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Quantitative Sandwich ELISA for the Determination of Lupine (*Lupinus* spp.) in Foods

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The use of lupine in foods has increased considerably during the past decade, reflected by a corresponding increase in reported lupine-induced allergic incidents. Lupine allergy may arise either by primary sensitization or by clinical cross-reactivity in peanut-allergic persons. Detection of lupine proteins in food has previously been based on the use of patient serum. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of lupine in processed foods was developed, using a polyclonal rabbit antilupine capture antibody and a biotinylated conjugate of the same antibody for detection. The antibody was highly specific for lupine, apart from minor cross-reactivities to other legumes. The assay had a detection limit of 1 μg/g and was successfully used to quantify lupine protein in various food matrixes. Recoveries ranged from 60 to 116%, while the intra- and interassay coefficients of variation were <6% and <21%, respectively.

KEYWORDS: Allergen; ELISA; food allergy; lupine

INTRODUCTION

Lupine, a plant of the Leguminosae family, belongs to the genus *Lupinus*, which includes 450 species. Lupine seeds have been used as human food and animal feed since ancient times. Years of selective breeding have led to lupine strains with reduced alkaloid content, the "sweet lupines" (*1*). Four species are of agricultural interest: the white lupine (*Lupinus albus*), the blue lupine (*Lupinus angustifolius*), the yellow lupine (*Lupinus luteus*), and the Andean lupine (*Lupinus mutabilis*).

The occurrence of lupine in food products has increased notably in many European countries during the past decade. Reasons for this development are, on one hand, the import of bakery goods from France, where up to 10% lupine flour in wheat flour was officially authorized in 1997 and, on the other hand, the spreading skepticism against potentially gene-modified soy protein and the use of lupine as a substitute. The addition of lupine protein or flour improves the nutritional value of a food product because of its high fiber and protein content (*2*). Lupine is used in different foods, such as bread, cookies, pastry, pasta, and sauces, and in beverages as a substitute for milk or soy, whereas the seeds are used as snacks (*3*−*6*). It is considered a source of low-cost protein and can be cultivated in problematic climates, making it attractive in comparison to other protein-rich plants, like soy.

Lupine, in the form of flour, seed, or dust, has been reported to produce a variety of different allergic responses such as urticaria and angioedema (*7*), contact urticaria (*8*), oral allergy syndrome (*9*), rhinoconjunctivitis (*10*, *11*), and anaphylaxis (*12*, *13*).

Lupine allergy apparently arises either by primary sensitization (*10*, *14*) or by clinical cross-reactivity in persons who are allergic to peanut (*15*). Immunoglobulin E (IgE)-mediated in vitro cross-reactivity within the legume family is frequent (*16*) but not necessarily of clinical relevance. When patients with one or more positive skin prick tests to legumes were orally challenged with peanut, soy, pea, or bean, the rate of cross-sensitivity was only 5% (*17*), although serious peanut−soy cross-reactions have been reported in youngsters (*18*). However, after oral challenge with lupine flour, 68% of the peanut-allergic patients showed positive clinical reactions (*19*). Because peanuts are among the foods most frequently associated with severe allergic reactions, including fatal food anaphylaxis (*20*), the possible risk of crossed peanut−lupine allergy should not be underestimated.

Lupine has now also been introduced into the Norwegian market, both in imported and domestic foods. Since the first documented report of lupine allergy, in a peanut-allergic patient who had eaten a hot dog bread containing lupine flour in 2003 (*21*), 10 new cases have been registered by the Norwegian National Register for Severe Allergic Reactions to Food (*22*).

The presence of hidden allergens because of contamination and the use of new, potentially allergenic proteins in food products represent an imminent risk for allergic consumers. Sensitive methods are needed to detect and quantify the presence of such food components. Currently, the enzyme-linked immunosorbent assay (ELISA) technique is the most commonly used method for food allergen analysis, as its sensitivity is good, it is simple to operate, and it has excellent potential for standardization (*23*). Various ELISA-based methods have been developed for the detection of trace amounts of several food allergens (*24*−*27*). Detection of lupine proteins in food has hitherto been based on the use of serum from allergic patients, in Western

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blot and radio-allergosorbent test (RAST) analyses (*7*). However, standardization of serum is difficult, and the access to suitable patient sera is limited. Furthermore, patient sera are potentially infectious and therefore are not useful in routine analyses. To the present date, no method suitable for the serial detection of lupine proteins in foods has been developed.

