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Novel Polyclonal−Monoclonal-Based ELISA Utilized To Examine Lupine (*Lupinus* Species) Content in Food Products

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Sweet lupines are increasingly used in food production. Cause for concern has been expressed due to the increase in reported lupine-induced allergic incidents and the association between lupine and peanut allergies. In the current study, a polyclonal−monoclonal antibody-based sandwich ELISA for the detection of lupine proteins in foods was developed. The assay was sensitive to both native and processed proteins from *Lupinus angustifolius* and *Lupinus albus* and had a detection limit of 1 μg/g. Intra- and interassay coefficients of variation were <5 and <17%, respectively. A selection of 112 food samples, both with and without lupine declaration, was evaluated for their content of lupine. The data showed that the majority were in agreement with the respective labeling. However, some inconsistency was seen, typically in bread/rolls and soy flours.

KEYWORDS: Conglutins; ELISA; food allergy; food labeling; globulins; lupine; monoclonal antibodies

INTRODUCTION

Food allergies affect 4−5% of the population in industrialized countries, and this number appears to be increasing (*1*, *2*). For these individuals, avoidance of foods eliciting their allergic reactions is currently the only possible recourse. The key source of information for allergen avoidance is the declaration on packaged foods. New legislation, which mandates clear labeling of specific food allergens, was recently implemented, both in the European Union (EU) and in the United States (*3*). In accordance with the EU Labeling Directive, all ingredients in a food product must now be declared in the ingredient list, including the potential allergenic ingredients listed in Annex IIIa of the directive, which must under all circumstances appear on the labeling of food products (*4*). The allergen list is under periodical re-evaluation and is, when necessary, updated on the basis of the most recent scientific findings.

Sweet lupines, such as *Lupinus angustifolius* and *Lupinus albus*, are increasingly being used in a variety of food products, mostly as an additive to wheat flour or as a substitute for soy flour, especially in bread and pasta products (*5*). In addition to being genetically unmodified, the use of lupine seeds is attractive due to their excellent nutritional properties, high protein content, ease of cultivation, low cost, and the absence of gluten (*5*, *6*). However, there have been a number of reports of allergic reactions to lupine, either as primary lupine allergy (*7*, *8*) or as a result of cross-reactivity to other legumes, especially peanut (*9*−*11*). Although no lupine allergens have yet been fully characterized, lupine globulins, and in particular the α-, β-, and γ-conglutins, have all been suggested as potential allergens in lupine (*12*−*14*). Concern for public health has been expressed because lupine is often a "hidden" component of food products and, more importantly, because of the association between lupine allergy and peanut allergy (*9*, *15*), the latter often being associated with severe allergic reactions (*16*). Some clinicians have therefore advised peanut-allergic individuals to avoid all lupine-containing products until these persons have been specifically tested (*11*), which may lead to an unnecessarily strict diet. The European Food Safety Authority (EFSA) recognized lupine as a food allergen in December 2005, and lupine was recently added to the EU food allergens list (*17*).

Reliable methods for the detection and quantification of allergens in food are necessary to ensure compliance with food-labeling regulations, for the disclosure of adulterations, and to provide consumer protection. Currently, enzyme-linked immunosorbant assay (ELISA) is the most commonly used technique, as it is simple to handle and relatively easily standardized (*18*). Previously, a quantitative sandwich ELISA for the detection of lupine proteins in food was developed in this laboratory (*19*), in which a polyclonal anti-lupine rabbit antibody was used as capture antibody and detection antibody. This ELISA was demonstrated to be sensitive for processed lupine proteins from *L. albus*, but recent observations indicate that the ELISA has a lower sensitivity for unprocessed lupine proteins (such as in flours) from *L. angustifolius*.

Monoclonal mouse antibodies (mAbs) against lupine globulins have recently been generated (*20*). Preliminary results showed that one of the mAbs (Lu11) was promising for use as a detection antibody in a sandwich ELISA format. In the current study, the characteristics and the validation parameters of this novel ELISA for lupine detection were determined. Furthermore, the extent to which lupine is found in commercial food products

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on the Norwegian market and whether this corresponds with the product labeling was evaluated. Various product categories, both with and without lupine as a labeled ingredient, were included in the survey.

