Gluten Analysis

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Abstract Wheat, barley and rye are sources of gluten and diverse food products are made from the grains of these cereals. Despite some species-specific differences, the molecular properties of the gluten proteins show similar characteristics in forming a unique protein network that has been extensively described in terms of its subunits and composition, its function in bakery products and its implications for human health.

There are many reasons for analysing gluten to serve purposes as diverse as assessing flour quality, selecting and breeding suitable cereal varieties, identifying varieties, identifying the source of gluten in a product, and quantifying gluten in food and drink, especially to protect gluten intolerant consumers.

The level of gluten in food and drink deemed to be safe for people with coeliac disease or non-coeliac gluten sensitivity is set in legislations. Various systems are in place worldwide to regulate food labelling and various testing methods are used or are available to quantify gluten, but cross-border standardisation to harmonize the quantification of gluten in food products has yet to be agreed.

To analyse gluten, the proteins must be separated from other possibly interfering food components. As it is difficult to solubilize gluten, gluten extraction is a critical part of the process. The level of gluten, the type of food matrix and the available technology impose further limitations and challenges.

In this chapter, we describe a strategy to select the most suitable gluten analysis approach according to the purpose, sample type, gluten level and performance characteristics required. The advantages and disadvantages of qualitative and quantitative

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gluten analysis techniques, such as gel electrophoresis, immunoassays, asymmetric field flow fractionation multi-angle laser light scattering, chromatography and chromatography coupled methods are covered.

1 The Purpose of the Gluten Analysis Defines the Right Technique

Reasons to analyse gluten may be to comply with food labelling legislation, to ensure food safety, to assess food quality (protein composition and functionality) or to identify and track varieties in breeding programs. There are already several qualitative and quantitative methods to serve the spectrum of needs for gluten analysis (Table 1).

Table 1 Overview of the most frequent purposes of gluten analysis in cereals and foods. Gluten levels vary from high (>100 mg/kg) to low (<100 mg/kg). RP-HPLC, reverse-phase high-performance liquid chromatography; SE-HPLC, size exclusion HPLC; PCR, polymerase chain reaction; LC-MS, liquid chromatography mass spectrometry, AFFFF-MALLS, asymmetrical flow field flow fractionation multi-angle laser light scattering; HMW, high molecular weight; LMW, low molecular weight; ELISA, enzyme-linked immunosorbent assay

Purpose		Gluten level in sample	Methods
Qualitative			
Breeding or quality assessments	Breeding new varieties	High	Gel electrophoresis, RP-HPLC
	Variety identification based on gluten composition		
Food safety	Presence/absence of gluten source, gluten speciation for labelling e.g. 'contains wheat'	Any	Gel electrophoresis, LC-MS, PCR
Methodological	Sample preparation for LC-MS analysis		Gel electrophoresis
	Checking enzymatic digestion efficiency prior to LC-MS analysis		
Clinical	Testing the immune response to gluten in serums	Low	Western blot
Quantitative			
Breeding or quality assessments	Quantification of total gluten	High	ELISA
	Gliadin to glutenin ratio		SE-HPLC
	HMW to LMW glutenin ratio		RP-HPLC
	Unextractable polymeric protein		SE-HPLC
	Size and mass of polymers	Any	AFFFF-MALLS
	Quantification of gluten in non-gluten containing food		ELISA, LC-MS
Food safety and labelling	Testing for gluten when used as a processing aid	Any	ELISA, LC-MS
	Testing for gluten in non-food materials		ELISA, LC-MS
	Food labelling – "Gluten free" (<20 mg/kg) and "very low gluten" (21–100 mg/kg)	Low	ELISA, LC-MS
	Quantification of partly or fully hydrated gluten		Competitive ELISA, LC-MS
	Validation of production line cleaning (swabs)		ELISA

Some of the methods are routinely used, while some require optimisation for use with gluten proteins. The purpose of the gluten analysis is an essential aspect to consider when first selecting an appropriate testing method. Other major determinants are the sample type and the expected or estimated level of gluten proteins in the samples. The diversity of food matrices that need to be dealt with is in itself a challenge and requires specific consideration for food analytics. The extraction of the target analyte, the gluten proteins, is more critical when the aim is quantification. Often a method is effective for samples with high levels of gluten, but less so for samples with only trace levels.

2 The Importance of Sample Type in Gluten Analysis

The grain quality of wheat, barley, rye and their cross varieties is often tested in cereal science and breeding. In these genetic materials, the level of gluten is high and the protein composition is the main characteristic of interest. Food products may contain gluten or gluten-containing cereals that have been added intentionally or may contain gluten due to unintentional contamination of raw materials during processing or product handling. Risk assessments of possible sources of contamination can be a way of estimating the expected level of gluten and gluten source before testing (Table 2).

Table 2 Overview of food sample types most often tested for gluten

Sample type	Gluten level	Foods	References (examples)
Gluten-containing cereals and cereal-based products (not heated)	High	Grains, flour, whole meal and other milling products from wheat, spelt, kamut, barley and rye	Batey et al. 1991; Bönick et al. 2017; Bromilow et al. 2017a; Colgrave et al. 2015; Cornec et al. 1994; DuPont et al. 2005; García-Molina and Barro 2017; Han et al. 2015; Labuschagne and Aucamp 2004; Larroque et al. 2007; Lexhaller et al. 2017; Lookhart et al. 1986, 1995, 2003; Martínez-Esteso et al. 2016, 2017; Peña et al. 2004; Qian et al. 2008; Schalk et al. 2017a, 2017b, 2018; Manfredi et al. 2015; Altenbach et al. 2010; Aghagholizadeh et al. 2017; Singh et al. 1991; Tatham et al. 2000; Zilic et al. 2011; Tanner et al. 2016; Guo et al. 2016
Cereal-based products (heat processed)		Bakery products, bread, pasta, noodles	García et al. 2005; Khamis 2014
Hydrolysed gluten content		Fermented products (e.g. beer, soy sauce, vinegar)	Allred et al. 2014; Cao et al. 2017; Sajic et al. 2017; Li et al. 2018

(continued)

Table 2 (continued)

Sample type	Gluten level	Foods	References (examples)
Gluten-free or low-gluten foods	Low	Non-gluten cereals and pseudo cereals (e.g. oat, maize, rice, sorghum, buckwheat, quinoa, amaranth and chia)	Real et al. 2012
		Soy, legumes, pulses	Melini et al. 2017
		Spices	
		Non-gluten containing foods and drinks	Taylor et al. 2018
		Fermented non-gluten products (e.g. soy sauce, vinegar, gluten-free beer, malt extracts and processed oats)	Panda et al. 2015
Process validation		Cleaning validation samples (swabs) Non-intentional gluten containing foods and drinks (when gluten is used as a processing aid)	
Non-food materials	Any	Binding agents or fillers, cosmetics, medicines, pet foods, children's toys	Hlywiak 2008

3 Gluten Analysis Methods

Many authors have published test protocols for gluten identification and quantification. Some methods are widely used, but often require optimisation for particular situations. Exact protocols for immunoassays, enzyme-linked immunosorbent assay (ELISAs) and Western blotting are not discussed here, as the manufacturer's instructions must be followed for each reagent kit. An overview of commercially available gluten ELISA kits was published recently with their specifications (Melini and Melini 2018). Rapid methods and convenient formats developed by some of the main ELISA manufacturers such as dip sticks (Glutentox from Biomedal (Biomedal 2017; Bromilow et al. 2017b), Rida-quick from R-bio-pharm, Veratox R5 from Neogen, etc.) and handheld devices (Taylor et al. 2018) are also available but are not discussed here in detail. The principle behind these methods is usually an immune reaction and they are less sensitive than standard ELISAs because the LOD is higher, but the assays are much faster to do.

