the superfamily of Bet v 1 homologous proteins, the major birch pollen allergen, and according to the structural classification of proteins, they are both members of the PR-10 family. In this context, Berkner et al. (2009) demonstrated sequence similarities of 74–84% between Gly m 4 (soybean) and the PR-10 proteins (lupine) responsible for lupine allergy. This fact explains the cross-reactivity obtained in our results (Fig. S2B and S2F, supplementary material).

3.3. Development of the quantitative real-time PCR method

3.3.1. Specificity

For the real-time PCR method, the use of a fluorescent probe rather than a universal dye was selected to enable high sensitivity, avoiding unspecific amplification products. The hydrolysis probe was designed based on the alignment with the available PR-10 sequences at NCBI of *L. albus* (accession no. AJ000108.1 and AB070618.1) and *L. luteus* (accession no. AF170091.1, AF322226.1, AY288355.1, AF322225.1 and AF170092.2), in order to be specific for *L. albus* combined with the La4-F2/La4-R2 primers (Fig. S1A, supplementary material). The choice of developing a method specific for *L. albus* was due to the fact that it is the most often cultivated lupine species in Mediterranean region for human nutrition and, consequently, with high probability on being present in foodstuffs as a potential allergen (Prusinski, 2017). The real-time PCR results with the designed new hydrolysis probe (La4-P) using the different *Lupinus* species confirmed that the assay was specific for *L.*

