Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Check for updates

Cite this: DOI: 10.1039/d4ay00852a

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

Detection of gluten peptides in human duodenal fluids with immuno-liquid chromatography-mass spectrometry†

Loïc Dayon, *[ab] Antonio Núñez Galindo,[a] Julien Chevalier,[c] Michèl Aquarius,[d] Britt Otten,[ef] Freddy J. Troost,[ef] Peter Duncan[c] and Michael Affolter [a]

Received 8th May 2024
Accepted 2nd September 2024

DOI: 10.1039/d4ay00852a

rsc.li/methods

Gluten proteins are storage proteins in wheat that exhibit a certain resistance to gastrointestinal digestion. To explore solutions to cope with accidental ingestion of gluten in individuals suffering from gluten-related disorders, it is essential to monitor the fate of gluten peptides in biological samples, *i.e.*, gastrointestinal juices, blood plasma or urine. In this work, we aimed at developing a mass spectrometry (MS)-based method for measuring gluten peptides in human duodenal fluids. Seven gluten peptides, including the well-documented 33-mer gluten peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), were selected after a literature review and characterization of a gluten-containing product. Isotopically labelled peptides were used as references and a targeted liquid chromatography (LC) MS assay based on high resolution parallel reaction monitoring (PRM) was designed. Despite iterative and fine tuning of the LC-PRM-MS method, the low level of endogenous gluten peptides in human duodenal fluid samples precluded their direct detection. Thus, an initial immunoprecipitation (IP) step was included. Several antibodies were tested, and one proved reliable for the enrichment of the 33-mer gluten peptide as well as a few additional gluten peptides. Figures-of-merits of the immuno-LC-PRM-MS assay were assessed with a focus on quantification trueness and precision. We have developed an MS-based method for measuring the 33-mer gluten peptide in human duodenal fluids. Based on isotopic dilution, the method relies on the combination of IP and LC-PRM-MS analysis. Measurements were shown to be sensitive, quantitative, and reproducible.

Introduction

Gliadins and glutenins, gathered under the umbrella of gluten proteins, are storage proteins in wheat. They contain high percentages of proline and glutamine amino acids, making them resistant to gastrointestinal digestion. Homologue proteins are found in other cereals, such as hordeins in barley or secalins in rye. Gluten-related disorders in individuals encompass celiac disease (CeD) and non-celiac wheat sensitivity (NCWS). For these individuals, consumption of gluten-free food is recommended to prevent gluten-associated symptoms. Yet, following a completely gluten-free diet can be challenging and accidental ingestion of gluten is a reality to cope with. Comprehensive analysis of gluten immunogenic sequences is of great relevance for understanding which peptides trigger the immune response, developing diagnostic tools, identifying therapeutic targets, informing personalized treatment strategies, and improving gluten-free food options.[1,2]

Probiotic solutions have been suggested as a way to mitigate the effects of hidden gluten in food.[3] To determine their effectiveness, it is important to understand their impact on the peptides from gluten that trigger an immune reaction. The measurement of levels of gluten peptides, for instance, in the duodenal fluids of human individuals is expected to help address this question. Such determination is also relevant to assess the efficacy of therapeutic approaches such as gluten-degrading enzymes.[4–6]

Enzyme-linked immunosorbent assay (ELISA) is an approved method for detecting the overall presence of gluten in food matrices. Reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS) has been used to quantitatively detect gluten peptides in wheat and in wheat-based food

[a]*Nestlé Institute of Food Safety & Analytical Sciences, Nestlé Research, Société des Produits Nestlé SA, EPFL Innovation Park, Bâtiment H, CH-1015 Lausanne, Switzerland. E-mail: loic.dayon@rd.nestle.com; Tel: +41 21 632 61 14*

[b]*Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland*

[c]*Nestlé Institute of Health Sciences, Nestlé Research, Lausanne, Switzerland*

[d]*Department of Gastroenterology, VieCuri Medical Center, Venlo, The Netherlands*

[e]*Department of Human Biology, NUTRIM, Institute of Nutrition and Translational Research in Metabolism, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, The Netherlands*

[f]*Food Innovation and Health, Centre for Healthy Eating and Food Innovation (HEFI), Maastricht University, Venlo, The Netherlands*

† Electronic supplementary information (ESI) available. See DOI: **https://doi.org/10.1039/d4ay00852a**

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

products.[7–9] Given the specificity of molecular mass measurements, MS methods are relevant for identifying different species and epitopes of gluten.[10,11] Yet, detecting and quantifying endogenously produced gluten peptides in human body fluids constitutes a significant challenge.[12] Some studies have characterized proteolytically resistant gluten-derived peptides using *in vitro* gastrointestinal digestion models.[8,13–15] However, characterizing potentially toxic or immunogenic peptides in human fluid samples from the gastrointestinal tract is much more difficult as the peptides are diluted in a complex matrix containing host, microbial and food proteins/peptides. While ELISA has been used to detect immunogenic gluten peptides in human stool,[16] urine,[17] stomach aspirates as well as *in vitro* gastric digestion,[4,18] there is still, to the best of our knowledge, no use of the method reported for detecting and quantifying gluten peptides in human duodenal fluid samples.

In the present work, we describe the development of an RP-LC high resolution MS/MS method relying on prior immuno-capture of relevant gluten peptides. This method is shown to be sensitive enough to quantitatively measure the immunodominant 33-mer gluten peptide in duodenal fluid samples. The deployment of such an assay could be crucial in evaluating the effects of solutions that aim at mitigating the effect of hidden gluten in individuals with CeD and NCWS.

Experimental

Chemicals and reagents

Formic acid (FA, 99%) and CH_3CN were obtained from BDH (VWR International, Poole, UK). H_2O (18.2 MΩ cm at 25 °C) was obtained from a Milli-Q apparatus (Merck Millipore, Billerica, MA). Trifluoroacetic acid (TFA) Uvasol was sourced from Merck Millipore. The bicinchoninic acid (BCA) protein assay and the Pierce MS-compatible Magnetic IP (protein A/G) kits were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Oasis HLB cartridges (1 cm^3, 30 mg) and RapiGest SF Surfactant (RapiGest) were acquired from Waters (Milford, MA, USA). Strata-X-C 33u Polymeric SCX cartridges (30 mg/1 mL) were from Phenomenex (Torrance, CA, USA).

Synthetic peptides

Synpeptide (Shanghai, China) provided peptides containing some isotopically labelled amino acids (*i.e.*, proline and/or phenylalanine) as indicated by asterisks in the following peptide sequences, where P* [+6 Da $^{13}C_5$,^{15}N] and F* [+10 Da $^{13}C_9$,^{15}N] are isotope-labeled with ^{13}C and ^{15}N: P*GQQQPFPPQQP*Y (13-mer; molecular weight (MW) = 1523.65 g mol^{-1}), P*GQQQPFPPQQPYPQPQP*F* (19-mer; MW = 2228.44 g mol^{-1}), LQLQP*F*PQPQLPYPQPQLPYPQPQLPYPQP*QP*F* (33-mer; MW = 3949.47g mol^{-1}), QLQP*F*PQPQLPYPQP*QL (17-mer; MW = 2041.31 g mol^{-1}), P*QQP*QQSFPQQQQP*A (15-mer; MW = 1754.86 g mol^{-1}), F*P*QPQQTFPQQPQLPFPQQPQQPF*P*Q (26-mer; MW = 3136.47 g mol^{-1}), and QLQP*FPQPQLP*Y (12-mer; MW = 1467.66 g mol^{-1}) (Table 1). Purity of these isotopically labelled peptides was stated to be above 97%.

