

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

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

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26 Abstract

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

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

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43 Chemical compounds studied in this article

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

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51 **1. Introduction**

52 Bitterness is an important flavour character of foods and beverages such as coffee, nuts,
53 fruits and beer (Lesschaeve & Noble, 2005). Whereas the bitterness flavour of tea and
54 red wine have been attributed mainly to flavonoid phenols, approximately 80% of beer
55 bitterness is derived from the addition of hops (*Humulus lupulus*) during the 'boiling
56 stage' of the brewing process (Arrieta, Rodríguez-Méndez, De Saja, Blanco, & Nimubona,
57 2010; Caballero, Blanco, & Porras, 2012). The lupulin glands of female hop cones
58 contain soft resins rich in phloroglucinol derivatives, namely α -acids (cohumulone,
59 humulone, adhumulone) and β -acids (colupulone, lupulone, adlupulone). These acids
60 undergo thermal isomerisation to give iso- α -acids, the major bitter compounds in beer
61 (Haseleu et al., 2010). Upon isomerisation, each iso- α -acid congener is present as
62 trans/cis stereoisomers with a ratio of approximately 3:7 in conventionally hopped beers
63 (Ch Schönberger & Kostelecky, 2011). In recent years beer-bittering practice has
64 diversified, with the development and usage of hop products in a variety of different
65 forms, and with varied points of addition to the brewing process (e.g. kettle addition,
66 post-fermentation bittering products, or dry hopping, which is feasible at a number of
67 different points). One such product is pre-isomerised iso- α -acids, widely available as an
68 aqueous extract or in pellet form, which are prepared from the chemical isomerisation of
69 α -acids outside of the brewhouse. These hop products usually have higher levels of cis-
70 isomers relative to trans-isomers thus, giving a lower trans/cis ratio (Schmidt et al.,
71 2014). Bitterness can also be achieved by the use of chemically reduced derivatives of
72 iso- α -acids, so called light stable hop products such as tetrahydro-iso-humulones (tetra)
73 and hexahydro-iso-humulones (hexa) which are prepared by hydrogenation and
74 reduction reactions, respectively. Advanced hop products are popular among brewers

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

77 Furthermore, hops available in various forms (cones, pellets, plugs) can be added at
78 different stages of the brewing process. Some brewers also soak hops in beer during
79 fermentation or conditioning to improve beer aroma in a technique known as 'dry-
80 hopping'. Dry-hopping imparts oxidised α -acids (known as humulinones) to beer.
81 Humulinones levels of 0.2 – 0.5% w/w have been reported in hop leaves and pellets
82 (Cocuzza & Mitter, 2008; Negri, di Santi, & Tabach, 2010; Wolfe, 2012). In addition to α -
83 acids, hops are also a source of polyphenols in beer although the amount of polyphenols
84 present in beer will depend on hop variety, form and the point at which the hops are
85 added during the brewing process. Furthermore, depending on hopping levels, brewing
86 malt usually represents the major source of polyphenols in beer (Aron & Shellhammer,
87 2010; Callemien & Collin, 2009).

88 Polyphenols contribute to bitterness, colour, body, and astringency in beer and other
89 beverages such as tea and wine, (Collin, Jerkovic, Bröhan, & Callemien, 2013) and have
90 been recognised to influence the acceptance of beverages (Drewnowski & Gomez-
91 Carneros, 2000). In beer they act as antioxidants, preventing oxidative degradation of
92 beer whilst also providing potential health benefits to consumers through their inhibitory
93 activity on certain mutagens and carcinogens (Floridi, Montanari, Marconi, & Fantozzi,
94 2003). These compounds are diverse in chemical structure and can be divided into
95 groups consisting of simple hydroxycinnamic and hydroxybenzoic acid derivatives
96 (phenolic acids), flavanols, flavanol glycosides and prenylated flavonoids (Goiris et al.,
97 2014). Flavanols are of particular interest to brewers because they form protein-
98 polyphenol complexes, leading to the formation of haze or turbidity in beer - brewers
99 consequently remove them by cold filtration or polyvinylpyrrolidone (PVPP) treatment
100 (Garcia, Grande, & Gándara, 2004). However, PVPP treatment is not selective for the
101 removal of haze active polyphenols only - leading to losses of other polyphenols that are
102 potentially beneficial to the flavour and stability of beer (Aron & Shellhammer, 2010;
103 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.