The aim of this study was therefore to develop and validate a sensitive, specific, and quantitative sandwich ELISA for the detection of lupine proteins in processed foods. The characteristics of the method and the validation parameters are presented here.

MATERIALS AND METHODS

Purification and Labeling of AntiLupine Antibody. A polyclonal antiserum was raised against $(NH_4)_2SO_4$-precipitated Lopino (used as lupine protein standard in the ELISA) in a rabbit as previously described (*21*). Lopino (Lupina, Visbek, Germany) is a processed crude protein preparation from *L. albus* seeds and is used in the manufacturing industry. The proteins (5 mg) in the lupine standard were covalently coupled to NHS-activated (Sepharose) HP columns (Amersham Biosciences) according to the manufacturer's instructions. The rabbit serum was desalted on PD-10 columns (Amersham Biosciences, Uppsala, Sweden) and then was passed through the protein-coupled (Sepharose) column to bind lupine-specific antibodies. Bound antibody was eluted in 100 mM glycine, pH 2.5, neutralized, and stored at 4 °C. Fractions were tested for binding activity using indirect ELISA, and total protein was determined by the Lowry method (DC Protein Assay, Bio-Rad, Hercules, CA). The purity of the IgG was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions. Pure antibody fractions were pooled and, following buffer exchange to 0.1 M sodium phosphate, 0.15 M NaCl, 0.1% sodium azide, pH 7.4, on PD-10 columns, were further concentrated using Vivaspin 6 concentrators with a 10 000 molecular weight cutoff membrane (Vivascience, Hannover, Germany) and finally were stored in aliquots at −80 °C until use. For covalent conjugation with biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (Sigma-Aldrich, Steinheim, Germany), the purified antibodies were dialyzed against 0.05 M carbonate-bicarbonate buffer, pH 9, overnight at 4 °C with a 3500 molecular weight cutoff membrane (Pierce, Rockford, IL). The final concentration was adjusted to 1 mg/mL. The biotin salt was then dissolved in deionized H_2O to a concentration of 1 mg/mL, and 1 part of the resulting solution was added to 6.67 parts of the antibody solution. The mixture was vortexed and subsequently rotated at room temperature (RT) for 4 h, followed by neutralization with 1 M NH_4Cl under rotation at RT for 10 min. After a buffer change to 0.1 M sodium phosphate, 0.15 M NaCl, 0.1% sodium azide, pH 7.4, using PD-10 columns, the total protein concentration was measured and the biotinylated antilupine antibody was stored at −20 °C until use.

Protein Extraction and Food Sample Preparation. Food samples purchased from local stores in Norway and Germany were homogenized in a mechanical blender (Retsch GmbH & Co, Haan, Germany). Homogenized samples (2 g) were extracted with 10 mL 0.1 M Tris, 0.5 M glycine, pH 8.7, overnight at 45 °C in a shaking water bath. Extracts were centrifuged at 39 200*g* for 25 min at 4 °C. Fat and larger particles from the matrix were then removed by filtration through glass wool, and the total protein concentrations were determined. Supernatants were stored at −20 °C or 4 °C or were used freshly. Extracts were diluted at least 1:20 in phosphate-buffered saline (PBS) (Oxoid, Basingstoke, U.K.) containing 1% bovine serum albumin (BSA) before analysis using the sandwich ELISA.