Antibodies

Polyclonal wheat gliadin antibody (cat. no. 70R-15482) (antibody 1) was obtained from Fitzgerald Industries International (Acton, MA, USA), anti-gliadin (wheat) antibody (cat. no. G9144) (antibody 2) from Sigma-Aldrich (Buchs, Switzerland), wheat gliadin antibody (cat. no. orb243824) (antibody 3) from Biorbyt (Cambridge, UK), G12 and A1 antibodies (antibody 4, specific for a hexapeptide with the amino acid sequence QPQLPY, and antibody 5, specific for a heptapeptide with the amino acid sequence QLPYPQP, respectively) from Hygiena (Camarillo, CA, USA), R5 monoclonal antibody (antibody 6, specific for pentapeptides with the amino acid sequences QQPFP, QQQFP, LQPFP, and QLPFP) from Ingenasa (Madrid, Spain), and gliadin peptide monoclonal antibody (14D5) (antibody 7) from Enzo Life Sciences (Lausen, Switzerland) (Table 2).

Human samples

Human duodenal fluid samples were collected during a randomized clinical trial at Maastricht University, The Netherlands. The study protocol was approved by the Independent Review Board Nijmegen (IRBN; Nijmegen, The Netherlands) on September 25, 2018, with the reference number IRBN2018005. It was performed in full accordance with the Declaration of Helsinki (latest amendment by the World Medical Association in 2013, Fortaleza, Brazil) and Dutch Regulations on Medical Research Involving Human Subjects (WMO, 1998). The study was registered at the Central Committee on Research Involving Human Subjects (CCMO) in The Netherlands, under reference number NL65701.072.18 and at https://ClinicalTrials.gov (ref. no. ID NCT03775499). All subjects were informed about the nature of the study and risks associated with participation before written informed consent was obtained. All participants were above 18 years of age. Briefly, clinically diagnosed CeD and self-reported NCWS patients were included in the study. A 270 cm long polyurethane single lumen naso-duodenal catheter (outer diameter 2.6 mm; Fresenius Kabi, Runcorn, United Kingdom) reaching the distal duodenum was placed by gastroduodenoscopy at the endoscopy unit in a medical center (VieCuri, Venlo, The Netherlands). Successful positioning of the catheter tip in the distal part of the duodenum was determined by measuring pH of the intestinal fluid. To confirm the accurate positioning of the catheter tip in the small intestine, the pH of the first fluid samples was assessed using pH-Fix indicator strips (Macherey Nagel, Düren, Germany). Two duodenal aspirate samples were collected at the baseline. Subsequently, a 3 g gluten challenge was administered. The gluten challenge consisted of a liquid gluten free breakfast meal (Nestlé Resource, Oosterhout, The Netherlands) with 3 g added gluten powder (Priméal, Peaugres, France). Duodenal aspirates were collected at 20 min intervals for a period of 370 min.

Samples used in this work were identified as indicated in ESI Table SI1† as sample A, sample B, sample C, sample D, and sample E from CeD individuals following the gluten challenge. A pooled sample (pool Y) was made with equal volumes of six other samples (from CeD individuals after the gluten challenge) and aliquoted. An additional pool of samples (pool Z) from

This journal is © The Royal Society of Chemistry 2024

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

Table 1 Selected peptides as main targets of an LC-PRM-MS assay. Some peptide sequences or part of them are present in the Immune Epitope Database and Analysis Resource (https://www.iedb.org),[19] *i.e.*, LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF, QLQPFPQPQLPYPQPQL, PQQPQQSFPQQQQPA, PQQTFPQQPQLPF, and QQPQQPFPQ. Gliadins GDB2 and GAD7, despite being abundant in the gluten product and presenting epitopes, were not followed as no clear existing peptide sequence was found in the literature. The potential celiac disease-relevant T-cell epitopes encoded within the peptides are indicated[20]

Peptide sequence	Peptide name	Protein	T-cell epitope
PGQQQPFPPQQPY	13-mer	GDA4, GDA9	
PGQQQPFPPQQPYPQPQPF	19-mer	GDA9	
LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	33-mer	GDA9	DQ2.5-glia-α1a/α1b/α2
QLQPFPQPQLPYPQPQL	17-mer	GDA4, GDA9	DQ2.5-glia-α1a
PQQPQQSFPQQQQPA	15-mer	GDBX	DQ2.5-glia-γ1; DQ8-glia-γ2; DQ8.5-glia-γ1
FPQPQQTFPQQPQLPFPQQPQQPFPQ	26-mer	GDBX	DQ8-glia-γ1a
QLQPFPQPQLPY	12-mer	GDA4, GDA9	DQ2.5-glia-α1a

Table 2 Identification of gluten peptides after IP in duodenal fluid sample A. LC-MS/MS using DDA revealed the capture of gluten peptides after IP with seven different antibodies (experiments of "Batch 1" as depicted in ESI Fig. SI4). To facilitate the antibody performance comparison, Mascot searches were performed in Swiss-Prot restrained to the Viridiplantae (green plants) taxonomy (see ESI files SI_Sample_pep_after+Ab_01-07). In the detected sequences, the epitope(s) relevant to the used antibody are displayed in bold

