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Rapid Shotgun Proteomic Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry-Based Method for the Lupin (*Lupinus albus* L.) Multi-allergen Determination in Foods

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ABSTRACT: Allergy to lupin is a growing food safety problem because this legume, increasingly exploited in the food industry, is one of the allergens that, according to law, must be declared on the labels of food products in the European Union. In this context, a rapid targeted proteomic approach based on liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) analysis was proposed and aimed to unequivocal confirmation and reliable determination of the major lupin allergens, i.e., conglutins, in pasta and biscuits. Detected concentrations were around 1 mg of lupin/kg of pasta and biscuits, proving the capabilities of the MS-based method in terms of the sensitive allergen screening method. Good precision was observed in terms of both intra- and interday repeatability, with relative standard deviation (RSD) lower than 23%. Recoveries from 95 ± 10 to 118 ± 12% and from 103 ± 1 to 110 ± 12% ranges were calculated for biscuits and pasta, respectively. Finally, the applicability of the devised method was investigated by analyzing market samples containing lupin and samples that may possibly contain traces of lupin deriving from cross-contamination between products and production lines.

KEYWORDS: *lupin allergens, liquid chromatography–ion-trap tandem mass spectrometry, quantitative analysis, pasta, biscuits*

INTRODUCTION

Lupin seed has traditionally been consumed as an appetizer and snack food for ages. During the past decade, lupin flour has been increasingly used as a food ingredient, especially in pasta and bakery products, because of its interesting nutritional, nutraceutical, and functional properties.[1−3] The high protein and fiber content makes lupin a good candidate as an alternative protein source in substitution of soy, egg, and animal proteins, also meeting demands of the vegetarian consumers. Because lupin flour does not contain gluten, it has also been proposed for the production of gluten-free food for patients with wheat allergies and celiac disease.[3] In addition, it has been demonstrated that a regular consumption of lupin may prevent diseases, such as obesity, diabetes, and also cardiovascular disease, thanks to its hypocholesterolemic and hypoglycaemic properties.[4] As for its functional properties, lupin flour acts as a taste and yellow color enhancer as well as a natural emulsifier, thus being used in not only baked products and pasta but also meat broth and soup, vegetable drinks, and creams. As a consequence of the growing introduction of lupin in the human diet, allergic reactions to lupin have been increasingly documented as either primary lupin allergy involving ingestion or inhalation of lupin flour[5−7] or a result of a cross-reactivity to the rest of the leguminous species, especially peanut.[8,9] Taking into account the opinions of the European Food Safety Authority (EFSA) related to the evaluation of lupin for labeling purposes,[10] European Commission Directive 2006/142/EC established that lupin should be included in Annex IIIa (Directive 2000/13/EC), which contains a list of the ingredients that must under all circumstances appear on the labeling of foodstuffs, because they are likely to cause adverse reactions in susceptible individuals.[11] However, lupin may be present in food products as hidden allergen, resulting from intentional and undeclared addition of lupin flour or from cross-contamination during food production processes. To assess the risks of allergic reactions, eliciting threshold doses (EDs) for lupin have been evaluated, but available ED values are still inconsistent.[2] On this regard, a recent study reported that lupin-allergic patients react at doses as low as 0.5 mg of lupin flour, pointing out that modest exposures could elicit reactions.[3]

Protein electrophoresis and immunoblotting as well as mass spectrometry (MS)-based studies[1,12−14] have been used to identify and characterize the seed storage proteins, conglutins in particular, as major allergens of the *Lupinus* species. The two major fractions are β-conglutin (vicilin-like protein or acid 7S globulin) and α-conglutin (legumin-like protein or 11S globulin), whereas the two minor components are δ-conglutin (2S sulfur-rich albumin) and γ-conglutin (basic 7S globulin).[1] Even though food processing, such as thermal treatments, can influence the allergenicity of food proteins,[15] it was observed that lupin allergens are relatively stable to heat.[16] Thus, to safeguard the health of consumers and to ensure compliance with food-labeling regulation, sensitive, accurate, and reliable analytical methods for the detection and quantitation of lupin allergens in food products are necessary. With this aim, in recent years, antibody sandwich enzyme-linked immunosorbent

