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Multiplex liquid chromatography-tandem mass spectrometry for the detection of wheat, oat, barley and rye prolamins towards the assessment of gluten-free product safety

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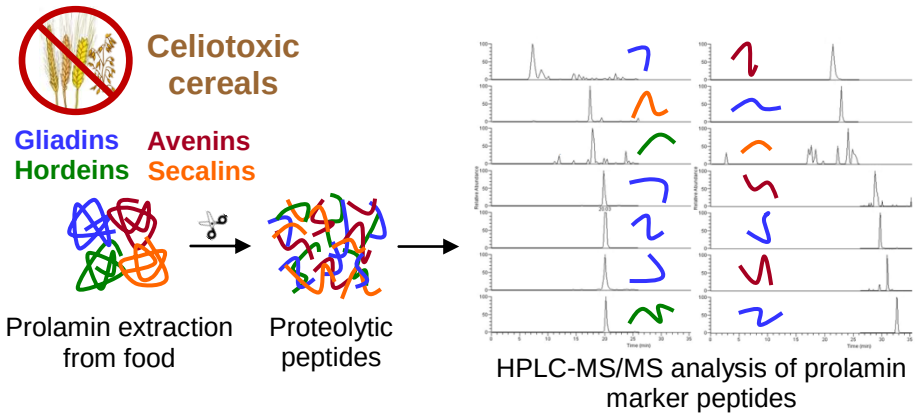
*Graphical Abstract

Graphical abstract



Highlights

- Multiplex LC-MS/MS detection of wheat, oats, barley and rye in food.
- Discrimination among celiotoxic cereals by selection of unique marker peptides.
- Defatting step for matrix complexity reduction and improved sensitivity.
- Investigation of gluten presence in different kinds of food product samples.

Abstract

Celiac patients should feel confident in the safety of foods labelled or expected to be gluten-free. In this context, a targeted shotgun proteomic approach based on liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) technique was proposed to assess the presence of celiotoxic cereals, namely wheat, oats, barley and rye, in raw and processed food products. To this aim, unique marker peptides were properly selected in order to distinguish between the different cereal types. A cocktail solution based on reducing and denaturing agents was exploited for prolamin extraction from raw and processed food; in addition, defatting with hexane was carried out for sample clean-up, allowing to largely reduce problems related to matrix effect. Method validation on fortified rice flour showed good analytical performance in terms of sensitivity (limits of detection in the 2-18 mg kg^{-1} range). However, poor trueness was calculated for self-made incurred bread (between 3 and 30 % depending on the peptide), probably due to baking processes, which reduce gluten extractability. Thus, it is evident that in the case of processed foods further insights into extraction efficiency and reference materials for protein calibration are required to obtain accurate gluten determination. Finally, the developed method was applied for the analysis of market food products, offering the possibility to discriminate among cereals, with good agreement with labelled ingredients for gluten-containing foodstuffs.

Keywords: Shotgun proteomic analysis; liquid chromatography-ion-trap tandem mass spectrometry; gluten-free food; celiac disease; celiotoxic cereals; gluten

1. Introduction

In individuals affected by celiac disease the ingestion of prolamins, the alcohol-soluble proteins of gluten [1], from wheat (gliadins), barley (hordeins), rye (secalins) and probably certain oat varieties (avenins) induces an immunologically mediated inflammatory damage to the small-intestinal mucosa [2, 3]. Taking into account that the only treatment of celiac disease is lifelong avoidance of

dietary gluten [4], in the last years, thanks to the growing awareness of celiac disease impact on social life, food industry has developed several lines of gluten-free food products, widely available at supermarkets. In order to avoid any confusion regarding foods formulated, processed or prepared to meet the special dietary needs of celiac people, the revised version of Codex Alimentarius Standard 118-1979 in 2008 recommended universally agreed definitions for the expressions "very low gluten" and "gluten-free" foods. In particular, very low-gluten foods are specially processed to reduce gluten content to a level above 20 up to 100 mg kg^{-1}; whereas, gluten-free foods are defined as dietary foods containing a gluten level not exceeding 20 mg kg^{-1}, regardless of whether the products naturally do not contain wheat, oats, barley, rye (or their crossbred varieties) or have been specially processed to remove gluten [5]. In 2009, European Commission adopted the Codex recommendations in Commission Regulation 41/2009, concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten [6]. In addition, a matter of great concern addressed by European legislation is the possible contamination at any point during the production cycle, such as crop growth, harvesting, transport, milling, storage and processing, especially for oat products. Actually, all specially-formulated gluten-free products could contain "hidden" gluten, due to grain contamination, cross-contact during production changeover and poor manufacturing practice.

In this context, the detection and quantification of trace amounts of gluten in food is of paramount importance to support regulatory requirements for gluten-free products by proper food labelling and to safeguard celiac patients' health. As recently reviewed [7-10], several methods have been developed for gluten content assessment, based on different techniques for the detection of related proteins [11-17], digested peptides [18-22] or DNA [23-25]. As for prolamins, the traditional extraction protocol involving aqueous alcohol, usually 40-70 % (v/v) ethanol, is effective only on raw materials, as cereal flours; in fact, food containing gluten usually undergo non-thermal (mechanical, chemical or enzymatic) and/or thermal processing, with modification of gluten chemical properties, affecting its solubility. In particular, high temperature treatments induce the

formation of isopeptide and disulphide bonds, resulting in the formation of polymerized gluten with reduced extractability [26, 27]. For these reasons, in the last years many efforts have been made for the development of extraction solutions, composed by denaturing and reducing agents, able to improve gluten recovery from processed foods respect to that reachable by the use of only aqueous alcohol [28].