129 **2. Materials and methods**

130 2.1 Materials

131 34 fresh commercial lager beers were sourced from 17 countries over 4 continents and
132 analysed within 8 weeks of production. For reasons of confidentiality the beers are not
133 identified but the countries from which they were sourced are as follows: Australia (2),
134 Belgium (1), Cuba (1), Czech Republic (6), Denmark (1), France (1), Germany (2),
135 Hungary (1), Italy (2), Netherlands (3), Poland (2), Peru (1), Romania (1), South Africa
136 (3), Turkey (1), UK (2) and USA (4).

137 2.2 Chemicals and reagents

138 Hydroquinone (99%), catechin (99%), epicatechin (98%), 4-hydroxybenzoic acid (99%),
139 caffeic acid (95%), vanillic acid (97%), syringic acid (95%), *p*-coumaric acid (98%),
140 sinapic acid (98%), ferulic acid (99%), 2,5-dihydroxybenzoic acid (98%), gallic acid
141 (98%), cinnamic acid (98%), salicylic acid (99%), 1,2-dihydroxybenzene (99%),
142 homovanillic (99%), gentisic acid (98%) and chlorogenic acid (99%) were all purchased
143 from Sigma-Aldrich (UK). Protocatechuic acid (99.6%) was acquired from HWI analytic
144 (Germany). Ethyl benzoate, isooctane and methanol (all HPLC grade) as well as
145 orthophosphoric acid 85% (ASC grade) were purchased from VWR (UK). Reverse
146 osmosis (RO) water was obtained from a Milli-Q water purification system by Millipore.
147 Carboxymethylcellulose (CMC), ethylenediamine tetraacetic acid (EDTA), ammonia and
148 ferric reagent solutions were all technical grade chemicals from VWR (UK). For
149 humulinone synthesis, CO₂ extract of α -acid resin (86%) was kindly donated by Botanix,
150 Paddock Wood, Kent. Cumene hydroperoxide (80% technical grade), diethyl ether,
151 sodium bicarbonate, hexane, phosphoric acid and hydrochloric acid (HCl) were all from
152 Sigma-Aldrich (UK) and of ASC reagent grades.

153 Iso- α -acid standard (ICE-3) containing trans-isocohumulone, trans-isohumulone, trans-
154 isoadhumulone (62.3% w/w), α - & β -acid (44.64%, 24.28% w/w), and tetra standard
155 (99.3% w/w) were purchased from Labor Veritas Co. (Switzerland).

156 2.3 Instrumentation

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

163 2.3 Analysis of hop bitter acids in beer

164 2.4.1 Extraction of hop bitter acids from beer

165 Cold beer was degassed by stirring for 1 h followed by the transfer of an aliquot (5 ml)
166 into a 50 ml centrifuge tube, the degassed beer was acidified with orthophosphoric acid
167 (100 μ l) and an internal standard (benzoic acid) was added (0.003 mg/L). The mixture
168 was then extracted into isooctane (10 ml) on a roller bed for 30 min. The isooctane
169 extract was transferred into a glass tube and evaporated under a controlled flow of
170 Nitrogen with a Visidry attachment coupled to a solid phase extraction manifold
171 (Supelco). The residue was dissolved in acetonitrile (2 ml) to give the HPLC sample.

172 2.4.2 HPLC-UV analysis of hop bitter acids

173 Hop acid separation was achieved with a binary mixture of (A) 1% v/v acetic acid and
174 (B) 0.1% v/v orthophosphoric acid in acetonitrile. The gradient elution was: 0-5 min:
175 30% A, 70% B; 15-24 min: 20% A, 80% B; 25 min: 10% A, 90% B; 30 min: 10% A,
176 90% B; 35 min: 0% A, 100% B; 44 min: 0% A, 100% B; 46 min: 30% A, 70% B; 55
177 min: 30% A, 70% B over a 55 min run time. Injection volume was 10 μ l, flow rate was
178 0.5 ml/min and column temperature was 25°C. The peak area of iso- α -acids,
179 humulinones were extracted at 270 nm and at 310 nm for tetrahydro-iso- α -acids.