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assays (ELISAs) for the determination of processed and native lupin proteins at trace levels in food matrices have been developed.[17−19] However, because ELISAs could be affected by cross-reactivity and unpredictable effect of processing on food matrices and/or protein epitopes, positive ELISA results preferably require confirmatory analysis by non-immunological techniques, such as polymerase chain reaction (PCR) or MS, to corroborate data and to improve detection specificity.[18]

Thus, a hybridization probe-based real-time PCR assay has been recently proposed as an alternative to ELISA methods for the detection of lupin DNA in foods[20] and for the study of the effect of processing on lupin detectability.[21] A duplex real-time PCR method has also been developed for the simultaneous detection of soy- and lupin-specific mtDNA sequences in a heat-treated model food matrix and in commercial food products.[22] Despite the very high specificity and sensitivity, the main disadvantage of PCR methods is that they detect DNA and not directly the target allergen proteins, therefore not allowing for the measurement of actual health risk. In fact, dependent upon protein expression, significant variations in the relationship between the quantity of DNA and the amount of allergen present may be observed. Moreover, the presence of DNA in a food product does not guarantee the presence of allergens and vice versa.

On the other hand, MS-based methods overcome the limits of both immunological and PCR techniques and play a pivotal role in proteomic research. In fact, thanks to their high specificity, sensitivity, and accuracy, MS-based methods are applied as confirmatory tools for unambiguous identification and characterization of proteins and peptides. In recent years, the development and application of liquid chromatography−electrospray ionization−tandem mass spectrometry (LC−ESI−MS/MS)-based techniques for the investigation of allergens in food is considerably increased.[23−28] Concerning lupin allergens, a method based on bioinformatic processing of high-performance liquid chromatography (HPLC)−ESI−MS/MS data for the detection and quantitation of the main storage proteins of *Lupinus albus* in lupin beverage and lupin protein isolates has been devised.[29] The same research group proposed the development of a nano-HPLC-chip−MS/MS label-free method for the simultaneous characterization and relative quantitation of *L. albus* seed storage proteins in protein extracts to profile their different expression in four lupin cultivars.[30] More recently, a HPLC-chip−ion-trap MS/MS method has been devised and applied for label-free absolute quantitation of γ-conglutin in lupin seed protein extract.[31]

When the growing necessity of sensitive and accurate determination of lupin content in processed food products is taken into account, in the present study, our attention was focused on the development and validation of a shotgun proteomic LC−ESI−MS/MS-based method for the simultaneous detection and quantitation of lupin allergens in biscuits and pasta in a single short run. The method involves the use of a sample treatment incorporating solid-phase extraction (SPE) with size-exclusion columns for sample cleanup. The allergenic proteins β-conglutin, α-conglutin, γ-conglutin, and δ-conglutin were investigated by selecting and monitoring specific and unique target tryptic peptides.

To the best of our knowledge, this is the first method based on SPE sample cleanup and tandem MS measurements of multiple lupin proteins, developed for rapid detection, unambiguous confirmation, and determination of lupin residues at trace levels in food products.

MATERIALS AND METHODS

Chemicals. Trizma hydrochloride (Tris-HCl, >99% purity), sodium chloride (>99.5% purity), ammonium hydrogen carbonate (99% purity), formic acid (>98%), acetonitrile (99.9% purity), trifluoroacetic acid (TFA, >98%), iodoacetamide (IAM, >99% purity), D,L-dithiothreitol (DTT, >99% purity), trypsin from bovine pancreas, Bradford reagent, and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). Sodium dihydrogen orthophosphate (>99% purity) was purchased from Carlo Erba (Milan, Italy). Buffered solutions and mobile phases were obtained in HPLC-grade water prepared with a Milli-Q element A10 system (Millipore, San Francisco, CA).

Lupin Samples. Dry sweet white lupin seeds (*L. albus*) were kindly provided by SA.DE.S. Olive (Parma, Italy).

Market Samples. Five food products containing lupin (pasta and biscuits) as an ingredient and five biscuit and pasta samples reporting the precautionary label "may contain traces of lupin" were obtained from a local food store. Biscuits and wheat pasta used as blank samples for method validation did not report the precautionary label "may contain traces of lupin".