The most common and recognized approach for the detection of gluten in food is the Enzyme-linked Immunosorbent Assay (ELISA), mainly based on antibody recognition of gliadin epitopes. Different set-up, such as sandwich or competitive formats, as well as several types of mono- and poly-clonal antibodies, have been proposed for ELISA immunological assays [29, 30], associated with different sample extraction methods [28, 31] and gluten/prolamin calibration standards [32, 33]. The sandwich R5 ELISA [11], validated in a collaborative trial conducted by Prolamin Working Group [34], has been endorsed by the Codex Alimentarius for gluten determination [35]: it exploits monoclonal R5 antibody recognition of QQPFP, QQQPFP, LQPFP and QLPFP epitopes present not only in all fractions of wheat gliadin, but also in barley hordeins and rye secalins. R5 does not react with oat avenins. This assay is used in combination with cocktail extraction solution, which is based on reducing 2-mercaptoethanol and disaggregating guanidine hydrochloride agents; it resulted suitable for gluten extraction both from natural and heat-processed foods [34]. However, Codex Alimentarius stated that "for the detection of hydrolysed gluten, a modification of the R5 assay (competitive ELISA) has to be applied" [35], since in case of hydrolysed food (such as beer, baby food, syrup) the sandwich R5 ELISA would underestimate gluten content. For this purpose, a competitive ELISA based on R5 antibody, together with a compatible extraction solution (named UPEX), has been proposed for the analysis of any kind of food, including heat-treated and/or hydrolysed products [31]. Actually, in a very recent study Diaz-Amigo et al. [36] claimed that quantitative ELISAs differ not only in their design and specificity, but also in the results they provide for the same samples. In fact, the compromised accuracy of existing methods is due to different factors: (1) lack of appropriate reference materials for intact and hydrolysed gluten

proteins; (2) unreliability of constant prolamin/gluten conversion factor (conventionally set to 2), making no discrimination between cereals and cultivars; (3) possible cross-reactivity of the antibody towards the glutelin fraction, likely present in sample extracts obtained under denaturing and reducing conditions; (4) differences between cereal gluten/prolamin groups; (5) chemical modification of gluten after food processing causing probable alteration, associated with partial or complete loss of immunoreactive epitopes [37].

As for the assessment of food safety, a growing area is the development of biosensors exhibiting high sensitivity, speed, portability and low-cost needs [38]: immuno- and genosensors have been recently devised for the assessment of gluten-free foodstuff compliance with the law [14-16, 25].

In the last years, mass spectrometry (MS) technology has shown great potential and versatility for allergen analysis, overcoming some limitations associated to antibody-based methods; in fact, the exploitation of liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) technique for targeted bottom-up proteomic approach permitted to considerably improve confidence in protein determination [19, 39-43]. In particular, MS-based methods may allow gluten determination avoiding antibody cross-reactivity problems, with also the great advantage of discriminating among wheat, oats, barley and rye on the basis of unique digested peptides.

Nevertheless, ESI source is prone to matrix effect, which affects method accuracy and analytical performances: in fact, both exogenous compounds, introduced by sample treatment and chromatography (such as salts, buffers, reducing and denaturing agents), and endogenous matrix components may suppress or enhance analyte ionization [44]. As a consequence, existing sample treatment protocols for gluten determination in food need to be re-evaluated in terms of their suitability for MS methods [45], introducing efficient sample clean-up steps to reduce matrix complexity and desalt the extracts.

Recently, Friedler and coworkers [19] identified many grain (wheat, barley and rye)-specific chymotryptic peptide markers by MS proteomic approach and developed a LC-MS/MS method to detect low parts per million wheat contamination of oat flour (used as blank matrix), by monitoring

only wheat marker peptides. In addition, the authors used 70 % (v/v) ethanol for protein extraction, thus limiting the applicability of the method to only unprocessed raw materials.

In this context, the present study deals on the development of a shotgun proteomic LC-ESI-MS/MS-based method for simultaneous determination of gluten from different celiotoxic cereals in food. To the best of our knowledge, this is the first LC-MS/MS method able to distinguish among wheat, oats, barley and rye in a single chromatographic run and that could be applied also to complex food matrices.

2. Materials and methods

2.1. Chemicals and materials

Gliadin from wheat (powder), ethanol (EtOH), ammonium bicarbonate, guanidine hydrochloride, 2-mercaptoethanol, dithiothreitol (DTT), iodoacetamide (IAA), trypsin, chymotrypsin, pepsin, formic acid (FA), hexane and supelclean™ ENVI™-18 SPE tubes were purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile (ACN) was obtained from VWR International (Milan, Italy). Deionized water was obtained from an in house Milli-Q water purification system Alpha Q-Water (Millipore, Billerica, MA, USA). RIDASCREEN® Gliadin ELISA was purchased from R-Biopharm AG (Darmstadt, Germany).

2.2. Food products

Unprocessed raw materials (flours and seeds) as well as processed foods (pasta, biscuits, cookies, crackers, beverages, breads, breakfast cereals, snacks) were obtained from local supermarkets. In particular, 13 products containing gluten, 5 products expected to be gluten-free and 11 declared gluten-free products were analysed.