Antibody	Identified peptide sequence(s) (protein(s))
Antibody 1 (Fitzgerald)	—
Antibody 2 (Sigma-Aldrich)	VRVPVPQLQPQNPSQQQPQEQVPLVQ (alpha/beta-gliadin A-I, A-V, MM1)
	VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF (alpha/beta-gliadin A-I, A-V, MM1)
Antibody 3 (Biorbyt)	VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF (alpha/beta-gliadin A-I, MM1, A-V)
	IILHQQHHHHQQQQQQQQQQPLSQ (alpha/beta-gliadin A-II)
	VRVPVPQLQPQNPSQQQPQKQVPLVQQQQF (alpha/beta-gliadin A-IV)
Antibody 4 (Hygiena)	VRVPVPQLQPQNPSQQQPQEQ (alpha/beta-gliadin MM1, A-I, A-III)
G12: QPQLPY	IILHQQQQQQQQQQQQQPLSQ (alpha/beta-gliadin MM1)
	LQLQPFP**QPQLPY**P**QPQLPY**P**QPQLPY**PQPQPFRPQQPYPQSQPQ (alpha/beta-gliadin MM1)[a,b,c]
	LQLQPFP**QPQLPY**P**QPQLPY**P**QPQLPY**PQPQPFRPQQPYPQSQPQY (alpha/beta-gliadin MM1)[a,b,c]
	LQLQPFL**QPQLPY**SQPQPFRPQQPYPQPQPQ (alpha/beta-gliadin A-I)
	VRVPVPQLQPQNPSQQQPQEQVPLM (alpha/beta-gliadin A-III)
	QLQPFP**QPQLPY**SQPQPFRPQQPYPQPQPQ (alpha/beta-gliadin)[c]
	SQQQQPVLPQQSPF (gamma-gliadin B-I, glutenin, low molecular weight subunit)
	LQPHQIAQL (gamma-gliadin (fragment))
Antibody 5 (Hygiena)	IILHQQQQQQQQQQQQQPLSQ (alpha/beta-gliadin MM1)
A1: QLPYPQP	VRVPVPQLQPQNPSQQQPQEQVPLVQ (alpha/beta-gliadin MM1)
	VRVPVPQLQPQNPSQQQPQEQVPLVQQQQ (alpha/beta-gliadin MM1)
	QPQQPFPQPQQPQQPFPQ (gamma-gliadin)
	QPQQPFPQPQQQFPQPQQPQQSFPQQQQPAIQSFLQQQM (gamma-gliadin)[d]
	VRVPVPQLQPQNPSQQQPQEQVPLM (alpha/beta-gliadin A-III)
	LQPHQIAQL (gamma-gliadin (fragment))
Antibody 6 (Ingenasa)	VRVPVPQLQPQNPSQQQPQEQVPLVQ (alpha/beta-gliadin A-I, A-V, MM1)
R5: QQPFP, QQQFP, LQPFP, QLPFP	VRVPVPQLQPQNPSQQQPQEQVPLVQQQQF (alpha/beta-gliadin A-I, A-V, MM1)
	QPFPQQP**QQPFP**QTQQ (gamma-gliadin)
	LQPHQIAQL (gamma-gliadin (fragment))
Antibody 7 (Enzo)	IILHQQQQQQQQQQQQQPLSQ (alpha/beta-gliadin MM1)
14D5: LQPFPQPELPYPQPQ used as antigen	

[a] Includes the sequence of 33-mer. [b] Includes the sequence of 17-mer. [c] Includes the sequence of 12-mer. [d] Includes the sequence of 15-mer.

NCWS individuals constituted a negative control where no gluten peptide was expected (no gluten consumption for the participants).

Sample preparation of synthetic peptides in a matrix-free buffer

The peptides were initially diluted to 1 mg mL^{-1} in H_2O/CH_3CN/FA 96.9/3/0.1 before further dilution in the same solution (*i.e.*, from 0.01 ng μL^{-1} to 10 ng μL^{-1} with subsequent injection of 5 μL for RP-LC-MS/MS analysis).

Sample preparation of duodenal fluids spiked with synthetic peptides

Protein/peptide concentrations in duodenal fluid samples were determined using the BCA protein assay. Volumes corresponding to different amounts of proteins/peptides (*i.e.*, 50 μg

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

or 100 μg) were spiked with isotopically labelled peptides (*i.e.*, from 2.5 ng to 2.5 μg) as indicated in the text and figures. Samples were then acidified by addition of 500 μL of H_2O/CH_3CN/FA 96.9/3/0.1. Solid-phase extraction (SPE) purifications (Oasis HLB and SCX) were performed using a 4-channel Microlab Star liquid handler (Hamilton, Bonaduz, Switzerland) according to a previously reported protocol.[21] Lyophilized samples were kept at −20 °C.

Sample preparation of duodenal fluids spiked with synthetic peptides using immunoprecipitation

Protein/peptide concentrations in duodenal fluid samples were determined using the BCA protein assay. Volumes corresponding to an amount of 1 mg of proteins/peptides were spiked with isotopically labelled peptides (*i.e.*, 0.05 ng, 0.5 ng, 5 ng, 25 ng or 100 ng; 5 ng being the determined optimal amount (see the Results & discussion section below)). Samples were acidified by addition of 500 μL of H_2O/CH_3CN/TFA 94.9/5/0.1. SPE purifications (Oasis HLB and SCX) were performed using a 4-channel Microlab Star liquid handler.[21]

The IP procedure was then performed following the manufacturer's instructions with slight modifications. The buffers were used directly from the Pierce MS-compatible Magnetic IP (protein A/G) kit. Removal and discarding of supernatants described in the protocol were performed with a magnetic stand. The IPs were performed in two days as follows. On the first day, amounts of 5 μg of each antibody (see the Materials subsection) were mixed with the purified proteins/peptides extracted from duodenal fluids and diluted with 500 μL of IP-MS cell lysis buffer. The whole mixture was incubated overnight at 4 °C with mixing to form the immune complex. The second day, protein A/G magnetic bead preparation, incubation with the immune complex, washes and final elution were performed according to the manufacturer's instructions. The immunoprecipitated samples were purified using SPE as described above.

Reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS)

The lyophilized samples were resuspended in 100 or 125 μL (25 μL when samples were immunoprecipitated) of H_2O/CH_3CN/FA 96.9/3/0.1 to be typically diluted at approximately 0.4 μg μL^{-1} to 1 μg μL^{-1} of total theoretical peptide content. RP-LC-MS/MS was performed with an Ultimate 3000 RSLC nanosystem and an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific). Peptides (injection of 2.5 to 5 μL) were trapped on an Acclaim PepMap 300 μm × 5 mm (C18, 5 μm, 100 Å) pre-column and separated on an Acclaim PepMap RSLC 75 μm × 50 cm (C18, 2 μm, 100 Å) column (Thermo Scientific) coupled to a stainless steel nanobore emitter (40 mm, OD 1/32″) (Thermo Scientific). The column was heated to 50 °C using a PRSO-V1 column oven (Sonation, Biberach, Germany).

Data-dependent acquisition (DDA)

Prior to MS, one-line peptide LC separation was performed with a gradient of mobile phase A (H_2O/CH_3CN/FA 97.9/2/0.1) and B (H_2O/CH_3CN/FA 19.92/80/0.08): from 6.3% (hold for 1 min) to 11% B over 3 min, from 11% to 17.5% B over 19.2 min, from 17.5% to 25.5% B over 11.4 min and from 25.5% to 40% B over 7.4 min, with final elution (98% B) and equilibration (6.3% B) for a further 23 min. The flow rate was 300 nL min^{-1} with a total analysis time of 65 min. A positive ion spray voltage of 1900 V and a transfer tube temperature of 275 °C were set up. For MS survey scans in profile mode, the Orbitrap resolution was 120 000 at $m/z = 200$ (automatic gain control (AGC) target of 2×10^5) with a m/z scan range from 300 to 1500, RF lens set at 30%, and a maximum injection time of 100 ms. For MS/MS with higher-energy collisional dissociation (HCD) at 30% of the normalized collision energy, the AGC target was set to 2×10^3 (isolation width of 1.2 in the quadrupole) with the ion-trap acquiring with a rapid scan rate in centroid mode. A duty cycle time of 3 s was used to determine the number of precursor ions to be selected for HCD-based MS/MS. Dynamic exclusion was set for 60 s within a ±10 ppm window. A lock mass of $m/z = 445.12002$ was used.

