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# DETECTION OF LUPINE (*Lupinus spp.* L.) AS A FOOD ALLERGEN USING THREE METHODS: END-POINT PCR, REAL-TIME PCR AND ELISA

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## ABSTRACT

The aim of this work was to compare three methods for the detection and quantification of lupine as an allergen in food. The methods that were used in this work were the direct method: ELISA and the indirect methods: end-point PCR and real-time PCR. We examined the detection limit (the sensitivity with which we can detect the presence of the allergen in a sample) and the reliability for performing an analysis. We used 17 samples of plant species from a processing plant for dehydrated soups production and lupine samples from lupine processing companies. Its practical use is wide and it is used mainly in the bakery industry, in the manufacture of confectionery, pasta, sauces, as a substitute for soy and also in the production of gluten-free food, because it does not contain gluten. Lupine, however, is also included in the list of 14 allergenic substances, which in accordance with the EU legislation must be listed on food labels. The high risk group, which suffers from primary sensitization or cross-reaction with peanuts, are allergic patients. In the EU, people who are allergic to peanuts range from 0.7 to 1.5%. In experiment 1, we detected the presence of lupine using primers for the detection of α- and δ-conglutine in the samples, using the end-point PCR method and the detection limit of this reaction was at the level of 100 ppm. For the vizualization of the DNA fragments, we used a 2% agarose gel and UV visualizer. In experiment 2 we detected lupine using the TaqMan real-time PCR reaction and primers for the detection of α and δ-conglutine at the level of 10 ppm of lupine in sample. The CP values of lupine using primers for the detection of α-conglutine was 24.85 ±0.12 and the reliability equation was $R^2$ = 0.9767. The CP lupine values using primers for the detection of δ-conglutine was 22.52 ±0.17 and the reliability equation was $R^2$ = 0.9925. In experiment 3, we used a sandwich ELISA method for the detection of lupine and the detection limit was within the range of 2-30 ppm and the reliability of the method according to the reliability equation was $R^2$ = 0.9975. The high sensitivity and equation of reliability justify the use of these methods for the detection and quantification of lupine in practice. The most sensitive indirect method for the detection of lupine in our study was the method of real-time TaqMan PCR with a detection limit 10 000-10 ppm of lupine. The most sensitive direct methot was ELISA with detection limit 2-30 ppm of lupine.

**Keywords**: lupine; food allergy; end-point PCR; real-time PCR; ELISA

## INTRODUCTION

Lupines are a relatively modern leguminous plants that can grow in a variety of soil conditions and can be particularly useful with regard to the fixation of nitrogen in the soil, respectively, in the decontamination of heavy metals from the soil **(Herridge and Doyle, 1988; Petterson and Harris, 1995)**. Lupine seeds are rich in protein, which can be used for food production, or the production of animal feed **(Edward and van Barneveld, 1998)**. There are four different types of lupines, which are of agricultural importance: White lupine (*Lupinus albus*), Narrow-leaf lupine (*Lupinus angustifolius*), Yellow lupine (*Lupinus luteus*), Andean lupine (*Lupinus mutabilis*). Lupine is considered as an inexpensive source of protein and can be grown in colder climates, which makes it an attractive crop in comparison to other protein-rich plants, such as soybean **(Holden et al., 2005)**. Lupine seeds are a rich source of non-carbohydrate polysaccharides (30-40%), oil (5-15%) with a high content of polyunsaturated fatty acids and proteins at approximately the same level as soybeans (30-40%) depending on the genotype and location **(Martínez -Villaluenga et al., 2006; Erbas et al., 2005)**. As a substitute for soybean, its popularity has grown in popularity mainly in Europe as lupine is not a genetically modified plant **(Peeters et al., 2009)**.Whereas lupine does not contain gluten and is also used in gluten-free diet and gluten-free products, for example in the productions bread or other bakery products **(Ziobro et al., 2013)**. Despite these positive aspects lupine was added to the list of allergens. Major allergens of Lupinus are storage proteins and conglutines. The two main factions are the α-and β-conglutine, the minor include γ- and δ-conglutine **(Melo et al., 1994)**. In recent years, increasingly lupine has been used mainly in the manufacture of bakery products, but also in the production of health-promoting foods in Europe. In parallel with this the number of cases of allergy to lupine also increases. Although the recorded primary sensitization to lupine occurs more often as cross-reaction in patients with pre-existing allergy to peanuts. Furthermore, a number of studies indicate that the risk of cross-reactions, clinically manifested in patients allergic to peanuts after exposure

lupine is relatively high **(Dooper et al., 2009)**. In recent years, primary and secondary sensitization of lupine was studied mostly through molecular approaches. These studies were conducted in patients allergic to peanut and lupine, and point out that a number of allergic polypeptides belongs to the following lupine protein families (α, β, γ-conglutines), which are responsible for allergic responses *in vitro* and *in vivo* **(Ballabio et al., 2013)**.

