ORIGINAL ARTICLE

Untargeted metabolomic profiling of 100% malt beers versus those containing barley adjunct

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Abstract

Why was the work done: The incorporation of unmalted barley at high proportion in the grist can introduce unfavourable bitter and astringent characteristics to beer, resulting in an upper limit on the proportion used. The flavour active compounds from raw barley which contribute these characteristics to the beer remain to be identified.

How was the work done: This study used non-targeted metabolomics to determine non-volatile metabolites which could contribute to flavour differences when brewing with barley. Three beers were analysed using ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole time-of-flight mass spectrometer (qTOF) with an electro-spray ionisation source (ESI). One beer was produced using 100% malt and two beers with a grist of 15% barley and 85% malt (beers A and B). The barley was used untreated (beer A) or treated using a proprietary process (beer B). The metabolomic profiles of the three beers were compared and statistically different molecular features were annotated via analysis of MS2 spectra.

What are the main findings: Several of the main differential molecular features were nitrogenous peptides and purine derivatives. This was attributed to the lack of the malting process and associated proteolytic enzyme activity reducing the extent of protein and peptide breakdown in the unmalted barley. Several of the identified peptides had amino acid residues which are known to cause bitter and kokumi (rich) taste in beer, which could explain the bitterness when brewing with unmalted barley.

Why is the work important: A non-targeted approach offers new insights into non-volatile molecular features in beer that have not been previously identified with targeted analyses. Accordingly, this work identifies metabolites and groups of compounds which have not been previously considered when investigating the unfavourable bitterness and astringency associated with the use of unmalted barley.

Keywords

unmalted barley, metabolomics, adjunct, flavour, UHPLC

Introduction

The brewing industry has focused goals on sustainability, seeking new approaches to reduce water consumption, energy use and greenhouse gas emissions. Reducing the requirement for malting could help improve sustainability, as the process contributes substantially to water and energy use (Krieger and Eiken 2020). There is a motivation to replace malt with unmalted barley (Aastrup 2010; Kok et al. 2019) as it has been estimated that non-renewable energy can be reduced by 56% when replacing malt completely with unmalted barley (Krieger and Eiken 2020). Indeed, brewing with 100% unmalted barley can be achieved using exogenous enzymes (Aastrup 2010; Steiner et al. 2012; Zhuang et al. 2017). However, key amylolytic, cytolytic and proteolytic enzymes required for barley degradation are synthesised during the malting process. Therefore, at high adjunct incorporation rates, exogenous enzymes are necessary to compensate for the enzymes that are synthesised de novo during malting.

The proportion of unmalted barley in the grist is not restricted by brewing technology, but rather by the sensory quality of the beer. Replacing a high proportion of malt in the grist bill with unmalted barley, results in a change in the bitterness and mouthfeel (Kunz et al. 2012; Yorke et al. 2021). Our previous study found that beer produced with 60% unmalted barley (but without exogenous enzymes) had increased bitterness and astringent character compared to an all-malt control. Indeed, the inclusion of unmalted barley had a notable influence on the temporal quality such that the beer had a high intensity of lingering bitterness (Yorke et al. 2021). Kunz et al (2012) found that beer produced with 90% raw barley showed a detectable change in bitterness, to a more astringent and abrasive bitterness. However, using a proprietary and confidential process, the unfavourable bitterness and astringency was minimised when using 60% unmalted processed barley into the grist bill (Yorke et al. 2021). Indeed, the bitterness and astringency characteristics in the beer produced with the processed unmalted barley were lower than those for an all-malt control. This suggests that beers can be produced using high levels of unmalted barley without negatively impacting the sensory profile (Yorke et al. 2021).

Polyphenols have been associated with bitterness in beer and around 70-80% of beer polyphenols are derived from the barley grain (Wannenmacher et al. 2018). Studies have used targeted high performance liquid chromatography (HPLC) to determine bitter phenolic compounds in beer (Goiris et al. 2014; Gribkova et al. 2022; Oladokun et al. 2016b). However, it has been reported that there is little variance in phenolic compounds between beers made with malt or with unmalted barley. Kunz et al (2012) found that beers produced with 0-50% unmalted barley showed comparable results in polyphenol and anthocyanin content with only a slight decrease in beer produced with 50-100% barley compared to an all-malt beer. Similarly, van Donkelaar et al (2016) found that increasing the proportion of unmalted barley marginally decreased the content of polyphenol and anthocyanogen. Accordingly, it is unlikely that phenolic compounds are responsible for the reported changes in bitterness and astringency when brewing with unmalted barley (Kunz et al. 2012; Yorke et al. 2021).

Metabolomics is the study of small molecular weight compounds (typically < 2000 Da) that are reactants or products of metabolism. Metabolites in beer originate from diverse sources including malted barley, adjuncts, hops and yeast (Heuberger et al. 2012). Metabolomic analyses can be classified as either 'targeted' or 'untargeted'. Targeted analyses focus on pre-defined groups of metabolites such as the flavour-active volatile metabolites in beer including aldehydes, higher alcohols and esters (Riu-Aumatell et al. 2014; Rossi et al. 2014). The non-volatile metabolites of beer including carbohydrates, phenolic compounds and hop acids have been determined by targeted liquid chromatography methods (Das et al. 2014; Oladokun et al. 2016a; Cheiran et al. 2019). In contrast, untargeted metabolomics focuses on the detection of many groups of metabolites to obtain a fingerprint of the sample without necessarily identifying or quantifying them. Statistical analysis can then be applied to the extracted molecular features to identify key differentially expressed molecular features between beer samples. Untargeted metabolomic profiling can provide a means to separate and identify both volatile and non-volatile flavour active fractions in beer, which can improve flavour detection and our understanding of flavour active compounds in beer.

Untargeted metabolomic profiling using ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) has been used to determine differential metabolites between alcoholic and non-alcoholic beer (Andrés-Iglesias et al. 2014), beers stored at different storage temperatures (Heuberger et al. 2012) and beers produced from different brewing procedures (Gallart-Ayala et al. 2016). Hughey et al (2016) showed electrospray ionisation quadrupole time of flight mass spectrometry (ESI q-TOF MS) to be a useful tool for identifying key differential compounds relating to flavour when investigating the use of different hop varieties in India pale ales. In this work, untargeted metabolomic analysis was used to identify previously unexplored compounds associated with the inclusion of unmalted barley in the grist which could contribute to the sensory differences found with adjunct beers.