Parallel reaction monitoring (PRM)

Only the final optimized LC-PRM-MS method is described herein. Prior to MS, one-line peptide LC separation was performed with a gradient of mobile phases A and B: from 6.3% (hold for 1 min) to 11% B over 3 min, from 11% to 25.5% B over 19.2 min, from 25.5% to 40% B over 11.4 min and from 40% to 80% B over 7.4 min, with final elution (98% B) and equilibration (6.3% B) for a further 23 min. The flow rate was 300 nL min^{-1} with a total analysis time of 65 min. A positive ion spray voltage of 1900 V and a transfer tube temperature of 275 °C were set up. Two MS experiments ran in parallel. An MS survey scan was performed in profile mode with an Orbitrap resolution of 15 000 at $m/z = 200$ (AGC of 4×10^5) with a m/z scan range from 300 to 1500, RF lens set at 30% and a maximum injection time of 50 ms. The second experiment performed MS/MS scans on a list of m/z targets using HCD at 31% of the NCE and according to a predefined scheduling over the LC elution (see Table 3). The AGC target was set to 5×10^4 (isolation width of 0.7 in the quadrupole), with a resolution of 120 000 at $m/z = 200$, and a maximum injection time of 246 ms with the Orbitrap acquiring in profile mode in a m/z scan range from 350 to 1000. A lock mass of $m/z = 445.12002$ was used.

Data processing and analysis

Protein and peptide identifications in duodenal fluids were performed against the Swiss-Prot database (05/2019 release) (560 118 sequences in total). Mascot[22] (version 2.6.1, Matrix Sciences, London, UK) was used as the search engine assuming the digestion enzyme to be non-specific. Variable amino acid modifications were oxidized methionine and deamidated asparagine/glutamine. Peptide and fragment-ion tolerances were set to 5 ppm and 0.8 Da, respectively. As the gluten product provided to the participants of the clinical trial was analysed using a classical shotgun proteomic approach (see the Results & discussion section for a brief experimental description), in that case, search parameters were digestion enzyme trypsin

 This journal is © The Royal Society of Chemistry 2024

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

Table 3 LC-PRM-MS assay parameters and measurement precision for 33-mer, 12-mer and additional gluten peptides using stable isotopic dilution and immuno-LC-PRM-MS. The parameters are given for the final immuno-PRM-MS assay that was retained for the measurement of seven peptides using stable isotopic dilution or label-free quantification. Experiments were performed in triplicate and each sample was measured in triplicate on two identical but independent LC-MS/MS systems. Averaged CVs are provided. NA: not available

Peptide sequence	Short name	Precursor *m/z*	Charge state	Retention time window	Fragment-ions used for quantification	Average CV% (LC-PRM-MS)	Average CV% (replication)
LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	33-mer	1304.017 1316.716	+3	39.5–49.5 min	y8, y6, y4, b6, b8	18%	18.5%
QLQPFPQPQLPYPQPQL	17-mer	1010.039 1021.066	+2	36.9–46.9 min	y8, y7, y5, y3, b3, b7	NA	NA
QLQPFPQPQLPY	12-mer	728.385 734.399	+2	35.2–45.2 min	y5, y3, b3, b5, b6, b7	16.5%	57.5%
LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPFRPQQPYPQSQPQ	NA	1336.940	+4	37.1–47.1 min	All available	17.5%	19%
LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPFRPQQPYPQSQPQY	NA	1377.706	+4	37.5–47.5 min	All available	10%	32.4%
LQLQPFLQPQLPYSQPQPFRPQQPYPQPQPQ	NA	1238.646	+3	34.7–44.7 min	All available	25.5%	27%
QLQPFPQPQLPYSQPQPFRPQQPYPQPQPQ	NA	1195.607	+3	31.8–41.8 min	All available	16%	31.6%

(maximum of two missed cleavages), a parent ion tolerance of 10 ppm, a fragment-ion mass tolerance of 0.6 Da, carbamidomethyl of cysteine as a fixed modification, and deamidation of asparagine and glutamine and oxidation of methionine as variable modifications.

All Mascot results files were initially loaded into Scaffold Q+S 4.8.8 and retrieved from Q+S 5.0.0 (Proteome Software, Portland, OR, USA). Peptide identifications were accepted if they could be established at greater than 95.0% probability. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 1 identified peptide (for the proteomic analysis of the gluten product the parameters were false discovery rates of at least 1% at peptide and protein levels and at least 2 identified peptides to report protein identification after a further search with X! Tandem (The GPM, **https://thegpm.org**; version Alanine (2017.2.1.4)). Protein probabilities were assigned by the Protein Prophet algorithm.[23] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Skyline software (version 4.2.0.19107) was used to treat LC-PRM-MS data. Fragment-ion signals were manually validated. Multiple *criteria* were checked for a given peptide using Skyline: identical retention times and transition profiles for endogenous and isotopically labelled peptides, co-elution of the selected transitions for a given precursor, and correct peak area integration with Skyline (if not, this was adjusted manually). Peak areas, ratios of endogenous peptides over isotopically labelled peptides (ratios L/H for light/heavy), and retention times were exported from Skyline. We used a single reference point quantitation approach.[24] The ratios of the light and heavy peak areas were used to estimate the unknown amount of endogenous peptide analytes.

Calculations and statistics were performed with Excel (Microsoft, Redmond, WA).

Results & discussion

Definition of gluten peptides to be measured with liquid chromatography tandem mass spectrometry

To detect gluten peptides in human duodenal fluid using LC-MS/MS, relevant peptide sequences need first to be defined. As the breakdown of food largely occurs in the duodenum of the small intestine, duodenal aspirate constitutes a relevant biofluid to look for the presence of gluten peptides. The peptidome of human duodenal fluids is composed of many peptides originating from humans, bacteria and essentially other organisms that are constituents of the ingested food. Some duodenal fluid samples were therefore first analysed with RP-LC-MS/MS after purification of the peptide fraction (see the Experimental section). An untargeted DDA method allowed the unbiased screening of the peptidome of two duodenal fluid samples, one collected 50 min after ingestion of a gluten product by a subject (sample A) and one collected 370 min after ingestion of a gluten product by a subject (sample B) (ESI Table SI1†). In sample A, the main detected peptides originated from bovine proteins and, to a lesser extent, from human proteins. Peptides of gliadins alpha/beta-gliadin (GDA0; P02863), alpha/beta-gliadin A-I (GDA1; P04721), alpha/beta-gliadin A-II (GDA2; P04722), alpha/beta-gliadin A-III (GDA3; P04723), alpha/beta-gliadin A-IV (GDA4; P04724), and alpha/beta-gliadin A-II (GDBX; P21292) from common wheat (*Triticum aestivum*) were evidenced (see ESI file SI_IDs_Sample_A†). None of the detected peptides covered already known immunogenic amino acid sequences (data not shown).[25–28] One peptide of glutenin, high molecular weight subunit DY10 (GLT0; P10387), and one peptide of glutenin, high molecular weight subunit DX5 (GLT5; P10388), were also detected. In sample B, the presence of peptides from wheat

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

proteins was not evidenced and the main peptides originated from human, bovine, pea, and chicken proteins (see ESI file SI_IDs_Sample_B†), in accordance with the previous consumption of a meal by the study participant.