## MATERIAL AND METHODOLOGY

### *Isolation DNA from samples*

DNA was isolated using commercial GeneMATRIX Food-Extrakt DNA Purification Kit (Molecular Genetic Resources, USA). DNA obtained from isolations were used in experiment 1 and 2. In the analysis we used 17 samples of plant species from a processing plant for dehydrated soups production and lupine samples from lupine processing companies (Table 1).

### *Organization of experiment 1*

In experiment 1 classical end-point PCR was used for detection lupine in samples. Primers for detection α- and δ-conglutine in lupine were used according to **Gomez-Galan et al., (2010)**. Length of base pair for α-conglutine was 153 bp and 150 bp for the δ-conglutine that serve to detect specific DNA species of the genus Lupinus (*Lupinus albus* and *Lupinus angustifolius*) (Table 2). Primers were manufactured by General Biotech (Czech Republic).

In the optimization of experiment 1 the mixture (mastermix) polymerase GoTaq® Hot Start Polymerase (Promega, Medison, USA) was used as follows in the production of the polymerase reaction. Another polymerase that was used in the preparation of the reaction mixture (mastermix) polymerase was Isis™ DNA polymerase (MP Biomedicals, Europe). To dye the agarose gel electrophoresis, we used the following dyes: Ethidium Bromide (EtBr) and GelRed™.

The mixture of classical (end point) PCR reaction using polymerase GoTaq ® Hot Start and primers for detection of α-and δ-conglutine contain the following ingredients: 2 μL of template DNA, 0.45 μL of dNTPs, 1.20 μL of $MgCl_2$, 1.50 μL of reverse and forward primers, 0.1 μL GoTaq® Hot Start polymerase (Promega, Medison, USA), 6 μL of buffer GoTaq Flexi Buffer (Promega, Medison, USA), made up to final volume of 30 μL by means of 17. 25 μL of PCR grade water.

The composition of the reaction mixture using Isis™ DNA polymerase was as follows: 2 μL of template DNA, 0.45 μL of dNTPs, 3 μL Isis Buffer 1x (20 mM Tris-HCl pH 9.0, 25 mM KCl, 10 mM $(NH_4)$ 2 $SO_4$, 1.5 mM $MgSO_4$, 0.1% Tween 20, and stabilizers), 1.50 μL of reverse and forward primers, 0.1 μL of Isis™ DNA polymerase (MP Biomedicals Europe) made up to the final volume of 30 μL with 21. 35 μL of PCR grade water.

The amplification was conducted with a thermal cycler (PCT-150™ MiniCycler, MJ Research, Watertown, USA). Course of the PCR reaction was as follows: The PCR cycle begins by pre-incubation at 95 °C for 5 min, followed by 40 cycles of its repetition with temperature profile: denaturation at 95 °C for 15 seconds, annealing, and polymerization at 62 °C for 30 seconds. The final step in the PCR reaction was the cooling to a temperature of 25 °C for 1 second.

**Table 1** Samples used for detection lupine

| Serial number | Sample | Country of origin |
|---|---|---|
| 1 | Lupine (A) | Austria |
| 2 | Lupine (NL) | Netherland |
| 3 | Lupine (SK) | Slovakia |
| 4 | Whole Soy chunks | Austria |
| 5 | Yellow pea flour milled | Germany |
| 6 | Chickpea flour milled | Germany |
| 7 | Green pea flour milled | Poland |
| 8 | Bean flour brown milled | Spain |
| 9 | Defatted soybean powder | Austria |
| 10 | White bean flour milled parboiled | Poland |
| 11 | The whole pre-cooked brown lentils | Germany |
| 12 | Dried bean pods | Germany |
| 13 | Whole dried green peas | Poland |
| 14 | Freeze-dried green peas | Germany |
| 15 | Yellow peas cooked dehydrated | Spain |
| 16 | The whole pre-cooked brown beans | Poland |
| 17 | Soy lecithin powder | Austria |

**Table 2** Primers used for lupine detection-experiment 1 and 2

| Primer | Sequence | ID NCBI |
|---|---|---|
| CongA-F (exp. 1) | 5'- AGAAACGACTTGAGGAGACA- 3' | NCBI U74384 |
| CongA-R (exp. 1) | 5'- AGCAGCAAGTCCAAGCCA- 3' | |
| CongD-F (exp. 1) | 5'- TGTGAGCAACTGAATGAGCTTAA-3' | NCBI X53523 |
| CongD-R (exp. 1) | 5'-AAACCCACAAGTCCTAGGCAAA3' | |
| Acon.-F (exp. 2) | 5'- AGAAACGGACTTGAGGAGACA -3' | NCBI U743844 |
| Acon.-R (exp. 2) | 5'- AGCAGCAAGTCCAAGCCA -3' | |
| Acon. probe (exp.2) | FAM-ACATCTCCTGATGCATACAACCCTCAAGCTGGTAGG-TAMRA | |
| Dcon.-F (exp. 2) | 5'- TGTGGAGCAACTGAATGAGCTTAA -3' | NCBI X53523 |
| Dcon.-R (exp. 2) | 5'- AAACCCACAAGTCCTAGGCAA -3' | |
| Dcon. Probe (exp. 2) | FAM-AGCCAGAGATGCCAGTGCCGTGCATT-TAMRA | |

