A comprehensive characterization of beer polyphenols by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS)

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Running title: **Characterization of beer polyphenols by high resolution mass spectrometry**

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ABSTRACT

Beer is the second most consumed alcoholic beverage in Europe and shown by the European Prospective Investigation into Cancer and Nutrition cohort study to be the main food contributor to hydroxybenzoic acid intake. About 70-80% of the total polyphenol content in beer comes from malt, and the remaining 30-20% from hops. In this work, liquid chromatography coupled with an electrospray ionization hybrid linear ion trap quadrupole Orbitrap mass spectrometry technique has been used for an accurate identification of beer polyphenols. 47 phenolic compounds were identified using high mass accuracy and confirmed by MS^2 experiments, including simple phenolic acids, hydroxycinnamoylquinics, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha- and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavanoids. As far as we know, 7 of these compounds have been recognized in beer for the first time: feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside.

KEYWORDS: beer, polyphenols, LTQ-Orbitrap, phenolic acids, high resolution mass spectrometry.

1. Introduction

Beer is the second most consumed alcoholic beverage in Europe, accounting for 37% of the total EU alcohol consumption, according to the European Spirits Organization. The average beer consumption per capita in Europe in 2009-2011 was 72.8 L. Beer contains carbohydrates, minerals (potassium, magnesium), vitamins (niacin, riboflavin, folate, cobalamin, pyridoxine) and amino acids. Additionally, beer contains polyphenols of which about 70-80% come from malt, and the remaining 30-20% come from hops (De Keukeleire, 2000). The main phenolic compounds are hydroxybenzoic acids, cinnamic acids, such as ferulic acid, and flavonols (Clarissa Gerhäuser, 2005). Hop polyphenol content depends on the type of beer and the quantity of hops added during production. Furthermore, during the brewing process and fermentation, some polyphenols undergo chemical changes, such as decarboxylation and isomerization. Beer constitutes a good source of polyphenols and was found to be the main food contributor to hydroxybenzoic acid intake in the European Prospective Investigation into Cancer and Nutrition cohort study (Zamora-Ros et al., 2013).

Although various phenolic compounds have been found in beer using different detectors, such as the coulometric array (Floridi, Montanari, Marconi, & Fantozzi, 2003; Jandera et al., 2005; Rehová, Skeríková, & Jandera, 2004), electrochemical (Madigan, McMurrough, & Smyth, 1994; Montanari, Perretti, Natella, Guidi, & Fantozzi, 1999; Nardini & Ghiselli, 2004; Piazzon, Forte, & Nardini, 2010; Vanbeneden, Delvaux, & Delvaux, 2006), photodiode array (Bartolomé, Peña-Neira, & Gómez-Cordovés, 2000), ultraviolet-visible spectrophotometry (Arts, van de Putte, & Hollman, 2000; McMurrough, Madigan, & Smyth, 1996) and low resolution mass spectrometry (Ceslova, Holcapek, Fidler, Drstickova, & Lisa, 2009; Vanhoenacker, De Keukeleire, & Sandra, 2004), a comprehensive identification of its phenolic profile by

high resolution mass spectrometry has been lacking. High-resolution/accurate mass measurement mass spectrometry techniques have proven to be a reliable tool for the structural elucidation of unknown compounds in complex samples. In this context, linear ion trap quadrupole-Orbitrap-mass spectrometry (LTQ-Orbitrap-MS) provides single-stage mass analysis that supplies molecular weight information, two-stage mass analysis (MS/MS) and multi-stage mass analysis (MS^n) that provides structural information. Exact mass measurements and molecular formula assignment are indispensable for the characterization of polyphenols. Moreover, accurate mass measurement of the product ions facilitates the elucidation of unknown compounds.

The aim of this work was to identify the full range of polyphenols found in beer. Therefore, a solid-phase extraction procedure was applied in order to increase sensitivity and lower the matrix effect. High mass accuracy was used to identify 47 phenolic compounds, confirmed by product ion scan experiments and high mass accuracy of the fragments. To our knowledge, 7 phenolic compounds are reported as being in beer for the first time.

2. Materials and methods

2.1. Chemicals and reagents

Gallic, caffeic, protocatechuic, ferulic, chlorogenic, sinapic, *p*-coumaric, vanillic and protocatechuic acids, quercetin-3-*O*-glucoside, catechin and epicatechin (97-99% purity, all) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Isoxanthohumol, 8-prenylnaringenin and xanthohumol (97-99% purity) were purchased from Enzo Life Science (Lausen, Switzerland). Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetic acid, formic acid and sodium acetate were purchased from Panreac Quimica S.A (Barcelona, Spain). Ultrapure water (MilliQ) was generated by the Millipore System

(Bedford, USA). SPE cartridges and Oasis MAX 96-well plate 30 µm (30 mg) were obtained from Waters (Milford, MA, USA).

2.2. Samples

Four types of beer were used for the identification of the phenolic compounds present in beer: lager, Pilsen, Märzenbier and non-alcoholic beer. All beers were produced in Spain and were obtained from the market.