Non-targeted metabolomics was used to determine differential non-volatile metabolites which could contribute to the differences in bitterness profile caused by the iuse of processed and unprocessed unmalted barley. Three beer samples (100% malt, 15% untreated barley (A)/85% malt and 15% treated barley (B)/85% malt) were analysed using ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole time-of-flight mass spectrometer with electrospray ionisation source (ESI). The 15% adjunct rate was used as it was high enough to give rise to readily measurable fold-changes in metabolite concentration, but low enough not to require substantial changes in brewing practice. Thus, the three beers were all brewed using identical protocols, such that their composition could be compared in terms of the raw materials used. The same barley was used with 'A' (untreated) and 'B' treated with the proprietary process. The metabolomic profiles of the three beers were compared, and analysis of MS^2 spectra used to putatively identify compounds that were novel in adjunct beers.

Materials and methods

Brewing materials

Lager malt (variety: Planet) was supplied by Soufflet Malt, Burton-on-Trent, UK. Unmalted barley (a blend of Planet and Laureate) was supplied by

Frontier Agriculture Ltd., Lincolnshire, UK. Zeus T90 hop pellets with 15.3% α-acid content and 30-40% co-humulone composition were sourced by Simply Hops (Tonbridge, UK). Saflager W34/70 dry lager yeast was obtained from Fermentis (France). The barley was used 'as is' (barley A) or treated using a (confidential) proprietary process (barley B).

Chemicals and reagents

LC-MS grade formic acid and acetonitrile were obtained from Fisher Scientific (Loughborough, UK). Type 1 high purity water was from a Lab Pro PURA Q+20 system (SLS, Nottingham, UK). Sodium phosphate dibasic dodecahydrate, potassium phosphate monobasic, ninhydrin, fructose, ethanol, potassium iodate and glycine were from Sigma-Aldrich (Dorset, UK).

Beer and sample preparation

The three beer samples used for this study were an all-malt control and two beers produced from a grist of 15% barley/85% malt. Beer A used untreated barley and beer B the barley was treated using a (confidential) proprietary process. The three beers are referred to as 100% malt, 15% (untreated) barley (A) and 15% (treated) barley (B). The 15% incorporation rates were chosen as these beers were produced without balancing the C:N ratio of wort or micronutrient levels, ensuring that any substantial metabolomic differences are likely to be due to changes in adjunct incorporation rates and not caused by free amino nitrogen (FAN) supplementation.

The test beers were produced at pilot scale using a 40 L Briggs brewline at the University of Nottingham. For the all-malt beer, 7 kg of malt was used and for the 15% barley beers, 5.95 kg of malt was mixed with 1.05 kg of unmalted barley. Grists were milled with a Roppi-250 roller mill (Robix, Veszprém, Hungary) at a gap setting of 0.4 mm. The liquor to grist ratio used was 2.5:1 and the mash-in temperature was 65°C with an addition of 90 mg/L calcium chloride. This was followed by an 85 min mash profile (65°C for 60 min, 72°C for 10 min and 78°C for 5 min). The mash was separated in a lauter tun and sparged with 8 L of water. The worts were boiled with T90 Zeus hop pellets for 60 min with a target final

bitterness of 20 BU. The wort was adjusted with brewing liquor to 16°P at the end of the kettle boil. The worts were cooled and transferred into single 28 L stainless steel conical fermenters (The Grainfather, New Zealand). SafLager W34/70 dried yeast was pitched at 1 g/L at 15°C with static fermentation for 10-12 days at 12-18°C, followed by maturation at 4°C for 4 days. All beer samples were diluted to a 4% ABV, with 100 mL of the green beer stored at -20°C for analysis.

The alcohol content (ABV), original gravity (OG) and final gravity (FG) were measured using an Alcolyzer Plus connected to a DMA 4500 densitometer (Anton Paar, Graz, Austria). The pH of the wort and beer was determined using a 3510 pH meter (Jenway, Stafford, UK). The wort and beer free amino nitrogen content was measured using the ninhydrin method (Analytica EBC 8.10.1). The physicochemical properties of the wort and beer samples are reported in Table 1.

Sample preparation

Sample preparation and acquiston of high-resolution MS data were based (with some modifications)on the approach of Gallart- Ayala et al (2016). The test beers were degassed via sonication (20 min), diluted 1:1 with distilled water in replicates of 5 and filtered using 0.2 µm Whatman puradisc syringe filters (Sigma-Aldrich, Dorset, UK). The filtered samples were stored at -20°C in 1.5 mL microcentrifuge tubes. On the day of analysis, samples were thawed at 5°C, vortexed for 1 minute and transferred to 2 mL amber HPLC vials and capped.

Metabolomic analysis by UHPLC-qTOF-MS

LC-MS/MS analysis was performed using an Agilent 1260 Infinity II UHPLC, coupled to an Agilent 6546 tandem Quadrupole – Time of Flight mass spectrometer (Agilent Technologies, Cheadle, UK). The chromatographic separation was performed using a Kinetex C18 column (2.6µm, 150 x 2.1mm; Phenomenex, Macclesfield, UK) held at 40°C. Solvents A and B comprised 5% versus 95% respectively of acetonitrile in water (v/v) plus 0.1% formic acid. The mobile phase flow rate was 0.3 ml/min, starting from 0% B, with a ramp to 20% B at 10 minutes and to 100% B at 13 minutes. This condition was held for 2 min, and then returned to starting conditions by 15.6 minutes, with a further 3.4 minutes of reconditioning.

The ESI source used drying gas and sheath gas temperatures of 320°C, and 350°C respectively, drying gas and sheath gas flows of 8 and 11 L/min, with the nebuliser set to 35 psi. VCAP and nozzle voltages were set to 3500 V and 1000 V respectively, with fragmenter, skimmer and octopole RF voltages of 110, 65 and 750 V respectively. MS1 data were acquired in both (+) and (-) ESI, between m/z 100-3200. MS2 data were acquired in auto MS-MS mode with collision energies fixed at 10, 20 and 40 V, using separate preferred ion lists to avoid co-elution of targets within 0.2 min.

A single pooled QC sample for column conditioning and quality assessment was created using equal volumes of beers brewed with barley adjuncts to 45% (w/w). For MS1 acquisition, the column was conditioned with 8 x 5 µL injections of the QC sample. Thereafter, samples were injected in a randomised order, with a QC sample injected every five analytical runs.

Table 1.

Analysis of wort and beer samples.

