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Development and Optimization of a LAMP Assay for Lupin Detection in Foods

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Abstract

Lupin (*Lupinus* spp.) is increasingly incorporated into processed foods as a gluten-free ingredient and alternative protein source, but it is also a regulated allergen in the European Union due to cross-reactivity with other legumes, especially peanut. Reliable methods for detecting undeclared lupin traces in complex food matrices are therefore essential for consumer protection. In this study, a loop-mediated isothermal amplification (LAMP) assay was developed for rapid and sensitive detection of lupin DNA. Several nuclear and chloroplast regions were evaluated for primer design, and gene encoding the Lup a 1 allergen was selected as the optimal target. Amplification was monitored by real-time fluorescence, agarose gel electrophoresis, and visual colorimetry. The selected primer set achieved a detection limit of 25 pg of lupin DNA and consistently detected lupin in binary mixtures down to 10 mg/kg, with no cross-reactivity against closely related legumes or tree nuts. Application to processed foods confirmed detection in products declaring lupin and revealed potential undeclared presence in some commercial samples. Colorimetric detection provided reliable results comparable to real-time monitoring, enabling simple readouts without specialized equipment. Overall, the developed LAMP assay represents a rapid, specific, and sensitive alternative to PCR-based methods for allergen monitoring and food safety management.

Keywords: lupin; LAMP assay; allergen-encoding gene; chloroplast marker; food safety

1. Introduction

Lupin (*Lupinus* spp.), a legume rich in antioxidants, fiber, and protein, is increasingly used in the food industry as a gluten-free ingredient and alternative protein source. However, lupin is a recognized allergen, particularly in individuals sensitized to peanut, due to its cross-reactivity. Although the prevalence of lupin allergy in the general population is still uncertain, it has been estimated at around 0.3–1%, with higher rates of sensitization in pediatric cohorts (≈4–5%) and up to ~34% among peanut-allergic children in some studies [1,2]. In *L. albus*, our group has characterized two principal allergens: Lup a 1 (7S protein, 34.5 kDa) and Lup a 2 (11S protein, 20 kDa). Both have been partially sequenced, revealing a high degree of homology with major allergens found in lentil, peanut, soybean, and pea, which likely accounts for their IgE cross-reactivity with these legumes [3].

Given its frequent use in a variety of processed foods, from bakery to dairy and meat products [4], concern about the undeclared presence of lupin as a hidden allergen is growing.

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In line with this, European Regulation (EU) No. 1169/2011 [5]. mandates the labeling of 14 allergenic ingredients, including lupin, regardless of their concentration in food products. To ensure consumer safety and facilitate allergen management under Hazard Analysis and Critical Control Points (HACCP) systems, the development of sensitive and reliable analytical methods for allergen detection is essential.

Methods to detect allergens in processed foods can be based on protein (immunoassays) or DNA. Immunoassays quantify allergenic proteins, while DNA-based techniques detect the genetic material of allergenic ingredients. In processed foods, DNA-based approaches present a key advantage over protein-based methods because DNA is generally more stable than proteins under industrial treatments such as heating, pressure or fermentation, which can denature allergens, alter epitopes, and reduce extraction efficiency, potentially compromising immunoassay performance [6]. Consequently, DNA-based techniques, such as polymerase chain reaction (PCR), are widely used due to their simplicity, specificity, sensitivity, and the inherent stability of DNA under processing conditions. However, PCR-based approaches can still be affected by inhibitors present in complex food matrices and often require high-purity DNA, especially for quantitative assays (qPCR).

Isothermal amplification techniques, such as loop-mediated isothermal amplification (LAMP) [7], represent an alternative within DNA-based allergen detection methods. In contrast to conventional PCR techniques, LAMP operates at a constant temperature (60–65 °C), enabling rapid amplification without thermal cycling and facilitating direct detection with simple heating devices. This method uses a core set of primers consisting of two pairs that recognize six distinct regions of the target sequence. The first pair includes internal primers, the forward internal primer (FIP) and the reverse internal primer (BIP), which play a key role in initiating the amplification process. The second pair consists of the outer primer, F3 and B3, which initiate strand displacement and facilitate the reaction. Additionally, a pair of loop primers, the forward loop primer (LF) and the reverse loop primer (LB), can be designed to bind to the loop structures formed during amplification, thereby accelerating the reaction [8]. Amplification products can be detected by agarose gel electrophoresis, fluorescence using real-time instruments, or endpoint analysis using turbidity or colorimetric indicators [9].

LAMP has several advantages: The isothermal format eliminates the need for a thermocycler and allows results in less than one hour, while its polymerase and primer architecture confer higher tolerance to matrix inhibitors, which is advantageous for on-site or low-tech screening in HACCP workflows [8,10,11]. It has been successfully applied to detect nucleic acids from a wide range of organisms—bacteria, viruses, animals, and plants—using both real-time and endpoint formats [11,12]. LAMP has shown robust performance in complex food matrices, with sensitivity comparable to qPCR in plant-derived allergens such as soybean and hazelnut, while offering a simpler and faster workflow suitable for routine screening [13–16]. Moreover, portable and integrated LAMP devices are increasingly being developed to enable simple and fast nucleic acid testing that can be implemented directly in field or industrial environments [17,18]. These features make LAMP highly suitable for rapid and field-deployable allergen detection.

