Journal of Chromatography B 1215 (2023) 123554

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

A chromatographic and immunoprofiling approach to optimising workflows for extraction of gluten proteins from flour

Matthew Daly [a], Xin Huang [a,b], Chiara Nitride [a,c], Olivier Tranquet [d,e], Adrian Rogers [f], Peter R. Shewry [g], Lee A. Gethings [a], E.N. Clare Mills [a,h,*]

[a] Manchester Institute of Biotechnology, Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK
[b] Department of Food and Nutrition, University of Helsinki, Agnes Sjöberginkatu 2, PL 66, FI-00014 Helsinki, Finland
[c] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy
[d] UR1268 BIA, Rue de la Géraudière, BP 71627, 44316 Nantes, France
[e] INRAE, Aix Marseille University, UMR1163 Biodiversité Et Biotechnologie Fongiques, (BBF), UMR1163, 13009 Marseille, France
[f] Bio-Check UK, St. Asaph, Wales, UK
[g] Rothamsted Research, Harpenden, UK
[h] School of Biosciences and Medicine, The University of Surrey, Guildford, UK

ARTICLE INFO

Keywords:
Gluten
Gluten-free
HPLC
SDS PAGE
Immunoblotitng

ABSTRACT

Ingestion of gluten proteins from wheat, and related prolamin proteins from barley, rye, and oats, can cause adverse reactions in individuals with coeliac disease and IgE-mediated allergies. As there is currently no cure for these conditions, patients must practice avoidance of gluten-containing foods. In order to support patients in making safe food choices, foods making a "gluten-free" claim must contain no more than 20 mg/Kg of gluten. Mass spectrometry methods have the potential to provide an alternative method for confirmatory analysis of gluten that is complementary to analysis currently undertaken by immunoassay. As part of the development of such methodology the effectiveness of two different extraction procedures was investigated using wholemeal wheat flour before and after defatting with water-saturated butan-1-ol. A single step extraction with 50 % (v/v) propan-2-ol containing 2 M urea and reducing agent (buffer 1) was compared with a two-step extraction using 60 % (v/v) aqueous ethanol (buffer 2) followed by re-extraction of the pellet using buffer 1, using either wheel mixing under ambient conditions (19 °C) or sonication at 60 °C. The procedures were compared based on total protein extraction efficiency and the composition of the extracts determined using a combination of HPLC, SDS-PAGE and immunoblotting with a panel of four gluten-specific monoclonal antibodies. Defatting generally had a detrimental effect on extraction efficiency and sonication at 60 °C only improved extraction efficiency with buffer 2. Although the single-step and two-step procedures were equally effective at extracting protein from the samples, analysis of extracts showed that the two-step method gave a more complete extraction of gluten proteins. Future studies will compare the effectiveness of these procedures when applied in the sample workflows for mass spectrometry based methods for determination of gluten in food.

1. Introduction

Coeliac disease affects approximately 1 % of the global population, a figure that has increased by a factor of 4–5 fold over the last 50 years, reflecting a true rise in incidence rather than increased awareness and detection [1,2]. Coeliac disease is a non-IgE, immune-mediated gastro-intestinal disorder which primarily affects those with either the Human Leukocyte Antigen (HLA) receptor DQ2 or –DQ8 alleles, although other

Abbreviations: ANOVA, One-way analysis of variance; AP, Alkaline phosphatase; ATIs, α-amylase/trypsin inhibitors; BSA, Bovine serum albumin; DTT, Dithiothreitol; ELISA, Enzyme-linked immunosorbent assay; HLA, Human Leukocyte Antigen; HMW, GS, High molecular weight glutenin subunits; IgE, Immunoglobulin E; IgG, Immunoglobulin G; LMW, GS, Low molecular weight glutenin subunit; Mr, Relative molecular mass; NBT/BCIP, Nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate; WDEIA, Wheat-dependent exercise-induced anaphylaxis; WTO, World Trade Organisation.

* Corresponding author.
E-mail address: clare.mills@manchester.ac.uk (E.N.C. Mills).

https://doi.org/10.1016/j.jchromb.2022.123554
Received 7 August 2022; Received in revised form 12 October 2022; Accepted 26 November 2022
Available online 10 December 2022
1570-0232/© 2022 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

HLA alleles have been implicated [3,4]. Symptoms of coeliac disease include increased risk of adenocarcinoma and lymphoma, and villous atrophy resulting in malabsorption and failure to thrive [5], although many can be asymptomatic in adulthood. Symptoms are elicited following ingestion of gluten, a proteinaceous fraction present in wheat, and related proteins from other cereal species. Gluten is therefore functionally defined as "a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 M NaCl." by both the Codex Standard [6] and the European Commission [7]. There is currently no cure for coeliac disease, and as such, individuals must practice strict avoidance of gluten-containing foods. Following the recommendation of the Codex Alimentarius Commission, many legislative bodies have enacted labelling laws specifying the amount of gluten that can be included in finished food products while maintaining a gluten-free claim [8]. Further, as the measures set out in the Codex Alimentarius were recognised by the World Trade Organisation (WTO) in the Agreement on Sanitary and Phytosanitary measures [9], any signatory of the WTO are "encouraged" to base their national measurements on this standard.

IgE-mediated allergy to wheat also exists although it is not as prevalent as coeliac disease [10]. This allergy can be categorised as either a food allergy caused by ingestion of wheat-containing foods, contact allergy to wheat, or an occupational asthma, often known as bakers' asthma, where individuals become allergic to wheat flour by inhalation but are usually tolerant to ingested wheat [11]. A well-defined subset of food allergic individuals only experience allergic reactions when wheat-containing foods are eaten in conjunction with exercise, a condition known as wheat-dependent exercise-induced anaphylaxis (WDEIA) [12]. People with IgE-mediated allergy to wheat are sensitised to gluten proteins but unlike individuals with coeliac disease, may also react to other flour proteins.

Although functionally described as gluten proteins, the gluten fraction is comprised primarily of seed storage proteins, which based on their solubility in different buffers can be subdivided into monomeric gliadins (classically named prolamins) and polymeric glutenins (glutelins). However, both of these fractions have been defined as prolamins based on their high contents of the amino acids proline and glutamine, their sequence similarities [13] and the fact that they are all soluble in alcohol-water mixtures. They also all carry coeliac toxic motifs, nine amino acid sequences that can elicit a reaction in patients with coeliac disease [14,15]. The prolamins account for up to 75–80 % of total flour protein in wheat, barley, and rye, and constitutes about 10 % of oat protein. Furthermore, the seed storage prolamins of wheat, unlike those from other cereal species, have the unique ability to form a visco-elastic gluten network which allows the production of leavened bread, a property not shared by prolamins from other cereals, and contributing to the importance of this crop. Indeed, bread consumption accounts for ~ 12 % of daily calorie intake of the UK population [16] but up to 50 % in parts of the Middle East, North Africa and Central Asia.

In order to comply with labelling laws, the amount of gluten in finished food products must be quantified to make a gluten-free claim. The Codex Alimentarius Commission definition of gluten-free foods follows a classical definition of prolamins as corresponding to wheat gliadins, based on their solubility in 40–70 % (v/v) aqueous ethanol, and makes the assumption that this fraction comprises half of the total gluten content [6]. This definition neglects the polymeric glutenins, which are insoluble unless reduced to release their component subunits but also carry coeliac toxic motifs [14]. The Codex recommendation also states that gluten must be determined in food and ingredients by use of an immunological method, or another method that provides at least equal sensitivity and specificity. One enzyme-linked immunosorbent assay (ELISA) which employs a monoclonal antibody (R5) raised against rye secalins [17] and is known as the R5 Mendez method [6] is mentioned specifically in the Codex Standard. Other ELISAs are also available that meet the performance requirements for gluten detection in foods as defined by the Codex Standard, including one based on a 33 residue coeliac-toxic peptide derived from α-gliadin, known as the G12 ELISA [18,19]. Both of these ELISA kits measure the gliadin content of a sample and so must use a conversion factor to arrive at a total gluten determination. Additionally, these antibodies vary in their reactivity to other cereals, such as barley and rye, limiting the accuracy of quantification of these cereals compared to wheat which can result in misrepresentation of the gluten content of a food product. These factors are exacerbated by the fact that the extraction buffer used in these assays can fail to extract all gliadins exhaustively, since some gliadin-like proteins form alcohol insoluble polymers [20]. Such methodological issues are compounded by the variability between cultivars and the effects environmental growing conditions on the gliadin content of gluten, which can range from 60 to 75 %, which can lead to overestimation of total gluten when applying a twofold conversion factor [21].

