The impact of hop bitter acid and polyphenol profiles on the perceived bitterness of beer.

Olayide Oladokun[a], Amparo Tarrega[a], Sue James[b], Katherine Smart[b], Joanne Hort[a], David Cook[§a]

[a]International Centre for Brewing Science, Bioenergy and Brewing Science Building, University of Nottingham, School of Biosciences, Division of Food Science, Sutton Bonington Campus, Loughborough, LE12 5RD, UK.

[b]SABMiller Plc, SABMiller House, Church Street West, Woking, Surrey, GU21 6HS.

Abbreviated running title: Impact of bitter congener profiles on perceived bitterness of beer

§ Corresponding author

e-mail: david.cook@nottingham.ac.uk
Tel: +44 (0)115 9516245

Abstract

Thirty-four commercial lager beers were analysed for their hop bitter acid, phenolic acid and polyphenol contents. Based on analytical data, it was evident that the beers had been produced using a range of different raw materials and hopping practices. Principal Components Analysis was used to select a sub-set of 10 beers that contained diverse concentrations of the analysed bitter compounds. These beers were appraised sensorially to determine the impacts of varying hop acid and polyphenolic profiles on perceived bitterness character. Beers high in polyphenol and hop acid contents were perceived as having 'harsh' and 'progressive' bitterness, whilst beers that had evidently been conventionally hopped were 'sharp' and 'instant' in their bitterness. Beers containing light-stable hop products (tetrahydro-iso-α-acids) were perceived as 'diminishing', 'rounded' and 'acidic' in bitterness. The hopping strategy adopted by brewers impacts on the nature, temporal profile and intensity of bitterness perception in beer.

Keywords: Beer, phenolic acids, total polyphenol content, hop acids, humulinones, tetrahydro-iso-humulones, bitterness quality.

Chemical compounds studied in this article

Protocatechuic acid (PubChem CID:72); Catechin (PubChem CID:73160); Epicatechin (PubChem CID:72276); Caffeic acid (PubChem CID:689043); Vanillic acid (PubChem CID:8468); Ferulic acid (PubChem CID:445858); *p*-coumaric acid (PubChem CID:637542); Cinnamic acid (PubChem CID:444539); 4-hydroxyphenylacetic acid (PubChem CID:127); Sinapic acid (PubChem CID:637775).

1. Introduction

Bitterness is an important flavour character of foods and beverages such as coffee, nuts, fruits and beer (Lesschaeve & Noble, 2005). Whereas the bitterness flavour of tea and red wine have been attributed mainly to flavonoid phenols, approximately 80% of beer bitterness is derived from the addition of hops (*Humulus lupulus*) during the 'boiling stage' of the brewing process (Arrieta, Rodríguez-Méndez, De Saja, Blanco, & Nimubona, 2010; Caballero, Blanco, & Porras, 2012). The lupulin glands of female hop cones contain soft resins rich in phloroglucinol derivatives, namely α-acids (cohumulone, humulone, adhumulone) and β-acids (colupulone, lupulone, adlupulone). These acids undergo thermal isomerisation to give iso-α-acids, the major bitter compounds in beer (Haseleu et al., 2010). Upon isomerisation, each iso-α-acid congener is present as trans/cis stereoisomers with a ratio of approximately 3:7 in conventionally hopped beers (Ch Schönberger & Kostelecky, 2011). In recent years beer-bittering practice has diversified, with the development and usage of hop products in a variety of different forms, and with varied points of addition to the brewing process (e.g. kettle addition, post-fermentation bittering products, or dry hopping, which is feasible at a number of different points). One such product is pre-isomerised iso-α-acids, widely available as an aqueous extract or in pellet form, which are prepared from the chemical isomerisation of α-acids outside of the brewhouse. These hop products usually have higher levels of cis-isomers relative to trans-isomers thus, giving a lower trans/cis ratio (Schmidt et al., 2014). Bitterness can also be achieved by the use of chemically reduced derivatives of iso-α-acids, so called light stable hop products such as tetrahydro-iso-humulones (tetra) and hexahydro-iso-humulones (hexa) which are prepared by hydrogenation and reduction reactions, respectively. Advanced hop products are popular among brewers

because they offer added flexibility in terms of their usage, and can be added downstream of the brewing process (De Keukeleire, 2000).

Furthermore, hops available in various forms (cones, pellets, plugs) can be added at different stages of the brewing process. Some brewers also soak hops in beer during fermentation or conditioning to improve beer aroma in a technique known as 'dry-hopping'. Dry-hopping imparts oxidised α-acids (known as humulinones) to beer. Humulinones levels of 0.2 – 0.5% w/w have been reported in hop leaves and pellets (Cocuzza & Mitter, 2008; Negri, di Santi, & Tabach, 2010; Wolfe, 2012). In addition to α-acids, hops are also a source of polyphenols in beer although the amount of polyphenols present in beer will depend on hop variety, form and the point at which the hops are added during the brewing process. Furthermore, depending on hopping levels, brewing malt usually represents the major source of polyphenols in beer (Aron & Shellhammer, 2010; Callemien & Collin, 2009).

Polyphenols contribute to bitterness, colour, body, and astringency in beer and other beverages such as tea and wine, (Collin, Jerkovic, Bröhan, & Callemien, 2013) and have been recognised to influence the acceptance of beverages (Drewnowski & Gomez-Carneros, 2000). In beer they act as antioxidants, preventing oxidative degradation of beer whilst also providing potential health benefits to consumers through their inhibitory activity on certain mutagens and carcinogens (Floridi, Montanari, Marconi, & Fantozzi, 2003). These compounds are diverse in chemical structure and can be divided into groups consisting of simple hydroxycinnamic and hydroxybenzoic acid derivatives (phenolic acids), flavanols, flavanol glycosides and prenylated flavonoids (Goiris et al., 2014). Flavanols are of particular interest to brewers because they form protein-polyphenol complexes, leading to the formation of haze or turbidity in beer - brewers consequently remove them by cold filtration or polypvinylpyrrolidine (PVPP) treatment (Garcia, Grande, & Gándara, 2004). However, PVPP treatment is not selective for the removal of haze active polyphenols only - leading to losses of other polyphenols that are potentially beneficial to the flavour and stability of beer (Aron & Shellhammer, 2010; Mikyška, Hrabak, Hašková, & Šrogl, 2002).