Despite these advantages, LAMP also presents some limitations. Primer design is more complex than in PCR or qPCR and requires the identification of suitable target regions that allow the design of multiple primers while ensuring species-specific discrimination. Additionally, analyzing LAMP products by gel electrophoresis carries a high risk of cross-contamination due to the large quantities of amplified DNA generated [19]. However, this issue can be mitigated through closed-tube detection strategies including the pre-reaction incorporation of colorimetric indicators such as phenol red or hydroxy naphthol blue [20]. Another limitation is that multiplexing is less straightforward than in qPCR,

although duplex or multi-allergen LAMP formats are starting to be explored for food allergens [16,21].

Several DNA-based real-time PCR assays and commercial kits are currently available for lupin detection in foods, including methods validated for quantification in cereal-based matrices and for the identification of trace contamination in processed products [22,23]. These qPCR approaches offer excellent sensitivity and specificity, but they still depend on thermocycling platforms and laboratory infrastructure, which can limit their implementation for rapid screening or on-site controls along complex production chains. Nevertheless, LAMP assays specifically optimized and fully validated for lupin detection in complex food matrices remain scarce. Therefore, there is a clear need for a rapid, robust and easily transferable lupin-targeted LAMP assay suitable for routine food-control workflows. In this study, we developed and evaluated a LAMP assay for the rapid, sensitive, and specific detection of lupin DNA in food products. Primer design was addressed targeting both nuclear and chloroplast sequences, and assay performance was assessed in terms of sensitivity, specificity, and applicability to commercially processed food products.

2. Materials and Methods

2.1. Plant Material and Commercial Samples

Seeds of *Lupinus albus* L. var. Orden Dorado, supplied by CICYTEX (Finca La Orden, Badajoz, Spain), were used in this study.

Genomic DNA from several plant species commonly used as food ingredients (Table 1) was extracted to evaluate the specificity of the primers. In addition, a variety of processed foods, including cereal bars, chocolate, cookies, bread, flour, muffins, and vegetable burgers, were purchased from local markets. These products had labels declaring the possible presence of lupin or other legumes and were used to evaluate the performance of the assay in real food matrices.

Table 1. Plant species used to assess specificity.

Common Name	Scientific Name
Peanut	*Arachis hypogaea*
Soya	*Glycine max*
Chickpea	*Cicer arietinum*
Pea	*Pisum sativum*
Green bean	*Phaseolus vulgaris*
Lentil	*Lens culinaris*
Pistachio	*Pistacia vera*
Cashew	*Anacardium occidentale*
Hazelnut	*Corylus avellana*
Almond	*Prunus dulcis*
Chestnut	*Castanea sativa*
Walnut	*Juglans regia*
Wheat	*Triticum spelta*

2.2. Samples

Binary mixtures were prepared by spiking known amounts of lupin flour into spelt wheat (*Triticum spelta* L.) flour to obtain the following concentrations: 100,000 mg/kg, 10,000 mg/kg, 1000 mg/kg, 100 mg/kg, 10 mg/kg, 1 mg/kg, and 0.1 mg/kg. The first mixture (5 g lupin flour + 45 g spelt flour) was homogenized using a food processor to obtain a total of 50 g, followed by 10-fold serial dilutions.

2.3. Primer Design

Two nuclear regions, Lup a 1 allergen-encoding gene (single-copy gene) and the multicopy intergenic spacer (IGS), and two chloroplast markers (*psbA-trnH* and *matK*) were selected. Sequences were retrieved from the Allergome and GenBank databases and analyzed using the nucleotide Basic Local Alignment Search Tool (BLASTn), available online at the NCBI website (accessed on 10 September 2023), to identify homologous regions. Sequences of each target region showing >80% identity with lupin were aligned with ClustalW using MEGA-11 [24] to identify regions of interspecific variability. PCR and sequencing confirmed that the sequences from our plant material matched the database entries.

LAMP primer sets were designed using the New England Biolabs LAMP Primer Design Tool, https://lamp.neb.com/#!/, (accessed on 1 October 2023) and PrimerExplorer V5, https://primerexplorer.eiken.co.jp/lampv5e/index.html (accessed 1 October 2023). From the sets generated, those with optimal thermodynamic parameters were selected (Table 2). Special attention was paid to the terminal stability of primers involved in initiating DNA synthesis, namely the 3′ ends of primers LF, LB, F2, B2, F3, and B3, and the 5′ ends of primers F1c and B1c, which later become the 3′ ends of the synthesized F1 and B1 primers. Terminal stability was evaluated using Gibbs free energy change (ΔG), and only those primers with values equal to or lower than −4 kcal/mol were retained, as this threshold ensures efficient and stable primer annealing. To minimize the risk of secondary structure formation, sets with less negative dimer ΔG values were preferred. Furthermore, at least one loop primer (LF or LB) was required in each set to accelerate the amplification process by introducing additional priming sites during the reaction [25].