3.1 Chromatography and Coupled Techniques for Gluten Analysis

Molecular profiling using reversed-phase (RP) or size exclusion (SE) high performance liquid chromatography (HPLC) has been widely used since the 1990s not only in wheat quality characterisation (Lookhart et al. 1986, 1995; Batey et al. 1991) but also for other applications after optimising the protocols (Table 3).

There are no standard methods for gluten analysis using liquid chromatography (LC) and LC coupled with mass spectrometry (MS) techniques. LC-MS or LC-MS/MS is considered to be a powerful and highly sensitive proteomics technique that is in high demand for food testing required for gluten-free labelling (Haraszi et al. 2011). There are several LC-MS platforms that differ in the technologies used for ionisation (e.g. electrospray ionisation (ESI) or matrix assisted laser desorption ionization (MALDI), fragmentation (e.g. triplequadrupole, quadrupole time of flight (QTOF) or Orbitrap), detection (e.g. collision induced dissociation, higher-energy collisional dissociation), acquisition modes (e.g. data dependent analysis, data independent analysis, multiple reaction monitoring) and data analysis tools (vendor specific search engines, databases and other bioinformatics packages) (Table 4). There is a definite need to standardise the different data analysis platforms and several researchers advise using multiple platforms to ensure the comparability of results (e.g. Fiedler et al. 2014; Bromilow et al. 2017b; Martínez-Esteso et al. 2016).

Several instruments and a range of extraction and digestion methods are used to identify proteins by LC-MS. A general workflow (e.g. Juhász et al. 2015a; Martínez-Esteso et al. 2016) and a table of published wheat gluten markers (Table 4) are evidence of the feasibility of using LC-MS or LC-MS/MS techniques for gluten analysis but to date these methods are not used routinely. As well as the need for expertise, the costs of instrumentation and maintenance are still limiting factors. Gluten quantification using LC-MS/MS requires that a set of peptide markers can

Table 3 Applications for which high performance liquid chromatography (HPLC) methods are used for gluten analysis. SE, size exclusion; RP, reversed phase; MALLS, multi-angle laser light scattering; MALDI-TOF, matrix assisted laser desorption ionisation - time of flight; LC-MS, liquid chromatography mass spectrometry, ELISA, enzyme-linked immunosorbent assay

Purpose	Technique	References (examples)
Characterisation of gluten sub-fractions	SE-HPLC	Cornec et al. 1994
Prediction of wheat quality	SE-HPLC	Labuschagne and Aucamp 2004
Molecular weight distribution of gluten proteins	RP-HPLC coupled with MALLS	Larroque et al. 2007
Characterisation and identification of wheat gluten proteins	RP-HPLC coupled with MALDI-TOF	Qian et al. 2008
Variety identification	RP-HPLC	Han et al. 2015
Fractionation of gluten proteins for subsequent LC-MS or ELISA testing to characterise the immunogenic fractions	RP-HPLC	Martínez-Esteso et al. 2017; Scherf 2016; Schalk et al. 2017b
Gluten quantification for studying the effect of N fertilisation	RP-HPLC	García-Molina and Barro 2017

Table 4 Published sequences of wheat gluten peptides obtained from LC-MS/MS studies that may be used for identification or quantification. Peptides were obtained with trypsin, chymotrypsin or thermolysin

Protein	Peptide sequence	Equipment	Bioinformatics	References
Alpha-gliadin	ALQTLPAMCNVY	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	CQAIHNVVHAIIL	Thermo Scientific LTQ XL linear ion trap	Bioworks 3.3, Xcalibur	Manfredi et al. 2015
	DVVLQQHNIAHGR	Agilent 6530 LC-ESI-QTOF	Spectrum Mill	Liao et al. 2017
	DVVLQQPNIAHASSK	Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	FQPSQQNPQAQGF	Bruker HCT-Ultra PTM iontrap MS, Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
	LQLQPFPQPQLPY	Waters Synapt G1 nanoLC-ESI-QTOF & Xevo TQS	PLGS, Skyline	van den Broeck et al. 2015
		Waters nanoLC -Thermo Scientific LTQ XL	Mascot, Scaffold, Skyline, Xcalibur	Fiedler et al. 2014
	LQLQPFPQPQLPYPQPHLPYPQPQPF	Waters Synapt G1 nanoLC-ESI-QTOF & Xevo TQS	PLGS, Skyline	van den Broeck et al. 2015
	LQLQPFPQPQLPYPQPQLPYPQPQ LPYPQPQPF			
	LQLQPFPQPQLPYPQPQLPYPQPQPF			
	LQLQPFPQPQLPYPQPQPF	Waters Synapt G1 nanoLC-ESI-QTOF & Xevo TQS	PLGS, Skyline	van den Broeck et al. 2015
		Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
	LWQIPEQSR	Agilent 6530 LC-ESI-QTOF	Spectrum Mill	Liao et al. 2017
	NLALQTLPAMCNVYIPPYCTIVPFGIFGTN	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	QIPEQSR	Thermo Scientific LTQ XL linear ion trap	Bioworks 3.3, Xcalibur	Manfredi et al. 2015
	QQILQQQLIPCRDVVL	Waters Synapt G1 nanoLC-ESI-QTOF & Xevo TQS	PLGS, Skyline	van den Broeck et al. 2015
	QQQLIPCRDVVL			

	RPQQPYPQPQPQY	Waters Synapt G1 nanoLC-ESI-QTOF & Xevo TQS	PLGS, Skyline	van den Broeck et al. 2015
		Bruker HCT-Ultra PTM iontrap MS, Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
		Waters nanoLC - Thermo Scientific LTQ XL	Mascot, Scaffold, Skyline, Xcalibur	Fiedler et al. 2014
	RPQQPYPQPQPQYSQPQHPIS QQQQQQQQQQQEQQIL	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	RPQQPYPQSQPQY	Waters Synapt G1 nanoLC-ESI-QTOF, Xevo TQS	PLGS, Skyline	van den Broeck et al. 2015
	VSQQSYQLLQQLCCLQLWQTPEQSR	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
		Thermo Scientific nanoLC-ESI- Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
Avenin-LIKE	SAWEPQHPSSPEHQPTPQPQEHPVPHQK	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	TAWEPHHPSSPEQQPTPQPQEQPVPHQK			
Gamma-gliadin	LQCQAIHNVVHAIILHQQQK	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	NYLLQQCDPVSLVSSLVSMILPR			
	RPLFQLIQGQGIIRPQQPAQLEVIR			
	AFPQPQQTFPHQPQQQVPQPQQPQQPF	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	ALRTLPTMCNVY	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	ANIDAGIGGQ	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	APFASIVADIGGQ	Agilent 6530 LC-ESI-QTOF	Spectrum Mill	Liao et al. 2017
		Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010

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Table 4 (continued)

Protein	Peptide sequence	Equipment	Bioinformatics	References
	APFASIVAGIGGQ	Agilent 6530 LC-ESI-QTOF	Spectrum Mill	Liao et al. 2017
		No MS	No MS	Srinivasan et al. 2015
		Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	ASIVADIGGQ	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	ASIVAGIGGQ	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
				Schalk et al. 2018
	ASIVAGISGQ	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	ASIVASIGGQ			
	CQQPQQTIPQPHQTF			
	FHQPQQQFPQPQQPQQ			
	FHQPQQQFPQPQQPQQSFPQQQRP			
	FRQPQQPFY			
	FRQPQQPFYQQPQQTFPQPQQ			
	FYQQPQQTFPQPQQ			
	GIIQPQQPAQLEGIRSLVL			
	HQPQQQFPQPQQPQQSFPQ			
	HQPQQQFPQPQQPQQSFPQQQRPF			
	IIMQQEQRQG			
	IIMQQEQRQGVQ			
	IIQPQQPAQYE			
	IIQPQQPAQYEVIRS			
	ILLPLSQQQQL			
	ILLPLSQQQQLGQGTL			