Sample Treatment. Manually peeled lupin seeds and samples were ground under liquid nitrogen to obtain a fine and homogeneous powder. A total of 1 g of ground sample was suspended in 25 mL of 0.2 M Tris-HCl buffer (pH 8.2), and protein extraction was carried out under magnetic stirring for 6 h at 60 °C. The efficiency of the protein extraction procedure was initially evaluated by varying the composition and concentration of extraction buffer and then quantitating the total protein content by the Bradford assay using BSA as a standard protein. The extraction buffers used were 0.2 M Tris-HCl (pH 8.2), 0.05 M Tris-HCl with 0.5 M NaCl (pH 8.0), 0.05 M NH_4HCO_3 (pH 8.0), and 0.1 M NaH_2PO_4 (pH 8.0). For each buffer, two independent extractions of 1 g of lupin powder were performed.

The extract was then centrifuged at 10 000 rpm for 20 min at 4 °C and then filtered on 0.2 μm regenerated cellulose filters (Econofilters, Agilent Technologies, Santa Clara, CA). A total of 5 μL of 200 mM DTT solution (in 50 mM NH_4HCO_3 buffer at pH 8.0) was added to 91 μL of extract for protein reduction (at 37 °C for 45 min). Subsequently, 4 μL of 1 M IAM solution (in 50 mM NH_4HCO_3 buffer at pH 8.0) was added for the protein alkylation purpose, and the reacting mixture was stored in the dark for 45 min at room temperature. A buffer exchange and sample purification step was carried out using size-exclusion columns (Bio-Rad Laboratories, Milan, Italy) with a 6 kDa exclusion limit. First, the column was centrifuged for 2 min in a swinging bucket centrifuge at 1000*g* to remove the packing buffer, and then it was conditioned 3 times with 500 μL of 50 mM NH_4HCO_3 buffer (pH 8.0) and centrifuged for 1 min. A total of 100 μL of the extract was loaded directly on the column and centrifuged for 4 min. The purified sample (100 μL) was thus collected in a 2.0 mL microcentrifuge tube. Finally, the enzymatic digestion was performed by adding 5 μL of 2 mg/mL trypsin solution to 100 μL of purified protein extract (protein/trypsin ratio of 50:1) and carried out at 50 °C for 24 h. The digestion reaction was quenched with 1 μL of TFA.

HPLC−Ion-Trap MS. LC separation was performed using a 100 × 2.1 mm inner diameter, 2.7 μm, Kinetex C_{18} column (Phenomenex, Torrance, CA) thermostatted at 25 °C on a HPLC system (Thermo Electron Corporation, San Jose, CA). The mobile phase was delivered by the Surveyor chromatographic system (Thermo Electron Corporation) equipped with a 200 vial capacity sample tray. All data were acquired using a LTQ XL linear ion-trap mass spectrometer (Thermo Electron Corporation) equipped with a pneumatically assisted ESI interface. The system was controlled by the Xcalibur software. A binary solvent gradient was used for the analysis of the tryptic digests. Solvent A consisted of 0.1% (v/v) formic acid aqueous solution, and solvent B was 0.08% (v/v) formic acid in acetonitrile. The sheath gas (nitrogen, 99.9% purity), the auxiliary gas (nitrogen, 99.9% purity), and the sweep gas (nitrogen, 99.9% purity) were delivered at flow rates of 50, 20, and 5 arbitrary units, respectively. Optimized conditions of the source were set as follows: ESI voltage,

Table 1. SRM Transitions Monitored for the Peptides from the Lupin Allergen Proteins Investigated (MS^2 Mode)

protein (UniProt ID)	peptide sequence	m/z precursor (charge state)	m/z product 1 (fragment type)/m/z product 2 (fragment type)
β-conglutin (Q53HY0)	ATITIVNPDRR	419.2 (+3)	542.5 (y_9^{+2})[a]/486.0 (y_8^{+2})
	IVEFQSKPNTLILPK	576.4 (+3)	758.1 (y_{13}^{+2})[a,b]/693.6 (y_{12}^{+2})
α-conglutin (Q53I54)	GLEETLCTMK	591.1 (+2)	582.4 (water loss)[a]/539.2 (y_4^{+1})
	VIIPPTMRPR	394.1 (+3)	484.3 (y_8^{+2})[a,b]/427.9 (y_3^{+1}, y_7^{+2})
δ-conglutin (Q333K7)	ALQQIYENQSEQCQGR	651.3 (+3)	700.0 (y_{11}^{+2})[a,b]/554.2 (b_5^{+1})
	QEEQLLEQELENLPR	623.3 (+3)	799.1 (b_{13}^{+2})[a]/741.4 (b_6^{+1})
γ-conglutin (Q9FSH9)	ISGGVPSVDLIMDK	715.8 (+2)	1017.6 (y_9^{+1})[a,b]/414.2 (b_5^{+1})
	VGFNTNSLK	490.2 (+2)	481.9 (water loss)[a,b]/412.4 (y_7^{+2})

[a]Most intense transition. [b]Transition monitored for the calculation of the validation parameters.