	Original Gravity (°P)	Final Gravity (°P)	High gravity ABV (%)	Final ABV (%)	Wort FAN (mg/L)	Beer FAN (mg/L)	Mash pH	Wort pH	Beer pH
100% Malt	15.9	2.4	7.39	4.01	261	110	5.44	5.39	4.45
15% Barley (A) - 85% Malt	15.6	2.8	6.83	4.08	218	104	5.36	5.34	4.28
15% Barley (B) - 85% Malt	16.6	3.2	7.43	4.07	210	109	5.37	5.34	4.27

For data analysis, the Total Ion Counts (TICs) of repeat QC injections were visually assessed to check comparability of runs throughout the data set. Global MS1 features (peak height >4000) were first extracted using Mass Profiler software (MP; v10, Agilent Technologies), and exported to a common .CEF file for each polarity. Thereafter, files for each replicate group were time aligned to the central QC sample in Profinder software (v10, Agient Technologies), then features extracted (peak height >3000) in the 'batch targeted' mode using the MP .CEF file as a template. Extracted files were then exported to Mass Profiler Professional (MPP; v15, Agilent Technologies) for statistical analysis, and initial principal component analysis (PCA) plots generated to assess data quality. To isolate features which differed according to adjunct usage, features were initially filtered on the basis of significant difference from control ($p<0.01$; one way ANOVA) and then fold change (>50 compared to control) to focus on compounds novel to adjunct beers. The shortlisted features were further limited to peak heights of >10,000 and exported as preferred inclusion lists for MS2 auto MS-MS analysis.

Following the second round of data acquisition, features triggering MS-MS acquisition were extracted using Mass Hunter Qualitative Analysis software (v10, Agilent Technologies), and the resulting spectra exported to Sirius GUI software (v4; Dührkop et al (2019) for putative identification. The Sirius, CSI:Finger ID (www.csi-fingerid.org/; Dührkop et al. 2015) and Canopus (Dührkop et al. 2021; Feunang et al. 2016) modules were respectively employed to provide a prediction of *de novo* chemical formulae, identity match scores and to predict chemical class. All searchable databases (as of Spring 2021) were employed for CSI:Finger ID analysis.

Results and discussion

Quality assurance

Once conditioning was complete, visual inspection of Total Ion Count (TIC) plots of QC samples confirmed there was no problematic drift in retention time or overall abundance during acquisition in + and − ESI modes. Principal component analysis on the initial extracted features (>3000 peak height, for 3D plot see Figure 1) showed a clear separation in the metabolomic features from the three beer samples, predominantly along the X and Y axes. As expected, features from the QC samples did not cluster centrally, as the sample included several other beers in addition to those investigated in the current study. A considerable number of features were identified in the initial pool (14723 in positive mode, and 6581 in negative mode) which required the application of criteria to shortlist key molecular features.

Figure 1.

3D Principal component analysis (PCA) plots for the UPLC-MS ESI+ data of the extracted features (>3000 peak height). QC samples are grey circles, 15% (untreated) barley (A) are yellow circles, 15% (treated) barley (B) are red circles and 100% malt are blue circles. The PCA plot reports the analysis of five replicates of samples.
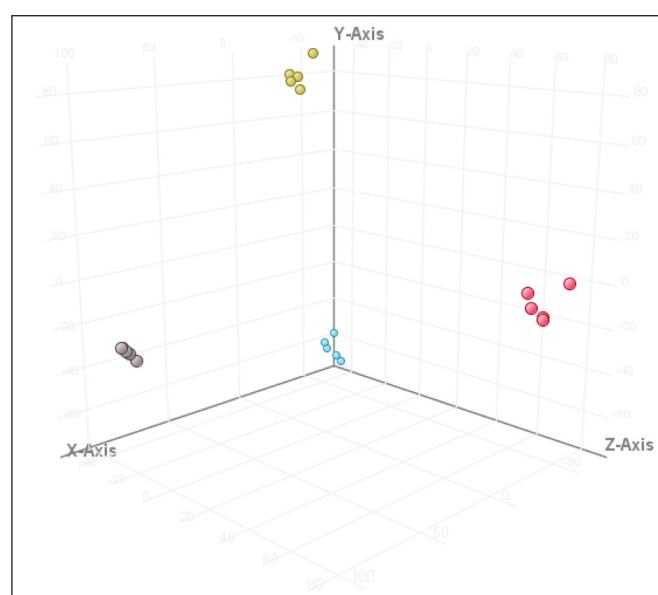
Identification of molecular features differentiating the beers

To determine molecular differences between the barley beers and the all-malt beer, analysis was applied to filter key differential metabolomic features. Three criteria were used in the analysis: (i) the feature is significantly different (p<0.01) to another sample in the sample set; (i) the peaks differed in intensity with a >50-fold change, (iii) and the feature had a peak height of >10,000. The most abundant features were focused on to facilitate downstream identification of the differentially expressed features. This does not mean that molecular features with low abundance would not be important for sensorial properties. However, molecular features in high abundance have a greater likelihood of positive identification.

Using these criteria, 87 features (68 ESI+ and 19 ESI-) were identified as significantly higher in 15% barley (B, treated) compared to 15% barley (A, untreated) and/or 100% malt. Further, it was determined that 92 features (79 ESI+ and 13 ESI-) were significantly higher in 15% barley (A) compared to 15% barley (B) and/or 100% malt.

So as to identify the shortlisted molecular features, the MS spectra were searched against Sirius GUI software and CSI:Finger ID scores were used to identity match scores for the predicted chemical formulae and chemical class compared to a searchable database. For the whole data set, the SIRIUS score ranged from 0-100% across all features, with 100% being the best match. The CSI:Finger ID score ranged from -23 to -580 across all features, with a score closest to zero being the best match. With the aim of consolidating the key differential metabolites, further investigation was only used with metabolites with a SIRIUS score over 50% or a CSI:Finger ID score better than -150. Based on these criteria, the pool of differential metabolites were narrowed down to 37 features (31 ESI+ and 6 ESI-) which were significantly higher in 15% barley (A) compared to 15% barley (B) and/or 100% malt. Additionally, 30 features (27 ESI+ and 3 ESI-) were significantly higher in 15% barley (B) compared to 15% barley (A) and/or 100% malt (Table 2 and 3). Differential metabolites between the malt control, 15% barley (A) and 15% barley (B) representative of a range of chemical classes including peptides,

purines, secondary alcohols, oligosaccharides, and benzoic acid derivatives. In this study, these components are 'putatively identified' (i.e. MSI level 2; Sumner et al. 2007) as it is acknowledged that additional analysis with independent standards is required to provide full identification. However, the above criteria assure a degree of confidence about the assignments reported in terms of chemical class and elemental composition.

Peptides

The most recurrent chemical class identified in the differential compounds were peptides (Tables 2 and 3). A range of di-, tri- and tetrapeptides were identified as differential compounds in positive ion mode. Table 4 shows the 11 peptide features that were identified as being significantly higher in the 15% barley (A) beer compared to 100% malt and the treated 15% barley (B). Table 5 reports the six peptides that were identified as being significantly higher in 15% barley (B) compared to 100% malt and 15% barley (A) beers.