MATERIALS AND METHODS

Materials. Processed proteins from *L. albus* seeds, in the form of a tofu-like product (Lopino; Lupina, Visbek, Germany) were purchased from a local store in Germany. This product consists of heat-treated proteins; that is, seeds were soaked and blended, and the filtrate was boiled and pressed. Native proteins from *L. angustifolius* seeds, in the form of lupine flour, were kindly provided by Soja Austria (Lupipan; Vienna, Austria).

Production of Anti-lupine Antibodies. A polyclonal rabbit antiserum against the processed lupine protein extract was raised and purified as previously described (*10*, *19*). The monoclonal antibody (mAb Lu11) was generated by hybridoma technology, using the processed lupine protein extract as immunogen, and purified as previously described (*20*).

Protein Extractions and Lupine Protein Fractions. All protein extracts, if not otherwise stated, were prepared as described previously (*19*). In brief, homogenized samples (2 g) were extracted with 10 mL of 0.1 M Tris−0.5 M glycine (pH 8.7) overnight at 45 °C and centrifuged at 39200*g* for 25 min at 4 °C. Protein extracts were stored at −20 °C. Detailed procedures for the isolation of globulins, albumins, and α-, *β*-, and *γ*-conglutins have been described previously (*20*). Total protein concentrations were determined according to the Lowry method (DC protein assay) (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Sandwich ELISA Procedures. *Polyclonal−Monoclonal (pAb−mAb) ELISA.* Flat-bottom, high-binding polystyrene 96-well microplates (Corning Inc., Corning, NY) were coated with 100 μL/well of 0.05 M carbonate−bicarbonate buffer, pH 9.6 (Sigma-Aldrich, Steinheim, Germany), containing 1.5 μg/mL purified polyclonal anti-lupine antibody. All wash steps were carried out with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T, pH 7.6), three times, using a programmable automatic plate washer (Skatron Instruments, Lier, Norway). PBS (Oxoid, Basingstoke, U.K.) containing 1% BSA and 0.1% Tween 20 was used as blocking and assay buffer for the ELISA. Wells were blocked with 250 μL/well and incubated for 1−2 h at room temperature (RT). After washing, 100 μL/well of a 2-fold serial dilution of the lupine protein standard [consisting of a 50:50 mixture of processed (*L. albus*) and native (*L. angustifolius*) lupine proteins] was added, in a concentration range from 1000 to 0.98 ng/mL. Triplicate sample extracts, diluted at minimum 1:10 (v/v), and assay buffer blanks were incubated for 1 h at room temperature under gentle shaking. After washing, bound lupine proteins were detected by adding 100 μL/well of mAb Lu11 (0.4 μg/mL) and incubated for 1 h at room temperature under gentle shaking. Plates were washed again and incubated with 100 μL/well of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig (DakoCytomatation, Glostrup, Denmark), diluted 1:6000, for 1 h at room temperature. After a final wash, 75 μL of K-Blue 3,3′,5,5′-tetramethylbenzidine substrate (Neogen, Lexington, KY) was added to each well. Color development was stopped by the addition of 50 μL/well of 2 M H_2SO_4, and optical density (OD) was read at 450 nm on a 1420 VICTOR^2 multilabel plate counter (Wallac, Turku, Finland).

Polyclonal−Polyclonal (pAb−pAb) ELISA. This ELISA procedure has been described in detail previously (*19*). In brief, plates were coated with 2 μg/mL purified rabbit anti-lupine pAb. Standard proteins [i.e., semipurified Tris−glycine Lopino extract (*10*)] at concentrations ranging from 269 to 0.26 ng/mL and samples, diluted at minimum 1:20 (v/v), were added to blocked plates. For detection of bound lupine proteins, wells were first incubated with biotinylated anti-lupine pAb, followed by incubation with HRP−streptavidin conjugate (Zymed, San Francisco, CA). Final steps were performed as described for the pAb−mAb ELISA.

Extraction Buffers. The following extraction buffers were tested in the study (**Table 1**): Tris−glycine, 0.1 M Tris, 0.5 M glycine, pH 8.7; citrate, 0.5 M citric acid monohydrate, 0.5 M sodium citrate dihydrate, pH 4; high-salt buffer (HSB), 20 mM $NaH_2PO_4 \cdot H_2O$, 1 M NaCl, pH 7.5; HSB, 20 mM $NaH_2PO_4 \cdot H_2O$, 1 M NaCl, pH 4.5; sodium carbonate, 50 mM, pH 11; urea, 6 M, pH 8.7; PBS, 0.172 M, pH 7.4 (Oxoid); sodium borate, 7 mM disodium tetraborate decahydrate, pH 9.2. The extraction procedure was performed as described above. Ionic strengths were high (>1 M), medium (0.5−1 M), and low (<0.5 M).