	INVPYANIDAGIGGQ			
	IQILRPLFQ			
	IQPSLQQR			
	IQPSLQQRL			
	KAPFASIVADIGGQ			
	LAQIPRQ			
	LPLSQQQQVGQGSLVQGQGIIQ PQQPAQL	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	LQPHQIAQL	Thermo Scientific LTQ XL linear ion trap	Bioworks 3.3, Xcalibur	Manfredi et al. 2015
	LQPHQPF	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
		Thermo Scientific LTQ XL linear ion trap	Bioworks 3.3, Xcalibur	Manfredi et al. 2015
	LQPHQPFSQQPQQ	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	LQPQQPQQSFPQQQQPL	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
		Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
				Schalk et al. 2018
	LQPQQPQQSFPQQQQPLIQ	Applied Biosystems QSTAR Oulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	LQPQQPQQSFPQQQQPLIQL			
	LQPQQPQQSFPQQQQPLIQLSL			
	LQQPQQPFPQPQQQLPQPQQPQQ			
	LQQQCSPVAMPQR	Thermo Scientific LTQ XL linear ion trap	Bioworks 3.3, Xcalibur	Manfredi et al. 2015
	NFLLQQCNHVSLVSSLVSIILPR	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	NFLLQQCNPVSLVSSLISMILPR			

(continued)

Table 4 (continued)

Protein	Peptide sequence	Equipment	Bioinformatics	References
	NIQVDPSGQVQW	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
				Schalk et al. 2018
	NIQVDPSGQVQWLQQQLVPQLQQPL	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	PFIQPSLQQR			
	QGVQILVPL			
	QLAQLEAIR			
	QLVQGQGIIQPQQPAQY	Waters nanoLC -Thermo Scientific LTQ XL	Mascot, Scaffold, Skyline, Xcalibur	Fiedler et al. 2014
	QPFPQQPQQPYPQQPQQPFPQT QQPQQPFPQSK	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	QQCCQQLAR	Shimadzu nano HPLC, SCIEX 5600 TripleTOF MS	Protein Pilot 4.0 (SCIEX)	Li et al. 2018
	QQLPQPQQPQQSFPQQQR	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	QSFPQQQRPF			
	QSFPQQQRPFIQPSLQQR			
	RQPQQPF			
	SDCQNMQQQCCQQLAQIPR	Thermo Scientific nanoLC-ESI- Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	SDCQVMQQQCCQQLAQIPR	Agilent 6530 LC-ESI-QTOF	Spectrum Mill	Liao et al. 2017
				Simonato et al. 2011
	SFPQQQPPF	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	SIIMQQEQRQGVQIRRPL			
	SQQPQQAFPQPQQTFPHQPQQQVPQ PQQPQQPF			
	SQQQQLGQGTL			
	SQQQQLGQGTLVQGQGIIQPQQL			

	SQQQQLGQGTLVQGQGIIQPQQLAQL			
	SQQQQLGQGTLVQGQGIIQPQQ LAQLEAIRSL			
	SQQQQVGQGIL			
	SQQQQVGQGILVQGQGIIQPQQPAQL			
	SQQQQVGQGSL			
	SQQQQVGQGSLVQGQGIIQPQQPAQL			
	TQQPQQPFPQFQQPHQPF			
	VDPGYQVHWPQQQPFPQPQQP			
	VHWPQQQPFPQPQQP			
	VPPECSIIRAPF			
	VPPECSIMR	Thermo Scientific LTQ XL linear ion trap	Bioworks 3.3, Xcalibur	Manfredi et al. 2015
	VPPNCSTINVPY	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	VPPNCSTINVPYANIDAGIGGQ6			
	VQGQGIIQPQQL	Waters nanoLC -Thermo Scientific LTQ XL	Mascot, Scaffold, Skyline, Xcalibur	Fiedler et al. 2014
	VQGQGIIQPQQLAQLEAIRSL	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Mascot Daemon, Scaffold, Protein Prophet	Altenbach et al. 2010
	VQQQIPIVQPSVL	Thermo Scientific LTQ XL linear ion trap	Bioworks 3.3, Xcalibur	Manfredi et al. 2015
	VSPDCSTINAPF	Applied Biosystems QSTAR Pulsar i MS	X!Tandem, Pascot Paemon, Pcaffold, Protein Prophet	Altenbach et al. 2010
	VSPDCSTINAPFASIVVGIGGQ			
	VTILRPLFQ			
	VYVPPYCST			
	YQQPQQTFPQPQ			
	YQQPQQTFPQPQQ			
	YQQQQVGQGTLVQGQGIIQPQQPAQL			
Gliadin/ avenin-like	TTISPSSDVTTDMGGY	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016

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Table 4 (continued)

Protein	Peptide sequence	Equipment	Bioinformatics	References
HMW-GS	HVSVEHQAASL	Bruker HCT-Ultra PTM iontrap MS, Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2018
				Schalk et al. 2017a
	LQPGQGQQGY	Bruker HCT-Ultra PTM iontrap MS, Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2018
				Schalk et al. 2017a
	QGQGPQGKQGY	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	QQPGQGQHPEQGK	Shimadzu nano HPLC, SCIEX 5600 TripleTOF MS	Protein Pilot 4.0 (SCIEX)	Li et al. 2018
	QQPGQGQQPEQGQQPGQGQQGYYP TFPQQPGQGK	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	QQPGQGQQTR	Shimadzu nano HPLC, SCIEX 5600 TripleTOF MS	Protein Pilot 4.0 (SCIEX)	Li et al. 2018
	QVVDQQLAGR			
	TASLQQPGQGQQGHYPASL	Bruker HCT-Ultra PTM iontrap MS, Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2018
				Schalk et al. 2017a
	TTSLQQSGQGQQGY	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	VAKNQQLAAQLPAMCR			
HMW-GS Ax2	QQDQQSGQGQQPGQR	Shimadzu nano HPLC, SCIEX 5600 TripleTOF MS	Protein Pilot 4.0 (SCIEX)	Li et al. 2018
	QQPGQGQQLR			
	YYPTSPQQPGQEQQPR			
HMW-GS Bx14	DVSPGCRPITVSPGTR	Shimadzu nano HPLC, SCIEX 5600 TripleTOF MS	Protein Pilot 4.0 (SCIEX)	Li et al. 2018
HMW-GS Bx17	AQQLAAQLPAMCRLEGSDALSTR LEGSDALSTR	Thermo Scientific nanoLC-ESI-Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	DVSPGCRPITVSPGTR			
	QDQQPGQR			
	QQGYYPTSPQQPGQGQQLGQGQPG YYPTSQQPGQK			

	QQSGQGQQPGQGQQSGQGQQPGQG QQAYYPTSSQQSR			
	QYEQQPVVPSK			
	RYYPSVTSSQQGSYYPGQASPQQSGQG QQPGQEQQPGQGQ			
HMW-GS By15	CCQQLR	Thermo Scientific nanoLC-ESI- Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	ELQESSLEACRQVVDQQLAGRLPWST GLQMR			
	ELQESSLEQCR			
	LPWSTGLQMR			
	QLQCERELQESSLEACR			
	QVVDQQLAGR			
	VQQPATQLPIMCR			
HMW-GS Dx5	ACQQVMDQQLR	Thermo Scientific nanoLC-ESI- Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	AQQLAAQLPAMCR			
	DISPECHPVVVSPVAGQYEQQIVVPPK			
	ELQELQER			
	GGSFYPGETTPPQQLQQR			
	IFWGIPALLK	AB SCIEX QTRAP® 4500	MIDAS™ workflow	Lock 2014
	IFWGIPALLKR	Thermo Scientific nanoLC-ESI- Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	PQQPGQWQQPEQGQPR			
	YYPSVTCPQQVSYYPGQASPQRPGQGQQ PGQGQQGYYPTS			

(continued)

Table 4 (continued)

