

SPECIAL GUEST EDITOR SECTION

Gluten Analysis in Processed Foodstuffs by a Multi-Allergens and Grain-Specific UHPLC-MS/MS Method: One Method to Detect Them All

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Background: Celiac disease, a complex, long-term autoimmune disorder and gluten intolerance, is estimated to affect from 1 to 5% of the world's population. **Objective:** As a consequence, to protect gluten-sensitive consumers, the development of reliable analytical methods allowing the detection of gluten in various food products is needed. **Methods:** Currently, ELISA is probably the most widespread used methodology. The method based on the R5 antibody has received type I status in Codex Alimentarius. However, the ELISA method suffers from some limitations, especially concerning quantification of nonwheat gluten. As a consequence, the development of another complementary methodology such as LC–tandem MS (MS/MS) is considered to be essential. Furthermore, this method could also be used for the simultaneous detection of gluten with other allergens, which will constitute a great additional benefit for producers of “free-from” food products and/or having a management policy integrated for several allergies and/or intolerances. **Results:** A multi-allergen and grain-specific ultra-HPLC coupled to MS/MS method allowing the identification and the discrimination of gluten from seven cereals, simultaneously with the detection and identification of 10 allergens in only one analysis, is thus described here. **Conclusions:** This method can be used for the analysis of a broad range of foodstuff matrices containing wheat and/or its derivatives, including cereals, flours, heat-treated and foodstuffs, but also more complex samples having undergone fermentation processes (such as beers).

CD is characterized by chronic inflammation of small intestinal mucosa, which may result in various symptoms such as diarrhea, abdominal pain (6), intestinal villi atrophy (2, 4–5), and extraintestinal symptoms (1, 5). CD and gluten intolerance are estimated to affect approximately 1 and 5% of the world's population, respectively (6). Dietary allergy to wheat is certainly undoubtedly less widespread in the population but may affect about 1% of children. In its extreme form, wheat allergy may lead to anaphylaxis and death (7). It is interesting to note that almost half of the patients outgrow the allergy to wheat by the age of 8 years (8).

Gluten, the main cause of these important public health issues, is defined by the Official Journal of the European Union and by the Food and Agriculture Organization and World Health Organization in the Codex Standard for gluten free foods (118–1979) as “the protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and which is insoluble in water and 0.5 M sodium chloride solution” (9, 10). Gluten is not an individual protein but rather a generic name given to a complex mixture of seed storage proteins. These gluten proteins are divided into several groups (also called as “Osborne fractions”) depending on their solubility, in which prolamins (soluble in water/alcohol mixtures) and glutenins (soluble in diluted acid) are the main constituents. Specific names are usually given to prolamins regarding the cereal, such as gliadins in wheat, hordeins in barley, secalins in rye, avenins in oats, and zeins in maize (2, 5, 7, 11–13).

It is worth noting that the chemical structure of the seed storage proteins and their tendency to trigger CD are closely related to the taxonomy of the cereals (5). Cereals belong to the Gramineae family, which is divided into several subfamilies, including Bambusoideae (rice), Panicoideae (millet, maize, and sorghum), and Pooideae, which is also divided in Aveneae (oats) and Triticeae (wheat, barley, rye, etc.; 14). Cereal grains that are known to trigger CD include wheat (meaning any triticum species, e.g., durum wheat, spelt, Kamut[®]), barley, rye, triticale, einkorn, emmer, bulgur, and farro. Oats in the diet of celiac individuals are still controversial because oats contain gluten proteins (avenins) that may induce CD (5, 14). Nevertheless, it is now well established that moderate amounts of pure oats (not contaminated with gluten from any triticum species) can be tolerated by most celiac individuals (9, 14, 15).

Because the only effective treatment for CD is a strict long-life gluten-free diet, worldwide regulations have been implemented for the labeling of foodstuffs potentially contaminated with gluten or processed to eliminate gluten. According to the

Celiac disease (CD), a complex, long-term autoimmune disorder, is observed in genetically susceptible individuals in response to exposure to dietary gluten (1–5). Described as a gastrointestinal tract disease,

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European Commission No. 828/2014, the Canadian Food and Drug Regulation, and the U.S. Food and Drug Administration, food products containing less than 100 mg/kg of gluten (in the food sold to the final consumer) can be labeled as “very-low-gluten” products, while those with a concentration equal to or lower than 20 mg/kg are labeled as “gluten-free” (9, 16).

In order to certify such labeling, several gluten detection methods have been developed. Among them, ELISA is probably the most widely used. ELISA based on the R5 antibody has obtained type I status in Codex (STAN 118-1979 from 2008) for the quantification of gluten (10, 17). Nevertheless, this ELISA methodology suffers from several limitations. ELISA detection methods focused on gluten proteins have been developed to detect very limited peptide sequences of gliadins. As an example, the target sequence of ELISA based on R5 antibody contains only five amino acids (QQPFP, which can be detected several times in some proteins, and which does not allow the detection of oat avenins), which may increase the susceptibility of false-positive and false-negative results. Additionally, most ELISA calculations are based on the assumption that gliadin represents 50% of the wheat gluten, irrespective of the cereal present in the sample. This proportion may vary with cereals, cultivars, harvest periods, processing of the product, wheat fractionation processes, extraction procedures, antibodies cross-reactivity, etc., therefore increasing the uncertainty of the method (10, 16–18). Finally, current gluten detection by ELISA does not differentiate the cereal source of gluten (17).

PCR, a highly sensitive method, was also developed as an alternative to ELISA. This analytical method targets DNA of gluten-containing cereals as a marker for the presence of allergenic material in the sample, and not directly the gluten proteins. As a consequence, PCR assays cannot discriminate gluten from other nonallergenic proteins of the same cereal, which can result in some false positives (16).

Based on the drawbacks of these methods, the development of another complementary methodology is considered essential. Numerous publications describe the development of LC–tandem MS (MS/MS) methods to detect specifically gluten proteins (6, 12, 17, 19–23). Indeed, LC–MS/MS seems to be better adapted to provide cereal identification because it uses multiple specific peptide markers with several points of confirmation, also reducing the susceptibility of false positives and false negatives (23).

Most of these publications describe complex extraction and enzymatic digestion (with chymotrypsin, pepsin, trypsin, trypsin/LysC, or trypsin/chymotrypsin) procedures mainly limited to some gluten cereals (12, 17, 19–22). Nevertheless, from the point of view of producers of “free-from” food products and/or having a management policy integrated for several allergies and/or intolerances, the simultaneous detection of gluten with other allergens could be a great additional benefit. In this publication, we describe the detection of gluten proteins from seven cereals (wheat, barley, rye, spelt, einkorn, Kamut, and oats) simultaneously with 10 other allergens [milk (casein and beta-lactoglobulin), egg, soy, peanut, and six tree nuts (almond, hazelnut, walnut, pecan, cashew, and pistachio)], using a simple extraction and tryptic digestion procedure based on the protocol by Planque et al. (24–26).

This multi-allergen and grain-specific ultra-HPLC (UHPLC)–MS/MS method allowing the discrimination of several cereals will be compared to ELISA methodology, by the analysis of in-house made and proficiency test samples, and commercially available food products.

Materials and Methods

Materials and Reagents

Tris(hydroxymethyl)aminomethane (tris), urea, dimethyl sulfoxide, DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, and trypsin from bovine pancreas (T8802) were from Sigma-Aldrich (Bornem, Belgium). Acetic acid was from Acros Organics (Geel, Belgium), and hydrochloric acid was obtained from Fisher Chemical (Loughborough, United Kingdom). Acetonitrile, 2-propanol, methanol (ULC-MS grade), and formic acid were obtained from Biosolve (Valkenswaard, the Netherlands). Standard Reference Materials including milk powder (NIST 1549a; 25.64% protein), whole egg (NIST 8445; 48% protein), peanut butter (NIST 2387; 22.2% protein), and soy flour (NIST 3234; 53.37% protein) were purchased from the National Institute of Standards and Technology (NIST; Gaithersburg, MD). Cereals [including common wheat, barley, rye, spelt, einkorn, Khorasan wheat (Kamut), oats, rice, and millet], grain-like seeds (used in bakery, including buckwheat, linseed, white sesame seed, and black sesame seed), and some other ingredients that could be used in bakery (including tapioca, tiger nut sedge, and chestnut) were purchased from local stores. To minimize potential cross-contaminations, all these cereal, grain-like seeds, and bakery ingredients were subsequently washed with cold water, dried at 37°C in an oven, sorted manually, and grounded to form the respective flours used in the following experiments. Sep-Pak C18 solid phase extraction (SPE) columns were used for peptide purification and enrichment (6 cc, 500 mg; WAT043395) and purchased from Waters (Milford, MA). Labeled peptides, FFVAPFPEVFGK [¹³C₆ ¹⁵N₂]-OH (Casein), LSF[Ring-D₅]NPTQLEEQCHI-OH (BLG), GGLEPINF[D₅]QTAADQAR-NH₂ (egg), EAFGV[D₈]NMQIVR-OH (soy), TANELNLLIL [¹³C₆ ¹⁵N]R-OH (peanut), ALPDEVL [¹³C₆ ¹⁵N]ANAYQISR (almond), GEEMEEMV [¹³C₆ ¹⁵N]QSAR (walnut), LV [¹³C₅ ¹⁵N]GFGINGK (pecan), AMTSPL [¹³C₆ ¹⁵N]AGR (cashew), ADIYTEQV [¹³C₅ ¹⁵N]GR (hazelnut), ALPLDV [¹³C₅ ¹⁵N]IK (pistachio), and, for gluten, LLVAPGQCENLATIHNV [¹³C₅ ¹⁵N]R, AQQLAAQL [¹³C₆ ¹⁵N]PAMCR, NVLLQQCSPVALV [¹³C₅ ¹⁵N]SSLR, and VFLQQCSPVV [¹³C₅ ¹⁵N]MPQR were purchased from Eurogentec (Seraing, Belgium) and used as internal standards.

