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Martina Koeberl, Michael F. Sharp, Rongkai Tian, Saman Buddhadasa, Dean Clarke, James ROBERTS

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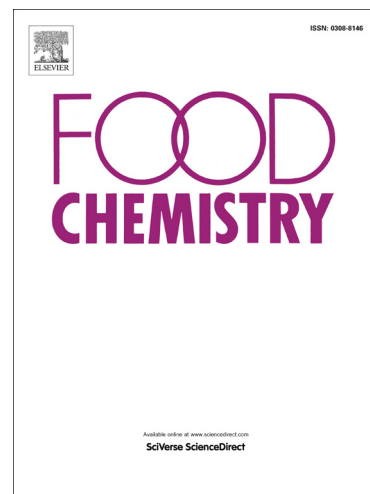
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TITLE:

Lupine allergen detecting capability and cross-reactivity of related legumes by ELISA.

AUTHORS:

Martina KOEBERL^{a,*}, Michael F. SHARP^a, Rongkai TIAN^{a,b}, Saman BUDDHADASA^a,
Dean CLARKE^a, James ROBERTS^a

Authors' Affiliation Address:

^aNational Measurement Institute, 1/153 Bertie Street, Port Melbourne, VIC, 3207,
Melbourne, Australia

Authors' Affiliation

^aNational Measurement Institute, 1/153 Bertie Street, Port Melbourne, VIC, 3207,
Melbourne, Australia

^bChemical and Biomedical Engineering, University of Melbourne, Grattan Street,
Parkville, VIC, 3010 Melbourne, Australia

martina.koeberl@measurement.gov.au, msharp@svi.edu.au, tianrongkai415@gmail.com,
saman.buddhadasa@measurement.gov.au, dean.clarke@measurement.gov.au,
james.roberts@measurement.gov.au

CORRESPONDING AUTHOR:

*Martina Koeberl, PhD

martina.koeberl@measurement.gov.au

Allergen Research Scientist, Analytical Service Branch, National Measurement Institute, 1/153
Bertie Street, Port Melbourne, VIC 3207, Melbourne, Australia

Email: Martina.Koeberl@measurement.gov.au

Phone: +61 3 9644 4888

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ABSTRACT

Lupine belongs to the genus *Lupinus* and includes three species commonly consumed by humans. The *Lupinus* genus is closely related to other legumes, such as peanuts, soya, chickpeas, peas, lentils and beans. However, the consumption of lupine (and related legumes) can cause severe allergenic reactions. Therefore, reliable analytical detection methods are required for the analysis of food samples. In this study three commercially available ELISA test kits were analyzed for the detection capability of three common lupine species, as well as cross-reactivity to related legumes. All three ELISA test kits could detect the lupine species, though with different sensitivities. Cross-reactivity varied for the ELISA test kits and all showed some cross-reactivity to related legume samples analyzed.

KEYWORDS:

Lupinus angustifolius (Blue Lupine or Australian Sweet Lupine)

Lupinus albus (White Lupine)

Lupinus luteus (Yellow Lupine)

ELISA test kit comparison

Lupine cross-reactivity

1. Introduction

Lupine belongs to the genus *Lupinus* and is part of the *Leguminosae* family. Other common foods belonging to the *Leguminosae* family are peanuts, soya, peas, beans, chickpeas and lentils. There are more than 450 different lupine species (Röder, Kleiner, Sachs, Keil, & Holzhauser, 2013), however, three main species have agricultural significance as they are used in human consumption, namely *Lupinus angustifolius* (Blue Lupine or Australian Sweet Lupine), *Lupinus albus* (White Lupine), and *Lupinus luteus* (Yellow Lupine) (de Jong et al., 2010; Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008; Ecker & Cichna-Markl, 2012; Shaw, Roberts, Grimshaw, White, & Hourihane, 2008). A fourth species, *Lupinus mutabilis* (South American Lupine), is uniquely produced and consumed in South America (Jappe & Vieths, 2010).

Traditionally lupine seeds were consumed in Mediterranean areas. More recently the consumption of lupine flour has vastly increased due to its nutritional value, especially in Australia, Europe and USA (Cabello-Hurtado et al., 2016; Jappe & Vieths, 2010). The usage of lupine flour in food products covers a wide range, e.g. pasta, bread, cake, pizza, cream cheese, tofu, sausage, spices, jam, noodles and ketchup (Cabello-Hurtado et al., 2016; de Jong et al., 2010; Guillamon et al., 2010; Jappe & Vieths, 2010; Röder et al., 2013; Shaw et al., 2008). Lupine is also used as a replacement for soya, milk and egg white in vegan food (de Jong et al., 2010; Jappe & Vieths, 2010) and/or gluten-free food products (Cabello-Hurtado et al., 2016; de Jong et al., 2010; Guillamon et al., 2010; Jappe & Vieths, 2010; Röder et al., 2013; Shaw et al., 2008). The different usages of lupine in food production often results in poor visibility of lupine when used as a food ingredient in food products. Although lupine has been traditionally used and cultivated in Europe and South America, Australia grows 75-80% of the total world lupine crop and therefore is the world's largest producer (Duranti et al., 2008; Jappe & Vieths, 2010).

Lupine is high in protein (20-48%), high in fibre, and low in fat (Cabello-Hurtado et al., 2016; de Jong et al., 2010; Röder et al., 2013). Reported protein content are for blue lupine 22-29.8%, white lupine 30.1-31.2% and yellow lupine 35.2-38.1% (Röder et al., 2013). The high protein content is composed of mainly seed storage proteins, which are globulins and represent approximately 87% of the total protein content (Guillamon et al., 2010; Jappe & Vieths, 2010). The globulins are further subdivided into α -conglutin (3-4%), β -conglutin (43-45%), γ -conglutin (5-6%), and δ -conglutin (3-13%) (Cabello-Hurtado et al., 2016; Guillamon et al., 2010; Jappe & Vieths, 2010).