2.3. Sample preparation

Samples were prepared in a darkened room with a red safety light to avoid analyte oxidation. Beer foam was removed by ultrasonication and agitation using a magnetic stirrer. Then, in order to improve sensitivity, a solid-phase extraction step was applied following the method developed by Medina-Remón *et al*. for urine samples (Medina-Remon et al., 2009) and then used and validated in tomato samples (Vallverdu-Queralt, Jauregui, Medina-Remon, & Lamuela-Raventos, 2012) with minor modifications. Briefly, beer ethanol was evaporated under nitrogen flow. Oasis® MAX cartridges were activated with 1 ml of methanol and subsequently 1 ml of sodium acetate 50 mM pH 7. Then 1 ml of dealcoholized beer acidified with 34 µl of hydrochloric acid at 35% was loaded into the cartridges. Cartridges were rinsed with sodium acetate 50 mM pH 7.0 containing 5% methanol. Polyphenols were eluted with 1800 µl of methanol with 2% formic acid. The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with water (0.1% formic acid) up to 100 µl and filtered through a 13 mm, 0.45 µm PTFE filter into an insert-amber vial for HPLC analysis. Samples were stored at -20 ºC until analysis. In order to prove that the beer matrix did not influence recovery of the analytes, a recovery assay was performed using 12 representative beer polyphenols (4-hydroxybenzoic, caffeic acid, catechin, epicatechin, chlorogenic, ferulic acid, kaempferol-*O*-glucoside, *p*-coumaric acid, protocatechuic acid, quercetin-3-*O*-

glucoside, sinapic and vanillic acids). The recoveries of the polyphenols in beer ranged from 78.3% ± 6.6% and 113.5% ± 8.6%.

2.4. LC-High resolution mass spectrometry and experimental conditions

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a setting of 30,000 resolution at *m/z* 400. Operation parameters were as follows: source voltage, 4 kV; sheath gas, 20 (arbitrary units); auxiliary gas, 10 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 275 °C. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target $5 \cdot 10^5$ for MS mode and $5 \cdot 10^4$ for MS^n mode). Beer samples were analyzed in full scan mode at a resolving power of 30,000 at *m/z* 400 and data-dependent MS/MS events acquired at a resolving power of 15,000. The most intense ions detected during full scan MS triggered data-dependent scanning. Data-dependent scanning was carried out without the use of a parent ion list. Ions that were not intense enough for a data-dependent scan were analyzed in MS^n mode with the Orbitrap resolution also set at 15,000 at *m/z* 400. An isolation width of 100 amu was used and precursors were fragmented by collision-induced dissociation C-trap (CID) with a normalized collision energy of 35 V and an activation time of 10 ms. The mass range in FTMS mode was from *m/z* 100 to 1000. The data analysis was achieved using XCalibur software v2.0.7 (Thermo Fisher Scientific). An external calibration for mass accuracy was carried out before the analysis.

Liquid chromatography analysis was performed using an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler. Chromatographic

separation was accomplished with a Phenomenex Luna C_{18} column 50 x 2.0 mm i.d, 3 µm (Phenomenex, Torrance, CA, USA). Gradient elution of analytes was carried out with water/0.1 % formic acid (solvent A) and acetonitrile/0.1 % formic acid (solvent B) at a constant flow rate of 0.4 l/min, and the injection volume was 5 µl. A non-linear gradient was applied: 0 min, 2% B; 0-2 min, 8% B; 2-12 min, 20% B; 12-13 min, 30% B; 13-14 min, 100% B; 14-17 min, 100% B; 17-18 min, 2% B and the column was equilibrated for 7 min to initial conditions.

3. Results and discussion

3.1. General

The data-dependent scan experiment was very useful for the identification of unknown phenolic compounds since it provides high resolution and accurate mass product ion spectra from precursor ions that are unknown beforehand within a single run. Combining data-dependent scan and MS^n experiments, 47 phenolic compounds were tentatively identified in beer including simple phenolic acids, hydroxycinnamoylquinic acids, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha- and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavanoids. As far as we know, 7 of these have never been deteremined before in beer: feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside.

Phenolic compounds were identified by generating the molecular formula using accurate mass with some restrictions (C=30, H=100, O=15), and matching with the isotopic pattern. This molecular formula was then identified using the Dictionary of Natural Products (Chapman & Hall/CRC). Lastly, analytes were confirmed using MS/MS data and comparing the fragments found with the literature.