Protein	Peptide sequence	Equipment	Bioinformatics	References
HMW-GS Dy10	AQQPATQLPTVCR	Thermo Scientific nanoLC-ESI-Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	CCQQLR			
	ELQESSLEACR			
	LPWSTGLQMR	AB SCIEX QTRAP® 4500	MIDAS™ workflow	Lock 2014
		Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	QLQCERELQESSLEACR	Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	QQPVQGQQPEQGQQPGQWQQGYYPTS PQQLGQGQQPR			
	QVVDQQLAGR	AB SCIEX QTRAP® 4500	MIDAS™ workflow	Lock 2014
	QVVDQQLAGRLPWSTGLQMR	Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	SVAVSQVAR	AB SCIEX QTRAP® 4500	MIDAS™ workflow	Lock 2014
		Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
HMW-GS Dy3	ACQQVMDQQLR	Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	AQQPATQLPTVCR			
	DISPECHPVVVSPVAGQYEQQIVVPPK GGSFYPGETTPPQQLQQR			
	ELKACQQVMDQQLR			
	GGSFYPGETTPPQQLQQR			
	IFWGIPALLKP			
LMW-d	QQHQIPQQPQQFPQQQQF	Shimadzu LC - SCIEX QTRAP 5500	Analyst 1.6.1, Skyline	Bönick et al. 2017
	SQQQISQQPQQLPQQQQIPQQPQQF			

LMW-GS	GQQPQQQQL	Bruker HCT-Ultra PTM iontrap MS, Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2018
				Schalk et al. 2017a
	GSSLTSIDGQ	Waters Synapt G2 nanoLC-ESIQTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	GVGTRVGAY			
	LQQCSQTAY			
	QLPQIPEQSR	Shimadzu nano HPLC, SCIEX 5600 TripleTOF MS	Protein Pilot 4.0 (SCIEX)	Li et al. 2018
	QQQPLPPQQTFPQQPL	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2018
				Schalk et al. 2017a
	SHHQQQPIQQQPQPF	Shimadzu nano HPLC SCIEX 5600 TripleTOF MS	Protein Pilot 4.0 (SCIEX)	Li et al. 2018
	SIILQEQQQGF	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2018
				Schalk et al. 2017a
	SQMLQQSICHVMQQQCRQQLR	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	VQQQIPVVQPSIL	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2018
	VQQQLPVVQPSIL	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
LMW-i	ILPTMCSVNNPLYR	Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	SQMLQQSICHVMQQQCCQQLPQIP QQSRYEAIR			
	TTTSVPFGVGTGVGAY`			
	VFLQQQCIPVAMQR			
LMW-m	QLPQIPEQSRYDAIR	Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	SQTLWQSSCHVMQQQCCR			
	VFLQQQCSPVAMPQSLAR			

(continued)

Table 4 (continued)

Protein	Peptide sequence	Equipment	Bioinformatics	References
LMW-s	SQMLQQSSCHVMQQQCCQQLPQIP QQSRYEAIR	Thermo Scientific nanoLC-ESI Q-Exactive hybrid quadrupole-orbitrap	PEAKS	Aghagholizadeh et al. 2017
	TTTSVPFDVGTGVGAY			
	VFLQQQCSPVAMPQSLAR			
Omega-gliadin	HQQPEQIISQQPQQPF	Waters Synapt G2 nanoLC-ESI-QTOF, Xevo-TQS	PLGS, Skyline	Martínez-Esteso et al. 2016
	ILQPQQPLPQQPQQPF			
	SPHQPQQPFPQPQRPTPL			
	HQQQLPQQQFPQQQFPQQQFPQ QQQFPQQQQL			
	FPHQSQQPF	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a
	GSSLTSIGGQ			
	QPHQPQQPYPQQ	Shimadzu LC - SCIEX QTRAP 5500	Analyst 1.6.1, Skyline	Bönick et al. 2017
	QQYPQQQPSGSDVISISGL	Bruker HCT-Ultra PTM iontrap MS Thermo Scientific TSQ Vantage	DataAnalysis 3.4, Mascot	Schalk et al. 2017a

be targeted and based on peptide fragmentation the amount of gluten can be detected. It is clear from Table 4 that only very few wheat gluten peptides have been identified in the multiple studies using different LC-MS platforms. LQLQPFP QPQLPY, LQLQPFPQPQLPYPQPQPF, RPQQPYPQPQPQY and VSQQSYQLL QQLCCLQLWQTPEQSR from alpha-gliadin, APFASIVADIGGQ, APFASIVAG IGGQ, LQPHQPF and LQPQQPQQSFPQQQQPL from gamma-gliadin, LPWST GLQMR and SVAVSQVAR from HMW-GS Dy10 were each found in at least two studies. The abundance of certain proteins or peptides may be as low as zero or below a detectable limit. Even if their abundance is sufficiently high to be detected, these peptides are obtained via enzymatic digestion after protein extraction. The use of chymotrypsin, as opposed to trypsin, proved to be more successful for gluten digestion due to the particular amino acid composition of gluten proteins and the limited number of trypsin specific cleavage sites (e.g. Sealey-Voyksner et al. 2010; Martínez-Esteso et al. 2016).

It is thus more feasible to use chromatography and coupled techniques as confirmatory or identification approaches in gluten analysis. Relative quantification of the different gluten protein types is routinely done by SE- and RP-HPLC, but absolute quantification of gluten components is not yet fully achievable using LC-MS platforms.

As well as SE-HPLC, it is highly advisable to use another analytical technique, asymmetrical flow field-flow fractionation multi-angle laser light scattering (AFFFF-MALLS) to fully characterise storage protein polymers that have accumulated in cereal grains and are present in flour. In this case, the molecular screening is performed in a trapezoidal shaped cell where polymers are subjected to a double cross-flow gradient followed by multi-angle detection of deviation of a laser beam (Lemelin et al. 2002). This technique does not involve a stationary phase so the absence of protein shearing forces offers the possibility of measuring several polymer parameters (such as molecular mass and radius of gyration) and hence knowing the distribution of these molecular characteristics within the sample analysed and calculating the polydispersity index. Such measurements are not possible with SE-HPLC separation, which often has a cut-off of about 1000 kDa. AFFFF-MALLS has proved useful for characterizing polymer masses in flour, for example, by showing which of their properties explain the environmental stability of bread making quality (Lemelin et al. 2005), that they are highly influenced when wheat grain is subjected to ozone treatment (Goze et al. 2017), and that they undergo the unfolded protein response caused by environmental stresses during protein accumulation (Branlard et al. 2015). AFFFF-MALLS is likely to be the tool of choice for further research especially that aimed at reducing the polymer masses to render the gluten better for consumer health.

3.2 Electrophoresis Techniques

Electrophoresis techniques for gluten analysis are very specific and widely used. Detailed protocols and highlighted applications are provided here for the most frequently used techniques (Table 5 and Annexes).

Table 5 Gel electrophoresis methods to study gluten proteins

Method	Purpose	Advantage	Disadvantage	References
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	To separate proteins based mainly on their molecular weights	Detecting small variations in protein size	Difficult to separate many proteins having similar molecular weights. Measured molecular weights are not accurate.	Singh et al. 1991; Peña et al. 2004; Ikeda et al. 2008
Acid polyacrylamide gel electrophoresis	To separate proteins based on their molecular weights and charges.	Useful to separate gliadins.	Gel handling is difficult. Interpretation of the banding patterns is difficult.	Metakovsky and Novoselskaya 2001; Branlard et al. 1990
Two-dimensional gel electrophoresis	To separate more proteins based on their isoelectric point and molecular weights	Separate more proteins. Usable for protein sequencing and MS analysis.	Time consuming. Immobilised IEF gels are more expensive.	Dumur et al. 2004; Ikeda et al. 2006; Liu et al. 2010

3.2.1 Sodium Dodecyl Sulphate Polyacrylamide Gel-Electrophoresis (SDS-PAGE)

SDS-PAGE is the most frequently used technique for HMW-GS analysis and is partly used for LMW-GS analysis. This technique has the advantage of allowing the detection of small size variations in HMW-GSs, but it is less useful for separating LMW-GSs and gliadins because they include many proteins with similar molecular weights. When using SDS-PAGE for gluten analysis, it is necessary to first block the free SH residues of the component proteins by alkylation with 4-vinylpyridine. The concentration of the bis-acrylamide cross-linker and the pH of the separation gel are also important aspects to optimise when separating gluten proteins. Better separation is obtained using a lower bis-acrylamide concentration (1.3%C) and lower pH (pH 8.5). A standard protocol used at the Wheat Chemistry and End–Use Quality Laboratory of CIMMYT is shown at the end of this chapter in Appendix I with methods for selective extraction then electrophoresis of gliadins and glutenins for SDS-PAGE.