Proteolytic activity during malting and mashing creates a broad range of peptides, of varying size, that are derived from barley proteins. It is logical that omitting the malting process for 15% of the grist would change the spectrum of peptides in beer. Those emphasised in the adjunct beers (Tables 4 and 5) would presumably result from breakdown of barley proteins under mashing conditions, as opposed to the blend of proteolytic enzymes active during malting. Many of the latter are inactivated by mash conditions and/or have specific inhibitors which limit their mash activity.

Amongst the 17 peptides identified in this study, 10 peptides contained glutamine or proline residues. Prolamins, also known as hordeins in barley, are proteins that are rich in proline and glutamine (20-55%) (Mickowska et al. 2012). The S-poor prolamin (C-hordein in barley) accounts for approximately 10-20% of the total prolamins with an amino acid composition of 40-50% glutamine, 20-30% proline and 7-9% phenylalanine (Strouhalova et al. 2012). During malting, hordeins are partially degraded to amino acids and small peptides by a range of proteolytic enzymes. The level of hordeins decrease during the malting process, and in the case of C-hordeins decrease by approximately 65%

Table 2.

Differential metabolites putatively identified by SIRIUS GUI software from analysis of positive [M+H]+ and negative [M-H]- ionisation of the beers. Differential metabolites reported are significantly higher in 15% (untreated) barley (A) compared to 100% malt or 15% (treated) barley (B).

Elemental composition corresponds to the [M+H]+ and negative [M-H]- ion. Retention time (RT) is the elution time in minutes. 'Beer' where the compound was significantly different to 15% (untreated) barley (A). The samples 100% malt and 15% (treated) barley (B) are reported as '100M' and '15B(B)' respectively.

Feature no	Mode	Predicted compound	Predicted class	Elemental composition	m/z	RT	SIRIUS score (%)	CSI finger score	Beer
1	[M + H]+	Compound 1	Amino acid	C7H15NO3	203.14	1.70	33	-100.49	100M 15B(B)
2	[M + H]+	Compound 2	Dipeptide	C7H12N2O3S	205.06	1.52	100	-107.06	100M 15B(B)
3	[M + H]+	2-(3-Amino-1H-1,2,4-triazol-1-yl)-N-(5-hydroxy-4-methylpentyl)acetamide	Monoamine	C10H19N5O2	242.16	1.56	100	-135.86	100M 15B(B)
4	[M - H]-	2-(hydroxymethyl)-6-methoxy-4,5-bis[[1-[(4-nitrophenyl)methyl]triazol-4-yl]methoxy]tetrahydropyran-3-ol	Peptide	C27H30N8O10	625.20	2.07	80	-371.66	100M 15B(B)
5	[M + H]+	α-Glutamylserylleucine	Oligopeptide	C14H25N3O7	389.20	3.48	33	-77.61	100M 15B(B)
6	[M + H]+	Alanylserylprolylproline	Oligopeptide	C16H26N4O6	389.20	3.11	94	-123.16	100M 15B(B)
7	[M + H]+	Valylalanylglycylglutamine	Oligopeptide	C15H27N5O6	374.20	2.03	25	-74.89	100M 15B(B)
8	[M + H]+	Adenine	6-aminopurine	C5H55N5	136.06	1.21	97	-37.74	100M 15B(B)
9	[M + H]+	6,7-dihydropurin-6-ylium	Purines and derivative	C5H3N4	137.05	1.73	99	-29.34	100M 15B(B)
10	[M + H]+	4-(3-hydroxypropylamino)butanoate	Fatty acid and conjugate	C7H15NO3	203.14	1.70	100	-95.74	100M 15B(B)
11	[M + H]+	(4-methoxyphenyl)-methylium	Benzene substituted derivative	C8H9O	203.12	2.31	100	-58.07	100M 15B(B)
12	[M + H]+	Compound 12	Benzoic acid derivative	C26H42O4	419.32	14.16	99	-56.32	100M 15B(B)
13	[M + H]+	Prostaglandin F1β	Eicosanoid	C20H36O5	339.25	13.66	100	-66.52	100M 15B(B)
14	[M + H]+	bis(2,5-dimethoxy-4-methylphenyl)methane	Prenol lipid	C19H24O4	335.19	13.72	50	-167.34	100M 15B(B)
15	[M + H]+	Compound 15	Cyclohexanone	C19H26O5	335.19	13.72	50	-172.44	100M 15B(B)
16	[M + H]+	Compound 16	Flavone	C24H24O4	399.16	14.01	98	-135.42	100M 15B(B)
17	[M + H]+	Compound 17	Secondary alcohol	C18H36N2O3	310.24	12.03	50	-106.09	100M
18	[M - H]-	Compound 18	Dicarboxylic acid	C19H26O5	393.19	11.90	33	-194.02	100M
19	[M - H]-	2,6-bis(1,1-dimethylethyl)-4-(methylthio)-phenol	Phenyl propane	C15H24OS	297.15	15.66	99	-88.05	100M
20	[M + H]+	Threonylthreonylleucylthreonine	Oligopeptide	C18H34N4O8	435.25	2.15	33	-61.14	100M
21	[M + H]+	Alanylvalylvalylalanine	Oligopeptide	C16H30N4O5	359.23	3.59	33	-64.05	100M
22	[M + H]+	Isoleucylprolylthreonine	Oligopeptide	C15H27N3O5	330.2	3.64	99	-84.87	100M
23	[M + H]+	L-Valyl-L-α-glutamyl-L-tryptophan	Oligopeptide	C21H28N4O6	433.21	3.93	88	-128.56	100M
24	[M + H]+	4-Methylpicolinic acid	Substituted benzene	C7H7NO2	198.11	1.44	33	-69.33	100M
25	[M + H]+	Compound 25	Secondary alcohol	C16H33NO3	310.24	13.61	50	-106.09	100M
26	[M + H]+	Compound 26	Secondary alcohol	C18H36N2O3	329.28	12.03	99	-86.19	100M
27	[M - H]-	Compound 27	Dicarboxylic acid	C19H26O5	393.19	11.90	33	-194.02	100M
28	[M - H]-	2,6-bis(1,1-dimethylethyl)-4-(methylthio)-phenol	Phenyl propane	C15H24OS	297.15	15.66	99	-88.05	100M
29	[M + H]+	Compound 29	Coumarins and derivative	C22H26O5	403.21	13.70	25	-141.09	15B(B)
30	[M + H]+	Compound 30	Alkyl glycoside	C26H48O8	511.33	12.81	78	-131.90	15B(B)
31	[M + H]+	Compound 31	Secondary alcohol	C20H28O6	347.19	13.02	33	-132.58	15B(B)
32	[M - H]-	Hexopyranosyl-(1->3)hexopyranosyl-(1->4)hexopyranose	Oligosaccharide	C18H32O16	503.16	1.05	99.7	-56.96	15B(B)
33	[M + H]+	α-Glutamylglutaminylvaline	Oligopeptide	C15H26N4O7	375.19	1.26	99	-126.44	15B(B)
34	[M + H]+	Leucylglutaminylglycylglutamic acid	Oligopeptide	C18H31N5O8	446.23	1.72	56	-87.95	15B(B)
35	[M + H]+	Leucylglycylthreonylglutamine	Oligopeptide	C17H31N5O7	418.23	3.73	33	-83.26	15B(B)
36	[M + H]+	α-Glutamylalanylleucine	Oligopeptide	C14H25N3O6	332.18	3.76	99	-57.78	15B(B)
37	[M + H]+	γ-Glutamyltyrosine	Gamma glutyl amino acid	C14H18N2O6	311.13	2.73	99	-23.10	15B(B)