Standard Solutions Preparation

Four allergen solutions were prepared by the extraction of NIST Standard Reference Materials [whole milk powder (NIST 1549a; 390 mg; 2.5 mg of total milk proteins/mL), spray-dried whole egg powder (NIST 8445; 417 mg; 5 mg of total egg proteins/mL), soy flour (NIST 3234; 375 mg; 5 mg of total soy proteins/mL), peanut butter (NIST 2387; 450 mg; 2.5 mg of total peanut proteins/mL)] in 40 mL tris-HCl (200 mM, pH 9.2) and urea (2 M). Once agitated (30 min; room temperature) and ultra-sonicated (15 min; 4°C), the solutions were centrifuged (14000 rpm; 10 min; 10°C), and the supernatants were used to spike food samples. For multi-allergen analysis, a NIST standard pool (10 mL) was prepared by diluting these supernatants [milk: 0.2 mL (0.05 mg of total milk proteins/mL); egg: 0.6 mL (0.3 mg of total egg proteins/mL); soy: 1 mL (0.5 mg of total soy proteins/mL); peanut: 1 mL (0.25 mg of total peanut

proteins/mL)] with 7.2 mL of tris-HCl (200 mM, pH 9.2) and urea (2 M).

Internal standards stock (1 mg/mL) and working (4, 8, or 10 µg/mL) solutions were prepared as described recently by Planque et al. (25, 26).

Standard solutions of the cereal flours, the grain-like seed flours, and the flours of some bakery ingredients were prepared individually at a theoretical concentration of 0.8 mg of total gluten proteins/mL according to the extraction of 50 mg of each flour in 5 mL of tris-HCl (200 mM, pH 9.2) and urea (2 M; based on the extraction protocol depicted before). This concentration was estimated according to the theoretical percentages depicted in literature (7, 11, 13, 16), by which about 10% of cereals are proteins and 80% of proteins are gluten proteins (which corresponds to 8% of gluten proteins in cereals; 16).

“Free-From” Matrix Preparation

Two allergen-free matrices, consisting of cookies and baby fruit compote, respectively, were prepared. “Free-from” cookie dough was prepared with maize flour (54.7%), sugar (16.9%), olive oil (14.9%), water (13%), salt (0.3%), ammonium bisulfate (0.1%), and sodium bicarbonate (0.1%) and baked at 200°C for 20 min (cookie thickness: about 8 mm; cookie weight: 21 ± 0.2 g). “Free-from” baby fruit compote was prepared with apple (87.9%), banana (12%), and ascorbic acid (0.1%). Once grounded, the mixture was baked at 80°C for 40 min.

Besides these in-house made matrices, some commercially available samples labeled as “gluten-free” products were also analyzed (e.g., gluten-free beer, rice cakes, etc.).

Contaminated Matrices Preparation

Contaminated matrices were also used in this study. Spiked cookies and spiked baby fruit compote were prepared by adding the pool-mix allergens solution and gluten-containing cereal extracts to “free-from” cookies or “free-from” baby fruit compote, respectively. The volumes spiked depend on the levels to target in the matrix (e.g., 20 ppm of gluten, which correspond to 20 µg of total gluten proteins per gram of matrix).

Some commercially available samples containing gluten were also used (beer, rice cake, etc.).

Marker Peptide Selection by Targeted Approach

The marker peptide(s) selection for gluten proteins was thus implemented according to the method already described by Planque et al. and where specific marker peptides were selected for egg, milk, soy, peanut, and six tree nuts allergens (25, 26).

First, gluten protein sequences were selected from the available sequences described in UniProt database. Second, the *in silico* enzymatic digestion of selected allergen proteins and the design of their respective MRM were obtained using the open source software Skyline. The following parameters were selected: peptide length of 7–25 amino acids, b or y fragmentations, carbamidomethylation modification on cysteines, precursor ion charge 2 or 3, and product ion charge 1. After *in silico* digestion, an experimental selection of gluten marker peptides in cereal flours was performed for each cereal, grain-like seed, and bakery ingredient solution prepared

individually at 0.1 mg of total gluten proteins/mL. Once extracted (agitation, ultra-sonication, centrifugation), proteins were subsequently reduced with DTT (1 mL; 200 mM; 45 min; room temperature), alkylated with IAA (1 mL; 400 mM; 45 min; room temperature; in the dark), and enzymatically digested with trypsin (1 mL; 1 mg/mL in acetic acid 50 mM; 1h; 37°C). The reaction was quenched with formic acid (300 µL; 20% volume) and centrifuged (4660 × g; 5 min; 10°C). The resulting solutions were analyzed by UHPLC-MS/MS without any further purification or concentration.

Once the most potent peptides were selected, the collision energy was optimized with Skyline for each MRM by automatically modifying this energy with a step size set to 1 eV. The optimum collision energy for each MRM is summarized in Table 1. Because the method developed here is a multi-target UHPLC-MS/MS, the number of peptides and MRM have to be limited to a maximum of three or four peptides per allergen and three or four MRM transitions for each of these selected peptides.

Sample Preparation Protocol

“Free-from” spiked and commercially available samples were finely ground and homogenized. Then, 2 g of those homogenates were subsequently extracted, reduced, alkylated, and digested according to the aforementioned protocol. The resulting centrifuged peptide solutions were purified using a Sep-Pak C18 SPE column (6 cc, 500 mg) and concentrated by evaporation of the elution solvent (N₂; 40°C). The extracts were then solubilized in 5% acetonitrile in 0.1% formic acid solution (600 µL), centrifuged (11 800 × g; 10 min; 10°C), and analyzed by UHPLC-MS/MS.

Multi-Allergen and Grain-Specific UHPLC-MS/MS Analysis

Peptide separation was performed with an Acquity system (Waters) on an Acquity UPLC peptide BEH C18 column (130 Å, 1.7 µm, 2.1 × 150 mm) at 50°C at 0.2 mL/min. Peptide elution was carried out for 26 min as follows: 0–3 min: 92% A; 3–18 min: 92–58% A; 18.0–18.1 min: 58–15% A; 18.1–22.5 min: 15% A; 22.5–22.6 min: 15–92% A; and 22.6–26 min: 92% A (solvent A: 0.1% formic acid; solvent B: acetonitrile plus 0.1% formic acid). After sample analysis, the UPLC column was flushed with methanol–isopropanol–acetonitrile–water, 1+1+1+1 (v/v/v/v), for 20 min before returning to the initial conditions (92% solvent A) for 5 min in order to avoid carry-over (peaks attributed to the previously analyzed sample may be observed in subsequent chromatograms). MRM detection in positive electrospray mode was performed with a Waters Xevo TQS triple quadrupole system. The cone nitrogen flow was set at 150 L/h, the collision gas flow at 0.12 mL/min, the capillary voltage at 2.0 kV, and the source temperature at 150°C. The desolvation temperature was set at 500°C and the nitrogen flow at 1200 L/h.