104 The oral sensation of astringency is perceived as a drying, puckering or rough mouth-
105 feel, resulting from the precipitation of proline-rich proteins in saliva by polyphenols
106 (McLaughlin, Lederer, & Shellhammer, 2008). Several phenolics including ferulic acid, *p*-
107 coumaric acid and protocatechuic acid have also been noted to elicit astringency
108 (Callemien & Collin, 2009). Flavanol monomers such as catechin and epicatechin were
109 found to be more bitter than astringent (Drewnowski & Gomez-Carneros, 2000; Peleg,
110 Gacon, Schlich, & Noble, 1999).
111 It is widely accepted within the brewing industry that the bitterness characteristics of
112 beers differ due to factors not determined using the simplistic analytical measurement of
113 bitterness units (BU). It is anticipated that this might relate to the diversity of hop
114 products and hopping strategies employed across the industry and the impacts which
115 this has on the relative concentrations of the array of compounds contributing to
116 bitterness perception. Whilst there is some knowledge of the individual bitterness
117 qualities which hop acid isomers impart to beer (Fritsch & Shellhammer, 2009), the links
118 between hopping practice, bittering congener profile and the perceived bitterness
119 characteristics of beers remains poorly understood. In this study we analysed the major
120 hop acid isomers and polyphenolic compounds present in 34 commercially significant
121 lager beers sourced from around the world. Having thus established the analytical
122 bittering profiles of these beers, 10 beers, which varied significantly in the congeners
123 present, were selected for sensory evaluation. A sensory lexicon for beer bitterness was
124 developed to adequately reflect the diversity of bitterness experienced by the panel and
125 was used to rate beer bitterness characteristics. Finally, correspondence analysis of the
126 sensory data set was used to explore links between the bitterness congener profiles and
127 perceived bitterness character of beers. This study thus represents a significant step
128 towards understanding how to control this important flavour attribute of beers.

2. Materials and methods

2.1 Materials

34 fresh commercial lager beers were sourced from 17 countries over 4 continents and analysed within 8 weeks of production. For reasons of confidentiality the beers are not identified but the countries from which they were sourced are as follows: Australia (2), Belgium (1), Cuba (1), Czech Republic (6), Denmark (1), France (1), Germany (2), Hungary (1), Italy (2), Netherlands (3), Poland (2), Peru (1), Romania (1), South Africa (3), Turkey (1), UK (2) and USA (4).

2.2 Chemicals and reagents

Hydroquinone (99%), catechin (99%), epicatechin (98%), 4-hydroxybenzoic acid (99%), caffeic acid (95%), vanillic acid (97%), syringic acid (95%), *p*-coumaric acid (98%), sinapic acid (98%), ferulic acid (99%), 2,5-dihydroxybenzoic acid (98%), gallic acid (98%), cinnamic acid (98%), salicylic acid (99%), 1,2-didydroxybenzene (99%), homovanillic (99%), gentisic acid (98%) and chlorogenic acid (99%) were all purchased from Sigma-Aldrich (UK). Protocatechuic acid (99.6%) was acquired from HWI analytic (Germany). Ethyl benzoate, isooctane and methanol (all HPLC grade) as well as orthophosphoric acid 85% (ASC grade) were purchased from VWR (UK). Reverse osmosis (RO) water was obtained from a Milli-Q water purification system by Millipore. Carboxymethylcellulose (CMC), ethylenediamine tetraacetic acid (EDTA), ammonia and ferric reagent solutions were all technical grade chemicals from VWR (UK). For humulinone synthesis, CO_2 extract of α-acid resin (86%) was kindly donated by Botanix, Paddock Wood, Kent. Cumene hydroperoxide (80% technical grade), diethyl ether, sodium bicarbonate, hexane, phosphoric acid and hydrochloric acid (HCl) were all from Sigma-Aldrich (UK) and of ASC reagent grades.

Iso-α-acid standard (ICE-3) containing trans-isocohumulone, trans-isohumulone, trans-isoadhumulone (62.3% w/w), α- & β-acid (44.64%, 24.28% w/w), and tetra standard (99.3% w/w) were purchased from Labor Veritas Co. (Switzerland).

2.3 Instrumentation

HPLC analysis was carried out on a Waters Alliance 2695 instrument equipped with a column heater and a membrane degasser. Detection was achieved with a UV detector and peak areas were processed with the operating HPLC software (Empower 2). Separation of polyphenols and hop acids was achieved with a Purospher STAR rp-18 endcapped column (250 X 4.6 mm, 3 µm) from Merck Millipore (UK) coupled with a C18 guard cartridge from Phenomenex (UK).

2.3 Analysis of hop bitter acids in beer

2.4.1 Extraction of hop bitter acids from beer

Cold beer was degassed by stirring for 1 h followed by the transfer of an aliquot (5 ml) into a 50 ml centrifuge tube, the degassed beer was acidified with orthophosphoric acid (100 µl) and an internal standard (benzoic acid) was added (0.003 mg/L). The mixture was then extracted into isooctane (10 ml) on a roller bed for 30 min. The isooctane extract was transferred into a glass tube and evaporated under a controlled flow of Nitrogen with a Visidry attachment coupled to a solid phase extraction manifold (Supelco). The residue was dissolved in acetonitrile (2 ml) to give the HPLC sample.

2.4.2 HPLC-UV analysis of hop bitter acids

Hop acid separation was achieved with a binary mixture of (A) 1% v/v acetic acid and (B) 0.1% v/v orthophosphoric acid in acetonitrile. The gradient elution was: 0-5 min: 30% A, 70% B; 15-24 min: 20% A, 80% B; 25 min: 10% A, 90% B; 30 min: 10% A, 90% B; 35 min: 0% A, 100% B; 44 min: 0% A, 100% B; 46 min: 30% A, 70% B; 55 min: 30% A, 70% B over a 55 min run time. Injection volume was 10 µl, flow rate was 0.5 ml/min and column temperature was 25°C. The peak area of iso-α-acids, humulinones were extracted at 270 nm and at 310 nm for tetrahydro-iso-α-acids.

2.4.3 Determination of bitterness units

Bitterness unit was determined according to ASBC method Beer-23A (ASBC Method of Analysis, 2011). Beer (5 ml) was transferred into a 50 ml centrifuge tube and acidified with 3N HCl (0.5 ml). Isooctane (10 ml) was added and the mixture was shaken by hand three times before extraction on a rolled bed for 15 min. The mixture was subsequently centrifuged at 400 x g twice for 5 min each time to aid phase separation. An aliquot of the clear isooctane layer was transferred into a cuvette and absorbance was measured with a spectrophotometer at 275 nm against a blank of orthophosphoric acid and isooctane. The recorded absorbance was multiplied by an empirical factor of 50 to give BU values in mg/L.