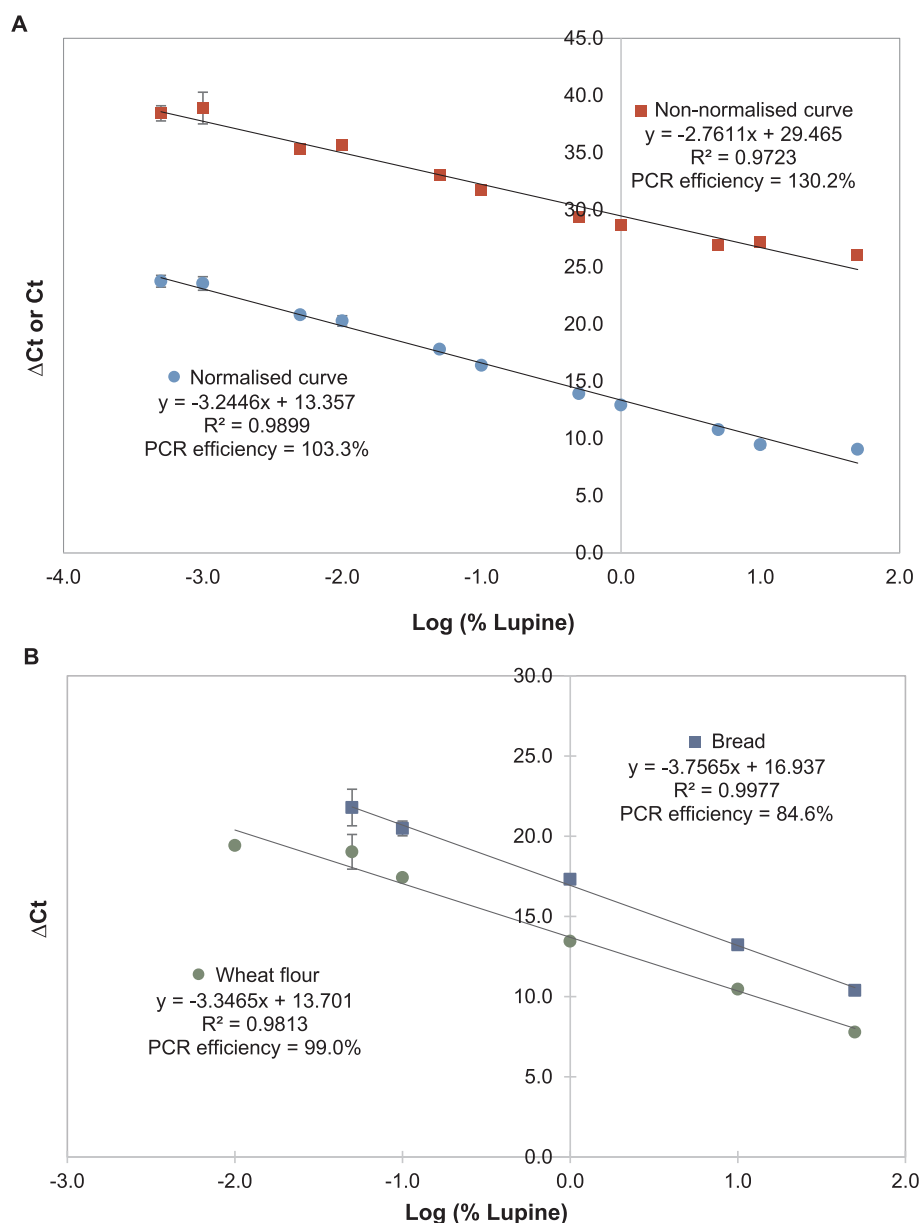


Fig. 2. Calibration curves obtained by real-time PCR targeting the *Lup 4* encoding gene of lupine. (A) Normalised and non-normalised calibration curves using the binary mixtures of lupine in rice flour (50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001% and 0.0005% (w/w)) in both models; (B) normalised calibration curves of lupine in wheat flour and breads (50%, 10%, 1%, 0.1%, 0.05% and 0.01% (w/w)). The normalised ΔCt method was performed by the parallel amplification of a eukaryotic gene (18S rRNA) as reference, using data from two independent real-time PCR runs ($n = 8$ replicates).

albus (Fig. S3B, supplementary material). Contrarily to the qualitative PCR results, in which all the four lupine species were amplified (Fig. S3A, supplementary material), the hydrolysis probe allowed increasing the specificity of the proposed real-time PCR system.

In order to identify the nucleotide differences among the species in the target Lup a 4 sequence, the 124 bp PCR products (obtained with primers La4-F2/La4-R2) of *L. albus*, *L. luteus*, *L. angustifolius* and *L. mutabilis* were sequenced. Firstly, the sequencing results (Fig. S1B, supplementary material) of *L. albus* showed four differences in the target fragment of 124 bp, one of them in the probe region (G → A), compared with the consensus sequence from the NCBI (accession no. AJ000108.1). This difference was common to all species, suggesting possible nucleotide mistakes in the deposited sequence at the database or no effect on the probe binding to the target region in the case of *L. albus*. In the same region of the probe, *L. angustifolius* and *L. mutabilis* showed five nucleotide differences in comparison with sequenced *L. albus*, while *L. luteus* evidenced only two nucleotide differences. In all cases, the nucleotide differences among species were sufficient to affect the efficiency of probe ligation, thus explaining the absence of amplification of *L. luteus*, *L. angustifolius* and *L. mutabilis* by real-time PCR (Fig. S3B, supplementary material). Moreover, the sequence of the forward primer in *L. angustifolius*, *L. mutabilis* and *L. luteus* presents two differences (C → A and C → T), which did not affect primer binding by qualitative PCR (Fig. S3A, supplementary material).

3.3.2. Sensitivity

The absolute LOD of the real-time PCR method was established by the use of 10-fold serially diluted *L. albus* DNA extracts to cover the amplification of 5 orders of magnitude of the target analyte (10 ng to 1 pg). According to the parameters required for method development and validation, the LOD has to be defined as the lowest concentration level of the analyte with positive amplification at least 95% of the times (Bustin et al., 2009; ENGL, 2015). In this study, the LOD was determined assuming the lowest amount of *L. albus* DNA with positive amplification in all replicates (8/8), which corresponded to the level of 1 pg of lupine DNA, equivalent to approximately 1.7 genomic DNA copies (Fig. 1). The estimation of the amplified DNA copies was calculated using the available prime estimate value of *L. albus* genome size (0.6 pg), obtained from the Plant DNA C-value database (Bennett, & Leitch, 2012), assuming that the targeted sequences are single copy genes. Since the lowest amplified level (1 pg) is within the linear range of the calibration curve, it is also considered as the LOQ. With the proposed real-time PCR system, the absolute sensitivity was improved when compared with other reports that targeted allergen encoding sequences of *L. angustifolius* (500 pg and 50 pg for Lup an α -conglutinin and Lup an δ -conglutinin genes, respectively) (Galan et al., 2010). The obtained absolute sensitivity (1 pg) was also higher than the one (7 pg) reported by Scarafoni et al. (2009) using a real-time PCR system based on SYBR Green dye to detect Lup a γ -conglutinin encoding sequence of *L. albus*. The TaqMan real-time PCR method reported by Galan et al. (2011) was able to reach a sensitivity of 1 pg of *L. angustifolius* DNA, but targeting a mitochondrial gene (tRNA-MET), which is a multicopy gene,

in opposition to the allergen encoding sequences that are single-copy genes.

According to the general guidelines described by Bustin et al. (2009) and ENGL (2015), other performance parameters of real-time PCR methods have to comply with the acceptance criteria established for this type of assay, namely PCR efficiency that should range between 90 and 110%, slope within -3.6 and -3.1 and correlation coefficient (R^2) above 0.98. Fig. 1 shows the amplification curves and the respective calibration curve, as an example run, highlighting its adequate performance in terms of R^2 (0.991), PCR efficiency (101.0%) and slope (-3.298).