The prevalence of lupine allergy in the general population is unknown. However, with the increased consumption of lupine, increased incidences of lupine allergy are being reported (Kaw, Hefle, & Taylor, 2008). Lupine allergy, in the lupine allergic population, can occur without documented evidence of clinical or laboratory cross-reactivity to other legumes (Peeters et al., 2007). The estimated prevalence of lupine allergy in the suspected food allergic population ranges from 0.3% to 8%, with the prevalence of children being 5% (de Jong et al., 2010; Hieta, Hasan, Makinen-Kiljunen, & Lammintausta, 2009; Jappe & Vieths, 2010). Lupine allergy can be a severe allergy, including the potential for anaphylactic shock and fatality (Jappe & Vieths, 2010; Kaw et al., 2008; Röder et al., 2013). Lupine allergy possibly equals peanut allergy in its sensitivity and severity (Peeters et al., 2007).

Lupine allergy is often reported in patients with allergies to other legumes, such as soya, pea, lentil, chickpea and particularly peanut (Jappe & Vieths, 2010; Kaw et al., 2008; Peeters et al., 2007). Food products from the *Leguminosae* family contain a high content of seed storage proteins, which share proteins with similar structures, including similar epitope regions. Therefore patients sensitized to one legume allergen could cross-react to a structurally similar protein from another legume (Berkner et al., 2009; Shaw et al., 2008). Clinical cross-reactivity between lupine and peanut is reported from 17.1% up to 65%

(Jappe & Vieths, 2010). Clinical cross-reactivity between lupine to other legumes (pea, lentil and bean) has been reported to be 31% in atopic children (Jappe & Vieths, 2010; Peeters et al., 2007). Lupine IgE cross-reactivity has been reported for peanut, soya, lentil, bean, chickpea and bean (Jappe & Vieths, 2010). The cross-reactivity of lupine sensitized patients has been reported to be 59-72% (de Jong et al., 2010; Hieta et al., 2009; Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017) and 52-55% (Hieta et al., 2009; Hoffmann et al., 2017) for soya and pea, respectively. Peanut allergy is currently the most prominent legume allergy, followed by soya (Peeters et al., 2007).

Due to the severity of lupine allergy, legume cross-reactivity and its abundance in processed food, the European Union (EU Regulation No. 1169/2011) has made lupine a mandatory allergen, to be labelled on all lupine containing food products (Council, 2011). Changes to the Australian and New Zealand Food Standards Code in May 2017 made lupine a mandatory allergen, now to be labelled on food products containing lupine in Australia and New Zealand ((FSANZ), 2017). The detection of allergens for mandatory labelling, supporting regulation, requires appropriate analytical tools. Accurate methods for food allergen analysis are essential, due to an increasing allergenic population. False negative results, in the worse-case scenario, could lead to death of allergic individuals. On the other side, false positive results lead to unwarranted recalls of food products, less food choices, increased food costs and a loss of trust and reputation of the analytical community. False positive results can occur when utilizing antibody-based methods, such as enzyme-linked immunosorbent assay (ELISA), due to cross-reactivity of antibodies between the target and related species with similar proteins. Analytical cross-reactivity is not always clinical relevant, e.g. almond with mahleb cross-reactivity (Daly, Ansari, Haubl, Rogers, & Brunner, 2018). In this study, investigating lupine ELISA test kit cross-reactivity with related legumes, the cross-reactivity is clinically relevant. Nevertheless, cross-

reactivity from a method accuracy and/or regulatory point of view are undesirable (Lacorn, Lindeke, Siebeneicher, & Weiss, 2018).

ELISA is the most widely used technique for the detection and identification of food allergens. Not all currently available lupine ELISA test kits detect all three common species of lupine (*Lupinus angustifolius*, *Lupinus albus*, *Lupinus luteus*). The aim of this study was to investigate these three lupine species and 24 commercial food products consisting of related legumes using three different lupine ELISA test kits for lupine quantification. Lupine quantification ELISA test kits have been assessed from five different manufacturers, namely ELISA Systems®, Immunolabs®, Neogen®, R-Biopharm® and Romer Labs®. Immunolabs® and Neogen® state that their ELISA test kit do not detect all three lupine species. Therefore test kits from ELISA Systems®, R-Biopharm® and Romer Labs® were used for analysis of three lupine species and 24 commercial food products from related legumes.

2. Materials and methods

2.1. Lupine ELISA test kits.

Three commercially available ELISA test kits were used in this study: Lupin residue from ELISA Systems® Pty Ltd. Australia (purchased through ELISA Systems®, Australia); RIDASCREEN® fast lupine from R-Biopharm®, Germany (purchased through R-Biopharm®, Australia); and AgraQuant® lupine from Romer Labs®, Austria (purchased through Arrow-Scientific, Australia).

2.2. Sample preparation.

The lupine species analyzed are summarized in table 1 and the product samples analyzed summarized in table 2. The different lupine species were supplied as whole lupine

beans/seeds. Lupine seeds were ground with a blender (Breville, Australia) to achieve a fine homogenized powder.

One negative and one positive control sample were analyzed with each ELISA test run. The negative control sample was commercially available custard powder. The positive control was the same custard powder, spiked with 10 mg/kg fine ground commercial available lupine flour.

To assess if different lupine species can be detected by the ELISA test kits in a matrix, one positive sample was generated by using finely ground lupine species powder. The lupine species mixture represents a total final spike of lupine species of 10 mg/kg in custard powder (negative control), consisting of equal amounts of blue lupine, white lupine and yellow lupine. It was ensured that the positive control and the lupine species mixture are well homogenized before applying the to the extraction process.

The powders of all samples or liquid samples were extracted according to the respective ELISA test kit manufacturer's protocols. All samples were analyzed directly without any further dilution, whereas the lupine species and lupine flour extracts were serially diluted: a) 1:10,000; b) 1:100,000 c) 1,000,000. The extracted solutions were analyzed in duplicate ELISA wells, following the individual ELISA test kit protocols. The absorbance of individual samples and ELISA test kits standards were measured at the wavelength specified by each test kit, using a microtiter plate reader (Benchmark Plus microplate spectrophotometer (Bio-Rad, Australia)).