3.2.2 Acid Polyacrylamide Gel-Electrophoresis (A-PAGE)

A-PAGE is currently only used for the advantages it offers for analysing gliadins due to the difficulty of handling the gels. A-PAGE separates gliadins better than SDS-PAGE, because it separates them based on their molecular weights and charges. There is a huge diversity of gliadins. Although it is difficult to interpret the banding

patterns, the catalog by Metakovsky et al. (2018) lists 182 alleles at the six *Gli* loci of common wheat that may be useful for genomic analysis of gliadin gene families. A protocol used at INRA (France) is shown at the end of this chapter in Appendix II.

3.2.3 Two-Dimensional Gel Electrophoresis (2-DE)

The 2-DE technique separates more proteins based on their isoelectric point and molecular weights. It has been used for gluten protein sequencing (Ikeda et al. 2006) and MS analysis (Liu et al. 2010). The cost of immobilised isolelectric focusing (IEF) gels and instruments for IEF is nevertheless a limiting factor.

3.3 Challenges in Gluten Analysis

Gluten testing is undoubtedly a challenge and has been recently reviewed (Melini and Melini 2018). Due to the unique properties of gluten proteins, routine methods that are suitable for general protein analysis have often been found to be unsuccessful or have required prior protocol modification. While immunoassays have been shown to be suitable for routine gluten analysis in relation to compliance with food legislation and labelling, the limitations and challenges of other methods such as LC-MS are apparent. Critical factors like the complexity of the food matrix, the type of antibody in immunoassays, gluten extraction procedures and lack of reference material can all impact the reliability of immune-detection of gluten proteins and the need for harmonisation has been clearly highlighted.

3.3.1 Definition of Gluten

One of the challenges of gluten analysis is the ambiguity with which it is defined. In bread-making, the gluten is obtained when flour is added to water then mixed and washed with salt solution until other flour compounds, particularly starch and soluble proteins, are removed. The remaining viscoelastic portion is classically called gluten. By contrast, the legislative definition of gluten in Europe encompasses oat as a gluten source and defines gluten proteins according to their insolubility in 0.5 M NaCl (Codex 2008). The properties of oat avenins are however distinctly different from the properties of wheat gluten, barley hordeins and rye secalins, especially from the point of view of their toxicity (Real et al. 2012). Wheat research most frequently refers to the Osborne definition of gluten. Historically, wheat proteins were classified as water-soluble albumins, salt-soluble globulins, alcohol-soluble gliadin (prolamins) and insoluble glutenin (glutelins) (Osborne 1924). There is now a need in food labelling to display the gluten source, whether wheat, barley or rye, as some consumers may suffer from food allergy. Differentiating between

wheat, barley and rye gluten is difficult, especially with certain methods (e.g. immunoassays) due to the similar sequence characteristics and solubility of gluten proteins. The definition of gluten is therefore specific for the selected extraction and analysis method so it is very important to state this especially in food safety applications. Conversely, if the purpose of gluten testing is for legislative labelling purposes, the legislative definition of gluten may determine what extraction and testing methods need to be used.

3.3.2 Solubility and Extractability of Gluten

The solubility of gluten proteins depends on the extraction solvents used (pH, ion strength, polarity) and the composition of the surrounding matrix. Gluten is most often extracted with either 60% or 80% ethanol (van den Broeck et al. 2009; Mena et al. 2012), 55% isopropanol (Colgrave et al. 2015), isopropanol and NaI (DuPont et al. 2005), or multi-step protocols based on the Osborne fractionation using a series of extraction solvents (Lookhart and Bean 1995; Zilic et al. 2011; Fallahbaghery et al. 2017).

The extraction efficiency of gluten proteins also depends on the fat and carbohydrate content of the matrix. In the future, it may be easier to design a standardised protocol for extracting gluten from wheat and other cereals than from food, especially processed food. For example, the presence of lipids and polyphenols influence protein solubility and the molecules can interfere with protein detection and identification when present in protein fractions.

Gluten solubility can be aided by converting the disulfide bonds into sulfhydryl groups using reducing agents such as dithiothreitol or beta-mercaptoethanol. In the presence of urea, proteins can be denatured and SDS can mask the surface charges of peptides and proteins. The use of polyvinylpyrrolidone was shown to aid gluten extraction from chocolate or cacao containing samples (Mena et al. 2012; Satsuki-Murakami et al. 2018). Fish gelatin, a reducing agent (Tris (2-carboxyethyl)-phosphine) and an anionic surfactant (N-lauroylsarcosine) are used in the universal prolamin and glutelin extractant solution (UPEX) before extraction with 80% ethanol, which is claimed to be suitable for all types of subsequent analysis techniques such as ELISA and LC-MS (Mena et al. 2012). Recently, a rapid, simple, and reproducible protocol for extraction and digestion of gluten proteins was published that is suitable for LC-MS quantification (Li et al. 2019).

The different extraction methods target various proportions of the different gluten protein types. The purity of the obtained gluten fractions can vary not only due to the presence of non-protein compounds but also of other non-target proteins. For example, the glutenin fraction contains gliadins while the LMW-GS fraction may contain omega-gliadins. The sequence homology between gliadins and LMW-GS means they have similar affinity for extraction buffers and is the main reason for their co-extraction.

3.3.3 Gluten Protein Sequences and Structure

Gluten proteins have a great amount of sequence homology within and between species. The secondary structure and conformation of the gluten protein chains differ however due to the presence of S-containing amino acids and the various polypeptide chain lengths. The S content of proteins makes them prone to disulfide bridge formation, which is a dynamic chemical bonding between the S-S and the reduced SH-SH forms. The sequence characteristics determine the physical and chemical properties of the proteins, which are very similar for the corresponding gluten protein subgroups of different species (e.g. HMW-GS in wheat and D-hordeins in barley). This homologue behavior can be advantageous and disadvantageous depending on the purpose of testing. When total gluten content is analysed, extraction is easier if the compounds of interest have similar properties. When the aim is to define the source of gluten (e.g. whether it is from wheat, barley or rye) or to characterise or quantify the different subgroups or even to target certain sequences, sequence homology is a major problem.

Accessibility of enzymes and antibodies to the target protein/peptide/epitope sequence is a substantial limiting factor in gluten detection methods. The use of different mono- or polyclonal antibodies in immunoassays, the specificity of antibodies, and the abundance of the immune-responsive protein sites are often the reason for variation in the performance of ELISA kits (Schopf and Scherf 2018). Enzymes can only cleave proteins if they have physical access to their specific cleavage sites on the relevant section of the polypeptide. Enzyme accessibility is therefore a major factor when producing peptides for LC-MS detection. Unfolding of the three-dimensional and secondary structure of the protein chain for digestion is a crucial step.