Binary model mixtures containing known amounts of *L. albus* flour (50.0%, 10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001%, w/w) in rice flour were used for the relative quantification of lupine. The real-time PCR system enabled a dynamic range of 5 orders of magnitude, covering the amplification down to 0.0005% (w/w) (5 mg/kg) of lupine in rice flour, which was within the linear range of the calibration curve, thus being considered as the relative LOD and LOQ (Fig. 2A). This value is comparable to the relative sensitivities reported by Röder et al. (2013) (1 mg/kg), Galan et al. (2010, 2011) (10 mg/kg and 2.5 mg/kg, respectively) and Demmel et al. (2008, 2011, 2012) (0.1–10 mg/kg), all using real-time PCR methods with TaqMan probes. However, it should be highlighted that the reports of Demmel et al. (2008, 2011, 2012) and Galan et al. (2011) targeted mitochondrial DNA sequences (multi-copy genes), which enable achieving higher sensitivities than the nuclear sequences such as the allergen encoding genes. It is also important to refer that the sensitivity reported by Röder et al. (2013) (1 mg/kg) was established based on an estimate of serially diluted lupine DNA and not using model mixtures, including much more interfering compounds and non-target DNA. Accordingly, the highest absolute and relative sensitivity for lupine detection using real-time PCR with TaqMan probes, targeting allergen-encoding sequences, was reached in the present work.

3.3.3. Model construction and validation

For the relative quantification of lupine, two strategies were evaluated using: i) a non-normalised real-time PCR calibration curve and ii) a normalised curve based on the parallel amplification of the target lupine sequence (specific amplification) and a universal eukaryotic region (reference gene) with approximately the same amplification efficiencies. Both calibration curves were constructed and the respective performance parameters were evaluated and compared. Non-normalised Ct values from the amplification of the binary reference mixtures targeting Lup a 4 gene were plotted against the logarithm of lupine content, providing a calibration curve with a slope of -2.76 ± 0.07 , a R^2 of 0.972 ± 0.002 and a PCR efficiency of $130.2 \pm 4.2\%$. Similarly, to improve the performance parameters of method, a normalised calibration curve was constructed using the Δ Ct values from the amplification of the target lupine sequence and a eukaryotic conserved DNA region of nuclear 18S rRNA as endogenous control, considering the following expression:

Table 2

Validation results obtained in normalised and non-normalised quantitative real-time PCR systems with model mixtures of lupine in rice flour.

Blind mixture	Actual value (%)	Normalised			Non-normalised		
		Estimated value ^a (%)	CV ^b (%)	Bias ^c (%)	Estimated value ^a (%)	CV ^b (%)	Bias ^c (%)
1	2.50	2.38 $\pm$ 0.18	7.6	-4.63	4.51 $\pm$ 0.41	9.0	80.3
2	0.80	0.96 $\pm$ 0.11	11.7	20.0	2.15 $\pm$ 0.17	8.1	168.6
3	0.25	0.28 $\pm$ 0.03	9.1	13.4	0.40 $\pm$ 0.04	9.1	61.1
4	0.025	0.012 $\pm$ 0.003	21.6	-53.4	0.006 $\pm$ 0.001	18.5	-76.8

^a Mean values $\pm$ standard deviation (SD) ($n = 8$) of two independent runs

^b CV – coefficient of variation.

^c Bias = ((mean estimated value-true value)/true value $\times$ 100).

$$\Delta Ct = Ct_{\text{lupine}} - Ct_{\text{euk}}$$

where Ct_{lupine} and Ct_{euk} are the Ct values for lupine and eukaryotic sequences, respectively. It can be noted that method normalisation leads to a clear improvement of the analytical performance since the parameters of PCR efficiency ($103.3 \pm 2.7\%$), R^2 (0.989 ± 0.001) and slope (-3.24 ± 0.07) were all within the acceptance criteria for this type of assays (Bustin et al., 2009; ENGL, 2015). Fig. 2A represents the normalised and non-normalised calibration curves, each one constructed with mean values of two independent real-time PCR runs ($n = 8$).