2.3. Sample treatment

Food samples, unless flours, were ground under liquid nitrogen to obtain a fine and homogeneous powder. The exploited prolamin extraction protocol was a revisiting of the method published by García et al. [28]. An amount of 200 mg of ground sample was defatted by adding 1 mL of hexan, then vortexed and spinned down. The defatting step was repeated for 3 times. Then the sample was suspended in 2 mL of cocktail solution (250 mM 2-mercaptoethanol and 2 M guanidine hydrochloride in 50 mM bicarbonate buffer pH 8.0) and incubated for 40 min at 50 °C, with intermittent vortexing every 10 min. After cooling down at room temperature (RT), 7 mL of 80 % (v/v) EtOH were added and the sample was incubated at RT for 1 h under agitation. Centrifugation at 5800 rpm for 10 min was performed, then the supernatant was filtered on 0.22 µm regenerated cellulose filter and dried under nitrogen flux. The sample was reconstituted in 4 mL of 50 mM bicarbonate buffer. Reduction was carried out by incubating the extract with 20 mM DTT for 40 min at 37 °C; then, for alkylation reaction 40 mM IAA was added, followed by incubation for 40 min at RT in the dark. The digestion was carried out by using both trypsin and chymotrypsin (1:100 enzyme-to-protein ratio for each enzyme) and keeping at RT for approximately 18 h. The digestion reaction was quenched by adding 100 % FA to achieve 5 % (v/v) FA as final concentration.

The digested extract was desalted with supelclean™ ENVI™-18 SPE tube: more precisely, the column was washed with 4 mL of ACN and 4 mL of 5 (v/v) % FA. After sample loading, 4 mL of 5 % (v/v) FA followed by 2 mL of hexan were fluxed onto the column, in order to remove salts and fat compound interferences. The elution was carried out with 2 mL of 70 % (v/v) ACN in water with 5 % (v/v) FA, followed by 1 mL of 100 % ACN. Finally, the two collected fractions were mixed. The desalted sample was dried under nitrogen flux and reconstituted in 100 µL of water containing 7 % (v/v) FA. After filtration on 0.22 µm water wettable polytetrafluoroethylene syringe filter, the sample was injected.

As for the choice of the best conditions for digestion reaction, preliminary experiments exploiting different proteolytic enzymes, alone or in combination, and different enzyme-to-protein ratios (1:20, 1:50 and 1:100) were carried out. The use of trypsin (at 37 °C for 18 h), chymotrypsin (at RT for 18

h) or a combination of them (at RT for 18 h) were investigated for protein digestion after reduction and alkylation reactions. Also a pepsin digestion, without reduction and alkylation steps, in 10 mM HCl at pH 2.0 for 3 h at 37 °C under agitation, was performed. The first three mentioned digestion reactions were quenched by adding 5 % (v/v) FA, while the latter one by adding 20 µL of 1 M NaOH.

2.4. Fortified samples in rice flour

Fortified samples, used as standards for method development and validation, were obtained by adding Sigma whole gliadin and ground oats, barley and rye to rice flour (blank matrix) and homogenizing the mixture before weighting and extraction. For standard preparation it was necessary to assess the content of soluble gliadins in whole Sigma gliadin as well as the amount of soluble avenins, hordeins and secalins in ground oats, barley and rye, respectively. The quantification of prolamin content in the extracts (gliadin, oat, barley and rye extracts) was performed by RIDASCREEN® Gliadin ELISA, applying a proper dilution factor so that the absorbance values fall into the assay calibration range. Since the R5 antibody of RIDASCREEN® Gliadin ELISA does not react with avenins and tends to overestimate hordein and secalin content, the soluble prolamin amount of the extracts was also quantified by NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmingon, Germany). The extractions were repeated seven times in different days: prolamin quantification permitted to calculate a correction factor for purity (i.e. 0.4 for gliadin and 0.1 for other prolamins), on the basis of which amounts of 5 g each of ground oats, barley, rye and 1.25 g of Sigma whole gliadin were added to 33.75 g of rice flour to prepare a 10000 mg kg^{-1} soluble prolamin stock mixture. After proper homogenization, subsequent dilutions with rice flour were made to produce working fortified mixtures.

2.5. Preparation of self-made rice breads

Incurred bread materials containing all the investigated prolamins were prepared as follows: 30 g of rice flour, 0.2 g each of Sigma whole gliadin, ground oats, barley and rye, 0.6 g of sodium chloride, 0.2 g of sucrose, 0.6 g of dried bakery yeast, 3 g of egg whites, 1 g of olive oil and 24 g of tap water were kneaded into a dough, fermented for 1 h at 40 °C and baked in a pre-heated oven at 220 °C for 20 min. After cooling at RT, the bread was weighed. Incurred breads containing only gliadin and negative control were prepared by following the same procedure, but adding only gliadin in the first case and without prolamin addition in the second case.

The final amount of prolamins in the breads was calculated by considering the correction factors for purity, as follows: gliadins (mg kg^{-1}) = (Sigma whole gliadin added (mg)/weight of bread (kg)) × 0.4; avenins, hordeins or secalins (mg kg^{-1}) = (ground grains added (mg)/weight of bread (kg)) × 0.1.

2.6. LC-MS/MS analysis

Chromatographic separation was performed on a HPLC system (Thermo Electron Corporation, San Josè, CA, USA) coupled with 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 (Thermo Electron Corporation). The mobile phase was delivered by the Surveyor chromatographic system (Thermo Electron Corporation). The LC separation was performed on a Kinetex C18 column (100 x 2.1 mm inner diameter, 2.6 µm) (Phenomenex, Torrance, CA), thermostated at 25 °C. A binary solvent gradient was used for the analysis: solvent A consisted of 0.1 % (v/v) FA aqueous solution, and solvent B was 0.08 % (v/v) FA in ACN.

LTQ operation parameters were set as follows: ESI voltage, 3.5 kV; capillary voltage, 20 V; tube lens, 100 V; capillary temperature, 200 °C; sheath gas, 50 arbitrary units; auxiliary gas, 20 arbitrary units, and sweep gas, 5 arbitrary units.

