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Review

Proteomics as a tool to understand the complexity of beer

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ABSTRACT

Recently “Omics” technologies such as genomics, proteomics, metabolomics and lipidomics have become widely popular as complementary technologies for conventional analytical methodologies, providing comprehensive and reliable results in a high throughput manner. Improvements and advances in technology have seen their application in the study of food science and nutrition in the field of “foodomics”. This relatively new discipline includes studies of food quality and safety, detection of food contamination and the presence of food allergens, compound profiling, authenticity and much more. Here, we discuss state-of-the-art proteomics techniques and their potential application within the food and beverage industry with a particular focus on beer analysis. Particular advantages of proteomics applications are the discovery of protein biomarkers, which can be used to validate the quality of raw ingredients and final products and monitor the type and amount of important proteinaceous components. An additional benefit of these techniques is their ability to provide insight into the effects of abiotic stress upon ingredient biochemistry during the brewing process. For example, protein structural changes and post-translational modification can modulate the flavour, texture and appearance of beer. A greater perception of these changes can be appreciated using a proteomics approach and this accumulated knowledge will help maltsters and brewers, in association with breeders, create better and safer products for consumers. Aside from routine identification and quantification workflows, it can also lead to the discovery and characterisation of novel proteins. Therefore, the application of proteomic techniques including both gel-based and mass spectrometric analyses is discussed in this paper, along with a recently published novel approach for the relative quantification of hordein in beer using mass spectrometry.

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1. Introduction

Written in the history of Ancient Egypt and Mesopotamia, ale (or beer) is one of the oldest beverages produced by humans (Arnold, 2005) with brewing described as the oldest biotechnological process.

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Considered a gift from the gods and suitable as a gift for the pharaohs of the time, beer was used for nutrition, in religious practices and also in the treatment of ailments. In those days, beer was lower in alcohol, higher in protein content, sweeter and thicker. In fact, it was drunk from a communal bowl using reeds to avoid drinking the sediment. Medieval ale (unhopped beer) spoiled easily. The addition of hops not only improved the taste, but also added antibacterial activity which inhibits Gram-positive bacteria, but does not affect brewer's yeast, making beer a safe drinking source. Prior to the use of hops, other bittering herbs, spices, and flowers were used in beer. The first documented hop cultivation was in the Hallertau region of Germany around 736 and the first recorded use in brewing was in a French monastery in 822 (Townsend & Shellhammer, 2012). In the 1530s King Henry VIII placed a ban on the use of hops, "a wicked and pernicious weed", in English ales as it was thought in those days that consuming beer brewed with hops caused insanity or drove those that consumed it to sinful behaviour. This law remained until 1552 when his son, King Edward VI, in a burst of sanity, repealed it.

Nowadays, beer primarily serves as a drink with occasional use in cooking and in shampoos. Beer is one of the most common alcoholic drinks in popular culture with global market revenue in excess of \$500 billion pa (Kirin, 2011). The European countries centred around Germany are reported to have the highest annual beer consumption per capita (Czech Republic, 158.6 L; Germany 110.0 L; Austria, 108.3 L), but China and the USA constituted the largest share of the annual beer consumption by nation in 2010 (28,640 ML and 23,974 ML respectively) (Kirin, 2011).

2. The brewing process

Beer is derived from malted cereals and grains, most commonly barley and wheat, along with water, hops and yeast. In the malting process, barley (or other cereals) undergoes a controlled germination at lower temperatures to minimise rootlet growth and respiratory loss of sugar. Germination activates degradative enzymes such as α -amylase, which later converts the stored starch into sugars needed for fermentation. The germinated grain (malt), is gently dried by roasting to stop germination, but allow retention of amylase activity. The malt is milled to grist and then mashed by mixing with hot water, and held near 60 °C (the amylase rest) to swell the starch granules and allow conversion by enzymes including α - and β -amylase, α -glucosidase and starch de-branching enzyme. The starch is degraded to oligosaccharides of up to four degrees of polymerisation (DP4). Often additional temperature steps are programmed to allow for instance, protease activity to proceed. The sugar containing liquid, or wort, is raised to boiling and hops are added. Hops are the conical flower of the *Humulus lupulus* vine – that give the beer a specific flavour and/or aroma. During boiling the majority of the protein is precipitated forming a curd – the "hot break". This enriches the wort for a specific suite of heat stable, water soluble protein families consisting mainly of serpin Z4 and lipid transferase proteins (LTP) (Perrocheau, Rogniaux, Boivin, & Marion, 2005). Following filtration through a bed formed by the spent grain husks (lautering) to remove the particulate matter, precipitated proteins and debris, the wort is rapidly cooled and oxygenated. It is then fermented in the presence of added yeast, converting the sugars to alcohol and carbon dioxide. The type of yeast used and the temperature of fermentation dictate whether the beer is an ale (warmer temperatures) or a lager (cooler temperatures). The final stage in brewing is conditioning in which the beer is stored at a temperature according to the style of beer for several weeks. Most styles of beers are then filtered and pasteurised to remove the yeast and stabilise the beer prior to packaging. Some traditional beers, e.g. traditional Bavarian *weiss bier*, are not filtered. Many beers are brewed from the four primary ingredients: barley, water, hops and yeast – as required under the German beer purity law of 1516, *Reinheitsgebot*, a law instituted to prevent unhealthy ingredients from being used, such as rushes, roots, mushrooms and in

some instances animal products. However, wheat, rye, oats, rice, maize and to a lesser extent sorghum, millet and teff have all been used to brew beer. Many of these grains, especially rice and maize are used as an adjunct – that is to supplement the primary mash ingredient, such as barley malt – to produce a more cost-effective or a more desirable product. For example, wheat has been used as an adjunct for its ability to promote foam stability.

3. Proteins in beer

Beer contains ~500 mg/L of proteinaceous material (Hejgaard & Kaersgaard, 1983), typically of size 5–100 kDa (Sorensen & Ottesen, 1978). These beer polypeptides originate from the cereal grains used in brewing (barley, wheat or other cereals). Protein constitutes approximately 10% of the total mass of barley grain with hordeins being the most abundant proteins (40–50% of protein) (Osman et al., 2002). The remaining protein content is predominantly comprised of albumins, globulins, serpins, amylase inhibitors, lipid binding proteins, chaperones and enzymes. It is well established that proteins undergo a number of modifications and hydrolysis during the brewing process, especially during malting and mashing (Gupta, Abu-Ghannam, & Gallagher, 2010; Steiner, Gastl, & Becker, 2011). Proteins may be modified by hydrolysis, glycosylation (enzymatic, covalent addition of a sugar) or glycation (the non-enzymatic, covalent addition of a sugar) particularly during malting and mashing. N-linked glycosylation occurs at the nitrogen of asparagine or arginine side chains, while O-linked glycosylation occurs at the hydroxyl oxygen of serine and threonine, and to a lesser extent to tyrosine and hydroxylysine or hydroxyproline side chains. Glycation occurs predominantly at lysine residues and to a lesser extent arginine residues, particularly during mashing (Bobalova, Petry-Podgorska, Lastovickova, & Chmelik, 2010; Petry-Podgorska, Zidkova, Flodrova, & Bobalova, 2010). A large proportion of the protein content is removed from wort during boiling as trub (hot break) and during wort cooling (cold break). The modifications that occur to the proteins during malting and mashing are largely responsible for the characteristics of the finished product – not only do they impart colour and flavour, but also they are involved with haze formation and foam stabilization (Bamforth, 1985; Didier & Benedicte, 2009; Steiner et al., 2011).