Results and Discussion

Cereal Selection

As depicted by the European Union Crops Market Observatory for cereals (Figure 1; 27), European agriculture in its 28 member states is dominated, by far, by three cereal

Table 1. MRM parameters for the identification of gluten and oat proteins by UHPLC-MS/MS^a

Gluten	Peptide	Protein	UniProt accession No.	Cereals	Retention time, min	Precursor	Charge state	Product ions	Collision En., V
Gluten	R.LLVAPGQCNLATIHNV.R.Y	Alpha-amylase/trypsin inhibitor CM3	P17314 P11643	Common wheat, barley, rye, spelt, and Kamut	11	626.0	3	740.4 810.45 199.1	14 23 24
	K.AQQLAAQLPAMQR.L ^b	HMW glutenin Dx5 unit	P10388 P02862 P02861 P08489	Common wheat, barley, rye, einkorn, spelt, and Kamut	10.8	729.4	2	634.3 946.5 747.4 634.3	21 23 22 12
	R.CGDLSSMLR.S	Alpha-amylase/trypsin inhibitor 0.28	P01083	Common wheat and Spelt (and to a lesser extent in rye)	10.85	519.7	2	593.3 821.4 706.4	18 18 18
	K.VFLQQQCSPVAMPQSLAR.S	LMW glutenin	A2TN61	Common wheat, spelt, and Kamut	12.3	687.3 1030.5 687.3	3 2 3	671.4 671.4 1069.6 873.5	19 29 20 21
	K.NVLLQQCSPVALYSSLR.S ^b	75k gamma secalin	E6KZP9	Rye	15	628.7	3	745.5 674.4 941.6 941.6	17 17 19 33
	R.IVPLAIDTR.V ^c	B3-hordein	P06471	Barley	11.6	499.3	2	785.5 575.3 688.4	13 18 19
	K.VFLQQQCSPVPVPQR.L ^{b,d}	B1-hordein	P06470	Barley	10.9	891.9	2	400.2 596.4 792.5	28 28 32
	R.LVVDQQLAGR.L	HMW glutenin	Q7XZB5	Einkorn (and to a lesser extent in Kamut, common wheat and spelt)	9.8	549.8	2	886.5 787.4 672.4	18 16 20
	K.VFLQQQCSPVMPQR.L ^b	LMW glutenin	A2TN60	Einkorn (and to a lesser extent in common wheat)	11.2	908.9 606.3	2 3	400.2 826.5 630.3 400.2	24 15 15 21
	K.NYLLQQCNPSLVSSLVSMILPR.S ^b	Gamma-Gliadin	B6UKL6	Common wheat, einkorn, spelt, Kamut	20.2	877.8 1316.2	3 2	1102.6 1034.5 1597.9 1401.8	28 29 43 43

Table 1. (continued)

Peptide	Protein	UniProt accession No.	Cereals	Retention time, min	Precursor	Charge state	Product ions	Collision En., V
Oats	R.QFLVQQCSPVAVPFLR.S	P80356	Oats	15.8	994.5	2	532.3	26
					663.4	3	532.2	13
							801.5	19
	R.QLEQIQEQLR.C	P80356	Oats	10	627.3	2	642.4	18
							755.4	20
							883.5	22
	R.QLAQIQEQLR.C ^b	Q09114	Oats	10.4	598.3	2	642.4	16
							755.4	19
							883.5	18

^a The cone voltage was fixed at 35 V.^b Peptides present in protein with some sequences known as having toxic/immunogenic sequences.^c IVPLAIDTR peptide is not detected in hydrolyzed samples, e.g., beers.^d VFLQQCSPVPQQR peptide is preferred to IVPLAIDTR for hydrolyzed samples (e.g., beers) because it is well detected.

crops, wheat, maize, and barley, which yield about 60 (barley), 65 (maize), and 145 (wheat) million tons per year. Other cereals and grain-like seeds are produced in a much lesser extent but are also used in the food industry, namely, in bakery, and are commonly found and sold in organic shops.

These cereals and grain-like seeds can be divided into three main classes. The first, which includes wheat (*Triticum aestivum*), barley (*Hordeum*), rye (*Secale*), spelt (*Triticum spelta*), einkorn (*Triticum monococcum*), and Kamut (Khorasan wheat; *Triticum turgidum* L. subsp. *turgidum*), are known to contain prolamin and gluten proteins (e.g., gliadins, glutenins, hordeins, secalins, etc.) and are therefore closely associated with gluten sensitivities. The second is represented by oats (*Avena sativa*), which contain gluten-like avenins (alcohol soluble prolamins). Even though avenins are described as having a possible, but controversial, association with gluten sensitivities, it is now well established that moderate amounts of pure oats (not contaminated with gluten from any *Triticum* species) can be tolerated by many CD patients (9, 14, 15). The third class includes cereals (maize, rice, millet), grain-like seeds (buckwheat, linseed, sesame), and some ingredients used in bakery (tapioca, chestnut, tiger nut sedge). All the ingredients of this third class do not contain any gluten proteins. Considered as nontoxic for celiac individuals, they can therefore be used as negative controls. Based on all these considerations, the cereals, the grain-like seeds, and the ingredients used in bakery selected for this study are wheat, barley, rye, spelt, einkorn, Kamut, oats, maize, rice, millet, buckwheat, linseed, white sesame seed, black sesame seed, tapioca, chestnut, and tiger nut sedge.

Cereal Marker Peptide Selection in Pure Cereal Samples

One of the main objectives is to develop a multi-target UHPLC-MS/MS method allowing the simultaneous detection of gluten proteins together with other allergens (such as egg, milk, soy, peanut, and six tree nuts) while identifying and discriminating the cereal(s) responsible for this potential contamination.

As a consequence, the choice of the enzyme used for protein digestion was fixed by the protocol already developed and validated for 10 allergens by Planque et al. (25) to which gluten could be added. In this protocol, the choice for trypsin was mainly guided by the number of lysine (K) and arginine (R) in the sequence of target proteins. This enzyme therefore generates peptides whose sequence size is compatible with LC-MS/MS analyses. Nevertheless, the number of lysine and arginine is very low in the sequence of the gluten proteins, therefore reducing the number of potential peptide targets. The challenge to find some specific peptides for gluten protein is thus more difficult than with chymotrypsin (or another enzyme such as pepsin), having a higher number of cleavage sites, but incompatible with the multi-target protocol already developed and validated.

To select potential specific marker peptides for the detection of gluten proteins, a large number of gluten proteins sequences, including namely glutenin [high-molecular-weight (HMW)-, low-molecular-weight (LMW)-], gliadin (α -, β -, γ -, ω -), alpha-amylase/trypsin inhibitor, secalins (γ -, ω -, etc.), hordeins (γ -, B-, C-, D-), and avenins (-3, -A, -E, -F, etc.), were selected for these cereals according to the UniProt database. Indeed, the screening of protein sequences was not limited to epitopes

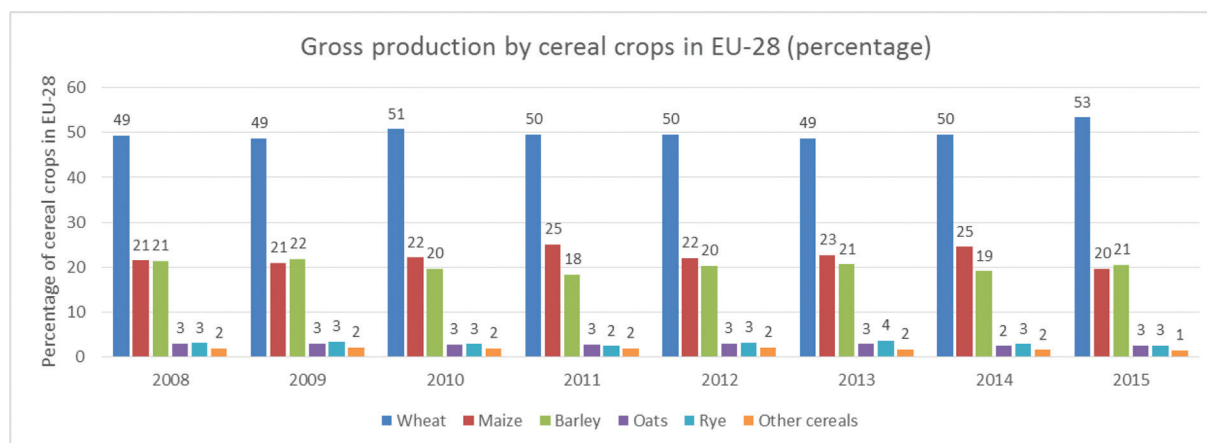


Figure 1. Distribution of the gross production by cereal crops in European Union and its 28 members from 2008 to 2015. Source: DG Agriculture and Rural Development based on Eurostat crop production annual data (27).

inducing potential toxic or immunogenic reactions because this method will be used as an analytical tool for gluten detection and not especially related to CD. Peptides and MRM transitions were then generated by Skyline in silico tryptic digestion of these protein sequences. In order to limit the number of both target peptides and MRM transitions, this in silico digestion was limited to (1) peptides with 7–25 amino acids, (2) b or y fragmentations, (3) precursor ion charge of 2 or 3, and (4) product ion charge of 1. According to these parameters, more than 180 proteins were preselected, which represent more than 435 potential peptides and 10854 MRM transitions (Figure 2).