ELISA test kits for lupine quantification have been assessed from commonly used or available in Australia ELISA test kit manufacturers, namely ELISA Systems®, Immunolabs®, Neogen®, R-Biopharm® and Romer Labs®. Two manufacturers state that the ELISA test kits are not capable of detecting different lupine species. Hence, experiments were carried out only using test kits from ELISA Systems®, R-Biopharm® and

Romer Labs®. To ensure this is an independent study the performance of the different test kits will not be revealed according to the manufacturers. The results and discussion of this manuscript will be referred to the three tested kits as ELISA test kit A, ELISA test kit B and ELISA test kit C.

The standard curves of the different test kits were analyzed in triplicate. The mean of the absorbance and the standard deviation (SD) were plotted in figure 1. The variation of the detection capability of different lupine species for the ELISA test kits was determined by calculating the SD, based on the absorbance, of the diluted lupine extract in the reportable range of the ELISA test kit.

According to the test kit manufacturer's instructions, the limit of quantification (LOQ) corresponds to the lowest standard provided. Samples that measured an absorbance (and calculated value) between the Limit of Detection (LOD) and LOQ were repeated. The measured absorbance from all samples analyzed was divided by the absorbance of the lowest standard provided by the test kit manufacturer. The obtained ratio of this division (will be referred as "fold increase" in the remaining article) was plotted as bar graphs in figure 2 and figure 3. The area between the calculated fold increases of 0.5-1 are shaded in grey (as shown in figure 2 and figure 3). The fold increase of 1 equals the LOQ for the test kits, and a fold increase of 0.5 equals half of the LOQ ($\frac{1}{2}$ LOQ). Fold increases higher than six are indicated with an arrow, as these values are outside the standard range. The SD, based on the absorbance, was calculated for all lupine species for each test kit to interpret the detection variability for different lupine species.

2.3. Calculation of relative percentages.

The related legume samples analyzed were pure food matrices. The relative percentage of the pure food product required to generate a positive result at the LOD level was calculated. Following is an example of the calculations. 100% pure peanut flour had a

response on ELISA test kit A of 1.12 (Factors shown in figure 3). To generate a response at the LOD level of ELISA kit A, 15.4% peanut flour in a food matrix would be required. Applying these calculations the matrix effect of other food ingredients is not considered.

2.4. Protein sequence alignment.

Protein sequences of proteins from lupine species and related legumes have been aligned and analyzed for their amino acid sequence homology using Clustal Omega (Clustal Omega multiple sequence alignment program; <https://www.ebi.ac.uk/Tools/msa/clustalo/>). Supplementary table 1 summarizes the details about the proteins used for this alignment. Selected proteins were reported by Foley et al. (Foley et al., 2011), the current registered allergens by the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Sub-committee (IUIS) (accessed 30. June 2017) and current available Pubmed sequences (<https://www.ncbi.nlm.nih.gov/pubmed/>, accessed 30. June 2017). The allergenic protein sequences available for chickpea are only predicted sequences (Pubmed) and have therefore not been analyzed in this study.

3. Results and discussion

3.1. Comparison of ELISA test kits.

The analysis of food allergens is very challenging; one reason being that ELISA test kits have several disadvantages (Koeberl et al., 2018). Nevertheless, antibody-based methods (ELISA) are currently the most common technique for the analysis of food allergens. Specific allergen quantification from the ELISA test kits cannot be compared as the ELISA test kit manufacturers generate their own antibodies, conjugates, standards and calibrants used in their respective ELISA test kits. This leads to different results and/or units when analyzing the same sample with different ELISA test kits. Due to intellectual property

concerns, detailed information about antibodies, conjugates, calibrants and standards are not provided by the manufacturer to the laboratory analyst. With current ELISA methods it is difficult to definitively quantify the real allergenic protein concentration in analysis. To improve measurements of food allergens (certified) reference materials are needed as well as harmonization of units (Koeberl et al., 2018).

The three lupine ELISA test kits tested in this study were used to detect the presence of lupine in food products. The three ELISA test kits differ from each other in the detection range, LOD, LOQ and sample extraction buffer ratio. The LOQ and LOD for the three different lupine ELISA test kits varied. As stated in the respective test kit instruction manuals, the standards and antibodies of R-Biopharm® and Romer Labs® are based on lupine proteins, whereas ELISA Systems® use lupine flour proteins. The specificity of the antibody is important for the detection capability of lupine species as well as cross-reactivity. However, the specificity of antibodies used in commercial available ELISA test kits is not available. Commercially available ELISA test kits from different manufactures are often based on different proteins for the same food allergen. This means that results between test kits can be difficult to compare (e.g. lupine protein or lupine flour protein).

Figure 1 shows the mean absorbance of the individual provided standards of the test kit (n=3), including the SD. The given concentration of the individual standards is displayed in figure 1 as dots. Visualized in figure 1, the targeted lupine content and the resulting lupine detection range are different for each test kit. Whereas the absorbance values measured for the provided standards in all test kits are similar. This highlights that the allergen quantification obtained by different ELISA test kits cannot be directly compared. The availability of (certified) reference materials would improve these discrepancies.

3.2. *Lupine species detection capability.*

Three lupine ELISA test kits were used in this study to detect the presence of lupine in food products. Lupine detection included the three most common lupine species in food and feed industry, namely blue lupine (one cultivated variety, grown in two different locations), white lupine (two different cultivated varieties, grown in two different locations) and yellow lupine (one cultivated variety, grown in one location). Lupine flour, a lupine species mixture (material and methods), a lupine food product, a negative control and a positive control were also analyzed. Figure 2 shows the fold increase for the different lupine samples analyzed. The region between the calculated fold increase of 0.5-1 is shaded in grey (as shown in figure 2 and figure 3). The fold increase of 1 equals the LOQ for the test kits, and a fold increase of 0.5 equals $\frac{1}{2}$ LOQ.

All three ELISA test kits investigated in this study positively detected each of the different lupine species. However, the response of lupine samples varied depending on the ELISA test kit. Similar variations in the response were reported by Roeder et al. (Röder et al., 2013). Possible explanations for variations are the antibodies used for the particular ELISA test kit and the difference in the protein profile for lupine species/cultivars. Moreover, the protein sequences for the same protein can vary between species and cultivars, e.g. blue lupine has been reported to be 60-70% identical in the protein sequences for β -conglutin with white lupine (Guillamon et al., 2010).