To assess the quantitative performance of both non-normalised and normalised models, a set of blind mixtures containing 0.025, 0.25, 0.8 and 2.5% (w/w) of lupine in rice flour was used. The obtained lupine estimates and comparative analysis with the actual values are presented in Table 2. All the estimates exhibited adequate values of coefficient of variation (CV), expressing the relative standard deviation of results that varied between 7.6% and 21.6%, which demonstrated acceptable precision ($\leq 25\%$) of both non-normalised and normalised methods over the tested dynamic range. In terms of trueness (bias), the estimates using the non-normalised curve exhibited bias between -76.8% and 168.6% (Table 2), which are greatly above the criterion of acceptance of $\pm 25\%$ of the actual value (ENGL, 2015). In opposition, with the normalised calibration curve, the accuracy data ranged from -4.63% to 20.0% for the three highest levels, which are within the acceptable bias. The mixture of 0.025% (w/w) of lupine in rice, revealed a bias higher than $\pm 25\%$, but at these low concentration levels the deviations from the true value are more likely to occur. Therefore, the method can provide acceptable quantitative results in the range of 0.25–2.5% of lupine in rice flour. The obtained results from the method validation and the performance parameters of real-time PCR assays clearly demonstrate that the normalisation improves the reliability and accuracy of the quantitative method. This fact is due to the use of a reference endogenous gene that accounts for possible amplification differences due to inconsistent DNA recovery and quality/degradation among extracts, which might affect PCR efficiency (Villa et al., 2017; Costa, Amaral, Grazina, Oliveira, & Mafra, 2017).

3.3.4. Effect of food matrix and processing

To evaluate the effect of food matrix and processing on the performance of the proposed method, the model mixtures of wheat flour and breads spiked with 50.0%, 10.0%, 1.0%, 0.05%, 0.01% and 0.001% (w/w) of lupine flour were used. Regarding the wheat flour models, the real-time PCR system enabled the amplification down to 0.01% (w/w) (100 mg/kg) of lupine in wheat flour, which can be considered as the relative LOD and LOQ. The normalised calibration curve constructed by plotting the ΔCt values against the logarithm of lupine content provided a slope of -3.35 , a R^2 of 0.981 and a PCR efficiency of 99.0% (Fig. 2B). On the other hand, the application of the normalised real-time PCR system to model breads enabled the lupine flour amplification down to 0.05% (w/w) (500 mg/kg). The mean values for PCR efficiency, slope and R^2 were 84.6%, -3.76 and 0.998, respectively (Fig. 2B). Comparing both calibration models, a difference of about 3.2 cycles can be noticed between the curve intercepts, which demonstrates a clear delay of the amplification of DNA from bread mixtures in relation to wheat flour.

Food matrix clearly affected the sensitivity of the method since the LOD of the assay using the wheat flour model mixtures was 20-fold higher than the one using rice flour mixtures. Waiblinger, Boernsen, Nümann and Koepfel (2014), who studied the application of a multiplex real-time PCR to detect different allergenic foods, including lupine, in a ring-trial involving several laboratories, reported the importance of matrix effect. The ring-trial results obtained by the analysis of different food matrices (rice cookies, wheat cookies and sauce hollandaise powder) spiked with known amounts of the allergen, showed that the effect of matrix cannot be neglected (Waiblinger et al., 2014).

The presence of components such as fats, carbohydrates or other plant metabolites might hamper the efficient DNA extraction from foods, which leads to a decrement on PCR efficiency, subsequently affecting the quantitative results (Costa, Melo, Santos, Oliveira, & Mafra, 2015). Thus, the assessment of different calibration models is fundamental for the adequate development of a quantitative method.

The influence of processing, more specifically baking, seems to have a negative effect on lupine detection demonstrated by the decrease of sensitivity of lupine detection in bread compared with rice or wheat flour models and by PCR efficiency (84.6%) that is slightly out the acceptable range (Bustin et al., 2009; ENGL, 2015). Thermal treatment may alter the structural properties of food components, namely the protein fraction (including allergens), representing one of the major problems for their analysis by immunochemical methods. DNA is more stable to this type of treatment, but high temperatures during long time may negatively affect the PCR performance, depending on the extent of DNA degradation (Costa, 2013; Costa, Amaral, et al., 2017; Costa, Oliveira, & Mafra, 2013).

A validation test to assess the quantitative performance of the two systems as affected by food matrix and processing was also performed with a set of blind mixtures (25%, 2.5% and 0.25%, w/w of lupine flour in wheat flour or breads). Table 3 shows the obtained results, which demonstrate the high precision and accuracy of the two methods since both displayed adequate values of coefficient of variation (CV) and bias within the criterion of acceptance of $\pm 25\%$ of the actual value (ENGL, 2015).