All the cereal, grain-like seed, and bakery ingredient flours were thus individually extracted by mixing and then ultra-sonicating (at 4°C) the sample in Tris-HCl-urea buffer (10 mg flour/mL). After the subsequent reduction of disulfide bonds with DTT at room temperature and carbamidomethylation of cysteins with IAA in the darkness, proteins of the resulting solutions were digested with trypsin at 37°C for 1 h. Trypsin digestion is stopped with formic acid, and the solution is centrifuged to eliminate potential precipitates. The digested extracts were then analyzed by UHPLC-MS/MS without any further purification or concentration. By this step, the most potent gluten target peptides were preselected regarding either their ability to be detected in a majority (or even in all) of the selected cereals or, on the other side, for their high specificity for one of these cereals (e.g., barley). Preselected peptides were then studied with negative control cereals (maize, rice, millet), grain-like seeds (buckwheat, linseed, white sesame seed, black sesame seed), and bakery ingredients (tapioca, chestnut, and tiger nut sedge) to confirm their specificity for gluten proteins.

It is worth noting that all peptides that are depicted in Figure 2 and allow the detection of gluten proteins in several cereals are not found in oats. This choice was justified by the fact that the safety of oats in a gluten-free diet remains controversial because this cereal contains gluten-like avenins, which may be tolerated by some CD patients. Nevertheless, some publications describe that cross-contaminations with gluten-containing cereals, which may occur at all stages of production, storage, and handling of seeds and flours, is the main problem facing people with CD. Consequently, it was more appropriate to target peptides for gluten-containing

cereals and some other highly specific peptides for oats in order to more easily discriminate those cereals and their respective potential toxicity for CD patients.

Among peptides detected in a majority of the gluten-containing cereal flour samples tested, LLVAPGQC[+57]NLATIHNV and AQLAALPAMC[+57]R are certainly two of the most promising peptides. Indeed, as depicted in Figure 3, LLVAPGQC[+57]NLATIHNV [from the α -amylase/trypsin inhibitor (ATI) proteins] is detected in all the gluten-containing cereals with the exception of einkorn, while AQLAALPAMC[+57]R (HMW glutenin protein) is detected in all the six gluten-containing flour samples, including einkorn, but with a lower intensity than LLVAPGQC[+57]NLATIHNV (Figure 3). Even though ATI proteins belong to the nongluten proteins family, they still constitute a good marker for gluten because ATIs are known for their implication in baker's asthma and rhinitis but also in gastrointestinal hypersensitivity to wheat and, of course, in CD (7, 28). Besides these two “universal” peptides, some other peptides such as VFLQQQC[+57]SPVAMPQSLAR, NYLLQQC[+57]NPVSLVSSLSMILPR, and C[+57]GDLSSMLR allow the detection of gluten proteins but only in some gluten-containing flour samples (Figure 3).

Nevertheless, among all the peptides derived from gluten-containing cereals selected here, none of them are obtained after the tryptic digestion of gliadin proteins. Indeed, the peptides detected after tryptic digestion of gliadin are found to be much less intense than those selected here for all these cereals and derived from other proteins. Therefore, Prolamin Working Group (PWG)-gliadin, which is usually used as reference standard for most of the ELISA kits, could not be used as reference standard for the multi-allergen and grain-specific UHPLC-MS/MS method. Therefore, in-house-produced cereal flour extract solutions will be prepared to fill that purpose. These standard solutions could also be used more adequate to estimate the concentration of the corresponding cereal (e.g., use of in-house-produced barley flour standard solution for barley-contaminated samples).

In order to confirm the presence of an allergen, the detection of one specific peptide can be confirmed by one or two other peptides. As a result, gluten target peptides specific to only one cereal were also investigated for barley, rye, einkorn (usually lesser detected with the “universal” peptides), and oats. As a

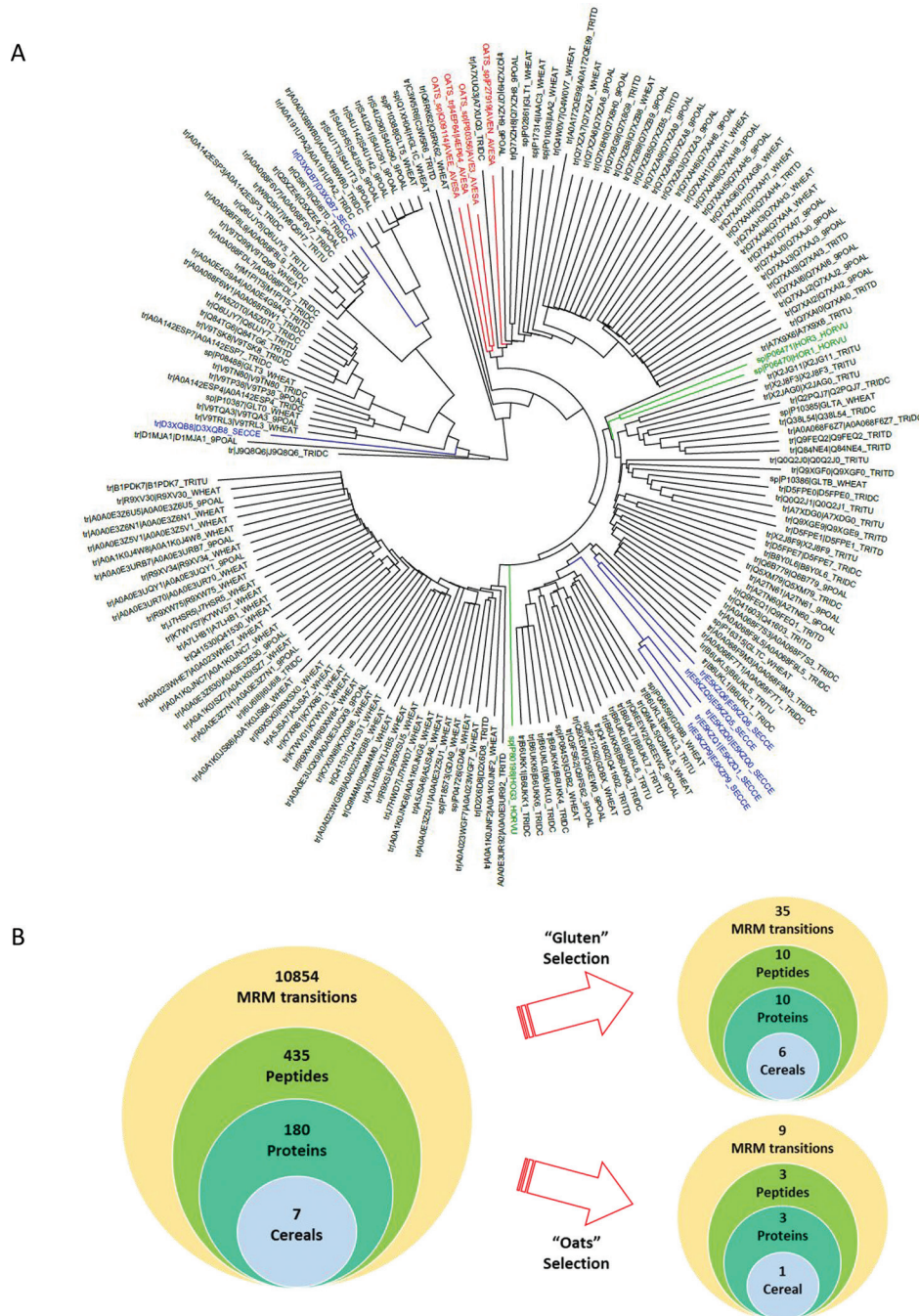


Figure 2. (A) Phylogenetic tree of the selected and analyzed gluten and oats proteins covered in this publication (in blue: sequences from rye proteins; in red: sequences from oats proteins; and in green: sequences from barley proteins). (B) Selection of MRM transitions for the specific detection of oats and six gluten-containing cereals by multi-allergen and grain-specific UHPLC-MS/MS method.

second advantage, these specific peptides will be used also to discriminate gluten-containing cereals, which is not possible using the currently available ELISA and PCR methods.

As depicted in Figure 4, IVPLAIDTR, a peptide derived from B3-hordein, was found to be highly specific for barley. Similarly, the presence of barley can also be specifically identified with VFLQQQC[+57]SPVPVPQR (from B1-hordein), while the presence of rye can be specifically detected with NVLLQQC[+57]SPVALVSSLR (from the 75k gamma secalin; Figure 3). Finally, avenins from oats were also specifically

detected with QFLVQQC[+57]SPVAVVPFLR, QLEIQEQLR (both from avenin-3) and QLAQIEQLR (avenin-E; Figure 3).

