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Analytical Methods

A real-time PCR method for the detection and quantification of lupin flour in wheat flour-based matrices

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ABSTRACT

Lupin flour is growingly being used in bakery products, mainly as a soybean protein substitute. The aim of the present work was to detect and quantify the presence of lupin flour in wheat-based foods using a newly set up qPCR system based on SYBR green. Although DNA sequence information for lupin is scarce, it has been possible to design a primer pair highly specific for the target gene and devoid of any primer-dimers amplification capacity. Lupin flour revealed to be a difficult matrix, since large amounts of compounds tend to co-purify with DNA, even adopting well established extraction protocols. Nonetheless, the primers used allowed to reach high PCR efficiencies and did not show any cross-reactivity with DNAs extracted from various plant and animal foods. The sensitivity achieved was 7 pg of lupin DNA, corresponding to a percentage of less than 0.1% of lupin flour in the foods.

1. Introduction

Lupin seeds have been eaten by humans since ancient times. White lupin (*Lupinus albus* L.) is a leguminous grain crop which represents an important source of proteins for human nutrition, since its seed is one of the richest in protein content (up to 44% on dry weight basis), with a biological value of 91% relative to egg proteins (Egaña, Uauy, Cassorla, Barrera, & Yañez, 1992). Other cultivated lupin species are the blue lupin (*L. angustifolius*), and the yellow lupins (*L. luteus*), mainly used for feed. Notably, in lupin seed the content of some antinutritional compounds, including lectins, hydrolase inhibitors, saponins and antimetabolites is lower than in soybeans and other grain legumes (Scarafoni, Magni, & Duranti, 2007).

Lupin flours are used in bakery products and pasta (Dervas, Doxastakis, Hadjisavva-Zinoviadi, & Triantafillakos, 1999; Pollard, Stoddart, Popineau, Wrigley, & MacRitchie, 2002; Sironi, Sessa, & Duranti, 2005), as well as a soybean substitute in sauces and other food preparations (Bez, Schott, & Seger, 2005; Dervas et al., 1999). Various studies have shown that lupin flour can successfully be incorporated into products, at up to 20% inclusion, to produce foods that rate higher than the controls, in terms of colour, texture, taste and overall acceptability (Doxastakis, Zafiriadis, Irakli, Marlani, & Tananaki, 2002; Pompei, Lucisano, & Ballini, 1985). A number of pasta products containing lupin flour are available on the European market (Capraro, Magni, Fontanesi, Budelli, & Duranti, 2008; Dervas et al., 1999; Holden, Moen, Sletten, & Dooper, 2007). As lupin flour does not contain gluten, it is sometimes used as functional ingredient in gluten-free foods (Sironi et al., 2005). However, allergic reactions to lupin have been reported in peanut-allergic individuals, with a cross-reactivity rate to lupin flour in peanut-allergic individuals of around 30% (Moneret-Vautrin et al., 1999; Magni et al., 2005; Parisot, Aparicio, Moneret-Vautrin, & Guerin, 2001). For this reason, lupin seeds and products thereof have been recently included in the Annex IIIa of Directive 2000/13/EC (Directive 2006/142/EC), which lists the ingredients which must under all circumstances appear on the labelling of foodstuffs.

Modern food safety criteria require very accurate food control. The Regulation (EC) number 178/2002 defines traceability as "the ability to trace and follow a food, feed, food-producing animal or substances intended to be, or expected to be incorporated into a food or feed, through all stages of production and distribution". Moreover, quantitative assays are required for the correctness of labelling procedures and prevention of food adulterations and contaminations. In this frame, and in view of an even more extensive use of lupin flour as food ingredients in the near future (Scarafoni et al., 2007; Sironi et al., 2005), it is crucial to set up and optimise methods aimed at tracing its presence in food formulations.