Table 2. DNA sequence of primers and ΔG values.

Primer Sets	Primers		Sequence (5′ → 3′)	ΔG5′	ΔG3′
Lup a 1_P48L3 (194 pb) Dimer ΔG: −1.52	F3		GAGAGTGTCAAGGGAACAG	−4.59	−4.51
	B3		AGGTGAGAGAGATATCCAAAT	−5.25	−4.16
	FIP	F1c	GACCAGATTGAGAGGGTTTGTCTT	−5.35	−4.41
		F2	ATCCAAGAATTGACAAAATATGCC	−4.41	−4.98
	BIP	B1c	AGCAATGAGCCCATATATTCAAACA	−4.79	−4.33
		B2	CCTGAACTTGAGGGTTTCT	−4.86	−4.01
	LB		GGAAACTTCTATGAGATCACCCCAG	−4.62	−6.29
Lup a 1_P1L9 (194 pb) Dimer ΔG: −1.75	F3		CATTCATTATTTTGGGATTGCA	−4.07	−4.96
	B3		AGTAGCCAGGATTGTTGATG	−4.81	−4.07
	FIP	F1c	CGCCATACTCAAGGTTATATGCT	−6.57	−4.37
		F2	GTAGAGCCACAATCACGAT	−3.92	−5.23
	BIP	B1c	CAGAATCCCAGCTGGCTCAAC	−3.90	−4.51
		B2	GGTATTGCGAGCTTGACTAC	−3.73	−4.08
	LB		CTTAACCCGGATGACAACCAGAAG	−3.34	−4.35
IGS_P68L2 (234 pb) Dimer ΔG: −1.07	F3		CGGGTCACACTAGAAGCCT	−6.63	−5.58
	B3		GGGCATATCCTTAGCACGAA	−6.24	−5.35
	FIP	F1c	GTCGCCTTGTCCCTCGGAAAAG	−7.03	−3.62
		F2	TGCCAACAGCTACCCATCT	−5.92	−4.74

Table 2. *Cont.*

Primer Sets	Primers		Sequence (5′ → 3′)	ΔG5′	ΔG3′
IGS_P68L2 (234 pb) Dimer ΔG: −1.07	BIP	B1c	GTCAAACACGGCCGCACCTT	−4.18	−5.00
		B2	GGGCAAGTTCCCAATCATCC	−6.36	−4.76
	LB		CCGCCCATGGTGCACTT	−7.97	−5.40
IGS_P44L1 (230 pb) Dimer ΔG: −2.36	F3		ACGGAAACCCCAAGGAGA	−5.69	−4.94
	B3		CTCACAGTGCCGAGTTGG	−4.91	−5.00
	FIP	F1c	GGATTGTGACTAGGTGCAGCCT	−4.51	−6.08
		F2	GCGTGGACAGAATTACCCG	−7.18	−5.86
	BIP	B1c	GGCTTAAGCTAGCGCCACATCC	−4.93	−4.90
		B2	TCGGACGTTCCTGGGATT	−6.04	−4.85
	LB		CCTTCCATAAATAACGGGTCACACA	−5.30	−5.22

2.4. DNA Extraction and PCR

DNA was extracted from 75 mg of each sample, either raw flour or processed food, using the SPEEDTOOLS FOOD DNA Extraction Kit (Biotools, B & M Labs, S.A. Madrid, Spain), with slight protocol modifications to improve yield. Briefly, the lysis buffer volume was increased to 650 µL, samples were homogenized by vortexing for 5 min (instead of 15 s), and the lysate was centrifuged for 12 min (instead of 10 min). The final elution step was also modified and performed twice by adding 50 µL of BCE buffer preheated to 65 °C, incubating for 5 min at room temperature, and centrifuging at 11,000 rpm for 1 min after each elution, yielding a total volume of 100 µL. All remaining steps were carried out according to the original protocol. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Purity ratios (A260/280 and A260/230) were used to assess sample quality, and integrity was checked by agarose gel electrophoresis using 0.8% agarose in 0.5× TBE buffer. All extractions were performed in triplicate.

Conventional PCR reactions were set up in a final volume of 20 µL using 25 ng of template DNA, 250 nM of each primer, and 1× FastStar PCR Master Mix (Biotools, B & M Labs, S.A. Madrid, Spain). Amplification was performed in a SensoQuest LabCycler (Progen Scientific Ltd., London, UK) with an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 3 min. The sequences of the primers used for conventional PCR are provided in Supplementary Table S1.

2.5. LAMP Assay Conditions

LAMP reactions were carried out in 15 µL final volumes using either purified DNA from raw materials or synthetic double-stranded DNA fragments (Bio Basic Inc., Markham, ON, Canada) corresponding to the target sequences. Prior to isothermal amplification, DNA was denatured at 95 °C for 5 min to enhance reaction efficiency.