(Flodrova et al. 2012; Tatham and Shewry 2012). Indeed, it has been shown that well-modified malt contains less than half the hordeins present in the original barley (Celus et al. 2006). The D-hordeins (sulphur rich proteins) are degraded more rapidly than B and C-hordeins during malting (Picariello et al. 2011). Colgrave et al (2012) identified C-hordein fragments in wort and trace levels of C-hordein peptides in an all-malt ale. Many of the peptide fragments contained sequences of glutamine and proline. When brewing with unmalted barley, the hordeins would not have been degraded during malting leading to increased proteins in the mash. Proteolytic enzymes from the malt in the mash will break down the intact proteins in the unmalted barley but not to the same extent as during the

Table 3.

Differential metabolites putatively identified by SIRIUS GUI software from positive [M+H]+ and negative [M-H]- ionisation analysis. Differential metabolites shown are of which significantly varied higher in 15% (treated) barley (B) compared to 100% malt or 15% (untreated) barley (A).

Elemental composition corresponds to the [M+H]+ and negative [M-H]- ion. Retention time (RT) is the elution time in minutes. 'Beer' where the compound was found to be a significantly different to 15% (treated) barley (B). The samples 100% malt and 15% (untreated) barley (A) are referred to as 100M and 15B(A).

Feature no	Mode	Predicted compound	Predicted class	Elemental composition	m/z	RT	SIRIUS score (%)	CSI finger score	Beer
38	[M + H]⁺	1-(cyclopropanecarbonyl)piperidine-4-carboxylate	Amino acid	C10H15NO3	198.11	1.45	100	-148.07	100M
39	[M + H]⁺	Compound 39	Dipeptide	C16H29N3O9	446.15	1.46	58	-144.03	100M
40	[M + H]⁺	Prolylprolylglycylserine	Oligopeptide	C15H24N4O6	389.20	2.09	10	-150.31	100M
41	[M + H]⁺	3-oxohex-5-enoate	Carboxylic acid ester	C6H8O3	170.08	3.09	100	-76.52	100M
42	[M + H]⁺	Isoleucylprolylthreonine	Oligopeptide	C15H27N3O5	330.20	3.64	99	-89.14	100M
43	[M + H]⁺	N-(4-Carboxy-3-hydroxy-3-methylbutanoyl)tryptophan	N-acyl-alpha amino acid	C17H20N2O6	349.14	3.66	50	-134.87	100M
44	[M + H]⁺	N-Acetylmethionylglutamic acid	Dipeptide	C12H20N2O6S	321.11	4.13	99	-129.5	100M
45	[M + H]⁺	Compound 45	n-acyl amides	C18H36N2O3	329.28	12.01	100	-125.67	100M
46	[M + H]⁺	Compound 46	Triterpene saponins	C37H62O10	689.42	12.52	44	-117.90	100M
47	[M + H]⁺	Compound 47	Fatty acid amide	C16H33NO3	310.24	13.60	50	-105.40	100M
48	[M + H]⁺	12-oxo-stearic acid	Cyclic alcohols and derivative	C18H30O3	317.21	13.77	99	-102.87	100M
49	[M - H]⁻	Compound 49	Disaccharide	C12H20O10	323.10	1.52	89	-106.12	100M
50	[M - H]⁻	2,6-bis(1,1-dimethylethyl)-4-(methylthio)-phenol	Phenyl propane	C15H24OS	297.15	15.28	98	-83.47	100M
51	[M + H]⁺	2-Acetamido-N-acetyl-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosylamine	Amino acid	C16H24N2O9	421.18	1.25	95	-228.25	15B(A)
52	[M + H]⁺	(2S,3S,4S,5S,6R)-2-[[(2R,3S,4R,5R)-5-(6-aminopurin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy]-6-(hydroxymethyl)oxane-3,4,5-triol	Purine nucleoside	C16H23N5O9	430.16	1.71	64	-108.04	15B(A)
53	[M + H]⁺	(1-{[(3-Cyanopropyl)sulfonyl]methyl}cyclopropyl)acetic acid	α-amino acid	C10H15NO4S	246.08	1.87	100	-161.85	15B(A)
54	[M + H]⁺	Prolylserylglycylproline	Oligopeptide	C15H24N4O6	389.20	2.09	99	-127.65	15B(A)
55	[M + H]⁺	Serylalanylalanylleucine	Oligopeptide	C15H28N4O6	361.21	3.21	25	-96.63	15B(A)
56	[M + H]⁺	Prolyl-α-aspartylthreonylproline	Oligopeptide	C18H28N4O8	447.21	3.52	96	-144.37	15B(A)
57	[M + H]⁺	Seryl-α-glutamylleucine	Oligopeptide	C14H25N3O7	348.18	3.57	49	-61.92	15B(A)
58	[M + H]⁺	α-Glutamylglycylleucine	Oligopeptide	C13H23N3O6	318.17	3.79	99	-81.60	15B(A)
59	[M + H]⁺	Compound 59	n-acyl amide	C22H42N2O3	383.33	12.08	99	-129.51	15B(A)
60	[M + H]⁺	9-Oxooctadecanedioic acid	Long chain fatty acid	C18H32O5	311.22	12.60	33	-59.71	15B(A)
61	[M + H]⁺	Compound 61	Sequiterpenoid	C12H18O2	195.14	12.61	50	-110.89	15B(A)
62	[M + H]⁺	[(1S,2S,6R)-2-Hydroxy-2,6-dimethyl-6-vinylcyclohexyl]acetic acid	Prenol lipid	C12H20O3	213.15	12.66	50	-90.58	15B(A)
63	[M + H]⁺	Compound 63	n-acyl amide	C18H37NO3	316.29	13.49	50	-89.49	15B(A)
64	[M + H]⁺	Compound 64	Cyclic alcohol and derivative	C20H30O6	389.19	13.59	80	-124.20	15B(A)
65	[M + H]⁺	3,5,11,14-Tetrahydroxy-19-oxocard-20(22)-enolide	Cardenolide	C23H32O7	403.21	13.70	25	-138.84	15B(A)
66	[M + H]⁺	15-Oxo-11,13-eicosadienoic acid	Linoleic acid and derivative	C20H34O3	341.27	13.77	25	-40.84	15B(A)
67	[M - H]⁻	9-Oxoheptadecanoic acid	Linoleic acid and derivative	C17H32O3	329.23	12.99	50	-73.09	15B(A)

malting process, explaining the higher concentration of peptides in beer produced with barley (A) and barley (B) compared to the all-malt control.