180 2.4.3 Determination of bitterness units

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

190 2.5 Analysis of phenolic/ polyphenol compounds in beer

191 2.5.1 Extraction of beer phenolic compounds

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

201 2.5.2 HPLC-UV analysis of beer phenolic compounds

202 The chromatographic method used a binary solvent system consisting of (A) 1.25 % v/v
203 acetic acid and (B) 0.1% v/v orthophosphoric acid in acetonitrile. The gradient elution
204 protocol was as follows: 0-25 min: 98% A, 2% B; 25-30 min: 76% A, 24% B; 35-40
205 min: 55% A, 45% B; 45 min: 15% A, 85% B; 50 min: 0% A, 100% B; 55-65 min: 98%
206 A, 2% B. Injection volume was 10 µl, flow rate was 0.5 ml/min and column temperature
207 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 CO₂ 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 (NaHCO₃, 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.

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

252 2.8 Quantitation and statistical analysis

253 External standard solutions of α -acids (0.5, 1, 2, 4 and 8 mg/L), iso- α -acids (1, 10, 20,
254 40 and 60 mg/L), tetra (0.5, 1, 2, 4 and 8 mg/L) and humulinones (0.5, 1, 2, 4 and 8
255 mg/L) were all prepared in acetonitrile. The mean values of triplicate injections were
256 used to plot calibration curves for the quantification of hop bitter acids in beers. A stock
257 solution (20 mg/L) of standard grade phenolic compounds was prepared by dissolving
258 (0.01 g) of the standards in a 500 ml volumetric flask containing a mixture of RO water
259 and methanol (~200 ml), before making up to mark with the same solvent mixture.
260 Serial dilution was made from the stock solution to achieve external standards of 10, 5,
261 2.5, 1 and 0.5 mg/L levels. Quantification was achieved from the standard calibration
262 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.

268 3. Results and discussion

269 3.1 Phenolic profiles of lager beers

270 The liquid-liquid extraction protocol using ethyl acetate and water enabled the effective
271 analysis of quantitatively significant phenolic compounds in beer. An example of the
272 chromatographic separation achieved with the described extraction protocol and HPLC
273 method for the Czech lager beer (L) is provided as complementary data. The HPLC
274 method described enabled the simultaneous separation and quantification of several
275 phenolics in beer. Where possible, the phenolic compounds were identified based both on
276 prior knowledge and by matching peaks against authentic standards run separately, and
277 with regard to both retention time and UV absorbance spectrum. Whilst not all peaks on
278 the trace could be identified, unknown peak areas were also integrated and included in
279 the analytical profiles of the beers labelled as unknown (U) 1,2....etc. The elution pattern
280 of phenolic acids in beer followed an order of decreasing polarity under RP-HPLC
281 conditions, thus phenolic acid derivatives of benzoic acid were eluted before the
282 hydroxycinnamic acid derivatives. The polarity of phenolic acids is increased mostly by
283 the hydroxyl group at the para-position, followed by the ortho- and meta-positions of the
284 benzene ring (Torres, Mau-Lastovicka, & Rezaaiyan, 1987). The phenolic profile of each
285 of the 34 beers was analysed, however, without further reference to beer brands it
286 would not be informative to publish this data for each 'blind-coded' beer. To illustrate the
287 variability present in the data set, we summed the total contents of the quantified
288 phenolic compounds in each beer (Table 1), which shows a substantial range of
289 concentrations (3.9 to 21.2 mg/L). Ferulic acid was the most abundant phenolic acid
290 present in the beers, with a concentration ranging from 0.98 mg/L in the Australian lager
291 (BB) to 7.61 mg/L in the American lager beer O (data not shown). *p*-coumaric acid is the
292 precursor compound to ferulic acid and is formed via the shikimic acid reaction pathway,
293 therefore the concentration of ferulic acid is usually greater than that of *p*-coumaric acid
294 in beer (Garcia et al., 2004). The concentrations of *p*-coumaric acid across the beers
295 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