166 Table 1 shows the list of 47 phenolic compounds tentatively identified using LC-ESI-
167 LTQ-Orbitrap, along with their retention time, accurate mass molecular formula and
168 error (mDa). No differences in the phenolic profile were observed among the 4 types of
169 beer, except for Märzenbier, which lacked catechin-*O*-hexoside.
170 Figure 1 shows an FTMS chromatogram of a beer sample.
171 *3.2. Phenolic acids*
172 *3.2.1. Hydroxybenzoic acids and derivatives*
173 Hydroxybenzoic acids have a C_6-C_1 chemical structure and show a characteristic loss of
174 CO_2 $[M-H-44]^-$ in MS^2 experiments (Vallverdú-Queralt, de Alvarenga, Estruch, &
175 Lamuela-Raventos, 2013). The examination of the chromatograms in FTMS mode and
176 the data-dependent scan revealed the presence of gallic acid (*m/z* 169.3014, 0.6 mDa)
177 protocatechuic acid (*m/z* 153.0193, 0.06 mDa), 4-hydroxybenzoic acid (*m/z* 137.0241,
178 0.20 mDa), vanillic acid (*m/z* 167.0349, 0.07 mDa) and dihydroxybenzoic acid (*m/z*
179 153.0187, 0.60 mDa). All ions showed a loss of 44 u in the MS^2 spectra, and vanillic
180 acid showed an extra fragmentation due to the loss of the methyl group $[M-H-15]^-$.
181 Moreover, protocatechuic acid-*O*-hexoside was also identified (*m/z* 315.0710, 1.10
182 mDa) and confirmed by MS^2 experiments, which showed the loss of the intact sugar
183 $[M-H-162]^-$ with an error below 0.30 mDa.
184 *3.2.2. Hydroxycinnamic acids and derivatives*
185 Hydroxycinnamic acids have a C_6-C_3 structure with a double bond in the side chain in
186 *cis* or *trans* configuration. Hydroxycinnamic acids are secondary metabolites generated
187 from phenylalanine and tyrosine and are the precursors of the other polyphenol classes
188 in plant biosynthetic pathways (El-Seedi et al., 2012). The examination of the
189 chromatograms led to the identification of a few hydroxycinnamic acids: caffeic acid
190 (*m/z* 179.0349, 0.06 mDa), *p*-coumaric acid (*m/z* 163.0400, 0.05 mDa), ferulic acid (*m/z*

193.0506, 0.06 mDa) and sinapic acid (*m/z* 223.0611, 0.08 mDa). The typical loss of CO_2 $[M-H-44]^-$ was observed for all hydroxycinnamic acids with an error below 0.40 mDa. Loss of a methyl group $[M-H-15]^-$ was also shown in ferulic and sinapic acid with an error of 0.1 mDa. Moreover, ferulic, *p*-coumaric and sinapic acid were confirmed comparing the retention time and the MS^2 spectrum with pure standards.

Hydroxycinnamic acid derivatives were also identified in beer samples. The MS^2 spectra showed the characteristic fragmentation involving cleavage of the sugar moiety $[M-H-162]^-$. Accurate mass measurement revealed the presence of two caffeic acid-*O*-hexosides (*m/z* 341.0877, 0.43 mDa), one coumaric acid-*O*-hexoside (*m/z* 325.0928, 0.10 mDa), one ferulic acid-*O*-hexoside (*m/z* 355.1034, 0.10 mDa), and two sinapic acid-*O*-hexosides (*m/z*385.1139, 0.12 mDa). While caffeic acid, coumaric acid and sinapic acid have been described in beer elsewhere (Bartolomé et al., 2000; Floridi et al., 2003; Jandera et al., 2005; Montanari et al., 1999; Nardini & Ghiselli, 2004; Rehová et al., 2004), as far as we know, this is the first time that these hexoside hydroxycinnamic acid derivatives have been reported in beer.

A peak showing *m/z* 353.0877 revealed the presence of four caffeoylquinic acids with an error below 0.13 mDa: neochlorogenic, chlorogenic, cryptochlorogenic and 1-caffeoylquinic acids. It was possible to differentiate the four isomers by their relative intensities in MS^2 spectra according to the method cited by other authors using mass spectrometry (Clifford, Johnston, Knight, & Kuhnert, 2003; Parejo et al., 2004). All caffeoylquinic acid isomers show a characteristic fragment of *m/z* 191.0561 with a 0.60 mDa of error which corresponds to the quinic acid.

Also found was feruloylquinic acid (*m/z* 367.1034, 0.10 mDa), which is the first time it has been detected in beer, to our knowledge. MS^2 spectra of feruloylquinic acid showed

215 the fragment of quinic acid (*m/z* 191.0506, 0.20 mDa) and ferulic acid (*m/z* 193.0506,
216 0.40 mDa).

217 *3.2.3. Hydroxyphenylacetic acids*

218 Hydrophenylacetic acids have a C_6-C_2 carbon structure and are characterized by loss of
219 a molecule of CO_2 in MS^2 experiments. Beer is one of the major sources of
220 hydroxyphenylacetic acids in European diets, along with olives, cider and wine
221 (Zamora-Ros et al., 2013).

222 Three peaks were identified in FTMS corresponding to three hydroxyphenylacetic acid
223 isomers (*m/z*151.0395, 0.05 mDa) confirmed by the exact mass. One isomer is expected
224 to be 4-hydroxyphenylacetic acid since this has been reported in beer previously
225 (Nardini, Natella, Scaccini, & Ghiselli, 2006).

226 *3.3. Flavonoids*

227 Flavonoids are a large family of compounds with a common chemical structure: a
228 diphenylpropane skeleton bearing two benzene rings (A and B) connected by a pyran
229 ring attached to the A ring. Flavonoids are further divided into subclasses
230 (anthocyanidins, chalcones, flavanols, flavanones, flavonols, flavones and
231 isoflavonoids). Many biological effects and health benefits have been associated with
232 flavonoid consumption, although these effects may be related to their ability to
233 modulate cell-signaling rather than their antioxidant activity (Williams, Spencer, &
234 Rice-Evans, 2004).