Many factors can impact the content, composition and distribution of hordein in unmalted barley. It has been previously shown that growth conditions and the processing of unmalted barley impacts the hordein content in the grain (Swanston et al. 1997; Molina-Cano et al. 2004). It has also been found that difference in hordein location can result from environmental impacts during growth. Hordeins are also distributed in different areas of the grain, B and C-hordeins are in the sub-aleurone cells while D hordein is mainly in the starchy endosperm cells (Shewry et al. 1980). Thus, any processing applied to the barley grain that degrades the aleurone layer

would reduce the B and C-hordein content being introduced to the mash. Cysteine proteinases play the biggest role in solubilising proteins during mashing (Jones and Budde 2005). Davy et al (1998) investigated two cysteine endoproteases, EP-A and EP-B, which play a central role in the breakdown of barley endosperm and storage proteins and are secreted into the starchy endosperm during germination. It was found that the cysteine endoproteases specified to hydrolytic sites with amino acids in the preferential order Leu > Phe > Val > Pro > Ser. This could explain the peptide sequences that were identified as differential peptides, as six of the peptides have terminal leucine residues (Table 4 and 5). This suggests that cysteine proteinases in the mash, breakdown intact hordeins forming different peptide sequences.

Table 4.

Differential peptides putatively identified from positive ionisation analysis of the three beers. Differential metabolites are significantly higher in 15% (untreated) barley (A) compared to 100% malt and/or 15% (treated) barley (B).

'Beer' where the compound was significantly different to 15% (untreated) barley (A). The samples 100% malt and 15% (treated) barley (B) are referred to as 100M and 15B(B).

Feature no.	Compound name (Amino acid residues)	Elemental composition	m/z	RT	SIRIUS score (%)	CSI finger score	Beer
5	α-Glutamylserylleucine (glu-ser-leu)	C14H25N3O7	389.2	3.48	33	-77.61	100M 15B(B)
6	Alanylserylprolylproline (ala-ser-pro-pro)	C16H26N4O6	389.2	3.11	94	-123.16	100M 15B(B)
7	Valylalanylglycylglutamine (val-ala-gly-gln)	C15H27N5O6	374.2	2.03	24.9	-74.89	100M 15B(B)
20	Threonylthreonylleucylthreonine (thr-thr-leu-thr)	C18H34N4O8	435.25	2.15	33	-61.14	100M
21	Alanylvalylvalylalanine (ala-val-val-ala)	C16H30N4O5	359.23	3.59	33	-64.05	100M
22	Isoleucylprolylthreonine (ile-pro-thr)	C15H27N3O5	330.2	3.64	99	-84.87	100M
23	L-valyl-L-α-glutamyl-L-tryptophan (val-glu-trp)	C21H28N4O6	433.21	3.93	88	-128.56	100M
33	α-Glutamylglutaminylvaline (glu-gln-val)	C15H26N4O7	375.19	1.26	99	-126.44	15B(B)
34	Leucylglutaminylglycylglutamic acid (leu-gln-gly-glu)	C18H31N5O8	446.23	1.72	56	-87.95	15B(B)
35	Leucylglycylthreonylglutamine (leu-gly-thr-gln)	C17H31N5O7	418.23	3.73	33	-83.26	15B(B)
36	α-Glutamylalanylleucine (glu-ala-leu)	C14H25N3O6	332.18	3.76	99	-57.78	15B(B)

Peptides can contribute taste and mouthfeel sensations in food and beverages with the taste depending on the amino acid composition and sequence (Zhao et al. 2016). The presence of glycine, alanine, valine, leucine, tyrosine and phenylalanine in peptides can impart bitterness (Zhao et al. 2016). Four of the tripeptides identified, Features 5, 36, 57 and 58 (Table 4 and 5) have a leucine residue located at the C-terminus of the tripeptide. Ishibashi et al (1987) found that peptides with a leucine residue at the C-terminus have a higher bitterness compared to peptides with leucine residues located at the N-terminus or in the middle. It was proposed that the hydrophobic residue of the peptide acts a binding site between bitter peptides and gustatory receptors. Similarly, the tripeptide val-glu-trp (Feature 23, Table 4) has a bulky hydrophobic tryptophan residue at the C-terminus of the tripeptide. This is important in providing bitterness to the tripeptide as the hydrophobicity of the C-terminus amino acid influences the bitterness of the tripeptide (Kim and Li-Chan 2006; Xu and Chung 2019). The hydrophobic valine residue at the N-terminal would also increase bitterness as the combination of hydrophobic amino acids at the C- and N-terminus can also increase bitterness in tripeptides (Kim and Li-Chan 2006).

For tetrapeptides, bulky hydrophobic amino acids at the C-terminus and bulky basic or hydrophobic amino acids at the N-terminal relate to bitterness (Kim and Li-Chan 2006). The tetrapeptide ala-ser-pro-pro (Feature 6, Table 4) consists of bulky hydrophobic proline residues and hydrophobic alanine. Proline is a major contributor to bitter taste in peptides (Ishibashi et al. 1988). Similarly, Features 40, 54 and 56 (Table 5) contain two proline residues which would increase the bittering effect of these four tetrapeptides. It has been proposed that bitter tetrapeptides produce bitterness due to a bulky hydrophobic group of the peptide functioning as a binding unit for the bitter taste receptor whilst the adjacent bulky basic or bulky hydrophobic group plays a role as a stimulating unit, increasing bitter taste (Maehashi and Huang 2009).

A range of bittering peptides were identified as differential features between the untreated 15% barley (A) and treated 15% barley (B) beers. This could contribute to the change in the quality of bitterness when brewing with unmalted barley (Yorke et al. 2021). The importance of utilising proteolytic enzymes to increase free amino nitrogen for the formation of flavour-active volatile compounds is well understood (Hill and Stewart 2019; Lei et al. 2013a, b; Lin et al. 2022). However, these results show the importance of proteolytic enzymes and the hydrolysis of proteins in the formation of taste inducing peptides in the beer. The known taste attributes of these tri- and tetrapeptides suggests that they may explain some of the differences in bitterness and astringency when using higher levels of unmalted barley in the grist.

Y-glutamyl peptides

In positive ion mode, a peak centred at 2.73 min was tentatively ascribed to γ-glutamyl tyrosine

Table 5.