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

326 3.3 Hop bitter acid profile of lager beers

327 For the bitterness profiles of these beers, the bitter tasting hop acids present in the lager
328 beers were evaluated using two separate analytical methods; firstly by HPLC as
329 described in section 2.4.2 and secondly by bitterness unit method (2.4.3). The latter
330 method has been suggested to yield inflated bitterness values due its susceptibility to
331 interference from other compounds present in beer that absorb light at the wavelength
332 of measurement (Schönberger, 2006; Tomlinson, Ormrod, & Sharpe, 1995). In contrast,
333 HPLC measurements are agreed to provide a better assessment of beer bitterness
334 because they allow for the selective quantification of iso- α -acids, the major bittering
335 principles in beer (Ting, Kay, & Ryder, 2007). A comparison of the hop bitter acid
336 concentrations in the beers by BU and HPLC methods is presented in Fig. 2. The results
337 showed that the BU of the lager beers ranged from 8 - 36 mg/L, although bitterness was
338 overestimated by the BU method in comparison to HPLC values in around 60% of the
339 samples. The sum of analysed iso- α -acid hop acid concentrations (HPLC) was between 8
340 and 41 mg/L. The average bitterness across all 34 beers in both methods was \sim 23 mg/L
341 and the most bitter beers had concentrations >30 mg/L (GG, AA and L). The latter beers
342 each had lower BU values in comparison to HPLC values, e.g. beer AA had a BU value of
343 34 mg/L but the HPLC value was 41 mg/L. Around 7 of the beers including U, J, I, E, C
344 and T had similar bitterness concentrations according to both methods. The American
345 light lager beer R contained the lowest amount of hop acids (8 mg/L) and did not contain
346 any reduced iso- α -acid products.

347 Inspection of the HPLC chromatograms revealed that both iso- α -acids and tetrahydro-
348 iso- α -acids were present in beers K, S, V, N, as well as the presence of humulinones at
349 up to 3 mg/L in beers O, Q, AA, T and V. The presence of tetrahydro-iso- α -acids in beers
350 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

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

380 Beers in quadrant 1 were generally lower in hop acid and polyphenol content compared
381 to beers in quadrant 4 which were characterised by high levels of these compounds. The
382 beers in quadrant 2 were correlated with high trans/cis ratio and residual α -acids which,
383 as noted, is indicative of conventional hopping techniques. Beers in quadrant 3 had lower
384 trans/cis ratios, (indicative of the use of pre-isomerised hops) as well as containing
385 tetrahydro-iso- α -acids. A total of 10 beers were selected from the 4 quadrants to
386 represent the diversity amongst the 34 beers: beers CC and V from quadrant 1, X and
387 GG from quadrant 2, S, N and BB from quadrant 3 and beers E, AA and T from quadrant
388 4.

389 3.5 Beer bitterness lexicon

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

398 3.6 Perceived bitterness character and correlation to bitterness and polyphenolic profile

399 The correspondence analysis of the sensory data is presented in Fig. 4. This revealed
400 that beer CC, selected from quadrant 1 of the PCA in figure 3, which had relatively low
401 hop bitter acid and polyphenol contents, was perceived as having an 'artificial', 'metallic'
402 and 'instant' bitterness. Beer V from the same quadrant (figure 3), but deduced to have
403 been dry-hopped from the presence of humulinones, had a 'rounded' and 'smooth'
404 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

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

434 **4. Conclusions**

435 The phenolic acid and total polyphenol contents of 34 lager beers brewed in different
436 geographical locations were determined. The former was achieved by a combination of
437 LLE and HPLC analysis while the latter was accomplished with an international global
438 assay method. Phenolic compound concentrations ranged from 3 – 12 mg/L and TPC was
439 between 74 - 256 mg/L, with the highest values identified in dry-hopped beers. No linear
440 relationship was found between total phenolic compound concentration and TPC although
441 dry-hopped beers were found to contain a greater amount of polyphenols in comparison
442 to beers that had not been dry-hopped (N.B. the usage of dry hopping was deduced
443 from the presence of significant concentrations of humulinones in the beer, but was not
444 verified by the manufacturers in all instances). Sensory analysis showed that beers with
445 varying profiles of bitter congeners (hop acids and phenolics) had distinctive bitterness
446 characters. These differences are believed to be driven by the selective usage of various
447 hop products and points of addition in the brewing process. Since the present work used
448 a survey of international lager brands, these factors are largely deduced, albeit logically
449 and based on obvious analytical differences between the finished beers; however, it
450 should be borne in mind that the manufacturing processes were not disclosed, nor were
451 they independent variables in the study. Dry-hopped beers generally contained more
452 polyphenol compounds and humulinones, and were sensorially perceived as having a
453 'harsh' and 'progressive' bitterness. In comparison, beers which had evidently been
454 conventionally bittered and as such contained relatively high residual amount of α -acids
455 and trans/cis ratio were perceived as having an 'instant' and 'sharp' bitterness. Beers
456 containing tetrahydro-iso- α -acids were rated as having a 'diminishing' temporal
457 character of bitterness. These results support the hypothesis that the production
458 processes employed by brewers in terms of hopping strategy, and the raw materials
459 used, give beers a distinct polyphenolic and bitterness fingerprint which influences the
460 overall bitterness impression of beer. Understanding the sensory character of bitterness
461 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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466 Nottingham in sponsoring this research.