Table 1. Effect of Extraction Buffers with Different pH and Ionic Strengths on the Total Protein and Lupine Protein Content Measured in a Lupine-Containing Food[a]

buffer	pH	ionic strength	total protein (mg/mL)	lupine protein (μg/g)
Tris–glycine	8.7	medium	2.8	1081
citrate	4.0	medium	2.4	726
HSB	7.5	medium	3.0	625
HSB	4.5	medium	2.0	558
sodium carbonate	11.0	low	5.5	346
urea	8.7	high	7.6	123
PBS	7.4	low	3.9	121
sodium borate	9.2	low	2.0	14

[a] Total protein concentrations were determined by the Lowry method, and lupine protein concentrations were determined using the polyclonal–monoclonal ELISA.

Recovery Experiments. Samples of lupine-free bread were spiked with extract of lupine flour or Lopino at levels of 1, 100, and 1000 μg/g of bread sample, in a total volume of 5 mL of extraction buffer. After a 15 min incubation at room temperature, an additional 5 mL of extraction buffer was added, and protein extraction was performed as described above. For determination of recovery rates three independent extractions were performed, extractable proteins were analyzed in both ELISAs, and the mean values for the recovery were calculated.

Immunoblotting. The NuPAGE Gel system (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein extracts by SDS-PAGE in accordance with the manufacturer's instructions. Protein extracts were loaded onto 4−12% Bis-Tris precast gels. Separated proteins were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad) and detected as described previously (*19*). In brief, immunoblots were blocked with Tris-buffered saline (TBS) containing 1% BSA and 0.1% Tween 20 and incubated with anti-lupine PAb (1:10000 diluted) and anti-lupine mAb (0.1 μg/mL), respectively. For detection, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Zymed) or HRP-conjugated rabbit anti-mouse Ig (DakoCytomation). Blots were developed with a 3,3′,5,5′-tetramethylbenzidine substrate solution (Zymed).

Specificity. Lupine protein fractions and native protein extracts from 28 non-lupine-containing foods and food ingredients were examined for cross-reactivity in the pAb−mAb ELISA. The lupine protein fractions were applied in the same concentration range as the lupine protein standard. The single food component extracts were diluted 1:10 (v/v) in ELISA assay buffer, which thus is equivalent to a portion of 100% in a typical food matrix, prior to analysis. Measurements of total protein were performed in cross-reacting extracts, which then were analyzed further at higher dilutions.

Food Survey. A total of 68 food products and 112 food samples, including cakes, bread, pasta, chocolate spread, biscuits, flour, and chips, were purchased at various locations in Oslo, Norway, in 2005−2006. Food samples with and without declared lupine in the ingredient list were included in the study. Food samples with no declared lupine content were mainly selected from companies also producing lupine-containing foods. Two different lots of each product were obtained when possible. The samples were homogenized and extracted in Tris−glycine buffer as described above and analyzed twice. If the labeling was not in agreement with the result obtained in the ELISA, an additional independent extraction and analysis was performed.

RESULTS

Characteristics of a Polyclonal−Monoclonal Antibody-Based ELISA for Detection of Lupine Proteins. A sandwich ELISA for the detection of lupine proteins in food samples was

Table 2. Comparison of Lupine Protein Recovery Rates between the Polyclonal–Polyclonal and Polyclonal–Monoclonal Sandwich ELISAs[a]

	recovery (%)					
	pAb–pAb			pAb–mAb		
food	1	100	1000	1	100	1000
bread spiked with lupine flour	14 ± 1.7	12 ± 0.6	16 ± 0.9	44 ± 3.6	60 ± 2.1	88 ± 1.9
bread spiked with Lopino	78 ± 5.8	101 ± 9.0	112 ± 5.5	85 ± 4.8	123 ± 9.2	150 ± 10.7