Agarose gel electrophoresis and - to visualize the DNA fragments of the PCR reaction - we used a 2% agarose gel. When optimizing, the methods we used were ethidium bromide dye and dye GelRed™. Electrophoresis was performed in electrophoresis trough the direct-current voltage of 75 V, for 60 minutes.

DNA samples were gel volume of 15 µL, 2 µL EtBr, or GelRed™ 1 µL weight DNA marker (ruler). In visual processing images electropherograms (Figures of agarose gel after visualization by UV emitter) were processed using the freeware software Irfanview version 4.28.

In identifying the the limit of detection, we used a decimal dilutions of samples. Decimal dilutions are made in the following way: we took 2 µL of DNA sample and have it mixed with µL ml of water bidestilovanej PCR. In this way we prepared by diluting the concentration of $10^{-1}$ to $10^{-6}$. These samples were used in determining the limit of detection in experiment 1 and 2.

***Organization of experiment 2***

In experiment 2, we have optimized the Taq Man® real-time PCR reaction. Primers for α- and δ-conglutine and Taq Man® probe were designed according to **Gomez- Galan et al., (2010)** (Table 2).

Mastermix for detection lupine - experiment 2: total reaction volume was 20 µL, PCR grade water -CYBR 10 µL $H_2O$, LightCycler® Taq Man® Master (Roche, Germany) 4 µL, Taq mix for uni (α and δ-conglutine) (Mastermix were used separately for detection of α and δ-conglutine) 4 µL (forward and reverse primer, 15 pmol.$\mu L^{-1}$ and probe 3 pmol.$\mu L^{-1}$), 2 µL of template DNA.

The analysis of samples using the method of Taq Man® real-time PCR, we used the LightCycler capillary Cycler® 1.5 (Roche, Germany) and results were analyzed using the LightCycler software version 4.5 (Roche, Germany). Protocol Taq® Man real-time PCR reaction was as follows: predenaturation 2 minutes at 50 °C, denaturation for 10 min at 95 °C, annealing and polymerization: 95 °C for 15 seconds, 63 °C for 1 minute, which were repeated 50 times, the last step was cooling to 37 °C for 1 second.

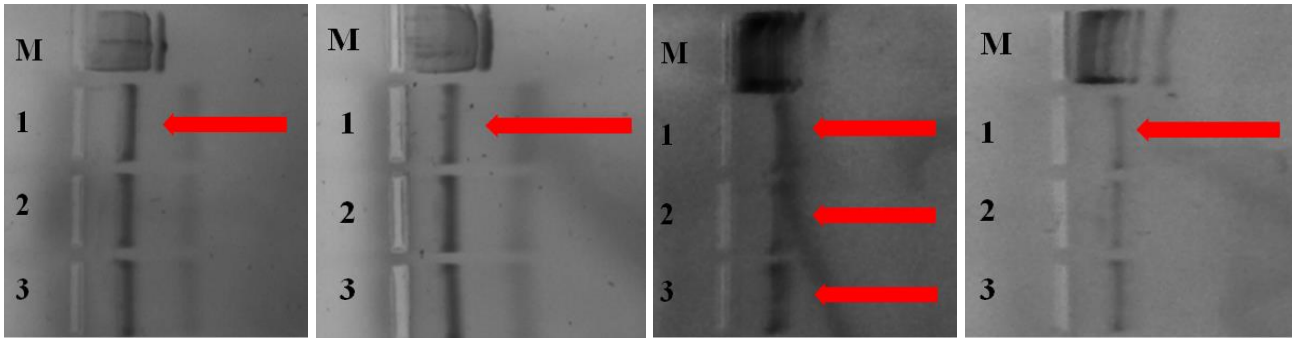
***Organization of experiment 3***

Experiment 3 focused on quantitative detection of lupine in samples using immuno-enzymatic assay ELISA. For the detection, we used 96-well ELISA (Immunolab GmbH, Germany), catalog number LUP-E01.

The limit of detection (i.e. sensitivity of the method) is 0.2 ppm for the standard curve. Limit of quantification of the assay is 2 ppm, and range generally from 2 ppm to 30 ppm. The optical density was measured at a wavelength of 450 nm at Neogen® Stat Fax® 303 ELISA reader. Calculation of concentration of lupine in the sample was performed by a calibration curve of well-defined standards, which have been a ELISA kit (n=2).