235 *3.3.1. Flavanols and derivatives*

236 Using LTQ-Orbitrap in FTMS and MS^2 modes, the comprehensive fragmentation
237 pathways of (+)-catechin and (-)-epicatechin (*m/z* 289.0717, 0.12 mDa) was
238 unambiguously determined. Additionally, (+)-catechin and (-)-epicatechin were
239 confirmed with pure standards.

Two types of hexoside derivatives were found in beer samples: catechin-*O*-hexoside (*m/z* 451.1245, 0.13 mDa) and catechin-*O*-dihexoside (*m/z*613.1773, 0.12 mDa). Both derivatives showed the loss of the sugar moiety in MS^2 spectra. Catechin-O-hexoside has been found in beer previously (C. Gerhäuser et al., 2002) but, to our knowledge, this is the first report of catechin-*O*-dihexoside in beer.

3.3.2. Flavonol derivatives

Kaempferol-3-*O*-glucoside (*m/z* 447.0932, 0.05 mDa) and quercetin-3-*O*-glucoside (*m/z* 463.088, 0.12 mDa) were found in beer by analyzing the chromatograms in FTMS, for which both analytes showed the loss of 162 u due to the sugar moiety. Quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside were further confirmed by comparing the chromatograms with pure standards. Kaempferol-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-arabinoside and quercetin-3-*O*-glucoside have been previously reported in beer (Clarissa Gerhäuser, 2005; Jandera et al., 2005; Rehová et al., 2004), but not kaempferol-3-*O*-glucoside. 3,7-dimethylquercetin (*m/z* 329.0666, 0.09 mDa) was also found and confirmed by MS^2 spectra showing the loss of the methyl groups $[M-H-15]^-$.

3.3.3. Flavones

The examination of the chromatograms in FTMS mode and dependent scan led to the identification of three apigenin derivatives. Apigenin-*C*-hexoside (*m/z* 431.0983, 0.20 mDa) showed a loss of 120 and 90 u in the MS^2 spectra characteristic of the cross-ring fissions in the sugar unit, as reported previously in apigenin-6,8-di-*C*-hexoside (Marín, Ferreres, Tomás-Barberán, & Gil, 2004; Parejo et al., 2004). Apigenin-*C*-hexoside-*O*-hexoside (*m/z* 593.1511, 0.30 mDa) was also detected and showed the two different fragmentation patterns of *C*-glycosides and *O*-glycosides in MS^2 experiments: [M-H-

120]$^-$ and [M-H-90]$^-$ which corresponds to the loss of *C*-glycosides, and [M-H-162]$^-$ which agrees with the loss of the intact *O*-glycoside.

Also found was apigenin-*C*-hexoside-*C*-pentoside (*m/z* 563.1406, 0.23 mDa), which showed an extra loss of 90, 120 and 60 u in MS^2 spectra characteristic of cross-ring cleavage of the *C*-pentoside unit as shown in Figure 2 and also reported previously (Marín et al., 2004; Vallverdú-Queralt, Jáuregui, Di Lecce, Andrés-Lacueva, & Lamuela-Raventós, 2011). Apigenin-*C*-glucoside and apigenin-*C*-diglucoside have been reported in beer previously (C. Gerhäuser et al., 2002), but as far as we know, this works demonstrates for the first time that apigenin-*C*-hexoside-*C*-pentoside is also present.

3.4. Bitter acids

Bitter acids are characteristic compounds of beer since they are synthesized in the lupulin glands of the hop plant. Bitter acids have a prenylated polyketide structure and there are two main subclasses: *α*-acids (humulones) and *β*-acids (lupulones). *α*-acids (n-, co-, and ad-humulone) undergo thermal isomerization during wort boiling and are transformed into iso-*α*-acids (isohumulone, isocohumulone and isoadhumulone) *via* an acyloin-type ring contraction (De Keukeleire, 2000). Iso-*α*-acids play an important role in beer organoleptic properties, since they contribute to its bitter flavour (Huvaere, Sinnaeve, Van Bocxlaer, & De Keukeleire, 2004) and the stability of the foam (Ferreira, Jorge, Nogueira, Silva, & Trugo, 2005; Kunimune & Shellhammer, 2008).

Three *α*-acids were identified in FTMS mode and dependent scan spectra: co-humulone (*m/z* 347.1864, 0.30 mDa), n-humulone and ad-humulone (*m/z* 361.2020, 1.11 mDa). All *α*-acids showed the same fragmentation pattern in MS^2 spectra: the loss of 69 u, which corresponds to the scission of the prenyl chain (3-methyl-but-2-en-1-yl, C_5H_9) with an error below 0.30 mDa. A fragment of 235.1335 (error of 0.30 mDa) was also

observed in some peaks, which might be related to ring fission. Ad-humulone and n-humulone were distinguished according to the intensity of the *m/z* 292.1312 fragment, as reported previously (Hofte & Van Der Hoeven, 1998), the peak with a greater intensity of the mass fragment being matched to n-humulone. A co-humulone isomer was also detected, although the two compounds could not be discriminated based on their mass fragment intensities.