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560 flavor when dry hopping beer.
- 561

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

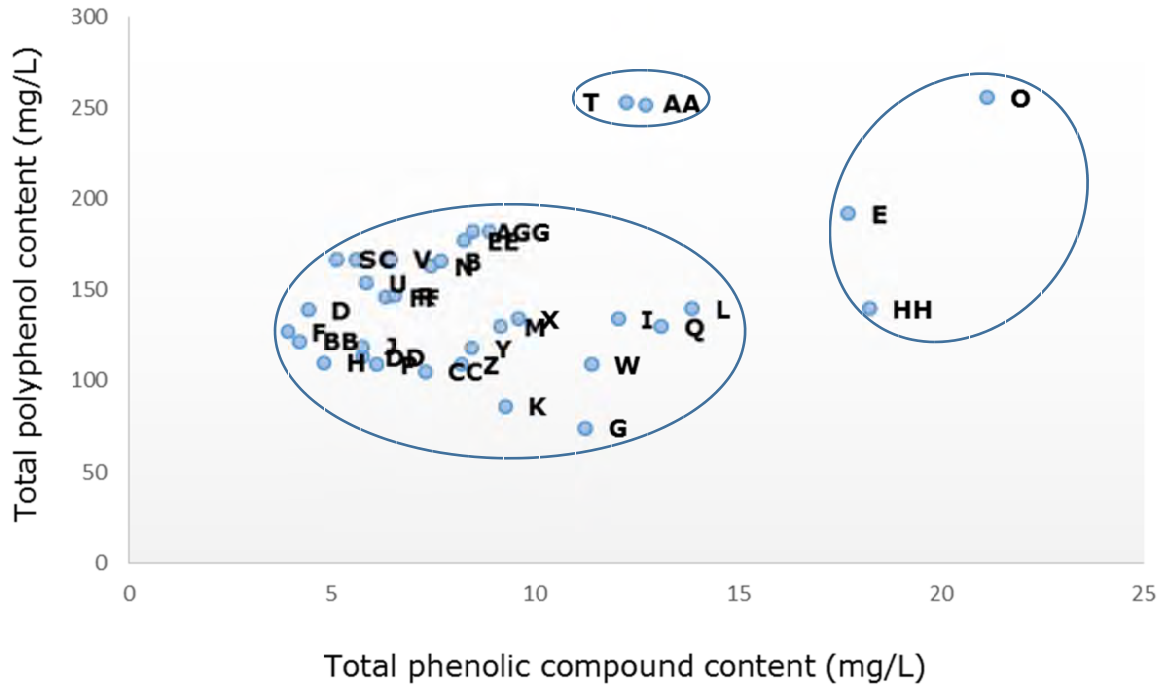
BEER	COUNTRY	TPCC* (mg/L)	
		SUM	SE
O	USA	21.17	1.5
Q	USA	13.12	0.8
HH	Czech republic	18.25	1.5
E	Czech republic	17.73	1.8
L	Czech republic	13.87	1.1
AA	UK	12.26	1.1
T	USA	12.73	0.6
I	Czech republic	12.05	1.0
W	Belgium	11.39	0.4
G	Hungary	11.26	0.8
X	Czech republic	9.61	0.2
M	Italy	9.17	1.0
K	Czech republic	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, syringic
 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.