The possibility to trace a food or an ingredient is based on the peculiar properties of one or a few molecules, which are clearly and unambiguously distinguishable from the several other food components. DNA is the macromolecule that fulfils this requirement. Real-time PCR is the technique of choice for nucleic acid quantification because it offers the most timely, sensitive, and practical way of meeting new detection standards (Engel, Moreano,

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Ehlert, & Busch, 2006). However, PCR can negatively be influenced either by the loss of DNA structural integrity, and by the possible presence of inhibitors. DNA of food ingredients undergoes physical and chemical injuries due to processing, which can damage DNA through depurination, cross-linking, and hydrolysis. DNA damage may also occur during the extraction procedure through oxidation reactions and mechanical shearing (Cankar, Štebih, Dreo, Žel, & Gruden, 2006; Terry, Harris, & Parkes, 2002). Several compounds which may co-purify from the food matrix, such as polysaccharides and polyphenols can reduce the overall efficiency of PCR since they may act as potent inhibitors of DNA-polymerases (Bickley & Hopkins, 1999). Most of these contaminant molecules can influence the correct quantification of the DNA solutions used as template in PCR reactions (Wilkie, Issac, & Slater, 1993). Amongst the other factors of inconvenience reminded above, it is well known that the method adopted for the extraction of DNA from food samples is of critical importance to yield high quality DNA (Terry et al., 2002), since the use of poor quality DNA may lead to impaired or completely failed amplification reactions, reducing the sensitivity of the method or even making target sequences undetectable (Cankar et al., 2006; Engel & Moreano, 2003).

Currently, there is an increasing interest in developing DNA-based detection methods for foods that may contain allergens, as supplement or an alternative to less sensitive immunologically based methods. The aim of the present work was to assess a polymerase chain reaction-based analytical approach as a tool to detect and quantify the presence of lupin flour in wheat-based foods.

2. Materials and methods

2.1. General

Lupinus albus L. seeds of the sweet Multitalia, Ares and Lux varieties were kindly provided by Dr. A. Conocchiari (Agroservice S.p.A., S. Severino Marche, Italy) and Dr. A. Seger (Terrena Lup'Ingredients, Martigne Ferchaud, France). *Lupinus luteus* seeds were provided by Dr. C. Bagger, Bioraf, Aakirkeby, Denmark). *Triticum durum* and dehulled *Triticum aestivum* seeds were kindly provided by Prof. A. Pagani and Dr. R. Caramanico (State University of Milan, Italy). *Glycine max, Arachis hypogea, Sesamum indicum* and *Zea mays* seeds, chicken eggs and bovine milk were purchased from local market. Food samples were provided by Dr. J. Bez (Fraunhofer IVV, Freising, Germany) and Dr. A. Seger (Terrena Lup'Ingredients, Martigne Ferchaud, France). The samples were kept at −80 °C in sealed bags until used.

All reagent grade chemicals were from Sigma–Aldrich (Milano, Italy) unless otherwise indicated. Oligonucleotides synthesis and DNA sequencing were performed by Primm s.r.l. (Milano, Italy).

DNA sequences were obtained from EMBL database (available on-line at <www.ebi.ac.uk>) and NCBI nucleotide sequence database (available on-line at <www.ncbi.nlm.nih.gov>).

2.2. DNA purification

DNA was extracted from lupin and soybean leaves by chaotropic solid phase extraction (SPE) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

DNA from all other samples were isolated by SPE using the NucleoSpin Food kit (Macherey-Nagel, Düren, Germany) essentially according to the instructions manual included in the kit, using 100 mg of starting material. Prior to DNA extraction, all the seeds were ground with a coffee mill to pass through a 60 mesh sieve, whereas fresh bovine milk and chicken egg were prepared according to Hermann (2001) and Hermann (2004), respectively.