## RESULTS AND DUSCUSSION

In experiment 1, we compared the use of polymerase Isis and GoTaq Hot Start GelRed combination of dyes and ethidium bromide. The results are documented in Figure 1. The combination of polymerase and dye Isis GelRed showed better quality of DNA fragments. These same results were obtained with the primers for the α-conglutine (Figure 1A), as well as the use of primers for the δ-conglutine (Figure 1B). Whereas the results using polymerase GoTag Hot Start and ethidium bromide did not show good results (Figure 1 C, D) such the results of the first combination, we did not use it further. The limit of detection end-point PCR using Isis polymerase and GelRed dye to detect lupine using primers to detect α- and δ-conglutine is documented in Figure 2, the detection limit using alpha conglutine stood at 0.01% (Figure 2 A). The same limit of detection was also detected using primers for the detection of δ-conglutine (Figure 2 B). The limit of detection end-point PCR reactions was at level 100 ppm lupine in the sample. The results of tested samples by the end-point PCR reaction (n=3) using the primers for the detection α- and δ-conglutine are shown in Figure 3. Red arrows indicate the DNA fragments of α or δ-conglutine of lupine. Yellow arrows indicate the DNA fragments that have a larger fragment of DNA fragments of DNA than of lupine. The blue arrow indicates the presence of dimer DNA. Green arrows indicate DNA fragments that have a lower DNA fragment as a DNA fragment of lupine.




**1 A** **1 B** **1 C** **1 D**

**Figure 1 A-D** Comparison of combination of polymerases Isis and GoTaq , dye's GelRed and Ethidium Bromide (EtBr). Electroforeogram of PCR: samples 1-3 using Isis and GelRed and primers for α-conglutine (A) δ-conglutine (B). Electroforeogram of PCR: samples 1-3 using GoTaq® HotStar and EtBr and primers for α-conglutine (C) δ-conglutine (D).
Legend: M lane - weight marker, lane 1 - sample lupine from Austria, lane 2 - sample lupine from the Netherlands, lane 3 - sample lupine from Slovakia, red arrow shows the DNA fragment

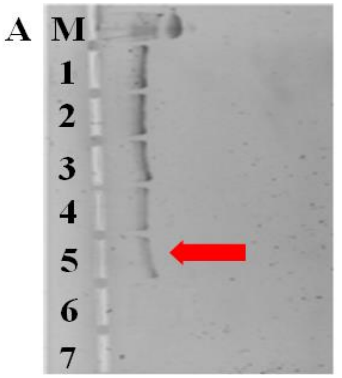



**Figure 2 A, B**

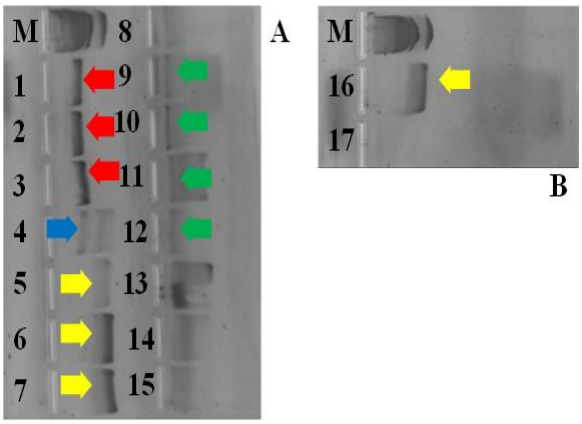



**Figure 3 A, B**

**Figure 2 A, B**
Electroforeogram of PCR: sample of lupine using Isis ™ polymerase and dye GelRed ™ and primers for α-conglutine (A) and δ-conglutine (B) decimal dilutions. Legend: lane M-weight marker, lane 1 - 100% of the DNA, lane 2 - 10% of the DNA, lane 3 - 1% of the DNA, lane 4 - 0.1% of the DNA, lane 5 - 0.01% of the DNA, lane 6 - 0.001% DNA, lane 7 to 0.000% 1 DNA lupine, red arrow indicates a DNA fragment of DNA concentration of 0.01% lupine (100 ppm).

**Figure 3 A, B**
Electroforeogram PCR samples 1-15 (A), 16-17 (B) using the polymerase Isis™ and dye GelRed™ and primers for α-conglutine. Legend: M-lane weight marker, lane 1 - lupine (Austria), lane 2 - lupine (Netherlands), lane 3 - lupine (Slovakia), lane 4 - soya chunks, lane 5 - yellow pea flour, lane 6 - chickpea flour ground, lane 7 - green pea flour, lane 8 - brown bean flour, lane 9 - defatted soybean powder, lane 10 - pre-cooked white bean flour, lane 11 - pre-cooked brown lentils, lane 12 - string beans dried, lane 13 - green peas dried, lane 14 - green peas freeze-dried, lane 15 - yellow peas cooked dehydrated, lane 16 - whole bean brown parboiled, lane 17 - soy lecithin powder, red arrows indicate the presence of DNA fragments of α-conglutine, yellow arrows show the DNA fragments are larger than fragments of α-conglutine green arrows indicate the DNA fragments that are smaller than α-conglutine, black arrow shows the sequence of dimer DNA of soy.