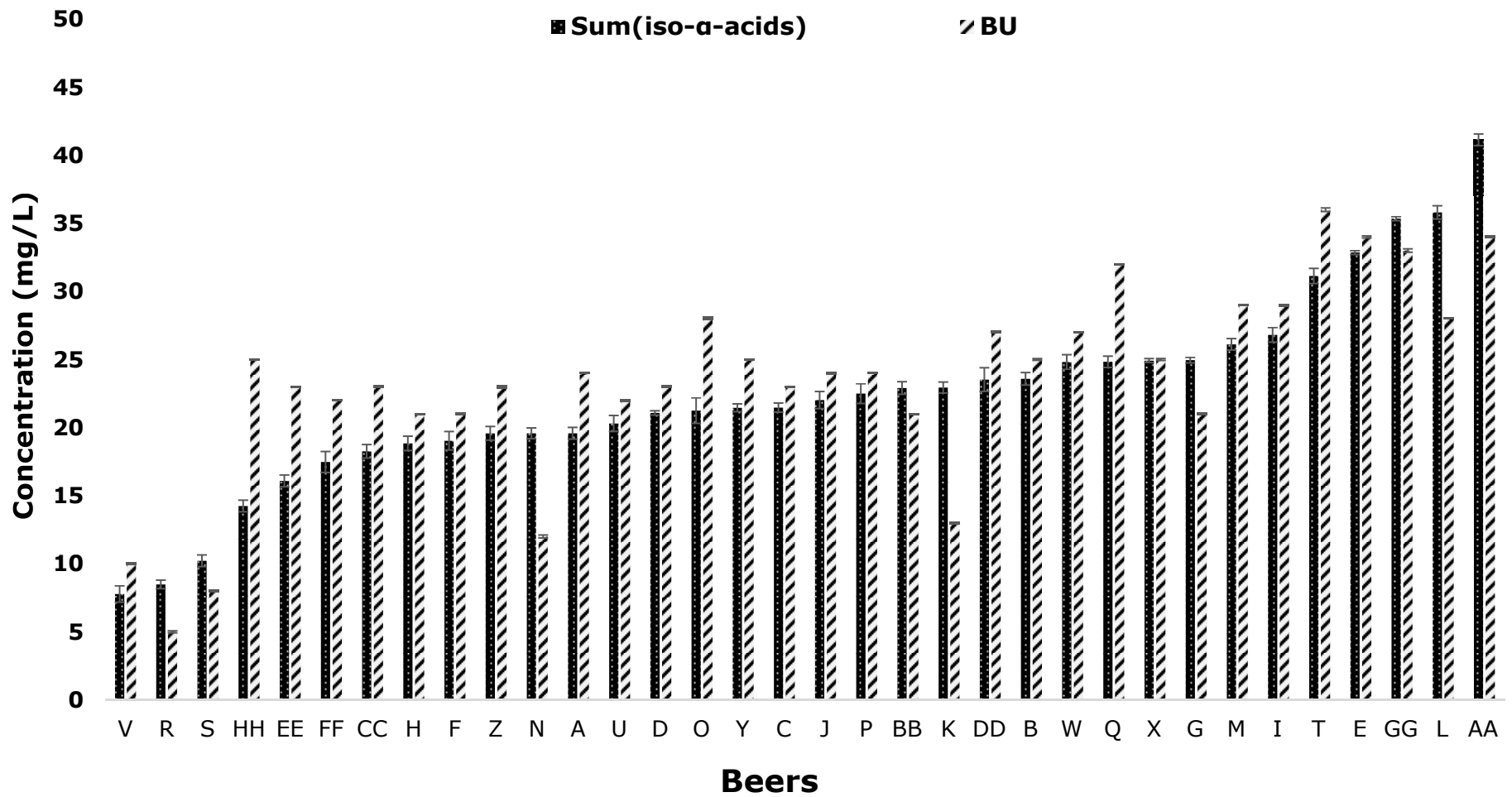
Attribute	Definition	p-value
Harsh	Tingly, painful, irritating, raspy	0.000*
Acidic	Vinegary, fruit-like acidity	0.491
Tart	Acidic with sour notes	0.219
Rounded	Pleasant, not spiky, not harsh	0.000*
Metallic	Tin/metal taste, silver coin taste	0.041*
Sharp	Instant, bitterness taste at tip of tongue	0.008*
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.

