1 **Occurrence of melatonin and indolic compounds derived from *L*-tryptophan yeast**

2 **metabolism in fermented wort and commercial beers**

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Highlights:

- PTFE filters were the most convenient to diminish losses of tryptophan metabolites
- Melatonin is stable in beer matrix for 30 days at -20ºC
- 5-HTRP, NA5-HT, 3-IAA and L-TRP EE described for the first time in commercial beers
- NA5-HT and 3-IAA produced during the alcoholic fermentation of wort

Abstract

Melatonin and serotonin are bioactive compounds present in foods and beverages and related to neuroprotection and anti-angiogenesis, among other activities. They have been described in wines and the role of yeast in their formation is clear. Thus, this study evaluates the content of these bioactives and other related indolic compounds in beer. For this purpose, commercial beers were analyzed by a validated UHPLC-HRMS method and sample treatment optimized due to the low concentrations expected. Moreover, a wort was fermented with different commercial beer yeast (Abbaye, Diamond, SafAle, SafLager) in order to monitor the formation of these bioactives during the elaboration process.

Results show that indolic compounds such as *N*-acetylserotonin and 3-indoleacetic acid are produced during the alcoholic fermentation of wort. Moreover, the occurrence of four indolic compounds (5-hydroxytryptophan, *N*-acetylserotonin, 3-indoleacetic acid, *L*-tryptophan ethyl ester) in commercial beers is reported for the first time.

Keywords: melatonin, 3-indoleacetic acid, beer, bioactive, HRMS, fermentation, *Saccharomyces cerevisiae*

45 **1. INTRODUCTION**

46 Beer is one of the most consumed alcoholic beverages in the European Union (EU 28),
47 being its consumption of 359,112 thousand of hL in 2016. Spain is the third country in
48 beer production (38,634 thousand of hL), being its consumption per capita of 46 L per
49 year (The Brewers of Europe, 2017).

50 Beer contains alcohol, amino acids, carbohydrates, vitamins and also bioactive
51 compounds such as polyphenols and melanoidins, mostly from hops and malt (González-
52 San José, Rodríguez, & Valls-Bellés, 2016). The amino acid *L*-tryptophan (L-TRP) is
53 considered as a non-preferred nitrogen source for yeast (Beltran, Novo, Rozès, Mas, &
54 Guillamón, 2004), therefore its presence in wort and beer usually goes unnoticed since it
55 is not crucial for the fermentation step. Nevertheless, L-TRP is the precursor of some
56 bioactive compounds such as melatonin (MEL), serotonin (5-HT) and 3-indoleacetic acid
57 (3-IAA) which has been reported in fermented beverages as wine (Rodriguez-Naranjo,
58 Gil-Izquierdo, Troncoso, Cantos, & García-Parrilla, 2011; Mihaljević Žulj, Tomaz,
59 Maslov Bandić, Puhelek, Jagatić Korenika, & Jeromel, 2015). Since yeast metabolism'
60 is essential for the synthesis of above-mentioned bioactive compounds in wine, it seems
61 reasonable to evaluate their presence in beer as well as to evaluate their production during
62 the alcoholic fermentation of wort.

63 The analysis of MEL in foods and beverages has been the focus of a number of studies.
64 Thus, it has been quantified by means of ELISA in grapes (Iriti, Rossoni, & Faoro, 2006),
65 and beers (Garcia-Moreno, Calvo, & Maldonado, 2013). However, ELISA shows a huge
66 variability when tested in complex matrices different from biological fluids (Rodriguez-
67 Naranjo et al., 2011). Recent advances in the analytical field mostly consist on the use of
68 ultra-high-performance liquid chromatography coupled to High Resolution Mass
69 Spectrometer (UHPLC-HRMS) has become the preferred technique for the analysis of

70 bioactive compounds in food. By these means it is possible to identify and quantify
71 metabolites unequivocally at trace levels (1-100 ng/mL), permitting the simultaneous
72 analysis of a number of different compounds involved in the synthetic pathways or at
73 least chemically related. Recently, our research group has used it to determine L-TRP
74 derived compounds related with MEL metabolism such as 5-hydroxytryptophan (5-
75 HTRP), *N*-acetylserotonin (NA5-HT), 5-HT and also others coming from different
76 pathways such as tryptamine (TRY) and *L*-tryptophan ethyl ester (L-TRP EE) in synthetic
77 grape must (SM) (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-
78 Parrilla, 2016). Kocadağlı, Yilmaz and Gökmen (2014) reported the presence of MEL in
79 beer at 94.5 ng/mL using HPLC-MS/MS. In any case, the main analytical challenging
80 issues include the low concentration of MEL found in foods, sometimes near to the limits
81 of detection, its amphipathic characteristics and the high reactivity of the molecule.