Each reaction mixture contained Bst 2.0 WarmStart® DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA) at a final concentration of 0.32 U/µL, dNTPs at 1.4 mM, $MgSO_4$ at 8 mM, and primers at optimized concentrations (1.6 µM for FIP and BIP, 0.2 µM for F3 and B3, and 0.4 µM for LF and/or LB) and 5 µL of DNA template at variable concentrations.

To evaluate primer performance, lupin genomic DNA from raw samples was first tested in a 10-fold serial dilution series ranging from 5 ng/µL to 0.0005 ng/µL. This dilution panel was used to compare amplification across the different primer sets. For

analytical sensitivity and a more accurate determination of the LOD of the selected primer set, additional intermediate dilutions close to the expected threshold (0.025 ng/µL and 0.0025 ng/µL) were also analyzed. Extracts from binary mixtures and commercial products were used at a 1:2 dilution. The reaction temperature was tested at 60 °C, 63 °C, and 65 °C to determine the optimal conditions for amplification. Primer sets that successfully amplified the target DNA under these conditions were selected for further analysis.

For sensitivity analyses, including genomic DNA dilutions and spiked samples, a minimum of 12 replicates per condition were included, combining independent DNA extractions and technical replicates. Specificity testing against non-target plant species, as well as commercial food product testing, was performed in at least six replicates per sample, always including a lupin positive control in the same run. In every LAMP run, 2–4 non-template controls (NTCs) were included to confirm the absence of contamination.

2.6. Detection of LAMP Products

LAMP amplification was assessed using three different detection methods. Agarose gel electrophoresis was employed as a conventional endpoint approach to visualize the characteristic ladder-like pattern of LAMP products. Samples were run on 1.8% agarose gels stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA) in TBE buffer.

To monitor amplification in real time, reactions included 1× LAMP Fluorescent Dye (New England Biolabs Inc., Ipswich, MA, USA) and were carried out using a CFX Duet Real-Time PCR instrument (Bio-Rad, Hercules, CA, USA). The LAMP reaction proceeded at 65 °C for 40 min, with fluorescence measurements recorded every minute. A final step at 85 °C for 5 min was applied to inactivate the polymerase, and product specificity was confirmed through melting curve analysis. For real-time fluorescence monitoring, positive reactions were defined by sigmoidal amplification curves crossing the fluorescence threshold (time-to-positivity, analogous to Ct in qPCR) and were further confirmed by melting curve analysis showing identical dissociation profiles among positives.

Colorimetric detection was performed using the WarmStart® Colorimetric LAMP 2× Master Mix with UDG (New England Biolabs Inc., Ipswich, MA, USA), which includes the pH-sensitive dye phenol red. Positive reactions were indicated by a clearly visible color change from pink to yellow, as observed by the naked eye, and were verified by agarose gel electrophoresis, showing the expected LAMP ladder pattern. These reactions were incubated under the same temperature and time conditions as the real-time assays to ensure comparability.

3. Results

3.1. Primer Design and Amplification Performance

To develop a specific LAMP-based method for the detection of lupin DNA, four genomic regions were evaluated as potential targets: the Lup a 1 allergen-encoding gene, a nuclear intergenic spacer (IGS), and two chloroplast regions (intergenic *psbA-trnH* and *matK*). The suitability of each region for optimal primer design was assessed based on two main criteria: the ability to meet the thermodynamic requirements necessary for efficient LAMP primer design, and the presence of sufficient sequence variability to avoid non-specific amplification in phylogenetically related species. This dual assessment ensured that selected targets supported both efficient amplification and high specificity for lupin DNA while avoiding false positives.

For Lup a 1, the coding sequence available in the Allergome database was used to locate and retrieve the full gene sequence from the *Lupinus albus* genome. Homologous sequences were identified using BLAST and the region of the gene showing the highest

interspecific variability was selected for primer design. Two primer sets, P1L9 and P48L3, were generated from this region, each including the four core primers (F3, B3, FIP, and BIP) and one loop primer (Table 2).

For the intergenic spacer region ETS/IGS, the GenBank sequence GU574606 was used to retrieve an extended flanking region (1 kbp) to explore suitable primer binding sites. Comparison of this region against sequences in public databases using BLAST revealed a high degree of variability; specifically, no sequences were found with a query coverage greater than 50% and sequence identity above 85%. Two primer sets were selected from this region, named P44L1 and P68L2 (Table 2).

In contrast, chloroplast regions proved to be less suitable. Although the *psbA-trnH* intergenic region showed high variability, the sequence was not appropriate for designing primers that met the required thermodynamic conditions. The *matK* gene showed low sequence variability, and no primer sets with sufficient specificity for lupin were identified.