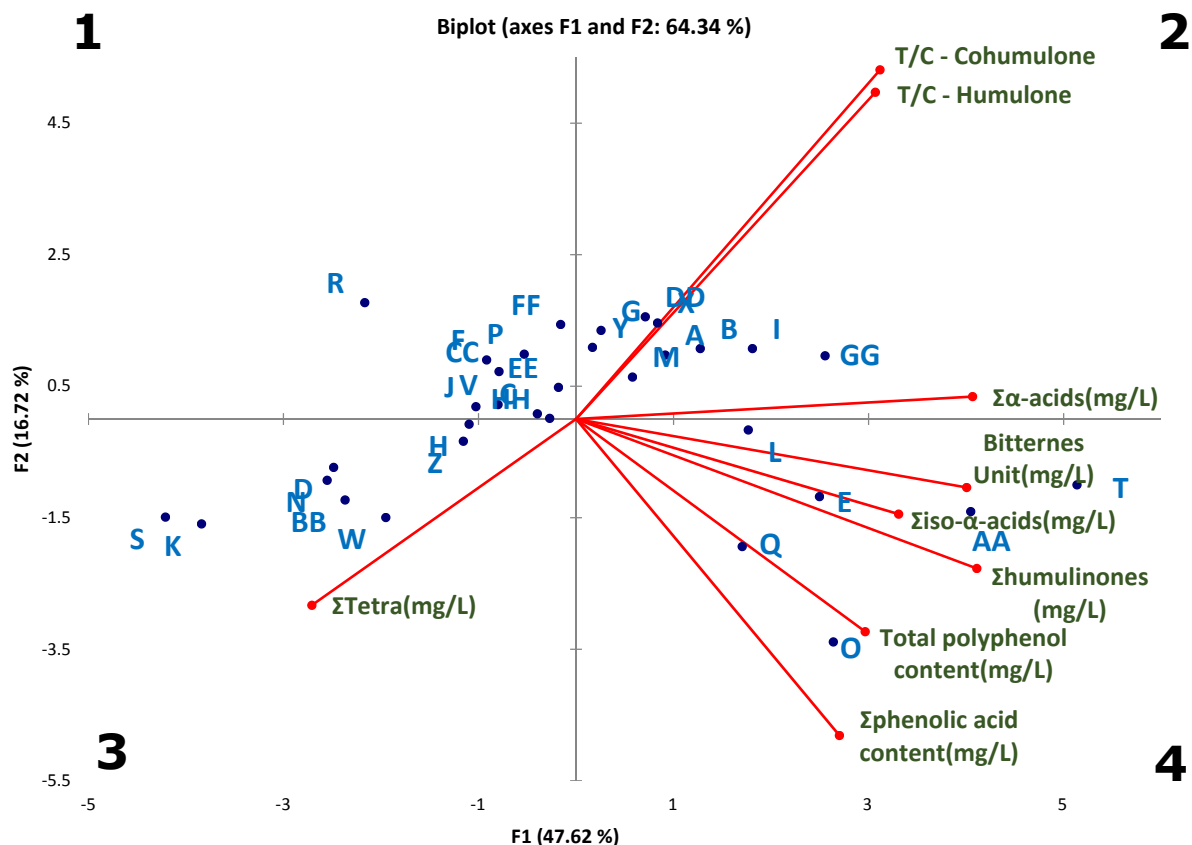


577

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

580 Data are ordered by increasing sum of iso-α-acids; error bars represent standard error values of 3 independent replicate analyses.

581 *Signifies beers containing tetrahydro-iso-α-acids.