As we aimed to deploy an LC-MS/MS assay in a clinical trial where participants received a specific gluten product (Gluten pur from Priméal), we analysed this food matrix with MS-based proteomics as well. The gluten product was analysed with LC-MS/MS using DDA after optimization of its dissolution in urea 8 M containing a RapiGest surfactant at 1%. Enzymatic digestion used trypsin/Lys-C following a previously published sample preparation protocol.[29] We identified nine gliadins: gamma-gliadin B-I (GDB1; P04729), gamma-gliadin (GDB2; P08453), gamma-gliadin (GDB3; P04730), gamma-gliadin B (GDBB; P06659), gamma-gliadin (GDBX; P21292), alpha/beta-gliadin A-IV (GDA4; P04724), alpha/beta-gliadin clone PW1215 (GDA6; P04726), alpha/beta-gliadin clone PW8142 (GDA7; P04727), and alpha/beta-gliadin MM1 (GDA9; P18573) (see ESI file SI_IDs_Gluten_Product†). Several immunogenic amino acid sequences are known to be present in these proteins but most of them were not overlapping with the tryptic peptide sequences detected herein (ESI Fig. SI1†).

Based on these experimental observations, we decided to (i) define the immunogenic amino acid sequences and gluten peptides mainly based on some previous literature studies[25–28] where recent results from *in vitro* simulation of gastrointestinal digestion were not included at the time of our study[30,31] and (ii) focus preferentially on sequences of the gliadin forms detected in the gluten product. Following these principles, we retained seven peptides (Table 1) as main targets for the development of a targeted LC-MS/MS assay. The peptides were synthesized with incorporation of isotopically labelled amino acids as indicated in the Experimental section.

Measurements of gluten peptides in duodenal fluid samples with liquid chromatography tandem mass spectrometry

In this part, we evaluated the detection of the seven selected peptides (Table 1). Following the classical development of a targeted LC-PRM-MS assay, we studied the isotopically labelled peptides, determining their retention time in LC as well as their most abundant charge state in ESI-MS. The retained charge states were +2 and +3. Given the rather hydrophobic nature of most of the selected peptides, we adapted the conventional LC gradient to optimize their elution (see the Experimental section). Calibration curves were successfully constructed in matrix-free buffer solutions, ranging from 0.05 to 50 ng of each peptide injected on the column. Nonetheless, some peptides, such as 33-mer, showed limited detectability with respect to shorter peptides (Fig. 1 and ESI Fig. SI2†). Importantly, we did not detect any significant amounts of unlabelled peptides in the isotopically labelled peptides (data not shown), validating their use as internal standards.

We then spiked the isotopically labelled peptides in duodenal fluid samples (*i.e.*, in sample A and sample B; see ESI Table SI1†). While the isotopically labelled peptides responded in the biofluid rather similarly as in the matrix-free buffer, we did not detect any endogenous counterparts in these fluids. We only detected a potential response for the 13-mer peptide in sample A (data not shown), but that needed to be confirmed in additional experiments (see below).

As a matter of fact, the LC-PRM-MS assay appeared to lack sensitivity for detecting endogenous gluten peptides, should they exist in the analysed duodenal fluid samples (of note: we confirmed the presence of other peptides from GDA4 and GBDX in sample A using untargeted DDA acquisition; see the previous subsection). To improve assay sensitivity, we optimized several MS acquisition parameters (MS/MS resolution, AGC, ion injection time and the isolation window).[32] Among them, increasing the MS/MS resolution improved the fragmentation pattern matching with that of the isotopically labelled reference standards (*e.g.*, enhanced specificity and reduced chemical noise), while increasing the AGC ion target only marginally affected the sensitivity. The PRM-MS optimized parameters are detailed in the Experimental section. In addition, we noticed that certain peptides (*i.e.*, 13-mer and 19-mer) presented RP-LC elution peaks split in two (ESI Fig. SI3†). This behaviour was attributed to the isomerization of prolines in such peptides with proline–proline bonds, as previously reported.[33] Despite further optimization attempts (*e.g.*, column heating to 60 °C and gradient

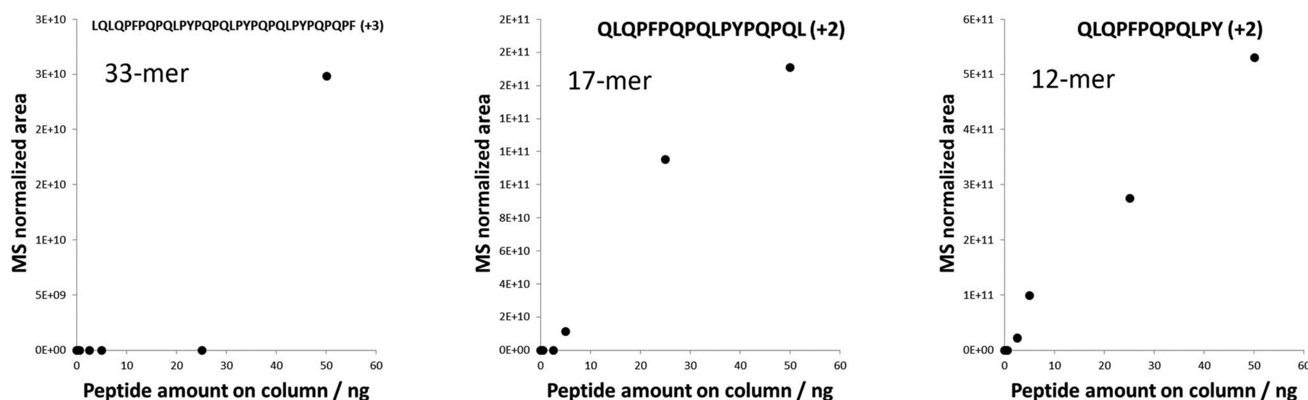

Fig. 1 Quantitative response of the isotopically labelled gluten peptides in a matrix-free buffer using LC-PRM-MS. LC-PRM-MS targets the triply charged ion of 33-mer, the doubly charged ion of 17-mer, and the doubly charged ion of 12-mer.