82 Beer presents in its composition some compounds from the starch sources such as dextrins
83 (González-San José et al., 2016) that could negatively affect the analytical determination
84 of bioactive compounds such as MEL and 5-HT which are expected in ng/mL. Sample
85 pre-treatment is an important step to avoid the presence of interfering substances in the
86 UHPLC-HRMS analysis. For this purpose, solid phase extraction (SPE) is the most
87 suitable technique since it is widely used for complex matrices as beer, due to its low
88 solvent consumption, sample clean-up, quickness and simplicity (Boyaci et al., 2015).
89 Besides, a prior filtration step is recommended to avoid clogging in the SPE cartridges
90 before loading samples (Boyaci et al., 2015). Nylon (NY), polytetrafluoroethylene
91 (PTFE) and cellulose acetate (CA) filters are used in the analysis of MEL in grape skin
92 and wine, and in metabolomic studies related with yeast (Iriti et al., 2006; Stege, Sombra,
93 Messina, Martinez, & Silva, 2010; Rodriguez-Naranjo et al., 2011; Smart, Aggio, Van
94 Houtte, & Villas-Bôas, 2010). Since filters can retain small compounds, their suitability

95 evaluation for the analysis of L-TRP metabolites in beer matrix is worthy for
96 methodology development. However, in order to quantify the expected trace levels, beer
97 samples have to be concentrated as seen in other food matrices such as wine (Rodriguez-
98 Naranjo et al., 2011).

99 Thus, the aims of this work are: (i) to optimize sample treatment in order to improve the
100 analysis of bioactive indolic compounds in beers, (ii) to study different conditions of
101 temperature and concentration during storage time in order to set the most suitable storage
102 conditions for beer samples, (iii) to unveil the production of indolic compounds during
103 alcoholic fermentation in wort, and (iv) to study the occurrence of different L-TRP
104 derived compounds in commercial beers, widely consumed in Spain, using a validated
105 UHPLC-HRMS method.

106 2. MATERIAL AND METHODS

107 *2.1 Beer samples*

108 Beer brands were selected as being representative of beer consumption in Spain. The
109 Brand Foodprint 2018 study (*Kantar Worldpanel*, 2018
110 https://www.kantarworldpanel.com) about the main food brands sold was used to extract
111 the most consumed beers in Spain by autonomous region, including alcohol-free beers.
112 As a result, 6 bottles of 19 different beer brands were purchased from local supermarkets
113 in the glass-bottle format. More details of each beer sample are described in Table 1. Most
114 beers were Lager type, with the exception of a Stout type brand, and had an alcoholic
115 content between 0.0 and 7.2º.

116 *2.2 Beer Sample treatment*

117 Commercial beers were degassed for 30 minutes in an UB-1488 ultrasonic bath
118 (J.P.Selecta, Barcelona, Spain). All samples were filtered before the solid phase

119 extraction (SPE) procedure. SPE was performed in C18 Bond Elut SPE cartridge
120 (VARIAN, Agilent) which has been widely used for sample cleaning to study MEL in
121 fermented beverages (Rodriguez-Naranjo et al., 2011) and indolic compounds in SM
122 (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-Parrilla, 2017) and
123 wines of different grape varieties (Fernández-Cruz, Cerezo, Cantos-Villar, Troncoso, &
124 García-Parrilla, 2019a).

125 All cartridges were conditioned with 2 mL of methanol and after that with 2 mL of milliQ
126 water. Then, 2 mL of samples were loaded. Cartridges were subsequently washed with 2
127 mL of a 10% methanol:water solution. The indolic compounds under study were eluted
128 with 1 mL of methanol in dark brown eppendorfs and dried in a vacuum concentrator
129 (HyperVACLITE, GYOZEN, Korea) at 30 ºC, 2000 rpm. Pellets were subsequently
130 rediluted in dark HPLC vials with 500 µL solution of 10% methanol:water with formic
131 acid (0.1 %) prior to UHPLC-HRMS analysis.

132 *2.3. Reagents*

133 Standards of 9 indolic compounds including 3-IAA, 5-HTRP, 5-HT, L-TRP, L-TRP EE,
134 MEL, NA5-HT, TRY and tryptophol (TOL) were supplied by Sigma Aldrich (Barcelona,
135 Spain). Merck (Darmstadt, Germany) provided methanol of LC/MS grade. Prolabo ®
136 (Obregon, Mexico) supplied formic acid for LC/MS with at 99% purity.