[a] Blank bread samples were spiked with extract of native lupine flour from *L. angustifolius* or extract from processed lupine protein (Lopino) from *L. albus* at levels of 1, 100, or 1000 μg/g of bread. Values represent the average of three spiking experiments and are reported as mean ± standard error of the mean (SEM).

established, using a polyclonal rabbit anti-lupine capture antibody and a monoclonal mouse anti-lupine detection antibody (Lu11). Optimal concentrations of the antibodies were determined using a lupine protein standard consisting of a 50:50 mixture of processed (*L. albus*) and native (*L. angustifolius*) lupine proteins, in the concentration range from 0.98 to 1000 ng/mL. The working range of the assay, defined as the linear part of the curve with a squared correlation coefficient (R^2) > 0.99, normally ranged from 7.8 to 125 ng/mL. For analysis, serial dilutions of food sample extracts were performed if necessary, and OD values closest to the midpoint of the linear portion of the standard curve were used to calculate lupine protein concentrations. The limit of detection (LOD), calculated for each assay as the mean plus 3 times the standard deviation of the mean buffer blank value, never exceeded an equivalent of 0.2 μg of lupine protein/g of sample. However, to reduce the possibility of false positive results, the LOD was defined as 1 μg/g.

Influence of Buffers on Extraction Efficiency. To optimize the extraction method for lupine proteins, a lupine-containing product (hot dog bread) was extracted with various buffers of different pH and ionic strengths (**Table 1**). Both the total protein content and lupine protein content were measured in the extracts. The data suggested that the extraction buffer used had a considerable effect on the amount of total protein and lupine protein extracted (**Table 1**). Total proteins were extracted most efficiently by a urea-based extraction buffer, whereas the highest yield of lupine proteins was observed with a Tris−glycine-based extraction buffer. No correlation was found between the yield of total protein and lupine protein. Buffers of medium ionic strength seemed to be more effective for lupine protein extraction than buffers of low or high ionic strengths. The buffer pH did not appear to affect the lupine protein yield. Because the most effective buffer with respect to lupine protein extraction was Tris−glycine, this buffer was used in subsequent studies.

Intra- and Interassay Precisions. Pasta and hot dog bread, declared to contain lupine, were found to contain high levels of lupine protein when analyzed in the pAb−mAb ELISA. These food products were therefore used for an in-house validation of the ELISA method to determine the intra- and interassay precisions. The intra-assay precisions, calculated from 10 replicates of the same extract and expressed as mean coefficients of variation (CVs), were 5% for pasta and 4.4% for hot dog bread. The interassay precisions, calculated from analysis of the same extract on different days (n), were 9% ($n = 15$) for pasta and 12% ($n = 18$) for hot dog bread. The interassay variation was further investigated using an extract containing blank (lupine-free) bread that was spiked with 1 μg of processed lupine proteins/g of sample. The variation at this level was found to be 17% ($n = 13$).

Comparison of Recovery Rates between the pAb−pAb and pAb−mAb Sandwich ELISAs. The measurement of low, intermediate, and high concentrations of processed lupine proteins in spiked blank bread samples demonstrated that the pAb−pAb and pAb−mAb sandwich ELISAs had comparable recovery rates for processed lupine proteins from *L. albus*, ranging from 78 to 112% in the pAb−pAb assay and from 85 to 150% in the pAb−mAb assay (**Table 2**). However, the recovery for bread spiked with lupine flour proteins (i.e., native lupine proteins from *L. angustifolius*) was poor in the pAb−pAb assay, with recoveries between 12 and 16%, independent of spiking level. In contrast, the recovery rate for lupine flour proteins was higher in the pAb−mAb assay, ranging from 44 to 88% (**Table 2**). In general, a higher concentration of lupine protein was found in food samples analyzed in the pAb−mAb ELISA, compared with the conventional pAb−pAb ELISA (data not shown). This can be exemplified by the pasta and hot dog bread samples used in the validation, which contained 400 and 900 μg of lupine protein/g, respectively, measured in the pAb−mAb assay, compared to 49 and 100 μg/g in the pAb−pAb assay.