3.4. Analysis of commercial samples

In order to evaluate the application of the developed method to real foods, several commercial samples labelled with lupine as an ingredient or at trace levels were tested for its presence. The summarised PCR results, together with the corresponding label information of samples, are presented in Table 4. Based on the qualitative PCR results, it is possible to verify that, from the 14 samples declaring the information “may contain traces of lupine”, only two tested positively for lupine (#2 and #15) by qualitative PCR. These results were further confirmed by real-time PCR, with estimated concentrations $< 0.25\%$, suggesting the common practice of the precautionary labelling. The samples declaring “sweet lupine flour” and presenting positive amplification by qualitative PCR were also tested by real-time PCR (#4, #25 and #26). Two samples (#4 and #25) showed low estimates ($< 0.25\%$), while in sample #26 the presence of lupine was not confirmed by real-time PCR, though with the universal primers the result was positive (Table 4). This finding might be due to the addition of sweet lupine flour as a minor ingredient and to the fact of being highly processed and complex products (cookies and wafers), which makes it very difficult or even disables lupine DNA amplification. Besides, the designation of “sweet

Table 3

Validation results based on the application of the normalised quantitative real-time PCR approach to blind mixtures of lupine flour in wheat flour and in breads.

Samples	Lupine (% w/w)		CV (%) ^b	Bias (%) ^c
	Actual value (%)	Estimated value ^a (%)		
<i>Wheat flour</i>				
A	25	23.5 ± 3.1	13.3	− 6.0
B	2.5	3.08 ± 0.25	8.0	23.1
<i>Bread</i>				
C	2.5	2.79 ± 0.18	6.4	11.6
D	0.25	0.258 ± 0.060	23.4	3.2

^a Mean values $\pm$ standard deviation (SD) ($n = 8$) of two independent runs.

^b CV – coefficient of variation.

^c Bias = ((mean estimated value-true value)/true value $\times 100$).

Table 4Results of analysed commercial samples by qualitative PCR and normalised quantitative real-time PCR targeting the *Lup a 4* encoding gene of lupine.

Samples		Relevant label information	Qualitative PCR		Real-time PCR		Estimated lupine (% w/w) (mean $\pm$ SD) ^b
			EG-F/EG-R	La4-F2/La4-R2	EG-F/EG-R (Ct $\pm$ SD) ^a	La4-F2/La4-R2 (Ct $\pm$ SD) ^a	
1	Apricot cookies	Without lupine addition	+	–	NA	NA	
2	Cookies	May contain traces of lupine	+	+	15.83 $\pm$ 0.29	37.12 $\pm$ 0.95	< 0.25%
3	Cookies	May contain traces of lupine	+	–	NA	NA	
4	Cookies	Sweet lupine flour	+	+	21.43 $\pm$ 4.40	40.53 $\pm$ 3.64	< 0.25%
5	Cookies	Sweet lupine flour	+	–	NA	NA	
6	Cookies	May contain traces of lupine	+	–	NA	NA	
7	Cookies	Without lupine addition	+	–	NA	NA	
8	Chocolate cookies	May contain traces of lupine	+	–	NA	NA	
9	Chocolate cookies	May contain traces of lupine	+	–	NA	NA	
10	Fior di Sole cookies	May contain traces of lupine	+	–	NA	NA	
11	Lupine cookies with lemon flavour	Lupine flour (4.9%)	+	+	17.49 $\pm$ 0.05	27.89 $\pm$ 0.21	7.82 $\pm$ 0.57
12	Lupine biscuits	Lupine flour (5.0%)	+	+	17.42 $\pm$ 0.08	27.74 $\pm$ 0.13	8.59 $\pm$ 0.41
13	Plain biscuit	May contain traces of lupine	+	–	NA	NA	
14	Bread	May contain traces of lupine	+	–	NA	NA	
15	Cereal bread	May contain traces of lupine	+	+	17.15 $\pm$ 0.03	37.76 $\pm$ 0.00	< 0.25%
16	Cereal rustic bread	May contain traces of lupine	+	–	NA	NA	
17	Crostini	Lupine protein	+	+	19.34 $\pm$ 0.33	30.64 $\pm$ 0.21	4.12 $\pm$ 0.53
18	Flour for bread	Lupine protein	+	+	17.37 $\pm$ 1.23	26.14 $\pm$ 0.06	22.9 $\pm$ 1.0
19	Pan Carré	Lupine protein	+	+	22.13 $\pm$ 0.20	33.15 $\pm$ 0.11	5.43 $\pm$ 0.27
20	Pan gratí	May contain traces of lupine	+	–	NA	NA	
21	Marble cake	May contain traces of lupine	+	–	NA	NA	
22	Cupcake	May contain traces of lupine	+	–	NA	NA	
23	Panzerottini	Lupine protein	+	–	NA	NA	
24	Wafers	May contain traces of lupine	+	–	NA	NA	
25	Chocolate wafers	Sweet lupine flour	+	+ / –	15.53 $\pm$ 0.00	37.14 $\pm$ 0.89	< 0.25%
26	Cream wafers	Sweet lupine flour	+	+ / –	20.76 $\pm$ 0.54	–	