Three iso-α-acids were also found in beer: iso-α-cohumulone, iso-α-adhuhulone and iso-α-humulone. Although iso-α-acids have the same molecular weight as α-acids, they could be distinguished by the different fragmentation pattern. Iso-α-acids showed a characteristic loss of 96 u, which corresponds to the scission of the side chain (4-methyl-pent-3-en-1-oxo, C_6H_8O) instead of 69 u (C_5H_9). Iso-α-acids were identified with an error below 0.40 mDa. Iso-α-adhumulone and iso-α-humulone could not be distinguished since both peaks had the same fragmentation pattern and intensities.

3.5. Prenylflavanoids

Prenylflavanoids are a sub-class of flavonoids, mainly found in hops, characterized by having a prenyl group attached in the flavanoid backbone (ring A). Female inflorescences of hops used in brewing are particularly rich in xanthohumol, although during the brewing process, xanthohumol isomerizes into isoxanthohumol (Stevens, Taylor, Clawson, & Deinzer, 1999). Other important hop flavanoids previously found in beer are desmethylxanthohumol, 6-prenylnaringenin 8-prenylnarigenin and 6-geranylnaringenin (Stevens, Taylor, & Deinzer, 1999).

All prenylflavanoids follow the same fragmentation pattern and show ions corresponding to retro-Diels Alder fragmentation in the C-ring, involving 1,3 scission in MS^2 spectra, as described previously in other flavonoids like naringenin (Vallverdu-Queralt, Jauregui, Medina-Remon, Andres-Lacueva, & Lamuela-Raventos, 2010).

Isoxanthohumol (*m/z* 353.1394, 0.07 mDa), 8-prenylnaringenin and 6-prenylnaringenin (*m/z* 339.1237, 0.22 mDa and 0.13 mDa, respectively) were found in beer samples and confirmed using MS^2 data. Isoxanthohumol and 8-prenylnaringenin were further confirmed by comparison with pure standards.

3.6. Alkylmethoxyphenols

Flavour-active volatile phenols, such as vanillin, acetovanillone, 4-vinylsyringol, 4-vinylguaiacol and 4-vinylphenol, have been described in beer previously (Vanbeneden, Gils, Delvaux, & Delvaux, 2008). In particular, ferulic acid releases 4-vinylguaicol through decarboxylation by thermal decomposition during the wort boiling (McMurrough, Madigan, Donnelly, et al., 1996) and by enzymatic reaction during fermentation and brewing (Coghe, Benoot, Delvaux, Vanderhaegen, & Delvaux, 2004)

The higher sensitivity of the LTQ-Orbitrap system enabled the identification of low-intensity signals, such as 4-vinylguaicol (*m/z* 149.0607, 0.80 mDa). MS^2 spectrum confirmed the presence of this compound, showing a loss of the methyl group $[M-H-15]^-$

3.7. Indole-based compounds

Indole-3-carboxylic acid (*m/z* 174.0560, 0.06 mDa) was also found in beer samples. MS^2 spectra showed a loss of CO_2 $[M-H-44]^-$. Indole-3-carboxylic acid is a product of the biotransformation of the indole alkaloid gramine, which is found in barley (Digenis, 1969).

4. Conclusion

Using an LTQ-Orbitrap high resolution mass spectrometer, we were able to identify 47 phenolic compounds in beer, seven of which, as far as we know, are reported for the first time. Most of these polyphenols are hexosides, dihexosides, pentosides and quinic conjugates, such as feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-

hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside. LC-ESI-LTQ-Orbitrap-MS allowed the characterization of the polyphenols present in beer, based on the accurate mass measurement with a low error (<1.1 mDa) and the MS^2 spectra data. The phenolic compounds were further confirmed by comparisons with pure standards whenever possible as well as with the literature.

This study broadens knowledge of beer polyphenols, which might be helpful for further research on the health and sensory properties of beer.