Therefore, the development of a method to determine both gliadins and glutenins should provide results that are more reliable and avoid the use of conversion factors. A prerequisite of such a method is the ability to extract both fractions, a subject of ongoing research [22–27]. Defatting prior to extraction has been shown to reduce sample complexity by removing interfering compounds such as polyphenols and may enhance protein extractability [28]. Buffers employing reducing agents (such as 2-mercaptoethanol, dithiothreitol and Tris(2-carboxyethyl)phosphine) and chaotropes such as guanidine hydrochloride [24,25] and urea [26,29] have been shown to improve the extractability of gliadins and glutenins, especially from thermally-processed foods. Indeed a combination of the guanidine-containing buffer known as "cocktail" followed by the addition of 80 % (v/v) ethanol has been used in combination with the R5 antibody, and has also been used to develop a next-generation ELISA which utilises four gluten monoclonal antibodies, including R5 [30]. This assay allows the quantification of the glutenin polymers and therefore overcomes the need for a factor to convert from gliadins to total gluten protein content. Comparison of the efficiency of different aqueous alcohol solutions to extract gluten proteins showed that 50 % (v/v) aqueous ethanol, propan-1-ol and propan-2-ol were equivalent but at 70 % (v/v), propan-1-ol may be the most effective solvent [27,31]. For routine analysis it is preferable to have a simple, single-step extraction that provides effective extraction of all gluten components. Therefore the composition and immunoreactivity of extracts prepared from a bread wheat cultivar using a reducing chaotropic buffer analogous to the Mendez cocktail buffer containing urea, dithiothreitol (DTT) and propan-2-ol [29] alone or as part of a sequential two-step extraction procedure in samples first extracted with 60 % (v/v) ethanol, a solvent consistent with those recommended in the Codex Standard. In this study, the efficiency of extraction and composition of extracts have been characterised using a combination of HPLC, and polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis to identify the optimal extraction conditions regarding both the composition and quantity of gluten proteins. The workflows chosen employ solvents compatible with MS analysis without the need for further treatments which are required when ionic detergents, such as SDS, are employed.

2. Materials and methods

2.1. Materials

All reagents used were analytical grade unless stated otherwise. *Triticum aestivum* cv Hereward grain was grown at Rothamsted Research (Harpenden, UK) and milled with a Bühler MLU-202 mill (Urzwil, Switzerland) at Campden BRI (Gloucestershire, UK) where an 81.4 % extraction of white flour from the grain was achieved. Formic acid, acetonitrile and water used in chromatography were all HPLC grade (Sigma-Aldrich, Dorset, UK). Urea, ethanol, propan-2-ol, *n*-butanol, Tris (hydroxymethyl) amino methane and dithiothrietol (DTT) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Reagents for gel electrophoresis were NuPAGE Bis-tris gels (4–12 %), NuPAGE lithium

dodecyl sulphate (LDS) buffer (4×, pH 8.4), 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (20 × concentrate), SYPRO™ Ruby Protein Gel Stain and Mark 12™ protein marker were from Invitrogen (Shropshire, UK). For immunoblotting, the pre-stained SeeBlue™ protein marker was used, also from Invitrogen (Shropshire, UK). Four anti-gluten monoclonal mouse antibodies were used (Supplementary Table 1) as follows; R5 anti-gliadin (Operon, Zaragoza, Spain) [17], G12 anti-33mer (gift of Adrian Rogers, Romer Labs, Runcorn, UK) [18], IFRN0610 anti-gliadin and –LMW glutenin [32], and ONT18A5 anti-ω5 gliadin [33]. The epitopes recognised by these antibodies are summarised in Supplementary Table 1. Goat anti-mouse IgG polyclonal antibody conjugated to alkaline phosphatase (AP) and 1-Step™ nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate (NBT/BCIP) Substrate Solution for immunoblotting were purchased from Thermo Fisher Scientific (Hertfordshire, UK).

2.2. Sample preparation

Briefly, Hereward flour was defatted by addition of water-saturated *n*-butanol (1:5, w:v, sample-to-buffer ratio) and stirred at ambient temperature (19 °C) for 3 h. The solvent was removed by filtering through Whatman™ qualitative filter paper (11 μm pore size), and the retained sample allowed to air dry for 1 h. The process was then repeated but extracting the sample for 18 h. Prolamin proteins were extracted using the method of Schalk, Lexhaller [22] with the following modifications: (1) albumins and globulins were not sequentially extracted with water and salt solution, respectively; (2) no repetitive extractions using the same extraction buffer were undertaken; and (3) the sample-to-buffer ratio was 1:20 (w:v). Two types of extraction were performed on 2 g lots of flour as follows:

(1) **Single step:** 50 % (v/v) propan-2-ol, 100 mM Tris-HCl, pH 7.5 containing 2 M urea and 60 mM DTT (Buffer 1; 40 mL).
(2) **Two-step:** Initial extraction with 60 % (v/v) aqueous ethanol (Buffer 2; 40 mL) followed by re-extraction of the resulting pellet with Buffer 1 (40 mL). The extracts from each step were analysed separately and not pooled.

Extractions were performed at either ambient temperature (19 °C) for 30 mins on a wheel mixer or at 60 °C with sonication for 15 mins and vortexing every 5 mins. Extracts were then clarified by centrifugation for 10 mins at 10,000 × *g*, the supernatants collected into fresh falcon tubes and stored at −20 °C (Supplementary Fig. 1).

2.3. Protein determination

Total flour protein was determined by measurement of total nitrogen using the Dumas combustion method with a Leco combustion analyzer (Leco Corp., St. Paul, MN, USA). A conversion factor of 5.7 was used from nitrogen to total protein [34]. The protein content of extracts were determined in duplicate using the RC DC™ Protein Assay (Bio-Rad, Watford, Hertfordshire, UK), a modified Lowry protein assay [35] which allows quantitation of protein in the presence of reducing agents and detergents. Briefly, proteins present in solution were precipitated and any soluble interfering substances were removed after centrifugation. After resuspension, alkaline copper tartrate solution and Folin reagent were added, and the absorbance recorded at 750 nm. Bovine serum albumin (BSA) was used as a standard [36] and although this may overestimate the prolamin content it allowed comparison of different extraction procedures.

2.4. Gel electrophoresis

Protein extracts were separated by SDS-PAGE [37]. Samples were mixed with NuPAGE LDS sample buffer (1:4 v/v) containing 50 mM DTT and heated for 10 mins at 80 °C to reduce any disulphide bonds present. Sample (5 μg protein/lane) and either the Mark 12™ protein ladder (5 μg protein/lane) or the SeeBlue™ prestained marker for immunoblots (10 μg/lane) were loaded on to a 4–12 % NuPAGE Bis-tris Mini protein gel and the proteins separated in MES buffer at 200 V, 350 mA and 100 W for 35 mins. After electrophoresis, gels were fixed with 50 % (v/v) methanol and 10 % (v/v) trichloroacetic acid for 30 min. Gels were subsequently transferred to clean containers, washed with 10 % (v/v) methanol and 7 % (v/v) trichloroacetic acid, rinsed with MilliQ water and stained with SYPRO™ Ruby Protein Gel Stain overnight in the dark with agitation. After destaining with MilliQ water, gels were imaged using a GE Healthcare Typhoon TRIO variable mode imager (GE Healthcare Lifesciences, Buckinghamshire, UK) with an excitation wavelength of 532 nm and emission wavelength of 605 nm.

2.5. Reversed phase high performance liquid chromatography RP-HPLC

Protein extracts were filtered (Merck Millipore Millex™ Nonsterile Syringe Filters 0.45 μm pore size, Fisher Scientific, Loughborough, UK) prior to reversed phase high performance liquid chromatography (RP-HPLC) analysis. Fifty microliters of extract (containing 1 mg mL^{-1} total protein) were loaded on to a Jupiter C18, 300 Å, 5 μm, 250 × 4.6 mm column attached to a Shimadzu Prominence HPLC system (Kyoto, Japan). The column was equilibrated in 0.1 % (v/v) formic acid in HPLC grade water (Buffer A), and a flow rate of 1 mL min^{-1} was used throughout. Proteins were eluted using a gradient of 0.1 % (v/v) formic acid in acetonitrile as buffer B. The elution gradient was as follows: 0 mins 0 % B, 0.5 mins 24 % B, 20 mins 56 % B, 20.1–24.1 mins 90 % B, 24.2–30 mins 0 % [22]. The absorbance of the eluate was monitored at 210 nm.