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) (n = 8) of two independent runs.^b Mean percentage (%) values $\pm$ SD (n = 8) of two independent runs; (+) Positive amplification; (–) Negative amplification; + / – Doubtful amplification; NA – not applicable.

lupine” is attributed to domesticated lupine species that contain low levels of bitter-tasting and potentially toxic alkaloids, which in practice refers to the three main species (*L. albus*, *L. luteus* and *L. angustifolius*). Therefore, the very low estimated content or undetectable lupine might be due to the specificity of the probe for *L. albus* species, which can be present at low quantities or even absent in sweet lupine flour. In the 6 samples stating “lupine flour” or “lupine protein” in their labels, lupine was detected, with the exception of sample #23 (Panzerottini), possibly because of being added at minor amounts and being a complex and processed product as previously referred for other samples. Thus, in the remaining 5 samples declaring lupine flour/protein (#11, #12, #17–#19), it was possible to quantify lupine in concentrations between 4.12 $\pm$ 0.53% and 22.9 $\pm$ 1.0%, which seems to be in good agreement with labelled information (Table 4).

4. Conclusions

The present work proposes a normalised real-time PCR approach using a hydrolysis probe based on the Δ Ct method to detect a sequence of the allergenic PR-10 protein (Lup a 4) encoding gene of *L. albus* in processed foods. The method allowed detecting and quantifying lupine as a potential allergenic food since both the LOD and LOQ were down to 0.0005% (5 mg/kg) of lupine in rice flour. Food matrix and thermal processing clearly affected the sensitivity of the method, which was 20-fold lower in wheat flour model mixtures than in rice flour. The baking process had also a negative effect on the sensitivity of lupine detection in bread compared with rice or wheat flour models, decreasing also the PCR efficiency to values slightly out of the acceptable range. The developed method was also successfully applied to 26 processed food samples, revealing that 2 out of 14 declaring that may contain traces of lupine were positive for lupine, suggesting the excessive use of the precautionary labelling. From five samples that declared lupine as an

ingredient, its content could be effectively estimated in the range 4.12–22.9%.

This study demonstrated that real-time PCR is an accurate and powerful tool for the detection and quantification of lupine as an allergen, which can contribute to a more effective management of allergens by the food industry and the regulatory agencies, thus helping protecting the health of sensitised/allergic consumers.