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501 **Figure captions**

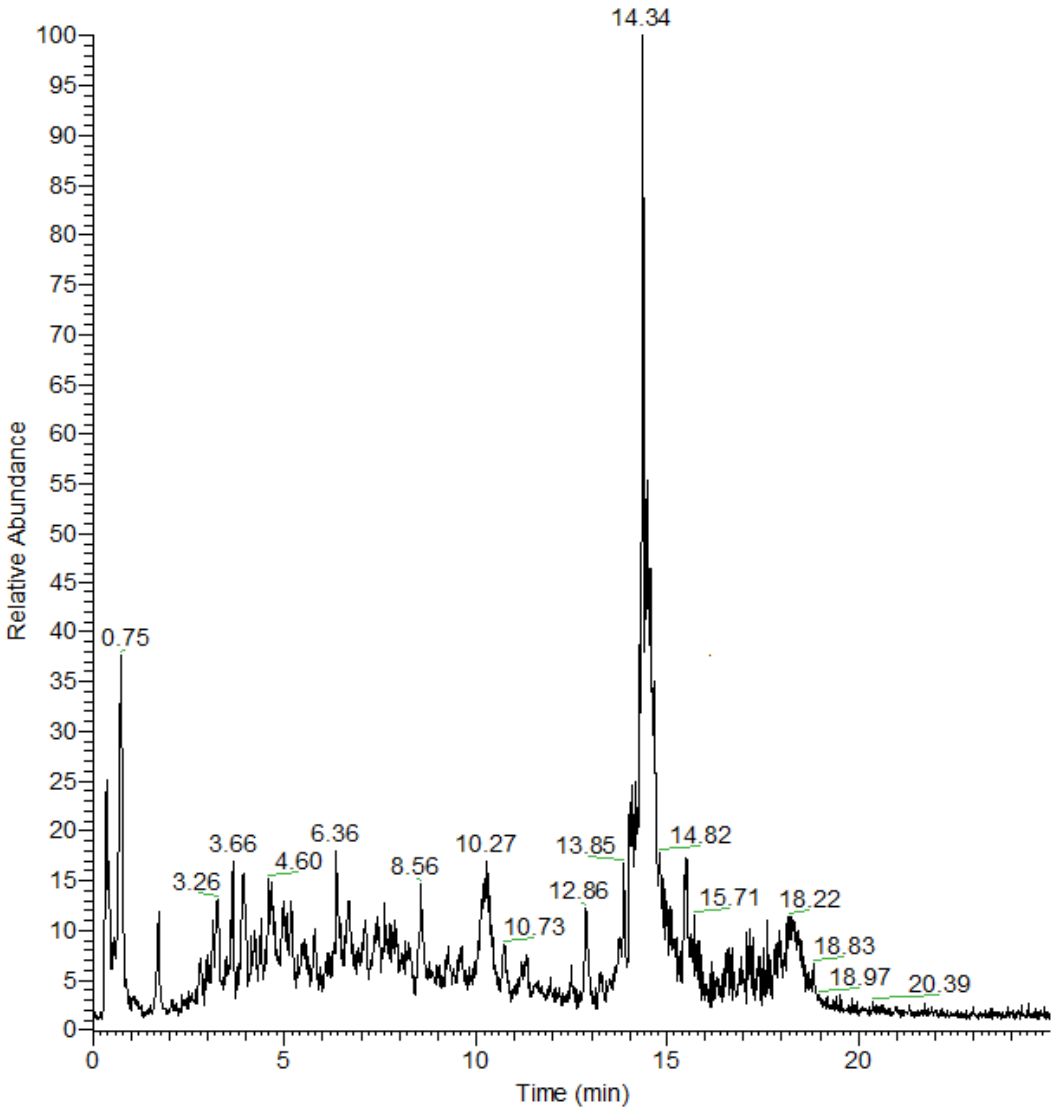
502 **Figure 1.** Beer FTMS chromatogram.

503 **Figure 2 .**MS^2 spectra of apigenin-*C*-hexoside-*O*-hexoside (A) and apigenin-*C*-
504 hexoside-*C*-pentoside (B).

505 **Tables**

506 **Table 1.** Phenolic acids identified in beer

507



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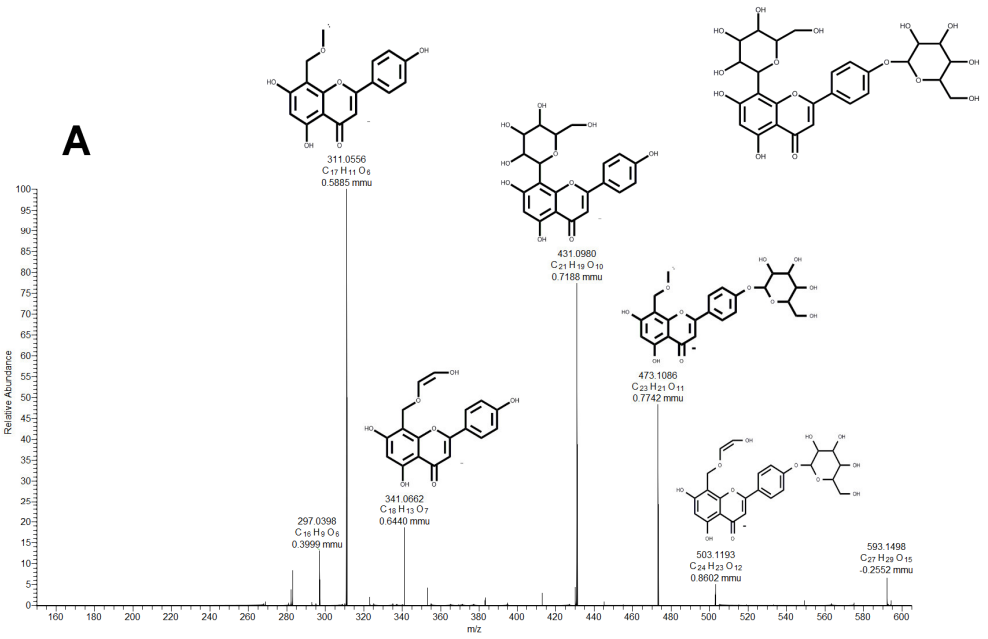
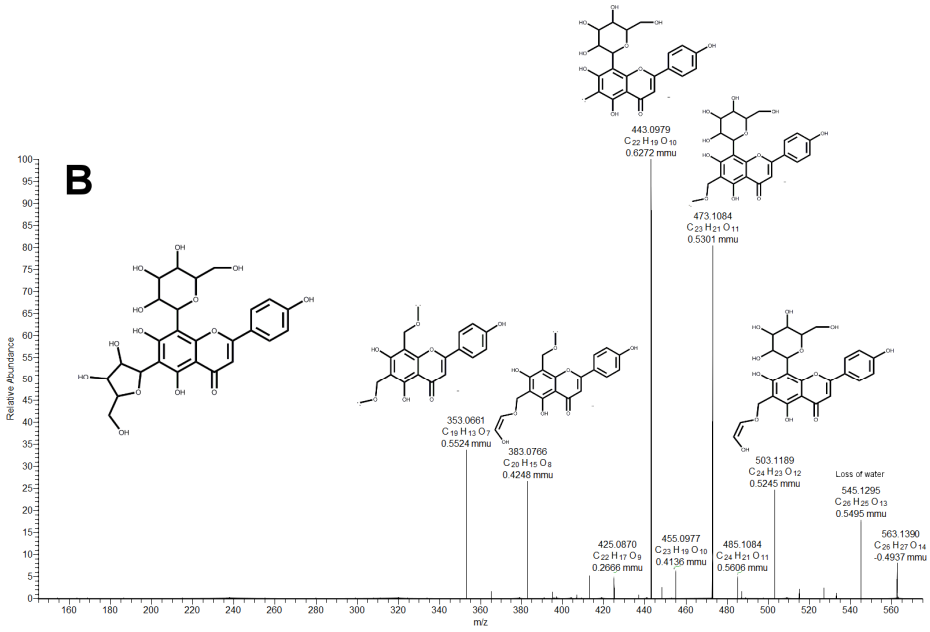