The data-dependent acquisition (DDA) mode was carried out using a linear gradient from 2 to 85 % of solvent B over 75 min, with column re-equilibration of 12 min. The flow rate was 200 µL min^{-1},

and the injection volume was 10 μL. The ion trap was set in order to ignore any single charged ions acquired in the 300-2000 amu mass range and to perform MS/MS analysis, by using a normalized collision energy of 30, only on eluting species that overcome a predefined threshold of 500 cps. The acquired spectra were submitted to Bioworks 3.3 software (Thermo Electron Corporation) to identify peptides and infer protein assignments from a database that consisted of reviewed and unreviewed entries for the Poaceae family listed in the Uniprot database (downloaded on January 29th, 2014 and containing about 562,000 entries). Bioinformatic search parameters included 1 missed cleavages, 1 amu peptide and fragment tolerance and carbamidomethyl as fixed modification on the cysteines caused by IAA treatment (+57). 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 and sequence specificity. The last criteria was verified by using a BLAST search with the following parameters: algorithm, blastp; MATRIX PAM30; word size, 2; E Value 20000. In addition, a multiple alignment between gliadins, avenins, hordeins and secalins was performed separately in order to choose the most representative peptides for each grain. In order to select peptides specific for each grain, peptide uniqueness was further verified by a global alignment. LC-MS/MS analysis of the selected peptides was carried out in time-scheduled pseudo-selected reaction monitoring (pSRM) mode: extracted ion chromatograms were obtained by extraction of individual fragment ion currents using Xcalibur software. In this way it is possible to record and visualize full MS/MS product ion spectra for each peptide. Precursor ions, normalized collision energies (CE) and the MS/MS transitions are given in Table 1. The optimized gradient is the following: solvent B was initially set at 2 % for 5 min and then delivered by a linear gradient from 2 to 30 % in 37 min and to 40 % in 4 min. Finally, column was re-equilibrated for 9 min. The flow rate was 200 μL min^{-1} and the injection volume was 30 μL. The first acquisition segment was from 0 to 26 min, and the second one from 26 to 55 min.

2.7. Method Validation

Validation of the analytical method was performed on fortified rice flour according to the Eurachem guidelines [46]. The absence of gluten in rice flour, used as blank matrix, was previously verified by RIDASCREEN® Gliadin ELISA. Validation was carried out by monitoring the most abundant MS/MS transition for the most sensitive peptide for each grain; however, for gliadins, two peptides belonging to different families (γ-gliadins and α/β-gliadins) were chosen. The detection (LOD) and quantification (LOQ) limits were calculated on matrix and were expressed as the concentration of analyte giving a signal that is $2ts_b$ and $10s_b$ above the mean blank signal, respectively, where s_b is the standard deviation of the blank signal obtained from ten independent blank measurements and t is the constant of the t-Student distribution (one-tailed) at 95 % confidence level. In particular, in order to assess the blank signal distribution, analytes were added before extraction, each in the amount proper to give the minimum integrable signal. LOD values were calculated for all the marker peptides reported in Table 1. Matrix-matched calibration curves were built up and linearity was assessed starting from LOQ values of each peptide. Concentration levels were analyzed performing three measurements at each concentration level. 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. Intra-day repeatability and intermediate precision were calculated in terms of RSD % on two concentration levels (LOQ and intermediate level over the calibration range). Intra-day repeatability was assessed by performing three independent extraction replicates at each level in the same day, whereas intermediate precision was estimated over five days by performing independent extractions; for each extract three LC-MS/MS injections were carried out. The matrix effect was assessed by a t-test ($\alpha = 0.05$, two-tailed) between curve slopes calculated on a matrix digest and on an aqueous digest; more precisely, the digested prolamin extracts from whole Sigma gliadin, ground oats, barley and rye were added to digested rice flour extract or to bicarbonate buffer. Trueness was calculated in terms of percent recovery as a ratio of determined and added prolamin content, both on fortified rice flour at two concentration levels (LOQ and intermediate calibration level) and on incurred bread materials.

3. Results and Discussion

3.1. Prolamin extraction

A challenging task in the safety assessment of gluten-free foods for celiac patients is the extraction of celiotoxic wheat, oat, barley and rye gluten components. Food matrix complexity and raw material transformation during industrial processing make particularly difficult prolamin isolation from food samples. For this aim, García et al. [28] proved that cocktail solution, based on 2-mercaptoethanol and guanidine hydrochloride in saline phosphate buffer, is suitable for prolamin extraction also from thermally processed foods. In addition, the inclusion of an aqueous alcohol extraction step (80 % (v/v) EtOH) is common place when ELISA assay is carried out for the analysis of wheat gluten proteins. In fact, such a procedure allows to extract the alcohol-soluble monomeric gliadin proteins from the remaining alcohol-insoluble proteins in the sample, thus improving the detection of the prolamin fraction. Taking into account the necessity to even out as much as possible the composition of buffers involved during the entire sample treatment protocol, the use of a cocktail solution based on 50 mM ammonium bicarbonate buffer pH 8.0, instead of saline phosphate buffer, was investigated. On the basis of DDA and spectrophotometric analysis, it was observed that this buffer change does not influence extraction efficiency.

Furthermore, in order to minimize food matrix interference on ESI ionization process, the complexity of the matrix was reduced by introducing a defatting step that involved the use of hexane prior to prolamin extraction. As a consequence, the devised method could be applied also for the analysis of very complex foodstuffs containing high fat level.