2.6. Immunoblotting

Electrophoretically separated proteins were transferred to nitrocellulose membrane (0.2 μm pore size, Bio-Rad, CA, USA) using a Bio-Rad Semi-Dry blotting apparatus (Bio-Rad, CA, USA). Parameters for the transfer were 15 V, 300 A, 100 W for 35 mins for two gels. Any free binding sites on the nitrocellulose membrane were blocked by incubation with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 0.05 % (v/v) Tween-20 and 5 % (w/v) skimmed milk powder (blocking buffer) overnight at 4 °C. After washing, membranes were incubated for 1 h at ambient temperature with primary anti-gluten antibodies with the following dilutions (v:v) in blocking buffer: R5 – 1:100, G12 – 1:1000, IFRN0610 – 1:100 and ONT18A5 – 1:500 [38]. After washing, membranes were incubated with secondary goat anti-mouse IgG antibody conjugated to AP (diluted 1:5000, v:v in blocking buffer) for 1 h at ambient temperature. After further washing, membranes were incubated with 1-Step™ NBT/BCIP Substrate Solution for 15 mins to allow for colour formation. The developed immunoblots were imaged using U:Genius 3 (Syngene, Cambridge).

2.7. Statistical analysis

GraphPad Prism version 7.04 for Windows (GraphPad Software, San Diego, California USA) was used for calculation of (1) extracted protein determined using the protein assay; (2) chromatogram peak areas; and (3) descriptive statistics. Significant differences between the amounts of extracted protein were calculated by multiple one-way analysis of variance (ANOVAs) where $p < 0.05$ was taken to be significant. Significant differences between the standard deviations of two extractions were calculated using the F test where $p < 0.05$ was determined to be significant.

3. Results

Improved extraction and determination of gluten by ELISA has been

reported using a proprietary buffer system known as the Mendez cocktail [30] based on a patented procedure [39] while other reports have used analogous buffers containing aqueous alcohol, a reducing agent and a chaotrope for fractionation of gluten and analysis of gluten in processed products [22,25]. This study has further adapted these approaches by including propan-2-ol and their effectiveness compared with 60 % (v/v) ethanol which is recommended in the Codex Standard.

3.1. Extraction efficiency

The effects of varying the extraction conditions on the amount of total protein extracted were initially compared (Fig. 1A, Table 1 and Supplementary Table 2). Defatting had no effect on the total protein content of the flour as determined by Dumas nitrogen analysis, with the total protein content being 12.7 % (w/w) for the defatted sample and 12.4 % (w/w) for the non-defatted sample. Since no replicate Dumas analysis was performed so no significance testing was undertaken. However, the difference is within the repeatability of the Dumas method for analysis of total protein nitrogen which is 0.41 % [40]. The different buffers and conditions used extracted between 16.6 % ± 3.6 (Step one of the two-step procedure performed at ambient temperature) and 85.5 % ± 6.8 (single step procedure performed at 60 °C) of the protein from the defatted samples when compared to the expected protein content based on Dumas analysis. In contrast, a greater amount of protein was extracted from the non-defatted sample, between 45.9 % ± 10.6 (step one of the two-step procedure performed at ambient temperature) to 125.3 % ± 26.8 (single step procedure performed at ambient temperature) being extracted. The difference between defatted and non-defatted samples was only statistically significant when using the single-step extraction at ambient temperature ($p = 0.0434$) (Fig. 1A, Table 1 and Supplementary Table 2) but the general trend was consistent with other findings [41,42].

Buffer 1 consistently extracted the greatest amount of protein under all the conditions tested compared to buffer 2 (corresponding to the Codex Standard extractant). Buffer 1 was therefore used to re-extract the pellet after extraction with buffer 2, recovering either a similar or greater amount of protein. The extraction conditions did not have statistically significant effects on protein extraction using either the single-step procedure or the individual steps in the two-step procedure. This lack of an effect was partially carried forward when summing the protein extracted in each step of the two-step extraction procedure. Defatting had no effect on the extraction efficiency except when the extraction was performed at 60 °C with sonication, which significantly increased extraction compared to wheel mixing at ambient temperature ($p = 0.007$).

3.2. Electrophoretic profiling of protein extracts

The protein profiles of the different extracts were then compared using SDS-PAGE (Fig. 1B). The extract prepared using the single-step extraction with buffer 1 under ambient conditions (Fig. 1B lanes 1–4) showed the presence of three high-molecular weight glutenin subunit (HMW-GS) bands with $M_r > 70{,}000$. These corresponded to the HMW-GS composition of cv Hereward, 1Dx3 + 1Dy12, 1Bx7 + 1By9, with subunits 1By9 and 1Dy12 co-migrating in the gel system used [43]. Other bands were present with M_r values of between ~ 38–60,000 with bands M_r ~ 38–50,000 probably corresponding to α- and γ-gliadins and LMW-GS that have similar electrophoretic mobilities. The two bands at M_r ~ 55,000 may correspond to ω1/2-gliadins. Bands corresponding to ω5-gliadin were not apparent, which may reflect their relative abundance and protein loading, as others have demonstrated their presence by SDS-PAGE in cv Hereward [44]. Defatting had little effect on the protein profile apart from improving the extractability of M_r ~ 10,000 polypeptides which probably correspond to α-amylase/trypsin inhibitors (ATIs) (Fig. 1B, lanes 3,4) that were not extracted from the non-defatted flour (Fig. 1B, lanes 1,2).

Extraction with buffer 2 in the first step of the two-step procedure at ambient temperature (Fig. 1B lanes 5–8) showed a protein profile

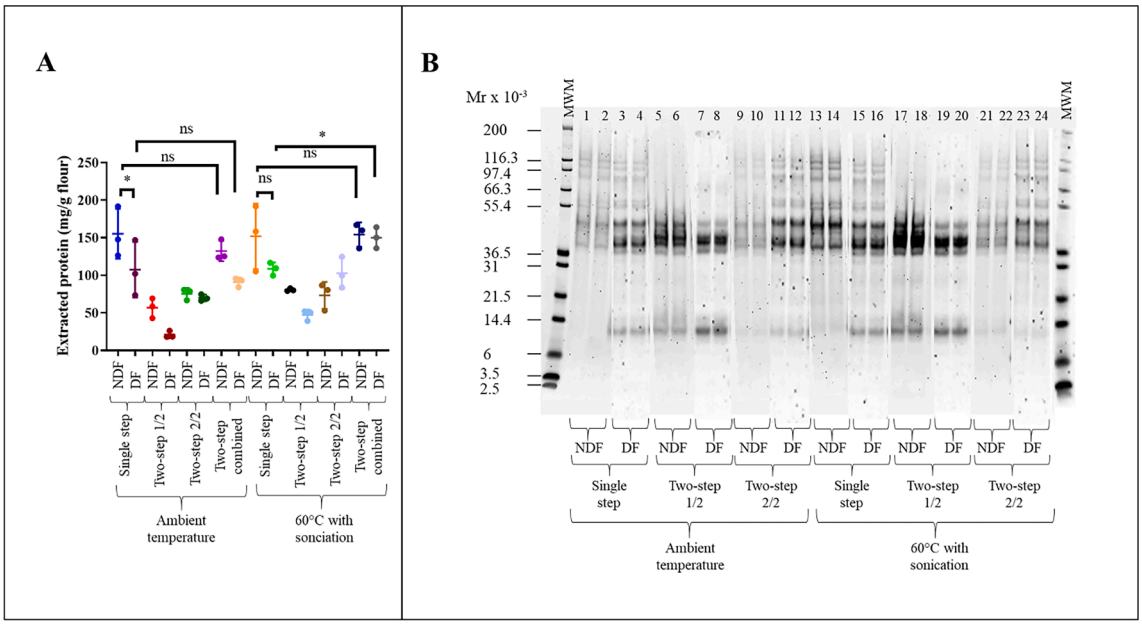

Fig. 1. Comparison of the efficiency of extraction using different conditions. (A) Total extracted protein from wheat flour under different extraction conditions. Error bars denote standard deviations of triplicate extracts analysed in duplicate. Significance was determined using multiple one-way ANOVAs. NDF indicated non-defatted sample, and DF indicates defatted sample. (B) Protein stained 1D-PAGE analysis of non-defatted and defatted wheat flour extracts prepared using different extraction conditions. Lanes 1–2, 5–6, 9–10, 13–14, 17–18, 21–22 are non-defatted samples (NDF), whereas lanes 3–4, 7–8, 11–12, 15–16, 19–20, 23–24 are defatted samples (DF). Samples in lanes 1 – 12 were extracted at ambient temperature, and those in lanes 13–24 were extracted at 60 °C with sonication. Ten microliters containing 5 μg protein was loaded per lane. Lanes are as follows: MWM - Mark12™ protein standards; 1–4, 13–16 – extracts prepared using a single step extraction with buffer 1; lanes 5–8, 17–20 – extracts prepared in step one of the two step extraction using buffer 2 ; lanes 9–12, 21–24 – extracts prepared in step two of the two step extraction using buffer 1.