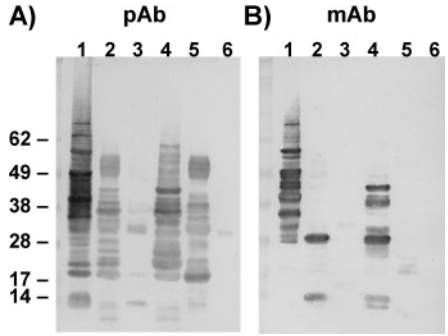

Figure 1. Specificity of anti-lupine pAb (**A**) and mAb Lu11 (**B**) for lupine protein fractions shown by immunoblotting. Lanes: (1) lupine protein standard; (2) globulin; (3) albumin; (4) α-conglutin; (5) β-conglutin; (6) γ-conglutin. Protein sizes (kDa) are indicated on the left side of the blots. Five micrograms of protein was applied in each lane. Anti-lupine pAb 1:10000 diluted; mAb Lu11 0.1 μg/mL.

Specificity of the pAb−mAb ELISA. The individual specificities of the anti-lupine antibodies used in the pAb−mAb ELISA were investigated by immunoblotting with lupine seed storage protein fractions. Both the polyclonal and monoclonal antibodies recognized the mixture of native and processed lupine proteins (used as lupine protein standard in the ELISA), globulins, and α-conglutins (**Figure 1**). The pAb also bound to β-conglutins and, to a lesser extent, to albumins (**Figure 1A**). None of the antibodies bound to γ-conglutin. Furthermore, titrations of the lupine seed storage protein fractions in the ELISA, at concentrations similar to the lupine protein standard, showed that the pAb−mAb ELISA was specific for globulin and α-conglutin, whereas albumin and β- and γ-conglutin were not detected at these concentrations (data not shown).

To determine the cross-reactivity of the pAb−mAb ELISA, nearly 30 legumes, tree nuts, seeds, and other common foods and food ingredients were analyzed as normal food samples.

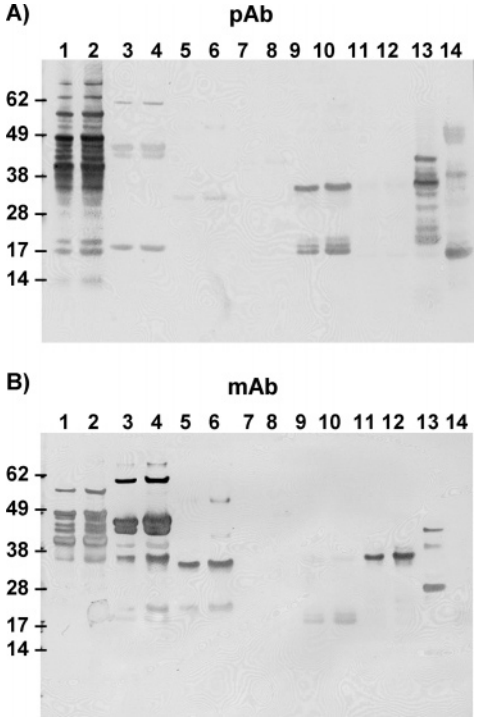

Figure 2. Specificity and cross-reactivity of anti-lupine pAb (**A**) and mAb Lu11 (**B**) shown by immunoblotting. Lanes: (1, 2) lupine protein standard (2.5, 5 μg); (3, 4) almond (2.5, 5 μg); (5, 6) cashew (2.5, 5 μg); (7, 8) fenugreek (2.5, 5 μg); (9, 10) pumpkin seed (2.5, 5 μg); (11, 12) sunflower seed (2.5, 5 μg); (13) α-conglutin (5 μg); (14) β-conglutin (5 μg). Protein sizes (kDa) are indicated on the left side of the blots. Anti-lupin pAb 1:10000 diluted; mAb Lu11 0.1 μg/mL.

No cross-reactivity was found in extracts from peanut, soybeans, chickpea, yellow pea, green lentil, hazelnut, Brazil nut, pine nut, sesame seed, shrimp, parvalbumin, ovalbumin, ovomucoid, dry milk, wheat flour, whole casein, and cinnamon. The method tested positive for extracts from brown bean, walnut, linseed, curry, and tandoori masala. However, when these extracts were diluted to 100 μg of protein/g of sample, no cross-reactivity was observed (i.e., all extracts gave signals below the LOD). In contrast, similarly diluted extracts from fenugreek, almond, cashew, pumpkin seed, and sunflower seed produced positive responses. Titrations of these cross-reactive extracts at protein concentrations equivalent to those of the lupine protein standard showed that the positive responses (defined as percent of maximum OD) were 88% for almond, 68% for pumpkin seed, 33% for fenugreek, 16% for sunflower seed, and 11% for cashew.