Two peptides, LVVDQQLAGR and VFLQQQC[+57]SPVMPQR, were also found to identify the presence of einkorn. Nevertheless, these two peptides were found to be less specific to this cereal. Indeed, the HMW-glutenin-derived peptide LVVDQQLAGR and the LMW-glutenin-derived peptide VFLQQQC[+57]SPVMPQR were also detected to a lesser extent (from 10 to 20 times lower in intensity) in Kamut and in common wheat. All these results are summarized in Table 1 with

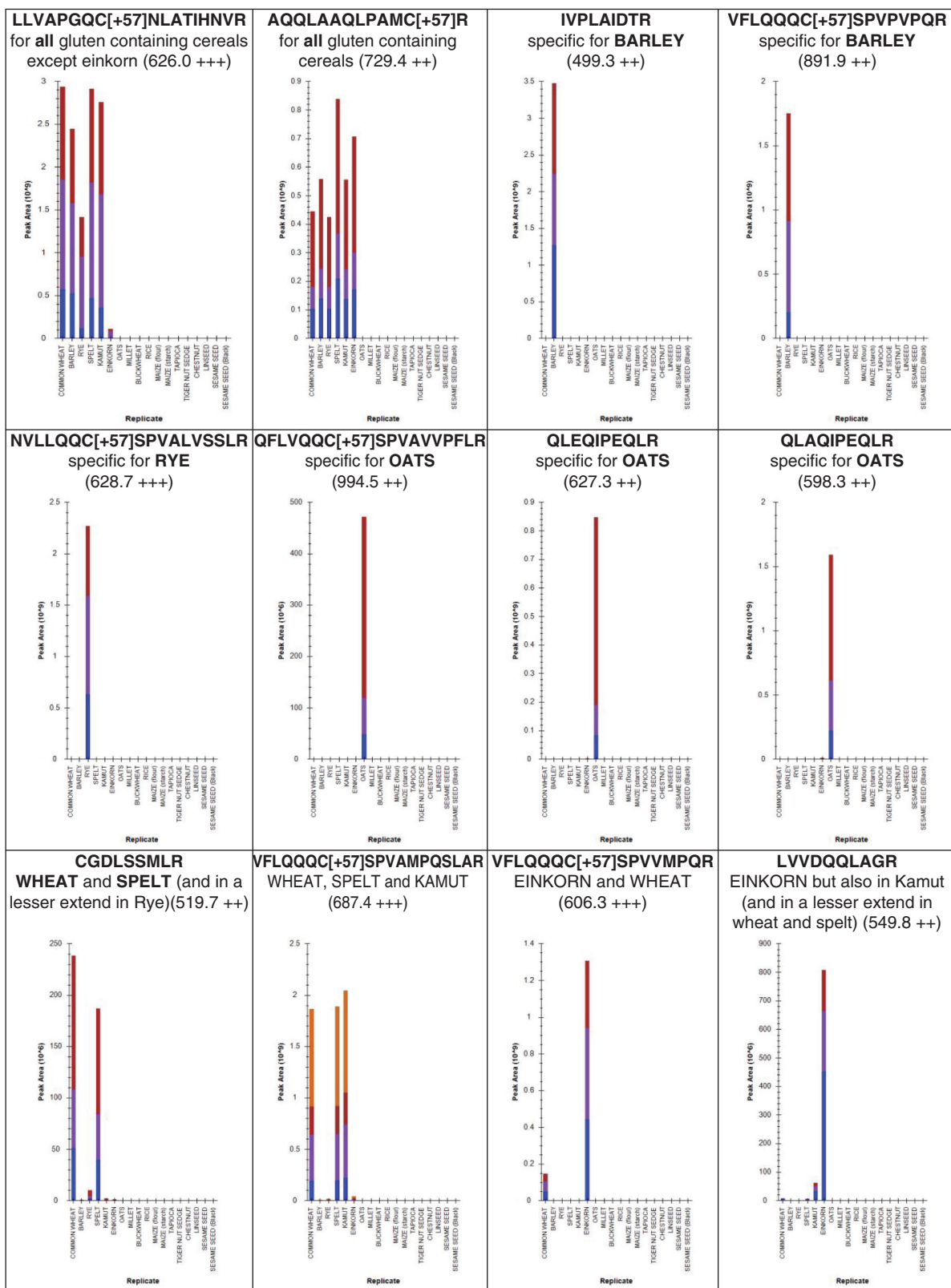


Figure 3. Specificity of some selected peptides for one or several cereals containing gluten or oat protein. These histograms depict the sum of the three (or four) highest MRM transitions of each peptide. These histograms were recorded with the Skyline software. Chromatograms illustrating this specificity for each targeted cereals, grain-like seeds, and bakery ingredients are depicted in the Supplemental Information.

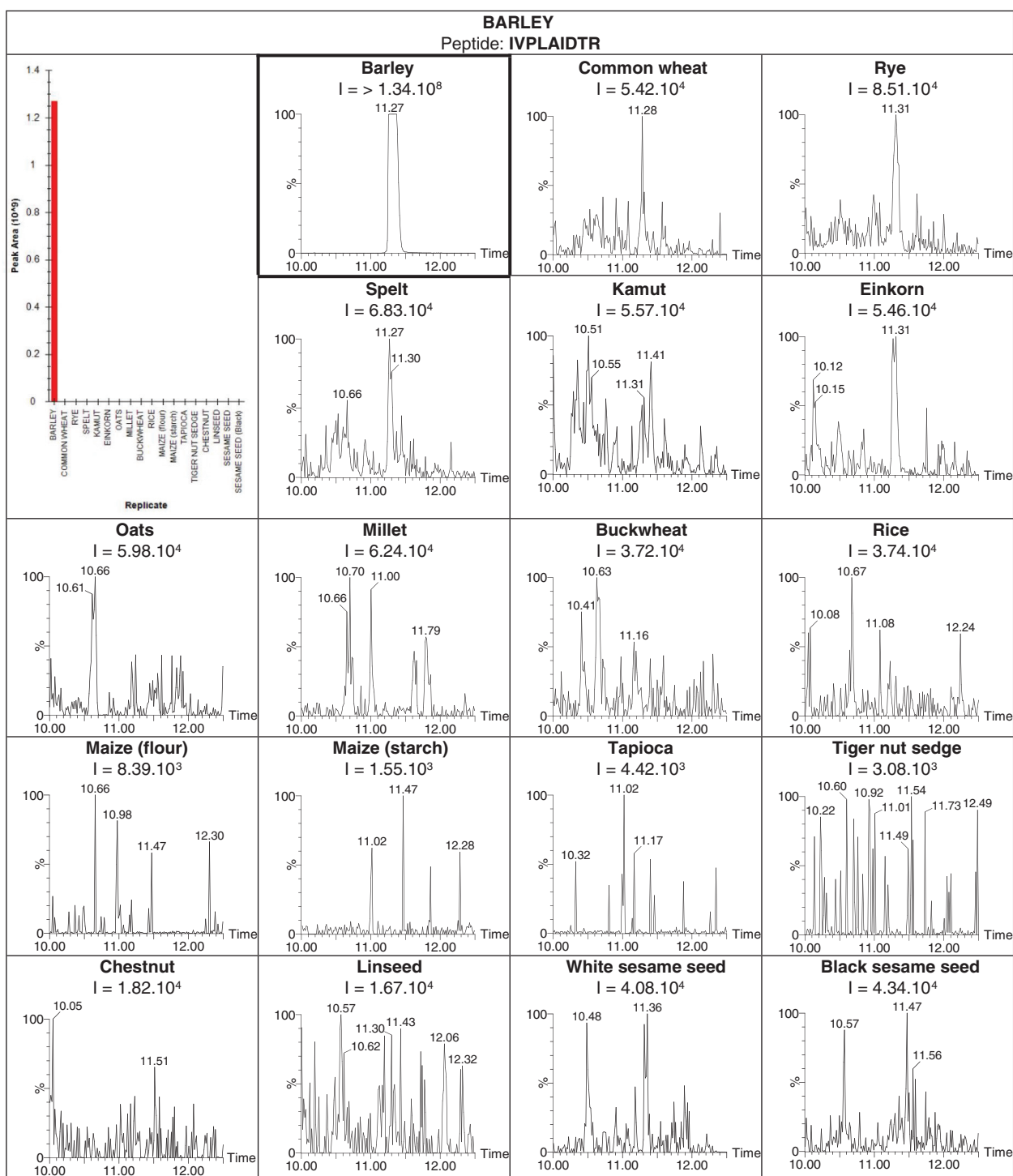


Figure 4. Histogram (top-left) and chromatograms of the highest MRM transition of barley B3-hordein peptide IVPLAIDTR (transition 499.3 > 785.5; expected retention time: 11.2 min), illustrating the specificity of this peptide in various cereals, grain-like seeds, and bakery ingredients. The specificity of the other selected peptides is depicted in the Supplemental Information.

all the MRM parameters required for their identification by UHPLC-MS/MS analysis.

Based on these results, some cereals turn out to be easily discriminated and/or identified. Those cereals are rye, barley, oats, and *Triticum*. Within *Triticum*, the different subspecies including wheat (*Triticum aestivum*), spelt (*Triticum spelta*), Kamut

(*Triticum turgidum* L. subsp. *turgidum*), and, to a lesser extent, einkorn (*Triticum monococcum*) are more difficult to discriminate because the target peptides are detected in at least two of these cereals and sometimes with very similar relative intensities (e.g., the peptide VFLQQQC[+57]SPVAMPQSLAR is detected with very similar intensities in wheat, spelt, and Kamut; Figure 3).