Table 1
Efficiency of extraction of prolamins from wheat flour using different extraction conditions. Three sample replicates for each extract were prepared in triplicate and each extract analysed in duplicate. Dumas combustion was completed on a single sample (N × 5.7).

Extraction buffer	Extraction temperature	Extracted protein(mg)/g flour ± S.D. (n = 3)			
		Non-defatted		Defatted	
		Extracted protein (mg/g flour) ± S.D. (n = 3)	Recovery ± S.D. (n = 3) (based on Dumas)	Extracted protein (mg/g flour) ± S.D. (n = 3)	Recovery ± S.D. (n = 3) (based on Dumas)
		Single step extraction			
Buffer 1	Ambient temperature	155.4 ± 33.2	125.3 ± 26.8	107.6 ± 37	84.7 ± 29.1
	60 °C	151.8 ± 43.6	122.5 ± 35.1	108.6 ± 8.6	85.5 ± 6.8
		Two-step extraction			
Step one: Buffer 2	Ambient temperature	57 ± 13.2	45.9 ± 10.6	21 ± 4.6	16.6 ± 3.6
	60 °C	80.8 ± 1.8	65.2 ± 1.4	47.4 ± 7.2	37.3 ± 5.6
Step two: re-extraction of pellet with buffer 1	Ambient temperature	75.4 ± 7.6	60.9 ± 6.1	70.2 ± 4.4	55.2 ± 3.4
	60 °C	73.4 ± 18	59.2 ± 14.5	102.8 ± 20.6	80.9 ± 16.3
Combined extraction Step one + Step two	Ambient temperature	132.4 ± 13.6	106.8 ± 11	91.2 ± 2.50	71.8 ± 4.7
	60 °C	154.2 ± 16.4	124.4 ± 13.2	150.1 ± 14	118.2 ± 11

characteristic of the monomeric gliadins with bands of M_r ~ 40,000, 50,000 and 55,000. Given their known solubility in 60 % ethanol, these are likely α- and γ-gliadins [23,45], proteins of M_r ~ 10,000 also probably corresponding to ATIs and other similar low M_r proteins such as puroindolines [46]. However, there was no evidence of bands corresponding to the ω-gliadins which have a M_r of ~ 50–66,000, again probably reflecting differences in protein loading and relative abundance [22,47,48]. The profile of the fraction extracted from the pellet using buffer 1 in the two-step procedure at ambient temperature (Fig. 1B lanes 9–12) was very similar to that of fraction resulting from the single step procedure except there was evidence of faintly staining polypeptides of M_r ~ 10,000 in the non-defatted sample. These data support the suggestion based on extraction efficiency that the two-step procedure provides a more complete extraction of the gluten proteins.

In general, extracts prepared at 60 °C with sonication (Fig. 1B lanes 13–24) had qualitatively similar profiles to the extracts prepared under ambient conditions except they were stained more intensely. This may either be the result of more complete denaturation, or due to the extraction procedure at this temperature combined with sonication affecting protein reactivity in the protein assay. These data also suggest that buffers 1 and 2 are complementary and can extract different gluten proteins although the variability in the amounts of protein extracted make it difficult to draw any firm conclusions.

Since extraction efficiency was greater when using the non-defatted flour and resolution of the SDS-PAGE protein profiles of extracts prepared at 60 °C with sonication was sharper, these extracts were taken

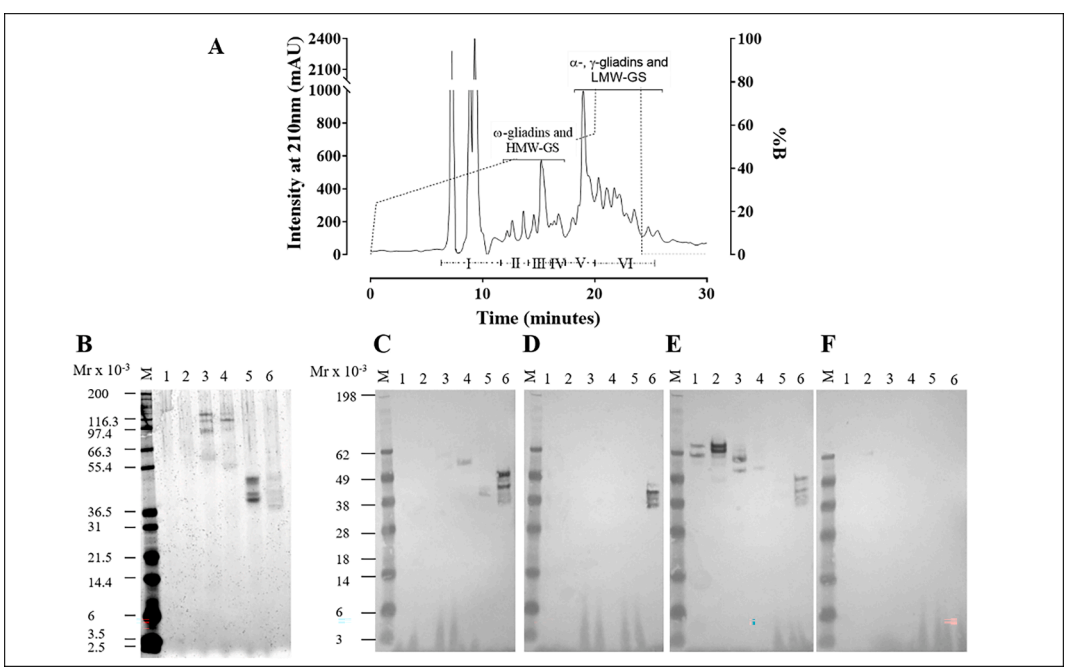

Fig. 2. Characterisation of an extract of non-defatted flour prepared using a single step extraction with buffer 1 performed at 60 °C with sonication (A) Reversed-phase HPLC chromatogram. Fractions collected corresponding to gliadin proteins are identified on the chromatograms by roman numerals. (B) Protein stained 1D-PAGE of RP-HPLC fractions stained. MWM indicates Mark12™ protein marker. Lanes were as follows: Lane 1 – fraction I (7 to 11 min); lane 2 – fraction II (11 to 13 min); lane 3 – fraction III (13 to 16 min); lane 4 – fraction IV (16 to 17 min); lane 5 – fraction V (17 to 20 min) and lane 6 – fraction VI (20 to 25 min). (C)-(F) Immunoblots were developed with the following antibody preparations: R5 (C), G12 (D), IFRN0610 (E) and ONT18A5 (F). MWM indicates SeeBlue™ pre-stained protein marker and lanes on immunoblots correspond to those in the protein stained gel presented in panel B.

forward for further analysis.

3.3. Reversed phase high performance liquid chromatography profiling of protein extracts

3.3.1. Analysis of buffer 1 extract (single step extraction procedure)

Gliadin and glutenin groups were not resolved by RP-HPLC in the buffer 1 extract, as reported by others [49]. Therefore, fractions representing five groups of RP-HPLC peaks were collected and further characterised using SDS-PAGE (Fig. 2B) and immunoblotting with four different anti-gluten antibody preparations (Fig. 2C-D) the specificity of which is summarised in Supplementary Table 1.