Results from the current study show, the lowest response for all samples was observed for ELISA test kit A. The samples were homogenized and diluted as described in the material and method section. Results obtained in the reportable range of ELISA test kits were diluted 1:10,000 for ELISA test kit A and diluted 1:100,000 for ELISA test kit B and ELISA test kit C. Based on the SD the lowest variation for blue lupine, white lupine and yellow lupine was observed with ELISA test kit C (SD 0.32), followed by ELISA test kit A (SD 0.58). The most variation showed ELISA test kit B (SD 1.00). This variability for different

lupine species was lower than reported by Ecker et al. (Ecker & Cichna-Markl, 2012; Ecker, Ertl, & Cichna-Markl, 2013), and similar to the investigation by Roeder et al. (Röder et al., 2013).

Overall, lupine flour gave the highest response for all samples investigated. This was independent of whether the lupine flour was analyzed with or without a matrix. The whole lupine seeds/lupine beans were used in this study to analyze the different lupine species. However, the commercial available lupine flour is generated by de-hulling lupine seeds before milling (de Jong et al., 2010). This manufacturing difference can explain the higher sensitivity for lupine flour. Lupine proteins and allergens have been reported to be stable towards thermal treatment (Christina Ecker et al., 2013; Jappe & Vieths, 2010). Similar results have been observed in this study for the analyzed lupine product (soaked and fermented lupine seeds in chili), assuming that food product had undergone heat sterilization.

3.3. Cross-reactivity of lupine related food commodities.

Twenty-four food samples, a negative control and a positive control were analyzed using three lupine test kits to investigate cross-reactivity, summarized in table 2. Figure 3 shows the fold increase based on the lowest standard for all samples analyzed. The area between LOQ and $\frac{1}{2}$ LOQ is highlighted in grey in figure 3; showing results that would need further investigation for specific ELISA test kits.

All samples investigated show variations in response and cross-reactivity for the three different ELISA test kits. In summary, ELISA test kit A showed positive cross-reactivity, between LOQ and $\frac{1}{2}$ LOQ and negative results of 16.6%, 33.3% and 50%, respectively. ELISA test kit B was cross-reactive, between LOQ and $\frac{1}{2}$ LOQ and negative results to 70.8%, 16.6% and 12.5%, respectively. ELISA test kit C detected positive cross-reactivity, between LOQ and $\frac{1}{2}$ LOQ and negative results of 71.6%, 25% and 33.3%, respectively.

In more detail, four samples (fenugreek, faba bean, whole chickpea beans and chickpea flour) showed cross-reactivity to all three ELISA test kits. Chickpea is known to cause cross-reactivity with lupine (Jappe & Vieths, 2010). Holden et al. (Holden, Faeste, & Egaas, 2005) found cross-reactivity in their developed competitive ELISA test kit to chickpea, pea, peanut, lentil and soya. In a further study by the same research group (Holden, Moen, Sletten, & Dooper, 2007), using a combination of monoclonal and polyclonal antibodies, the cross-reactivity to peanut, chickpea and soya could be eliminated, however, this assay still showed cross-reactivity to brown bean and fenugreek. Ecker et al. (Ecker & Cichna-Markl, 2012; C. Ecker et al., 2013) and Kaw et al. (Kaw et al., 2008) did not detect any cross-reactivity towards chickpea and pea using ELISA test kits they developed.

While whole chickpea and chickpea flour were cross-reactive on all three ELISA test kits, canned chickpea was negative on all three ELISA test kits. Additionally, canned pea was negative on all three ELISA test kits. The three main factors that significantly influence allergen analysis by ELISA are food matrix, level of food processing and ELISA test kit selection (Török et al., 2015). The canned samples were the most processed food samples in this study and therefore it is possible that canned chickpea did not return a positive result due to the impact of food processing or the proteins detected by the test kits.

The link between food allergen detectability and clinical data is unfortunately often missing. The clinically relevant epitopes for allergens are not always known. The target and characteristics of the antibodies of the different ELISA test kits are unknown, due to intellectual property of various test kit manufacturers. To complete the discussion in this study, we include references for changes in allergenicity due to thermal treatment. In case of chickpea, it has been reported that boiling chickpea had no effect on allergenicity but

autoclaving reduces the allergenicity significantly (Gupta et al., 2017; Verma, Kumar, Das, & Dwivedi, 2013).

Five samples (white kidney bean, adzuki bean, soya milk, soya sauce and sweet paprika) were either negative or below the LOQ on all three ELISA test kits. Significantly, the later three items were highly processed food samples. The allergenicity of soya decreased after boiling and microwaving (Alvarez-Alvarez et al., 2005). Although the highly processed soya products showed a response below the LOQ on all three ELISA test kits, the unprocessed soya products (soya flour and whole soya bean) showed high cross-reactivity on two ELISA test kits. Even lecithin from soya showed cross-reactivity on one ELISA test kit. Ecker et al. (Ecker & Cichna-Markl, 2012; C. Ecker et al., 2013) did not detect any cross-reactivity for soya, whereas Kaw et al. (Kaw et al., 2008) reported weak cross-reactivity for soya and black bean.

All tested peanut samples showed cross-reactivity on ELISA test kit B. In contrast, ELISA test kit A showed no cross-reactivity to peanut samples. ELISA test kit C showed cross-reactivity for only raw peanuts. The majority of developed ELISA test kits in literature do not report cross-reactivity for peanut or lentil (Ecker & Cichna-Markl, 2012; C. Ecker et al., 2013; Holden et al., 2007; Kaw et al., 2008). However, in this study, cross-reactivity was observed for all three lentil samples investigated, two test kits with positive results and one showing results between the LOQ and $\frac{1}{2}$ LOQ. Literature described that boiling increases allergenicity of lentils (Alvarez-Alvarez et al., 2005). It is recommended that cross-reactivity of lentil should be further investigated, for clinical and ELISA test kits studies, as neither of the tested lentil samples were processed.

3.4. Determination of possible relevance of cross-reactivity in an analytical context.

All related legume species analyzed in this study were pure matrices. However, food samples tested in an analytical setting are usually not pure samples as they would contain

more than one component. To determine how much of the pure sample needs to be present in a food matrix, the relative percentage was calculated as described in the materials and methods section. For these calculations it is assumed that other food components do not have a matrix effect when tested with the relevant ELISA test kit. Table 3 summarizes the calculated percent of a food product required in a mixed food to generate a positive result at LOD level. The products with the lower relative percentages give the strongest cross-reactivity response. These food products, or products containing these ingredients, potentially generate false positive responses. We recommend when these food matrices are analyzed, that the results should be carefully investigated.

Low level cross-contamination is not likely to be detected by these tests, as a minimum of 14% legume related species is required for a positive response. False positive responses will most likely relate to intentional additions of related products and should be reflected in the ingredients list. For example, gluten-free muffins can contain high percentages of chickpea/soya flour. When testing a food product for lupine by these ELISA test kits, the ingredients list should be checked for related legumes.

3.5. Protein homology between legumes.

Although literature reports the highest sensitization and clinical relevant cross-reactivity to occur between lupine and peanut (Guillamon et al., 2010; Jappe & Vieths, 2010; Kaw et al., 2008; Peeters et al., 2007), the cross-reactivity of tested peanut samples is low compared to other samples analyzed in this study. The highest cross-reactivity in this study was observed for chickpea, followed by soya. To further analyze our analytical data and relate it to reported clinical data, an amino acid sequence alignment was performed with allergenic legume proteins (Supplementary table 1). For cross-reactivity between allergens more than 35-40% amino acid sequence identity is necessary (Breiteneder & Mills, 2005). However, the structure of the allergen is also important for the possibility of

being cross-reactive. It was reported, when proteins share the same tertiary structure, the amino acid identity can be as low as 20-30% and still be cross-reactive (Pomes, 2008).

Seed storage proteins (α -conglutin, β -conglutin, γ -conglutin, and δ -conglutin) account for the main proteins fractions in legumes (Cabello-Hurtado et al., 2016; Guillaumon et al., 2010; Jappe & Vieths, 2010). The allergens registered with the IUIS and protein sequences reported in literature (Foley et al., 2011) belong to different protein types, including α -conglutin, β -conglutin, δ -conglutin, non-specific lipid transfer proteins (nsLTP) pathogenesis-related protein-10 (PR-10) and profilins. Supplementary table 1 summarizes the protein sequences aligned in this study and provided the registered allergen name with the IUIS. There are currently no protein sequences available for nsLTP from any lupine species and only PR-10 proteins from yellow lupine. Nevertheless, PR-10 proteins from yellow lupine have been reported to be 75-95% similar with white lupine (Berkner et al., 2009). The protein sequences available for chickpea for allergenic proteins are only predicted sequences and have therefore not been analyzed for this study.

The various proteins are similar between legume species and share common epitopes, resulting in cross-reactivity as seen in detection test kits and patients (Christina Ecker et al., 2013; Hoffmann et al., 2017; Jappe & Vieths, 2010). Based on a protein sequence alignment performed in this study, the highest homology was found between PR-10 proteins from yellow lupine and Gly m 4 (53-72%) (Jappe & Vieths, 2010), Vir g 1 (53-77%) and Ara h 8 (49-68%) (Ecker & Cichna-Markl, 2012; Holden, Sletten, Lindvik, Faeste, & Dooper, 2008; Shaw et al., 2008), including similar surface of the molecule (Shaw et al., 2008). Similar identity (40-60%) was found for α -conglutins from lupine with Ara h 3 (Foley et al., 2011) and Gly m 6 (Kaw et al., 2008). The β -conglutins from lupine share 47-50%, 20-60% and 33-55% amino acid homology with Ara h 1, Ara h 2 and Ara h 3, respectively. Of these three peanut proteins only Ara h 1 is a β -conglutin. Literature

reports similar homology (Rojas-Hijazo, Garces, Caballero, Alloza, & Moneo, 2006) and sequence identity (47%) (Peeters et al., 2007) for Ara h 1 (Ecker & Cichna-Markl, 2012; Foley et al., 2011; Holden et al., 2008; Shaw et al., 2008), Gly m 4 (51-58%) (Cabello-Hurtado et al., 2016; Guillamon et al., 2010; Kaw et al., 2008; Peeters et al., 2007), pea (46-59%) (Guillamon et al., 2010), bean (50-56%) and Len c 1 (53-58%) with β -conglutins from lupine, though the latter is a γ -conglutin. The lowest sequence identity (29-50%) for δ -conglutins from lupine was found for δ -conglutin of peanut (Foley et al., 2011; Rojas-Hijazo et al., 2006).

In summary, the protein alignment shows that the same protein types of other legumes share high amino acid homology when compared to the lupine protein homologues. This high amino acid sequence identity explains high cross-reactivity of lupine to other legumes according to Breiteneder & Mills (Breiteneder & Mills, 2005) for clinical and analytical data.

The overall sequence identity of allergenic proteins between lupine and soya is slightly higher when compared with lupine and peanut. This may explain why the three tested ELISA test kits in this study cross-reacted more with tested soya products compared to peanut. The major allergen reported for soya is Gly m 1, which is not a food allergen but an airborne soya-hull allergen (Wilson, Blaschek, & de Mejia, 2005). In contrast, all peanut allergens are food allergens, including the major peanut allergens Ara h 2 and Ara h 6 (previous major peanut allergens are Ara h 1, Ara h 2 and Ara h 3) (Zhuang & Dreskin, 2013). We hypothesize that the higher clinical cross-reactivity for lupine and peanut, compared to lupine and soya, can be explained by the origin of the exposure pathway (airborne or food) for their relevant major allergens.

In summary, we presume that the different observed cross-reactivity between analytical and clinical data could be explained by the two reasons discussed above; the higher

amino acid homology between soya and lupine compared to lupine and peanut; and the origin of the exposure of the major allergens from soya and peanut.

4. Conclusion

Three commercially available ELISA test kits were analyzed for their capability to detect the three most commonly consumed lupine species. Various related legume products were also tested for cross-reactivity. The calculated concentration of lupine for all samples varied for the test kits due to different standards, highlighting the importance of a more comparable analytical method for food allergen detection. Currently alternative methods, e.g. mass spectrometry, are being investigated to achieve more consistent and comparable results in food allergen detection. Further improvements for the quantification of food allergens should be provided by (certified) reference materials for food allergens, which are currently insufficient available.

In this current study, all three ELISA test kits were able to detect the three lupine species, with ELISA test kit A showing the lowest response. ELISA test kit A showed the lowest cross-reactivity and ELISA test kit B showed the highest cross-reactivity. The test kit cross-reactivity for soya was higher compared to peanut, although the clinical lupine cross-reactivity is highest for peanut. This highlights that further research is required on comparing and determination of analytical detection methods with clinical relevant proteins. Overall, this study suggests that various ELISA test kits were capable to detect different lupine species. However, the selection and interpretation of the results of ELISA test kits for lupine allergen analysis should consider the sample matrix, as the cross-reactivity of related legume samples varied for different ELISA test kits.

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Abbreviaton

Ara h 1 (Ara h 2, Ara h 3, Ara h 5, Ara h 6, Ara h 7, Ara h 8, Ara h 9, Ara h 10, Ara h 11, and Ara h 14) Registered peanut (*Arachis hypogaea*) allergen with IUIS;

CV Cultivated Variety;

DF Dilution Factor;

ELISA Enzyme-Linked Immunosorbent Assay;

Gly m 1 (Gly m , Gly m 4, Gly m 5, Gly m 6, Gly m 7 and Gly m 8) Registered soya (*Glycine max*) allergen with IUIS;

IgE Immunoglobulin E;

IUIS World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee,

Len c 1 (Len c 3) Registered lentil (*Lens culinaris*) allergen with IUIS;

LOD Limit of Detection,

LOQ Limit of Quantification,

Lup an 1 Registered blue lupine (*Lupinus angustifolius*) allergen with IUIS

nsLTP non-specific lipid transfer proteins;

Pha v 3 Registered green bean (*Phaseolus vulgaris*) allergen with IUIS

Pis s 1 (Pis s 2, Pis s 3) Registered pea (*Pisum sativum*) allergen with IUIS

PR-10 Pathogenesis-related Protein-10;

SD Standard Deviation;

Vir g 1 (Vir g 2, Vir g 4, Vir g 6) Registered mung bean (*Vigna radiata*) allergen with IUIS

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Conflict of interest

The authors declare no conflict of interest.

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Figures captions

Figure 1. ELISA test kit specific standards analyzed in triplicate. The mean absorbance $\pm$ standard deviation (n=6) is shown on the left y-axis. The given concentration of the specific ELISA test kit standards is illustrated as dots on the graph and the concentration in ppm is given on the right y-axis.

Figure 2. Lupine species and lupine products analyzed by three different commercial available ELISA test kits. DF: Dilution factor (1:10,000); DF*: Dilution factor (1:100,000); DF**: Dilution factor (1:1,000,000); CV: Cultivated Variety; *L. alb.*: *Lupinus albus*; *L. ang.*: *Lupinus angustifolius*; *L. lut.*: *Lupinus luteus*. Arrows indicate a fold increase of higher than six, being outside the standard range.

Figure 3. Food products analyzed by three different commercial available ELISA test kits. Arrows indicate a fold increase of higher than six, being outside the standard range.

Tables captions

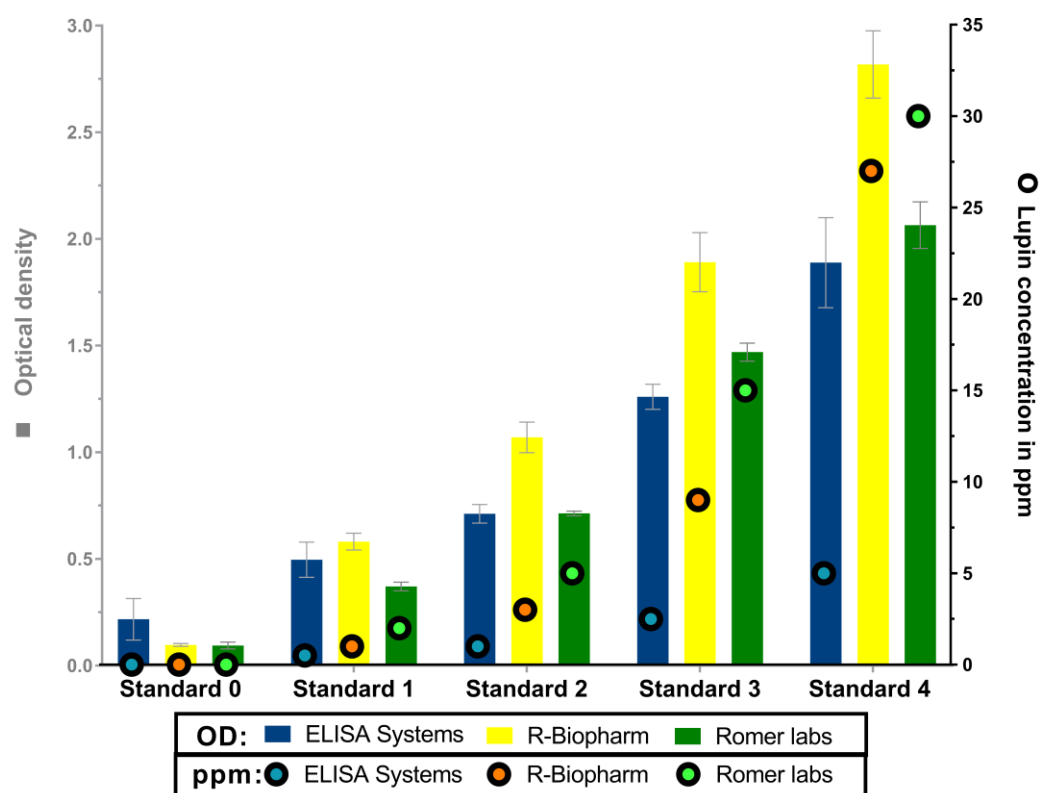
Table 1. Summary of different lupine species analyzed in this study, including the cultivated variety, grown location and harvesting time.

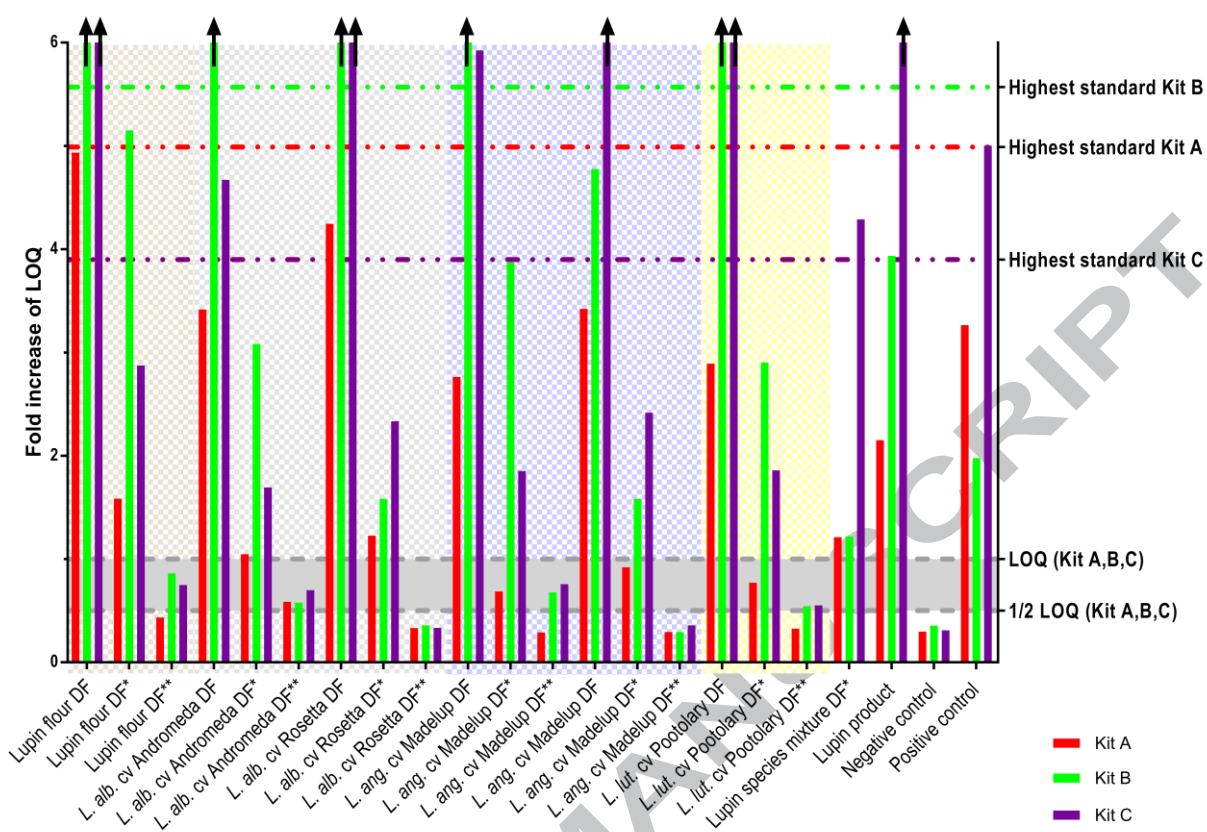
Table 2. Summary of samples analyzed in this study, including the food processing state.

Table 3. Component percentage required for positive result from related legume species. Calculated relative percentage of the food type required in a mixed food matrix to generate a positive result by the relevant ELISA test kit at the Limit of Detection. ND stands for not detected i.e. no cross-reactivity.

Supplementary table 1. Protein sequences analyzed for amino acid sequence identity between proteins of different legumes. The table summarizes the common name and

scientific name of the legume, the protein type, if the protein is registered as allergen with IUIS, including the allergen name and the Gen-bank accession number. IUIS stands for World Health Organization and International Union of Immunological Societies Allergen Nomenclature Sub-committee; PR-10 for pathogenesis-related protein-10; and nsLTP for non-specific lipid transfer proteins.