A
311.0556
C17 H11 O8
0.5885 mmu
431.0980
C21 H19 O10
0.7188 mmu
473.1086
C23 H21 O11
0.7742 mmu
341.0662
C18 H13 O7
0.6440 mmu
297.0398
C15 H9 O6
0.3999 mmu
503.1193
C24 H23 O12
0.8602 mmu
593.1498
C27 H29 O15
-0.2552 mmu
Relative Abundance
m/z


B
443.0979
C22 H19 O10
0.6272 mmu
473.1084
C23 H21 O11
0.5301 mmu
353.0661
C18 H13 O7
0.5524 mmu
383.0766
C20 H15 O8
0.4248 mmu
503.1189
C24 H23 O12
0.5245 mmu
Loss of water
545.1295
C26 H25 O13
0.5495 mmu
563.1390
C26 H27 O14
-0.4937 mmu
425.0870
C22 H17 O9
0.2686 mmu
455.0977
C23 H19 O10
0.4136 mmu
485.1084
C24 H21 O11
0.5606 mmu
Relative Abundance
m/z

510

511

TABLES

Table 1. Phenolic acids tentatively identified in beer.

Compound	Rt (min)	Accurate mass [M-H-]$^-$	Fragments *m/z* (% intensities)	Error (mDa)	Molecular Formula
Gallic acid*	1.16	169.0142	125.0241 (100)	0.60	$C_7H_6O_5$
4-vinylguaiacol	1.46	149.0607	134.0362 (100)	0.80	$C_9H_{10}O_2$
Caffeic acid-*O*-hexoside I	1.55	341.0877	179.0345 (100), 135.0400 (10)	0.40	$C_{15}H_{18}O_9$
Caffeic acid-*O*-hexosideII	2.23	341.0877	179.0344 (100), 135.0400 (10)	0.43	$C_{15}H_{18}O_9$
Protocatechuic acid-*O*-hexoside	2.3	315.0710	153.0190 (100), 109.0291 (10)	1.10	$C_{13}H_{16}O_9$
Protocatechuic acid*	2.45	153.0193	109.0292 (100)	0.06	$C_7H_6O_4$
Catechin*	2.96	289.0717	245.0814 (100), 205.0502 (50),179.0345 (20)	0.12	$C_{15}H_{14}O_6$
Catechin-*O*-hexoside I	3.06	451.1245	289.0710, (100) 245.0817(10)	0.13	$C_{21}H_{24}O_{11}$
Neochlorogenic acid 3-caffeoylquinic acid	3.24	353.0877	191.0557 (100), 179.0349 (40), 135.0448 (10)	0.13	$C_{16}H_{18}O_9$
Hydroxyphenyl acetic acid I	3.29	151.0395	107.0499 (100)	0.05	$C_8H_8O_3$
Catechin-*O*-dihexoside	3.33	613.1773	451.1242 (100) 289.0714 (50)	0.12	$C_{27}H_{34}O_{16}$
Coumaric acid-*O*-hexoside	3.5	325.0928	163.0402 (100)	0.10	$C_{15}H_{18}O_8$
4-Hydroxybenzoic acid*	3.54	137.0241	93.0343 (100)	0.20	$C_7H_6O_3$
Hydroxyphenyl acetic acid II	3.82	151.0395	107.0498 (100)	0.05	$C_8H_8O_3$
Epicatechin*	4.19	289.0717	245.0814 (100), 205.0502 (50),179.0345 (20)	0.12	$C_{15}H_{14}O_6$
1-caffeoylquinic acid	4.27	353.0877	191.0555 (100), 179.0343 (10)	0.11	$C_{16}H_{18}O_9$
Vanillic acid*	4.5	167.0349	152.0108 (100), 123.0447 (90), 108.0211 (30)	0.07	$C_8H_8O_4$
Chlorogenic acid 5-caffeoylquinic acid*	4.53	353.0877	191.0557 (100), 179.0346 (10), 135.0448 (1)	0.09	$C_{16}H_{18}O_9$
Catechin-*O*-hexoside II	4.56	451.1245	289.0710, (100) 245.0816 (10)	0.12	$C_{21}H_{24}O_{11}$
Caffeic acid*	4.6	179.0349	134.9875 (100)	0.06	$C_9H_8O_4$
Feruloylquinic acid	4.91	367.1034	193.0502 (100), 191.0557 (5)	0.10	$C_{17}H_{20}O_{15}$