Western Blotting. The NuPAGE Gel System (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein sample extracts by SDS−PAGE, in accordance with the manufacturer's instructions. All protein samples were applied in equal amounts (1 μg). Separation was performed under reducing conditions for 40 min at 200 V in 2-(*N*-morpholino) ethane sulfonic acid (MES) SDS running buffer, using 4−12% Bis-Tris gels and SeeBluePlus2 prestained reference standard. Samples were prepared with lithium dodecyl sulfate (LDS) sample buffer and dithiothreitol (DDT) reducing agent (all from Invitrogen). The proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane (Bio-Rad) for 60 min at 30 V with transfer buffer using a XCell II Blot Module (Invitrogen). Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.6) was used as washing buffer. TBS-T containing 1% BSA was used as blocking and assay buffer for the Western blots. After blocking for 30−60 min, the blot was incubated overnight at 4 °C with antilupine antibody (1.5 mg/mL) diluted 1:1 250 000 in assay buffer. The blot was washed (3 × 15 min) and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody (Zymed, San Francisco, CA) diluted 1:5000 in assay buffer. After washing (3 × 10 min), the membrane was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Zymed) until bands of satisfactory intensity appeared (2−10 min). All washing and incubation steps were performed at RT with gentle shaking, if not otherwise stated.

Sandwich ELISA Procedure. Flat-bottom polystyrene 96-well microplates (Corning Inc., Corning, NY) were coated overnight at 4 °C with 100 μL/well of 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma-Aldrich), containing 2 μg/mL purified rabbit antilupine antibody. All wash steps were carried out with PBS containing 0.05% Tween 20 (PBS-T, pH 7.6), three times, using a programmable automatic plate washer (Skatron Instuments, Lier, Norway). PBS containing 1% BSA was used as blocking and assay buffer for the ELISA. The wells were blocked with 250 μL/well of the blocking/assay buffer and were incubated for 1 h at RT. After washing, 100 μL/well of a 2-fold serial dilution of the previously described lupine protein standard was added in a concentration range from 0.26 to 269 ng/mL. Standards, buffer blanks (assay buffer), and sample extracts, at minimum 1:20 diluted, were incubated for 1 h at RT under gentle shaking in triplicates on each plate. After washing, bound lupine proteins were detected by adding 100 μL/well of biotinylated rabbit antilupine antibody diluted 1:100 000 and incubated for 1 h at RT under gentle shaking. Plates were washed again and were subsequently incubated with 100 μL/well of HRP-streptavidin conjugate (Zymed), diluted 1:5000 for 1 h at RT. All dilutions were performed in assay buffer, and plates were sealed with plate-sealing film during incubations. After a final wash, each well was incubated with 75 μL K-Blue TMB substrate (Neogen, Lexington, KY). Color development was stopped by the addition of 50 μL/well of 2 M H_2SO_4. Absorbance was read at 450 nm on a 1420 VICTOR^2 multilabel plate counter (Wallac, Turku, Finland).

Assay Validation: Specificity, Accuracy, Precision, Limit of Detection, and Limit of Quantification. The specificity of the method was assessed in cross-reactivity studies**.** Extracts from peanut (*Arachis hypogaea*), lentil (*Lens culinaris*), white bean (*Phaseolus vulgaris*)**,** common pea (*Pisum sativum*), soy (*Glycine max*), chick-pea (*Cicer arietinum*), hazelnut (*Corylus avellana*), dried milk, and wheat flour were prepared as previously described. All extracts were diluted 1:20 in ELISA assay buffer, which corresponds to a portion of 100% in a typical food matrix, prior to analysis with the sandwich ELISA.

The accuracy of the method was evaluated by performing recovery studies**.** Four different lupine-free food samples (hot dog bread, pasta, vegetarian sausage, and chocolate spread) were spiked with the lupine protein standard at levels of 1, 100, or 1000 μg lupine protein/g sample, in a total volume of 5 mL extraction buffer. After incubation for 15 min at RT, an additional 5 mL extraction buffer was added, and the extraction was performed as described previously. For the determination of recovery rates, the extractions were done in triplicate, the extractable proteins were analyzed by the sandwich ELISA, and the mean values for the recovery were calculated.