3.2. Prolamin digestion and extract purification

The optimization of the digestion reaction represents an important step for the development of global and targeted bottom-up proteomic approaches. Thanks to its reliability and specificity, trypsin is the most common proteolytic enzyme, which cleaves peptides on the C-terminal side of

lysine and arginine amino acid residues. As well known, the problem encountered during proteomic analysis of prolamins is the low amount of lysine and arginine amino acids, producing a low number of tryptic peptides and characterized by high molecular weight; for this reason, sequence analysis results complicated, as the most peptides are outside the working range to perform efficient fragmentation by MS/MS [10]. Chymotrypsin represents an alternative enzyme that provides an efficient digestion of prolamins giving a greater number of medium-sized peptides, with respect to trypsin. Furthermore, pepsin is another proteolytic enzyme used to digest proteins in order to simulate the gastric and duodenal digestion in humans.

As a consequence, digestion strategies based on a single enzyme or on a multiple-enzyme combination have been reported [19, 20]. In this study, digestion experiments using pepsin, trypsin and chymotrypsin alone as well as a trypsin/chymotrypsin combination were carried out. On the basis of DDA acquisition, the enzyme-to-substrate ratios did not significantly influence the number of proteolytic peptides, thus a 1:100 ratio was chosen to minimize costs. As for proteolysis, the combination of trypsin and chymotrypsin yielded the largest number of unique gluten peptide identifications resulting to be the optimal conditions for reproducible generation of prolamin peptides, as shown in Table 2 for gliadins.

3.3. Prolamin characterization and selection of marker peptides

Each prolamin extract was analyzed in a Top3 data-dependent mode, followed by bioinformatic processing. For each type of grain, the most abundant proteins resulted to be the specific prolamin type. Target method development required to select only few marker peptides to be monitored, as the most representative and reproducible, among a list of identified unique peptides. Thus, for each cereal, a ClustalW multiple alignment between all manually annotated and reviewed prolamins present in Swiss-Prot uniprot database was run in order to choose peptides common to the majority of proteins belonging to the same family (Table 3).

3.4. Method Validation

Method validation was carried out in terms of limits of detection and quantification, linear dynamic ranges, accuracy and recovery. LOD values for all the marker peptides are reported in Table 4, whereas Table 5 shows validation results for peptides used for quantification purposes. Considering a gliadin/gluten conversion factor of 2, the achieved sensitivity is suitable for the assessment of food legal compliance according to European legislation. A good precision was obtained in terms of both intra-day repeatability with RSD in the 6-12 % range (n = 9) and inter-day repeatability with RSD in the 9-21 % range (n = 15). Linearity was proved up to two orders of magnitude.

Matrix effect was investigated to assess influence of interfering extract components not completely removed by clean-up on response factors. In particular, signal enhancement of 27 % for VPPHCSVATTP and signal suppression of 25 % for SQLEVVR were observed, whereas for VQQQIPIVQPSVL, CQAIHNVVHAIIL and PLQPHQPY no significant matrix effect was measured ($p > 0.05$). It has to be highlighted that experiments performed without sample defatting steps with hexane showed a very strong matrix effect in terms of signal suppression for all the peptides, negatively compromising sensitivity. In fact, the use of hexane permitted to remove lingering hydrophobic interfering compounds from the final extract, allowing to reach good analytical performance. Matrix-matched calibration curves, with fortification of rice flour before extraction, were built up to perform a label-free quantification method. In the case of fortified rice flour blank matrix, recovery values were in the 90(±4)-107(±3) % range, except for PLQPHQPY for which 75(±3) % value was obtained.

Method trueness was assessed also by performing quantitative analysis of multi-cereal incurred self-made bread materials. It has to be pointed out that recoveries between 3 and 30 %, depending on the peptide, were calculated; as a consequence, further experiments were performed in order to investigate the reasons for the observed discrepancy between the calculated and the true values. For this aim, a first attempt was the quantification of the same incurred bread also by RIDASCREEN® Gliadin ELISA: however, since R5 antibody cross-reacts towards hordeins and secalins, it did not

permit to calculate individual concentration level for each cereal. To overcome this problem, incurred bread fortified only with Sigma whole gliadin was prepared and analysed both by LC-MS/MS and ELISA assay after proper dilutions. Trueness calculated from LC-MS/MS analysis was not satisfactory as in the case of multi-cereal bread analysis; in addition, poor trueness value (about 50 %) was calculated also with ELISA kit. In order to hypothesize possible causes of the observed signal decrease for incurred rice bread respect to fortified rice flour, experiments involving the baking of Sigma whole gliadin at the heating conditions used for incurred bread preparation (220 °C for 20 min) were performed. On the basis of LC-MS/MS signals, it was observed that the heating treatment affects the recorded signals to an extent compatible with the % trueness values previously calculated on incurred materials. Probably, as recently discussed [26, 27, 47], the high temperature treatment results in the formation of polymerized gluten with reduced extractability, giving different efficiency of prolamin extraction from processed food samples respect to calibration mixtures based on raw materials.

Reduced gluten extractability due to food processing is not the only limitation for quantitative analysis. In fact, also the absence of a reference material that may be used for calibration purpose is a problem that need to be solved. The variability between different grain cultivars and different compositions within prolamin families [48] likely prevents accurate quantitation results. In addition, it has to be highlighted that regulatory limits are expressed in terms of gluten, and are not referred to peptide content; thus, methods exploiting synthesized standard peptides for calibration are focused on selection of low celiac disease-immunogenic wheat varieties and on varietal screening [47, 48], but they do not allow assessment of "gluten-free" food product safety.