Table 2. Sequence Coverage Percentages Obtained for Each Protein Using Different Extraction Buffers (n = 2)[a]

protein	0.2 M Tris-HCl at pH 8.2 (%)	0.05 M Tris-HCl and 0.5 M NaCl at pH 8.0 (%)	0.05 M NH_4HCO_3 at pH 8.0 (%)	0.1 M NaH_2PO_4 at pH 8.0 (%)
β-conglutin	73 ± 3	51 ± 3	60 ± 4	65 ± 4
α-conglutin	22 ± 2	26 ± 3	11 ± 1	25 ± 2
δ-conglutin	67 ± 3	57 ± 2	66 ± 4	57 ± 3
γ-conglutin	40 ± 3	26 ± 2	30 ± 4	25 ± 2

[a]n = number of independent samples.

3.5 kV; capillary voltage, 20 V; tube lens, 100 V; and capillary temperature, 200 °C.

The following gradient elution was carried out under data-dependent acquisition (DDA) mode: solvent B was set at 2% for 5 min and then delivered by a linear gradient from 2 to 40% in 63 min and to 85% in 2 min. Solvent B was maintained at 85% for 5 min before column re-equilibration (15 min). The flow rate was 200 μL/min, and the injection volume was 10 μL. Using this acquisition mode, the ion trap was programmed to ignore any singly charged species acquired in the 300−1200 amu mass range and to perform MS/MS analysis (collision energy of 30 eV) only on eluting species that overcome a predefined threshold of 500 cps. Then, the peptide fragment ion list generated was processed by searching sequence database Bioworks 3.3 software (Thermo Electron Corporation) with stringent criteria, against a FASTA database containing only β-conglutin, α-conglutin, γ-conglutin, and δ-conglutin sequences and specifying the iodoacetamine derivative of cysteine (+57) as a fixed modification. For each conglutin, two marker peptides were selected in terms of peptide probability (<0.5), Xcorr (>1.5), signal intensity of the most abundant fragment of MS/MS spectrum, no post-translational modification sites, and sequence specificity (BLAST search; algorithm, blastp; MATRIX PAM 30; GAP COSTS, existence 10, extension 1; DATABASE, nonredundant protein sequences).

LC−MS^2 separation of the peptides selected was carried out in selected reaction monitoring (SRM) mode under the following optimized gradient: solvent B was initially set at 2% for 2 min and then delivered by a linear gradient from 2 to 25% in 9 min and to 60% in 1 min. Solvent B was maintained at 60% for 1 min before column re-equilibration (7 min). The flow rate was 200 μL/min, and the injection volume was 10 μL.

The mass spectrometer was operated in time-scheduled SRM mode by monitoring the peptides ATITIVNPDRR, VIIPPTMRPR, VGFNTNSLK, and ALQQIYENQSEQCQGR from 0 to 10.9 min (segment 1) and the peptides GLEETLCTMK, IVEFQSKPNTLILPK, ISGGVPSVDLIMDK, and QEEQLLEQELENLPR from 10.9 to 20 min (segment 2).

The optimized collision energy was 30 eV for all peptides, except for VGFNTNSLK and GLEETLCTMK, for which collision energy was set to 15 eV. The target peptides with the monitored SRM transitions are reported in Table 1.