As shown in Figures 3 A, B using the polymerase Isis™ and dye GelRed ™ and primers for α-conglutine are securely detect the presence of the samples of lupines. In lane 4, we recorded dimer. It was a sample of soy. In assessing the need to monitor very closely the position and size of the fragments. In lanes 5, 6, 7 and 16, we have seen more than a fragment of fragments of α-conglutine, the size if which is 153 bp. In lanes 9, 10, 11 and 12 are recorded in reverse fragments, which are less than a length of said fragment of the α-conglutine. Using the same combination using primers for the δ-conglutine are shown in Figure 4 A, B.

Using primers for alpha and delta conglutine brought the same results in detection of lupine. The result of the experiment 1 was to reach the detection limit at 100 ppm level of lupine.

In experiment 2 we use Taq Man real-time PCR method for detection of lupine. We used primers for α- and

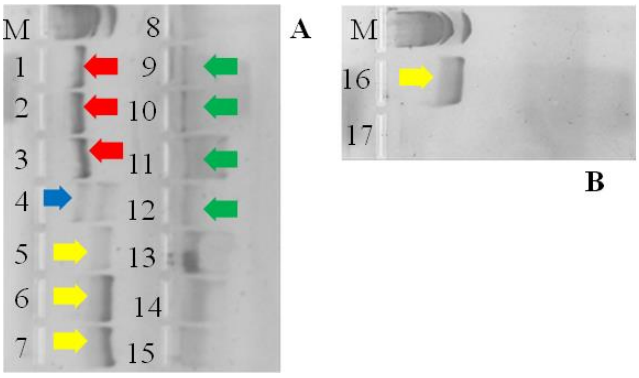



**Figure 6 A, B**
Electroforeogram PCR samples 1-15 (A), 16-17 (B) using the polymerase Isis™ and dye GelRed™ and primers for δ-conglutine. Legend: M-lane weight marker, lane 1 - lupine (Austria), lane 2 - lupine (Netherlands), lane 3 - lupine (Slovakia), lane 4 - soya chunks, lane 5 - yellow pea flour, lane 6 - chickpea flour ground, lane 7 - green pea flour, lane 8 - brown bean flour, lane 9 - defatted soybean powder, lane 10 - pre-cooked white bean flour, lane 11 - pre-cooked brown lentils, lane 12 - string beans dried, lane 13 - green peas dried, lane 14 - green peas freeze-dried, lane 15 - yellow peas cooked dehydrated, lane 16 - whole bean brown parboiled, lane 17 - soy lecithin powder, red arrows indicate the presence of DNA fragments of δ-conglutine, yellow arrows show the DNA fragments are larger than fragments of α-conglutine green arrows indicate the DNA fragments that are smaller than δ-conglutine, black arrow shows the sequence of dimer DNA of soy.

δ-conglutine and Taq Man probe to detect lupine. As in experiment 1, we used a decimal dilutions to determine the detection limit for the detection of lupine in samples. The result of detection limit using α-conglutine and Taq Man probe show Figure 5. Figure 6 show reliability of used method (primers for α-conglutine and Tag Man probe).

As Figure 5 demonstrates, the reaction showed a very high reliability, the reliability of which is documented by the equation $R^2$ = 0.9767. Thus, a sample of lupine concentration of 0.001% (10 ppm) can be detected with reliability of 97.67% using primers for the detection of α-conglutine. For the detection of lupine by Taq Man real-time method, we used the primers for the detection of delta conglutine. Figure 6 shows the progress of the reaction, where we have used a decimal dilutions of the sample for the detection of lupine.

As seen in Figure 6 as well as the use of primers for the detection of delta conglutine, we obtained the same result; that is, 0.0001% (10 ppm). Reliability of the method is presented in Figure 6.

When comparing the use of primers for the detection of α and δ-conglutine, the delta conglutine we achieved better results (better than CP values) and also better values of reliability $R^2$, than when we used primers for detection α-conglutine.

Table 3 is a table of values of CP (i.e. crossing-point values that indicate where within the cycle of the PCR reaction nonspecific background was exceeded) that we obtained a PCR reaction in all tested samples.

The result of experiment 2 was to achieve a detection limit of 10 ppm using primers alpha and delta conglutine. The CP values of lupine using primers for the detection of α-conglutine was 24.85 ±0.12 and the reliability equation was $R^2$ = 0.9767. The CP lupine values using primers for the detection of δ-conglutine was 22.52 ±0.17 and the reliability equation was $R^2$ = 0.9925.