Fraction I comprised material eluting at 7 – 11 mins. Although the fraction contained a peak showing high absorbance (~2400 mAU) at 210 nm, only limited staining was observed at M_r > 115,000, (lane 1, Fig. 2B) suggesting this fraction largely comprises non-proteinaceous UV absorbing material, possibly phenolic acids [50]. Only one antibody, IFRN0610, reacted with the fraction, binding to two proteins of M_r ~ 60–65,000 which did not stain well with SYPRO™ ruby (Lane 1, Fig. 2E). These may correspond to ω-gliadins identified previously by others, who also found they did not stain well with SYPRO™ ruby [51] and is consistent with the known reactivity of IFRN0610 with both fast (ω1-2) and slow (ω5) gliadins, notably the sequence LQSPQQSFHQQ found in ω1-gliadins [32]. Their mobility is different to that observed by some and may be due to the anomalous mobility on SDS-PAGE of these proteins [22,33,44,46]. The second peak fraction II comprised three distinct peaks eluting at 11–13 mins and, like fraction I, showed limited staining following separation by SDS-PAGE (only diffuse staining at M_r ~ 66,000) (Lane 2, Fig. 2B). However, immunoblotting with antibody IFRN0610 (Lane 2, Fig. 2E) showed two intensely staining bands of M_r ~ 62 and 65,000, probably also ω-gliadins, together with several faintly staining polypeptides of lower M_r in this fraction. Blotting with the ω5-gliadin specific antibody ONT18A5 also showed a faintly stained band of M_r ~ 62,000 indicating that a form of ω5-gliadin was present in this fraction (lane 2 of Fig. 2F). The third fraction (fraction III) eluted at 13–16 mins and comprised one large, and one small peak. SDS-PAGE analysis showed the presence of well-resolved protein-staining bands of M_r 97 – 120,000 corresponding to HMW-GS, together with faintly staining of bands of M_r ~ 60,000, again consistent with the mobility of ω-gliadins (lane 3, Fig. 2B). Immunoblotting of this fraction showed binding of R5 as illustrated by indistinct staining around M_r 60,000 (lane 3, Fig. 2C), and by IFRN0160, as illustrated by two well-resolved bands at M_r ~ 50 and 60,000 (lane 3, Fig. 2E). This is consistent with the expected molecular weight of ω-gliadins, which are also recognised by IFRN0160 [32]. These data indicate that fractions I, II and III largely comprise ω-type gliadins and HMW-GS, which is consistent with their previously reported chromatographic behaviour (Supplementary Table 3) [22,49].

The remaining peaks eluted between 16 and 25 mins (Supplementary Table 3) [22,49] and are likely to correspond to α- and γ-gliadins, and LMW-GS. The first of these fractions (fraction IV) contained several small poorly resolved peaks eluting between 16 and 17 mins, SDS-PAGE showed a similar protein profile to fraction III with three bands of M_r ~ 120–77,000 but also an additional band of M_r 55,000 (lane 4, Fig. 2B) which was recognised by the R5 antibody (lane 4, Fig. 2C). The fraction also contained a band of M_r 52,000 which reacted weakly with IFRN0610 (lane 4, Fig. 2E). Fraction V comprised material eluting between 17 and 20 mins (one small and one very large peak) whilst fraction VI comprised material which eluted between 20 and 25 mins (several defined peaks of decreasing height). SDS-PAGE showed that these fractions comprised a complex mixture of bands of M_r ~ 35 – 50,000 consistent with their being a mixture of α- and γ-gliadins and LMW-GS (lanes 5, 6 Fig. 2B). Only the R5 antibody reacted with fraction V, weakly recognising a M_r ~ 40,000 band (Fig. 2C, lane 5). Such poor immunorecognition of the prominently staining protein bands indicates that either the gliadin species recognised by the antibodies are present at low levels or the epitopes have been denatured or modified by the extraction procedure. In contrast, the antibodies R5, G12 and IFRN0610 all recognised several bands in fraction VI (Lanes 6 in Fig. 2C, D and E respectively), R5 and IFRN0610 binding strongly to two bands at M_r ~ 45 and 50,000 whilst G12 bound to slightly smaller polypeptides of ~ M_r 35 – 40,000. These binding patterns are consistent with these fractions comprising α- and γ-gliadins, and LMW-GS.

3.3.2. Analysis of buffer 2 extract (two-step extraction procedure)

RP-HPLC analysis of wheat flour extracted with 60 % (v/v) ethanol (buffer 2) showed a similar pattern to those reported previously for gliadins (Fig. 3A) [22,52,53]. Weakly-bound material eluting at ~ 3.5–7 mins formed a broad, poorly resolved series of peaks which were pooled to give fraction I. SDS-PAGE analysis of this fraction showed it comprised a range of bands of M_r ~ 10,000 – 90,000, with intense staining at ~ M_r 40,000 (lane 1, Fig. 3B). This fraction also contained components of M_r 10,000 which are likely to be ATIs (which are not recognised by the antibodies used), and bands of M_r 40–90,000 which reacted with antibodies R5, G12 and IFRN0610 on immunoblotting (Lane 1 of Fig. 3C, D and E respectively). Thus, R5 showed faint staining of bands at M_r ~ 60,000 and ~ 40,000 whereas the G12 antibody demonstrated strong staining to a M_r ~ 40,000 band likely to correspond to α- and/or γ-gliadins based on their solubility in 60 % ethanol and known M_r on SDS-PAGE. The IFRN0610 antibody also showed strong staining of bands of M_r 55–65,000 corresponding to ω-type gliadins together with M_r 40–50,000 bands likely to correspond to α- and/or γ-gliadins. ONT18A5 also bound, but only very faintly, to a band of M_r ~ 65,000 (Fig. 3F lane 1). Fractions II and III represented minor peaks eluting at 9 and 10.5 mins and did not contain any protein-staining material on SDS-PAGE (Fig. 3B, lanes 2 and 3). However, the IFRN0160 antibody bound to proteins present in separations of these fractions, notably a M_r 60,000 band in fraction II and a M_r ~ 65,000 band in fraction 3, which are likely to correspond to different ω-type gliadin polypeptides. The failure of the ONT18A5 antibody to bind to these components in the extract maybe due to their being present at low concentrations and/or the ω-type gliadins were not all extracted by 60 % (v/v) ethanol.

Although eluting as a small broad peak, fraction IV was found to comprise a poorly-resolved series of components ranging from M_r 30 to 115,000, indicating either possible protein aggregation or partial degradation of proteins (Fig. 3B, lane 4). The presence of bands at M_r ~ 100,000 suggested that either some HMW-GS were extracted using 60 % (v/v) ethanol or the ω-type gliadin polypeptides have become aggregated, purified ω-type gliadin forming SDS-resistant aggregates of M_r ~ 100,000 (Tranquet, unpublished observations) and following dough mixing [54]. The complexity of the protein profile was not reflected in antibody binding, with only IFRN0610 showing binding (Fig. 3E) to one band at M_r ~ 60,000 which is likely to correspond to a ω-type gliadin.

The remaining components were fractioned into a broad, moderate intensity group of peaks eluting at ~ 13 mins (collected as fraction IV), one high intensity well resolved peak eluting at around 17 mins (fraction VI) and three further peaks eluting at between 19.5 and 23.5 mins which were pooled as fraction VII. Fraction V (Fig. 3B, lane 5) contained bands with M_r ~ 30–66,000, including a clearly resolved band at M_r ~ 40,000 and poorly resolved bands of M_r ~ 36–66,000. Antibodies R5, G12 and IFRN0610 (Fig. 3C, D and E respectively) bound to components in this fraction with the R5 antibody showing weak binding to a range of bands with M_r between 40 and 55,000, whilst G12 and IFRN0610 showed strong binding to a M_r ~ 40,000 polypeptide. IFRN0610 also bound to a M_r ~ 50 and 60,000 bands likely to correspond to ω-gliadins. These data are consistent with fraction V comprising mainly α- and γ-gliadins.

SDS-PAGE of fraction VI (Lane 6, Fig. 3B) revealed strong staining of two bands of M_r ~ 40,000 and ~ 50,000 which may correspond to the components recognised by R5, which are likely to be α-gliadins, (Fig. 3C, lane 6). The G12 antibody showed similar reactivity to fraction V, binding to a band at M_r ~ 40,000 (Fig. 3D, lane 6) whilst IFRN0610