Differential peptides putatively identified from positive ionisation analysis of the three beers. Differential metabolites shown are significantly higher in 15% (treated) barley (B) compared to 100% malt and 15% (untreated) barley (A). 'Beer' indicates the beers for which the compound was found to be significantly different compared to 15% (treated) (B) barley. The samples 100% malt and 15% barley (A) are referred to as 100M and 15B(A).

Feature no.	Compound name (Amino acid residues)	Elemental composition	m/z	RT	SIRIUS score (%)	CSI finger score	Beer
40	Prolylprolylglycylserine (pro-pro-gly-ser)	C15H24N4O6	389.2	2.09	99.98	150.31	100M
42	Isoleucylprolylthreonine (ile-pro-thr)	C15H27N3O5	330.2	3.64	99	-89.14	100M
54	Prolylserylglycylproline (pro-ser-gly-pro)	C15H24N4O6	389.2	2.09	99.8	127.65	15B(A)
56	Prolyl-α-aspartylthreonylproline (pro-asp-thr-pro)	C18H28N4O8	447.21	3.52	96	144.37	15B(A)
57	Seryl-α-glutamylleucine (ser-glu-leu)	C14H25N3O7	348.18	3.57	49	-61.92	15B(A)
58	α-Glutamylglycylleucine (glu-gly-leu)	C13H23N3O6	318.17	3.79	99	-81.60	15B(A)

(Feature 37, Table 2, Figure 2), with a strong SIRIUS match score of 99% and CSIfinger score of -23.10. The compound was present at statistically significantly higher amounts in the untreated 15% barley (A) beer compared to the 15% barley (B) beer ($p<0.01$). γ-glutamyl peptides are small peptides that contain a γ-carboxyl group of glutamic acid at the N terminus of the peptide. Many γ-glutamyl di- and tri-peptides including γ-glutamyl tyrosine have been detected in fermented foods such as cheese, soy sauce and fermented fish (Zhao et al. 2016). Further, γ-glutamyl peptides have been identified in beer (Miyamura et al. 2015) with the kokumi ('rich taste') peptide γ-glu-val-gly at 0.08-0.18 mg/L.

The γ-glutamyl peptides can be enzymatically generated by γ-glutamyl transpeptidase or γ-glutamyl transferase (GGT). γ-glutamyl peptides can be formed by the enzyme binding to glutamate to form a γ-glutamyl-enzyme conjugate, which can react with other free amino acids in producing the corresponding γ-glutamyl dipeptides (Toelstede et al. 2009; Sofyanovich et al. 2019). Previous studies have reported that *Saccharomyces cerevisiae* can synthesise γ-glutamyl peptides intracellularly (Li et al. 2022; Sofyanovich et al. 2019). Accordingly, it is suggested that an increased tyrosine content in the wort could lead to the production of γ-glutamyl tyrosine during fermentation.

Kokumi compounds such as γ-glutamyl peptides impart minimal flavour in water, however when introduced into food products they can modify the perception of other basic tastes, contributing to

a more fuller, complex sensation (Li et al. 2020). Kokumi is expressed as providing a rich complexity, long lasting consistent aftertaste and overall mouthfulness (Li et al. 2020). The compounds are referred to as kokumi-active substances (Miyaki et al. 2015). The functional taste properties of γ-glutamyl peptides involve their ability to activate the calcium-sensing receptor (CaSR) channels on the tongue leading to a release of intracellular $Ca2^+$ in the surrounding taste cells (Ohsu et al. 2010). Although kokumi substances tend not to have a specific taste at typical concentrations in food, some γ-glutamyl peptides can exhibit bitterness and/or astringency in aqueous solutions depending on the C-terminus amino acid residue in the peptide (Liu et al. 2015; Yang et al. 2019). γ-glutamyl tyrosine can impart astringency and bitterness in aqueous solutions. Toelstede et al (2009) determined the intrinsic orosensory threshold concentration for γ-glutamyl tyrosine and reported a slight astringent mouth coating characteristic at 2.5 mM and bitterness at 5 mM. This agrees with Liu et al (2015) who determined that γ-glutamyl tyrosine imparts bitterness at 5 mM in water. Thus, the sensory properties of γ-glutamyl tyrosine could contribute to a more lingering bitterness or astringency in beer.

Purines

Three differential features were identified from the purine chemical class. Adenine (purine) and the purine derivative 6,7-dihydropurin-6-ylium were tentatively identified as features that were significantly higher in 15% barley (A) compared to

both 100% malt and 15% barley (B) (Table 2, Figure 3a and 3b). Feature 52 was determined as a purine nucleoside and was significantly higher in the treated (15%) barley (B) compared to 15% barley (A) (Table 3, Figure 3c).
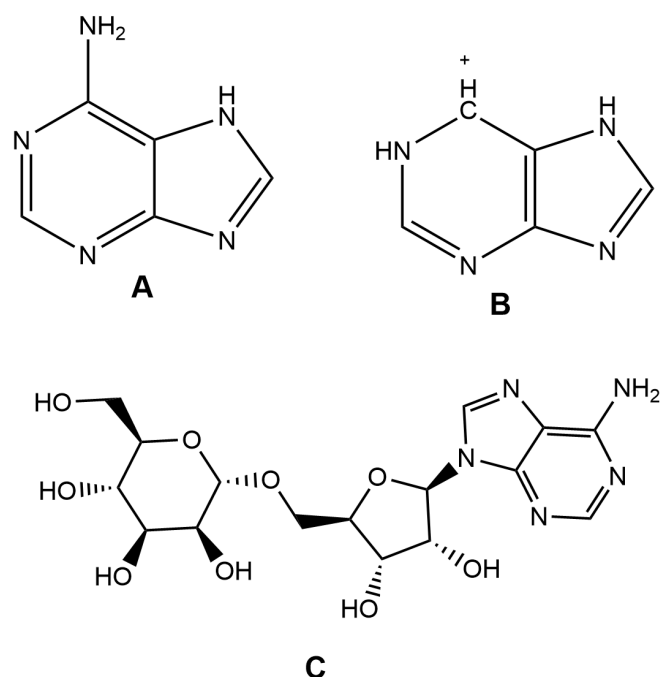
Figure 2.

Molecular structure of γ-glutamyltyrosine.
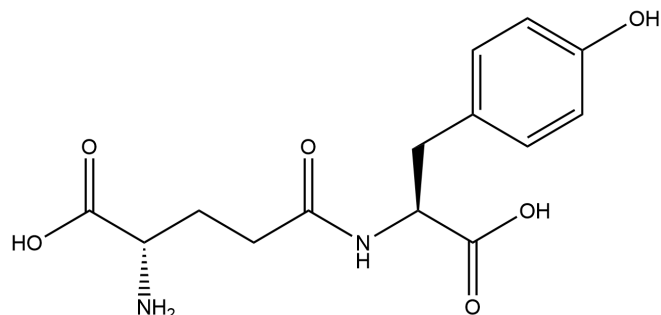
Figure 3.