 This journal is © The Royal Society of Chemistry 2024

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

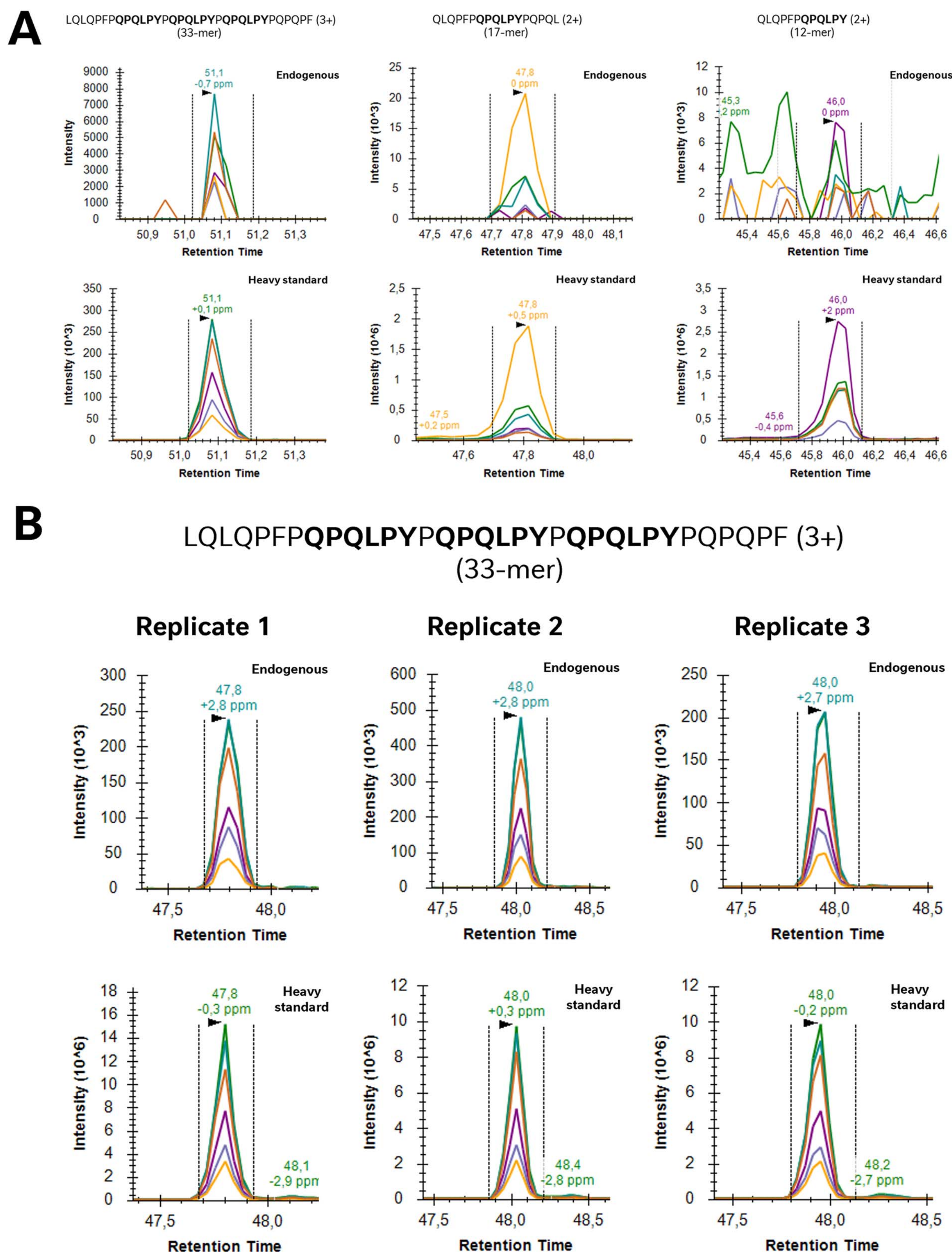

Fig. 2 Detection of gluten peptides (endogenous and isotopically labelled peptides) in duodenal fluid sample A using immuno-LC-PRM-MS. Using IP with antibody 4 and LC-PRM-MS (see experiments of "Batch 2" as depicted in ESI Fig. SI4†), 33-mer, 17-mer, and 12-mer gluten peptides could be detected (A). Detection of 33-mer was respectively demonstrated using such an approach in additional replication experiments (B).

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

shortening), the peak splitting remained (ESI Fig. SI3†). Consequently, we concluded that measuring these peptides posed challenges and that the detection of the endogenous 13-mer mentioned above in duodenal fluids was likely an artifact.

Detection of gluten peptides with immunoprecipitation coupled to liquid chromatography tandem mass spectrometry

After the first attempt at assay development, we were forced to acknowledge some limitations of our approach. While the LC-PRM-MS could detect and potentially quantify the different gluten peptides, the presence of these peptides in duodenal fluid was not proven and the sensitivity of the method was insufficient. The enrichment of the targeted immunogenic gluten peptides using an immuno-affinity capture could help address both of these issues.

We evaluated the incorporation of an immunoprecipitation (IP) step before LC-PRM-MS. Seven antibodies were tested for their ability to capture relevant gluten peptides on agarose-based beads (see Materials in the Experimental section). The immunoprecipitated samples were then analysed using LC-MS/MS with both DDA and PRM acquisitions. The overall experimental approach is illustrated in ESI Fig. SI4,† with the goal of selecting the most appropriate antibody/ies for gluten peptide enrichment. Table 2 summarizes the results of peptide detections with untargeted DDA after IP.

Different peptides were captured with each of the antibodies, and we successfully detected endogenous peptides containing immunogenic amino acid sequences of interest in human duodenal fluid (sample A). Antibody 4 appeared to be the most appropriate antibody for our application as it properly enriched the *QPQLPY* epitope and allowed the putative detection of three endogenous peptides that included sequences of interest, *i.e.*, 12-mer, 17-mer and 33-mer. Yet, to confirm the presence of these pre-selected peptides, a targeted LC-PRM-MS approach was necessary (Fig. 2A). This performance was repeated in triplicate sample preparations including IP and LC-PRM-MS analyses of the sample (Fig. 2B) for 12-mer and especially the 33-mer peptide.

Within this series of experiments, we also confirmed that higher amounts of isotopically labelled peptides (*e.g.*, 100 ng) spiked before IP induced their unspecific binding and capture during IP (experiments of "Batch 3" as depicted in ESI Fig. SI4†) as revealed by the systematic detection of the complete set of these isotopically labelled standards with LC-PRM-MS (data not shown), though this still permitted the detection of endogenous 12-mer and 33-mer peptides. In all these experiments, we noted that the 13-mer peptide was never detected endogenously following IP (particularly when using antibody 6 (*i.e.*, R5 monoclonal antibody from Ingenasa), suggesting that its previous detection with LC-PRM-MS may have been spurious indeed (see discussion in the previous subsection).

Interestingly, four specific peptides containing epitope *QPQLPY* were detected after IP using antibody 4 in sample A. We decided to further include those peptides in the LC-PRM-MS assay and quantify them semi-quantitatively without the use of isotopically labelled reference standards (ESI Fig. SI5†).

At this stage, our assay consisted of the spiking of 12-mer, 17-mer, and 33-mer isotopically labelled peptides into a human duodenal fluid sample, purification of the peptide fraction, IP of the peptides (both endogenous and isotopically labelled peptides) using an antibody capture with antibody 4, further sample clean-up and finally LC-PRM-MS analysis. Several parameters still needed to be optimized as well as some of the figure-of-merits of the assay to be determined (see ESI Fig. SI6†).

Quantification of gluten peptides with immunoprecipitation coupled to liquid chromatography tandem mass spectrometry

To optimise our method for quantification, we evaluated the amount of spiked isotopically labelled standards. We suspected that excessive amounts of heavy standards could potentially affect the efficiency of IP for the endogenous gluten peptides (see ESI Fig. SI6†).