The cross-reactive extracts were further investigated by immunoblotting with the anti-lupine pAb and mAb (**Figure 2**). The anti-lupine pAb recognized almond and pumpkin seed, whereas cashew was only weakly recognized, and sunflower seed and fenugreek were not recognized by the pAb (**Figure 2A**). The mAb bound to almond and, to a lesser extent, to cashew, pumpkin seed, and sunflower seed, but not to fenugreek (**Figure 2B**). Overall, the number and intensity of the bands and the size of the proteins recognized from these cross-reacting products differed from those observed with lupine proteins.

Survey of Lupine in Domestic and Imported Foods. To evaluate the extent to which lupine is used in commercial food products on the Norwegian market and to investigate whether the product labeling corresponds with the lupine protein content, a survey was carried out. A number of product categories, such as cakes, breads/rolls (including hot dog and hamburger breads), pasta, chocolate spread, biscuits, flour/mix, and potato chips, both with and without declared lupine, were included in the survey (**Table 3**). Two lots of each product were purchased when possible. In total, 68 food products (resulting in 112 food samples) were analyzed with the pAb−mAb ELISA. An additional extraction and analysis was performed if the labeling and ELISA results were not in agreement.

Sixteen of the 24 food samples (67%) with declared lupine content were positive in the pAb−mAb ELISA. The remaining 8 samples (33%), all breads, gave readings below the LOD of the assay. Discrepancies between the lupine protein content and the labeling were also found in the products without declared lupine in the ingredient list; of the 88 samples without a lupine declaration, 29 appeared to contain detectable lupine proteins. However, 11 of these undeclared products (9 breads and 2 cakes), which all contained trace levels of lupine (1−10 μg/g), were declared with the cross-reactive proteins sunflower seed or almond. These food samples were therefore further investigated with immunoblot using the mAb, and all appeared to contain the declared cross-reacting proteins (data not shown). Additionally, a weakly stained band of approximately 55 kDa, which is similar in size with one of the bands in the lupine protein standard, but not in any of the cross-reactive extracts (**Figure 2**), was observed in six of the products (four breads and two cakes), indicating the presence of small amounts of lupine proteins in these products (data not shown). The level of lupine detected in the undeclared but positive samples varied between 1 and >1000 μg/g of food, with nine (chocolate spread, biscuits, and flour/mix) between 100 and 1000 μg/g of food. When these samples were analyzed in the pAb−pAb ELISA, only the two flours (both labeled as pure soy flours) were positive. To further investigate this discrepancy, all of the ingredients of one of the chocolate spreads were obtained from the manufacturer (sugar, whey powder, cocoa, soy lecithin, dry milk, and roasted hazelnut paste) and analyzed using both ELISAs. Roasted hazelnut paste was found to be positive in the pAb−mAb, but not in the pAb−pAb ELISA, whereas all other ingredients were found to be negative in both assays. Only the seven products (chocolate spreads and biscuits) containing > 100 μg/g lupine protein in the pAb−mAb assay contained hazelnut by declaration. However, the degrees of processing of these hazelnuts are not known.

DISCUSSION

In the present study, a novel sandwich ELISA for the detection of lupine proteins in food products was developed and validated in-house, using a polyclonal rabbit antibody for capture and a monoclonal mouse antibody for detection.

The validation data of the polyclonal−monoclonal ELISA presented here are satisfactory, indicating that the method works reliably. The obtained LOD of the assay was 0.2 μg of lupine protein/g of sample, but in order to avoid false positive results and to improve the reliability of the assay the LOD was defined as 1 μg/g. Ideally, assay LODs should be low enough to detect allergen concentrations that might trigger allergic reactions in humans. The amount of the offending food able to elicit symptoms is variable with respect to both allergen and food matrix and also depends on the individual (*21*). The establishment of safe threshold values for allergens in food has therefore been difficult (*22*, *23*). However, because clinical data collected so far do not indicate the need for an LOD of < 1−5 μg/g (*24*), the LOD of 1 μg/g for the currently described lupine assay is likely to be sufficient to detect trace levels that might present a risk for allergic consumers.