In MS-based proteomics, the identification of protein/peptide sequence is based on using a protein sequence database and comparing it to the detected mass of an ionised peptide fragment. Identification is based on known amino acid residue masses, cleavage rules of the applied enzyme(s) and allowed missed cleavage(s). The proteins may have post-translational modifications that could themselves be modified during processing (e.g. deamidation). Modifications can be fixed or variable and can affect all or just some of the amino acid residues. Consequently, identification is limited by the number and completeness of sequences available in the database. It is only recently that the wheat genome sequencing project was completed, and a reference genome became available (International Wheat Genome Sequence Consortium 2018). Once the contents of the genome database are converted into searchable expressed protein sequences, then the capabilities of MS based protein identification methods will improve. Correct annotations are also important when identifying proteins or the plant source. Annotations of gluten proteins in the current databases (e.g. www.uniprot.org) are often incorrect but a manually curated prolamin sequence database (including gluten) has now been created (www.propepper.net, Juhász et al. 2015b). A similar database dedicated to gluten has been developed as a tool for proteomic studies (Glu.Pro V1.0, Bromilow et al. 2017b).

The quantities of individual gluten proteins in a sample might be low and the peptide quantities even lower. The expression level of individual proteins are species

and variety specific and will differ depending on the growing/environmental conditions. Biotic and abiotic stresses have an impact on the expression levels of proteins and protein groups (see 'Effects of environmental changes on the allergen content of wheat grain' chapter). In any gluten analysis method that relies on sequence data for identification or quantification, it is crucial to select abundant target peptides/proteins that are unique for the species, the total gluten content or a particular variety independent of the possible effects of stresses.

3.3.4 Method Performance Characteristics

The aim of gluten analysis determines the required sensitivity of a method. The surrounding food matrix is often the limiting factor in the achievable LOD or lower limit of quantification.

In immunoassays, the antibodies selected to target gluten peptides may cross-react with other non-target proteins creating false positive results or have more affinity for certain proteins perhaps from other species. In gluten ELISAs, overestimation and underestimation of gluten from one or other species are known issues. Indeed the antibodies in certain kits (e.g. R5) were developed against peptides/proteins of a particular species (e.g. barley hordein) and therefore the assay overestimates the quantity of proteins from that species. Continuing with the example of the R5 ELISA, test results are an underestimation of the actual level of wheat gluten because glutenin detection is not accounted for (e.g. Dostalek et al. 2006). Recent developments to detect total gluten content in oat by using a multiplex assay showed that it is possible to overcome this issue by selecting a better set of antibodies raised against gliadins and glutenins (Boison et al. 2018).

In MS-based gluten identification, if the target peptide for quantification is selected carefully and is unique for the gluten or its specific fraction, the possibility of cross reactivity can be excluded. In LC-MS/MS methods, the difficulty is to achieve limits of detection that are similar or lower than those for ELISAs.

The performance of commercially available ELISA kits was investigated by some researchers who mostly concurred on the need to improve gluten extraction, gluten peptide detection and calibrants, while debating the use of a suitable reference sample in the assays (Sharma 2012; Diaz-Amigo and Popping 2012; Bruins Slot et al. 2015; Bugyi et al. 2012; Torok et al. 2015; Panda et al. 2015; Martínez-Esteso et al. 2017; Rzychon et al. 2017; Lexhaller et al. 2017).

3.3.5 Standardisation and Harmonisation of Gluten Analysis

There is a lack of agreement on the level of performance necessary for gluten detection methods employed to comply with food safety legislation. Standardisation would bring gluten testing results into conformity with a standard. To arrive at an agreement would need acceptance of the use of a certified reference material (CRM) or a specific calibrant, not only for immunoassays but for any other suitable methodologies such as LC-MS/MS protocols.

Harmonisation of gluten detection would have to involve consideration of any processes that could contribute to making the results of different measurement procedures comparable by recognizing, understanding and explaining any disparities to generate uniform data or reliably convert it. The analytics community is well aware of the need for harmonisation and standardisation, but it is acknowledged not to be a straightforward exercise.

The outcome of various gluten analysis techniques (immunoassays, chromatography or MS) may be based on detection of a single peptide (e.g. a 33-mer), an individual protein (e.g. P18573 alpha-gliadin), a protein group (e.g. alpha-gliadins) or total gluten. It is difficult to determine accurate or meaningful conversion factors between

– peptides and individual proteins
– peptides and gluten
– individual proteins and gluten or
– gluten and the plant species of its origin.

For example, in ELISA methods gliadin is often measured and the data converted to represent gluten by using a conversion factor of 2. It is well known that the variation of the gliadin to glutenin ratio is variety dependent and also influenced by the environmental stresses. Although, it is not accurate, the use of a single conversion factor is currently the best approximation. Standardisation efforts may help to overcome the inaccuracies caused by using this factor of 2 when measuring gliadins and expressing gluten levels (Wieser and Koehler 2009; Diaz-Amigo and Popping 2013; Koerner et al. 2013; Bruins Slot et al. 2015).

Regardless of what aspect of gluten is measured, there should be a single agreed compound, a robust marker, that any method refers or converts to when expressing gluten analysis results. Options include the use of the same calibrants or standards or reference materials, although the ultimate solution may be the use of multiple techniques or more than one standard. Publications that compare method performances, reviewed the status of standardisation and harmonisation efforts of gluten analysis providing a high-resolution picture of the state of the art (Haraszi et al. 2011; Bugyi et al. 2013; Mena and Sousa 2015; Bruins Slot et al. 2015, 2016; Martínez-Esteso et al. 2017; Rzychon et al. 2017; Deora 2018; Alves et al. 2017; Melini and Melini 2018).

To date, standardisation and harmonisation of gluten detection remains unresolved, but two priorities are clear. An agreement on the specific analyte(s)/target(s)/set of markers is required to improve and make gluten measurements comparable (Martínez-Esteso et al. 2016). Well-characterised reference materials representative of all the different subgroups of gluten proteins are required (Martínez-Esteso et al. 2017).

Standardisation and harmonisation of analysis methods in gluten detection would also trigger a smoother implementation of the various food safety legislations world-wide helping people to consume gluten-free or low gluten foods safely. Last but not least, harmonisation would allow the food industry to better deal with gluten risk assessment, allergen management and communication of the associated issues (Melini and Melini 2018).

Appendix I

SDS-PAGE protocol used by the Wheat Chemistry and End–Use Quality Laboratory of CIMMYT

Reagents and main steps of the procedure were published in Peña et al. (2004, revised in 2018).

PREPARATION OF REAGENTS

Reagent 1. 1 M Tris pH 8.5

Dissolve 30.3 g of Tris in 220 ml of distilled water. Adjust pH to 8.5 with concentrated HCl. Bring the total volume up to 250 ml with distilled water. Store in the refrigerator.

Reagent 2. 1 M Tris pH 6.8

Dissolve 12.1 g of Tris in 64 ml of distilled water. Adjust pH to 6.8 with concentrated HCl. Bring the total volume up to 100 ml with distilled water. Store in the refrigerator.

Reagent 3. 10% sodium dodecyl sulfate (SDS)

Dissolve 5 g of SDS in 40 ml distilled water and bring the total volume up to 50 ml with distilled water. Store at room temperature (20–25 °C).

Reagent 4. Stock acrylamide solution (40%T and 1.3%C) for running and stacking gels

Weigh 0.52 g of bis-acrylamide and 39.5 g of acrylamide. Dissolve in approximately 70 ml of distilled water and bring the total volume up to 100 ml. Homogenize and filter the solution before use. Store in a dark (translucent) container in the refrigerator.

CAUTION: use gloves and mask when working with acrylamide.

Reagent 5. Stock sample buffer for total protein extracts

Dissolve 12 g of glycerol in 36 ml of distilled water. Add 0.76 g of Tris, 4.0 g of SDS, 750 mg of dithiothreitol (1.5% DTT) and 6.0 mg of bromophenol blue. Adjust to pH 6.8 with concentrated HCl and bring the total volume up to 50 ml with distilled water.

Reagent 6. Tris-glycine stock for running buffer

Put 30.0 g of Tris in a 1-L beaker. Add 887 ml of distilled water and stir. While stirring the solution first add 144 g of glycine then 10 g of SDS. The pH of the solution should be 8.3. **If the pH is higher, do not adjust it with HCl as it would alter the desired ionic strength.**

Reagent 7. Staining and destaining solutions
Solution 1 is a 12% trichloroacetic acid solution. Dilute 120 g of trichloroacetic acid in distilled water and make up to 1 L.
Solution 2 is the staining solution. Mix 400 ml of methanol, 100 ml of glacial acetic acid, 0.1 g of Coomassie Brilliant Blue R-250, and 500 ml of distilled water (1 L in total).

Reagent 8. 1.5% ammonium persulfate
Dissolve 150 mg of ammonium persulfate in 10 ml of distilled water. Prepare fresh immediately before use.

Procedures

Extraction of total proteins
Weigh 20 mg of sample into a microcentrifuge tube. Add 300 µl of sample buffer (reagent 5) and incubate for 5 min in an Eppendorf Thermomixer comfort at 90 °C and 1400 rpm. Centrifuge for 5 min at 13000 rpm in an Eppendorf Microcentrifuge 5415C (Brinkmann Instruments Inc., NY).

Gels for total protein extracts from durum wheat and bread wheat
The final acrylamide concentration to be used in the gel will depend on the size and type of bands to be examined in detail. For the running gel, 9–10% acrylamide is the most common gel concentration when examining high molecular weight glutenins from whole grain protein extracts. However, for better separation of subunits 2 and 2*, gels of around 13% acrylamide are more appropriate.

Formulas to determine gel concentrations

$$\text{Concentration of acrylamide}(\%\text{T}) = \frac{\text{g of acrylamide} + \text{g of bis} - \text{acrylamide}}{\text{total volume of solution}} \; 100$$

$$\text{Concentration of bis-acrylamide}(\%\text{C}) = \frac{\text{g of bis} - \text{acrylamide}}{\text{g of Acrylamide} - \text{g of bis} - \text{acrylamide}} \; 100$$

Staining and destaining gels
Submerge the gels for 5 min in a 12% trichloroacetic acid solution (reagent 1), then place them in staining solution (solution 2) for 4 h, and finally for destaining place them in distilled water for 24 h.

Reagents for glutenin and gliadin extracts
Solution 1.1. 0.08 M Tris-HCl buffer pH 8.0
Weigh 4.8 g of Tris and 20 g of SDS and dissolve them in 500 ml of distilled water, adjusting the pH to 8.0 with HCl.
Solution 1.2. 50% propanol
Solution 1.3. Mix 500 ml of each of solution 1.1 and 1.2
Solution 1.4. For 10 ml of solution 1.3, mix in 200 mg of DTT. This reagent should be prepared the same day.
Solution 1.5. For 10 ml of solution 1.3, add 140 µl of 4-vinyl-pyridine and mix. This reagent should be prepared the same day.
Solution 1.6. Glutenin extraction buffer
2% SDS (w/v), 40% glycerol (w/v), 0.02% (w/v) bromophenol blue.
Dissolve 20 g of glycerol in 36 ml of distilled water. Add 0.76 g of Tris, 1.0 g of SDS, and 10.0 mg of Coomassie Brilliant Blue R. Adjust the pH to 6.8 with concentrated HCl. Make the volume up to 50 ml with distilled water.
Solution 1.7. Gliadin extraction buffer
2% SDS (w/v), 40% glycerol (w/v), 0.02% (w/v) bromophenol blue. Dissolve 20 g of glycerol in 36 ml of distilled water. Add 0.76 g of Tris, 1.0 g of SDS, and 10.0 mg of Coomassie Brilliant Blue R. Adjust the pH to 8.0 with concentrated HCl. Make the volume up to 50 ml with distilled water.

Running gel (For two 15%T gels, 17 cm high and 1 mm thick)

24.7 ml of Tris pH 8.5 (Reagent 1)
24.4 ml of Acrylamide Stock for Running Gel (Reagent 4)
650 µl of 10% SDS.
14.0 ml of Distilled Water

This mixture is deaerated with a Sonics Branson 5510 for 2–4 min. Immediately filter the mix through coarse filter paper. Add 1.3 ml of fresh 1.5% ammonium persulfate (reagent 8). Assemble frames for pouring gels then add 50 µl of TEMED to the mixture. Mix gently for 5 seconds and pour the gel solution into the frames. Immediately apply 1 ml of butyl alcohol to the top of the gel to prevent the formation of a meniscus. The alcohol should be applied slowly with a syringe.

Stacking gel (For two 4.8%T gels, 3.0 cm high and 1.0 mm thick)
Before adding the stacking gel solution, remove the butyl alcohol from the top of the running gel.

6.12 ml of Distilled Water
1.10 ml of Tris pH 6.8 (Reagent 2)
1.05 ml of acrylamide stock for stacking gel (reagent 4).
90 µl of 10% SDS.
380 µl of 1.5% ammonium persulfate

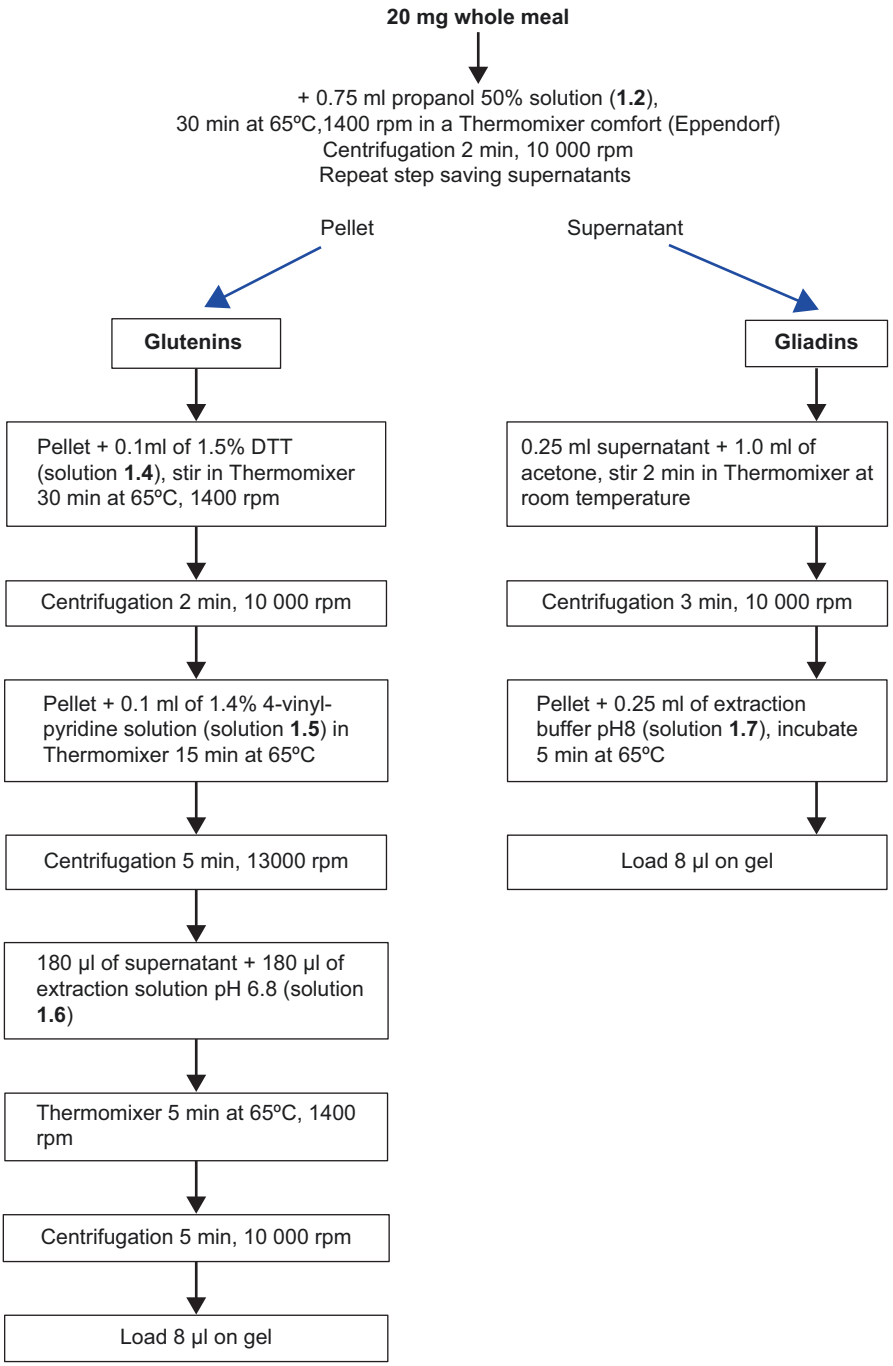
Mix reagents together gently, add 50 µl of TEMED, and mix again. Apply to the top of the running gel, being careful that there are no bubbles in the lanes of the Teflon lane former (comb). This can be achieved by inserting the Teflon comb at an angle of approximately 30°. Let the gel stand for 30 min to 1 hr. Very carefully remove the Teflon comb and with a syringe remove the excess solution which did not polymerise. Fill the lanes with running buffer (see below).

Running buffer
Mix 400 ml of stock for running buffer (reagent 6) and 3.6 L of distilled water. Use approximately 2.5 L of buffer per tank (e.g. Protean BIO-RAD equipment, Bio-Rad Laboratories, Richmond, California) for 2 gels (4.5 L for a buffer tank for 6 gels).

Running the gel
Load the samples in each lane (4–8 µl of extracted protein). Assemble the equipment, connect the hoses of the cooling system (15 °C) to running water and turn on the power. The running time depends on the current (mA) and the porosity (%T) of the gel. Electrophoretic separation can be run at 12.5 mA per gel for about 19 h (overnight). When using more than 25 mA per gel (runs of approx. 8 h), it is necessary to use a cooling apparatus to maintain temperature at around 15 °C. For a shorter running time of 4–5 h, run the electrophoresis at 35–40 mA per gel at 15 °C until the colored line arrives at the bottom edge of the gel. In order to maintain ionic strength, it is recommended to use a small pump connecting the lower to the upper buffer containers to recirculate the running buffer.

Preserving gels
Fresh gels may be kept for limited time if placed in polyethylene plastic bags in the refrigerator. Drying the gels with a gel drier is more convenient to preserve the gels for longer.

Selective extraction of gliadins and glutenins for SDS-PAGE
According to Singh et al. (1991) with modifications.



Appendix II

A-PAGE protocol for gliadin analysis

The main steps are described in Metakovsky and Novoselskaya (2001) for gels of standard size (18 × 20 cm). For long acrylamide gels (18 × 32 cm) it is advisable to use the protocol previously described by Branlard et al. (1990).

Reagents

1. 70% ethanol
2. 1.5% aluminium lactate pH 3.1

Dissolve 15 g of aluminium lactate in 800 ml of distilled water∗. Adjust pH to 3.1 with lactic acid. Bring the total volume to 1 L with distilled water.

3. 1% ascorbic acid

Dissolve 0.1 g of ascorbic acid in 10 ml of distilled water. Prepare fresh for each use.

4. 1% $Fe(SO_4)_3$

Dissolve 0.1 g of $Fe(SO_4)_3$ in 10 ml of distilled water.

5. 40% acrylamide

It is highly advisable to purchase 40% acrylamide solution ready to use. Alternatively, dissolve 40 g of high quality acrylamide in 100 ml of distilled water. Store at 4 °C.

6. 2% bis-acrylamide.

It is highly advisable to purchase 2% bis-acrylamide solution ready to use.
Alternatively, dissolve 2 g of high quality bis-acrylamide in 100 ml of distilled water. Store at 4 °C.

7. 0.33% hydrogen peroxide

Purchase 3% hydrogen peroxide (10 volumes) from a pharmacy or store and keep it at 4 °C. Make 1.1 ml of 3% hydrogen peroxide (10 volumes) up to 10 ml with distilled water. Prepare fresh for each use.

8. Stock sample buffer

Dissolve 3 ml of glycerol in 1 ml of 1.5% aluminum lactate pH 3.1. Add a small portion (a few grains of powder) of pyronin or methyl green. Bring the total volume up to 10 ml with distilled water.

9. Stock running buffer (0.15% aluminium lactate pH 3.1)

Dilute 100 ml of 1.5% aluminum lactate with 900 ml of distilled water.

∗Dr. Metakovsky consistently uses distilled water. The classic ultra-pure water influences the resolution of the gliadins and may alter the mobility of some gliadin alleles. Twice-deionized water (today referred to as ultra-pure water) was used by Branlard et al. (1990).

Procedures

Gel preparation

8.3% Acrylamide, 0.42% Bis-Acrylamide, 0.1% Ascorbic Acid, 0.00067% $Fe(SO_4)_3$, 0.15% Aluminum Lactate, pH 3.1

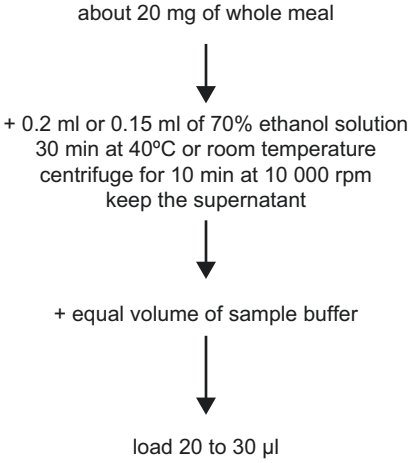
The glass plates (20 × 20 cm and 2 mm thickness for a normal gel or 30 × 20 cm and 2 mm thickness for a long gel for higher resolution) are cleaned with 70% ethanol then by spreading droplets of glycerol until a thin film forms and drying with KimWipes. This treatment makes it easier to remove gels from plates.

Mix 21 ml of 2% bis-acrylamide and 20.8 ml of 40% acrylamide. Add 67 µl of 1% $Fe(SO_4)_3$, 10 ml of 1% ascorbic acid, 10 ml of 1.5% aluminum lactate, and bring the total volume up to 100 ml, filter through filter paper and degas, then cool on ice before use. Add 0.4 ml of 0.33% hydrogen peroxide, mix well and pour rapidly into the glass plate assembly. The comb is inserted and the gel is polymerized for 10 min.

Gliadin extraction

Weigh about 20 mg of sample into a microcentrifuge tube. Add 150 µl of 70% ethanol and incubate for 30 min at room temperature. Centrifuge the sample for 5 min at 12500 rpm. Transfer the supernatant to a microcentrifuge tube and mix with an equal volume of the sample buffer. Freshly prepared (within two days) samples should be used to obtain well resolved electrophoretic profiles.

EXTRACTION OF GLIADINS FOR A-PAGE



Electrophoresis

After rinsing the wells with running buffer, the glass plates containing the gel are placed in the vertical slab gel apparatus. Then the wells are filled with the buffer and 20 to 30 µl of the samples are slowly loaded. Electrophoresis from the anode (the upper buffer) to the cathode (the lower buffer) is performed for 10–20 min at 220 V and then for 2.5–3 h at 550 V without buffer circulation. The lower tank of the vertical apparatus is cooled with tap water or in a cooling system at 10 °C.

Staining/destaining

The gels are kept on one of the glass plates to reduce the risk of damaging the gels. The staining procedure is the same as for the SDS-PAGE method.

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