We determined that quantification of the endogenous 33-mer gluten peptide in a pool of duodenal fluid samples (pool Y) was minimally affected in terms of the determined amount, but measurement precision decreased with increasing spiking (Fig. 3). In contrast, 17-mer and 12-mer peptides were strongly affected. Spiking of the isotopically labelled standards at 5 ng for 1 mg of duodenal proteins/peptides was suitable. As a matter of fact, we also questioned the reliability of the measurements of the 17-mer and 12-mer gluten peptides. Spiking amounts below 5 ng down to 0.05 ng were detrimental to the measurements as the isotopically labelled standards could not be detected efficiently anymore (data not shown).

To finalize the method development and test its applicability, three independent samples (*i.e.*, samples C–E), the pool of samples (pool Y) described above, and a negative control (pool Z; see the Experimental section and ESI Table SI1†) were analysed in triplicate using immuno-LC-PRM-MS. In addition, each prepared sample was measured in triplicate on two independent LC-MS/MS systems and the pool of samples on two different days.

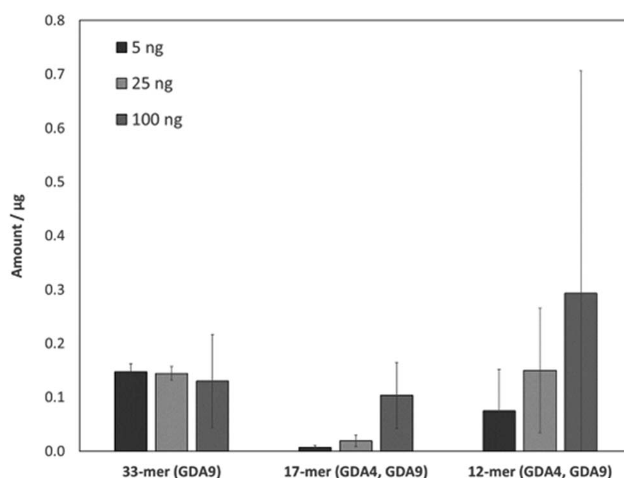

Fig. 3 Effect of isotopically labelled standard spiking on the determination of 33-mer, 17-mer and 12-mer gluten peptides. Isotopically labelled standards were spiked at 5 ng, 25 ng, and 100 ng in 1 mg proteins/peptides of a pool of duodenal fluid samples (pool Y). IP was used to enrich the peptides before LC-PRM-MS. Experiments were performed in triplicate.

 This journal is © The Royal Society of Chemistry 2024

View Article Online

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

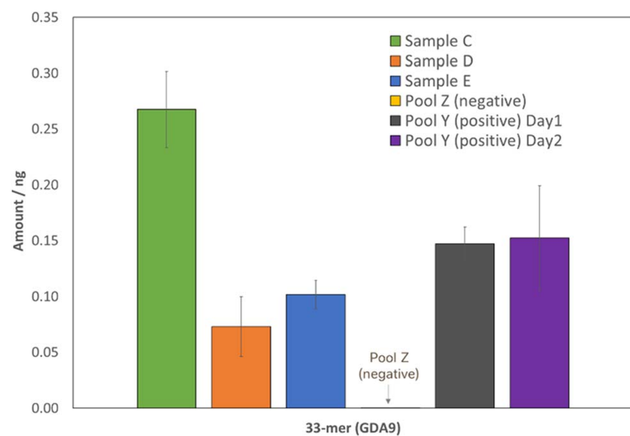

Fig. 4 Quantification of the 33-mer gluten peptide in various duodenal fluid samples using stable isotopic dilution and immuno-LC-PRM-MS. Experiments were performed in triplicate and each sample was measured in triplicate on the same LC-MS/MS system.

We confirmed that the detection level of 17-mer was too low for quantification in these samples (data not shown). While technical replication at the LC-PRM-MS level was adequate for 12-mer (coefficient of variation (CV) of 16.5%), the sample preparation reproducibility was insufficient (Table 3). As expected, 33-mer showed the absence of signals in the negative control sample (Fig. 4). The 33-mer gluten peptide displayed acceptable measurement reproducibility and precision (Fig. 4 and Table 3). The additional peptides that we included previously using a label-free quantification approach gave mixed results. For instance, peptide LQLQPFPQPQLPYPQPQL-PYPQPQLPYPQPQPFRPQQPYPQSQPQ reached sufficient performance to be considered for label-free quantification (Table 3).

In conclusion, our immuno-LC-PRM-MS assay reliably measured the 33-mer gluten peptide using stable isotopic dilution. Additionally, other peptides could be monitored using a label-free quantification that came at no extra cost.

Conclusions

We have developed a method capable of measuring 33-mer and other gluten peptides in human duodenal fluids using MS. Our immuno-LC-PRM-MS assay relies on the combination of peptide enrichment using specific antibody and peptide specific detection using sensitive high-resolution MS/MS (PRM acquisition mode).

During the development of this assay to measure gluten peptides, we faced certain limitations with the available technologies, which are worth mentioning. One challenge was the length of many measured gluten peptides, exceeding 20 amino acids. This posed difficulties in finding a suitable technology and supplier for synthesizing these peptides. Additionally, when designing the isotopically labelled standards, we opted to incorporate multiple isotopically labelled amino acids to prevent overlap with the isotopic distributions of the endogenous peptides. Consequently, the isotopic distribution patterns of the endogenous and isotopically labelled peptides could exhibit slight differences. For instance, the most intense isotope might differ. While LC-PRM-MS can easily target the most intense isotope for MS/MS analysis of such long peptides (where the most intense isotope is not the first isotope), conventional software tools seemed to be primarily designed to work with a monoisotopic peak as the most intense ion species. We observed that this had a controlled and systematic impact on quantification, but it likely compromised sensitivity. The last challenge we encountered was the significant hydrophobicity of the gluten peptides. In this regard, it may be worthwhile to evaluate alternative column phase materials.

Data availability

The data supporting this article have been included as part of the ESI.† The raw MS files can be made available upon request.

Author contributions

LD, PD and MA conceived and designed the study. JC, MA and PD provided the samples, reagents and their associated data. LD and ANG developed the methodology and performed the experiments. LD wrote the original draft of the manuscript. ANG, BO, FJT, PD, and MA reviewed and edited the manuscript. BO, FJT, and PD conceived, designed and supervised the clinical study.

Conflicts of interest

L. Dayon, A. Núñez Galindo, J. Chevalier, P. Duncan, and M. Affolter are employees of the Société des Produits Nestlé SA.

Acknowledgements

We thank Marjorie Guitard Uldry, Alexandre Panchaud and Gabriela Bergonzelli Degonda for their continuous support, encouragement, and very fruitful discussions.