The performance of the P1L9 and P48L3 primer sets targeting Lup a 1 allergen-encoding gene was evaluated using a synthetic DNA fragment containing both target sequences. Reactions were tested under various conditions including three different temperatures (60 °C, 63 °C, and 65 °C), two magnesium concentrations (4 mM and 8 mM), and with or without the addition of betaine (0.8 M). An initial DNA denaturation step was included in some experiments. The best amplification results were obtained at 63 °C with 8 mM $MgSO_4$ and previous DNA denaturation. The presence of betaine did not affect the outcome. Each primer set generated amplification curves in real-time fluorescence detection (Figure 1). Both primer sets generated sigmoidal amplification curves in real-time fluorescence detection and the melting curve analysis revealed identical dissociation profiles, confirming the specificity of the amplified products (Figure 1). For P48L3, fluorescence crossed the threshold, indicating the start of the exponential amplification phase, at approximately minute 20 and allowed detection down to 50 fg of synthetic DNA. In contrast, P1L9 showed a lower detection time, with fluorescence exceeding the threshold at around minute 10, detecting down to 5 fg. Given its higher amplification efficiency and earlier signal onset, P1L9 was selected for further validation.

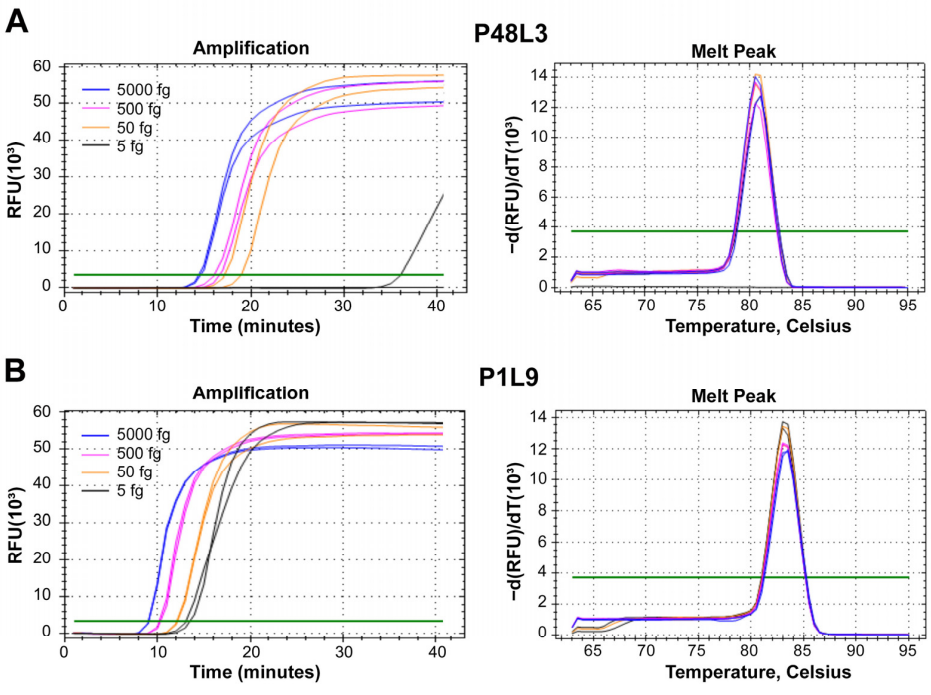

Figure 1. Real-time LAMP amplification of a synthetic DNA fragment using Lup a 1-targeting primer set P48L3 (**A**) and P1L9 (**B**). Reactions were carried out at 63 °C with 8 mM $MgSO_4$, without betaine.

Amplification curves (relative fluorescence units (RFU) versus cycles) and melting profiles are shown for 10-fold serial dilutions of lupin DNA. The green line represents the fluorescence threshold (threshold line).

Primer sets targeting the ETS/IGS region were evaluated using genomic lupin DNA. Although these sets met the thermodynamic requirements for LAMP, none produced consistent or specific amplification signals under the tested conditions, indicating that this genomic region was unsuitable for LAMP-based detection of lupin (Figure 2).