LOD Determination in Spiked Matrices

The UHPLC-MS/MS parameters were improved for all the selected peptides (summarized in Table 1). Then, these peptides were studied with in-house-made cookies and in-house-made fruit compote, which are representative of processed and polyphenol-rich matrices, respectively. This study allows the determination of the respective LOD ($S/N > 10$) for each of these peptides and in each of these two matrices. Because there is at least one peptide for each cereal that is detected in these two matrices at 10 ppm, a general LOD for gluten and oat peptides can be set to 10 ppm of total gluten proteins. This corresponds to half the value recommended by the European Commission No. 828/2014 to label foodstuff as “gluten-free” (<20 mg/kg; Table 2; Figures 5 and 6; 9).

It is important to note that in function of the number of peptides required to confirm the presence of a cereal (or gluten) in a sample, the LOD ($S/N > 10$) could be fixed at different levels, as shown in Table 2 and Figure 5. As an example, for oats, the LOD could be fixed from 2.5 ppm (if only one peptide is required) to 10 ppm (if three peptides are needed). At present, a LOD of 10 ppm will be applied for oats peptides because all of the three specific peptides for oats are detected with a S/N higher than 10 at this level (Figures 5 and 6), and this identification criteria of the number of required peptide is still not fixed up to now.

As depicted in Figures 5 and 6, these peptides and their corresponding MRM transitions can be added to those already validated for milk, egg, soy, peanut, and six tree nuts in a single analytical run. This will therefore allow the simultaneous detection and identification of 10 allergens and gluten (from seven cereals) according to a multi-allergens and grain-specific UHPLC-MS/MS analysis.

Because the analytical UHPLC-MS/MS method is now developed for the detection and the identification of gluten, the recovery will be evaluated on in-house-prepared samples, and analytical results will be compared with those of ELISA method on a proficiency test sample as well as on samples bought in supermarket. These will be described in the following parts.

Evaluation of the Method Performance: Recovery

Once the LOD was determined, the standard addition method was applied to estimate the trueness of the method for in-house-made cookies samples. This method was already implemented by Planque et al. (26) for the quantitative estimation of allergens in several matrices, including egg, milk, peanut, and soy in cookies, ice cream, chocolate, and sauce.

The recovery was tested on spiked cookies. For each of the seven cereals, three “free-from” cookie samples were spiked at a theoretical concentration of 20 ppm of gluten in the matrix (which corresponds to 20 μ g of total gluten proteins per gram of matrix). Two of these spiked samples were then added with wheat (or oats) standard solution at 10 ppm and 100 ppm, which corresponds respectively to 1 and 10 times the LOD for LLVAPGQCNLATIHNVN [or 2 and 20 times the LOD for QLEQIPEQLR (avenin, oats), respectively]. The three samples were then analyzed by the UHPLC-MS/MS method. A linear regression curve was fitted on these three samples and used to determine the concentration of the sample. As depicted in Figure 7, the concentration of gluten and avenins in spiked sample can be estimated by whatever cereal is present in the sample. Furthermore, the recovery, which varies from 88.5 to 101% depending on the cereals, is always within the acceptable range recommended by the AOAC INTERNATIONAL guideline 2016.002, summarizing the *Standard Method Performance Requirement* (SMPR[®]) for the detection and quantification of allergens in food products (between 60 and 120%; 29), and by the AOAC guideline 2017.021, describing the SMPR for the quantification of wheat, rye, and barley gluten in oats (between 50 and 200%; 30).

Evaluation of the Method Performance: Comparison with ELISA

The method was then tested for the quantification of an incurred FAPAS proficiency test sample. Assigned values

Table 2. LOD ($S/N > 10$) for peptides allowing the detection of gluten and oats in spiked cookies and fruit compote

Peptide	Cereals	LOD
LLVAPGQCNLATIHNVN ^a	Wheat, barley, rye, spelt, and Kamut	10 ppm ^a
AQQLAAQLPAMCR	Wheat, barley, rye, spelt, Kamut, and einkorn	50 ppm
CGDLSSMLR	Wheat and spelt	10 ppm
VFLQQQCSPVAMPQSLAR	Wheat, spelt, and Kamut	10 ppm
NVLLQQCSPVALVSSLR ^a	Rye	10 ppm ^a
IVPLAIDTR ^a	Barley	2.5 ppm ^a
VFLQQQCSPVPVPPQR ^b	Barley	20 ppm
LVVDQQLAGR	Einkorn	10 ppm
VFLQQQCSPVMPQR	Einkorn	5 ppm
NYLLQQCNVPSLVSSLVSMILPR	Wheat, spelt, Kamut, and einkorn	50 ppm
QLAQIPEQLR ^a	Oats	2.5 ppm ^a
QFLVQQCSPVAVVPFLR	Oats	10 ppm
QLEQIPEQLR	Oats	5 ppm

^a The most sensitive peptides, which can be used in priority to discriminate and identify cereal.

^b VFLQQQCSPVPVPPQR peptide is preferred to IVPLAIDTR for hydrolyzed samples (e.g., beers) because IVPLAIDTR is not detected in such samples.

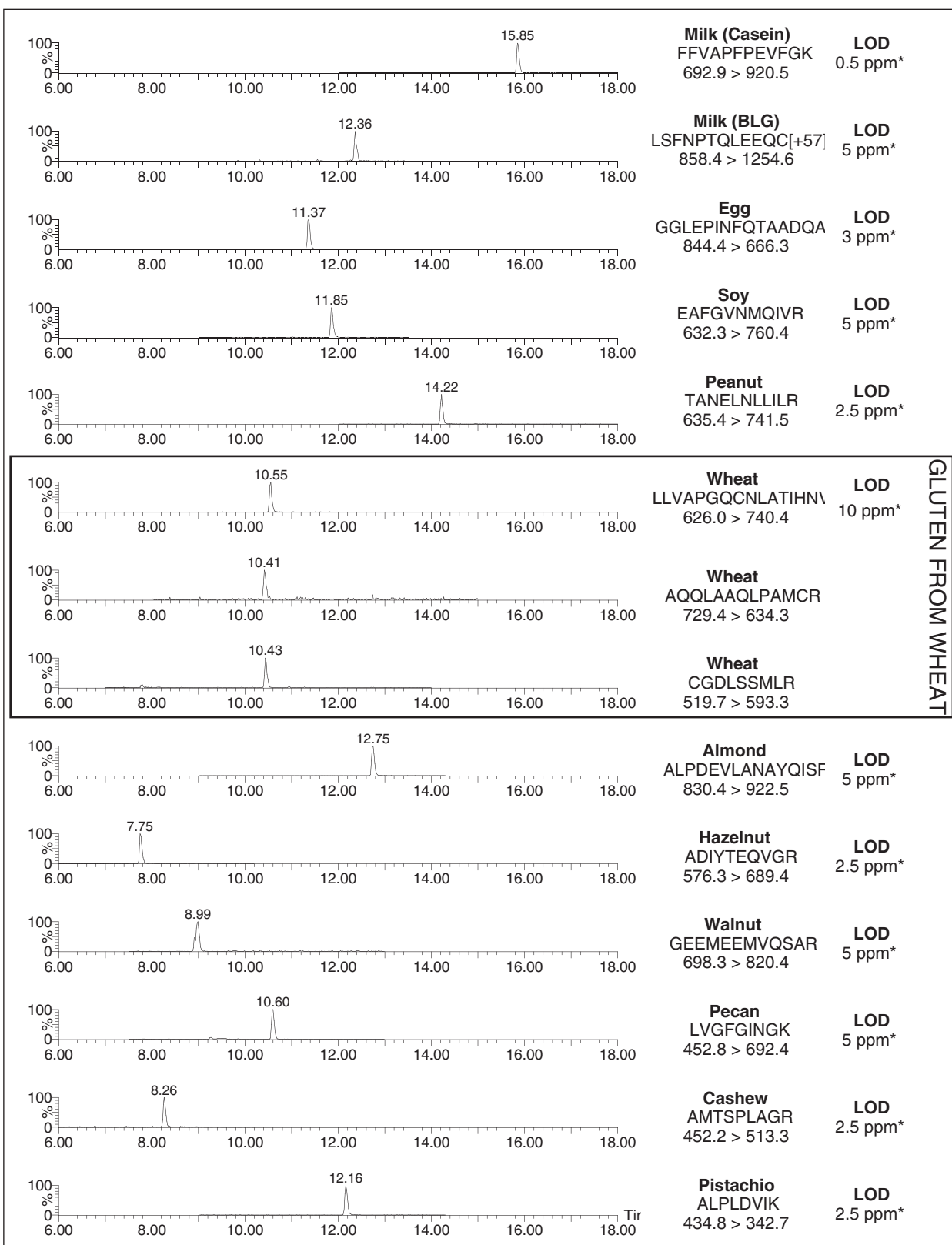


Figure 5. Multi-allergen and grain-specific UHPLC-MS/MS chromatograms of the respective main target peptides for whole milk, egg, soy, peanut, almond, hazelnut, walnut, pecan, cashew, pistachio, and common wheat, each spiked at 10 times the LOD in “free-from” cookies. *LOD is expressed in ppm, which corresponds to micrograms of total allergen proteins per gram of matrix.