Precision within and between assays was estimated using extracts from four different products (originating from different lupine species) containing lupine (hot dog bread, pasta, vegetarian sausage, and chocolate spread). The extracted samples were stored in aliquots at −20 °C and each analysis was performed with freshly thawed extract. For determination of intra-assay precision, the mean coefficients of variation (CVs) were based on 10 replicates. Interassay precision was determined as the mean CVs on the basis of triplicate analyses on 10 different days.

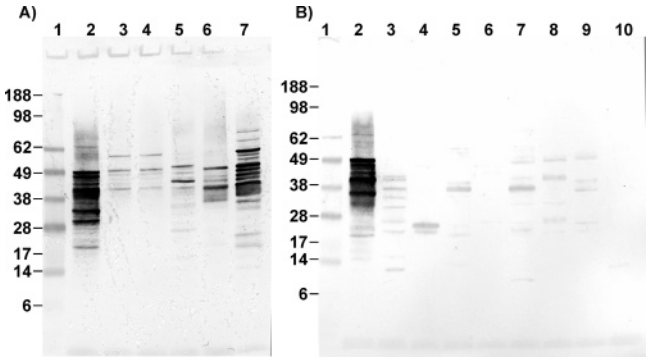

Figure 1. Western blot analysis of various foods using purified polyclonal antilupine antibody. Lane 1 in both panels (**A** and **B**): molecular weight marker, protein sizes (kDa) are indicated on the left side of the gels. (**A**) Detection of lupine proteins in lupine-containing foods. Lane 2: lupine protein standard; lane 3: hot dog bread; lane 4: pasta; lane 5: vegetarian sausage; lane 6: chocolate spread; lane 7: lupine flour. (**B**) Potentially cross-reactive foods. Lane 2: lupine protein standard; lane 3: peanut; lane 4: hazelnut; lane 5: chick-pea; lane 6: bean; lane 7: soy; lane 8: pea; lane 9: lentil; lane 10: wheat flour.

The limit of detection (LOD) for the sandwich ELISA was calculated as 3 times the standard deviation (SD) of the buffer blank mean value after 25 experiments. The limit of quantification (LOQ) was calculated as 10 times the SD of the buffer blank mean value after 25 experiments. Additionally, the LOD in four lupine-free complex food matrixes (hot dog bread, pasta, vegetarian sausage, and chocolate spread) was determined in the same way, on the basis of five experiments.

RESULTS

Detection of Lupine Proteins in Various Foods. The polyclonal antilupine antibody was used to determine the IgG-binding protein bands in a Lupino extract using Western blot analysis (**Figure 1A**). Major bands were found at 29, 33, 41, 43, 47, and 49 kDa, whereas additional bands between 17 and 63 kDa showed IgG binding to a lesser extent. Extracts of various foods, labeled as containing lupine, were also analyzed by Western blot (**Figure 1A**). Lupine proteins, originating from *L. albus* and *L. angustifolius*, were detected in hot dog bread, pasta, vegetarian sausage, chocolate spread, and lupine flour (*L. angustifolius*: Soja Austria, Vienna, Austria). Similar IgG-binding bands with molecular weights from 41 to 49 kDa appeared in all food samples labeled as containing lupine, thus confirming the choice of Lupino as a suitable lupine protein standard for a sandwich ELISA.

Sandwich ELISA Standard Curve. The lupine protein standard was used in a standard curve with concentrations from 0.26 ng/mL to 269 ng/mL. The working range of the assay was defined as the linear part of the curve with a squared correlation coefficient (R^2) > 0.99. The six-point calibration curves ranged in general from 1 ng/mL to 34 ng/mL (**Figure 2**). For the determination of lupine protein concentrations in food, serial dilutions of extracts were performed when necessary, and those that gave optical density (OD) values closest to the midpoint of the linear part of the standard curve were used to calculate the lupine protein concentrations.

Specificity. A preliminary cross-reactivity screening, analyzing peanut, hazelnut, brazil nut, almond, walnut, cashew, pea, chick-pea, pine nut, lentil, soy, casein, shrimp, cod, ovomucoid, and ovalbumin in a nonoptimized competitive ELISA, showed no inhibition with up to 40 μg/mL of total protein. The lupine protein standard at the same concentration gave total inhibition (data not shown).