Lupin and wheat DNA from flours have also been extracted with a standard liquid-phase CTAB-based method essentially according to Roger and Bendih (1985). One millilitre of pre-warmed (65 °C) isolation buffer (100 mM Tris–HCl, pH 8.0, 2% CTAB, 1.4 M NaCl, 1% PVP) and 50 μl of RNAse (10 mg/ml) were added to 200 mg of flour. The suspension was incubated at 65 °C with occasional shaking. After 30 min, 20 μl of Proteinase K (20 mg/ml) were added to the mixture and the sample incubated at 65 °C for another 10 min. After cooling to room temperature, two chloroform/isoamyl alcohol extractions (24:1 v/v) were performed. After the final centrifugation, the upper aqueous phase was transferred to a clean tube and 1 ml of precipitation buffer (25 mM Tris-HCl, pH 8.0, 1% CTAB, 1 mM EDTA) was added, followed by gentle mixing. The pellet was resuspended by adding 250–300 μl of hydration buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA) and incubated at 37 °C until it was completely dissolved. The DNA was then precipitated with 2.5 volumes of −20 °C absolute ethanol. After spinning at 10,000 rpm for 6 min, the pellet was washed with 70% ethanol and air dried. Finally, the DNA was dissolved in 50 μl of TE buffer.

2.3. DNA quantification

DNA was quantified using the DNA Quantitation Kit (Sigma–Aldrich, Milano, Italy) according to the kit instructions. The method employs the fluorescent dye bisbenzimide H 33258, which binds to the minor groove of dsDNA; the results are thus not influenced by the presence of RNA, proteins and other low molecular weight compounds (Labarca & Paigen, 1980; Moe, Garbarsch, & Kirkeby, 1994), allowing correct determinations. Quantified calf thymus DNA ranging in concentration from 5 to 500 ng/ml was used as a standard. Sample extracts were diluted 1:100 in distilled water to reduce interferences from other substances. Assays were performed directly in quartz fluorescence cuvettes (Hellma, Müllheim, Germany) by adding aliquots of standard or unknown DNA solutions to 2 ml of dye solution (0.1 μg/ml bisbenzimide H 33258 dissolved in 100 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA and 2 M NaCl). Fluorescence was measured with a Perkin–Elmer fluorometer (model LS50), using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

DNA concentrations were also estimated by UV absorption spectrophotometry at a wavelength of 260 nm using an Eppendorf Biophotometer, assuming that a solution of 50 μg of dsDNA in 1 ml of water absorbs 1 OD. The ratios OD_{260}/OD_{280} and OD_{260}/OD_{230} have been determined for all preparations.

2.4. End-point PCR

Amplificability of isolated plant DNAs was determined by PCR using primers specific for chloroplast *trnL* (UAA) gene. The amplicon encompasses the entire *trnL* (UAA) intron plus a few base pairs on each side belonging to the *trnL* (UAA) gene itself (Dahinden, von Büren, & Lüthy, 2001). The primers used were 5'-CGAAATCGGTAGACGCTACG-3' (sense) and 5'-GGGGATAGAGGGATTGAAC-3' (antisense) (Dahinden et al., 2001). For milk and egg samples, primers were synthesized according to the sequences indicated by Hermann (2001). PCR was performed in a volume of 25 μl. Each reaction contained 5 μl of template DNA solution (usually 65 ng or less, see Section 3 for details), 1X reaction buffer supplied by the enzyme manufacturer containing 1.5 mM $MgCl_2$, 200 μM of each dNTP (GeHealthcare, Milano, Italy), 0.5 μM each primer. The reaction was performed in a PCR Mastercycler (Eppendorf) device. The PCR program consisted of an initial denaturation at 96 °C for 5 min followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 52 °C for 30 s and polymerisation at 72 °C for 30 s.

2.5. Real-time quantitative PCR (qPCR)

Reactions were carried out in triplicate using an iCycler thermocycler equipped with the MyiQ detection system (Biorad, Milano, Italy). Reactions were set up in a final volume of 20 μl using iQ SYBR green Supermix (Biorad, Milano, Italy) with each primer added to a final concentration of 0.25 μM. For the quantification of food samples, 65 ng of total DNA were used. The cycling conditions were as follows: 1 cycle at 95 °C for 3 min; 40 cycles of 95 °C for 15 s (denaturation), 62 °C for 30 s (annealing and extension). The fluorescence signal was captured at the end of each cycle using the SYBR channel (490 nm excitation and 525 nm emission wavelengths). Melting curves were obtained by progressive heating at 0.3 °C every 15 s. Data were collected and processed, including baselines subtraction and threshold definition, with iQ5 software (Biorad, Milano, Italy).