4. Proteins in beer – health implications

Beer has served various medicinal roles, including as a mouthwash, enema, vaginal douche and applicant to wounds (Darby, Ghalioungi, & Grivetti, 1977). The use of beer as a medicine lacks firm evidence, but its nutritional benefits include a contribution to the diet particularly with relation to B vitamins, minerals and antioxidants and a similar role to wine in protection from coronary heart disease (Bamforth, 2002). In the middle ages, ale was cloudy and full of protein and carbohydrate, making it a good source of nutrition for peasants and noble alike. Historically, beer was a safer and more palatable option than the available drinking water which was often drawn from polluted rivers. In fact, at the end of the 17th century, the weekly allowance for pupils of all ages at one English school was two bottles a day. Beer was also common in the workplace. Benjamin Franklin, who lived in London from 1757 to 1774, recorded the daily beer consumption in a London printing house in which the employees each had a pint before breakfast, a pint between breakfast and dinner, a pint at dinner, a pint at six o'clock and a pint when they finished work.

Today, beer is considered relatively safe to drink in moderation, but excess consumption is linked with cirrhosis, cancer and alcohol-related injuries. The two predominant proteins in beer, serpin-Z4 (45 kDa) and lipid transfer protein 1 (LTP1, 9 kDa), have been implicated as the major beer allergens causing urticaria (hives) and IgE-mediated anaphylaxis (Baker's asthma) (García-Casado, Crespo, Rodríguez, & Salcedo, 2001). Coeliac disease is a well understood, non-IgE mediated autoimmune disorder that results in gradual chronic gastrointestinal

inflammation as a result of exposure to gluten proteins. Comprehensive and quantitative peptide epitope mapping experiments using T-cells isolated from coeliacs have identified three key immuno-reactive peptides. These highly immunogenic peptides were derived from α -gliadin (ELQPFQPELPYPQ), ω -gliadin/C-hordein (EQPFQPEQPFQWQP), and B-hordein (EPEQPIEQQYPYPQ) and account for 90% of the coeliac-specific response elicited by the full complement of wheat, barley and rye proteins (Tye-Din et al., 2010). IgE mediated gluten-allergy, has a rapid onset and may be life threatening. Gluten intolerance has a largely unknown aetiology, but is an area currently subject to active research (Biesiekierski et al., 2011). All three conditions require maintenance of a lifelong gluten-free diet. Gluten is the general name for related water-insoluble, but alcohol-soluble grain storage protein families from wheat (glutenin and gliadins), barley (hordeins), rye (secalins) and oats (avenins). These proteins are collectively known as prolamins, due to a very high proportion of proline and glutamine which renders them recalcitrant to intestinal digestion. The lack of water solubility limits the carry-over of prolamins in beer to trace levels, measured in the tens of parts per million ($\mu\text{g/mL}$, ppm) level. However, the presence of gluten proteins in the finished product is still too high for safe consumption by coeliacs and gluten intolerants. Moreover, while the intact proteins have low solubility in water and thus may be removed by filtration, peptides liberated during malting and brewing may have increased solubility and remain in solution in the final product.

5. Proteins in beer – measurement

The accurate measurement of gluten levels in beverages and food-stuffs is not straightforward. A variety of methods have been developed (Schubert-Ullrich et al., 2009). Antibody-mediated methods, such as enzyme-linked immunosorbent assays (ELISA), are the accepted methods for measuring prolamins concentrations (more below). Electrochemical detection has also been reported in which an electrochemical immunosensor exploiting an antibody raised against the putative immunodominant coeliac disease epitope was developed (Nassef et al., 2008). A quartz crystal microbalance biosensor was successfully applied to the sensitive measurement of gliadin (Chu, Lin, Chen, Chen, & Wen, 2012) in ten commercial food products. Real-time PCR systems for the detection of gluten containing cereals (Zeltner, Glomb, & Maede, 2009) as well as wheat, rye and barley DNA as a measure of cereal contamination have been developed and are considered a highly sensitive method for gluten analysis complementary to immunological techniques (Mujico, Lombardia, Mena, Mendez, & Albar, 2011). Despite the success of PCR techniques in food products, similar studies in beer

tested positive for yeast DNA, but were negative for plant DNA (Hotzel, Müller, & Sachse, 1999), limiting their application in highly processed products such as beer. Additionally, fluorescence correlation spectroscopy (Varriale et al., 2007) and flow cytometry (Capparelli, Ventimiglia, Longobardo, & Iannelli, 2005) have also been used to measure gliadins in food. Many of these methods suffer from the lack of suitable calibration standards. Antibody based techniques are sensitive, but are limited to the detection of specific amino-acid epitopes which may not be present to the same degree in all prolamins. Conversely, mass spectrometry based methods deliver unsurpassed sensitivity and confirmation of the amino-acid sequence of the analyte and rapid progress has been made in this area.

6. Proteomics and mass spectrometry in food and beverage analysis

Proteomics is generally defined as the large scale study of proteins by mass spectrometry and has been readily adopted for the characterization and quantification of proteins, in particular within the field of disease biomarker evaluation (Carr & Anderson, 2008). The role of mass spectrometry in the analysis of food proteins and peptides has been comprehensively reviewed (Herrero, Simo, Garcia-Canas, Ibanez, & Cifuentes, 2012; Mamone, Picariello, Cairra, Addeo, & Ferranti, 2009; Picariello, Mamone, Addeo, & Ferranti, 2011). It is a powerful tool that can be readily applied to the food and beverage industries to address questions of food quality, safety and nutritional assessment.

6.1. Gel-based proteomics

Initially, the standard method for studying the proteome of a cell, tissue or organism was by using two-dimensional electrophoresis (2-DE) (O'Farrell, 1975) to separate proteins. The first dimension involves isoelectric focussing; proteins remain charged at all pH values other than their isoelectric point and when an electric potential is applied to the gel, typically containing an immobilised pH gradient (an IPG strip), the proteins migrate to their pI. The second dimension involves sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), usually in the presence of a strong reducing agent such as DTT. Under these strongly denaturing conditions, secondary and tertiary structural elements and protein complexes are fully disrupted, allowing the proteins to be separated by molecular weight alone. Smaller proteins migrate faster through the acrylamide gel which is composed of a molecular sieve which slows the larger proteins. The end result is a two-dimensional map of protein spots, with each protein resolved to a

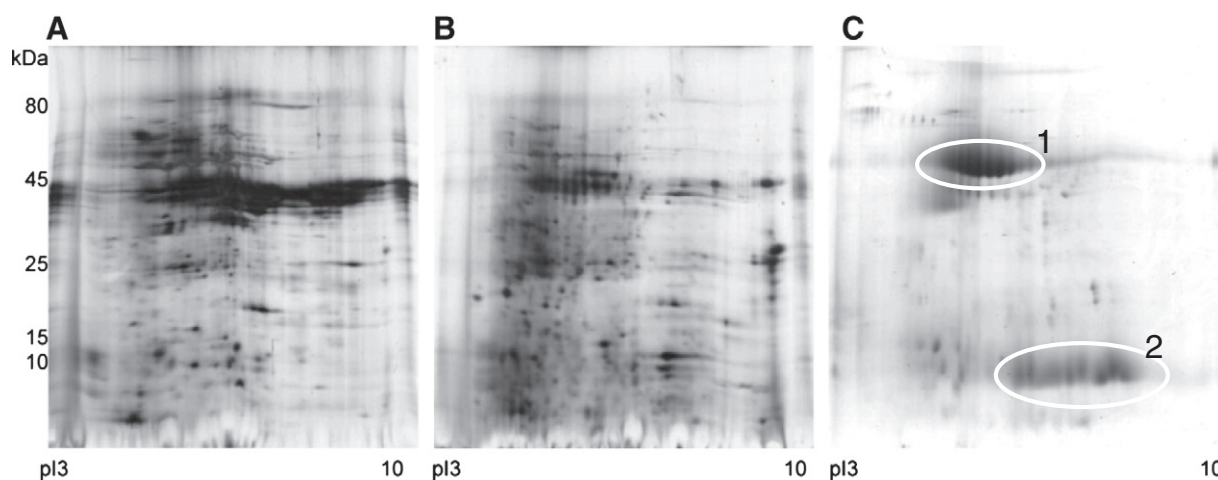


Fig. 1. 2D gel protein patterns of water-soluble proteins of barley (A), malt (B) and beer (C). Proteins were separated in the first dimension on an IPG strip pI 3–10 and in the second dimension on a 15% acrylamide SDS gel. The proteins were silver stained. The position of the two dominant protein families, serpin Z4 (1) and LTP (2) are seen as “protein trains” – a series of spots with similar molecular weight, but increasing pI due to glycation of lysines.