2.5 Analysis of phenolic/ polyphenol compounds in beer

2.5.1 Extraction of beer phenolic compounds

The phenolic compounds listed in section 2.2 were extracted from beer using liquid-liquid extraction. Degassed beer (5 ml) was transferred into a 50 ml centrifuge tube before acidification with orthophosphoric acid (250 µl). Ethyl acetate (10 ml) was then added before extraction on a roller bed for 30 min. After extraction, the residual beer from the bilayer mixture was discarded and RO water (5 ml) was added and further extracted on the roller bed for 15 min. The water layer was removed and discarded while the extract in ethyl acetate was transferred into a glass tube and dried down under controlled flow of Nitrogen using a Visidry attachment coupled to a SPE manifold (Supelco). The residue was reconstituted in a fixed volume of methanol (2 ml) prior to HPLC analysis.

2.5.2 HPLC-UV analysis of beer phenolic compounds

The chromatographic method used a binary solvent system consisting of (A) 1.25 % v/v acetic acid and (B) 0.1% v/v orthophosphoric acid in acetonitrile. The gradient elution protocol was as follows: 0-25 min: 98% A, 2% B; 25-30 min: 76% A, 24% B; 35-40 min: 55% A, 45% B; 45 min: 15% A, 85% B; 50 min: 0% A, 100% B; 55-65 min: 98% A, 2% B. Injection volume was 10 µl, flow rate was 0.5 ml/min and column temperature was set at 30°C. Peak areas were extracted at 280 nm and total run time was 65 min.

208 2.5.3 Determination of beer total polyphenol content

209 Beer total polyphenol content (TPC) was determined according to ASBC method Beer-35
210 (ASBC Method of Analysis, 1978), involving the reaction of polyphenols with ferric ion in
211 an alkaline solution. Beer (10 ml) was mixed with a preparation of
212 carboxymethylcellulose (CMC, 1%) and ethylenediamine tetraacetic acid (EDTA, 0.2%)
213 (8 ml) in a 25 ml volumetric flask. Ferric acid (0.5 ml) was added, followed by ammonia
214 (0.5 ml) with mixing after each addition. The solution was then made up to mark with
215 RO water and left to stand at room temperature for 10 min before an absorption
216 measurement was taken at 600 nm. The recorded absorbance was multiplied by 820 to
217 give total polyphenol values in mg/L.

218 2.6 Synthesis of humulinones from humulones

219 Humulinones were synthesised from humulone resin prepared from CO_2 extract of hops
220 (86.3% α-acids) using a modified version of a reported method (Taniguchi, Matsukura,
221 Ozaki, Nishimura, & Shindo, 2013). Humulone (1.41 g) and cumene hydroperoxide (0.7
222 ml) were dissolved in diethyl ether (7 ml). A solution of saturated sodium bicarbonate
223 ($NaHCO_3$, 6 g dissolved slowly in 40 ml RO water) was added to the solution and kept at
224 room temperature in a sealed vessel for 5 days, after which the sodium salt of
225 humulinones was generated. The salt was filtered and washed with water (150 ml x 2)
226 and diethyl ether (150 ml x 2) under vacuum in a Buchner flask and funnel. The crude
227 extract (1.45 g) was subsequently dissolved in methanol (100 ml) containing 1% v/v
228 phosphoric acid before the addition of a 0.5 N HCl solution (800 ml). The mixture was
229 partitioned with hexane (1 L x 2) before the hexane layer was evaporated to dryness
230 with a rotary evaporator to yield humulinones (0.95 g) of 99% purity (by HPLC).

231 2.7 Sensory evaluation of bitterness

232 Ethical approval for the sensory element of this investigation was obtained from the
233 University of Nottingham Medical Ethics Committee (J12022015) and all participants
234 gave written informed consent to participate in the study.

The qualitative aspects of bitterness were evaluated by experienced panellists from the University of Nottingham trained beer panel (n=6) using descriptive analysis. First, panellists were presented with a subset of 10 of the 34 beers to generate and define a bitterness lexicon. These beers represented extreme variation in analytical variables and were selected based on a PCA plot from the analytical concentration of their hop acid and polyphenol contents. Panellists then attended a further 2 2h sessions during which they tasted and described the bitterness of 10 ml samples of each beer and participated in group discussions to agree a final list of clearly defined bitterness related terms. Beer samples (10 ml) were then evaluated in 2 further sessions using a Check–All-That-Apply (CATA) technique, (Dooley, Lee, & Meullenet, 2010) where panellists were asked to indicate which of the terms in the lexicon were relevant to each sample. Data was collected with Fizz software (Biosystèmes, France). Each sample was presented individually and assessed in triplicate following a randomised balanced order based on a partial latin square design and served at 4±1°C. During sample evaluation, panellists were given 5 min to evaluate each sample followed by a 3 min break in order to minimise bitterness carry over. Water (Evian, Danone, France) and crackers (Rakusen's, UK) were provided for palate cleansing.

2.8 Quantitation and statistical analysis

External standard solutions of α-acids (0.5, 1, 2, 4 and 8 mg/L), iso-α-acids (1, 10, 20, 40 and 60 mg/L), tetra (0.5, 1, 2, 4 and 8 mg/L) and humulinones (0.5, 1, 2, 4 and 8 mg/L) were all prepared in acetonitrile. The mean values of triplicate injections were used to plot calibration curves for the quantification of hop bitter acids in beers. A stock solution (20 mg/L) of standard grade phenolic compounds was prepared by dissolving (0.01 g) of the standards in a 500 ml volumetric flask containing a mixture of RO water and methanol (~200 ml), before making up to mark with the same solvent mixture. Serial dilution was made from the stock solution to achieve external standards of 10, 5, 2.5, 1 and 0.5 mg/L levels. Quantification was achieved from the standard calibration curves. Statistical analysis including Cochran's Q test was used to determine which of

263 the attributes were significantly different between the beers. Correspondence analysis
264 was used to process the frequency data of bitterness attributes for each beers. Principal
265 component analysis (PCA) was used to aid the selection of beers samples for sensory
266 analysis based on analytical measurements of hop acid and polyphenol contents. All
267 statistical analyses were performed with the XLSTAT, v2015 package.