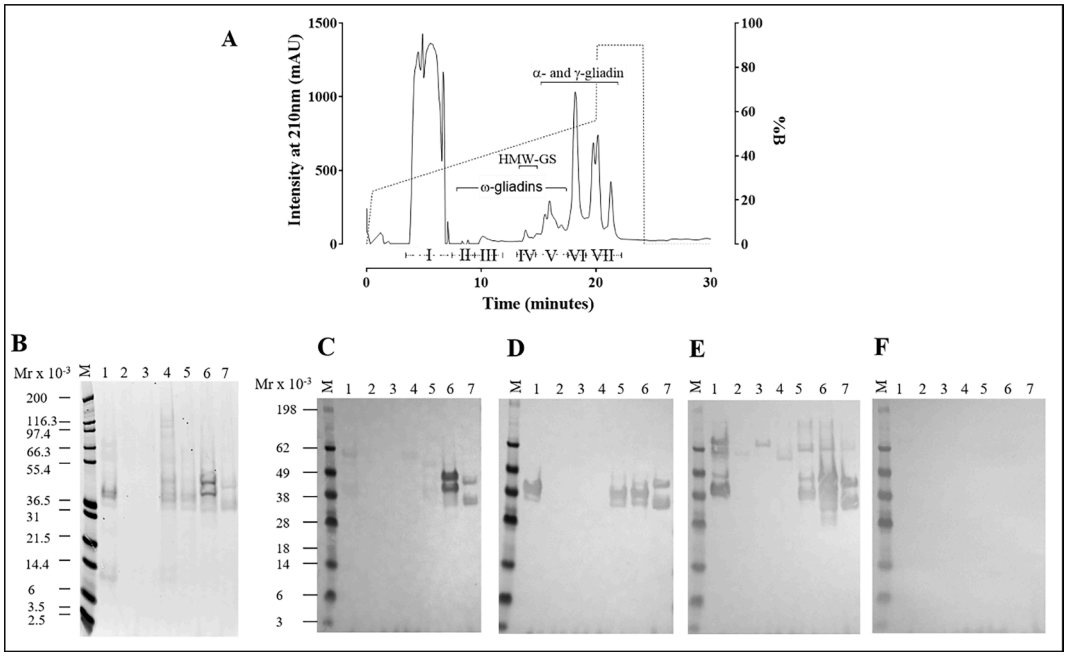

Fig. 3. Characterisation of an extract of non-defatted flour prepared in step one of the two step extraction procedure using buffer 2 performed at 60 °C with sonication (A) Reversed-phase HPLC chromatogram. Fractions collected corresponding to gliadin proteins are identified on the chromatograms by roman numerals. (B) Protein stained 1D-PAGE of RP-HPLC fractions stained. MWM indicates Mark12™ protein marker. Lanes were as follows: Lane 1 – fraction I (3.5 to 7 min); lane 2 – fraction II (7 to 9.5 min); lane 3 – fraction III (9.5 to 11.5 min); lane 4 – fraction IV (14 to 15 min); lane 5 – fraction V (15 to 17.5 min); lane 6 – fraction V (17.5 to 19 min); and lane 7 – fraction VI (19 to 22 min). (C)-(F). Immunoblots were developed with the following antibody preparations R5 (C), G12 (D), IFRN0610 (E) and ONT18A5 (F). MWM indicates SeeBlue™ pre-stained protein marker and lanes on immunoblots correspond to those in the protein stained gel presented in panel B.

demonstrated intense binding to M_r ~ 30 and 50,000 polypeptides accompanied by weaker binding to a M_r 60,000 (Fig. 3E, lane 6). SDS-PAGE of fraction VII showed differences in composition to fraction IV with staining of two bands at M_r ~ 30 and ~ 45,000 which may correspond to γ-gliadins (Fig. 3B, lane 7). All three antibodies showed a similar pattern of binding to this fraction recognising bands at Mr ~ 36 and 45 kDa, with IFRN0610 also binding to a M_r ~ 60 kDa polypeptide, indicating the presence of ω-gliadin in this fraction (Fig. 3 C-E, lane 7).

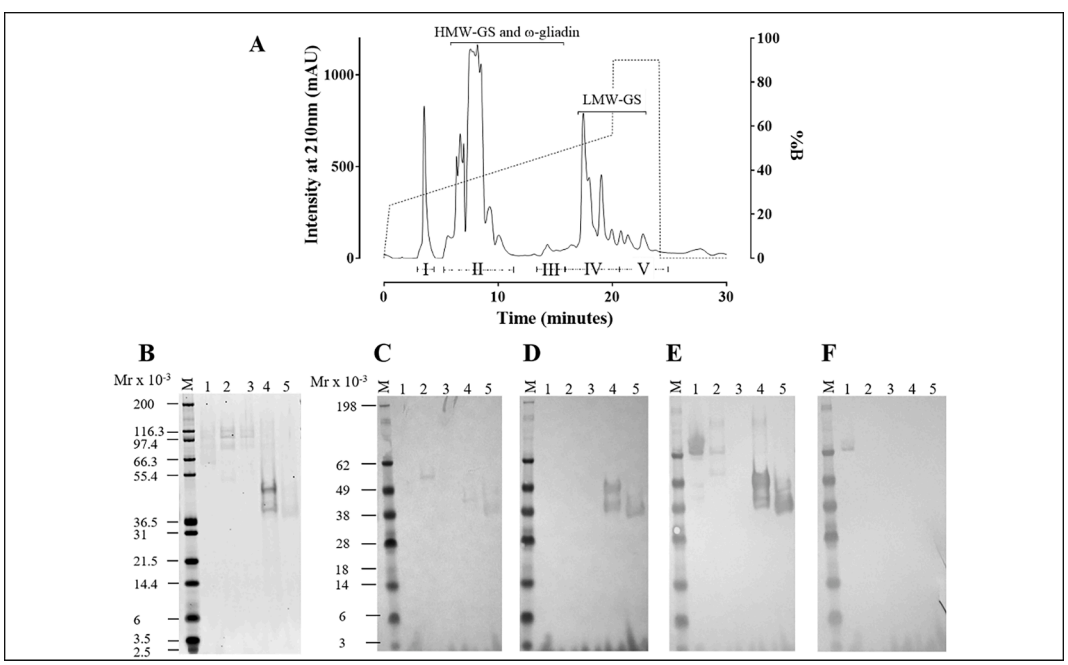

Fig. 4. Characterisation of an extract of non-defatted flour prepared in step two of the two step extraction with buffer 1 performed at 60 °C with sonication (A) Reversed-phase HPLC chromatogram. Fractions collected corresponding to gliadin proteins are identified on the chromatograms by roman numerals. (B) Protein stained 1D-PAGE of RP-HPLC fractions. MWM indicates Mark12™ protein marker. Lanes were as follows: lane 1 – fraction I (2 to 4 min); lane 2 – fraction II (5 to 11 min); lane 3 – fraction III (13 to 16 min); lane 4 – fraction IV (16 to 20.5 min); and lane 5 – fraction V (20.5 to 24 min). (C)-(F) Immunoblots developed with the following antibody preparations R5 (C), G12 (D), IFRN0610 (E) and ONT18A5 (F). MWM indicates SeeBlue™ pre-stained protein marker and lanes on immunoblots correspond to those in the protein stained gel presented in panel B.

3.3.3. Analysis of buffer one extract (two-step extraction procedure)

Re-extraction of the pellet remaining after extraction with 60 % ethanol (buffer 2) with buffer 1 (50 % propan-2-ol (v/v), 100 mM Tris-HCl, pH 7.5 containing 2 M urea and 60 mM DTT) extracted a mixture of components. These were separated by RP-HPLC (Fig. 4A), showing a profile consistent with that reported for the glutenin fraction of wheat [22,43,52]. Peaks eluting at 2–4 mins and 5–11 mins, respectively were collected as fractions I and II which were found to comprise a complex mixture of M_r of 66 – 115,000 components indicating the presence of ω-type gliadins and HMW-GS, the smeary in appearance of the gel track suggesting this was accompanied by protein modification or aggregation (Fig. 4B, lane 1). Immunoblotting showed that only antibodies IFRN0610 and ONT18A5 bound this fraction (Fig. 4E lane 1) recognising bands of $M_r \sim 65$ to 100,000 consistent with the presence of ω5-gliadins. Fraction II contained bands with masses consistent with those of HMW-GS but also components ~ 55 kDa, which may have been ω1, 2-gliadins (Fig. 4B lane 2). The incorporation of mutant forms of ω-gliadins with single cysteine residues (known as D-type LMW subunits of glutenin) into the glutenin polymer is well known [13] and therefore the presence of these proteins in this extract was to be expected. Both R5 and IFRN0610 antibodies (Lane 2, Fig. 4C and E, respectively) showed binding to bands of $M_r \sim 55{,}000$ in this fraction, which may have been ω1,2-gliadins, whilst the IFRN0610 antibody showed further binding to components at $M_r \sim 66{,}000$ consistent with ω5-gliadin, and bands corresponding to aggregated proteins $M_r > 115{,}000$.