Method Validation. Validation of the whole analytical method was performed on fortified samples of biscuits and wheat pasta according to Eurachem guidelines.[32] For this purpose, food samples were fortified with different amounts of ground lupin before extraction, and measurements were carried out by monitoring the most abundant MS^2 transition for each peptide (Table 1), i.e., the transition from the precursor ion to product ion 1, whereas the transition from the precursor ion to product ion 2 is used for confirmatory purposes. The detection limits (LOD) and the quantitation limits (LOQ) were calculated from the calibration curve as 3 s/slope and 10 s/slope, respectively, where s is the standard deviation of the blank signal obtained from 10 independent blank measurements. Linearity was investigated starting from LOQ values of each peptide to 25 000 mg/kg (eight concentration levels, two replicates for each level, and two injections for each extract). Mandel's fitting test was performed to check linearity. The significance of the intercept (significance level of 5%) was established by running a t test. Precision was assessed as relative standard deviation (RSD) for each peptide in terms of intra- and interday repeatability. For this aim, the intraday repeatability was evaluated by performing three independent extractions of the matrix fortified at two concentration levels (first level, LOQ value of each peptide; second level, 2 g of lupin/kg of matrix in the case of pasta or 5 g of lupin/kg of matrix in the case of the biscuit sample) and three LC−MS^2 injections for each extract in the same day. The interday repeatability was calculated on 5 days by performing five independent extractions of the matrix fortified at the same two concentration levels and three LC−MS^2 injections for each extract. The matrix effect was assessed by a t test (α = 0.05, two-tailed) between curve slopes calculated on a matrix tryptic digest and on an aqueous tryptic digest. Recovery of the whole method was calculated by analyzing a matrix-fortified sample at two concentration levels, i.e., LOQ of each peptide and 1 g of lupin/kg of matrix in the case of pasta or 5 g of lupin/kg of matrix in the case of the biscuit sample. Trueness was measured in terms of percent recovery, which was calculated as a ratio of determined and added lupin content (=determined milligram of lupin/kilogram of matrix/added milligram of lupin/kilogram of matrix × 100).

RESULTS AND DISCUSSION

Sample Treatment, Marker Peptide Selection, and LC−Ion-Trap MS/MS. Crucial steps in method development are the sample treatment and the selection of proper tryptic peptides to be monitored. Protein extraction requires the choice of the proper buffer system able to efficiently solubilize food sample proteins. For this purpose, four buffer solutions, selected among the most widely used for protein extraction, were evaluated in terms of the total protein extraction yield.

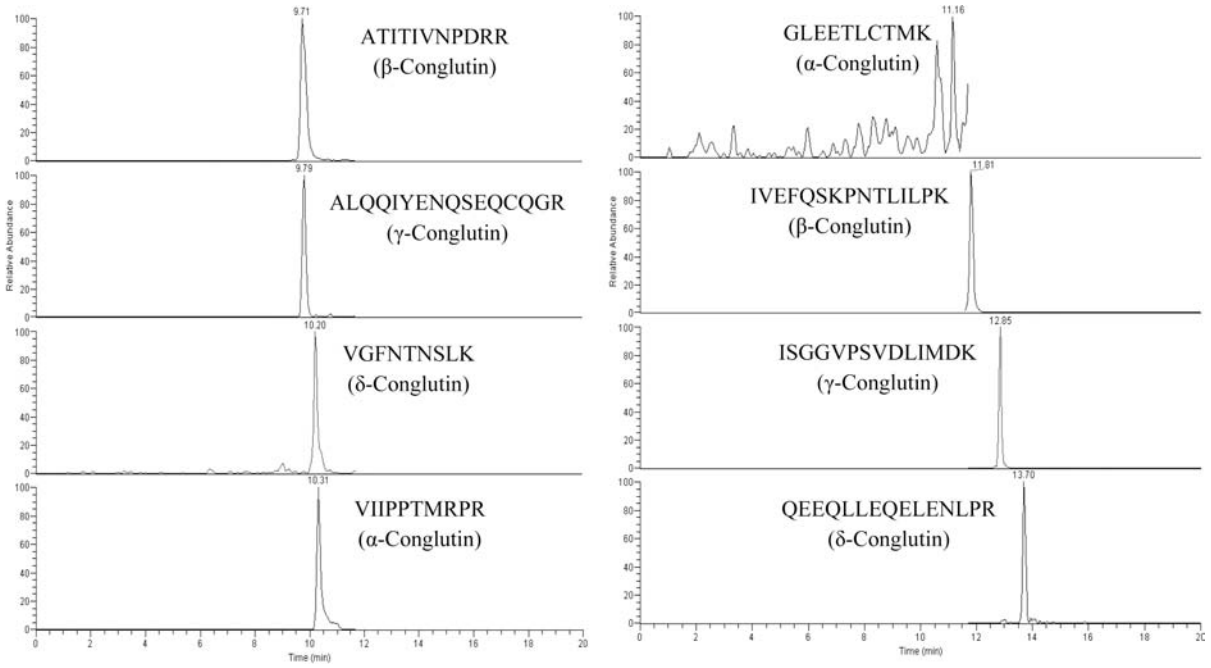

Figure 1. SRM LC−ESI−MS^2 chromatograms of the eight targeted peptides from a tryptic digest of a purified biscuit sample. The blank matrix was fortified with 1% (w/w) lupin.