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Table 1
Barley proteins identified in proteomics studies of beer.

Accession (Uniprot)	Accession (NCBI/other)	Protein name	Sample type (beer/wort)	References
<i>Protein Z (serpins)</i>				
Q9ST57	gi 75313847	Serpin-ZZA	Beer	6
P06293	gi 1310677	Serpin-Z4	Beer and wort	1, 2, 3, 6, 7, 8
Q43492	gi 75282567	Serpin-Z7	Beer and wort	2, 3, 4, 6, 7, 8
Q40066	gi 75281963	Serpin-ZX	Beer	3, 8
<i>Lipid transfer proteins</i>				
P07597*	gi 19039	Non-specific lipid-transfer protein 1 (LTP1)	Beer and wort	1–8
	gi 47168353			
F2ED95*	gi 326533572	Non-specific lipid-transfer protein (LTP6)	Wort	6
Q5UNP2	gi 75251746	Lipid-transfer protein (LTP)	Beer	8
Q9SES6	gi 6492243	Predicted protein (Similar to LTP)	Beer	6, 8
F2EE76	gi 326491097	Non-specific lipid-transfer protein 2 (LTP2)	Beer and wort	1,2,6, 8
P20145	gi 128377			
<i>Hordeins</i>				
F2EGD5	gi 326501830	Predicted protein (similar to avenin like-A protein)	Beer and wort	6, 8
P06471	gi 123459*	B-hordein	Beer and wort	2, 6, 8
	gi 224385*			
N/A	gi 224386	B-hordein	Beer	2
Q0PIV6	gi 110832715	B-hordein	Beer	5
Q670S1	gi 51556918	B-hordein	Beer	5
Q40026	gi 18929	B-hordein	Beer and wort	2, 6
P06470	gi 123458	B1-hordein	Wort	6
Q40021	gi 809031	B1-hordein	Beer	5
Q40022	gi 75220900	B1-hordein	Beer	6
Q4G3S5	gi 122220131	B3-hordein	Beer	6
P17991	gi 123461	C-hordein	Beer and wort	6
Q40055	gi 75220910	C-hordein	Wort	6
Q41210	gi 75102504	C-hordein	Wort	6
Q40053	gi 75102206	Hor1-17 C-hordein	Wort	6
Q84LE9	gi 671537	D-hordein	Beer and wort	2, 6, 8
	gi 30421167			
P17990	gi 226755	Gamma-hordein-1	Beer	2, 6, 8
P80198	gi 1708280*	Gamma-hordein-3	Beer and wort	1, 2, 3, 5, 6
	gi 288709*			
Q6EEY9	gi 34329257	Gamma-hordein-3	Beer	5
Q6EEZ0	gi 75254865	Gamma-hordein-3	Beer	6
<i>Trypsin/α-amylase inhibitors</i>				
P13691*	gi 123970	Alpha-amylase inhibitor BDAI-1	Beer and wort	1, 3, 4, 6, 7, 8
Q546U1*	gi 75275305			
P16968	gi 2506771	Alpha-amylase inhibitor BMAI-1	Beer	3, 6, 7, 8
P28041	gi 18955	Alpha-amylase/trypsin inhibitor CMA	Beer and wort	5–8
	gi 585289			
P32936	gi 585290	Alpha-amylase/trypsin inhibitor CMB	Beer and wort	1, 2, 4, 6, 7, 8
P11643	gi 585291	Alpha-amylase/trypsin inhibitor CMD	Beer and wort	2, 3, 5, 6, 7, 8
O23982	gi 75219274	Alpha-amylase/trypsin inhibitor CMD	Beer	6
P01086	gi 19009	Alpha-amylase/trypsin inhibitor CME	Beer and wort	3, 5, 6, 8
	gi 1405736			
N/A	CONTIG2069	BTICMc	Beer	7
O49861	gi 2707916	BTI-CMe2.1	Beer	1
N/A	gi 2707924	BTI-CMe3.1	Beer	4, 7
N/A	gi 225102	Trypsin/amylase inhibitor pUP13	Beer	1, 2, 4, 7, 8
<i>Other inhibitors</i>				
Q40059	gi 19005	Chymotrypsin inhibitor 2	Beer	8
Q1ENF2	gi 109238647	Cystatin Hv-CPI6	Beer	8
P22244	gi 132577	Protein synthesis inhibitor I	Wort	6
P01053	gi 124122	Subtilisin-chymotrypsin inhibitor-2A	Beer	8
P16062	gi 124125	Subtilisin-chymotrypsin inhibitor CI-1A	Beer	6
N/A	CONTIG3945	Subtilisin-chymotrypsin inhibitor CI-1B	Beer	4
P01054	gi 124129	Subtilisin-chymotrypsin inhibitor CI-1C	Beer	6
<i>Defence proteins, stress response proteins and chaperones</i>				
Q40057	gi 509070	18 kd heat shock protein	Beer	8
Q96458	gi 1536911	17 kDa class I small heat shock protein	Beer	6
O22462	gi 2454602	Barperm 1	Wort	7
P28814	gi 114832	Barwin	Beer and wort	3, 6, 7
Q9M4E3*	gi 75264682	Hordoindoline-A	Beer	3, 6
Q5URW5*	gi 75287023			
Q9FSI9	gi 75172332	Hordoindoline-B1	Beer	3, 6, 8
	gi 54661047			
Q9LEH8	gi 75173928	Hordoindoline-B2	Beer	3

Table 1 (continued)