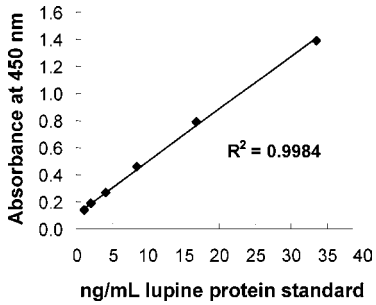

Figure 2. Representative linear six-point calibration curve based on the standard curve obtained using the lupine protein standard in the sandwich ELISA.

Table 1. Cross-Reactivity of Selected Foods and Food Ingredients in the Lupine Sandwich ELISA

food/ingredient[a]	total protein [mg/mL][b]	equivalent lupine protein [μg/g]
bean	12.5	<0.1
hazelnut	26.0	<0.1
dried milk	38.7	<0.1
wheat flour	5.3	<0.1
chick-pea	20.7	<0.4
peanut	43.1	<0.4
pea	9.7	<0.3
soy	23.0	<0.2
lentil	27.1	<0.2

[a] All food and food ingredients were extracted 1:5 (w/v) as described in Materials and Methods and were diluted 1:20 (minimum dilution in ELISA). [b] Values were obtained using the Lowry protein determination method.

Table 2. Recovery (%) of Lupine Proteins from Various Blank Food Samples Spiked with 1, 100, or 1000 μg/g of Lupine Protein Standard[a]

	recovery (%) at indicated amount of lupine protein added in μg/g		
blank food	1	100	1000
hot dog bread	116 ± 38	81 ± 11	80 ± 14
vegetarian sausage	64 ± 11	60 ± 3	64 ± 8
pasta	88 ± 25	98 ± 14	116 ± 11
chocolate spread	84 ± 21	70 ± 13	61 ± 11

[a] Values represent the average of three spiking experiments and are reported as mean ± standard error of the mean (SEM).

Extracts from potentially cross-reactive members of the Leguminosae family, a tree nut (hazelnut) and two common food ingredients (dried milk and wheat flour), were further analyzed in the optimized sandwich ELISA (**Table 1**). No cross-reactivity was found in bean, hazelnut, dried milk, or wheat flour at lupine protein concentrations corresponding to >0.1 μg/g. Minor cross-reactivity was observed for chick-pea, peanut, pea, soy, and lentil, none exceeding a lupine protein concentration corresponding to <0.4 μg/g. Further investigation using Western blot (**Figure 1B**), applying equal protein amounts, showed that chick-pea, peanut, pea, soy, lentil, and hazelnut were in fact recognized by the antilupine antibody to a minor degree. However, the number and intensity of bands were less than those observed for the lupine protein standard and lupine-containing products.

Accuracy. Blank food samples from hot dog bread, pasta, vegetarian sausage, and chocolate spread, spiked with three different amounts of lupine protein standard before sample extraction, were used to determine recovery rates (**Table 2**). In hot dog bread and pasta, the recovery of the lupine proteins

Table 3. Intra- and Interassay Variances (%CV) Determined for the Lupine Sandwich ELISA Using Lupine Containing Foods[a]

food	lupine protein [mean μg/g]	intra-assav variance (%CV)	interassay variance (%CV) ($n = 10$)
hot dog bread	100	5	14
vegetarian sausage	364	3	20
pasta	49	6	21
chocolate spread	10135	4	14

[a] The intra-assay variances were calculated from 10 replicates on the same extract, and the interassay variances were calculated from triplicate analysis of the same extract on 10 different days.

was between 80 and 116%, independent of the spiking level, whereas in vegetarian sausage and chocolate spread, the recovery was between 60 and 84%. The blank matrix extracts without lupine protein gave results below 0.1 μg/g.

Intra- and Interassay Precision. Hot dog bread, pasta, vegetarian sausage, and chocolate spread with lupine as a labeled ingredient were analyzed with the sandwich ELISA and were found to contain lupine proteins in amounts ranging from 50 μg/g to 10 000 μg/g. These products were used for the determination of the intra-assay precision and the interassay precision of the sandwich ELISA (**Table 3**). The intra-assay precision, expressed as %CV, was 3% in vegetarian sausage, 4% in chocolate spread, 5% in hot dog bread, and 6% in pasta. Interassay precision was 14% in hot dog bread and chocolate spread, 20% in vegetarian sausage, and 21% in pasta.