Table 3. Validation Data for the Quantitative Analysis of Lupin Allergens in Pasta and Biscuits under SRM MS^2 Acquisition Mode

matrix	protein	peptide sequence	LOD (mg/kg)[a]	LOQ (mg/kg)[a]	linear range (mg/kg)[a]	calibration curve $y = a \pm sx$	r^2 (n)
pasta	β-conglutin	IVEFQSKPNTLILPK	1	4	LOQ−1250	$37.6 \pm 0.2x$	0.999 (16)
	α-conglutin	VIIPPTMRPR	4	14	LOQ−25000	$6.58 \pm 0.03x$	0.999 (32)
	δ-conglutin	ALQQIYENQSEQCQGR	5	19	LOQ−2500	$1.25 \pm 0.02x$	0.992 (16)
	γ-conglutin	ISGGVPSVDLIMDK	13	42	LOQ−12500	$0.168 \pm 0.002x$	0.996 (20)
biscuit	β-conglutin	IVEFQSKPNTLILPK	1	4	10−1000	$25.5 \pm 0.3x$	0.997 (12)
	α-conglutin	VIIPPTMRPR	2	6	10−5000	$3.36 \pm 0.07x$	0.990 (16)
	δ-conglutin	ALQQIYENQSEQCQR	18	60	LOQ−5000	$0.55 \pm 0.01x$	0.990 (16)
	γ-conglutin	VGFNTNSLK	24	80	100−5000	$0.45 \pm 0.01x$	0.975 (12)

[a]Concentrations (milligram of lupin/kilogram of matrix) are referred to the matrix fortified with lupin.

Statistical analysis [analysis of variance (ANOVA)] showed no significant differences ($p > 0.05$) between the mean values of the extraction yields (~ 64%) for the buffer solutions under investigation. Thus, the best extraction conditions were chosen on the basis of the highest sequence coverage for the selected lupin proteins. To this aim, DDA data were processed to identify lupin tryptic peptides and to assess the sequence coverage for each conglutin. The obtained sequence coverage percentages are reported in Table 2. Because 0.2 M Tris-HCl showed the best sequence coverage for the most of the investigated proteins, it was chosen as the default extraction buffer. When the complexity of food matrices was taken into account, a rapid and effective purification step on protein extract was performed using 6 kDa size-exclusion cartridges to collect samples free from low-molecular-weight compounds, such as carbohydrates and lipids.

The next step was the selection of two marker peptides for each lupin protein able to unequivocally target allergens under investigation, i.e., occurring in every tryptic digest and satisfying stringent criteria as good ESI sensitivity, no post-translational modification sites, and sequence specificity (Table 1). To avoid false positive errors, BLAST searches were performed to verify that the peptide amino acid sequences are unequivocal for the protein of interest.

In the method development, particular attention was devoted to the separation of the lupin marker peptides. A rapid tryptic peptide separation is very important for the development of a reliable LC−ESI−MS/MS method for screening quantitative analysis in food control quality. Gradient optimization, involving acetonitrile as an organic-phase modifier in water, allowed for peptide elution within 15 min. As an example, LC−ESI−MS/MS chromatograms relative to a fortified biscuit sample are reported in Figure 1.

Method Validation. Validation of the whole analytical procedure was performed on the fortified samples of biscuit and pasta samples. Table 3 reports validation results related to the most intense peptide for each allergen. As expected, the different relative abundance of the conglutins in lupin seed[1] was found to affect the LOD and LOQ values calculated for the marker peptides. Correspondingly, peptides from the two major lupin seed storage proteins, i.e., β-conglutin (43.4%) and α-conglutin (33%), showed lower LODs and LOQs with respect to those from δ-conglutin (12.5%) and γ-conglutin (6%), allowing for the detection and quantitation of the lupin content

at trace levels. Linearity was proven up to 2 and 3 orders of magnitude for biscuit ($r^2 \geq 0.975$) and pasta ($r^2 \geq 0.992$) samples, respectively. In addition, no significant intercepts confirmed the absence of constant systematic errors at a confidence level of 95%. A good precision was proven in terms of both intraday repeatability [RSD in the 2−16% range (n = 18)] and interday repeatability [RSD in the 7−23% range (n = 75)].