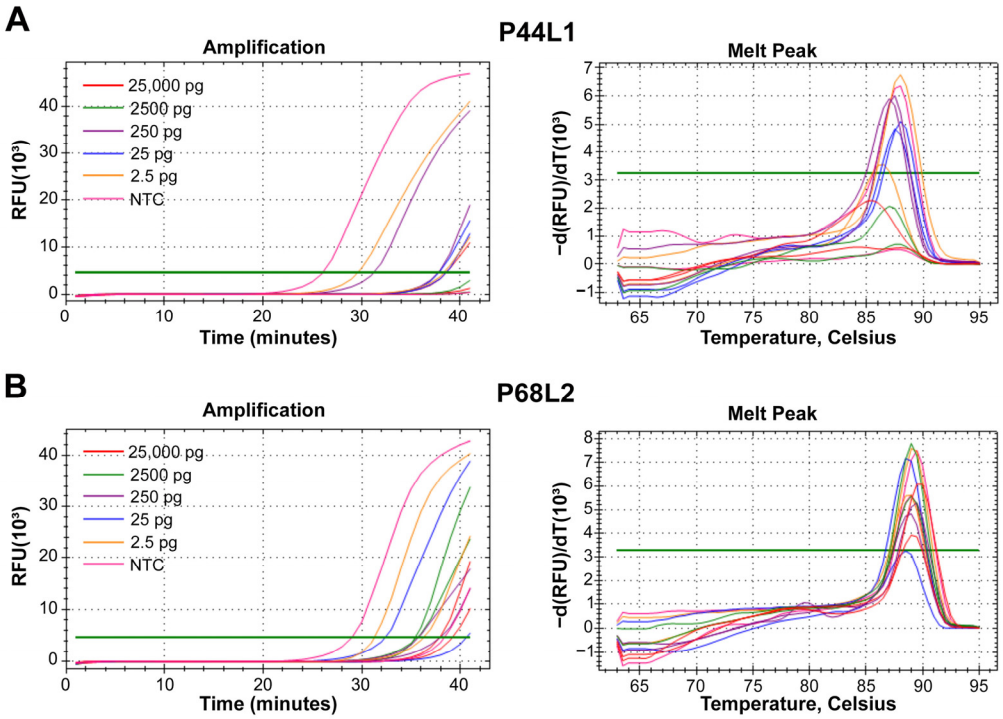

Figure 2. Real-time LAMP amplification of genomic lupin DNA using primer sets designed for the intergenic spacer ETS/IGS region: P44L1 (**A**) and P68L2 (**B**). Reactions were carried out at 63 °C with 8 mM $MgSO_4$, without betaine. Amplification curves (relative fluorescence units (RFU) versus cycles) and melting profiles are shown for 10-fold serial dilutions of lupin DNA. The green line represents the fluorescence threshold (threshold line).

3.2. Specificity and Sensitivity of the LAMP Assay

The P1L9 primer set, which demonstrated the most favorable amplification dynamics, was selected to further evaluate the assay's sensitivity and specificity. The specificity was confirmed using genomic DNA from various species (Table 1). Only lupin DNA yielded amplification, and no false positives were observed from related species, supporting the assay's specificity.

To evaluate sensitivity, *L. albus* genomic DNA was tested in a 10-fold serial dilution series (5 ng/µL–0.5 pg/µL), and additional intermediate dilutions near the detection threshold (25 and 2.5 pg/µL) were also included, covering a range of target DNA from 25 ng to 2.5 pg per reaction. The limit of detection (LOD) was defined as the lowest amount of target DNA that yielded positive amplification in at least 95% of replicates [26]. Reactions were assessed by agarose gel electrophoresis, real-time fluorescence, and visual colorimetric readout. For real-time LAMP reactions with P1L9, the lowest amount of *L. albus* DNA that met this criterion was 25 pg per reaction, with all replicates positive (16/16), whereas amplification at 12.5 pg was sporadic (40% positive reactions) and therefore did not meet the LOD criterion (Figure 3A). Using the same dilution panel for direct comparison, the reproducible detection limit in the colorimetric format was 125 pg, since results at 25 pg

were inconsistently positive (50% of replicates) (Figure 3B). No amplification was observed in non-template controls or in DNA from non-target plant species, confirming the assay's specificity and reliability.

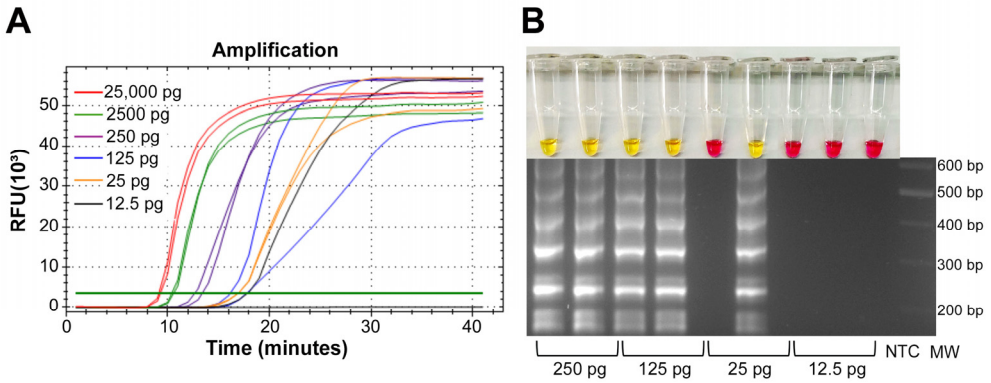

Figure 3. LAMP detection of lupin genomic DNA using the P1L9 primer set. (**A**) Real-time fluorescence amplification curves obtained at 63 °C for decreasing amounts of lupin genomic DNA (250 pg to 12.5 pg per reaction). (**B**) Colorimetric LAMP reactions incubated at 63 °C and the corresponding agarose gel electrophoresis for a subset of DNA amounts (250, 125, 25, and 12.5 pg per reaction), showing the pink (negative) to yellow (positive) color change. RFU, relative fluorescence units; NTC, non-template control; MW, 100 bp weight marker. The green line represents the fluorescence threshold (threshold line).