Fraction III corresponded to a small peak which eluted at 13–16 mins whilst fraction IV corresponded to a series of peaks eluting between 16 and 24 mins, which were putatively identified as HMW-GS and LMW-GS respectively, based on their elution times (Supplementary Table 3) [22]. SDS-PAGE analysis confirmed that Fraction III comprises only bands of $M_r > 100{,}000$, none of which were recognised by any of the antibodies, suggesting these proteins corresponded to HMW-GS. Fraction IV contained proteins eluting between 16 and 20.5 mins and demonstrated intense staining of two bands at $M_r \sim 40{,}000$ and 50,000 (Fig. 4B, lane 4) which is consistent with their being B-type LMW-GS [22]. This pattern of staining was consistent with the specificities observed for the G12 and IFRN0610 antibodies, although immuno-staining was more intense for IFRN0610, the R5 antibody only showing only very weak binding to a band at Mr ~ 45 kDa (Lane 4, Fig. 4C, D and E, respectively). Fraction V comprised proteins eluting between 20.5 and 24 mins. SDS-PAGE showed only faint, weakly staining and poorly resolved bands (lane 5, Fig. 4B), of a M_r 36 and 40,000 which is slighter lower than those present in lane 4. These may correspond to C-type LMW-GS, which are essentially mutant forms of α- and γ-gliadins. All three antibodies showed similar binding to a band at ~ 40 kDa with R5 showing the weakest binding, followed by G12 with IFRN0610 showing the most intense binding (Lane Fig. 4C, D and E, respectively).

4. Discussion

In this study, we demonstrated that defatting of flour samples with water-saturated butan-1-ol prior to extraction significantly reduced the total extracted protein, as reported by others using either gel-based [55] or mass spectrometry approaches for protein identification [26]. Such effects have been ascribed to the loss of more hydrophobic natgliadins and glutenins through interactions with lipid components including glycolipids [41,42]. It is of note that buffer 1 was only able to extract components of $M_r \sim 12{,}000$ likely to correspond to ATIs in the non-defatted samples indicating these were either removed in the defatting process or rendered intractable to the extraction procedures used in this study. It was also observed that 60 % (v/v) aqueous ethanol, when used alone, had a similar extraction efficiency to that previously reported when extraction was performed under ambient conditions [24]. Interestingly when followed by re-extraction of the pellet with buffer 1, the combined two-step extraction procedure was as effective in extracting protein as the single step extraction with buffer 1. Both procedures extracted more protein than has been reported for buffers employing guanidine hydrochloride as a chaotrope instead of urea [24]. A large variance between replicate extractions was observed for some procedures, likely due to the formation of a dough on addition of the extractant which made it difficult for the solvent to penetrate and solvate the protein. The data presented in this report suggest that methods that base their measurement of total gluten on 60 % (v/v) ethanol extracts, especially when prepared under ambient conditions, could under-represent the actual gluten present in a sample.

Qualitative analysis of the extracts by RP-HPLC, SDS-PAGE and immunoblotting using four anti-gluten antibodies which together recognised all types of gluten proteins were extracted by both procedures, although there were small differences in gluten protein composition of the different extracts and was exemplified by the ω-type gliadins. Thus, although the wheat cv. Hereward has previously been shown to have at least five ω-type gliadins these were reported to have higher M_r values than were observed in this study [44]. It is clear that differences in extraction conditions make precise comparisons difficult, but it appears that buffer 1 used in the single step extraction procedure extracted at least five polypeptides that appeared to be ω-type gliadins. Consequently, it is likely that the lower M_r values observed in the current report may have resulted from the inclusion of urea but a lower concentration of reducing agent than in the previous report [43]. Variation in the electrophoretic mobility of gluten proteins is also well established [22,33,44,46].

5. Conclusion

Previous work has demonstrated that almost all gluten fractions contain coeliac toxic motifs, and therefore methods, such as those based on antibodies that measure only gliadins, cannot sufficiently quantify the risk posed by residual gluten in gluten-free foods [14,15]. The data presented here show that both the single step extraction with buffer 1 and the two-step extraction procedure can effectively extract a high proportion of wheat flour gluten and is superior to a single step extraction using 60 % (v/v) ethanol, one of the solvents recommended by Codex [6]. However, no single extraction procedure was able to completely extract all gluten proteins, an observation that needs to be taken into consideration when developing sample preparation procedures for all methods but particularly when quantifying gluten in foods. Compromises always have to be made over time and complexity for sample preparation against completeness of extraction. Use of the single step buffer extracted more protein and a more complete protein profile than an extraction employing 60 % ethanol while maintaining method simplicity, and therefore would be preferable for high throughput analysis. The two-step extraction procedure used in this study, although more time consuming, has the potential to provide a more robust sample extraction for gluten analysis, which can be applied to thermally processed products. For example, analysis of baked products such as bread using a 60 % (v/v) ethanol extraction under-estimates gluten content [24], demonstrating the need for a more effective extraction buffer which is able to disrupt and dissolve proteins which are denatured and aggregated during processing.

Although other solvents, such as propan-2-ol, and chaotropes, such as urea, pose greater restrictions with immunoassay based methods, alternative analytical methods, such as those based on mass spectrometry (MS), are more amenable to the use of solvents and chaotropes in sample preparation. The sample amounts and extraction volumes used in this study are large, taking account of the need to extract representative samples for food analysis but this can be scaled down to the small amounts and volumes depending on the application [56]. Furthermore, the application of MS to the analysis of gluten provides an opportunity to take a fresh, molecular approach by quantifying all relevant sub-groups of gluten proteins that contain coeliac toxic motifs. Future studies will apply the single and two-step sample workflows for analysis of gluten in food using MS which could provide a comprehensive, robust, and

complementary alternative to immunoassay analysis of total gluten in gluten-free products, ultimately providing improved protection for consumers relying on gluten–free diets for their health and well-being.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK CASE (Grant No: 2113362) awarded to MD. Rothamsted Research receives grant-aided support from BBSRC of the UK and the work forms part of the Designing Future Wheat strategic programme (BB/P016855/1).

CRediT authorship contribution statement

MD, XH, LG and ENCM were involved in conceptualization of the study. MD was responsible for the investigation (execution) of the study, writing the manuscript and visualisaiton (preparation of figures). ENCM was also involved in writing the manuscript and all authors were involved in writing (critical review and editing) of the published work (both pre- and postpublicaiton stages).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lee A Gethings is an employee of Waters Corporation, a vendor of analytical instruments who are the industrial sponsor of the BBSRC CASE to Matthew Daly. Adrian Rogers is an employee of an immunoassay test kit vendor, Bio-Check UK Ltd. Matthew Daly undertook this work whilst a BBSRC-funded postgraduate student and is now an employee of Waters Corporation. The other authors have no other known competing financial interests for the published work

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123554.