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Supplementary material

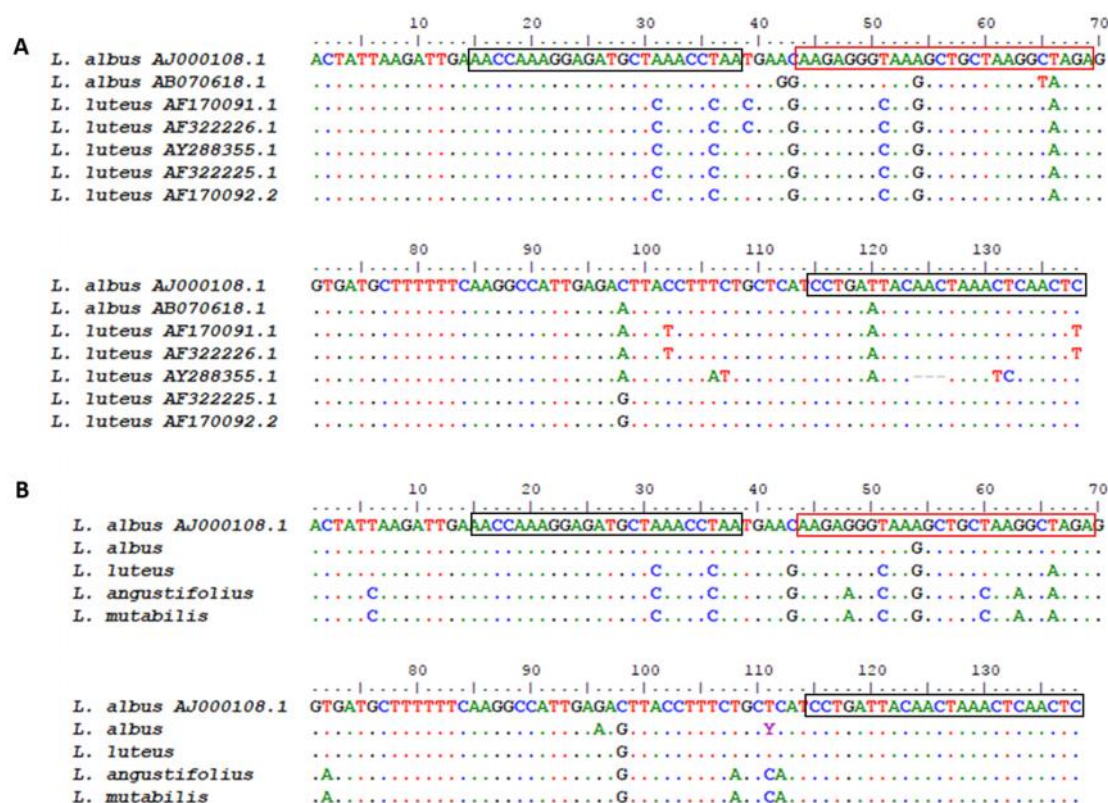


Fig. S1. Alignment of the available PR-10 protein encoding sequences available at NCBI database for the species *L. albus* and *L. luteus* (A) and alignment of sequencing products of *Lupinus* spp. (B). Black boxes represent La4-F2/La4R2 primers and red boxes represent the hydrolysis probe (La4-P).

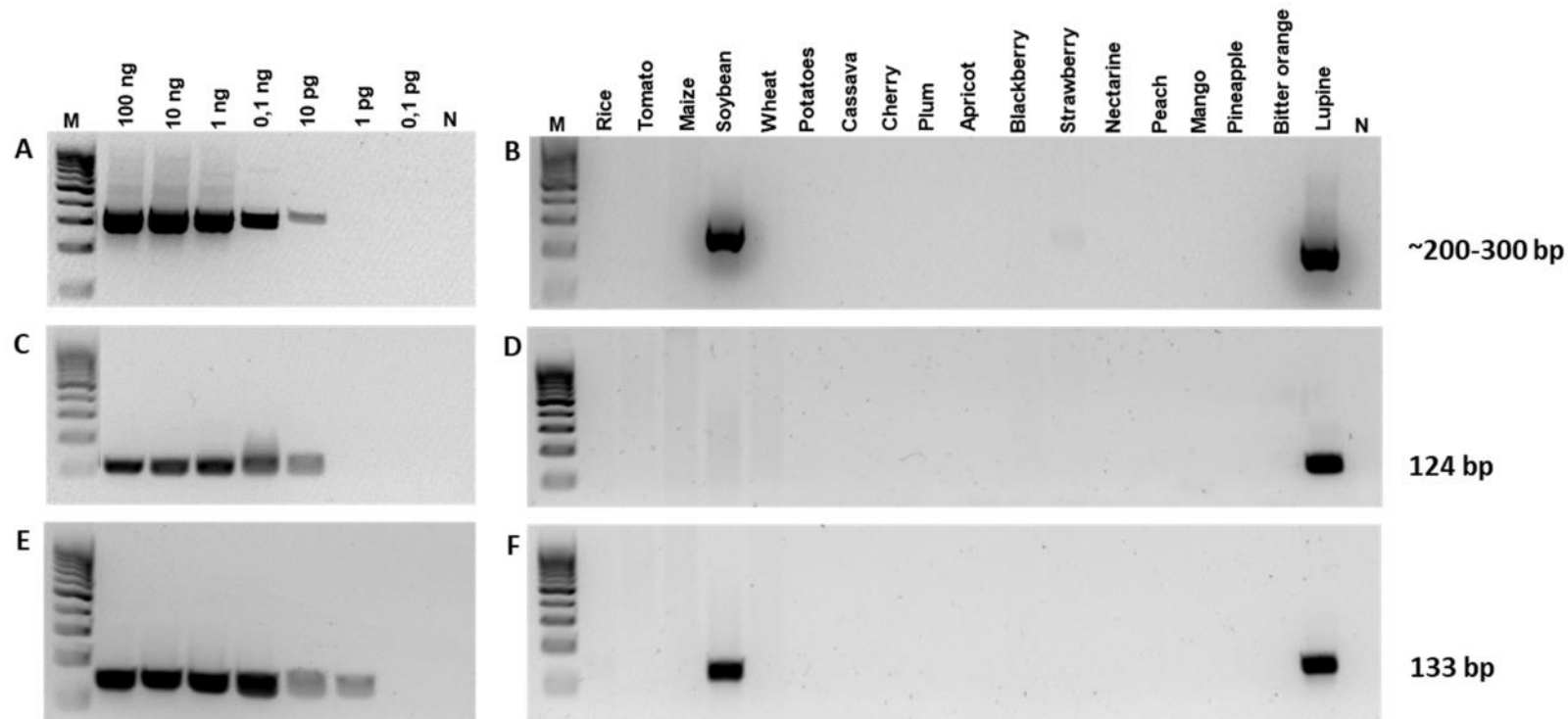


Fig. S2. Agarose gel electrophoresis of PCR products obtained with La4-F1/La4-R1 (A, B), La4-F2/La4-R2 (C, D) and La4-F3/La4-R3 (E, F) primers targeting the gene encoding for the Lup a 4 allergen. A, C and E correspond to serially diluted lupine (*L. albus*) DNA extracts and B, D and F are some of the tested species for cross-reactivity studies. Legend: N, negative control; M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany).