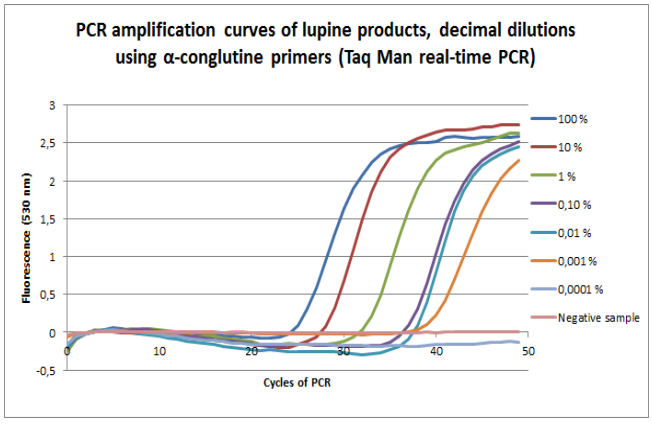



**Figure 4** Progress of the increase in fluorescence of PCR products samples of lupine using α-conglutine and Taq Man probe

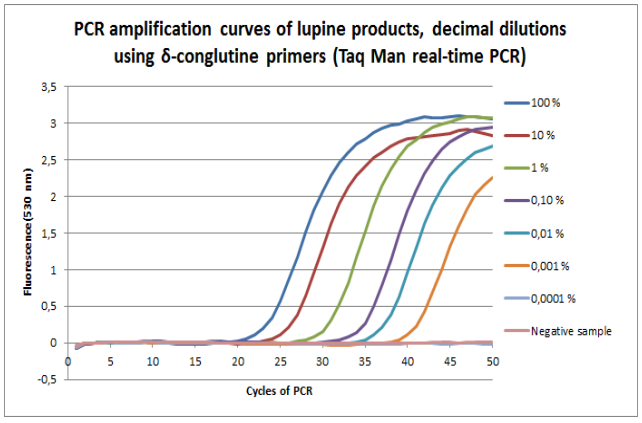



**Figure 5** Progress of the increase in fluorescence of PCR products samples of lupine using δ-conglutine and Taq Man probe

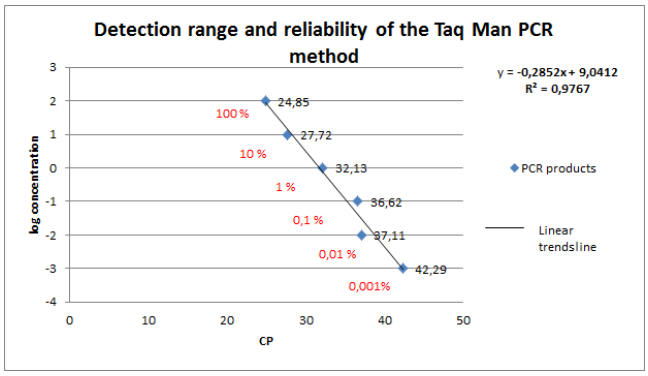



**Figure 7**

Detection range and reliability Taq Man method using α-conglutine for the detection of of lupine

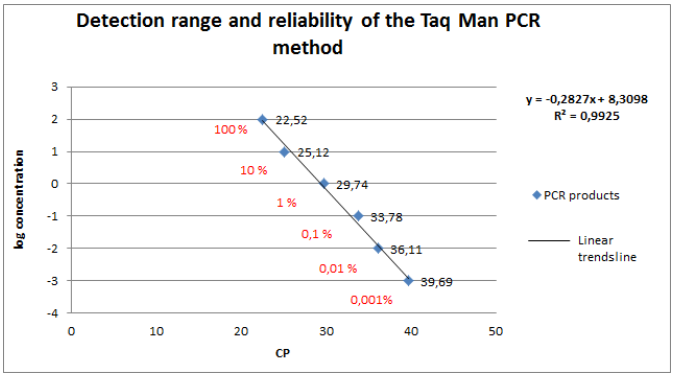



**Figure 8**

Detection range and reliability Taq Man method using α-conglutine for the detection of of lupine