Accession (Uniprot)	Accession (NCBI/other)	Protein name	Sample type (beer/wort)	References
<i>Defence proteins, stress response proteins and chaperones</i>				
Q5IU17	gi 75105477	Hordoin-doline-B2	Beer and wort	6
Q5IUJ4	gi 75105495	Hordoin-doline-B2	Beer and wort	6
P08772	gi 1351242	Leaf-specific thionin DB4	Beer	3
P09617	gi 135794	Leaf-specific thionin	Beer	3
P93180	gi 1808651	Pathogenesis-related protein 4	Beer and wort	7, 8
F2EH52	gi 326504766	Predicted protein (similar to class I heat shock protein)	Beer	6
F2EEH7	gi 326491299	Predicted protein (similar to endosperm transfer cell specific PR60)	Wort	6
F2DYL9	gi 326519636	Predicted protein (similar to heat shock protein)	Beer	6
F2DGA8	gi 326490111	Predicted protein (similar to heat shock protein 17)	Beer	6
F2EHN7	gi 326513238	Predicted protein (similar to late embryogenesis abundant protein B19.1)	Beer	6
B5TWD1*	gi 199582501	Predicted protein (similar to late embryogenesis abundant protein 3)	Beer	6, 8
F2DW12*	gi 326503316			
F2EKY2	gi 326527459	Predicted protein (similar to late embryogenesis abundant protein)	Beer	6
Q94620	gi 14164979	Thaumatococcus-like protein TLP6	Wort	7
Q946Y8	gi 75164490	Thaumatococcus-like protein TLP8	Beer	6
<i>Seed storage proteins</i>				
Q9SBH7	gi 75336940	Beta-amylase	Beer and wort	6
Q03678	gi 75101315	Embryo globulin	Beer	6
F2EBM4	gi 326529599	Predicted protein (similar to cupin family protein)	Beer	6
<i>Enzymes</i>				
P23951	gi 116316	26 kDa endochitinase 2	Beer	6
Q40069	gi 157830301	Barley grain peroxidase 1	Wort	7
Q8W011	gi 18025342	Beta-D-xylosidase	Wort	7
B2CHJ4	gi 169666634	Calcium-dependent protein kinase	Beer	8
F2CXV3	gi 326511321	Nucleoside diphosphate kinase	Beer	6
F2CQP8	gi 326493416	Predicted protein (Similar to lactoylglutathione lyase)	Beer	6
Q5EXM3	gi 55832255	Putative glutamate decarboxylase	Beer	5
Q42829	gi 3913438	S-adenosylmethionine decarboxylase	Beer	5
P34937	gi 2507469	Triosephosphate isomerase, cytosolic	Beer	8
<i>Other barley proteins</i>				
Q949H0	gi 49036474	40S ribosomal protein S7	Beer	3
P62162	gi 49037476	Calmodulin	Beer	3
F2CSK4	gi 7431022	Glucose and ribitol dehydrogenase homologue barley	Beer	8
Q43472	gi 75319742	Glycine-rich RNA-binding protein blt801	Beer	3
Q5ITH7	gi 54661662	Grain softness protein	Beer	8
F2EL93	gi 326527681	Predicted protein	Beer	6
Q94IM5	gi 14275758	P-type ATPase	Beer	5
F2DHH7	gi 326494858	Superoxide dismutase [Cu–Zn]	Beer	8

The references are as follows: (1) Perrocheau et al. (2005); (2) Weber et al. (2009); (3) Fasoli et al. (2010); (4) Jimure et al. (2010); (5) Picariello, Bonomi et al. (2011); (6) Colgrave et al. (2012); (7) Jimure, Nankaku et al. (2012); and (8) Konecna et al. (2012).

* represents protein isoforms identified in two or more proteomics experiments that share high homology, but that were reported with different accession numbers.

particular set of coordinates, dependent on the pI and molecular weight (MW). The location of the protein is visualised by staining the proteins with a variety of protein reactive dyes such as Coomassie Blue or silver. Protein spots in gels can be automatically detected and excised from the gel for subsequent mass spectrometry (MS) based analysis in a high throughput manner (Page, Amess, Rohlf, Stubberfield, & Parekh, 1999). The disadvantage of this workflow is that only 30–50% of a proteome can be visualised using a typical 2-DE gel (Baggerman, Vierstraete, De Loof, & Schoofs, 2005). Moreover, membrane proteins are particularly difficult to solubilise (Molloy, 2000).

The identification of beer proteins has been undertaken using a range of analytical techniques. Early experiments using gel filtration, isoelectric focusing (Mazanec, Bobalova, & Slais, 2009) and ion exchange chromatography (Gorinstein et al., 1999) revealed the presence of two major classes of proteins of sizes ~44 kDa and ~10 kDa (Sorensen & Ottesen, 1978). The high molecular weight fraction was noted to be rich in carbohydrate and a large proportion of the lysines present were noted to be blocked suggesting glycation of the proteins at lysine (Sorensen & Ottesen, 1978). It has been estimated that up to 16% of the lysine content of the 44 kDa protein is glycosylated through Maillard reaction (Hejgaard & Kaersgaard, 1983). SDS-PAGE has been used to further examine the major proteins of beer and revealed that two polypeptides of size ~40 kDa were in fact protein Z (Curioni, Pressi, Furegon, & Peruffo, 1995), now known as serpin-Z4 and

serpin-Z7. Advances in separation science for complex samples have been reported with the application of divergent flow isoelectric focusing to the study of barley grain, malt and beer (Mazanec et al., 2009).

Fig. 1 shows the application of 2D gel electrophoresis to the examination of barley, malt and beer (Perrocheau et al., 2005). The loss of proteins in beer is apparent with the major proteins remaining belonging to the serpin (protein Z) and LTP families.

6.2. Mass spectrometry

A modern mass spectrometer consists of an ionisation source to introduce the sample into the mass spectrometer as a series of ions, one or more mass analysers to separate the ions according to their mass-to-charge (m/z) ratio and a detector to count the number of ions being transmitted. There are two main types of ionisation used in proteomics. Matrix-assisted laser desorption/ionisation (MALDI) (Karas & Hillenkamp, 1988) is most commonly coupled to time-of-flight (TOF) mass analysers and is particularly useful for the analysis of intact proteins, but is also applied to the study of peptides resulting from proteolytic digestion. Electrospray ionisation (ESI) (Fenn, Mann, Meng, Wong, & Whitehouse, 1989) is commonly used to couple high performance liquid chromatography (HPLC) directly with the mass spectrometer in the hyphenated technique LC–MS and is used with quadrupole (Q), TOF or hybrid, e.g. Q-TOF mass analysers. The use of a single mass

analyser allows the measurement of the m/z ratio and in turn the molecular weight determination. Peptide mass fingerprinting (PMF) was commonly employed in the past on MALDI instruments to determine the mass of each proteolytic peptide and the mass list was used to interrogate a theoretical list of peptide masses produced from *in silico* digestion of all proteins in a given database.

Tandem mass spectrometry (MS/MS) which involves multiple stages of mass selection enables mass determination as well as peptide sequencing through fragment ion analysis. Fragmentation is achieved by collision-induced dissociation (CID or collisionally-activated dissociation, CAD), a mechanism in which gas-phase peptides are accelerated through an electrical potential to high kinetic energy and then allowed to collide with neutral gas molecules (often helium, nitrogen or argon). In the collision some of the kinetic energy is converted into internal energy which results in bond breakage and the fragmentation of the molecular ion into smaller fragments. These fragment ions are then transmitted to a second stage mass analyser and then on to the detector. The analysis of a peptide mixture results in the generation of a spectral dataset that is searched against a database and the peptide identifications are used to reconstruct the original protein(s). It is also possible to identify individual peptides from spectra in a process known as *de novo* sequencing. However, this is somewhat time consuming and not applicable for complex samples.

The first comprehensive proteomic profiling of beer was conducted in 2005 using the combination of 2-DE (Fig. 1) and mass spectrometry (Perrocheau et al., 2005). The study investigated the major proteins found in barley, malt and finally beer and revealed that the proteins identified in beer were heat-stable and hence recovered in beer. In addition to proteins from the known barley albumins, disulfide-rich proteins implicated in plant defence survived the brewing process. The hordeins were detected in barley and malt, but were absent from gels run using beer with the exception of γ 3-hordein (Perrocheau et al., 2005).

Table 1 summarises the barley proteins identified in proteomic profiling studies of beer and wort. As the various studies have utilised

different protein databases (primarily versions of Swissprot and/or NCBI), each protein identified was interrogated by BLAST searching and assigned an NCBI accession (gi|number) or Uniprot accession. In a number of instances, similar proteins were identified and these have been grouped to aid in protein profile comparison. The lack of peptide identification information did not allow the peptide evidence to be mapped back to proteins, so it is possible that more isoforms have been reported than are actually present. The non-barley proteins identified in beer (proteins derived from wheat, rice, maize and other cereals) are presented in Table 2 and finally proteins from yeast are denoted in Table 3.

The major focus of most proteomic studies examining beer has been in regard to beer quality and characteristics (Steiner et al., 2011), with an emphasis on beer haze stability (Bei et al., 2011, 2012; Iimure et al., 2009; Robinson et al., 2007), foaming (Iimure, Kimura et al., 2012) and gushing (Hegrova, Farkova, Macuchova, Havel, & Preisler, 2009). Proteomic approaches using SDS-PAGE, RP-HPLC and capillary electrophoresis, have been used to compare the proteins present in malts, worts and beers made from barley (Cortacero-Ramirez, Hernainz-Bermudez de Castro, Segura-Carretero, Cruces-Blanco, & Fernandez-Gutierrez, 2003; Lookhart, Bean, & Jones, 1999; Silva et al., 2008). Furthermore, a recent study by Iimure and co-workers examined the changes to proteins throughout the wort boiling process using 2-DE combined with MALDI-TOF mass analysis (Iimure, Nankaku, Kihara, Yamada, & Sato, 2012). Konečná et al. recently reported the use of OFFGEL prefractionation in combination with 2D-gel electrophoresis and MALDI-MS/MS for the analysis of four lager beers (Konečna et al., 2012). This unique combination of techniques facilitated the identification of 30 barley proteins, 31 yeast proteins and 9 proteins from other sources.

6.3. Gel-free proteomics

There are two major workflows within gel-free proteomics: top-down and bottom-up. Top down proteomics refers to protein identification based on the intact protein and is typically performed on Fourier

Table 2
Cereal proteins identified in proteomics studies of beer.

Accession (Uniprot)	Accession (NCBI/other)	Protein name	Sample type (beer/wort)	References
Q9XFM4	gi 29839419	13S globulin seed storage protein 3	Beer	2
Q2PS07	gi 83416591	16 kDa allergen	Beer	2
Q2QL53	gi 122202965	Alpha-gliadin storage protein	Wort	6
Q94ML6	gi 7209265	Alpha-gliadin	Beer	2
Q41540	gi 21711	CM17 protein precursor	Beer	8
Q19A44	gi 108597921	Dimeric alpha-amylase inhibitor	Beer	2
B6UKV5	gi 209972037	Gamma-gliadin	Wort	6
Q9FS58	gi 10638433	Gamma-gliadin	Beer	2
N/A	gi 281335542	Gliadin/avenin-like seed protein	Beer	8
B7U6L5	gi 215398472	Globulin 3B	Beer	8
P14323	gi 20210	Glutelin	Beer	2
Q94IL5	gi 75250230	HMW glutenin subunit x	Beer	6
A9ZMG8	gi 164457873	HMW glutenin subunit y	Beer	2
F5A7G6	gi 329745049	Low molecular weight glutenin subunit	Beer	6
Q8W3V2	gi 17425212	Low molecular weight glutenin subunit	Beer	2
Q2PCC5	gi 84617213	Type 2 non-specific lipid-transfer protein	Beer	2
C4NFP4	gi 229610215	Omega-secalin	Wort	6
Q0DAI8	gi 297606280	Os06g0650100	Beer	8
A6N0C7	gi 149391359	Polyubiquitin containing 7 ubiquitin monomers	Beer	8
Q2A784	gi 89143120	Putative avenin-like A protein	Beer	2
Q571R3	gi 62484809*	Putative gamma-gliadin	Beer and wort	2, 6
	gi 75218679*			
P29385	gi 20159	Putative globulin	Beer	2
Q8W0H5	gi 18461191	Putative glutamate transporter	Beer	1
Q7XZB7	gi 32328625	High molecular weight glutenin subunit	Beer	8
Q84QY3	gi 115453373	Os03g0393900 (patatin-like phospholipase family protein)	Beer	8
Q8RZX3	gi 297720697	Os01g0915900	Beer	8

The references are as follows: (1) Perrocheau et al. (2005); (2) Weber et al. (2009); (3) Fasoli et al. (2010); (4) Iimure et al. (2010); (5) Picariello, Bonomi et al. (2011); (6) Colgrave et al. (2012); (7) Iimure, Nankaku et al. (2012); and (8) Konečna et al. (2012).

* represents protein isoforms identified in two or more proteomics experiments that share high homology, but that were reported with different accession numbers.

Table 3

Yeast proteins identified in proteomics studies of beer.

Accession (Uniprot)	Accession (NCBI/other)	Protein name	Sample type (beer/wort)	References
P61867	gi 48428723	Acyl-CoA-binding protein 2	Beer	8
A6ZTT5	gi 206558328	Alcohol dehydrogenase	Beer	3
P00330	gi 308153603	Alcohol dehydrogenase 1	Beer	3
P38772	gi 731652	Box C/D snoRNA protein 1	Beer	3
B5VL27	gi 6322303	Cell wall mannoprotein CIS3	Beer	3, 8
P38248	gi 187608884	Cell wall protein ECM33	Beer	3
P11353	gi 1708178	Coproporphyrinogen-III oxidase	Beer	3, 8
P00924	gi 171455	Enolase 1	Beer	3, 4, 8
P00925	gi 6321968	Enolase 2	Beer	1, 3, 8
P15703	gi 6321721	Glucan 1,3-beta-glucosidase	Beer	3, 8
Q876J3	gi 46395590	Glucan 1,3-beta-glucosidase	Beer	3, 8
P23776	gi 6323331	Glucan 1,3-beta-glucosidase I/II	Beer	3, 8
P12709	gi 6319673	Glucose-6-phosphate isomerase	Beer	3, 8
B7X717	gi 219564313	Glyceraldehyde-3-phosphate dehydrogenase	Beer	8
P00358	gi 219564301	Glyceraldehyde-3-phosphate dehydrogenase 2	Beer	3, 8
P00360	gi 6322409	Glyceraldehyde-3-phosphate dehydrogenase 1	Beer	3, 8
E7Q722	gi 3730	Glycolipid-anchored surface protein	Beer	8
P10591	gi 349747	Heat shock protein of HSP70 family	Beer	8
P54839	gi 1708241	Hydroxymethylglutaryl-CoA synthase	Beer	3
P10594	gi 124702	Invertase 1	Beer	3
P00724	gi 124703	Invertase 2	Beer	3
P47912	gi 1346423	Long-chain-fatty-acid-CoA ligase 4	Beer	3
Q03558	gi 6321973	Oye2p	Beer	8
P38013	gi 6323138	Peroxisomal protein type-2	Beer	8
P00560	gi 129930*	Phosphoglycerate kinase	Beer	3, 8
	gi 10383781*			
P00950	gi 6322967	Phosphoglycerate mutase 1	Beer	3, 8
Q07688	gi 6435746	Phosphorelay intermediate protein YPD1	Beer	4
Q07930	gi 13432019	Pre-mRNA leakage protein 1	Beer	3
Q03655	gi 2494678	Probable 1,3-beta-glucanotransferase GAS3	Beer	3
P53334	gi 6321718	Probable family 17 glucosidase SCW4	Beer	3, 8
Q04951	gi 6323964	Probable family 17 glucosidase SCW10	Beer	3, 8
P53301	gi 1723734	Probable glycosidase CRH1	Beer	3
P39709	gi 731298	Probable transporter SEO1	Beer	3
P07274	gi 6324696	Profilin	Beer	3, 8
P42835	gi 347595651	Protein EGT2	Beer	3
P46955	gi 968906	Protein NCA3, mitochondrial	Beer	3, 8
P47032	gi 6322382	Protein PRY1	Beer	8
P40472	gi 347595710	Protein SIM1	Beer	3
Q02457	gi 1729852	Protein TBF1	Beer	3
P38288	gi 6319638	Protein TOS1	Beer	3, 8
P36135	gi 486485	Protein UTH1	Beer	3, 8
A6ZTT3	gi 6321179	Protein VEL1	Beer	3, 8
P40103	gi 731388	Putative uncharacterized protein YER188W	Beer	8
P00549	gi 125609	Pyruvate kinase	Beer	3
P07267	gi 6325103	Saccharopepsin	Beer	3, 8
P22803	gi 6321648	Thioredoxin-2	Beer	3, 4, 8
P00942	gi 230406	Triosephosphate isomerase	Beer	1, 4, 8
	gi 6320255			
Q08960	gi 2501612	tRNA wybutosine-synthesizing protein 1	Beer	3
P50089	gi 1723755	Uncharacterized protein YGR237C	Beer	3
Q3E824	gi 97218363	Uncharacterized protein YOR020W-A	Beer	3
P09232	gi 6320775	Vacuolar proteinase B	Beer	8

The references are as follows: (1) Perrocheau et al. (2005); (2) Weber et al. (2009); (3) Fasoli et al. (2010); (4) Iimure et al. (2010); (5) Picariello, Bonomi et al. (2011); (6) Colgrave et al. (2012); (7) Iimure, Nankaku et al. (2012); and (8) Konecna et al. (2012).

* represents protein isoforms identified in two or more proteomics experiments that share high homology, but that were reported with different accession numbers.

transform ion cyclotron resonance instruments capable of high resolution. The more common bottom-up, or shotgun, proteomics involves the identification of peptides derived from an enzymatic digest, typically trypsin, of an entire protein extract. Drawing on the higher MS sensitivity for the detection of peptides compared to proteins, these peptide-centric approaches (Duncan, Aebersold, & Caprioli, 2010) enable a deeper proteome analysis than can be achieved by protein-level studies. These gel-free approaches may use multiple orthogonal separation strategies, such as the combination of strong cation exchange (SCX) chromatography followed by reversed phase HPLC to reduce the chance of peptide co-elution during mass spectrometric analysis. The advent of faster scanning instrumentation has circumvented the need for extensive peptide separation and it is now possible to identify thousands of peptides in a single LC-MS/MS analysis. The major

obstacle in gel-free proteomics is the correct reconstruction of proteins in complex mixtures from their tryptic end-products. This is particularly problematic where the genome has not been sequenced and not all proteins are known (or predicted), such that protein identification relies on the identification of orthologous proteins from other species (Pedreschi, Hertog, Lilley, & Nicolai, 2012). Furthermore, in plants such as wheat and barley many closely related protein isoforms exist complicating the bioinformatic analysis. A further limitation is the lack of quantitative information that may be obtained particularly in relation to post-translational modifications and/or protein isoforms. A number of labelling strategies have been employed to overcome this limitation, including isobaric labelling (e.g. ICAT, iTRAQ), however in this review we will focus the discussion on label-free approaches to peptide quantification.

Intact protein analysis has been used to study glycation of low molecular weight barley proteins occurring during the malting process (Bobalova & Chmelik, 2007; Lastovickova, Mazanec, Benkovska, & Bobal'ova, 2011). The final malts were noted to demonstrate marked differences in the presence of glycosylated forms of the proteins with LTP1 being modified by up to four hexose additions. Two-dimensional (2D)-HPLC and MALDI-TOF MS were used to monitor protein glycation during brewing by comparing barley grain and malt. An examination of the intact proteins, focusing on LTP1, and chymotryptic digests revealed a high level of glycosylated protein in malt, presumably due to the elevated temperature and the availability of glucose and maltose that the grains were exposed to during malting (Petry-Podgorska et al., 2010). MALDI-TOF MS fingerprinting of untreated beers, in which the intact protein and maltooligosaccharides were analysed, was a useful tool for distinguishing beers of the same brand that originated from different breweries (Sedo, Marova, & Zdrahal, 2010). The study of the oligosaccharide components of beers was demonstrated in a relatively new application of MALDI-TOF MS (Park, Yang, Kim, & Kim, 2012).

The beer proteome has been examined by a number of laboratories using gel-free approaches, particularly LC-MS/MS analyses. An evaluation of the proteome content of beer was undertaken after peptide capture using combinatorial peptide libraries (Fasoli et al., 2010). Twenty different barley protein families were identified, of which the Z-serpins and LTP1 were again found to be the most abundant (Table 1), as well as identification of 40 gene products from yeast (Table 3). A recent study by Picariello and co-workers (Picariello, Bonomi et al., 2011) utilised a range of proteomic techniques to examine two commercial Italian barley malt beers. They demonstrate that very few proteins survive the malting and the brewing process with the exception of serpin-Z4 and LTP1. LTP1 has been shown to be exceptionally resistant towards denaturants, heat and proteases (Lindorff-Larsen & Winther, 2001; Perrocheau, Bakan, Boivin, & Marion, 2006). Using nano-HPLC-ESI-MS/MS Picariello et al. demonstrated the presence of peptide fragments arising from the two dominant barley albumins, but additionally from hordein proteins, some of which contained potentially coeliac-toxic epitopes (Picariello, Bonomi et al., 2011). No

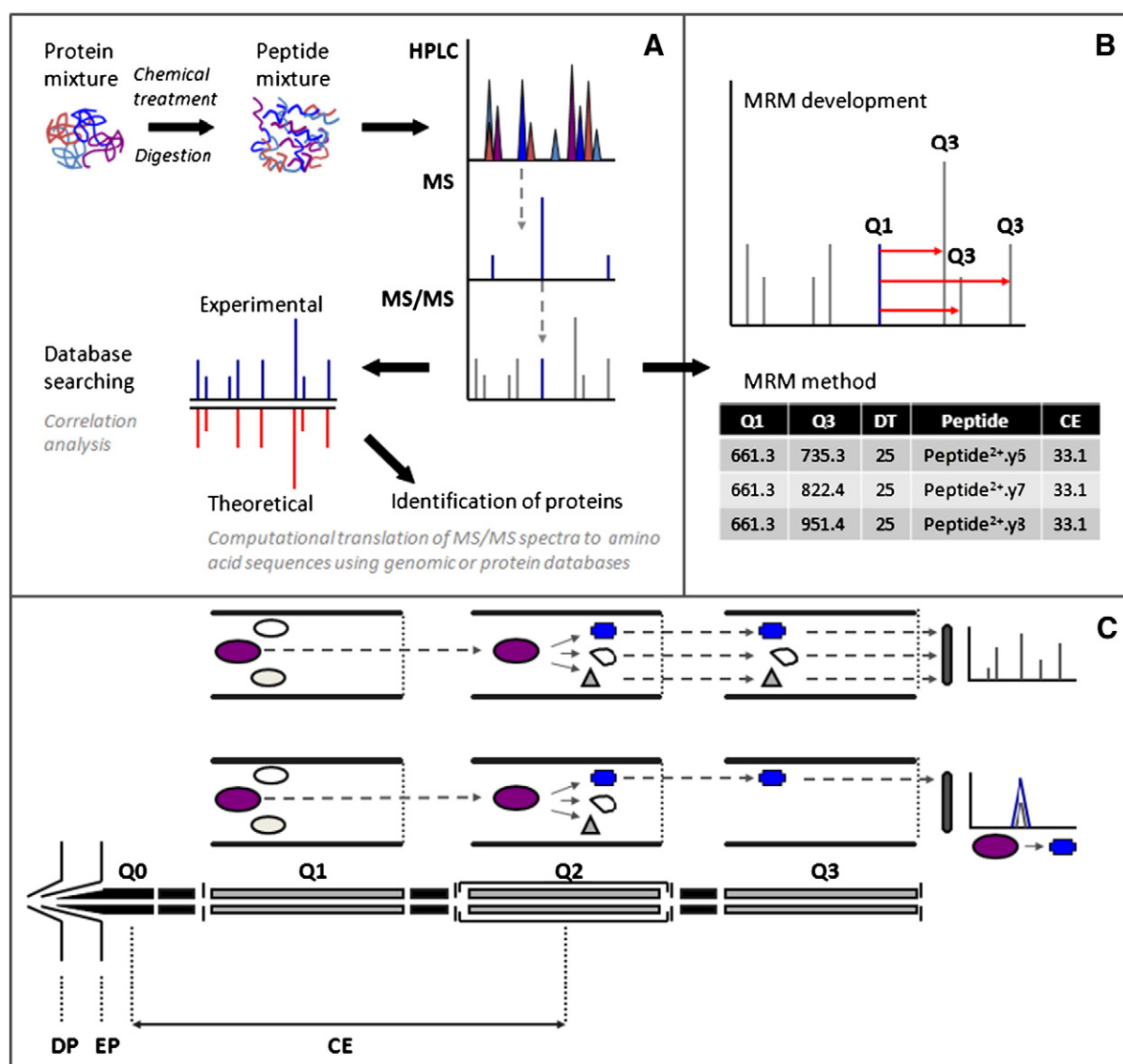


Fig. 2. General procedure for the identification of proteins and MRM development and analysis as performed on a triple quadrupole instrument. (A) After chemical processing and enzymatic digestion, the peptide mixture is subjected to chromatographic separation and mass analysis. MS/MS spectra are searched against an in silico generated theoretical mass spectra and peptide spectrum matches are used for protein identification. (B) Using the experimental MS/MS spectra, a list of MRM transitions is generated comprised of the Q1 m/z (precursor), Q3 m/z (fragment ion), dwell time (DT, ms), peptide ID and collision energy (CE, V). (C) A triple quadrupole mass spectrometer may be used in two modes: full scan MS/MS (top) or MRM (bottom). In both modes Q1 is set to a fixed m/z and Q2 is used for peptide fragmentation. In full scan mode, Q3 is scanned across a mass range, whereas in MRM mode, Q3 is fixed on the m/z of the pre-determined fragment ion.

intact hordein proteins were detected, but a number of peptide fragments derived from γ - and B-hordeins were present leading them to speculate that hordein are only present in trace amounts in beer.

As can be seen in Tables 1 and 2, the major proteins identified in beer belong to the serpin (protein Z) family, the lipid transfer proteins, hordeins, trypsin/ α -amylase inhibitors, defence proteins, stress response proteins and seed storage proteins. These protein families are again represented in the non-barley orthologs (Table 2). The MS-based analyses also yielded a large number of protein identifications derived from the yeast used in brewing. Among these, the enolases, phosphorylation related enzymes and heat shock proteins were abundant. A comprehensive map of ale-brewing yeast has been published and reports on the dynamics of the proteome during fermentation (Kobi, Zugmeyer, Potier, & Jaquet-Gutfreund, 2004).

6.4. Targeted mass spectrometry – quantification

The approaches described using MS as a tool to study gluten proteins have been global analyses, that is, they aim to identify all proteins present in a given sample. Multiple reaction monitoring (MRM or selective reaction monitoring, SRM) (Domon & Aebersold, 2006) mass spectrometry is a targeted approach that aims to identify and quantify a specific protein based on the peptides resulting from enzymatic digestion. MRM relies upon the inherent chemical and physical properties of the analyte, that is, its mass and its fragmentation pattern. MRM experiments are implemented on a triple quadrupole (QQQ)-based instrument. The first quadrupole (Q1) is used to select the peptide precursor ion which is then transmitted to the second quadrupole (Q2). The peptide precursor ion undergoes collision-induced dissociation (CID) yielding fragment ions that are transmitted to quadrupole 3 (Q3) for a second stage of mass selection. Only the fragment ions of interest are transmitted to the detector, all other ions are excluded (Domon & Aebersold, 2006) (Fig. 2).

One of the driving forces behind protein quantification in beer is the requirement for accurate measurement of gluten proteins. Coeliac disease (CD) occurs in approximately 1% of all populations studied worldwide and the only treatment involves the lifelong dietary exclusion of gluten proteins in wheat (gliadin and glutenins), barley (hordeins), rye (avenins) and in some individuals, oats (secalins) (Rocher, Calero, Soriano, & Mendez, 1996). Untreated coeliacs face a raft of adverse health outcomes including low bone mineral density and increased intestinal malignancy. Furthermore, an increasing number of the population appear to suffer from wheat and/or gluten intolerance. The current analytical techniques for gluten measurement are based on ELISA technology. The reliability of immunological methods depends on the specificity of the employed antibodies. For example, cross-reactivity with matrix proteins can result in false positives. The reactivity of the antibody may be altered by protein modifications that occur on heating or during processing. Protein hydrolysis may release peptides that retain their allergenic potential, but are unable to be detected by sandwich-ELISA based tests. The commercial antibodies were based on peptide epitopes from secalins (Mendez R5 antibody) (Kahlenberg et al., 2006) and ω -gliadin (Skerritt antibody) (Hill & Skerritt, 1990). These kits suffer from a variety of issues, including the under- and over-representation of hordein families. The R5 antibody is unable to accurately detect and quantify barley gluten (hordeins) in beer (Thompson & Mendez, 2008). The sandwich R5 ELISA is able to quantify native and heated gluten, but over-estimates hordeins (Kanerva, Sontag-Strohm, Ryöppy, Alho-Lehto, & Salovaara, 2006). The use of competitive ELISA is more sensitive, but it is difficult to match the prolamins being measured with the competitor prolamins supplied in the ELISA kit. Recently, the use of the competitive R5 ELISA method for measuring the level of gliadins in 28 commercial beers was described. However, this study did not assess the hordein proteins derived from barley, only proteins containing the QQFPF motif (Guerdrum & Bamforth, 2011). Second generation sandwich ELISA methods have been developed using antibodies raised against specific

immuno-dominant peptides involved in the biological response of coeliac disease, e.g. G12 and A1 monoclonal antibodies have been raised against the toxic 33-mer of α -gliadin (Moron, Bethune et al., 2008; Moron, Cebolla et al., 2008). The use of LC-MS/MS in the study of gluten proteins in beer has been recently reported (Weber, Cleroux, & Godefroy, 2009) and this technology was proposed as a feasible alternative to ELISA-based methodologies.

In our laboratory, we have comprehensively characterized the proteome of wort and beer with an emphasis on identification on the suite of prolamins proteins present (Colgrave, Goswami, Howitt, & Tanner, 2012). In this study, we provided spectral evidence for three previously characterized prolamins, eight prolamins with only transcript evidence and 19 predicted prolamins. A further nine prolamins were identified by searching the complete spectral set against a translated EST database. Additionally, this study included a comprehensive characterisation of all proteins in barley flour, wort and beer from a wild-type barley line (cv Sloop) and two hordein null lines. The proteins detected, in wort and beer, have been included in Tables 1 and 2. Using the protein profiles, an MRM LC-MS method was developed and applied to three beers brewed from selectively-bred barley lines and 60 commercial beers. This was the first application of MRM-MS to the relative quantification of the major hordein protein families. The technology was successfully used to confirm the presence of hordeins in beer and the absence of hordeins in commercial gluten-free beers. The B-hordeins, D-hordein and γ -hordeins were detected in the majority of beers. An avenin-like A protein, previously reported in beer by Picariello, Bonomi et al. (2011), showing homology to B- and γ -hordeins was additionally detected in ~50% of the beers. Traces of C-hordein proteins were detected in undigested beer as proteolytic fragments resulting from the brewing process at very low levels, but these could not be quantified owing to the random nature (non-tryptic) of the cleavage events. Many of these proteolytic end-products present in untreated beer contained potentially immunotoxic epitopes (QQFPF, QQLFP) and that may react with antibody-based assays.

One of the most exciting studies of recent years utilised the power of LC-MS/MS for the quantification of immunogenic wheat peptides (Sealey-Voyksner, Khosla, Voyksner, & Jorgenson, 2010). In this work, a cocktail of proteolytic enzymes (pepsin, trypsin and chymotrypsin) were employed to model gastric and duodenal protein digestion and resulted in the release of 25 target peptides derived from wheat gluten. MRM mass spectrometry combined with the use of synthetic peptide standards enabled the quantification of six of the potentially immunogenic peptides in a range of native and processed products, including a light beer.

6.5. Data interpretation and database searching

The current proteomic guidelines, as decided at the Paris workshop (Bradshaw, Burlingame, Carr, & Aebersold, 2006), are that all protein identifications must be supported by experimental evidence and detailed information about how the identifications are made. The generally accepted criteria for the positive identification of a protein are that it is identified by two peptides with at least 95% confidence. For single peptide identifications and peptides containing post-translational modifications, manual validation and spectral inclusion are required for publication. One of the current challenges for high-throughput proteomics is to use database search results from large numbers of MS/MS spectra in order to derive a list of identified peptides and their corresponding proteins. This task necessarily entails distinguishing correct peptide assignments from false identifications among database search results. For small datasets, manual validation and removal of false identifications may be possible, but for large datasets, automation of this process is necessary. To this end, comprehensive proteomic profiling experiments are typically accompanied by a false discovery rate (FDR) analysis in which the spectral dataset is searched against a forward target (true) database and against a reversed or shuffled decoy (false)

database (target-decoy approach (Elias & Gygi, 2007)). The number of true positives is divided by the total number of identifications (true and false) to yield the FDR. The majority of proteomics studies report proteins identified at a 1% global FDR.

One of the major challenges with bottom-up proteomic studies is the protein inference problem (Nesvizhskii & Aebersold, 2005). Introducing a digestion strategy early on in the analysis results in a loss of connectivity between proteins (the targets) and peptides (the analytes). The same peptide sequence can occur in multiple different proteins, either in unrelated proteins or in protein isoforms. The identification of these shared peptides leads to ambiguity in protein identification. Distinguishing between proteins of high sequence similarity relies on the identification of unique or distinct peptides (often referred to as prototypic peptides), but this requires high protein sequence coverage and is not always achievable in complex proteomics analyses.

This problem is exacerbated in proteomics studies of plants, in particular wheat and barley, wherein large numbers of closely-related protein isoforms are expressed. Barley (*Hordeum vulgare*) is a diploid organism with a single copy of each gene. However, the genetics of bread wheat (*Triticum aestivum*), a hexaploid, is much more difficult. Modern bread wheat arose some 10,000 years ago due to a spontaneous hybridisation event between a tetraploid and a diploid ancestor (Matsuoka, 2011). That is, bread wheat contains three separate and interacting genomes, originally from three diploid ancestors. There is a high degree of synteny (the physical co-localization of genetic loci on the same chromosome within species), homology (identity at the DNA and protein level) and duplication between the separate genomes, making molecular identification of a specific protein locus difficult. Furthermore, many of the cereal prolamins are rich in proline and glutamine. These proteins have a few basic residues and trypsin digestion may yield no usable peptides (C-hordein, Q41210), a few (3) usable peptides (B-hordein, P06470) or a good selection (10, D-hordein, Q84LE9). The number of available peptides is dictated not only by digestion constraints, but also by the size of the protein. In these cases, alternate proteases, such as chymotrypsin, which cleaves hydrophobic proteins more frequently than trypsin, or protease cocktails may be employed to increase the sequence coverage and decrease the ambiguity of protein identification.

As detailed above, large scale proteomics studies rely on identification of proteins by reconstruction from peptide spectrum matches (PSMs). If all peptides are distinct, that is they map to a single protein entry in the database, the correct number of proteins will be called, but if shared peptide evidence is used to identify multiple proteins, then the final protein list will be inflated. A minimalist approach is recommended whereby only proteins sufficient to explain all peptide evidence are reported. While the identification of multiple peptides mapping to a single protein may be enough to confirm its existence, it does not provide information as to the proteins state or form. To conclusively identify a protein in its mature form, complete sequence coverage is required and is infrequently obtained. Thus the identification of proteins in beer and other heavily processed products must be limited to the identification of the region of the protein for which there is coverage. In this respect approaches such as 2D-gels and top-down proteomics that examine intact proteins are particularly useful.

The choice of database is critical to the success of the proteomics study. One of the underlying assumptions in proteomics is that the databases interrogated are complete and contain all proteins and variants, however, this is rarely the case (Duncan et al., 2010). Database searching relies on the quality of the database searched – a protein can only be identified if it is present in the database. In the case of unsequenced or poorly sequenced genomes and therefore incomplete proteomes, many spectra may not be matched, despite their high quality, or may be assigned to an incorrect sequence. In these circumstances, the use of species specific databases will result in a decreased number of protein identifications as the proteins identified are limited to those present in the database. To overcome this issue, searches are carried

out against a higher taxonomy level, wherein protein identification relies on the identification of orthologous proteins in related species. For example, in the case of beer, which may contain proteins from barley, wheat, other cereal adjuncts, hops and yeast, we chose to search all proteins in the grass family Poaceae (Uniprot–Poaceae: 348,777 proteins) rather than restricting the search to *Hordeum* (annual and perennial grasses including barley; Uniprot–*Hordeum*: 26,060 proteins). This strategy will increase the number of redundant proteins that are identified, but enabled the highest number of identified proteins. A further complication is sequence redundancy as databases may contain additional proteins resulting from sequencing errors or from partial mRNA sequencing efforts. Databases such as Swissprot (Uniprot) and RefSeq (NCBI) are highly curated and have minimal redundancy.

7. Conclusions and future directions

In this review, we have discussed the application of mass spectrometry and proteomics to the study of beer, from humble beginnings as a grain to the final drinkable product. The major contributions to this field have focused on the characterisation of changes in proteins during brewing and the importance of individual beer components to beer haze formation and stability, foaming and gushing. The advent of faster, higher performance mass spectrometers has led to an increase in the number of studies exploring the beer proteome. Over the last few years, a number of studies have sought to examine the beer proteome for the presence of potential allergens, especially the proteinaceous gluten components, an area of analysis that has been exclusively undertaken using an antibody-based screening. While ELISA technology has been successfully applied to the quantification of wheat gluten in underivatised products, a number of issues limit the usefulness of ELISA in the analysis of hydrolysed and/or modified gluten present in food and beverages. Mass spectrometry is the only non-immunological method presently available to detect (with high specificity) glutenins, gliadins and related prolamins in flours and in food samples. Quantitative proteomics may overcome some of the issues with ELISA, however, there are still many significant challenges to overcome, namely the unambiguous identification of proteins and the definitive characterisation of the mature form of the protein identifications.

It seems that we have gone full circle. While beer was considered a safe alternative to water and a good source of nutrition in the pre-industrial era, we now concern ourselves with an understanding and limitation of the potential health problems associated with its consumption. Mass spectrometry is a key analytical tool and holds great promise in its application to food safety through the detection of microbial contamination and the occurrence of allergens, food quality and for monitoring key components (Aiello et al., 2011; Mamone et al., 2009; Picariello, Mamone et al., 2011). Food proteomics is beginning to exert influence across multiple aspects of the food chain (agriculture, food production, food safety and quality assurance) (Mamone et al., 2009). Mass spectrometry offers a highly sensitive, accurate and high throughput alternative to antibody-based screening methods and is expected to play a major role in food and beverage regulation in the coming years.

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