Cryptochlorogenic acid (4-caffeoylquinic acid)	4.91	353.0877	191.0557 (100), 179.0344, 173	0.11	$C_{16}H_{18}O_9$
Hydroxyphenyl acetic acid III	5.61	151.0395	107.0498 (100)	0.05	$C_8H_8O_3$
p-coumaric acid*	5.94	163.0400	119.0498 (100)	0.05	C9H8O3
Sinapic acid-*O*-hexoside I	6.29	385.1139	223.0610 (100), 208.0400 (20), 179.0698 (10)	0.11	$C_{17}H_{22}O_{10}$
Ferulic acid-*O*-hexoside	6.42	355.1034	193.0507 (100), 178.0280 (10)	0.10	$C_{16}H_{20}O_9$
Indole-3-carboxylic acid	6.8	174.056	130,0657 (100)	0.06	$C_{10}H_9NO_2$
Sinapic acid-*O*-hexoside II	6.89	385.1139	223.0610 (100), 208.0403 (20), 179.0698 (10)	0.12	$C_{17}H_{22}O_{10}$
Ferulic acid*	7.03	193.0506	149.0604 (100), 178.0267 (70), 134.0370 (40)	0.06	$C_{10}H_{10}O_4$
Apigenin-C-hexoside-O-hexoside	7.39	593.1511	311.0556 (100), 431.0980 (80), 473.1086 (50), 341.0662 (20), 297.0401 (10)	0.30	$C_{27}H_{30}O_{15}$
Apigenin-C-hexoside-C-pentoside	7.93	563.1406	443.0982 (100), 473.1087 (80), 353.0664 (40), 383.0769 (30), 503.1192 (30), 545.1300(20)	0.23	$C_{26}H_{28}O_{14}$

Sinapic acid*	8.37	223.0611	208.0371 (100), 179.0708 (40), 164.0474 (20)	0.08	$C_{11}H_{12}O_5$
Apigenin-C-hexoside	9.29	431.0983	311.0551 (100), 341.0658 (40), 413.0871 (10)	0.2	$C_{21}H_{20}O_{10}$
Quercetin-3-O-glucoside*	9.57	463.0881	301.0348 (100)	0.12	$C_{21}H_{20}O_{12}$
Kaempferol-3-O-glucoside*	11.1	447.0932	285.0403 (100)	0.05	$C_{21}H_{20}O_{11}$
3,7-dimethylquercetin	14.3	329.0666	314.0422 (100), 299.0200 (10)	0.09	$C_{17}H_{14}O_7$
Isoxanthohumol*	14.4	353.1394	233.0814 (100), 119.0499 (10)	0.07	$C_{21}H_{22}O_5$
8-prenylnaringenin*	14.6	339.1237	219.0655 (100), 245.0811(10)	0.22	$C_{20}H_{20}O_5$
6-prenylnaringenin	14.7	339.1237	219.0656 (100), 245.0812(10)	0.13	$C_{20}H_{20}O_5$
Cohumulone I	14.8	347.1864	235.1337 (100)	0.3	$C_{20}H_{28}O_5$
ad-Humulone	14.9	361.2020	235.1337 (100), 292.1313 (10)	1	$C_{21}H_{30}O_5$
Cohumulone II	15.3	347.1864	278.1158(100)	0.14	$C_{20}H_{28}O_5$
n-Humulone	15.5	361.2020	292.1312 (100), 343.1910 (10)	1.11	$C_{21}H_{30}O_5$
iso-α-cohumulone	16	347.1864	251.1286(100), 329.1754(30)	0.3	$C_{20}H_{28}O_7$
iso-α-ad/n-humulone	16.2	361.2020	265.1445 (100), 343.1916 (40), 235.1339 (10)	0.1	$C_{21}H_{30}O_7$
iso-α-ad/n-humulone	16.4	361.2020	265.1442(100), 343.1910(40), 235.1336(10)	0.2	$C_{21}H_{30}O_8$

*Analytes confirmed by comparing with pure standards.

512

513 HIGHLIGHTS

514 - Phenolic profile of 4 types of beer has been analyzed by high resolution mass spectrometry

515 -Phenolic profile among the four types of beers was very similar.

516 - 47 phenolic compounds were identified, 7 of them are described in beer for the first time.

517