References

[1] M. Ribeiro, F.M. Nunes, We might have got it wrong: Modern wheat is not more toxic for celiac patients, Food Chem. 278 (2019) 820–822.
[2] P. Singh, et al., Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis, Clin. Gastroenterol. Hepatol. 16 (6) (2018) 823–836 e2.
[3] K. Karell, et al., Hla types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the european genetics cluster on celiac disease, Hum. Immunol. 64 (4) (2003) 469–477.
[4] L.M. Almeida, et al., Presence of DQ2.2 Associated with DQ2.5 Increases the Risk for Celiac Disease, Autoimmune Dis. 2016 (2016).
[5] M.N. Marsh, Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology **102**(1) (1992) 330-354.
[6] Codex Alimentarius Commission, *Foods for Special Dietary Use for Persons Intolerant to Gluten*, Rome, Italy, 2008.
[7] European Commission, *Commission Notice 2017/C 428/01 of 13.7.2017 relating to the provision of information on substances or products causing allergies or intolerances as listed in Annex II of Regulation (EU) No 1169/2011 on the provision of food information to consumers.*, E. Commission, Editor, 2017.
[8] L. Bucchini, M. Daly, E.N.C. Mills, *Chapter 7. Food Allergen Labelling Regulation*, in *Health Claims and Food Labelling*, Royal Society of Chemistry, 2019, p. 107-126.
[9] WTO, Agreement on the Application of Sanitary and Phytosanitary Measures, WTO, Editor, 1994.
[10] B.I. Nwaru, et al., Prevalence of common food allergies in Europe: a systematic review and meta-analysis, Allergy 69 (8) (2014) 992–1007.
[11] A. Sapone, et al., Spectrum of gluten-related disorders: consensus on new nomenclature and classification, BMC Med. 10 (2012) 13.
[12] K.A. Scherf, et al., Wheat-dependent exercise-induced anaphylaxis, Clin. Exp. Allergy 46 (1) (2016) 10–20.
[13] P.R. Shewry et al., *Wheat grain proteins*, in: K. Khan, P.R. Shewry (Eds.), *Wheat: Chemistry and Technology*, AACC International, Saint Paul, MN, USA, 2009, pp. 223-298.
[14] M. Daly, et al., *Mapping Coeliac Toxic Motifs in the Prolamin Seed Storage Proteins of Barley, Rye, and Oats Using a Curated Sequence Database*, Front. Nutrition 7 (2020).
[15] S.N. Bromilow, et al., A curated gluten protein sequence database to support development of proteomics methods for determination of gluten in gluten-free foods, J. Proteomics 163 (2017) 67–75.
[16] C. Roberts et al., *National Diet and Nutrition Survey: Results from Years 7-8 (combined) of the Rolling Programme (2014/15 to 2015/16)*, L. Public Health England, UK, 2018.
[17] I. Valdés, et al., Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol, Eur. J. Gastroenterol. Hepatol. 15 (5) (2003) 465–474.
[18] B. Morón, et al., Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide, Am. J. Clin. Nutr. 87 (2) (2008) 405–414.
[19] K.A. Scherf, et al., Recent Progress and Recommendations on Celiac Disease From the Working Group on Prolamin Analysis and Toxicity, Front. Nutr. 7 (2020) 29.
[20] P.R. Shewry, N.G. Halford, Cereal seed storage proteins: structures, properties and role in grain utilization, J. Exp. Bot. 53 (370) (2002) 947–958.
[21] H. Wieser, P. Koehler, Is the calculation of the gluten content by multiplying the prolamin content by a factor of 2 valid? Eur. Food Res. Technol. 229 (1) (2009) 9–13.
[22] K. Schalk, et al., Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials, PLoS One 12 (2) (2017).
[23] S.N. Bromilow, et al., Comprehensive Proteomic Profiling of Wheat Gluten Using a Combination of Data-Independent and Data-Dependent Acquisition, Front. Plant Sci. 7 (2017) 2020.
[24] B. Gessendorfer, H. Wieser, P. Koehler, Optimisation of a solvent for the complete extraction of prolamins from heated foods, J. Cereal Sci. 52 (2) (2010) 331–332.
[25] E. Garcia, et al., Development of a general procedure for complete extraction of gliadins for heat processed and unheated foods, Eur. J. Gastroenterol. Hepatol. 17 (5) (2005) 529–539.
[26] U. Bose, et al., Optimisation of protein extraction for in-depth profiling of the cereal grain proteome, J. Proteomics 197 (2019) 23–33.
[27] M. Byers, B.J. Miflin, S.J. Smith, A Quantitative Comparison of the Extraction of Protein-Fractions from Wheat-Grain by Different Solvents, and of the Polypeptide and Amino-Acid-Composition of the Alcohol-Soluble Proteins, J. Sci. Food Agric. 34 (5) (1983) 447–462.
[28] A. Manfredi, et al., Multiplex liquid chromatography-tandem mass spectrometry for the detection of wheat, oat, barley and rye prolamins towards the assessment of gluten-free product safety, Anal. Chim. Acta 895 (2015) 62–70.
[29] K. Schalk, et al., Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials, PLoS One 12 (2) (2017) e0172819.
[30] M. Lacorn et al., *Quantification of Wheat, Rye, and Barley Gluten in Oat and Oat Products by ELISA RIDASCREEN(R) Total Gluten: Collaborative Study, First Action 2018.15.* J. AOAC Int. **102**(5) (2019) 1535-1543.
[31] P. Kanerva, T. Sontag-Strohm, O. Brinck, Improved extraction of prolamins for gluten detection in processed foods, Agric. Food Sci. 20 (3) (2011) 206.
[32] G.M. Brett, et al., Epitope Mapping Studies of Broad Specificity Monoclonal Antibodies to Cereal Prolamins, J. Cereal Sci. 29 (2) (1999) 117–128.
[33] S.B. Altenbach, et al., Towards reducing the immunogenic potential of wheat flour: omega gliadins encoded by the D genome of hexaploid wheat may also harbor epitopes for the serious food allergy WDEIA, BMC Plant Biol. 18 (1) (2018).
[34] Food and Agriculture Organisation of the United Nations, *Food energy – methods of analysis and conversion factors*, Rome, 2002.
[35] O.H. Lowry, et al., Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1) (1951) 265–275.
[36] M.J. Martinez-Esteso, et al., Defining the wheat gluten peptide fingerprint via a discovery and targeted proteomics approach, J. Proteomics 147 (2016) 156–168.
[37] F. Smith, et al., Digestibility of gluten proteins is reduced by baking and enhanced by starch digestion, Mol. Nutr. Food Res. 59 (10) (2015) 2034–2043.
[38] F. Smith, *Patterns and Pathways of Proteolysis of Gluten Proteins in the Gastrointestinal Tract*, in: *Faculty of Biology, Medicine and Health*, University of Manchester, 2016, p. 269.
[39] M.E. Elena Lopez Villar, M.E. Mercedes Llorente Gomez, M.E. Enrique Mendez Corman, *Process to extract gluten from food products and kit for achieving same*, 2009.
[40] B. Beljkaš, et al., Rapid method for determination of protein content in cereals and oilseeds: validation, measurement uncertainty and comparison with the Kjeldahl method, Accred. Qual. Assur. 15 (10) (2010) 555–561.
[41] J.A. Bietz, et al., Gliadin analysis by reversed-phase high-performance liquid chromatography: optimization of extraction conditions, Cereal Chem. 2 (61) (1984) 124–129.
[42] T.H. McCann, et al., Protein–lipid interactions in gluten elucidated using acetic acid fractionation, Food Chem. 115 (1) (2009) 105–112.
[43] L. Gao, et al., Characterization and comparative analysis of wheat high molecular weight glutenin subunits by SDS-PAGE, RP-HPLC, HPCE, and MALDI-TOF-MS, J. Agric. Food Chem. 58 (5) (2010) 2777–2786.
[44] Y. Wan, et al., Effects of nitrogen nutrition on the synthesis and deposition of the omega-gliadins of wheat, Ann. Bot. 113 (4) (2014) 607–615.
[45] F.M. DuPont, et al., Sequential extraction and quantitative recovery of gliadins, glutenins, and other proteins from small samples of wheat flour, J. Agric. Food Chem. 53 (5) (2005) 1575–1584.

[46] F.M. Dupont, et al., Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry, Proteome Sci. 9 (2011) 10.
[47] S.B. Altenbach, et al., Elimination of Omega-1,2 Gliadins From Bread Wheat (Triticum aestivum) Flour: Effects on Immunogenic Potential and End-Use Quality, Front. Plant Sci. 10 (2019) 580.
[48] S.B. Altenbach, C.K. Tanaka, B.W. Seabourn, Silencing of omega-5 gliadins in transgenic wheat eliminates a major source of environmental variability and improves dough mixing properties of flour, BMC Plant Biol. 14 (2014) 393.
[49] Y. Qian, et al., Characterization of wheat gluten proteins by HPLC and MALDI TOF mass spectrometry, J. Am. Soc. Mass Spectrom. 19 (10) (2008) 1542–1550.
[50] A. Brandolini, et al., Phenolic acids composition, total polyphenols content and antioxidant activity of Triticum monococcum, Triticum turgidum and Triticum aestivum: A two-years evaluation, J. Cereal Sci. 58 (1) (2013) 123–131.
[51] H.C. van den Broeck, et al., Staining efficiency of specific proteins depends on the staining method: wheat gluten proteins, Proteomics 8 (9) (2008) 1880–1884.
[52] K.A. Scherf, Impact of the preparation procedure on gliadin, glutenin and gluten contents of wheat starches determined by RP-HPLC and ELISA, Eur. Food Res. Technol. 242 (11) (2016) 1837–1848.
[53] H. Wieser, S. Antes, W. Seilmeier, Quantitative Determination of Gluten Protein Types in Wheat Flour by Reversed-Phase High-Performance Liquid Chromatography, Cereal Chem. J. 75 (5) (1998) 644–650.
[54] S. Iwaki, et al., Changes in Hydrophobic Interactions among Gluten Proteins during Dough Formation, Processes 9 (7) (2021).
[55] M. Katyal, et al., Comparative analysis of native and defatted flour from hard, extraordinarily soft, and medium-hard wheat varieties for protein solvation, pasting, mixing, and dough rheological behavior, J. Food Sci. 85 (1) (2020) 65–76.
[56] X. Mao, et al., Assessment of homogeneity and minimum sample mass for cadmium analysis in powdered certified reference materials and real rice samples by solid sampling electrothermal vaporization atomic fluorescence spectrometry, J. Agric. Food Chem. 61 (4) (2013) 848–853.