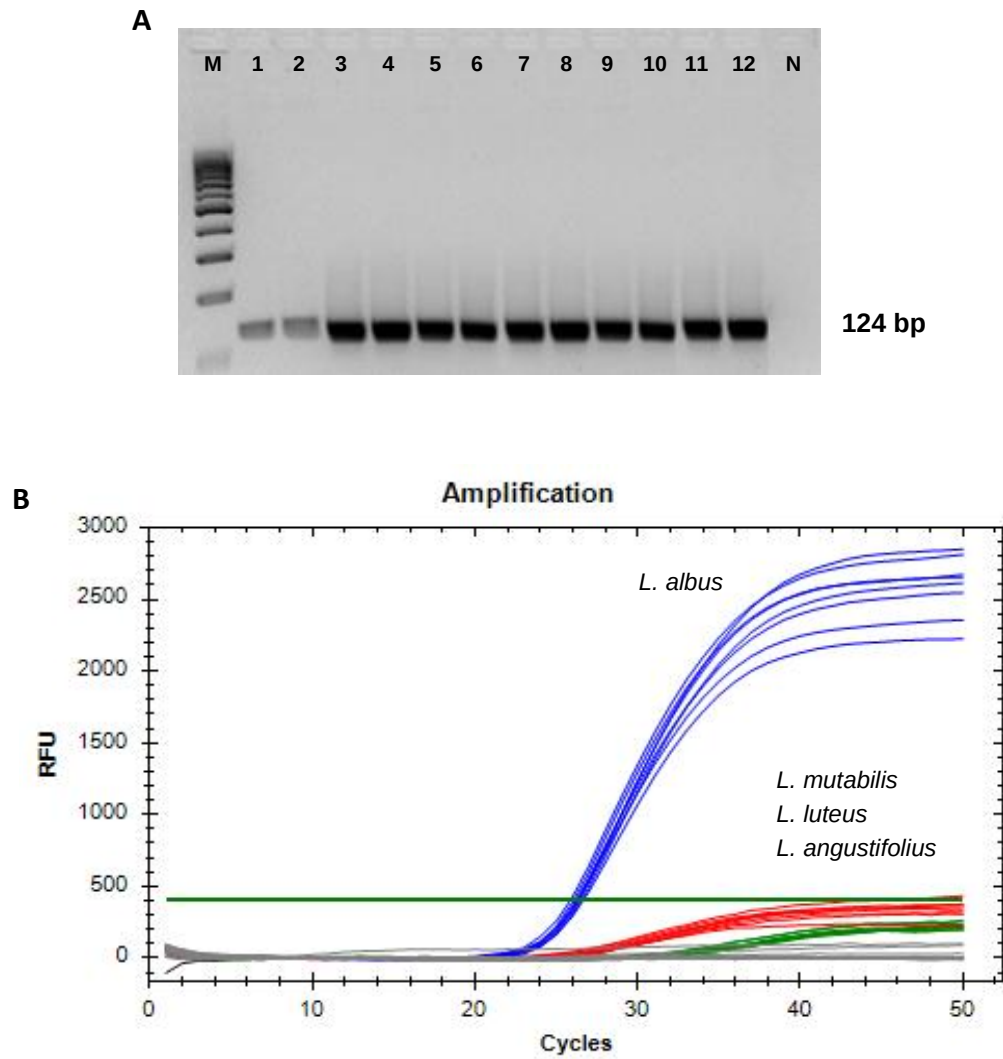


Fig. S3. Results obtained after the amplification of *Lupinus* spp. with La4-F2/La4-R2 targeting *Lup a 4* gene, using two specimens for each of the four species. (A) Agarose gel electrophoresis of qualitative PCR products; (B) amplification curves obtained by real-time PCR with a hydrolysis probe (La4-P) ($n=4$). Legend: lanes 1 and 2, *L. mutabilis*; lanes 3 and 4, *L. albus*; lanes 5 and 6, *L. luteus*; lanes 7 to 12, *L. angustifolius*; N, negative control; M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany).