



Analytical Methods

Development of a real-time PCR method for the simultaneous detection of soya and lupin mitochondrial DNA as markers for the presence of allergens in processed food

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ARTICLE INFO

Article history:

Received 13 August 2010

Received in revised form 7 December 2010

Accepted 1 January 2011

Available online 9 January 2011

Keywords:

Food allergens

Peanut

DNA isolation

Mitochondrial DNA

Real-time PCR

Duplex method

ABSTRACT

Lupin and soya are members of the *Leguminosae* family which are recognised as some of the richest source of vegetable proteins. Lupin- and soya-containing products are available on the EU market and could cause severe adverse reactions in allergic individuals, even if consumed at low concentrations. In this context the development of methods for reliable detection of these allergens in food products is a useful tool for the surveillance of established legislation on food labelling within the EU. This work described the development of a duplex real-time PCR method allowing the simultaneous detection of traces of lupin and soya in processed food based on a specific TaqMan[®] probe designed on a mitochondrial tRNA-MET gene. A set of primers and probes was designed for the amplification of a 168 and 175 bp fragment of lupin and soya mitochondrial DNA, respectively. The performance of the method was established using lupin and soya flours and cookies baked from lupin- and soya-containing dough (different concentrations and baking times). The PCR platform yielded consistent and repeatable results. The specificity of the system was tested with DNA from 28 plant species. The sensitivity of the method was suitable to detect allergenic ingredients in the low mg per kg range. Both lupin and soya at a level of 2.5 mg per kg food matrix could be detected in cookies baked at 180 °C for 10 min. The method was successfully applied to bakery (e.g. bread) and vegetarian (e.g. non-meat sausages) food products that contain or may contain soya and/or lupin as ingredient or contaminant (according to the declaration on the product label).

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1. Introduction

In industrialised countries, food allergies represent a health problem of considerable and increasing relevance. Food allergies affect up to 2% of the adult population and up to 8% of children (Hughes & Mills, 2001). Allergens are either deliberately used as ingredients or are present as contaminants in food products. To date, the only effective method to prevent adverse reactions to food allergens is strict allergen avoidance, requiring correct and integral declaration of products and the availability of products not inadvertently contaminated with allergens. The problem of the detection of declared or hidden allergens in food is a major concern for both the food industry and consumers. The most significant cause of uncontrolled exposure to food allergens is incorrect

labelling or contamination of a product that the consumer has considered safe.

Among food allergies, lupin and soya allergies are becoming an important public health concern because it affects an increasing number of children and adults in Europe (Hieta, Hasan, Mäkinen-Kiljunen, & Lammintausta, 2009; Lindvik, Holden, Løvik, Cvancarova, & Halvorsen, 2008; Reis et al., 2007; Shaw, Roberts, Grimshaw, White, & Hourihane, 2008) and the ingestion of even minute amounts of lupin or soya, often found as declared ingredients or contaminants in processed foods, may trigger allergic reactions including digestive disorders, respiratory (rhinitis and asthma) and skin reactions (urticaria and atopic dermatitis) (Poms, Anklam, & Kuhn, 2004; Łoza & Lampart-Szczapa, 2008). The estimated prevalence of soya allergy ranges from 0.5% in the general population to 3–6% in children (Article 6, 2000). The number of allergic reactions to lupin and soya in the general EU population is unknown (Ballmer-Weber et al., 2007), although a growing incidence of legume allergy is observed because of its increasing consumption

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(Holden, Fæste, & Egaas, 2005; Peeters, Nordlee, Hefle, Bruijnzeel-Koomen, & Knulst, 2005).

Therefore, EU legislation has been issued to inform the consumer about the content of food products by means of a mandatory labelling of food ingredients. The presence of the most important potential allergens needs to be declared on the label of food products whenever they are used as ingredients as required by Directive 2000/13/EC (2000), which was amended by (Directive 2003/89/EC (2003). Lupin and soya are recognised as food allergens by the European Food Safety Authority (EFSA) and belong to the EU food allergen list. While soya was included in the EU foodstuffs allergen list from its origin in 2000, mandatory declaration of lupin was first required by Directive 2006/142/EC, (2006) and was maintained in the recent Directive 2007/68/EC, (2007).

Thus, as far as allergenic contaminants are concerned, there are no legal concentration limits to date. There are only indications that there are levels of allergens (thresholds) below which an allergen poses only a small risk of causing harm to an allergic consumer (Yanez, Ivnovic, Owen, & Ballester, 1983). Due to the variation in individual responses it is not possible to say what constitutes a safe level. To date no threshold level for lupin and soya bean has been established. A study of the severity of soya allergy and the threshold level of soyabean protein inducing allergy symptoms established threshold doses ranging from 10 mg to 50 g of soya for subjective symptoms and from 454 mg to 50 g for objective symptoms (Ballmer-Weber et al., 2007). Regarding lupin, a recent study of the Allergy Vigilance Network (Gayraud et al., 2009) determined the reactivity threshold to lupin to be low, between 625 and 965 mg, which may correspond to amounts found in market products (few mg of soya or lupin per kg final product).

Soya (*Glycine max*) and lupin (*Lupinus*) are recognised as valuable sources of vegetable proteins. They are used by the food industry for the production of bakery products (e.g. bread and cookies), pastry, pasta, sauces, and vegetarian products (e.g. non-meat sausages). The main reasons for possible hidden allergens in food are contamination from previously or simultaneously produced products in the same factory. Contamination of food by either lupin or soya may take place at the stage of the production of the final product. Since lupin and soya are used by the food industry in the same class of products the simultaneously detection of soya and lupin ingredients in a single assay is of great interest.

Reliable methods must be put in place for the specific and sensitive detection of potential allergens in food to ensure compliance with food-labelling regulations and to provide consumer protection (van Hengel, 2007). Because allergens are specific proteins within a food, they are the primary analytes to be targeted.

Immunochemical methods, which exploit the specificity and affinity interactions of antibodies with proteins, such as enzyme-linked immunosorbent assays (ELISAs) have therefore been favoured in allergen detection (Kerbach et al., 2009). An alternative approach which detects the presence of the allergenic food species uses sensitive and specific DNA-based methods like real-time polymerase chain reaction (real-time PCR). The development of methods based on the detection of DNA sequences encoding for allergens or a comparable protein is becoming increasingly popular. The effect on DNA during food processing steps (such as heat treatment) is relatively minor compared to the effect on the expressed protein (Bauer, Weller, Hammes, & Hertel, 2003). Real-time PCR technique has been successfully used to detect genomic DNA from members of the legume family, lupin (Demmel, Hupfer, Hampe, Busch, & Engel, 2008; Scarafoni, Ronchi, & Duranti, 2009), soya (Abdullah, Radu, Hassan, & Khair Hashim, 2006; Gryson, Messens, & Dewettinck, 2008; Pedersen et al., 2008), pea (Brežná, Hudecová, & Kuchta, 2006) and peanut (Hird, Lloyd, Goodier, Brown, & Reece, 2003; Scaravelli, Brohee, Marchelli, & van Hengel,

2008) in food matrices. Limits of detection could be established for some allergens, including soya and lupin, and were in the range of 10–50 mg allergen per kg of food for soya (Köppel et al., 2010) and of 1000 mg allergen per kg of food for lupin (Scarafoni et al., 2009).

Duplex or multiplex PCR methods can be a valuable tool to indicate the presence of allergens if multi-target screening of several allergenic foods is required, even if many allergen combinations are unlikely to be present in certain food products. Previous studies using a duplex real-time PCR approach have been successfully used to simultaneously detect sesame and hazelnut in food (Schöringhumer, Redl, & Cichna-Markl, 2009). More recently two tetraplex real-time PCR assays have shown positive identification of hazelnut, peanut, celery, soya, sesame, milk, almonds and egg at levels of 10–100 mg allergen per kg of food (Köppel et al., 2010). To our knowledge, no simultaneous method for soya and lupin detection in food has been developed. In addition, the detection limits of individual methods are not sufficient for the detection of potentially hazardous lupin and soya residues of undeclared allergens in food. As members of the legume family, lupin and soya have been shown to have allergenic cross-reactivity (Guarneri, Guarneri, & Benvenega, 2005; Moneret-Vautrin et al., 1999; Peeters et al., 2007), therefore the development of a novel, sensitive and specific real-time PCR for the simultaneously detection of lupin and soya in processed foods is of valuable interest to adequately protect lupin or soya allergic patients.

In our previous work we used genomic DNA for the specific detection of lupin traces in a model cookie matrix and in food products currently available on the market (Gomez Galan, Brohee, Scaravelli, van Hengel, & Chassaigne, 2010). The α -conglutinin method allowed the detection of 10 mg lupin flour per kg food matrix in cookies baked at 180 °C for 10 min. In this work the selection of mitochondrial DNA (mtDNA) over genomic DNA as the target for the detection of lupin and soya is advantageous because its presence in multiple copies per cell increases the probability of achieving a positive result, even in the case of samples undergoing partial DNA fragmentation due to severe processing conditions (Verkaar, Nijman, Boutaga, & Lenstra, 2002). Large phylogenetic variability of mtDNA compared with nuclear sequences, which undergo a less rapid evolution, is likely to facilitate authenticity studies (Parties et al., 2000).

We report here the development of a duplex real-time PCR method for the simultaneous detection of soya and lupin specific mtDNA sequences in a heat treated model food matrix and in commercial food products. In this study, TaqMan® primers and probes were designed for amplification and detection of the target DNA. Two real-time PCR assays targeting DNA sequences coding for mitochondrial gene for initiator tRNA-MET in the case of lupin, and a sequence almost identical of soya, obtained via inverse PCR, have been developed in-house. Cookies containing both soya and lupin were prepared as a heat treated model matrix material, based on a recipe already used in our previous work (Gomez Galan et al., 2010). The objective of this work was to develop a method with detection limits for lupin and soya in the low mg ingredient per kg range of food product.

2. Materials and methods

2.1. Lupin and soya flours

Raw lupin flour from *Lupinus angustifolius* species (FRALU-NT) was kindly provided by Frank & Miedendorp (Twello, The Netherlands). Soya bean samples were obtained from the Reference Materials Unit of the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium).

2.2. Preparation of a model processed food matrix

Cookies were prepared as a model matrix material for the detection of lupin and soya traces. Wheat flour (1000 g $x-y$), 360 g butter, 374 g sugar, 120 g skimmed milk powder, 6 g sodium chloride, 2 g sodium hydrogen carbonate, 3 g ammonium bisulphate and 135 g water were mixed to obtain 2 kg dough. For lupin and soya spiked doughs x g of wheat flour was replaced by FRALU-NT and y g by soya to obtain the desired concentration of lupin and soya per kg dough. The final lupin and soya content in the cookies was 10 ($x = y = 0.020$), 7.5 ($x = y = 0.015$), 5 ($x = y = 0.010$), 2.5 ($x = y = 0.005$) and 1 ($x = y = 0.002$) mg per kg. All cookies were baked at 180 °C for different periods of time (5, 10, 15, 20 and 30 min). In addition to this, blank cookie dough was prepared without lupin or soya. All the spiked and blank cookies were ground separately and the resulting powder was stored at –20 °C until DNA extraction.

2.3. Commercial food products

Twenty nine food products containing lupin (“tofu-like” products, vegetarian sausage, bakery products comprising cookies and breads) or soya (cookies and bread) as a declared ingredient were obtained from local supermarkets in Belgium, The Netherlands and Germany.

2.4. DNA extraction and quantification

Total DNA was extracted from 1 g aliquots of lupin and soya samples (lupin and soya flours, cookies or market samples) using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1999). The precipitated DNA was resuspended with 100 µL of DNase, RNase free water (Sigma–Aldrich, St Louis, MO). Subsequently DNA was purified using the Wizard® DNA clean-up system (Lipp, Brodmann, Pietsch, Pauwels, & Anklam, 1999) (Promega, Southampton, UK) following the manufactures’ instructions and eluted with 100 µL of DNase and RNase free water preheated at 65 °C. Total DNA quantification was determined spectrophotometrically using a 3300 Ultraspect Spectrophotometer (GE Healthcare, Piscataway, NJ). DNA extracts were adjusted to a concentration of 40 ng/µL in DNase and RNase free water.

2.5. Design of primers and probe sets

TaqMan® primers and probes were designed for amplification and detection of the target DNA, a region within the *Lupinus angustifolius* mitochondrial gene for initiator tRNA-MET, amplicon size 168 bp (NCBI entry X04377). Identification of a homologous soya gene was achieved by inverse PCR (Ochman, Gerber, & Hartl, 1988; Offringa & van der Lee, 1996). Inverse PCR allows the in vitro amplification of DNA sequences that flank the region of known sequence (NCBI entry X04377). Inverse PCR products previously purified using the Gen Elute™ PCR Clean-up kit (Sigma–Aldrich, St Louis, MO), were digested with Xho (Sigma–Aldrich, St Louis, MO), HindIII (Sigma–Aldrich, St Louis, MO) and EcoRI (Sigma–

Aldrich, St Louis, MO) endonucleases and sequenced. The sequencing reactions were performed using the BigDye® Terminator v1.1 Ready Reaction Cycle Sequencing kit from Applied Biosystems Europe (Warrington, UK) and the run was done in an ABI Prism 3130® Genetic Analyser filled with the Performance Optimised POP-7™ Polymer and a 50 cm capillary array. The sequencing reactions were set up with 8–10 ng of PCR product, 3.2 pmol of primer in a final reaction volume of 20 µL. The thermal cycle started with 1 min of initial denaturation at 96 °C followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C for primer annealing and 150 s at 60 °C for elongation. Amplification reaction products were purified by ethanol precipitation prior to the sequencing run. The soya sequence obtained was almost identical to lupin but having seven bases and had three polymorphisms (Fig. 1).

Lupin and soya primers and probes were designed manually and checked with Primer Express (Applied Biosystems, Warrington, UK) and Integrated DNA technologies (<http://www.idtdna.com>) programs. Primers and probes sequences for this target DNA were: forward primer 5'-ACTAAGCAGAAGCAAGACGGA-3'; reverse primer 5'-ATGAACGCAGAGTTGGAACAG-3'; Lupin probe FAM-GGGCAGTTT GATGGCTATGATAGGCGCG-TAMRA; Soya probe VIC-AGGGCAGTTT CCTGATGGCTATGAATGGCG-TAMRA. Specificity of the primers and probes was checked using NCBI software (<http://www.ncbi.nlm.nih.gov/BLAST/>) where no homology with mitochondrial DNA from other plant species was found. Primers and probes were purchased from Applied Biosystems Europe (Warrington, UK).

2.6. Real-time PCR

Real-time PCR measurements were performed in triplicate in 96 well optical reaction plates using optical and adhesive covers (Applied Biosystems, Warrington, UK) run on a ABI Prism® 7900HT (Applied Biosystems, Warrington, UK) sequence detection system with the following thermal cycling protocol: 50 °C for 2 min, 95 °C for 10 min followed by 40 or 50 cycles of 95 °C for 15 s and 64 °C, for 1 min.

Optimal amplification and detection was obtained by employing an annealing temperature of 64 °C, a primer concentration of 900 nM, and a probe concentration of 500 nM. The reaction volume was set at 25 µL, and each reaction contained 1 × TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK) containing AmpliTaq Gold® DNA polymerase, AmpErase® UNG, dNTPs (with dUTP), DNA template (equivalent of 100 ng of DNA), primers and probe. Uracil-N-glycosylase was used to avoid false positive results due to carry-over contaminations from previous amplification reactions.

3. Results and discussion

3.1. Design of the real-time PCR assay for the simultaneous detection of soya and lupin

The increasing awareness of the potential of lupin and soya to trigger allergic reactions has led to efforts in developing real-time

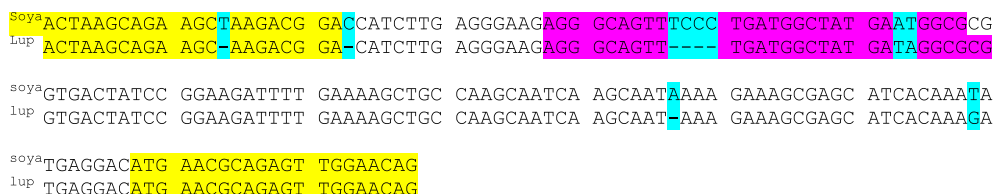


Fig. 1. Alignment of lupin (*Lupinus angustifolius*) mitochondrial gene for initiator tRNA-MET (NCBI X04377) and soya (*Glycine max*) mtDNA sequences showing variations between the two closely related species. Differences in nucleotide composition are highlighted in blue colour. Amplicon size is 168 and 175 bp for lupin and soya, respectively. The sequence of the forward primer and complementary sequence to the reverse primer (in yellow colour) as well as the sequence of the specific TaqMan® probes (in purple colour) are also indicated.

PCR methods for detecting simultaneously the presence of these two ingredients at trace levels in food. A DNA sequence encoding for initiator tRNA-MET was chosen as target for the detection of lupin by real-time PCR. A set of primers was designed for the amplification of a 168 bp fragment of lupin mitochondrial DNA and a complementary probe was designed for the real-time detection of this amplicon.

The performed TaqMan® specificity assays revealed that the set of primers developed for targeting lupin mitochondrial DNA also amplified soya DNA. In order to identify the homology between the two different species, sequencing of the amplicon detected in soya was performed and amplified products were compared with DNA databases. Notably, soya and lupin sequences had high homology with each other. The lupin gene was almost identical to the one of soya, however missing the bases T, C, T, C, C, C, A and presenting three polymorphisms T, A, G (differences in nucleotide composition highlighted in blue colour in Fig. 1).

To specifically detect the two plant species a duplex assay was designed using the same set of forward and reverse primers, plus two different probes labelled with two different fluorescent reporter dyes which are spectrally resolved: FAM™ dye for lupin detection and VIC™ dye for soya detection (Applied Biosystems, Warrington, UK).

3.2. Method efficiency and linearity

To obtain accurate results, the efficiency of the PCR reaction should be as close to 100% as possible. The analysis of samples covering a range of DNA amounts ranging from 0.001 to 100 ng allowed the establishment of a standard curve (Fig. 2). PCR measurements were made in triplicate ($n = 3$). The efficiency of the real-time PCR methods was calculated from the slope determined by the instrument software. The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/\text{slope}]-1}$ and was close to 1 (equivalent of theoretical slope of -3.3).

Efficiency was slightly better for the soya (97%) efficiency as compared to 94% for lupin in the investigated range. The assay was able to amplify 0.001 ng DNA of both lupin and soya DNA with Ct values <40. The linearity of the method was also assessed. The correlation coefficient (r^2) of the calibration curve for soya and lupin was 0.992 and 0.996 respectively, indicating a high correlation between soya and lupin DNA amounts and their corresponding Ct values.

3.3. Method specificity

The specificity of the primer and probe sets was checked by homology searches to DNA sequences present in the NCBI database. In addition to this, we assessed the specificity of the method by using DNA extracts from a variety of different foods. For this purpose a broad spectrum of samples was selected. DNA originating from the following species was used: barley, millet, buckwheat, oat, rye, spelt, rice, corn, wheat, green peas, lentils, mung beans, chick peas, white beans, sunflower seeds, rape seeds, olives, potatoes, almonds, Brazil nuts, cashew nuts, hazelnuts, macadamia, pecans, pistachios, walnuts, cacao beans and sesame seeds. In order to check the quality of the DNA non-coding regions of chloroplast DNA were amplified (Scaravelli et al., 2008).

Real-time PCR method failed to amplify most of the samples tested but amplified DNA from barley in the soya detection system only and from buckwheat in both lupin and soya detection systems. Only cross-reactivity above 1% signal was considered as significant. Barley led to a 7.8% signal in the soya system and in case of buckwheat to 8.6% signal in the lupin and soya system. Usually, allergens are found either as contamination or declared ingredient.

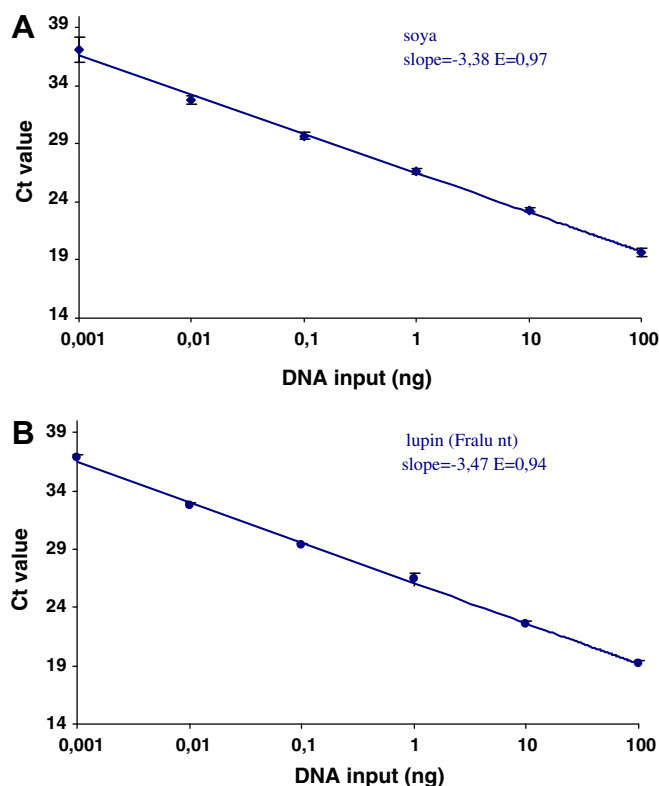


Fig. 2. Standard curves of the duplex real-time PCR method. The standard curve was based on the analysis of ten fold dilutions of DNA extracted either from soya (*Glycine max*) (A) and lupin (*Lupinus angustifolius*) (B). PCR was performed on DNA aliquots ranging from 0.001 to 100 ng. DNA samples were diluted with DNase and RNase free water. Ct values (mean and SD; three replicate measurements) are plotted against the DNA amount input to calculate the slope and the corresponding real-time PCR efficiencies (according to the equation: $E = 10^{[-1/\text{slope}]-1}$).

It is unlikely that bakery or vegetarian products become contaminated by raw barley. However, malted barley may be a labelled ingredient in blended flours typically used for breads and other bakery products. Protein is degraded by the malting process whereas DNA may remain sufficiently intact for PCR assays. In Europe, buckwheat flour is used, but for limited food application, e.g. for the preparation of pancakes. Furthermore, buckwheat is gluten-free and can be a good substitute for people who are allergic to gluten. Results indicate that the region of amplification chosen for the duplex real-time PCR may be a conserved region and therefore these cross-reactivities have to be considered even if it is unlikely they happen.

3.4. Analysis of a model processed food incurred with lupin and soya

In order to assess the applicability of the real-time PCR assay to detect lupin and soya used as ingredients present in the low mg per kg range in bakery products, a model food material was prepared. Lupin and soya containing cookies were made by spiking raw lupin and soya flours into a cookie dough. It has been shown that thermal processing has an effect on lupin and soya detectability and allergenicity (Alvarez-Alvarez et al., 2005). Therefore cookies were baked at 180 °C for different baking times (5, 10, 15, 20 and 30 min) to obtain a series of cookie samples that could be used to investigate the effect of heat treatment both on lupin and soya detection.

The quality of the extracted DNA was assessed by analysing aliquots of 100 ng DNA isolated from cookies spiked with 10 mg lupin and soya flour per kg and baked for different times using the

duplex real-time PCR method. For each sample, the reported cycle threshold (Ct) values refer to the mean value of nine replicates (three replicates of DNA extraction and three replicates of PCR amplification). Table 1 shows that increasing the baking time from 5 to 20 min had a clear effect on the DNA concentration in cookie extracts; however, it did not affect the quality of the extracted DNA since comparable Ct values were obtained (mean values and standard deviation from nine replicates).

Further increase of the baking time inhibited lupin and soya detection. After 30 min baking, no DNA was obtained from cookie extracts and the assay failed. Comparison of the Ct values obtained in the range 5–20 min revealed that the detection of lupin is more sensitive than the detection of soya in the temperature range studied (Table 1). In food industry reduced baking time and increased cookie production can be accomplished with new technology ovens. Ten to twenty minutes baking time at a temperature of 180 °C reflect realistic conditions.

The detection limits of the PCR assay targeting both soya and lupin mtDNA sequences were evaluated. Cookies spiked with 1, 2.5, 5, 7.5 and 10 mg per kg with lupin and soya flour, which were baked for 10 min at 180 °C, were analysed. DNA extraction at low concentrations is usually the critical step of the method. The repeatability of the DNA extraction for the lowest concentrations was evaluated by eight independent extractions from cookies (Table 2). The repeatability of PCR amplification refers to the mean value and standard deviation of 24 replicates (eight replicates of DNA extraction and three replicates of PCR amplification).

As expected, all eight extracts had a comparable DNA content (mean value) even if the standard deviation was higher than for the concentration level 10 mg per kg (Table 1). Table 2 shows the DNA content and Ct values obtained for the samples containing lu-

pin and soya at nominal concentrations of 2.5 mg per kg food matrix, which was the minimum concentration that yielded Ct values <40 and no false negative results. For samples containing only 1 mg per kg of the allergen the false-negative rates for lupin was 12.5%, and for soya 37.5%. Consequently, the limit of detection for the simultaneous determination of lupin and soya traces was set to 2.5 mg lupin and soya flour per kg matrix in cookies baked at 180 °C for 10 min.

The developed method, which targets mitochondrial lupin DNA (mtDNA), had a lower detection limit than our previously developed method based on nuclear DNA (nDNA) (Gomez Galan et al., 2010). The latter allowed the detection of 10 mg lupin flour per kg food matrix in cookies baked at 180 °C for 10 min, when raw and toasted lupin flour was used (Gomez Galan et al., 2010). For soya, the nDNA-based method (lectin (Le1) assay) developed by Köppel et al. (2010) has 10–50 times higher detection limits than our mtDNA based method. The difference in sensitivity between the two methods is explained by the high number of mitochondria and mtDNA copies per mitochondria in soya and lupin seeds, whereas a single nDNA copy is present per plant cell.

3.5. Analysis of commercial food products

To demonstrate the applicability of the duplex method to commercially available foods such as “tofu” like products, vegetarian sausages, cookies and bread that, according to their label, contained lupin or soya were analysed using the duplex real-time PCR method (in total 29 products, Table 3). The problem of the detection of declared or hidden allergens in food is a major concern for both the food industry and consumers. Since lupin and soya are used by the food industry in the same class of products the simultaneous detection of soya and lupin ingredients in a single analysis is necessary. In any case, results obtained with our detection method should correspond qualitatively with the declaration made on the package.

The results of the duplex real-time PCR analyses of commercial products are shown in Table 3. For lupin, data obtained with the duplex mtDNA method were compared to the ones obtained with the δ - and α -conglutin methods in our previous work (Gomez Galan et al., 2010). Results obtained with the three methods are expressed as the mean Ct value with their respective standard deviation (three replicates of DNA extraction and three replicates of PCR amplification) for samples with Ct values <40 (cut-off value for both soya and lupin detection).

In our previous work, both real-time PCR assays based on nDNA (α - and δ -conglutin) were capable of detecting lupin as an ingredient in the “tofu-like” products with low Ct values (23 and 26 cycles

Table 1

Lupin and soya detection in cookies baked at 180 °C for different periods of time (5–30 min). The analysed samples were representative for cookies containing lupin and soya flours at a level of 10 mg per kg food. The detection was performed by employing real-time PCR assays on aliquots of 100 ng DNA extracted from the cookies. The VIC-TAMRA and FAM-TAMRA detector is specific for soya and lupin, respectively. For each sample, the Ct values refer to the mean value of nine replicates (three replicates of DNA extraction and three replicates of PCR amplification).

Baking time (min)	DNA (ng/ μ L)	(Ct values)	
		VIC-TAMRA	FAM-TAMRA
		(soya detection)	(lupin detection)
5	133 $\pm$ 15	37.9 $\pm$ 0.9	36.1 $\pm$ 0.8
10	97 $\pm$ 20	36.1 $\pm$ 1.0	34.6 $\pm$ 0.1
15	89 $\pm$ 13	36.8 $\pm$ 1.1	35.3 $\pm$ 0.9
20	39 $\pm$ 6	39.4 $\pm$ 0.2	38.8 $\pm$ 0.6
30	Undetermined	Undetermined	Undetermined

Table 2

Comparison of soya and lupin detection in cookies containing lupin and soya flour at a concentration of 1 and 2.5 mg per kg food. The analysed cookies were baked at 180 °C for 10 min and the detection was performed by employing the duplex real-time PCR assay on aliquots of 100 ng DNA extracted from the cookies. VIC-TAMRA and FAM-TAMRA detector is specific for soya and lupin, respectively. For each sample, the Ct values refer to the mean of 24 replicates (eight replicates of DNA extraction and three replicates of PCR amplification).

Extract	Total DNA (ng/ μ L)	Duplex real-time PCR assay			
		1 mg per kg soya and lupin cookie		2.5 mg per kg soya and lupin cookie	
		VIC-TAMRA detector	FAM-TAMRA detector	VIC-TAMRA detector	FAM-TAMRA detector
1	115	Undetermined	38.69 $\pm$ 0.08	38.49 $\pm$ 0.41	36.66 $\pm$ 1
2	126	38.53 $\pm$ 0.76	Undetermined	36.47 $\pm$ 1.64	36.05 $\pm$ 0.71
3	139	39.10 $\pm$ 0.42	37.77 $\pm$ 1.77	37.70 $\pm$ 0.22	36.89 $\pm$ 0.34
4	140	Undetermined	35.93 $\pm$ 0.75	37.94 $\pm$ 0.35	38.19 $\pm$ 0.72
5	135	38.57 $\pm$ 1.06	38.02 $\pm$ 0.16	36.34 $\pm$ 0.65	37.22 $\pm$ 0.83
6	111	Undetermined	37.73 $\pm$ 0.42	36.40 $\pm$ 0.82	37.54 $\pm$ 0.10
7	88	39.29 $\pm$ 0.52	38.49 $\pm$ 1.10	36.03 $\pm$ 0.11	37.38 $\pm$ 1.04
8	119	37.91 $\pm$ 1.54	38.35 $\pm$ 1.06	36.92 $\pm$ 0.33	38.01 $\pm$ 0.5
Mean value (SD)	122 $\pm$ 17				

Table 3

Analysis of 29 commercial food products listing either lupin or soya amongst their ingredients. The detection was performed by employing 1) the duplex real-time PCR assay on aliquots of 100 ng DNA extracted from samples 1 and 2) the single real-time PCR assay on aliquots of 100 ng nDNA extracted from samples (α -conglutin and δ -conglutin methods are both specific for lupin). For each sample, the Ct values refer to the mean value of nine replicates (three replicates of DNA extraction and three replicates of PCR amplification).

Market samples		Duplex real-time PCR assay		Single real-time PCR assay (lupin)	
		Soya (VIC-TAMRA)	Lupin (FAM-TAMRA)	α -conglutin	δ -conglutin
"tofu" like lupin products	Sample 1	Undetermined	18.41 $\pm$ 0.42	26.1 $\pm$ 0.1	23.2 $\pm$ 0.1
	Sample 2	Undetermined	18.9 $\pm$ 0.11	26.3 $\pm$ 0.04	23.2 $\pm$ 1.2
Lupin vegetarian sausage	Sample 3	Undetermined	29.21 $\pm$ 0.37	37.1 $\pm$ 0.9	36.0
Lupin cookies	Sample 4	Undetermined	28.51 $\pm$ 0.21	34.2 $\pm$ 0.3	34.2 $\pm$ 0.3
	Sample 5	Undetermined	24.29 $\pm$ 0.21	30.3 $\pm$ 0.3	30.3 $\pm$ 0.3
	Sample 6	Undetermined	25.38 $\pm$ 0.34	33.9 $\pm$ 2.0	34.1 $\pm$ 1.3
	Sample 7	Undetermined	27.02 $\pm$ 0.14	37.0 $\pm$ 2.2	35.7 $\pm$ 0.7
	Sample 8	Undetermined	29.86 $\pm$ 2.09	Undetermined	37.6 $\pm$ 0.7
	Sample 9	Undetermined	28.3 $\pm$ 0.68	Undetermined	38.4 $\pm$ 0.3
	Sample 10	Undetermined	29.17 $\pm$ 0.47	38.7 $\pm$ 0.6	Undetermined
	Sample 11	Undetermined	28.97 $\pm$ 0.55	36.5 $\pm$ 0.2	37.0 $\pm$ 0.4
	Sample 12	Undetermined	31.18 $\pm$ 0.08	Undetermined	Undetermined
	Sample 13	Undetermined	26 $\pm$ 0.08	31.9 $\pm$ 0.04	32.8 $\pm$ 1.1
Lupin breads	Sample 14	Undetermined	31.17 $\pm$ 0.6	36.8 $\pm$ 0.2	36.3 $\pm$ 0.1
	Sample 15	Undetermined	35.53 $\pm$ 1.05	Undetermined	Undetermined
	Sample 16	Undetermined	33.63 $\pm$ 1.77	Undetermined	38.8 $\pm$ 0.05
	Sample 17	Undetermined	35.44 $\pm$ 0.2	Undetermined	Undetermined
	Sample 18	Undetermined	30.05 $\pm$ 0.67	36.6 $\pm$ 0.6	35.4 $\pm$ 1.8
	Sample 19	Undetermined	33.46 $\pm$ 0.09	Undetermined	37.3 $\pm$ 1.1
	Sample 20	29.7 $\pm$ 0.65	Undetermined	Undetermined	Undetermined
	Sample 21	29.12 $\pm$ 0.26	Undetermined	Undetermined	Undetermined
	Sample 22	Undetermined	34.12 $\pm$ 0.25	undetermined	37.09 $\pm$ 0.05
	Sample 23	23.27 $\pm$ 0.35	undetermined	undetermined	undetermined
Soya cookies	Sample 24	Undetermined	27.53 $\pm$ 0.27	34.4 $\pm$ 0.4	36.4 $\pm$ 1.7
	Sample 25	24.02 $\pm$ 0.69	Undetermined	Undetermined	Undetermined
	Sample 26	24.02 $\pm$ 0.92	Undetermined	Undetermined	Undetermined
	Sample 27	31.24 $\pm$ 2.22	Undetermined	Undetermined	Undetermined
	Sample 28	31.51 $\pm$ 1.01	Undetermined	Undetermined	Undetermined
Soya breads	Sample 29	31.28 $\pm$ 0.83	Undetermined	Undetermined	Undetermined

for the δ - and α -conglutin method, respectively) (Table 3). In these products (samples 1 and 2) lupin is a major ingredients with lupin levels around 30%. Therefore, it is not surprising that the mtDNA real-time PCR assay was capable of detecting this ingredient with very low Ct values (18 cycles) (Table 3). Soya detection returned negative results, which corresponded with the declaration made on the package. Lupin traces could also be detected in vegetarian sausages (sample 3) whereas soya detection returned a negative result. Results were in agreement with the declaration made on the label.

We also analysed 19 samples of bakery products, comprising cookies and breads, which listed lupin amongst the ingredients. In the case of lupin containing cookies, more reliable results were obtained with the mtDNA- than with the nDNA-based method (Table 3). On one hand, samples 4–13 returned 100% positive results when the lupin specific detector was used (26–29 cycles). On the other hand, the α - and δ -conglutin method yielded higher number of cycles and returned false-negative rates (37.5% and 25% for α - and δ -conglutin, respectively). Eight out of ten lupin containing bread samples returned positive results when the lupin specific detector was used. The two samples that failed to yield positive test results for lupin yielded positive results when the soya specific detector was used. Results may indicate that the samples were wrongly labelled or contaminated as no lupin but soya traces could be detected. This finding was verified against data obtained with the δ - and α -conglutin methods (lupin-specific) and a soya-specific ELISA test (Soya protein assay kit, Tepnel Biosystems Limited, UK).

Analysis of soya containing cookie and bread samples yielded similar results. Six out of seven samples of baked food products which listed soya amongst the ingredients yielded positive results when using the soya specific detector and negative results with the lupin specific detector, indicating the presence of traces of soya in

those samples. Sample 24 in which the soya specific detector showed negative results yielded positive results with the lupin specific detector, indicating the presence of lupin and not soya in the sample. Once more this finding was verified against data obtained with the δ - and α -conglutin methods (Table 3) and a soya-specific ELISA test performed on the samples (data not shown). In all cases, Ct values obtained in the case of lupin detection with the duplex method targeting mtDNA were lower than those obtained with the nDNA detection method previously developed (Table 3).

4. Conclusion

Duplex or multiplex PCR is a powerful technique enabling amplification of two or more products in a single reaction. The qualitative duplex real-time PCR assay that was developed in this work allows the detection of potentially allergenic ingredients in processed foods in a very efficient manner, producing results in the low mg per kg range for lupin and soya at once. Therefore, moving from single method to duplex and possibly multiplex PCR is a promising approach to produce results in a more economic and faster way.

The method is specific for lupin and soya since no significant amplification of DNA from related plants was observed. Many allergen combinations are unlikely to be present in certain commercial food, e.g. bakery products. In some cases, the cross reactivity between soya, lupin, buckwheat and barley has to be considered, however in the context of very specific samples.

One main advantage of the real-time PCR method based on the detection of specific mitochondrial DNA sequences is its sensitivity. The cookie model material used represents a DNA-rich food matrix which was likely to negatively impact the sensitivity that

could be achieved by real-time PCR. Despite this, the limit of detection for the duplex method was 2.5 mg per kg food products for both soya and lupin. The level of 2.5 mg per kg is within the range of detection limits that are relevant for the detection of potentially hazardous lupin and soya residues of undeclared allergens in food (wrong label or contamination).

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), but higher (68%) rates have been reported (Opinion of the scientific panel on dietetic products, 2005). A method for allergen analysis should ideally be able to reliably detect the allergen at the threshold dose level. For peanut, the lowest absolute dose eliciting allergic reactions has been determined to 1 mg (Taylor et al., 2002). Ingested doses of lupin flour reported to have triggered clinical reactions range from 265 to 1000 mg of lupin flour, but the lowest dose triggering reactions has not yet been established (Opinion of the scientific panel on dietetic products, 2005). Additionally, no threshold dose level has been estimated in the case of cross-reactivity.

In this frame, and in view of an even more extensive use of lupin and soy flour as food ingredients in the near future (Scarafoni et al., 2009), it is crucial to set up and optimise methods aimed at tracing its presence in food. In our work, the developed method is very sensitive yet not quantitative. Obviously, the development of acceptable thresholds is an urgent challenge in the context of international harmonisation.

The analysis of market samples is more challenging due to the complexity and diversity of the food matrices (vegetarian sausages, “tofu” like and bakery products) and food processing effects. The majority of samples that declared to contain lupin or soya as an ingredient indeed tested positive and the presence of soya or lupin in those samples was confirmed by positive PCR results. In the case of lupin containing breads, two of the market samples where lupin but not soya was declared as an ingredient tested positive when the soya specific detector was used. In the case of cookies and bread containing soya, six out of seven samples of baked food products which listed soya amongst the ingredients yield positive results when using the soya specific detector while yielding negative results with the lupin specific detector, indicating the presence of soya and not lupin in those samples. In all cases, findings were verified against data obtained with δ - and α -conglutinin methods (lupin-specific) or a soya ELISA test performed on the samples. These results showed that the duplex real-time PCR methods developed in this study is useful to support efforts to ascertain that food products are labelled correctly in order to protect the health of the allergic consumer.

The development of specific and sensitive multianalyte methods for the detection of declared, hidden or wrongly labelled allergens in food is a great challenge. Further work has to be done for quantification of allergens in a variety of food products. mtDNA based methods offer the advantage of great sensitivity, while nDNA methods offer the opportunity to quantify the allergenic ingredient. A compromise between sensitivity and accuracy of the method has to be found in order to be used in the range of threshold doses eliciting allergic reactions in sensitised individuals.

Acknowledgments

The authors would like to thank FRANK&MIEDENDORP (Twello, The Netherlands) and the Reference Materials Unit of the IRMM (Geel, Belgium) for providing the lupin and soy flour samples used in this study. The authors would like to thank Dr. Franz Ulberth and Dr. Alain Maquet for valuable comments.

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