3. Results and discussion

3.1 Phenolic profiles of lager beers

The liquid-liquid extraction protocol using ethyl acetate and water enabled the effective analysis of quantitatively significant phenolic compounds in beer. An example of the chromatographic separation achieved with the described extraction protocol and HPLC method for the Czech lager beer (L) is provided as complementary data. The HPLC method described enabled the simultaneous separation and quantification of several phenolics in beer. Where possible, the phenolic compounds were identified based both on prior knowledge and by matching peaks against authentic standards run separately, and with regard to both retention time and UV absorbance spectrum. Whilst not all peaks on the trace could be identified, unknown peak areas were also integrated and included in the analytical profiles of the beers labelled as unknown (U) 1,2….etc. The elution pattern of phenolic acids in beer followed an order of decreasing polarity under RP-HPLC conditions, thus phenolic acid derivatives of benzoic acid were eluted before the hydroxycinnamic acid derivatives. The polarity of phenolic acids is increased mostly by the hydroxyl group at the para-position, followed by the ortho- and meta-positions of the benzene ring (Torres, Mau-Lastovicka, & Rezaaiyan, 1987). The phenolic profile of each of the 34 beers was analysed, however, without further reference to beer brands it would not be informative to publish this data for each 'blind-coded' beer. To illustrate the variability present in the data set, we summed the total contents of the quantified phenolic compounds in each beer (Table 1), which shows a substantial range of concentrations (3.9 to 21.2 mg/L). Ferulic acid was the most abundant phenolic acid present in the beers, with a concentration ranging from 0.98 mg/L in the Australian lager (BB) to 7.61 mg/L in the American lager beer O (data not shown). *p*-coumaric acid is the precursor compound to ferulic acid and is formed via the shikimic acid reaction pathway, therefore the concentration of ferulic acid is usually greater than that of *p*-coumaric acid in beer (Garcia et al., 2004). The concentrations of *p*-coumaric acid across the beers followed a similar pattern as observed for ferulic acid, with beers BB and O containing

296 0.37 mg/L and 3.07 mg/L respectively (data not shown). Beer O was also found to
297 contain the highest amount of phenolic compounds overall (Table 1; 21.17 mg/L), while
298 beer F a South African lager beer had the lowest concentration at 3.91 mg/L. Beers
299 brewed in Germany (J, P), South Africa (F, C, D), Denmark (S) and Australia (BB, DD)
300 all had phenolic compound concentrations below 8 mg/L whilst the Czech beers (I, E, L,
301 HH) and American lagers (O, T, Q) all had phenolic compound concentrations of >10
302 mg/L. These values represent the total free phenolic acid content of beer which is
303 reported to be approximately 10 - 20% of total beer polyphenol content, since a
304 significant portion of beer phenolics are suggested to exist in bound form (Floridi et al.,
305 2003). The Czech beer (HH) was the only sample that contained gallic acid whilst
306 catechol, chlorogenic, salicylic, homovanillic and gentisic acids were not detected in the
307 beers, in agreement with reports from other studies (Garcia et al., 2004; Jandera et al.,
308 2005).

309 3.2 The relationship between phenolic acid and total polyphenol content of beer

310 The TPC of the beers was found to range between 74 and 256 mg/L. Similar values (70 –
311 240 mg/L) were reported by Dvorakova et al. (2007). The lowest concentration was
312 found in the Hungarian lager beer (G) whilst beers O and T, both American lagers and
313 the British lager beer (AA) all had polyphenol contents greater than 250 mg/L. Beer O
314 contained the highest amount of phenolic compounds and total polyphenol content. A
315 plot of total phenolic compound concentration versus TPC is displayed in Fig. 1. The plot
316 has been annotated to show three main clusters. The first cluster is of beers
317 characterised by TPC values of approximately 74 – 180 mg/L and phenolic compound
318 contents ranging between 3 and 15 mg/L. Beers AA and T formed a separate cluster,
319 due to the high TPC in these beers (>250 mg/L). In the last cluster, consisting of beers
320 E, O and HH, TPC ranged from 145 – 253 mg/L and phenolic compounds were in excess
321 of 15 mg/L. Interestingly, this data shows that there was no linear relationship between
322 TPC and the sum of phenolic compounds quantified. This is most likely due to the highly
323 varied brewing techniques and ingredients employed in the industry. The observed

higher TPC concentrations (>250 mg/L) in beers T, AA and O indicates that these beers were dry-hopped products.

3.3 Hop bitter acid profile of lager beers

For the bitterness profiles of these beers, the bitter tasting hop acids present in the lager beers were evaluated using two separate analytical methods; firstly by HPLC as described in section 2.4.2 and secondly by bitterness unit method (2.4.3). The latter method has been suggested to yield inflated bitterness values due its susceptibility to interference from other compounds present in beer that absorb light at the wavelength of measurement (Schönberger, 2006; Tomlinson, Ormrod, & Sharpe, 1995). In contrast, HPLC measurements are agreed to provide a better assessment of beer bitterness because they allow for the selective quantification of iso-α-acids, the major bittering principles in beer (Ting, Kay, & Ryder, 2007). A comparison of the hop bitter acid concentrations in the beers by BU and HPLC methods is presented in Fig. 2. The results showed that the BU of the lager beers ranged from 8 - 36 mg/L, although bitterness was overestimated by the BU method in comparison to HPLC values in around 60% of the samples. The sum of analysed iso-α-acid hop acid concentrations (HPLC) was between 8 and 41 mg/L. The average bitterness across all 34 beers in both methods was ~23 mg/L and the most bitter beers had concentrations >30 mg/L (GG, AA and L). The latter beers each had lower BU values in comparison to HPLC values, e.g. beer AA had a BU value of 34 mg/L but the HPLC value was 41 mg/L. Around 7 of the beers including U, J, I, E, C and T had similar bitterness concentrations according to both methods. The American light lager beer R contained the lowest amount of hop acids (8 mg/L) and did not contain any reduced iso-α-acid products.

Inspection of the HPLC chromatograms revealed that both iso-α-acids and tetrahydro-iso-α-acids were present in beers K, S, V, N, as well as the presence of humulinones at up to 3 mg/L in beers O, Q, AA, T and V. The presence of tetrahydro-iso-α-acids in beers K, S, V, N explains the lower BU values attained in these beers relative to the values

351 determined by HPLC, since BU absorbance is taken at a lower wavelength (275 nm) to
352 the absorption maxima of tetrahydro-iso-α-acids (310 nm). Significantly, of these four
353 beers the BU value of beer V which contained humulinones was the only one greater
354 than the attained HPLC value. This was also the case in the other beers containing
355 humulinones (O, Q and T) except for beer AA. This perhaps suggests that humulinones
356 contribute to the bitterness values attained with BU method as has been previously
357 observed (Parkin, 2014), although the contribution of beer polyphenols to BU values
358 cannot be totally excluded either. The presence of humulinones as well as relatively high
359 polyphenol content in beers O, Q, AA, T and V further supports the hypothesis that these
360 beers were dry-hopped. The low TPC observed in beer Q in comparison to the other dry-
361 hopped beers could be explained by a lower rate of dry-hopping or dry-hopping with
362 different hop products, i.e. hop pellets which contain relatively lower polyphenol content
363 instead of whole hop cones. Beers that contained tetra hop products (K, S, N, V) and
364 those dry-hopped (Q, AA, T, O) both displayed distinctive polyphenol and bitter acid
365 profiles. The highly dry-hopped beers (T, AA, Q) and high bitterness Czech lagers (E and
366 L) all had correspondingly high contents of phenolic compounds (see Table 1 and Figure
367 2).

368 3.4 Selection of exemplar beers for sensory assessment

369 In order to understand how the varying contents of hop acid isomers and phenolic
370 compounds impact on perceived sensory bitterness, a sub-set of 'exemplar beers' were
371 selected with the aid of a PCA plot of the analytical data. The PCA bi-plot shown in Fig. 3
372 accounted for about 65% of variation within the data set. A negative loading on PC 1
373 (47.6% of variation) was associated with the use of tetrahydro-iso-α-acids, whilst
374 positive loadings on this axis were related to high levels of iso-α-acids, α-acids,
375 humulinones and phenolic compounds. A positive loading on PC2 identified beers with a
376 high trans/cis ratio and residual α-acids – i.e. those which had used conventional
377 hopping practice as opposed to pre-isomerised or light stable products. Negative

loadings on this axis were driven largely by phenolic compounds (quadrant 4) or tetrahydro-iso-α-acids (quadrant 3).

Beers in quadrant 1 were generally lower in hop acid and polyphenol content compared to beers in quadrant 4 which were characterised by high levels of these compounds. The beers in quadrant 2 were correlated with high trans/cis ratio and residual α-acids which, as noted, is indicative of conventional hopping techniques. Beers in quadrant 3 had lower trans/cis ratios, (indicative of the use of pre-isomerised hops) as well as containing tetrahydro-iso-α-acids. A total of 10 beers were selected from the 4 quadrants to represent the diversity amongst the 34 beers: beers CC and V from quadrant 1, X and GG from quadrant 2, S, N and BB from quadrant 3 and beers E, AA and T from quadrant 4.

3.5 Beer bitterness lexicon

A total of 13 bitterness descriptors were generated by the trained panel of beer tasters following concept alignment. These attributes as well as their definitions are presented in Table 2, with some of the attributes e.g. instant, diminishing and progressive notably related to the temporal character of bitterness. Cochran's Q test analysis of the CATA frequency data showed that only 4 of the 13 bitterness attributes (acidic, tart, astringent and artificial) did not significantly differentiate across the sample set ($p > 0.05$) (Table 2). The temporal descriptors as well as descriptors such as harsh, rounded, metallic and smooth were all rated significantly differently amongst the 10 beers ($p < 0.05$).

3.6 Perceived bitterness character and correlation to bitterness and polyphenolic profile

The correspondence analysis of the sensory data is presented in Fig. 4. This revealed that beer CC, selected from quadrant 1 of the PCA in figure 3, which had relatively low hop bitter acid and polyphenol contents, was perceived as having an 'artificial', 'metallic' and 'instant' bitterness. Beer V from the same quadrant (figure 3), but deduced to have been dry-hopped from the presence of humulinones, had a 'rounded' and 'smooth' bitterness character; temporally this beer was 'diminishing' in bitterness. Conventionally

405 bittered beer GG with a high trans/cis ratio (selected from quadrant 2 of the PCA) was
406 perceived as having a 'sharp' and 'instant' bitterness. Beers N and S from quadrant 3 of
407 the PCA which were bittered with a blend of tetra as well as iso-α-acids were described
408 as 'diminishing' and somewhat 'acidic' in bitterness. Considering the trained panel was
409 not aware of the analytical bitterness fingerprint of these beers, it is interesting that all
410 the beers containing tetra (S, N, and V) are grouped together based on their sensory
411 bitterness character in the upper right quadrant of Fig. 4. Furthermore, they were
412 negatively correlated in this plot with beers AA, T and E which were relatively high in
413 levels of humulinones, iso-α-acids and polyphenols. This further supports the notion that
414 besides the intensity of bitterness, the character of bitterness in beer is also impacted by
415 the type of hop product used for bittering. The contribution of humulinones to beer
416 bitterness character is not yet fully understood. They were first thought not to contribute
417 significantly to bitterness (Verzele, 1986) but recent publications have associated the
418 presence of humulinones in beer with an increased sensation of bitterness and
419 potentially the source of harsh bitterness character often present in dry-hopped beers
420 (McLaughlin et al., 2008; Parkin, 2014). A recent report by Hopsteiner suggested that
421 humulinones are approximately 65% as bitter as iso-α-acids, thus representing a
422 significant additional source of bitterness in beer (Steiner, 2015).

423 The third beer selected from quadrant 3 (BB) which was exclusively bittered with pre-
424 isomerised products (based on the analytical profile and lower trans/cis ratio) was
425 described as being 'vegetative' in bitterness. Beers AA, E and T from quadrant 4 of the
426 PCA, containing the highest concentrations of hop acids, humulinones and polyphenols
427 were described as having a 'harsh' and 'progressive' bitterness character. A study of the
428 interaction between iso-α-acids and hop polyphenols by time-intensity (TI) and free
429 choice profiling (FCP) as reported by McLaughlin et al. (2008) found a significant effect
430 of polyphenols on perceived intensity, as well as character, of bitterness. In the study,
431 samples high in polyphenols were higher in intensities of 'harsh', 'medicinal', and

'metallic' (McLaughlin et al., 2008). Our results for the bitterness characters of beers AA, E and T in particular are in agreement with those findings.

4. Conclusions

The phenolic acid and total polyphenol contents of 34 lager beers brewed in different geographical locations were determined. The former was achieved by a combination of LLE and HPLC analysis while the latter was accomplished with an international global assay method. Phenolic compound concentrations ranged from 3 – 12 mg/L and TPC was between 74 - 256 mg/L, with the highest values identified in dry-hopped beers. No linear relationship was found between total phenolic compound concentration and TPC although dry-hopped beers were found to contain a greater amount of polyphenols in comparison to beers that had not been dry-hopped (N.B. the usage of dry hopping was deduced from the presence of significant concentrations of humulinones in the beer, but was not verified by the manufacturers in all instances). Sensory analysis showed that beers with varying profiles of bitter congeners (hop acids and phenolics) had distinctive bitterness characters. These differences are believed to be driven by the selective usage of various hop products and points of addition in the brewing process. Since the present work used a survey of international lager brands, these factors are largely deduced, albeit logically and based on obvious analytical differences between the finished beers; however, it should be borne in mind that the manufacturing processes were not disclosed, nor were they independent variables in the study. Dry-hopped beers generally contained more polyphenol compounds and humulinones, and were sensorially perceived as having a 'harsh' and 'progressive' bitterness. In comparison, beers which had evidently been conventionally bittered and as such contained relatively high residual amount of α-acids and trans/cis ratio were perceived as having an 'instant' and 'sharp' bitterness. Beers containing tetrahydro-iso-α-acids were rated as having a 'diminishing' temporal character of bitterness. These results support the hypothesis that the production processes employed by brewers in terms of hopping strategy, and the raw materials used, give beers a distinct polyphenolic and bitterness fingerprint which influences the overall bitterness impression of beer. Understanding the sensory character of bitterness in beers, and how that relates to their analytical bitterness fingerprint is of significant

462 value in order to both understand consumer response to beer bitterness and to optimise
463 production processes in this regard.

464 **Acknowledgement:**

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562 Table 1: Variation in the total analysed concentrations of phenolic compounds in 34
563 commercal lager beers.
564

		TPCC* (mg/L)	
BEER	**COUNTRY**	**SUM**	**SE**
O	USA	21.17	1.5
Q	USA	13.12	0.8
HH	Czech republic	18.25	1.5
E	Czech republic	17.73	1.8
L	Czech republic	13.87	1.1
AA	UK	12.26	1.1
T	USA	12.73	0.6
I	Czech republic	12.05	1.0
W	Belgium	11.39	0.4
G	Hungary	11.26	0.8
X	Czech republic	9.61	0.2
M	Italy	9.17	1.0
K	Czech republic	9.28	1.5
Y	Netherlands	8.45	0.7
Z	Turkey	8.18	0.4
GG	Romania	8.89	0.5
A	Poland	8.47	0.4
EE	Peru	8.26	0.8
N	France	7.44	0.7
CC	Italy	7.30	0.5
B	Poland	7.67	0.5
R	USA	6.55	1.0
DD	Australia	5.75	0.5
V	UK	6.45	0.5
FF	Cuba	6.32	0.7
S	Denmark	5.12	0.2
P	Germany	6.10	0.2
J	Germany	5.75	0.6
H	Netherlands	4.79	0.5
U	Netherlands	5.84	1.0
C	South Africa	5.59	0.2
BB	Australia	4.21	0.5
D	South Africa	4.42	0.3
F	South Africa	3.91	0.1

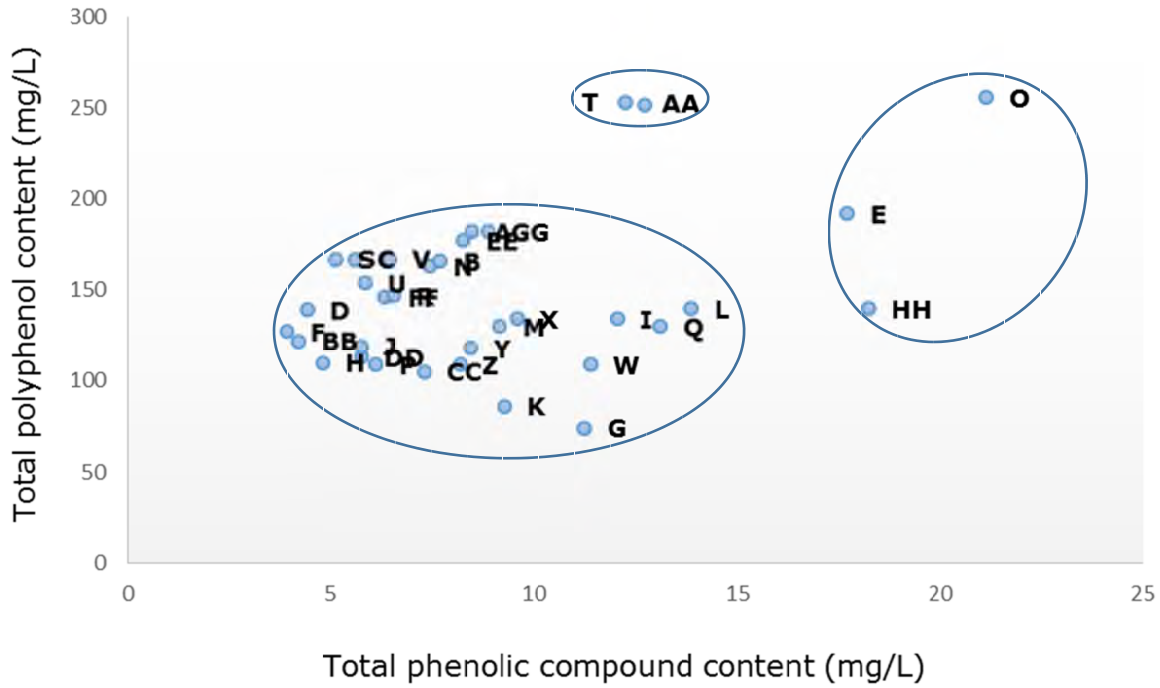
565

566 * TPCC = total phenolic compound concentration of gallic acid, hydroquinone, protocatechuic acid, catechin,
567 epicatechin, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, caffeic acid, vanillic acid, sinapic acid, syrignic
568 acid, p-coumaric acid, ferulic acid and cinnamic acid quantified in beer by HPLC.
569 SE is standard error of three independent replicate analyses.

570 Table 2: Beer bitterness descriptors (and their definitions) which were used for sensory
571 evaluation.

Attribute	**Definition**	***p*-value**
Harsh	Tingly, painful, irritating, raspy	0.000*
Acidic	Vinegary, fruit-like acidity	0.491
Tart	Acidic with sour notes	0.219
Rounded	Pleasant, not spiky, not harsh	0.000*
Metallic	Tin/metal taste, silver coin taste	0.041*
Sharp	Instant, bitterness taste at tip of tongue	0.008*
Smooth	Velvety	0.006*
Astringent	Dry, causing drying of the mouth	0.659
Artificial	Chemically, unnatural beer taste	0.517
Vegetative	Cabbage, sprout-like bitterness, hop-tea	0.000*
Progressive	Bitterness perception increases gradually	0.009*
Instant	Instantaneous bitterness	0.020*
Diminishing	Bitterness perception decreases quickly after ingestion	0.002*

572 *p-values* are from Cochran's Q-test. **P<0.05* indicates that the term was scored significantly
573 differently amongst the 10 lager beers used for sensory bitterness characterisation.



577

581 Figure 1: Plot of total phenolic compound concentrations (HPLC) versus total polyphenol

582 content (according to ASBC method Beer-35) for 34 commercial lager beers.

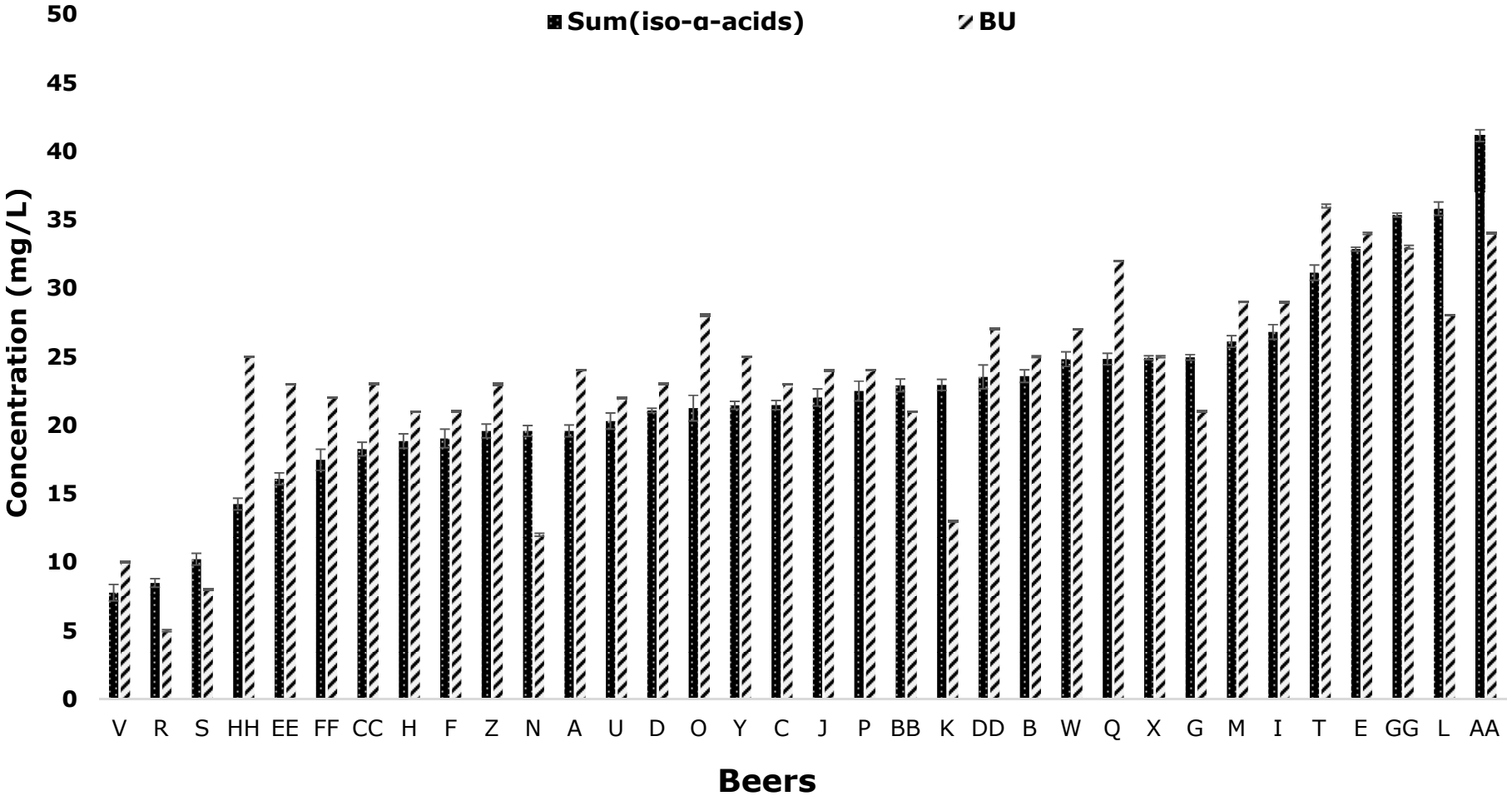
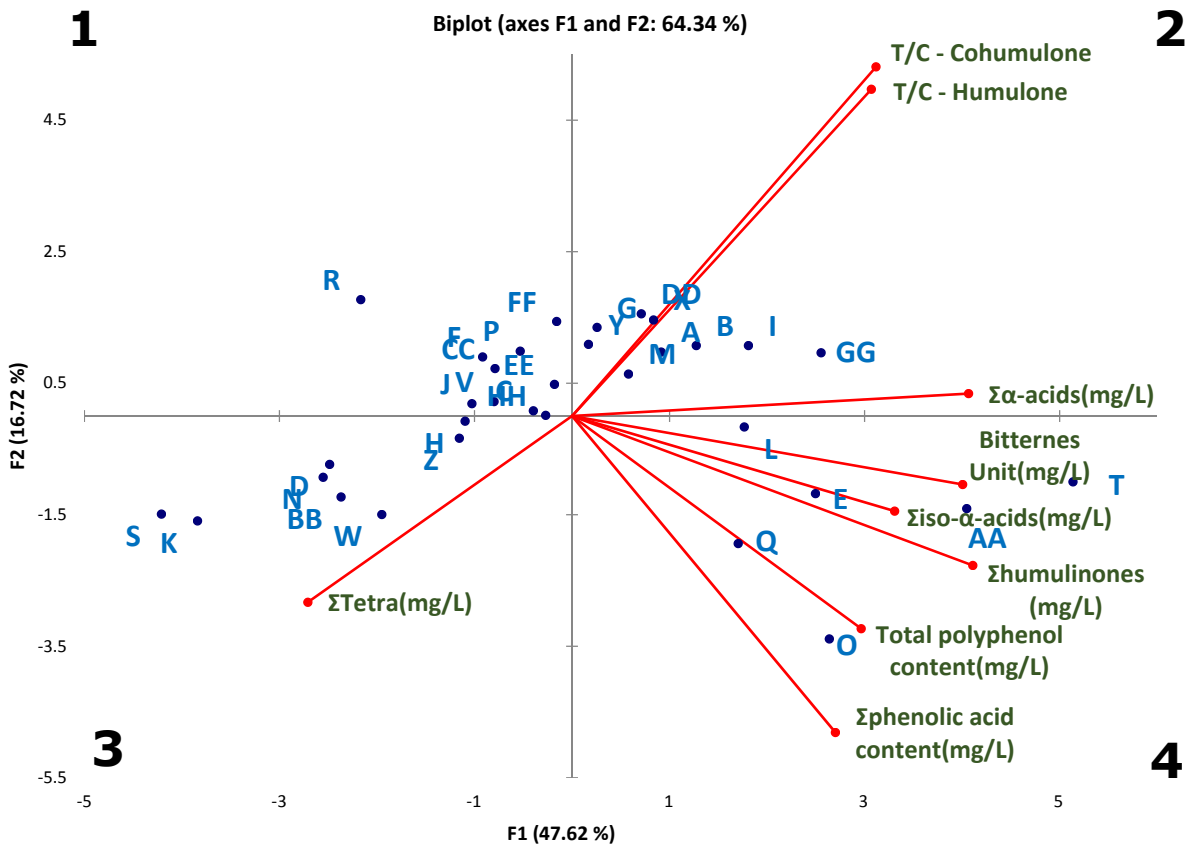


Figure 2: A comparison of the bitterness of 34 commercial lager beers as determined both by spectrophotometric BU values (striped bars) and the sum of iso-humulones determined by HPLC analysis (black bars).

Data are ordered by increasing sum of iso-α-acids; error bars represent standard error values of 3 independent replicate analyses.
*Signifies beers containing tetrahydro-iso-α-acids.



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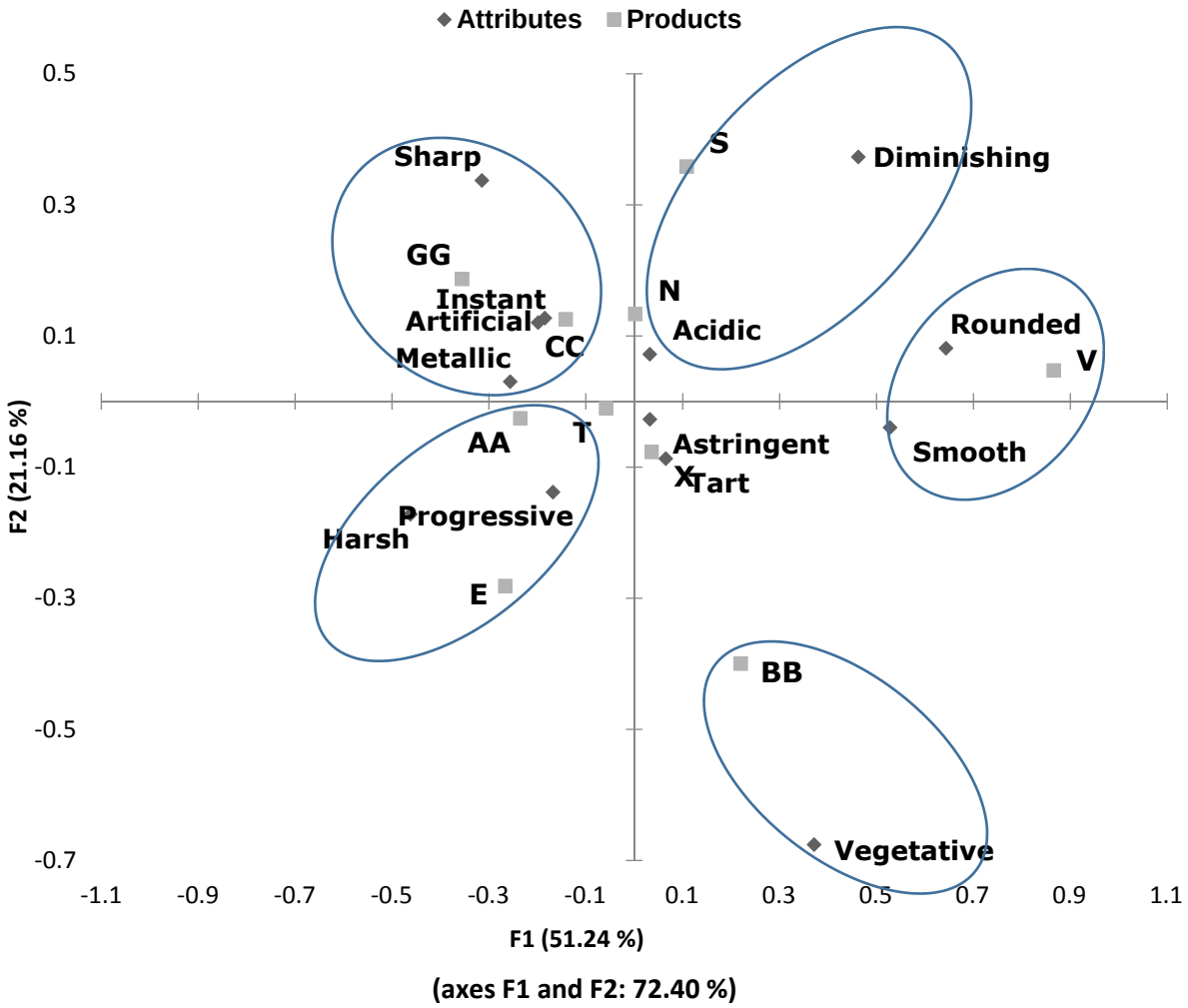
Beer	BU(mg/L)	Bitterness profile (mg/L)				TPC(mg/L)	TPCC(mg/L)*	T/C ratio(%)*
		Humulinones	Iso-α-acids	α-acids	Tetra			
CC	23.0	0.0	18	2.0	0.0	105.0	7.30	37
V	10.0	1.5	8	1.8	0.0	167.0	6.45	38
GG	33.0	0.5	35	3.5	0.0	182.0	8.89	47
X	25.0	0.0	25	1.6	0.0	134.0	9.61	48
S	8.0	0.0	10	0.0	3.8	167.0	5.12	27
N	12.0	0.0	20	0.0	3.1	163.0	7.44	34
BB	21.0	0.0	23	0.0	0.0	122.0	4.21	23
E	34.0	0.0	33	2.2	0.0	192.0	17.73	40
AA	34.0	3.0	41	3.8	0.0	253.0	12.26	43
T	36.0	2.5	31	5.3	0.0	252.0	12.73	44

583

584 Figure 3: PCA plot of 34 commercial lager beers according to their analysed contents of
585 hop acid isomers and phenolic compounds. Tabulated data provides a summary of the
586 analytical profile of the beer samples selected for sensory analysis.

587 *TPCC = total phenolic compound concentration of each beer by HPLC.

588 *T/C ratio = trans/cis ratio.



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590 Figure 4: Correspondence analysis symmetric plot of bitterness attributes (diamonds)

591 and beers (squares).

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593