The matrix effect was investigated for both matrices to assess the presence of systematic proportional errors. In the case of pasta, significant ion suppression ranging from 20 to 58% for the tryptic peptides ALQQIYENQSEQCQGR, VIIPPTMRPR, and ISGGVPSVDLIMDK was calculated, whereas significant signal enhancement of 23% for IVEFQSKPNTLILPK was observed. In the case of biscuit, significant signal enhancement in the 10−42% range for IVEFQSKPNTLILPK, VIIPPTMRPR, and ALQQIYENQSEQCQR was obtained, whereas the VGFNTNSLK peptide did not show a significant matrix effect, resulting from the absence of the significant difference between the slopes of aqueous and matrix-matched standard curves ($p > 0.05$). Thus, the matrix effect was overcome by performing a label-free quantitation using calibration curves built on matrix tryptic digests.

Method recoveries obtained in the case of biscuits were in the 95 ± 10 to 118 ± 12% range; similarly, recoveries from pasta were found to vary between 103 ± 1 and 110 ± 12%.

Method Application. Reliability of the developed method for the analysis of samples purchased from a company specialized in the production of gluten-free products was assessed. For qualitative investigation and confirmation of lupin content, all of the eight peptides were monitored, whereas for quantitative purposes, only the signal area of the IVEFQSKPNTLILPK peptide resulting from digestion of the most abundant β-conglutin was used. First, the method was applied for the quantitation of the lupin content in market samples declaring lupin among their ingredients (Table 4). All of these tested samples resulted to be positive with some differences in the content. In the pasta samples, lupin is one of major ingredients, with lupin levels around 6% (sample 1), 7% (sample 2), and 10% (sample 3). Lupin was also detected in biscuit samples but at levels around 0.03% (sample 6) and 2.5% (sample 7).

Table 4. LC−ESI−SRM−MS^2 Determination of the Lupin Content in the Food Samples Investigated (n = 3)[a]

	market sample	lupin declaration	lupin (%)
pasta	1	lupin flour	5.8 ± 0.3
	2	lupin flour	7.1 ± 0.3
	3	lupin flour	10.4 ± 0.5
	4	may contain traces of lupin	nd[b]
	5	may contain traces of lupin	nd[b]
biscuits	6	lupin flour	$2.9 \pm 0.5 \times 10^{-2}$
	7	lupin flour	2.5 ± 0.2
	8	may contain traces of lupin	nd[b]
	9	may contain traces of lupin	$1.6 \pm 0.2 \times 10^{-3}$
	10	may contain traces of lupin	$0.55 \pm 0.09 \times 10^{-3}$

[a] n = number of independent samples. [b] nd = not detected.

In the further step of the work, a possible cross-contamination that may occur during the production process was investigated by analyzing five samples not containing lupin as ingredient but reporting on their labels "may contain traces of lupine". As shown in Table 4, lupin was detected in two of three biscuit samples, whereas the other pasta and biscuit sample results were negative to lupin, proving the capability of the LC−MS/MS method to detect and quantitate lupin at trace levels. The sensitivity of the developed method was comparable to currently available lupin-check ELISAs[17,18,33] and PCR-based methods,[20−22] showing LOD values of about 1 mg of lupin/kg of food; on the other hand, a hybridization probe-based real-time PCR method reached a LOD value of 0.1 mg/kg. However, it has to be pointed out that the MS/MS approach followed in the present work is suitable for the unambiguous determination of lupin allergens in complex food matrices, overcoming the main limitations of ELISAs and PCR methods in terms of selectivity, accuracy, and reliability. In fact, the use of marker peptides of major lupin allergens gives multiple chances of identification of allergen contamination in food, and because these peptides are unique, false-positive allergen detection is dramatically reduced. To our knowledge, no published data are available regarding lupin content investigation and determination in the same food matrices using MS-based methods. In conclusion, the sensitivity and selectivity reached by the sample treatment method coupled with the targeted proteomic-based LC−ESI−MS/MS approach allow us to propose a powerful quantitative confirmatory method for quality food control.

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Notes

The authors declare no competing financial interest.

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