In a Western blot analysis with the four lupine-containing samples (**Figure 1A**), good correlations were seen between the amounts of lupine protein found in the ELISA and the band intensities on the blot. Pasta, containing 50 μg/g lupine proteins, showed the weakest band intensities, whereas chocolate spread, containing 10 000 μg/g, had the bands with the highest intensities.

Limit of Detection and Limit of Quantification. The LOD for the sandwich ELISA was <1 ng/mL lupine protein standard in assay buffer, equivalent to 0.1 μg/g sample, taking the sample dilution into account. The LOQ was <4 ng/mL lupine protein standard in assay buffer, equivalent to 0.4 μg/g sample. Furthermore, it was found that the LOD of the assay varied in the different blank food matrixes. In pasta and vegetarian sausage, the LOD was 0.1 μg/g, in hot dog bread it was 0.2 μg/g, and in chocolate spread it was 0.4 μg/g.

DISCUSSION

In the present study, a specific and sensitive sandwich ELISA for the detection of lupine protein in processed foods was developed and validated with four different complex food matrixes. The ELISA was used successfully to detect and quantify extractable lupine proteins, both in lupine-spiked foods and in various commercial foods known to contain lupine of two commercially used lupine species.

The sandwich ELISA was constructed using a purified polyclonal antibody for capture and a biotin conjugate of the same antibody for detection. The antilupine antibody recognized lupine proteins with molecular weights from 17 kDa to 63 kDa, as it was raised against several proteins in the $(NH_4)_2SO_4$-precipitated Lopino extract. Although the use of polyclonal antibodies directed against a single, purified protein or the use of monoclonal antibodies could have resulted in higher specificity, an antibody with high versatility was selected, thus maximizing the detection of lupine protein in complex food matrixes and favoring optimal consumer protection. Furthermore, by using a polyclonal antibody, the probability of detecting proteins which are altered or denatured is increased, as the antibody recognizes several different proteins and epitopes. Different food processing techniques may affect proteins, for example, by destroying specific epitopes or exposing hidden ones because of protein unfolding (*28*, *29*). For lupine, this remains to be evaluated, but in general, it is accepted that the use of polyclonal antibodies is favorable for food analysis.

Major proteins were present from 41 to 49 kDa in the different lupine-containing food products used in this study. Independently of matrix (e.g., pasta or chocolate spread) and processing, the proteins were identifiable on SDS−PAGE (data not shown) and were recognized by the antilupine antibody on Western blot and in the ELISA. The observed molecular weights of these proteins agree with published data where sera from patients allergic to lupine have been used. A 43 kDa protein is reported to be the major IgE binding protein (*15*).

The specificity of the antilupine antibody was tested both in ELISA and Western blot, using extracts from several other plants within the Leguminosae family and other commonly used food ingredients. The test concentrations used in the sandwich ELISA were selected as equivalent to those in raw food ingredients, so that the actual situation in multiple-component matrixes was overestimated by far, and the assay for the determination of any cross-reactivity was run under especially discriminating and challenging circumstances. The few positive responses observed all came from plants taxonomically closely related to lupine. Nevertheless, they produced only signals corresponding to less than 0.4 μg/g lupine protein in the ELISA. Considering the high protein concentrations used in the test, the presence of these proteins probably has no significance in analyses of actual food samples. Yet, the possibility of cross-reaction with other less commonly used legumes or other ingredients cannot be excluded and remains to be examined. Western blot analysis using the antilupine antibody confirmed the ELISA data. The observed cross-reactions are probably due to similar epitopes on the different legumes, which could not be totally discriminated by the polyclonal antilupine antibody. Similar observations have been found in in vitro studies using serum IgE from patients reacting to a specific legume, demonstrating cross-reaction to other legumes (*16*, *17*). The clinical relevance of such cross-reactions is generally low, with the exception of lupine/peanut-crossed reactions (*15*, *19*). However, with the new polyclonal antilupine antibody, a suitable tool is now available, which allows the differentiation between lupine and peanut proteins in food matrixes, thus facilitating the identification of the eliciting allergen in affected patients.