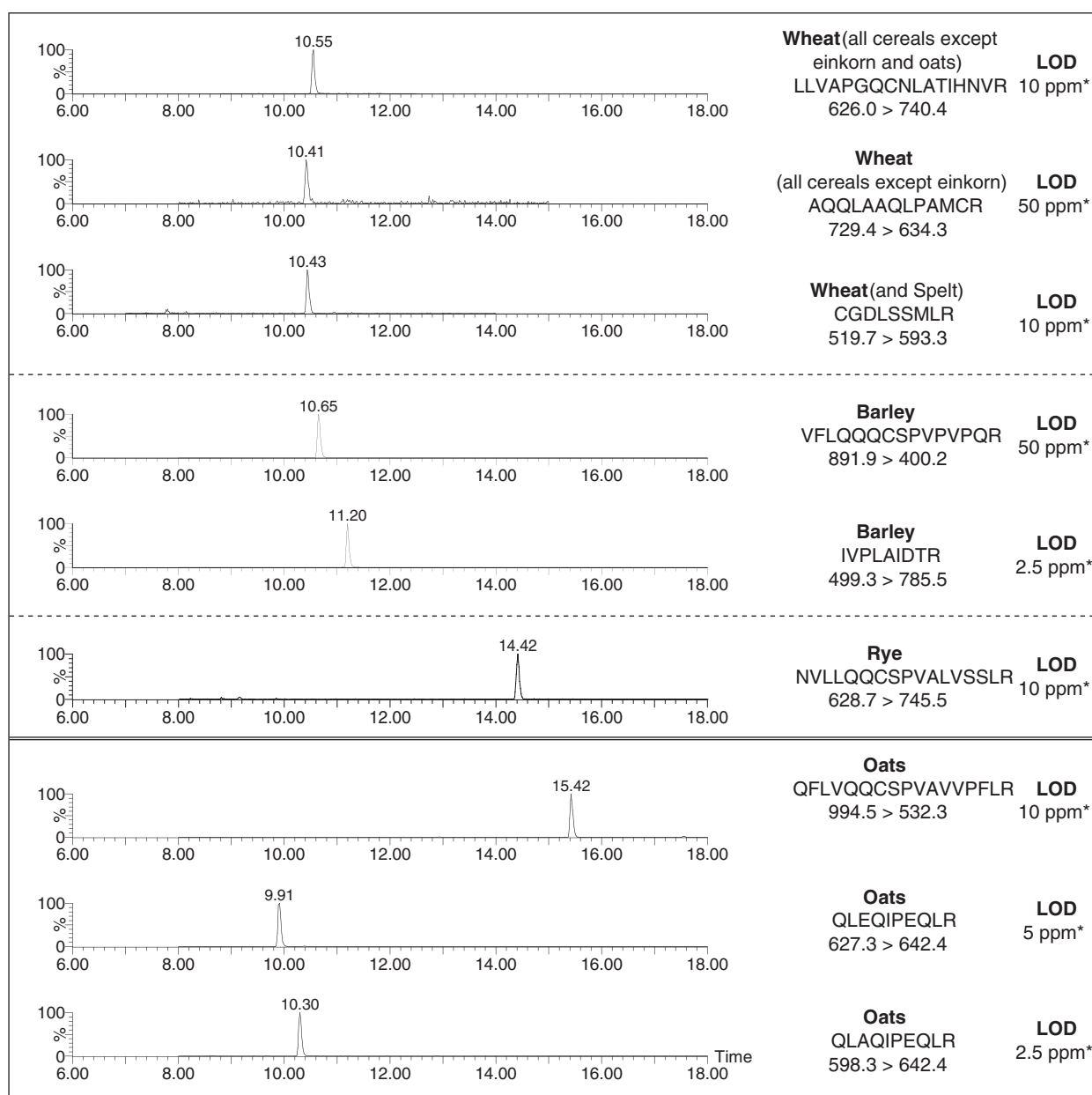


Figure 6. Multi-allergen and grain-specific UHPLC-MS/MS chromatograms of the respective main target peptides for common wheat, barley, rye, and oats, each spiked at 100 ppm in "free-from" cookies. *LOD is expressed in ppm, which corresponds to micrograms of total allergen proteins per gram of matrix.

determined for gluten concentration by the different ELISA kits varied from 37.3 to 56 ppm in this sample (about 50 ppm for R7001 Ridascreen Gliadin kits from R-Biopharm). Our multi-allergens and grain-specific UHPLC-MS/MS method estimated the gluten concentration in this sample at 33 ppm (expressed as total gluten proteins). While this estimation of the gluten concentration in this incurred sample is slightly underestimated compared with ELISA kits (Table 3), it should be noted that the concentration range for ELISA is quite high. Indeed, the assigned gluten concentrations as determined by ELISA vary by approximately 20 ppm in the sample. Therefore, a result of 33 ppm is considered well within the range of results.

Samples bought in supermarket were also compared with ELISA kits for their quantitative estimation to demonstrate the ability of the developed UHPLC-MS/MS method to identify and estimate the amount of gluten-containing cereals present in the samples (Table 3).

The estimation of the gluten concentration by the standard addition method was also tested for some commercially available samples (Table 3). In order to determine which kind of cereal is present in a sample, a table as that depicted in Table 4 can be used. Based on this table, it is quite easy to know if rye, barley, oats, and/or *Triticum* are present in the sample because at least one specific peptide was found for these cereals. For example, barley is the only cereal present in the Belgian

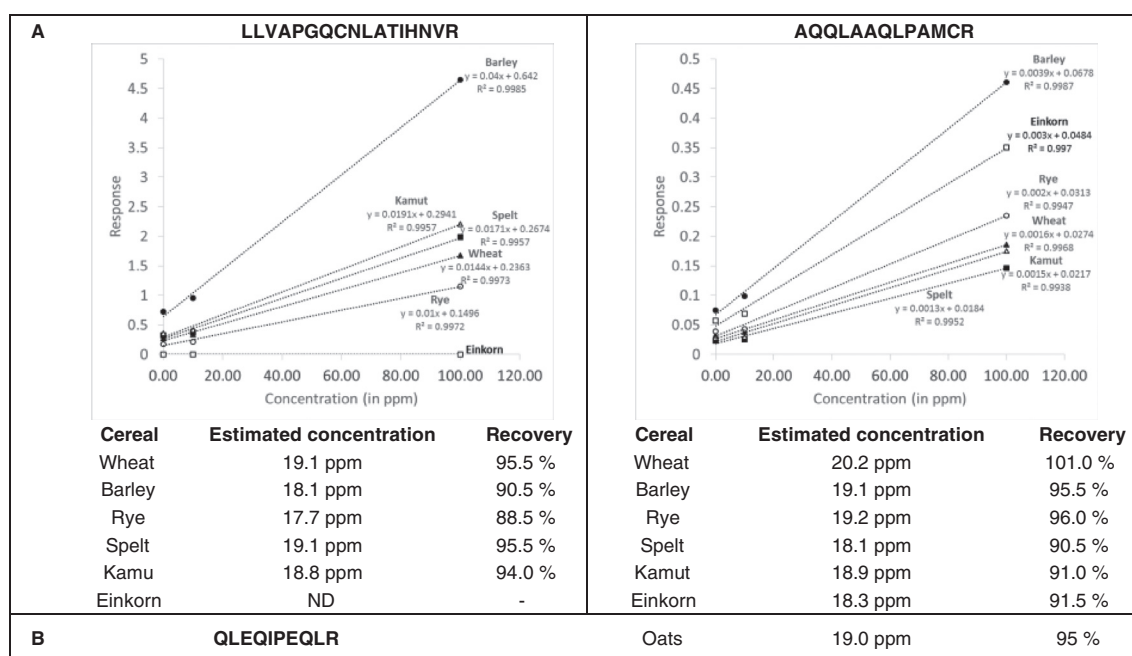


Figure 7. (A) Estimation of the total gluten proteins concentration in gluten-free cookies individually spiked at 20 ppm in each respective cereal by multi-allergen and grain-specific UHPLC-MS/MS analysis. ND: as depicted in Table 1, this peptide is not detected in einkorn. **(B)** Estimation of the total avenin concentration in gluten-free cookies spiked at 20 ppm in oats by multi-allergen and grain-specific UHPLC-MS/MS analysis.