2.6. Electrophoretic analysis of DNA extracts

DNA samples were analysed on 0.8% agarose gels run in 40 mM Tris–acetate buffer, pH 7.7, containing 1 mM EDTA, in a MiniGel apparatus (Biorad, Milano, Italy), at 70 V. Gels were stained with ethidium bromide and visualised with a Versadoc 4000 Imaging System (Biorad, Milano, Italy).

3. Results and discussion

3.1. Primers design and specificity

To develop the qPCR method, analyses of all *Lupinus spp.* nucleotide sequences available in databases have been carried out one by one with the aid of the primer design software Primer3 (available on the WEB at <http://frodo.wi.mit.edu/cg-bin/primer3/primer3_www.cgi>). The sequence to be amplified was pitched on the *L. albus* CγA32 gene (EMBL accession number: CAC16394), encoding for the seed glycoprotein γ-conglutin (Scarafoni et al., 2001). Proteins homologous to γ-conglutin have been found expressed and characterised in diverse plant species, including legume seeds (Hirano, Kagawa, & Okubo, 1992), wheat, tomato, soybean, corn, rice, (Qin et al., 2003). Their nucleotide sequences are available. The oligonucleotides were selected to specifically anneal only on the CγA32 gene, namely in regions of the coding frame where the nucleotide sequence is different amongst the Cγ homologous genes present in the public databases. Moreover, the primers have been deigned to have identical T_m, to have no more than two G or C nucleotides at the 3' terminus, to be predicted to form no secondary structures; iv) to produce the smallest possible amplicon. The forward primer (5'-ATGGTGTACACCCCTTAACC-3') was named cγ32-5f and anneals at position 1206 to 1227 of the target gene, whereas the antisense primer (5'-GGTATGAAGATGATGATGATGATG-3'), named cγ32-3r, matches between positions 1341 and 1365. The theoretical temperature of melting was 58.4 °C and 58.2 °C, respectively for sense and antisense primers. Potential primer-dimers formation was checked with the Operon Oligo Analysis and Plotting tool (<www.operon.com/oligos/toolkit/php>) and resulted negative.

Specificity of the primers was checked by BLAST searches through the databases. Only *L. albus* γ-conglutin A32 and *L. angustifolius* conglutin γ gene sequences produced alignments with both primers. No other significant similarities were found.

To experimentally test the *in silico* predictions, amplifications by real-time PCR of leaf lupin DNA was performed, using SYBR green I. This molecule fluoresces upon binding to dsDNA, allowing the detection of any products accumulated during amplification, including non-specific reaction products, such as primer-dimers. The evaluation of the dissociation curves makes it however possible to identify specific amplicons from other products, by assessing their own melting temperatures, contrary to other technologies such as Taqman. Other advantage of SYBR green is that specifically labelled probes are not required, thus reducing time assay set-up and running costs (Vanguiler, Vrana, & Freeman, 2008). Recently, a real-time PCR for the detection of lupin DNA in foods based on two primers and a fluorescent-labelled probe has been developed (Demmel, Hupfer, Hampe, Busch, & Engel, 2008).

The final PCR conditions were determined by testing several template DNA quantities, primers concentrations, duration of each PCR step and annealing temperatures. The optimised reaction parameters are those indicated in the Section 2. The post-amplification melting curve (Fig. 1) showed a sole, symmetric and sharp curve, indicating that only one product was accumulated. Primer-dimers products were virtually absent. The T_m of cγ32 amplicon was calculated to be 81 °C. The identity of the PCR product has been checked by nucleic acid sequencing, which confirmed the amplification of the expected region on the template DNA (not shown).

In order to assess the specificity of the selected primer pair, amplifications have been carried out using DNAs extracted from various plant and animal foods (Table 1). The results indicate that no amplification was obtained with non-lupin samples. The list of the selected foods includes the most common ingredients of lupin-containing bakery products (Bez et al., 2005). Soybean is the closest lupin-related plant and is widely used as a food ingredient. Peanut is one of the strongest lupin cross-reacting allergen (Moneret-Vautrin et al., 1999). Sesame and almond are also potent food allergens (Leduc et al., 2006; Holden, Sletten, Lindvik, Faeste, & Dooper, 2008). The three assayed varieties of *L. albus* and the other lupin species produced amplicons with identical dimension and T_m (not shown).

We concluded that the primer pair cγ32-5f/cγ32-3r can reliably be defined specific for lupin DNA.

3.2. DNA purification, quantification and quality

Two purification methods, namely a CTAB-based procedure and a SPE commercial kit, have been used. Quantifications of DNA solutions have been then carried out with the fluorescent dye bisbenzimide H 33258 and allowed us to determine the quantity of DNA

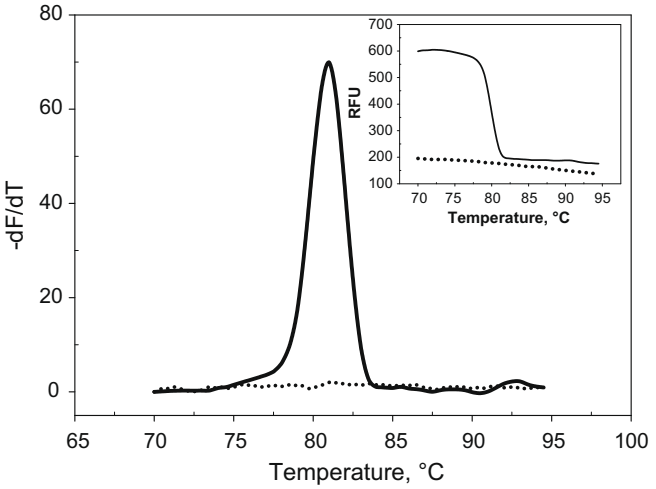

Fig. 1. Post-amplification melting curves of amplicon obtained by real-time PCR using cγ32-5f/cγ32-3r primer pair. Solid line indicates the sample where lupin DNA was used as template, whereas dotted line refers to the wheat DNA sample. In the inset, the course of fluorescence during progressive heating is reported.

Table 1
Specificity of cγ32-f5/cγ32-3r primers pair as experimentally assessed by amplification of plant and animal food ingredient.

Sample		PCR amplification
White lupin	*L. albus* Multitalia	+
White lupin	*L. albus* Ares	+
White lupin	*L. albus* Lux	+
Yellow lupin	*L. luteus*	+
Blue lupin	*L. angustifolius*	+
Soybean	*Glycine max*	–
Peanut	*Arachis hypogea*	–
Almond	*Prunus dulcis*	–
Corn	*Zea mays*	–
Soft wheat	*Triticum aestivum*	–
Durum wheat	*Triticum durum*	–
Sesame	*Sesamum indicum*	–
Chicken egg	*Gallus gallus*	–
Bovine milk	*Bos taurus*	–

Table 2
DNA extraction yields using two purification methods.

	CTAB[a] (ng/mg of flour)		Nucleospin Food[a] (ng/mg of flour)	
	F[b]	OD_{260}	F[b]	OD_{260}
Lupin	6.8 ± 0.4	900.7 ± 124.1	6.3 ± 0.2	950.3 ± 76.4
Wheat	30.5 ± 1.9	222.5 ± 30.7	47.7 ± 1.5	305.4 ± 22.8

[a] Determinations have been carried out in quadruplicate.
[b] Fluorometric determination with bisbenzimide H33258.

actually extractable from each matrix. The results are summarised in Table 2. The DNA yields using the two protocols were essentially the same in the case of lupin, whereas for wheat they were slightly higher if the SPE protocol was used. All considered, for the subsequent experimentation, DNA purifications from flours and food samples were performed using the NucleoSpin Food kit.

A remarkable difference of the DNA quantities obtainable from the two flours is evident. It means that, given an equal quantity of lupin or wheat flours, the amount of yielded DNA is intrinsically different. This remark is important when absolute quantifications have to be carried out. The issue has been taken into account and discussed in the qPCR approach described hereafter. The OD_{260} readings give an indirect indication on the presence and amounts of co-purified substances. In our case, the spectrophotometric determinations (Table 2) indicate a remarkable overestimation of the DNA's quantity extracted from the lupin matrix, strongly suggesting the presence of substances other than dsDNA. The OD_{260}/OD_{280} ratio was routinely 1.5–1.6, whereas OD_{260}/OD_{230} was around 1.8 (not shown). One of the contaminants in lupin flour sample was certainly RNA, as indicated by gel electrophoresis (Fig. 2A), but the presence of other compounds can not be excluded, as also suggested by the amplificability trials (see below). As far as wheat flour concerns, both the two purification protocols have been previously applied for real-time PCR quantifications (Olexová, Dovičovičová, & Kuchta, 2004; Terzi, Malnati, Barbanera, Stanca, & Faccioli, 2003). In our case the OD_{260} indicates a small presence of co-purified compounds which, however, did not affect the amplificability of the DNA. In Fig. 2B the results concerning the quantification of DNAs from the two flours samples (0% for wheat and 100% for lupin) are plotted along with those obtained from mixed flours (10%, 20% and 50% of lupin). The fluorimetric quantifications are indicated by the black triangles. The total amounts of dsDNA extracted from each mix coincided with the theoretical expected quantities. The amounts of co-purified substances, appraised by the ODs_{260}, are directly proportional to the lupin flour content (Fig. 2B, circles). The different chemical nature of the two matrices likely influences the performances, in terms of purity, of

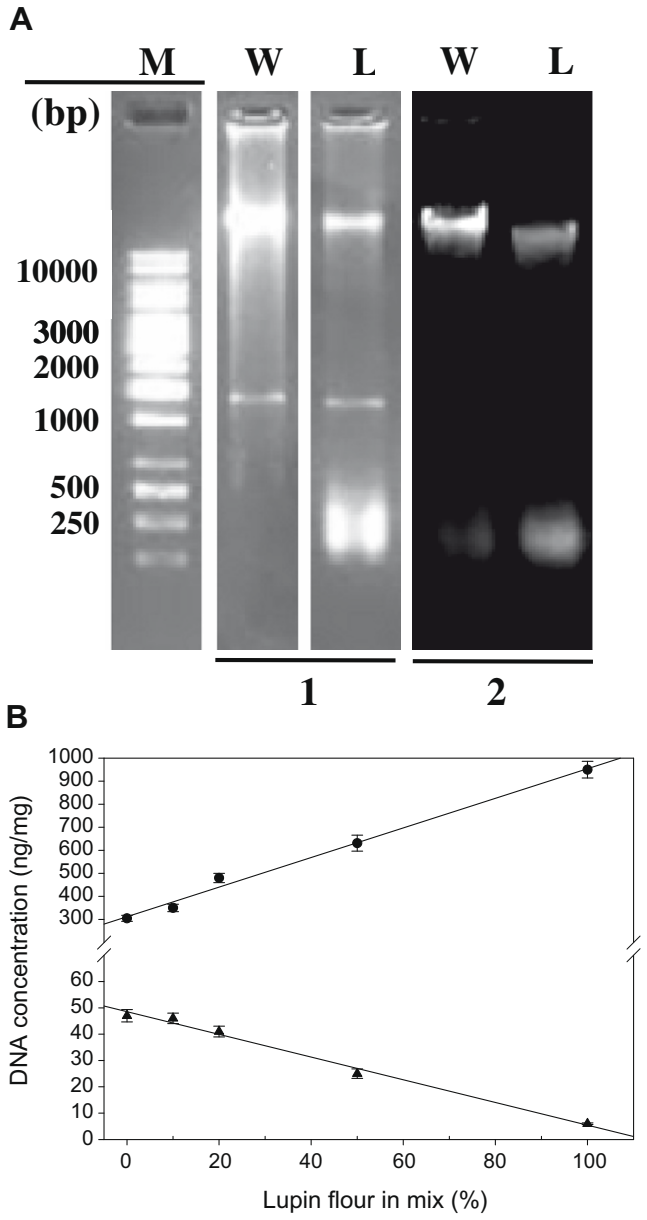

Fig. 2. Panel A: electrophoretic analysis of total DNA extracted with from wheat (W) and lupin (L) flours. DNA purifications were performed using the NucleoSpin Food kit (1) and the CTAB protocol (2). In each well have been loaded 2 μl of stock DNA solution. See text for details. Panel B: quantification of total DNA extracted from lupin–wheat mixed flours at various percentages using the NucleoSpin Food kit. Lupin and wheat unmixed flours are indicated with 100% and 0%, respectively. Concentrations of DNA solutions have been measured by the fluorescent dye bisbenzimide H33258 (triangles) and spectrophotometric absorbance at 260 nm (circles). Results are expressed as ng of DNA extracted per mg of flour. Different scaling ordinates have been used to emphasise the differences between the two methods. Experimental details in the text.

the isolation protocols. In fact, lupin seeds contain proteins up to 44% of its dry weight (Sironi et al., 2005), whereas soft wheat seeds mainly accumulate starch (about 70%) (Hoseney, 1986).

Amplificability of the isolated DNAs was determined by PCR using primers specific for chloroplast *trnL* (UAA) gene. This primer pair has extensively been used for identifying plant species (Taberlet et al., 2007). Samples from lupin flour were amplifiable only when the stock solutions (as eluted from SPE, usually DNA the concentration was around 0.4 μg/μl) were diluted at least 1:50 with sterile distilled water, confirming the presence of contaminant compounds in the DNA extracts which negatively interfered with

the polymerase reaction. On the other hand, the DNA samples prepared from leaves to assess the specificity of qPCR primers or to draw the calibration curves (see below) and from the food samples did not contain any inhibitory substance, since all of them were fully amplifiable.

3.3. qPCR

The slope of the standard curve measures the efficiency of the amplification process, according to the equation: $\eta = [10^{(-1/\text{slope})}]-1$, where η is the efficiency (Pfaffl, 2001). It follows that, for $\eta = 1$ (namely 100% efficiency), a the standard curve reaches a slope of −3.32 and that η is not dependent from the dilution factor of the standard. For a dilution series it will be: $Ct_2-Ct_1 = \log(N_1/N_2)/\log(1+\eta)$, where Ct is the threshold cycle and N_1/N_2 is the dilution factor (Rutledge & Côté, 2003). The result is that every tenfold difference in quantity translates to a difference of 3.32 cycles. In the present experimental setting the Ct shift between every dilution is 3, since a dilution factor of 1:8 was adopted. Thus, eightfold serial dilutions of purified genomic leaf lupin DNA (Fig. 3, circles) have been prepared and amplified with the set-up protocol. Standard curves have been produced showing linear correlation coefficients (R^2) ranging between 0.996 and 0.999 (over more than 15 runs). Reaction efficiencies, determined from the standard dilution series spanning 5 orders of magnitude, ranged between 96% and 100%. By and large, the results indicated that the designed primers and the protocol of amplification may be used for quantification purposes.

Further, the system has been tested with lupin DNA extracted from flour. The standard curves obtained in this case (Fig. 3, triangles) showed linear correlation coefficients ranging between 0.994 and 0.997 (over 10 runs). Reaction efficiencies ranged between 95% and 101%, indicating no inhibition of reaction due to contaminants in the DNA solutions. It is however worth to remark that DNAs from lupin flours following SPE was amplifiable by traditional PCR only upon dilution (Section 3.2).

The results of quantification of DNAs extracted from mixed flours are summarised in Table 3. The experimental data obtained were in good agreement with theoretical values calculated from the DNA quantity actually extractable from lupin flour. The detection limit, calculated by using the mixed flours, was 7 pg of lupin DNA. It is remarkable to note that the dynamic range of the method allows to detect and correctly quantify either high percentages (50%) and very low amounts of lupin flour in samples. Table 3 shows also the DNA quantifications of some bakery products prepared with different amounts of lupin flour: two kind of biscuits containing 7.5% and 15% lupin flour, respectively, and added with sesame; two breads made with the same amount lupin flour but of different varieties; an extruded snack (39% lupin flour); a crispy toast-like bread found on the local market whose the lupin content was unknown; flaked de-oiled lupin seeds. In all cases the experimental results are concordant, except for one sample, with the expected amounts calculated according to the respective ingredient list.