Notes and references

1 J. A. Tye-Din, J. A. Stewart, J. A. Dromey, T. Beissbarth, D. A. Van Heel, A. Tatham, K. Henderson, S. I. Mannering, C. Gianfrani, D. P. Jewell, A. V. S. Hill, J. McCluskey, J. Rossjohn and R. P. Anderson, *Sci. Transl. Med.*, 2010, **2**, 41ra51.
2 M. Y. Hardy, A. Girardin, C. Pizzey, D. J. Cameron, K. A. Watson, S. Picascia, R. Auricchio, L. Greco, C. Gianfrani, N. L. La Gruta, R. P. Anderson and J. A. Tye-Din, *Gastroenterology*, 2015, **149**, 1541–1552.e1542.
3 J. L. McCarville, J. Dong, A. Caminero, M. Bermudez-Brito, J. Jury, J. A. Murray, S. Duboux, M. Steinmann, M. Delley, M. Tangyu, P. Langella, A. Mercenier, G. Bergonzelli and E. F. Verdu, *Appl. Environ. Microbiol.*, 2017, **83**, e01323.
4 I. S. Pultz, M. Hill, J. M. Vitanza, C. Wolf, L. Saaby, T. Liu, P. Winkle and D. A. Leffler, *Gastroenterology*, 2021, **161**, 81–93.e83.

Published on 03 September 2024. Downloaded on 9/16/2024 3:31:51 AM.

5 G. Mamone, M. C. Comelli, S. Vitale, L. Di Stasio, K. Kessler, I. Mottola, F. Siano, L. Cavaletti and C. Gianfrani, *Front. Nutr.*, 2022, **9**, 974771.
6 G. Wei, E. J. Helmerhorst, G. Darwish, G. Blumenkranz and D. Schuppan, *Nutrients*, 2020, **12**, 1–15.
7 H. C. van Den Broeck, J. H. G. Cordewener, M. A. Nessen, A. H. P. America and I. M. van der Meer, *J. Chromatogr. A*, 2015, **1391**, 60–71.
8 O. Ogilvie, N. Larsen, K. Sutton, L. Domigan, J. Gerrard, N. Demarais and S. Roberts, *MethodsX*, 2020, **7**, 101076.
9 K. Schalk, C. Lang, H. Wieser, P. Koehler and K. A. Scherf, *Sci. Rep.*, 2017, **7**, 45092.
10 M. J. Martínez-Esteso, J. Nørgaard, M. Brohée, R. Haraszi, A. Maquet and G. O'Connor, *J. Proteomics*, 2016, **147**, 156–168.
11 T. O. Alves, C. T. S. D'Almeida, K. A. Scherf and M. S. L. Ferreira, *Front. Plant Sci.*, 2019, **10**, 1470.
12 B. A. Palanski, N. Weng, L. Zhang, A. J. Hilmer, L. A. Fall, K. Swaminathan, B. Jabri, C. Sousa, N. Q. Fernandez-Becker, C. Khosla and J. E. Elias, *Nat. Commun.*, 2022, **13**, 888.
13 F. Boukid, B. Prandi, A. Faccini and S. Sforza, *J. Am. Soc. Mass Spectrom.*, 2019, **30**, 1481–1490.
14 O. Ogilvie, S. Roberts, K. Sutton, L. Domigan, N. Larsen, J. Gerrard and N. Demarais, *Food Chem.*, 2020, **333**, 127466.
15 M. R. Perez-Gregorio, R. Días, N. Mateus and V. De Freitas, *Food Funct.*, 2018, **9**, 1726–1735.
16 L. Vaquero, I. Comino, S. Vivas, L. Rodríguez-Martín, M. J. Giménez, J. Pastor, C. Sousa and F. Barro, *J. Sci. Food Agric.*, 2018, **98**, 2201–2209.
17 M. D. L. Moreno, Á. Cebolla, A. Munõz-Suano, C. Carrillo-Carrion, I. Comino, Á. Pizarro, F. León, A. Rodríguez-Herrera and C. Sousa, *Gut*, 2017, **66**, 250–257.
18 G. Mamone, L. Di Stasio, S. Vitale, S. Picascia and C. Gianfrani, *Front. Nutr.*, 2023, **9**, 1049623.
19 R. Vita, S. Mahajan, J. A. Overton, S. K. Dhanda, S. Martini, J. R. Cantrell, D. K. Wheeler, A. Sette and B. Peters, *Nucleic Acids Res.*, 2019, **47**, D339–D343.
20 L. M. Sollid, J. A. Tye-Din, S. W. Qiao, R. P. Anderson, C. Gianfrani and F. Koning, *Immunogenetics*, 2020, **72**, 85–88.
21 L. Dayon, A. Núñez Galindo, O. Cominetti, J. Corthésy and M. Kussmann, in *Methods in Molecular Biology*, Humana Press Inc., 2017, vol. 1619, pp. 433–449.
22 D. N. Perkins, D. J. C. Pappin, D. M. Creasy and J. S. Cottrell, *Electrophoresis*, 1999, **20**, 3551–3567.
23 A. I. Nesvizhskii, A. Keller, E. Kolker and R. Aebersold, *Anal. Chem.*, 2003, **75**, 4646–4658.
24 J. Campbell, T. Rezai, A. Prakash, B. Krastins, L. Dayon, M. Ward, S. Robinson and M. Lopez, *Proteomics*, 2011, **11**, 1148–1152.
25 S. Gutiérrez, J. Pérez-Andrés, H. Martínez-Blanco, M. A. Ferrero, L. Vaquero, S. Vivas, J. Casqueiro and L. B. Rodríguez-Aparicio, *Mol. Metab.*, 2017, **6**, 693–702.
26 L. Shan, S. W. Qiao, H. Arentz-Hansen, Ø. Molberg, G. M. Gray, L. M. Sollid and C. Khosla, *J. Proteome Res.*, 2005, **4**, 1732–1741.
27 N. Tian, G. Wei, E. J. Helmerhorst, D. Schuppan and D. Schuppan, *Am. J. Physiol.: Gastrointest. Liver Physiol.*, 2014, **307**, G769–G776.
28 A. Camarca, R. P. Anderson, G. Mamone, O. Fierro, A. Facchiano, S. Costantini, D. Zanzi, J. Sidney, S. Auricchio, A. Sette, R. Troncone and C. Gianfrani, *J. Immunol.*, 2009, **182**, 4158–4166.
29 C. Haller, P. Chaskar, J. Piccand, O. Cominetti, C. Macron, L. Dayon and M. R. C. Kraus, *Proteomics: Clin. Appl.*, 2018, **12**.
30 L. Di Stasio, S. Picascia, R. Auricchio, S. Vitale, L. Gazza, G. Picariello, C. Gianfrani and G. Mamone, *Front. Nutr.*, 2020, **7**.
31 C. Gianfrani, A. Camarca, G. Mazzarella, L. Di stasio, N. Giardullo, P. Ferranti, G. Picariello, V. Rotondi Aufiero, S. Picascia, R. Troncone, N. Pogna, S. Auricchio and G. Mamone, *Mol. Nutr. Food Res.*, 2015, **59**, 1844–1854.
32 C. D. L. Nguyen, S. Malchow, S. Reich, S. Steltgens, K. V. Shuvaev, S. Loroch, C. Lorenz, A. Sickmann, C. B. Knobbe-Thomsen, B. Tews, J. Medenbach and R. Ahrends, *Sci. Rep.*, 2019, **9**.
33 J. C. Gesquiere, E. Diesis, M. T. Cung and A. Tartar, *J. Chromatogr. A*, 1989, **478**, 121–129.

 This journal is © The Royal Society of Chemistry 2024