3.5. Sample analysis

Taking into account the above discussed limitations for quantitation analysis, method reliability was assessed for the detection of prolamins in different types of food products by monitoring all the marker peptides. More precisely, the method was applied both to market samples containing gluten

among ingredients and to foodstuffs declared or expected to be "gluten-free". All the samples not reporting celiotoxic cereals among ingredients were analysed also by ELISA assay. Results from the analysis of all twenty-nine samples are reported in Table 6, expressed as the number of detected peptides *vs* the number of monitored marker peptides for each prolamin, showing good accordance between identified prolamins and labelled ingredients. For example, the analysis of five-cereals biscuits permitted to identify the presence of all the investigated cereals, as shown in Fig. 1. However, it has to be pointed out that in this sample the ASIVTGIVGH peptide of secalins (retention time: 24.1 min) was not detected. All other products not reporting celiotoxic cereals among ingredients resulted negative on the basis of both LC-MS/MS and ELISA immunoassay analysis.

4. Conclusions

LC-MS/MS technique represents a good alternative to the most exploited ELISA assays for assessment of gluten-free product safety. In fact, contrary to antibody-based assays, shotgun MS-based proteomic approach makes possible to detect individually wheat, oats, barley and rye by monitoring unique marker peptides in a single chromatographic run. Matrix complexity can be reduced through inclusion of defatting steps during sample preparation, with advantages in terms of sensitivity. Potentiality of the developed method was proved by celiotoxic cereal detection on different kinds of market food products. Finally, it has to be stated that accurate quantitative determination requires further insights to improve prolamin extraction efficiency from processed foodstuffs and to select proper reference materials for protein-level calibration purposes. However, the present approach opens the way to the possibility to perform a MS-based multiplex screening for the assessment of gluten-free product safety.

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Table 1

List of the selected peptides for each grain and MS/MS transitions monitored.

Proteins	Peptide sequence	*m/z* precursor (charge state)	*m/z* monitored fragment ions (fragment type)	Normalized collision energy
γ-gliadins	LQPHQPF	433.7 (+2)	625.3 (y_5^+)[a]/242.0 (b_2^+)	35
	VPPECSIMR	544.6 (+2)	495.1 (y_8^{+2})[a]/892.2 (y_7^+)	25
	LQQQCSPVAMPQR	771.8 (+2)	400.1 (y_3^+/ y_7^{+2})[a]/1143.2 (b_{10}^+)	25
	VQQQIPIVQPSVL	725.1 (+2)	852.3 (y_8^+)[a,b]/597.3 (b_5^+)	25
	LQPHQIAQL[c]	524.7 (+2)	806.2 (y_7^+)[a]/916.7 (b_8^+)	25
α/β-gliadins	CQAIHNVVHAIIL	744.5 (+2)	622.2 (b_{11}^{+2}) [a,b]/678.1 (b_{12}^{+2})	25
	QIPEQSR	429.1 (+2)	308.6 (y_5^{+2})/616.0 (y_5^+)[a]	35
Avenins	VPPHCSVATTPL	640.1 (+2)	1050.4 (b_{10}^+)[a,b]/229.1 (y_2^+)	25
	VPPHCPVATAPLGGF	760.2 (+2)	1030.5 (b_{10}^+)[a]/490.2 (y_5^+)	35
	VPPHCPVATVPLGGF	774.1 (+2)	1058.1 (b_{10}^+)[a]/490.0 (y_6^+)	35
Hordeins	PLQPHQPY	490.1 (+2)	641.2 (y_5^+)[a,b]/701.3 (b_6^+)	25
	AQQQPSIEEQHQL	768.9 (+2)	1080.5 (y_9^+)[a]/456.1 (b_4^+)	25
Secalins	ASIVTGIVGH	477.1 (+2)	583.3 (y_6^+)[a]/682.1 (y_7^+)	25
	SQLEVVR	415.6 (+2)	615.6 (y_5^+)[a,b]/216.1 (b_2^+)	25

[a] Transition monitored for LOD calculation.

[b] Transition monitored for calculation of validation parameters for quantitation purposes.

[c] Peptide in common between γ-gliadins and hordeins (see also [19])

Table 2

Number of gliadin proteins and peptides identified with different proteolytic enzymes (ratio 1:100) (n = 3).

	Trypsin	Chymotrypsin	Trypsin/Chymotrypsin	Pepsin
Proteins	12(±1)	23(±1)	26(±1)	8(±3)
Peptides	14(±1)	56(±3)	61(±1)	9(±2)

Table 3

Peptide attribution to proteins belonging to the same family.

Prolamins	Peptide	Protein name
Gliadins	LQPHQPF	GDBB
	VPPECSIMR	GDB2
	LQQQCSPVAMPQR	GDB1/GDB3
	VQQQIPIVQPSVL	GDB1
	CQAIHNVVHAIIL	GDA0/GDA1/GDA2/GDA3/GDA4/GDA6/GDA7/GDA8/GDA9
	QIPEQSR	GDA2/GDA3/GDA4/GDA6/GDA7/GDA9/GDB1
	LQPHQIAQL	GDB3/HOR1/HOR3
Avenins	VPPHCSVATTPL	AVEF
	VPPHCPVATAPLGGF	AVE3
	VPPHCPVATVPLGGF	L0L5I0
Hordeins	PLQPHQPY	HOR7
	AQQQPSIEEQHQL	HOG3
Secalins	ASIVTGIVGH	A4GU92
	SQLEVVR	A4GU92

Table 4

LOD values in fortified rice flour samples.

Prolamins	Peptide sequence	LOD (mg kg^{-1})
Gliadins	LQPHQPF	5
	VPPECSIMR	13
	LQQQCSPVAMPQR	3
	VQQQIPIVQPSVL	2
	CQAIHNVVHAIIL	3
	QIPEQSR	8
	LQPHQIAQL	2
Avenins	VPPHCSVATTPL	4
	VPPHCPVATAPLGGF	14
	VPPHCPVATVPLGGF	18
Hordeins	PLQPHQPY	9
	AQQQPSIEEQHQL	9
Secalins	ASIVTGIVGH	13
	SQLEVVR	9

Table 5

Performance of the method for quantification purposes in fortified rice flour samples.