To evaluate the assay's potential for detecting lupin in complex food matrices, binary mixtures of lupin flour in spelt wheat flour were prepared with known concentrations ranging from 100,000 mg/kg to 0.1 mg/kg. The performance of the P1L9 primer set was assessed across this range using endpoint agarose gel electrophoresis, real-time fluorescence, and colorimetric detection. All three detection methods showed consistent amplification (18/18) down to 10 mg/kg of lupin flour, establishing this value as the relative limit of detection (LOD) for the assay in binary mixtures (Figure 4). Notably, amplification was also observed at 5 mg/kg (14/18), although this level did not meet the criteria to be defined as the LOD, the lowest concentration detected in ≥95% of replicates.

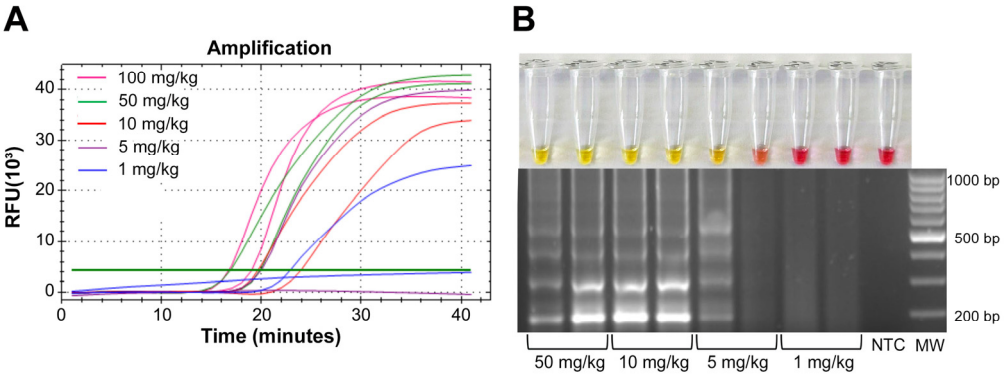

Figure 4. LAMP detection of lupin in binary mixtures using the P1L9 primer set. (**A**) Real-time fluorescence amplification curves obtained at 63 °C for binary mixtures containing 100 to 1 mg/kg lupin flour (*w*/*w*). (**B**) Colorimetric LAMP reactions incubated at 63 °C, showing the pink (negative) to yellow (positive) color change, and the corresponding agarose gel electrophoresis for a subset of samples (50 to 1 mg/kg). RFU, relative fluorescence units; NTC, non-template control; MW, 100 bp weight marker. The green line represents the fluorescence threshold (threshold line).

3.3. Applicability to Commercial Food Products

To evaluate the practical utility of the developed method, a set of commercial food products, including baked goods, flour blends, and plant-based preparations, were tested using both real-time and colorimetric LAMP. DNA from commercial food samples yielded the expected 18S PCR amplicon prior to LAMP analysis, indicating adequate DNA quality and absence of relevant matrix inhibition. Products were selected based on their labeling, which either declared lupin as an ingredient, indicated possible trace presence, or made no reference to lupin. The results, summarized in Table 3, were compared with the information provided on product labels.

Table 3. Detection of lupin in commercial food products using the LAMP assay with the P1L9 primer set. Each product was tested using both real-time fluorescence and colorimetric detection. Results are expressed as success rate (%) and, in parentheses, the number of positive replicates out of the total reactions (n = 6); ND: Not Detected. The label declaration for each product regarding lupin or other legume content is included for comparison.

Commercial Food	Label Declaration	Fluorescence	Colorimetric
Lupin snack	Lupin (38%), Beans (37%), soya	100% (6/6)	100% (6/6)
Muffin	Lupin (10%)	100% (6/6)	100% (6/6)
Bread	Soya, lupin traces	50% (3/6)	ND (0/6)
Chocolate with hazelnut	Hazelnut, soya	ND (0/6)	ND (0/6)
Crackers	Soya	ND (0/6)	ND (0/6)
Chocolate with pistachio	Pistachio, Almond, soya, Hazelnut	ND (0/6)	ND (0/6)
Chocolate	Almond, Hazelnut and soya traces	ND (0/6)	ND (0/6)
María cookies	May content soya traces	ND (0/6)	ND (0/6)
Wheat flour	May content soya traces	ND (0/6)	ND (0/6)
Rice flour	May content soya, tree nuts traces	ND (0/6)	ND (0/6)
Buckwheat flour	May content soya, tree nuts traces	83% (5/6)	50% (3/6)
Cereal Bar II	Almond and tree nuts	ND (0/6)	ND (0/5)
Chickpea flour	Not declared	67% (4/6)	50% (3/6)
Corn flour	Not declared	33% (2/6)	50% (3/6)
Rice and lentil pancake	Not declared	67% (4/6)	50% (3/6)

Samples explicitly containing lupin (muffins and lupin snacks) yielded positive results in all replicates across both detection formats. The product labeled as "may contain traces of lupin" tested positive in some replicates only under fluorescence detection. A subset of products not declaring lupin content showed variable outcomes, with occasional positive reactions observed in both real-time and colorimetric assays, suggesting potential undeclared contamination or cross-contact during production.