Table 3. Survey of Commercial Food Products with and without Lupine Declaration[a]

			lupine declared			lupine not declared		
product	no. of products	no. of samples	no.	positive	negative	no.	positive	negative
cakes	5	10	4	4		6	**4**	2
bread/rolls	26	46	9	1	**8**	37	**6**	31
pasta	7	9	1	1		8		8
chocolate spread	5	10	2	2		8	**6**	2
biscuits	12	18	8	8		10	**6**	4
flour/mix	7	13				13	**2**	11
chips	6	6				6		6
total	68	112	24	16	**8**	88	**24**	64

[a] The samples were analyzed using the polyclonal–monoclonal ELISA. Bold numbers represent products in which the declaration was not in accordance with the lupine protein content.

Although the recently published polyclonal−polyclonal lupine sandwich ELISA was described to be sensitive, specific, and quantitative to a satisfying degree (*19*), this assay also appeared to have some limitations. Solely processed lupine proteins from *L. albus* (Lopino), which may have altered structures compared with native seed proteins (*25*), were used both as immunogen and standard, as well as for spiking experiments (*19*). The lack of inclusion of native proteins and proteins from another lupine species in the establishment and validation of this ELISA seems to have resulted in unsatisfactory recovery and thereby an underestimation of native lupine proteins from *L. angustifolius*, as shown in the current study. In contrast, in the currently described pAb−mAb ELISA, the recovery of native lupine proteins from *L. angustifolius* was demonstrated to be considerably higher. The enhanced recovery of both processed and native proteins from two different lupine species implies that the sensitivity of the novel assay is substantially increased, compared to the previously described assay. This improvement in lupine quantification may be due to the choice of lupine protein standard and/or the choice of detection antibody. A protein standard consisting of a mixture of native and processed proteins was chosen, which reflects the fact that food products in general contain a combination of native and altered or processed proteins (*26*). In addition, the standard consists of a mixture of proteins from *L. angustifolius* and *L. albus*, which is a benefit because both lupine species are commercially used in food production. However, certified reference material for lupine proteins would have been desirable, but is not available at the moment. A well-characterized monoclonal anti-lupine antibody (Lu11) was used as detection antibody (*20*). An advantage of the use of mAbs in immunoassays is that they represent a homogeneous and unlimited source of antibodies.

A comprehensive cross-reactivity study was performed to investigate the specificity of the pAb−mAb ELISA. Of the nearly 30 (non-lupine-containing) foods and food ingredients analyzed, 5 extracts produced cross-reactive responses, and these were further examined by immunoblot. Of the tested extracts belonging to the Leguminosae family, only fenugreek was positive in the specificity test. However, this might have been caused by interfering substances, because the extract was highly viscous, and, furthermore, fenugreek was not recognized by any of the antibodies in the immunoblot. On the other hand, the possibility that a loss of binding was induced by the reducing effects under the procedure cannot be excluded. In the analysis of actual food samples, fenugreek will probably not constitute a problem because it is used in small amounts as a spice. Furthermore, both curry powder and tandoori masala, spices containing fenugreek, were negative in the cross-reactivity study. The traditional approach of investigating the cross-reactivity of a newly developed ELISA by analyzing foods belonging to the same plant family is inadequate when potential cross-reactions due to similarities in residue identities and/or structures are considered (*27*). Therefore, in addition to several legumes, a variety of tree nuts and seeds commonly used in foods, such as bakery products and pastries, were examined. The observed cross-reactivity to almond, cashew, sunflower seed, and pumpkin seed was unexpected, considering the fact that mAbs are monospecific in nature, targeting a single epitope. The mAbs affinity to almond was highly predominant, compared to the other proteins. Cross-reactivities to tree nuts and sesame have previously been reported in an ELISA for detection of almond (*28*). The cross-reactions observed in the current study are probably due to recognition of a similar well-conserved epitope and/or structure among these proteins, with the highest degree of resemblance in the lupine and almond epitopes. Furthermore, the apparent reactivity to roasted hazelnut paste, but not to native hazelnut in the pAb−mAb ELISA, suggests that this epitope was exposed following processing. The previously reported 30% inhibition of human IgE binding to lupine proteins by mAb Lu11 (*20*) might suggest a possible clinical relevance for the cross-reactive epitope. Characterization of this epitope is currently under investigation in our laboratory. The mAb Lu11, and thereby the pAb−mAb ELISA, specifically detects α-conglutins, which are members of the legumin (11S globulin) protein family (*25*). The 11S globulins have been identified as allergens in both almond (amandin) and cashew (Ana o 2) (*29*, *30*). Amandin is reported to be a 37 kDa polypeptide (*29*), whereas Ana o 2 contains a major band of approximately 33 kDa (*30*). These two polypeptides correspond in size to bands recognized by the mAb Lu11 in the immunoblot.