Prolamins	Marker peptide sequence	LOQ (mg kg^{-1})	Linear range (mg kg^{-1})	Calibration curve $y = a(\pm s_a)x$	R^2 (*n*)
Gliadins	VQQQIPIVQPSVL	3	LOQ-100	y = 106 (±4) x	0.980 (12)
	CQAIHNVVHAIIL	5	LOQ-500	y = 65 (±2) x	0.990 (15)
Avenins	VPPHCSVATTPL	6	LOQ-500	y = 122 (±3) x	0.990 (15)
Hordeins	PLQPHQPY	18	LOQ-500	y = 9.4 (±0.3) x	0.991 (12)
Secalins	SQLEVVR	17	LOQ-100	y = 64 (±3) x	0.967 (12)

Table 6

Results from LC-MS/MS analysis of market foodstuffs.

Food product		Celiotoxic cereal ingredients[a]	Detected peptides/Marker peptides			
			Gliadins	Avenins	Hordeins	Secalins
"Gluten-free" products[b]						
Raw materials	Buckwheat flour	none	0/7	0/3	0/2	0/2
	Corn flour	none	0/7	0/3	0/2	0/2
	Rice flour	none	0/7	0/3	0/2	0/2
Processed food	Crackers (1)	none	0/7	0/3	0/2	0/2
	Crackers (2)	none	0/7	0/3	0/2	0/2
	Cookies	none	0/7	0/3	0/2	0/2
	Rice cake	none	0/7	0/3	0/2	0/2
	Rice & corn cake	none	0/7	0/3	0/2	0/2
	Corn pasta	none	0/7	0/3	0/2	0/2
	Rice pasta	none	0/7	0/3	0/2	0/2
	Peach juice	none	0/7	0/3	0/2	0/2
Expected "Gluten-free" products[c]						0/2
Raw materials	Potato flour	none	0/7	0/3	0/2	0/2

	Mile	none	0/7	0/3	0/2	0/2
Processed food	Potato chips	none	0/7	0/3	0/2	0/2
	Cheese flavoured corn chips	none	0/7	0/3	0/2	0/2
	Vanilla pudding mix	none	0/7	0/3	0/2	0/2
"Gluten-containing" products						0/2
Raw materials	Wheat flour	wheat	7/7	0/3	0/2	0/2
	Spelt flour	spelt	7/7	0/3	0/2	0/2
	Kamut flour	kamut	7/7	0/3	0/2	0/2
	Couscous	wheat	6/7	0/3	0/2	0/2
Processed food	Crackers	wheat, barley malt	7/7	0/3	1/2	0/2
	Biscuits (1)	kamut, barley malt	5/7	0/3	2/2	0/2
	Biscuits (2)	barley, kamut, oats fiber	6/7	1/3	2/2	0/2
	Cereal cookies	oats, wheat	7/7	2/3	0/2	0/2
	Five cereal biscuits	wheat, barley, rye, oats	7/7	3/3	2/2	1/2
	Cereal snack chips	wheat, oats, barley	7/7	3/3	2/2	0/2
	Breakfast cereals	wheat, oats, barley	7/7	2/3	2/2	0/2
	Multigrain bread	rye, barley, oats	2[d]/7	2/3	1/2	2/2
	Beer	barley	1[d]/7	0/3	2/2	0/2

[a] Cereals are ordered as they appear in the labelled ingredient list.

[b] No celiotoxic cereals among ingredients and with the “Gluten-free” label.

[c] No celiotoxic cereals among ingredients and with the “May contain traces of gluten” or “May contain traces of wheat” label.

[d] Although the absence of wheat among ingredients, LQPHQIAQL peptide (belonging both to γ-gliadins and hordeins) was detected.

Figure captions

Fig. 1. LC-MS/MS extracted chromatograms of the marker peptides from the analysis of five-cereals biscuits (1:20 dilution) (in this sample ASIVTGIVGH peptide of secalins was not detected).