4. Discussion

The reliable detection of hidden traces of allergens is essential to ensure food safety and consumer protection. In this context, lupin is among the allergenic species that must be declared on food labels under Regulation (EU) No. 1169/2011 [5]. Conventional PCR-based molecular methods, while highly sensitive and specific, are time-consuming, labor-intensive, and require specialized laboratory infrastructure. Although LAMP has emerged as a simpler and faster alternative to PCR, its main challenge lies in assay optimization. The identification of specific genomic regions suitable for primer design, together with the evaluation of multiple primer sets to determine those with the best amplification performance, are crucial steps to ensure assay reliability requirements [27]. Nevertheless, once appropriate primer sets are identified and validated, the method can be adapted

to straightforward visual detection approaches, such as colorimetry, which facilitates its transferability to routine analyses.

In this study, we carried out the process of target selection and primer evaluation, leading to the establishment of an optimized LAMP assay for the rapid and reliable detection of lupin in food products. Four candidate genomic regions were evaluated in this work. Chloroplast sequences (*psbA-trnH* and *matK*), frequently used for plant species identification [28,29], were found to be unsuitable due to high conservation or thermodynamic constraints that prevented functional primer design. Similarly, the nuclear intergenic spacer (IGS) region did not yield consistent or specific amplification under the tested conditions. In contrast, the gene encoding the Lup a 1 allergen offered the optimal balance between appropriate regions for stable primer annealing, and interspecific variability, which ensured specificity. Among the two primer sets designed for this target, P1L9 displayed superior amplification kinetics and higher sensitivity and was therefore selected for full method validation. The assay demonstrated high specificity, with amplification exclusively for lupin DNA and no cross-reactivity with closely related legumes or tree nuts, confirming the suitability of Lup a 1 allergen-encoding gene as a species-specific target.

In terms of sensitivity, the assay consistently detected as little as 25 pg of lupin DNA and achieved reliable detection in binary mixtures down to 10 mg/kg of lupin flour, which was established as the relative limit of detection. To our knowledge, no previous LAMP-based assays for lupin detection have been reported. Comparable studies on other legumes support our findings: for instance, in soybean, targeting the multicopy ORF160b gene achieved the same limit of detection [13]. In comparison with previously published real-time PCR assays for lupin detection, the absolute sensitivity obtained here (25 pg) is somewhat lower than the 1 pg limit reported [30]. However, the relative LOD in binary model mixtures containing known amounts of *L. albus* flour (10 mg/kg) is similar to that reported for qPCR assays [31]. Multicopy nuclear (ITS1) or organellar targets have reached 0.1–2 mg/kg sensitivity [31–34], whereas single-copy nuclear targets typically show one order of magnitude lower sensitivity (10 mg/kg) [35]. Notably, Villa et al. [30] achieved a detection limit of 5 mg/kg of lupin in rice flour by targeting the gene encoding the Lup a 4 allergen. In addition, ELISA methods developed for lupin detection generally report LODs in the low mg/kg range, often below 10 mg/kg in different food matrices [36]. Taken together, these data indicate that the relative LOD obtained here falls within the sensitivity range of both qPCR- and ELISA-based screening methods. Our results confirm that equivalent sensitivity can also be achieved through LAMP, even with colorimetric detection, offering a faster and more cost-effective alternative to qPCR for routine allergen monitoring. Based on the published reference dose of 2.6 mg of lupin protein [37,38], a level below which most lupin-allergic individuals are unlikely to react, a sensitive method must detect amounts corresponding to approximately 65 mg of lupin flour per kilogram of food (assuming 40% protein content) [39]. This confirms the method is sufficiently sensitive for food safety applications, while offering a faster and more cost-effective alternative to qPCR for routine allergen monitoring, particularly when using the colorimetric LAMP format.

The robustness of the assay was confirmed by its successful performance across a variety of processed foods with complex matrices rich in carbohydrates, proteins, and fats. Lupin was consistently detected in all products declaring its presence on the label, confirming the reliability of the assay in varied matrices. These results support previous reports that LAMP is less susceptible to inhibition by food-derived compounds [17], confirming its suitability for allergen detection in complex processed foods. Although inhibition was excluded by successful amplification of an 18S rRNA fragment by conventional PCR in all commercial food extracts, it would be of interest in future work to compare the performance of the LAMP assay with a reference qPCR method using the same set of samples.

In a few products declaring only traces, lupin was also detected, although additional replicates would be required to confirm low-level cross-contamination close to or below the 95% detection threshold. In such matrices, the DNA extraction step may still require matrix-specific optimization, and this should be taken into account when extending the assay to new product categories. In conclusion, the optimized LAMP assay targeting the gene encoding the Lup a 1 allergen provides a rapid and specific alternative to PCR-based methods, with sensitivity compatible with food safety thresholds and applicability across diverse commercial products. The real-time and colorimetric formats make the assay suitable both for implementation in routine laboratory workflows and for scaled-up screening in industrial settings, and future work may focus on adapting the protocol to additional food matrices and integrating it into HACCP-based allergen control programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/allergies6010001/s1, Table S1. Primers used for conventional PCR assays in this study.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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