582

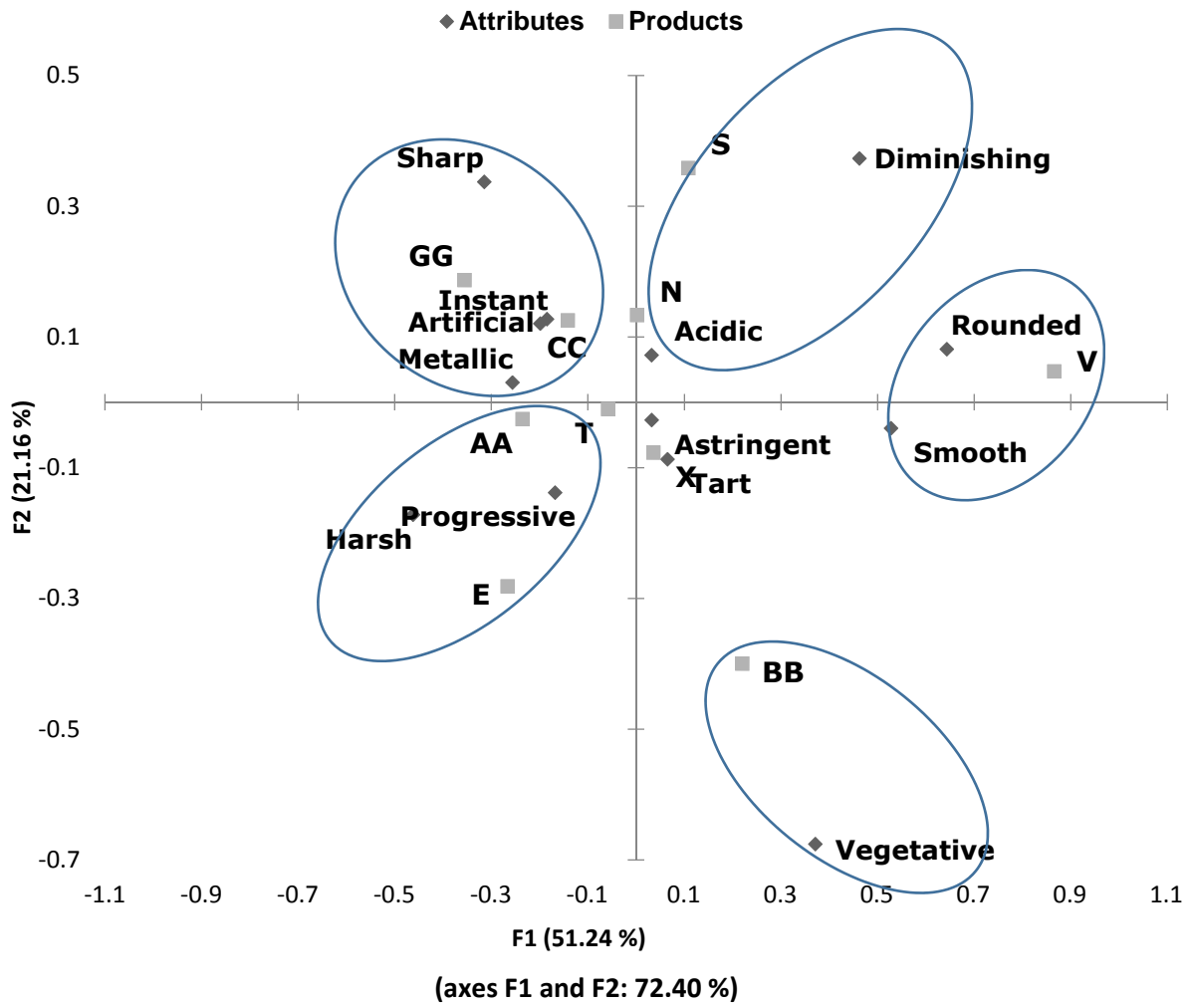
Beer	BU(mg/L)	Bitterness profile (mg/L)				TPC(mg/L)	TPCC(mg/L)*	T/C ratio(%)*
		Humulinones	Iso- α -acids	α -acids	Tetra			
CC	23.0	0.0	18	2.0	0.0	105.0	7.30	37
V	10.0	1.5	8	1.8	0.0	167.0	6.45	38
GG	33.0	0.5	35	3.5	0.0	182.0	8.89	47
X	25.0	0.0	25	1.6	0.0	134.0	9.61	48
S	8.0	0.0	10	0.0	3.8	167.0	5.12	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.



589

590 Figure 4: Correspondence analysis symmetric plot of bitterness attributes (diamonds)
 591 and beers (squares).

592
 593