Hazelnut protein was the only other food ingredient besides the legumes that gave a positive response in the selectivity test, though only on Western blot and not with the ELISA. This may be caused by the unfolding of proteins as a result of the denaturing conditions prior to and during the electrophoresis and thereby exposing epitopes formerly hidden in the native protein. Moreover, the corylin preparation used in these studies seemed to be unstable following repeated thawing and freezing and showed nonspecific binding to several different, unrelated antifood antibodies (data not shown).

The key parameters and characteristics of the lupine sandwich ELISA were further determined in an in-house validation, performed according to the recommended harmonized guidelines for single laboratory validation (*30*). The accuracy of the method was studied with recovery experiments, adding lupine protein

standard in varying amounts to four different blank food matrixes. For all four, the variances were highest at the lowest amounts of spiked lupine protein, probably because of poor sample homogeneity at this low level. The recovery of lupine protein from mainly cereal flour containing matrixes such as bread and pasta was almost complete, whereas it was reduced by about 20% from multicomponent food products such as vegetarian sausage and chocolate spread. These results agree well with the common assumption that food matrix components may interfere with either the extraction or the immunoassay procedure leading to suboptimal recovery rates. A less-than-optimal extraction procedure may partially explain the underestimation in some of the analyses. A full validation of the extraction procedure remains to be performed. For the lupine ELISA presented here, the recoveries obtained were considered to be satisfactory. However, the trueness of the extraction and the method could not be determined because a certified reference material for lupine protein is not available, and validation by an alternative analytical method was not possible either.

The intra-assay and interassay precision data for the ELISA were obtained by using four relevant lupine-containing foods. Lupine is added to wheat flour and is mostly used in bread or pasta products, but other applications, for example, as a protein source in vegetarian products or spreads, are also increasing. The lupine protein contents in the foods tested ranged from 50 μg/g in pasta to more than 10 000 μg/g in chocolate spread. The repeatability of the sandwich ELISA as measured by intra-assay precision was <6%, and the reproducibility as measured by interassay precision was <21%. The method worked reliably, was shown to be robust, and the performance precision was regarded as satisfactory.

With a detection limit of 0.1 μg lupine protein/g sample and a quantification limit of 0.4 μg/g, the performance of the new ELISA in terms of sensitivity is better than 1 μg/g, a concentration that gives a safety margin to the majority of consumers with food allergy (*31*). Although as little as 100 μg peanut protein was enough to trigger mild allergic reactions in extremely sensitive persons, no life-threatening incidents have been recorded at such low doses (*32*). The minimal provoking dose of lupine protein is unknown and is probably subject to considerable individual variation. However, because of the observed similarities among the legume proteins, it may be assumed that the no-effect level is likely to be similar to that of peanut. Consequently, the newly developed lupine sandwich ELISA is sensitive enough to detect lupine protein traces in food which might elicit allergic reactions in most of the sensitized consumers.

In a final evaluation of the validation data for the lupine sandwich ELISA and its application, the limit of detection for routine analysis was set to 1 μg lupine protein/g sample. This will reduce the probability of false positive results because of weak cross-reactions and will further increase the reliability of the assay, which is important for surveys and for the determination of hidden allergens in industrially manufactured food products.

In conclusion, a specific sandwich ELISA for the detection of lupine protein in processed food was successfully developed and validated with four different complex food matrixes. To our knowledge, this is the first method described for the detection of lupine protein that does not depend on the use of patient serum. The new sandwich ELISA is suitable for applications in the food industry, for example, the control of raw ingredients, processing equipment, and final products for contamination, and for food surveys commissioned by food authorities. Increased food safety is a current issue, and the protection of consumers with food allergy from the danger of hidden allergens is an important part of this policy.

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