Table 3
qPCR quantification of DNA extracted from lupin-wheat mixed flours and bakery products.

Sample		Lupin DNA quantity (pg/mg of product)	
		Expected	Measured[a]
Lupin flour	0%	0[b]	n.a.
	0.1%	6.3[b]	6.5 ± 0.5
	0.5%	31[b]	30 ± 1
	1%	63[b]	62 ± 2
	2%	126[b]	119 ± 4
	10%	630[b]	635 ± 22
	20%	1260[b]	1245 ± 38
	50%	3150[b]	3218 ± 104
	100%	6300[c]	6200 ± 200
Biscuit		472.5[d]	489 ± 25
Biscuit		945[d]	941 ± 36
Bread[f]		50[d]	49 ± 2
Bread[g]		50[d]	51 ± 2
Crispy toast-like bread		unknown	78 ± 2
Snack		2457[d]	2062 ± 77[e]
White flakes		8500[d]	8364 ± 345

n.a.: no amplification.
[a] Values are the mean of 5 assays.
[b] Theoretically calculated from the 100% sample value.
[c] Experimental, from Table 2.
[d] Deduced from the product recipe (list of ingredients).
[e] Values are the mean of 3 assays.
[f] Bread made with *L. albus* var. Ares.
[g] Bread made with *L. albus* var. Lux.

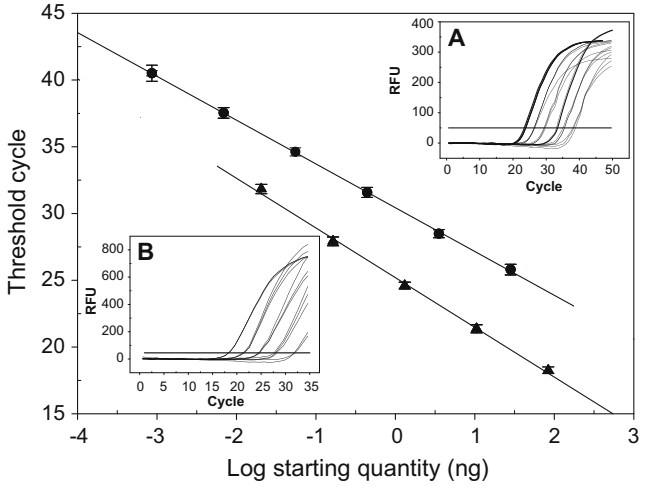

Fig. 3. Calibration curves obtained with eightfold serial dilutions of lupin DNA extracted from leaves (circles) and flour (triangles), as determined by real-time PCR. Details about experimental and statistical parameters of the best fitting lines are specified in the text. In the insets, the amplification plots of leaves (A) and flour (B) DNA samples are reported.

4. Conclusions

In food analysis, the need to purify DNAs from ingredients of different origin at the same time makes the extraction procedure and total DNA quantification challenging. DNA extraction methods must be efficient, yielding as much DNA as possible from any of the sample components. Wheat flour-based food are the main products were lupin flour is used as ingredient (Bez et al., 2005; Dervas et al., 1999; Doxastakis et al., 2002; Pollard et al., 2002) Following this consideration, prior to design and develop the qPCR methodology, the present work has evaluated the DNA extraction yields from the two different matrices, namely lupin and wheat flours, and from wheat-lupin composite flours prepared with various percentages of lupin. The quality of the purified DNAs has then been assessed in terms of reliability of quantification and amplificability.

Lupin flour turned out to be a difficult matrix, since large amounts of compounds are co-purified with DNA, even adopting well established extraction protocols. This, results in potential overestimations in DNA direct quantifications and consequently underestimations in PCR reactions.

Although DNA sequence information for lupin is relatively scarce it has been possible to design a primer pair highly specific for the target gene to be employed to analyse food matrices. The primers allowed reaching high efficiencies in qPCR.

By and large, the presented PCR method can be usefully used for sensitive and selective detection of lupin flour in food samples, such as bakery products. The limit of detection achieved was about 7 pg of lupin DNA, corresponding to a percentage of less than 0.1% of lupin flour in foods.

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