Table 3. Estimation of gluten concentration and labeling status of incurred and commercially available samples according to UHPLC-MS/MS and ELISA analyses

Matrix	ELISA kits range	Estimated concn ^a by R7001 kit (R-Biopharm)	Estimated concn by UHPLC-MS/MS	
Food chemistry proficiency test	37.3–56 ppm	50.0 ppm	33 ppm ^b	
Commercially available matrices	Estimated concn by R7001 kit (R-Biopharm)	Labeling status	Estimated concn by UHPLC-MS/MS	Labeling status
Salted rice cake	14 ppm	"Gluten-free"	<10 ppm (8 ppm ^c)	"Gluten-free"
Brawn rice cake	5 ppm	"Gluten-free"	<10 ppm (2 ppm ^c)	"Gluten-free"
Buckwheat cake	7 ppm	"Gluten-free"	<10 ppm (6.7 ppm ^c)	"Gluten-free"
Bolognese sauce	105 ppm	Contains gluten	117 ppm ^c	Contains gluten
Chocolate rice cake	40 ppm	"Very low gluten" content	<10 ppm (7 ppm ^c)	"Gluten-free"
Buckwheat and quinoa cake	16 ppm	"Gluten-free"	<10 ppm (4 ppm ^c)	"Gluten-free"

^a The concentration estimated is expressed in parts per million or in micrograms of total gluten proteins per gram of sample.

^b Sample contaminated with wheat flour.

^c Concentration estimated by UHPLC-MS/MS according to wheat standard solution addition (at 10 ppm and 100 ppm).

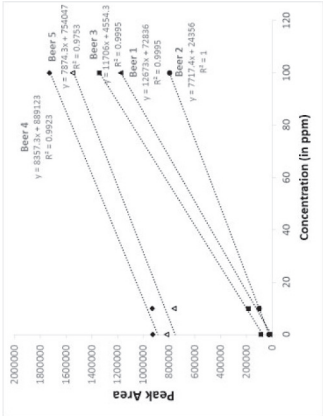
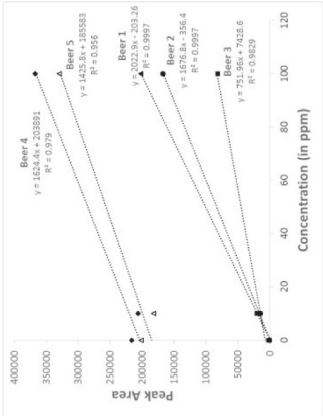
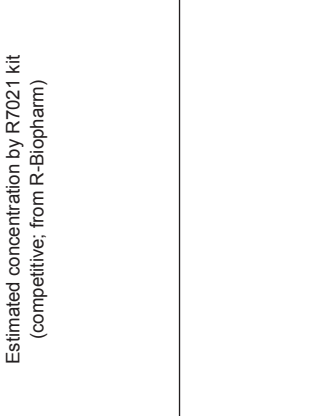
organic blond beer. On the other hand, it is a more difficult to differentiate *Triticum* subspecies from each other because they are closer genetically, and most of the peptides selected here are not exclusively detected in one of these cereals.

Furthermore, this UHPLC-MS/MS method allows for the detection and the estimation of the concentration of hydrolyzed gluten proteins in beers. Indeed, as described in Table 5, several beers have been analyzed to estimate their content in gluten proteins. This constitutes a real advantage for this method regarding ELISA kits because hydrolyzed gluten protein (namely hordeins) fragments show suppression of ELISA measurement of gluten when using the usual R7001 kits,

which requires the use of competitive R7021 ELISA kit (R-Biopharm) instead of R7001 kit when hydrolyzed beverages need to be analyzed (6). Nevertheless, for such hydrolyzed samples, only VFLQQCSPVPVPQR peptide has been used because IVPLAIDTR peptide is not detected in such samples.

Because some ELISA kits may under- or overestimate the gluten concentration when the contamination originates from a cereal other than wheat, gluten concentration was estimated by UHPLC-MS/MS for these beer samples for which the main gluten contamination comes from barley (Table 4). This estimation was done according to the standard addition of either wheat (generally used for standard additions) or barley

Table 5. Estimation of gluten concentration and labeling status of beers according to UHPLC-MS/MS and competitive ELISA analyses

	Beer 1, gluten-free organic amber beer	Beer 2, gluten-free blond beer	Beer 3, gluten-free millet malt-based beer	Beer 4, American Trappist beer	Beer 5, Belgian organic blond beer
UHPLC-MS/MS	1.6 ppm	3.3 ppm	6.9 ppm	111 ppm	141 ppm
	Gluten concn				
					
UHPLC-MS/MS	0.7 ppm	0.6 ppm	3 ppm	133 ppm	141 ppm
	Gluten concn				
					
ELISA	5.5 ppm	Not analyzed	Not analyzed	>100 ppm	>100 ppm
	Gluten concn				
					
ELISA	"Gluten free"	"Gluten free"	"Gluten free"	Contains gluten	Contains gluten
	Labeling status				
	Estimated concentration by R7021 kit (competitive; from R-Biopharm)				

extract standard solution (which is closer to the real gluten contamination source for these samples). The results obtained are very close to each other, regardless of the standard solution used (Table 5). This tends to confirm that the use of a standard solution corresponding to the main gluten contaminant source seems to not be mandatory for UHPLC-MS/MS method.

Nevertheless, even though most of the aforementioned results are in quite good concordance with ELISA measurements, the estimation of the gluten concentration by UHPLC-MS/MS method seems to be lower than that estimated by ELISA kits (Table 3).

Three important factors could explain the differences between ELISA and UHPLC-MS/MS methods for the estimation of the gluten concentration.

First, these methods do not use the same analytical standard (PWG-gliadin for ELISA and several in-house cereal flour extracts for UHPLC-MS/MS). As previously explained, PWG-gliadin cannot be used as standard reference for UHPLC-MS/MS because none of “gluten” peptides coming from the tryptic digestion of gliadin proteins were found to be sufficiently intense to be used for this purpose.

Second, they also use two different conversion factors with a factor 2 to obtain gluten proteins from PWG-gliadin for ELISA and a theoretical percentage of 8% for the content of gluten proteins in wheat flour for UHPLC-MS/MS.

Third, the estimation procedure is also different. ELISA kits use standard calibration curve (in solvent), while UHPLC-MS/MS method prefers standard additions in the matrix to analyze. Indeed, if a single calibration curve (in solvent or in a matrix) is used for the quantification of allergens in foodstuffs by UHPLC-MS/MS method (26), this curve does not fit adequately for all the matrices tested. Therefore, it results in a less accurate estimation of the gluten concentration than the standard addition methodology, which better takes into account all the interactions undergone by the proteins in the matrix to analyze.

Consequently, to allow more accurate quantitative estimation of the gluten protein concentration in processed samples, more works will be required, especially on the standards characterization (percentage of protein, protein profile of each reference standard, etc.), which may lead to a more accurate conversion factor and a better correlation and harmonization between existing methods.

Regarding all the results depicted in Tables 3 and 5, only one sample (chocolate rice cake; Table 3) is estimated as a “gluten-free” product by UHPLC-MS/MS (7 ppm) while being estimated as a “very-low-gluten” product by R7001 ELISA kit (40 ppm). In this particular case, besides the use of a different analytical standard than that of the ELISA kit, this underestimation could be explained by a significant matrix effect for this chocolate rice cake (very low S/N for these peaks), which causes lower peak intensity and underestimation of the total gluten proteins concentration. In all the other cases, the gluten concentration estimated by UHPLC-MS/MS is slightly lower than those estimated by the ELISA kit R7001. However, it is important to point out that both methods can only estimate the “true” and theoretical concentration, which is unknown for such commercially available samples. Nevertheless, both methods agree for those samples that the gluten concentration is lower than the 20 ppm, and that they are in accordance with European Commission No. 828/2014 for gluten-free labeling (9).

Conclusions

Such results show that a semiquantitative multi-allergen and grain-specific UHPLC-MS/MS method turned out to be complementary to ELISA kits. Its main advantage regarding ELISA kits is its ability to detect about 10 different allergens and gluten from seven cereals in only one analysis. Indeed, this method allows the detection and the discrimination of several cereals, including rye, barley, oats, and *Triticum* species. However, *Triticum* subspecies are trickier to differentiate because they are very close genetically.

Furthermore, this single multi-allergen and grain-specific UHPLC-MS/MS method can be used for a broader range of foodstuffs, including hydrolyzed samples, while specific individual kits could be required for ELISA kits. This includes cereals and flours but also simple transformed matrices (e.g., heat-treated foodstuffs such as cakes and sauces) and more complex samples having undergone the fermentation processes (e.g., beers).

This multi-allergen and grain-specific UHPLC-MS/MS detection method could thus be a great benefit for all three stakeholders: (1) the cereal producers to confirm the “purity” of their cereals and flours, (2) the food manufacturers to permit a more accurate precautionary allergen labeling, and (3) allergic consumers, allowing them to make an informed choice based on accurate labeling.

Nevertheless, for more perspective and to obtain the harmonization of analytical results, more work will be required, especially on reference materials, standards characterization, and conversion factors.

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