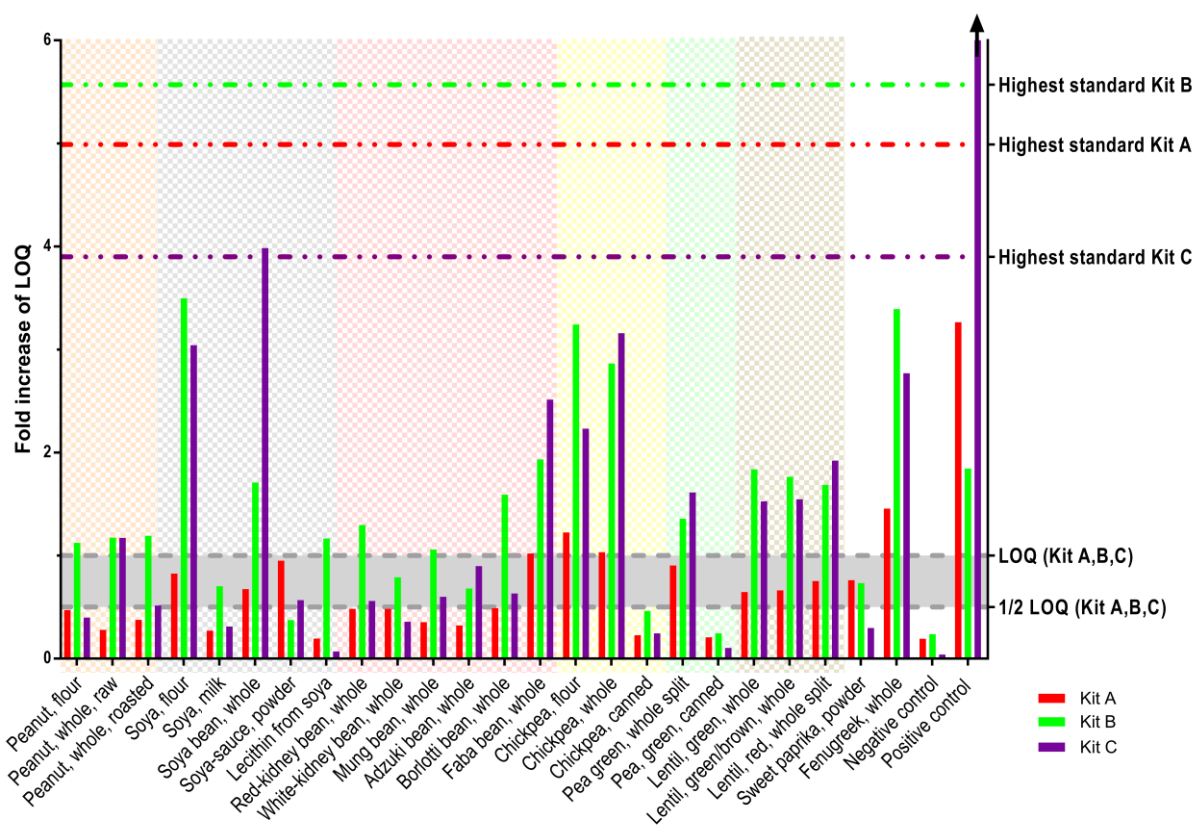


Table 1. Summary of different lupine species analyzed in this study, including the cultivated variety, grown location and harvesting time.

Common name	Scientific name	Cultivated variety	Location of growth	Harvested	Kindly provided by
Blue Lupine	<i>Lupinus angustifolius</i>	Madelup	Western Australia	2015	Mark
Blue Lupine	<i>Lupinus angustifolius</i>	Madelup	New South Wales	2016	Phil
White Lupine	<i>Lupinus albus</i>	Andromeda	Western Australia	2015	Mark
White Lupine	<i>Lupinus albus</i>	Rosetta	New South Wales	2016	Phil
Yellow Lupine	<i>Lupinus luteus</i>	Pootolary	Western Australia	2015	Mark

Table 2. Summary of samples analyzed in this study, including the food processing state.

Sample	Origin	Food processed	Processed by
Peanut, flour	Local supermarket	yes	Manufacturer; The fat was extracted by in-house SOXHLET fat extraction method
Peanut, whole, raw	Local supermarket	no	Ground for this study
Peanut, whole, roasted	Local supermarket	yes	Manufacturer; Ground for this study
Soya, flour	Local supermarket	yes	Manufacturer
Soya, milk	Local supermarket	yes	Manufacturer
Soya bean, whole	Local supermarket	no	Ground for this study
Soya-sauce, fermented, powder	Local supermarket	yes	Manufacturer
Lecithin from soya	Local supermarket	yes	Manufacturer
Red-kidney bean, whole	Local supermarket	no	Ground for this study
White-kidney bean, whole	Local supermarket	no	Ground for this study
Mung bean, whole	Local supermarket	no	Ground for this study
Adzuki bean, whole	Local supermarket	no	Ground for this study
Borlotti bean, whole	Local supermarket	no	Ground for this study
Faba bean, whole	Kindly provided by Phil, grown in New South Wales, harvested in 2016	no	Ground for this study
Chickpea, flour	Local supermarket	yes	Manufacturer
Chickpea, whole	Local supermarket	no	Ground for this study
Chickpea, canned	Local supermarket	yes	Manufacturer; Ground for this study
Pea, green, whole split	Local supermarket	no	Ground for this study
Pea, green, canned	Local supermarket	yes	Manufacturer; Ground for this study
Lentil, green, whole	Local supermarket	no	Ground for this study
Lentil, green/brown, whole	Local supermarket	no	Ground for this study
Lentil, red, whole split	Local supermarket	no	Ground for this study
Sweet paprika, powder	Local supermarket	yes	Manufacturer
Fenugreek, whole	Local supermarket	no	Ground for this study
Custard powder, powder	Local supermarket	yes	Manufacturer
Lupine, flour	Local supermarket	yes	Manufacturer

Lupine product, Soaked and fermented lupine seeds in chili paste (assumed to have undergone sterility heat treatment)	Local supermarket	yes	Manufacturer
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Table 3. Component percentage required for positive result from related legume species. Calculated relative percentage of the food type required in a mixed food matrix to generate a positive result by the relevant ELISA test kit at the Limit of Detection. ND stands for not detected i.e. no cross-reactivity.

Sample type	ELISA test kit A	ELISA test kit B	ELISA test kit C
Peanut, flour	15.4 %	40.9%	22.4%
Peanut, whole, raw	42.6 %	ND	42.7%
Peanut, whole, roasted	42.0%	ND	97.2%
Soya, flour	14.3%	60.6%	16.4%
Soya, milk	71.2%	ND	ND
Soya bean, whole	29.2%	74.1%	15.0%
Soya-sauce, fermented, powder	ND	52.7%	88.0%
Lecithin from soya	42.9%	ND	ND
Red-kidney bean, whole	38.5%	ND	89.5%
White-kidney bean, whole	63.4%	ND	ND
Mung bean, whole	47.2%	ND	83.4%
Adzuki bean, whole	73.6%	ND	55.6%
Borlotti bean, whole	31.4%	ND	79.2%
Faba bean, whole	25.9%	49.0%	19.9%
Chickpea, flour	15.4%	40.9%	22.4%
Chickpea, whole	17.5%	48.4%	15.8%
Chickpea, canned	ND	ND	ND
Pea green, whole split	36.8%	55.3%	31.0%
Pea, green, canned	ND	ND	ND
Lentil, green, whole	27.2%	77.4%	32.8%
Lentil, green/brown, whole	28.4%	75.6%	32.3%
Lentil, red, whole split	29.7%	66.4%	26.0%
Sweet paprika, powder	68.2%	65.8%	ND
Fenugreek, whole	14.7%	34.4%	18.1%

HIGHLIGHTS:

- Comparison of enzyme-linked immunosorbent assays for lupine allergen detection
- Determination of detection capability for three different lupine species
- Investigation of lupine cross-reactivity to related legumes