Molecular structures of A) adenine, B) 6,7-dihydropurin-6-ylium and C) Feature No. 52.

Nucleic acids account for 0.2-0.3% of barley but little survive malting and mashing with their degradation products - nucleotides, nucleosides and bases (purine and pyrimidine) present in beer (Briggs et al. 2004). During malting and mashing, nucleotidases catalyse the degradation of nucleic acids to nucleosides, with the glycosidic linkages between the purine and the sugar moieties cleaved by nucleosidases. Accordingly, the inclusion of unmalted barley will increase the nucleic acid content in the mash as there is no breakdown from

the malting process. Harris and Parsons (1958) found that nucleotidases in the mash function near optimally at normal infusion mash temperatures (e.g. 65°C). So, nucleotidases from the malted barley in the grist will enable the breakdown of nucleic acids in the unmalted barley resulting in a different mixture of nucleic acid breakdown products compared to an all-malt wort.

Some nucleic acid breakdown products have flavour enhancing properties (Lee et al. 1986) with purine and purine nucleotides recognised as influencing flavour. Bettenhausen et al (2018) found that purine compounds in malt and beer were associated with 'corn chip' flavour in beer. Further, the purine nucleosides in their study were negatively correlated with fruity characteristics (e.g. green apple and watermelon rind) but positively correlated with staling characteristics such as cardboard, bitter and astringent. Therefore, the purine based differential features putatively identified in this study may influence the sensory perception of beers produced with unmalted barley.
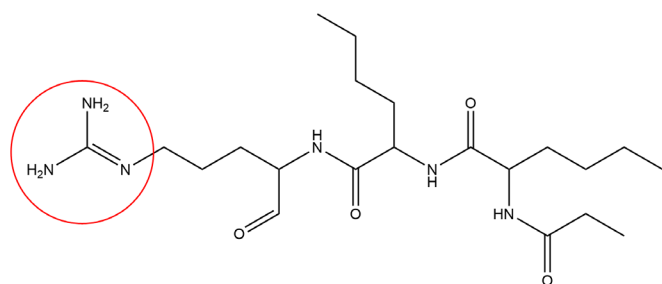
Hordatines

Hordatines are one of the most abundant but least studied group of phenolic substances in beer. Hordatines consist of polyamides conjugated with hydroxycinnamic acids to form phenolamides (Pihlava 2014). The hordatines in barley have been investigated to understand the astringent aftertaste in beer (Kageyama et al. 2011; Kageyama et al. 2013; Kohyama and Ono 2013). Kageyama et al (2011) fractionated malt acrospires by preparative HPLC and determined that a fraction exhibited astringency in a sensory evaluation. The fraction consisted of three compounds 4'-O-β-D-glucopyranosyl hordatine A, 4'-O-β-D glucopyranosyl hordatine B and 4'-O-β-D-maltosyl hordatine A, all of which exhibit astringent properties. Physical removal of acrospires from the malt by polishing led to reduced astringency and improved beer flavour. Kohyama and Ono (2013) isolated hordatine A-β-D glucopyranoside from ungerminated barley grains and found it localised in the aleurone layer.

Hordatines are synthesised from cinnamoyl-CoA and agmatine by agmatine coumaroyl transferase and subsequent peroxidase-catalysed dimerisation (Wannenmacher et al. 2018). In this study, a peptide

containing an agmatine residue was identified as being significantly higher in the untreated 15% barley/85% malt (A) beer compared to treated 15% barley/85% malt (B) and the all-malt control beer (Figure 4). However, the SIRIUS score and CSI:Finger score (0.2% and -154.65 respectively) failed to satisfy the match criteria for further investigation.

Figure 4.

Molecular structure of the tentatively identified peptide containing an agamatine residue (circled in red) at 14.58 min in the ESI+ mode.

However, peptides with agmatine residues would be an interesting area for investigation regarding their occurrence in beers and potential impacts on taste, due to their analogous chemical features to hordatine. It would be of interest to conduct further metabolomic analysis of beers produced with greater proportions of barley to determine if hordatine structures in the beer significantly change with increased adjunct incorporation.

Conclusions

Using non-targeted metabolomic profiling we putatively identified differential metabolites present when comparing all malt beer to beers produced from 15% unmalted barley and 85% malt. The large number of features analysed were reduced to focus on those which i) differed significantly between samples, ii) were present above a minimum threshold signal level and iii) could be identified with a reasonable degree of confidence using mass spectral data. The results illustrate that even a moderate proportion of unmalted adjunct can impact the metabolomic profile of beer. However, many of the features could not be assigned to identifiable molecules. Further work is required to confirm the chemical structures and properties of the differential compounds and to confirm if these compounds are important to the sensory profile of adjunct-based beers. Despite this, the non-targeted approach offers new insight into non-volatile

molecular features that have been overlooked with standardised targeted analyses.

Peptides and purine derivatives are suggested to be taste active. As significant breakdown of proteins and nucleotides occurs during malting, the level of these compounds together with nucleic acids will differ with a proportion of unmalted barley in the grist. Further, during mashing, nucleotidases are active on nucleic acids resulting in a different mixture of breakdown products compared to an all-malt wort.

Several of the identified peptides have bitter inducing properties, such as γ-glutamyl tyrosine which can contribute bitterness, astringency and induce kokumi taste in beer (Liu et al. 2015). Eleven of the 17 peptides identified were significantly higher in the (untreated) 15% barley (A) compared to (treated) 15% barley (B). Accordingly, processing the barley before use changed the protein and peptide composition of the beer. This could explain the increased bitter and astringency sensory characteristics that were observed when brewing with barley (A), but which were less pronounced in beers produced with barley (B) (Yorke et al. 2021).

Overall, the results reported here identify key metabolites which have not been considered previously when investigating the unfavourable bitterness and astringency characteristics found in beers containing unmalted barley. Processing the barley before use or supplementing with exogenous proteases to increase proteolytic activity in the mash could help reduce the identified bitter peptides and improve the sensory profile of beer produced with unmalted barley. This work suggests further opportunities to develop sustainable brewing practices without sacrificing the quality of beer.

Author Contributions

Joanna Yorke: Conceptualisation, methodology, formal analysis, investigation, writing (original draft).
Tristan Dew: Conceptualisation, methodology, formal analysis, investigation, resources, writing (reviewing and editing), supervision.
David Cook: Conceptualisation, methodology, resources, writing (reviewing and editing), supervision, funding acquisition.

Acknowledgements

Conflict of interest

The authors declare there are no conflicts of interest.

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