In the analysis of complex food samples, false positive results might in theory be obtained in the pAb−mAb ELISA if traces of lupine were detected in, for example, an almond-containing matrix. In these circumstances, immunoblotting with both anti-lupine antibodies can be used as a verifying method, because the cross-reacting proteins can be distinguished from lupine by their binding patterns in the immunoblot. The pAb−pAb ELISA may be used to exclude the possibility of false positive responses when foods containing processed hazelnut are analyzed.

The testing and selection of an appropriate extraction buffer may be as important as the selection of specific antibodies and protein standards. Of the eight buffers with different pH and ionic strengths examined in the present study, urea was shown to be the most effective in terms of total protein yield from the food matrix. However, the amount of total protein extracted was not correlated with the amount of lupine protein detected as urea gave a relatively poor yield of lupine protein measured using the pAb−mAb ELISA, possibly due to its denaturing

effect on the protein epitopes. Furthermore, the possibility of interference of the individual extraction buffers in the ELISA could be excluded, because all buffers gave signals below the background signal when tested in the assay. The most significant factor for lupine protein extraction appeared to be the ionic strength of the buffer in question, as buffers of medium ionic strength extracted more lupine protein than buffers of low or high ionic strengths. However, the buffer pH did not appear to affect the extraction efficiency of lupine proteins in the current study, as the same HSB buffer at neutral and low pH gave similar lupine protein yields. Overall, an extraction buffer based on Tris−glycine gave the greatest yield of detectable lupine, thereby contributing to an optimal ELISA. The ELISA detects lupine globulins, which are readily extracted in saline solutions (*26*), and therefore it was not unexpected that solutions with high salt concentrations were the most effective in the extraction of lupine proteins. The fact that the Tris−glycine-based buffer was the most efficient may also be explained by the presence of an amino acid, which may facilitate extraction.

The pAb−mAb assay was used to survey the extent to which lupine is found in commercial food products and how this relates to labeling practices. The investigation of 112 food samples, both with declared and undeclared lupine, showed that lupine is used in a wide variety of commercial food products on the Norwegian market. The results indicated that the lupine content was mainly in agreement with the respective labeling but that inadequate labeling of lupine also occurred. Trace amounts of lupine were found in chocolate spread and biscuits, which might indicate cross-contamination from other activities during the production process. In the chocolate industry, cross-contamination is known to be problematic, due to inadequate cleaning routines when using shared equipment (*31*). Current results also indicate that breads/rolls are a product category with inconsistencies in lupine declaration, because declared breads were found to be negative and undeclared breads were found to be positive. Interestingly, two flours, both labeled as pure soy flours, were found to contain large amounts of lupine proteins, indicating a problem with adulteration in such flours. Manufacturers need to be aware of the problem of potential cross-contaminants in raw materials and require documentation from their suppliers.

The choice of antibodies is critical and represents a challenge in the development of immunological-based food detection methods. The use of mAbs has improved the sensitivity of the novel pAb−mAb ELISA compared to the pAb−pAb ELISA. However, the monospecificity of mAbs may also be a drawback in food analysis if the actual epitope/structure that is recognized is conserved within a protein superfamily. Another challenge in food analysis is the unpredictable effect of processing on food matrices and/or proteins, which might destroy or expose epitopes. Positive ELISA results should preferably be further investigated by the separate analysis of all ingredients of a complex food product. Nonimmunological methods, such as Polymerase Chain Reaction (PCR) or mass spectrometry (MS), could potentially be applied as confirmatory methods.

In conclusion, the combination of an optimized and validated sandwich ELISA, together with immunoblot analyses, represents a considerable improvement in the detection and quantification of lupine proteins in foods. Both native and highly processed lupine proteins from *L. angustifolius* and *L. albus* are detected in different food matrices using the ELISA, which contributes to an increase in sensitivity. However, there is still a need for nonimmunological, confirmatory tests for the presence of allergenic residues in food products.

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