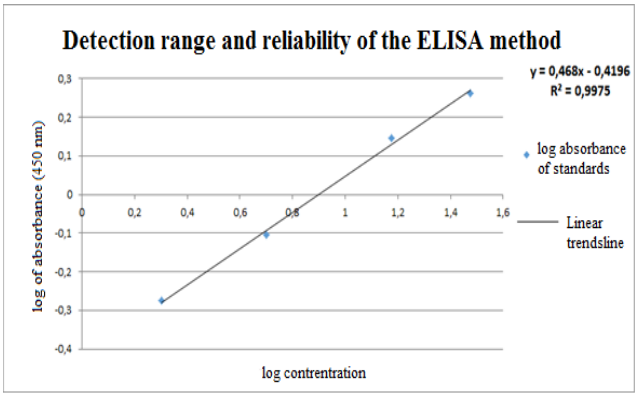



**Figure 9** Reliability and detection limit of the ELISA method (n=2)

In experiment 3 we used commercial ELISA kit for the detection of lupine in food. We measured the absorbance of the sample and then interpolated the results after which we got lupine concentration in ppm ($mg.kg^{-1}$). The number of measurements was (n = 2). Measurement results shown in Table 4. Samples 1-3 had more than 30 ppm of lupine in the sample, because these samples were samples of lupine. Sample 4 had more than 30 ppm although they were soybean, and thus presents a potential risk. We found traces of lupine in sample 7 pea - green (2.6 ppm), 12 - dried bean pods (2.3 ppm). Sample 12 - white bean was 22.55 ppm lupine in the sample, which also carries a potential risk.

Red numbers in Table 4 indicate values under detection limit. The result of this ELISA test was to confirm the presence of the traces of lupine, which we already observed in experiments 1 and 2.

The result is 3 experiment detected the presence of lupine traces in 5 samples, which could potentially pose a risk to sensitive consumers. High detection sensitivity and detection limit of 2-30 ppm predisposes ELISA as a highly effective tool in the detection of lupine in samples. High level of reliability ($R^2$ = 0.9975) makes ELISA highly reproducible and an accurate method for the determining of the presence of lupine as a food allergen.

Tools for the detection and quantification of lupine have been developed in various forms such as food matrix, using wheat flour **(Scarafoni et al, 2009, Demmel, et al., 2012)**, detection of traces of lupine in food **(Gomez-Galan et al., 2010)**, the methods for simultaneous detection of lupine and soybean using the mitochondrial DNA in processed foods **(Gomez-Galan et al., 2011)**. Methods have been developed for the detection of lupine flour as **(Gomez-Galan et al., 2010)**, but also as processed foods containing lupine examples: "Lupine tofu" cookies containing lupine bread containing lupine **(Gomez-Galan et al., 2010)** ice cream containing lupine **(Demmel, 2013)** or pizza **(Demmel et al., 2011)**.

Suitability using primers for the detection of α-and δ- conglutine was confirmed in the work of **Gomez-Galan et al., (2010)**. Besides the above mentioned primers **Demmel, et al. (2011)** used for the construction of the target DNA molecules with overlapping ends of the amplicons primers Limo-162 and ov'-Limo-62 to identify *Lupinus angustifolius* and primer length of the product was 101 bp.

Compared with the results of the authors **Scarafoni et al., (2009)**, who used to detect primer pair for the detection of γ-conglutine in the sample, we have achieved better results. We used a modified end-point PCR method and sensitivity reached, as mentioned above, a 100 ppm detection of lupine in the sample using a primer pair for both δ-conglutine, as well as α-conglutine. When using γ-conglutine, **Scarafoni et al. (2009)** were able to detect more types of lupines in samples - *Lupinus angustifolius*, *Lupinus albus* and *Lupinus luteus* (our chosen method is capable of detecting the presence of *Lupinus albus* and *Lupinus angustifolius*). With their choice using a primer

**Table 3** CP values of samples using primers for α and δ-conglutine

| Nr. | Sample | CP value α-conglutine | CP value δ-conglutine |
|---|---|---|---|
| 1 | Lupine (A) | **24.92 ±0.38** | **22,62 ±0.12** |
| 2 | Lupine (NL) | **25.64 ±0.57** | **23.15 ±0.49** |
| 3 | Lupine (SK) | **24.85 ±0.12** | **22.52 ±0.17** |
| 4 | Whole Soy chunks | not detected | 39.60 ±0.59 |
| 5 | Yellow pea flour milled | not detected | not detected |
| 6 | Chickpea flour milled | 35. 94 ±0.45 | 35.88 ±0.47 |
| 7 | Green pea flour milled | 35.6 ±0.35 | 36.02 ±0.38 |
| 8 | Bean flour brown milled | not detected | not detected |
| 9 | Defatted soybean powder | not detected | not detected |
| 10 | White bean flour milled | 34.58 ±0.55 | 35.39 ±0.25 |
| 11 | The whole pre-cooked lentils | not detected | not detected |
| 12 | Dried bean pods | not detected | not detected |
| 13 | Whole dried green peas | 37.45 ±0.35 | 36.85 ±0.60 |
| 14 | Freeze-dried green peas | not detected | not detected |
| 15 | Yellow peas cooked | not detected | not detected |
| 16 | The whole pre-cooked beans | not detected | >45 |
| 17 | Soy lecithin powder | 38.12 ±0.47 | 37.57 ±0.64 |