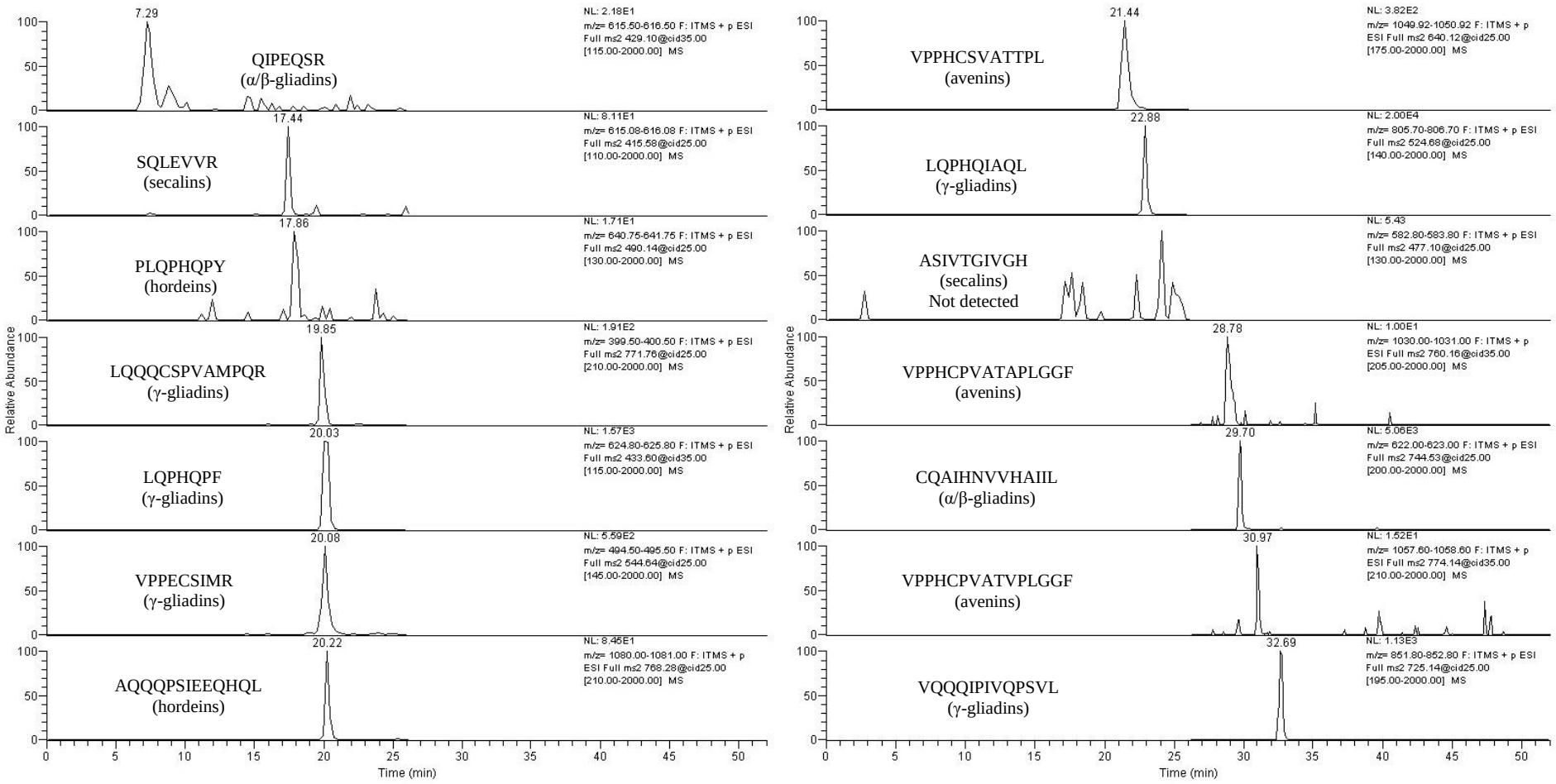
7.29
QIPEQSR
(α/β-gliadins)
NL: 2.18E1
m/z= 615.50-616.50 F: ITMS + p ESI
Full ms2 429.10@cid35.00
[115.00-2000.00] MS
17.44
SQLEVVR
(secalins)
NL: 8.11E1
m/z= 615.08-616.08 F: ITMS + p ESI
Full ms2 415.58@cid25.00
[110.00-2000.00] MS
17.86
PLQPHQPY
(hordeins)
NL: 1.71E1
m/z= 640.75-641.75 F: ITMS + p ESI
Full ms2 490.14@cid25.00
[130.00-2000.00] MS
19.85
LQQQCSPVAMPQR
(γ-gliadins)
NL: 1.91E2
m/z= 399.50-400.50 F: ITMS + p ESI
Full ms2 771.76@cid25.00
[210.00-2000.00] MS
20.03
LQPHQPF
(γ-gliadins)
NL: 1.57E3
m/z= 624.80-625.80 F: ITMS + p ESI
Full ms2 433.60@cid35.00
[115.00-2000.00] MS
20.08
VPPECSIMR
(γ-gliadins)
NL: 5.59E2
m/z= 494.50-495.50 F: ITMS + p ESI
Full ms2 544.64@cid25.00
[145.00-2000.00] MS
20.22
AQQQPSIEEQHQL
(hordeins)
NL: 8.45E1
m/z= 1080.00-1081.00 F: ITMS + p
ESI Full ms2 768.28@cid25.00
[210.00-2000.00] MS
Relative Abundance
Time (min)
21.44
VPPHCSVATTPL
(avenins)
NL: 3.82E2
m/z= 1049.92-1050.92 F: ITMS + p
ESI Full ms2 640.12@cid25.00
[175.00-2000.00] MS
22.88
LQPHQIAQL
(γ-gliadins)
NL: 2.00E4
m/z= 805.70-806.70 F: ITMS + p ESI
Full ms2 524.68@cid25.00
[140.00-2000.00] MS
ASIVTGIVGH
(secalins)
Not detected
NL: 5.43
m/z= 582.80-583.80 F: ITMS + p ESI
Full ms2 477.10@cid25.00
[130.00-2000.00] MS
28.78
VPPHCPVATAPLGGF
(avenins)
NL: 1.00E1
m/z= 1030.00-1031.00 F: ITMS + p
ESI Full ms2 760.16@cid35.00
[205.00-2000.00] MS
29.70
CQAIHNVVHAIIL
(α/β-gliadins)
NL: 5.06E3
m/z= 622.00-623.00 F: ITMS + p ESI
Full ms2 744.53@cid25.00
[200.00-2000.00] MS
30.97
VPPHCPVATVPLGGF
(avenins)
NL: 1.52E1
m/z= 1057.60-1058.60 F: ITMS + p
ESI Full ms2 774.14@cid35.00
[210.00-2000.00] MS
32.69
VQQQIPIVQPSVL
(γ-gliadins)
NL: 1.13E3
m/z= 851.80-852.80 F: ITMS + p ESI
Full ms2 725.14@cid25.00
[195.00-2000.00] MS
Relative Abundance
Time (min)

Fig. 1.