**Table 4** Absorbance and concentration of lupine (ppm) in samples

| Nr. | Sample | **Absorbance at 450 nm** (n=2) | **ppm (mg.kg$^{-1}$)** (n=2) |
|---|---|---|---|
| 1 | Lupine (A) | >3 | >30 |
| 2 | Lupine (NL) | >3 | >30 |
| 3 | Lupine (SK) | >3 | >30 |
| 4 | Whole Soy chunks | >3 | >30 |
| 5 | Yellow pea flour milled | 0.551 | 2.213 |
| 6 | Chickpea flour milled | 0.352 | 0.852 |
| 7 | Green pea flour milled | 0.5995 | **2.650** |
| 8 | Bean flour brown milled | 0.4055 | 1.186 |
| 9 | Defatted soybean powder | 0.482 | 1.669 |
| 10 | White bean flour milled | 1.1635 | **22.554** |
| 11 | Pre-cooked lentils | 0.5355 | 2.103 |
| 12 | Dried bean pods | 0.5585 | **2.344** |
| 13 | Whole dried green peas | 0.493 | 1.751 |
| 14 | Freeze-dried green peas | 0.55 | 2.214 |
| 15 | Yellow peas cooked | 0.469 | 1.571 |
| 16 | Pre-cooked beans | 0.489 | 1.760 |
| 17 | Soy lecithin powder | 0.877 | **6.042** |

pair for the detection of γ-conglutine it was possible to detect not only the sample to a concentration of 0.1%, equivalent to 1000 ppm, and so that we used the method of one, respectively two, logarithmic sensitive board.

At present, despite its relatively good detection capability the end point PCR reaction is not used very often. It is mainly used for the validation of specificity primer real-time PCR.

The disadvantage of this method is that in a single reaction we can look at more allergens at the same time and its elaborateness: the method requires a very high quality of DNA extraction, making the reaction mixture, producing an agarose gel and applying the PCR products

and the visualization by UV visualizer respectively, a computer program for figure processing. Therefore at present among the PCR reactions are the most common real-time PCR reaction. To speed up the analysis and of course, the number of samples analysed, we used duplexes **(Gomez-Galan et al., 2011)**, tetraplex **(Köppel et al., 2010)**, or other multiple access **(Waiblinger et al., 2014),** making it possible to analyze large amounts of allergens in real time. The most commonly used by real-time PCR reactions are Taq Man **(Gomez-Galan et al., 2011)**, SYBR Green I real-time PCR **(Scarafoni et al., 2009)**, methods based on FRET (fluorescence resonance energy transfer) **(Mair et al., 2014)** and others. Their advantage is to reduce the analysis time by about half compared with end-point PCR.

In the study by **Waiblinger et al. (2014)** interlaboratory tests were conducted (interlaboratory tests) between 17 participating laboratories, where using a single multiplex determined the presence of the following allergenic ingredients: sesame seeds, almonds, Brazil nuts and lupine. These tests were carried out and compared with each other. Using real-time PCR methods proved similarly as we detected in the sample, lupine, six of the seven PCR System at 10 ppm. Like us, most laboratories have reached the level of reliability equation $R^2$ = 0.99. Based on their work and results, we would like to note that our results, both the qualitative detection of lupine using real-time PCR results were closer to certified laboratories abroad. **Ecker et al. (2013)** used two ELISA tests in their work to detect and quantify lupine competitively. These products have two types of antibodies for the detection of lupine IgG antibody produced in rabbit and the body IgY antibodies produced in the body of a chicken. 32 plant samples were tested and found that both types of ELISA assays showed cross-reaction with pecans. Although the method used by us was not competitive with the ELISA method, but the sandwich ELISA method and kit according to the manufacturer set ELISA detected cross-reaction with chickpeas, lentils, soy flour, and a cooked and handled heat-treated flour showed greater cross-reaction by the manufacturer. Their ELISAs were able to detect the presence of *Lupinus albus*, *Lupinus luteus* and *Lupinus angustilofius* in the samples. We were able to detect the presence of *Lupinus albus* and *Lupinus angustifolius* as we had only these two types of lupines available. Through IgG ELISA they were able to detect 50 ppm of lupine in bread, vegetarian burgers and biscuits. The detection limit for IgY test was 50 ppm for vegetarian meatballs and bread, and 100 ppm for crackers. When we used the sandwich ELISA method we detected the presence of lupine ranging from 2.3 to 22.5 ppm.

## CONCLUSION

Using end-point, we were able to detect the presence lupine at 100 ppm. Using Taq Man real-time, we were able to detect the presence of 10 ppm of lupine in the sample. Using a commercial ELISA kit, we were able to detect the presence of lupin in the range from 2 to 30 ppm in the sample. The most sensitive indirect method for the detection of lupine in our study was the method of real-time TaqMan PCR with a detection limit 10 000-10 ppm of lupine. The most sensitive direct method was ELISA with detection limit 2-30 ppm of lupine.

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