137 *2.4. Filtration optimization*

138 In order to elucidate the effect that filtration caused on the concentrations of indolic
139 compounds, most usual filters were tested such as NY and PTFE. Additionally, CA filters
140 were also included since they are used to perform the quenching step for extracellular
141 matrices fermented with yeast (Smart et al., 2010). For the sake of comparison, three
142 different stock solutions (LOQ, 1.5 x LOQ and 3x LOQ) of the 9 indolic compounds were

143 prepared. Values based on the LOQ concentrations (Table 1 of supplementary material)
144 previously described by Fernández-Cruz et al. 2016. The stock solutions were filtered
145 with the different membrane materials and were dried in a vacuum concentrator
146 (HyperVACLITE, GYOZEN, Korea) at 30º C, 2000 rpm. Pellets were subsequently
147 rediluted in dark HPLC vials with 500 µL solution of 10% methanol:water with formic
148 acid (0.1 %) prior to UHPLC-HRMS analysis (Fernández-Cruz et al., 2016) Stock
149 solutions at LOQ, 1.5x LOQ and 3x LOQ values no filtrated were also analysed as
150 control. Results are expressed as a rate between concentration of samples after use of
151 different filter materials (PTFE, NY and CA) and samples with no filtration step.

152 *2.5. Stability of samples and storage conditions*

153 A standard pilsner beer was used to monitor likely alterations on indolic compounds
154 concentration along storage of samples at different temperatures. Previously, beer was
155 degassed 30 minutes by stirring to remove the carbon dioxide. Beer samples were
156 enriched with 200 ng/mL of stock solutions. Then, 1 mL of each solution was placed in
157 dark vials. This cluster was prepared in triplicate in order to storage beer matrix at three
158 temperatures: 4 ºC, -20 ºC and -80 ºC. Sampling (1 mL) was performed at the initial point,
159 and then after 7, 15 and 30 days of storage period.

160 *2.6. Wort alcoholic fermentation*

161 The wort was prepared by mixing a liophylized commercial blonde barley malt extract (8
162 EBC, *La tienda del cervecero*, La Palma, Cartagena, Spain) with sterile water up to a
163 density of 1050 g/cm^3. Subsequently, wort was brought to boil for 5 minutes and cooled
164 down in order to add hop up to reach 12 IBUs (isomerized hop extract 6%, *La tienda del*
165 *cervecero*, La Palma, Cartagena, Spain). Four fermentation experiments were conducted
166 as follows: 750 mL of wort was placed 1L-bottles with an initial concentration of 10^6

167 cel/mL of the different *Saccharomyces cerevisiae* yeast Abbaye and Diamond were
168 purchased from Lallemand (Bayern, Germany); SafLager and SafAle were supplied by
169 Fermentis (Marcq-en-Baroeul, France). Each yeast was provided as active dried yeast
170 (ADY), being rehydrated before inoculation, following the manufacturer's instructions.
171 Fermentations were carried out at 22ºC for ale type (Abbaye, SafAle) and at 16ºC for
172 lager type yeast (Diamond, SafLager). An initial sample of the inoculum was taken before
173 inoculation, and then, sampling was performed daily for seven days until the end of
174 alcoholic fermentation. A volume enough to reach $2x10^9$ cells was taken from bottles and
175 centrifuged at room temperature for 5 minutes at 7600 rpm to separate supernatant from
176 cell pellet. The former was stored at -80º C until UHPLC-HRMS analysis.

177 *2.7. Quenching and intracellular metabolites extraction*

178 Each cell pellet was submitted to a quenching procedure according to Álvarez-Fernández,
179 Fernández-Cruz, Valero, Troncoso and García-Parrilla (2019). Briefly, the resuspended
180 pellet, in milliQ water, was mixed with a pre-cooled quenching solution (-23º C)
181 (glycerol:saline solution, 3:2, v/v) in a 1:4 proportion. Then, cells were centrifuged at
182 36036 *g* for 20 minutes at -20º C and the resulting supernatant was discarded. The pellets
183 were mixed with a cold-washing solution (glycerol:saline solution, 1:1, v/v) and
184 centrifuged again with the same conditions previously described. Eventually, pellets were
185 stored at -80º C before intracellular metabolites extraction.

186 Intracellular extraction was performed based on previous works (Álvarez-Fernández et
187 al., 2019). The procedure consisted on mixing pellet with a cold extraction solution (-30º
188 C) (methanol:milliQ water, 1:1, v/v) and centrifuged twice at 36086 *g* for 20 minutes at -
189 20º C, preserving the supernatant. Resulting extracts were stored at -80º C until SPE
190 procedure and UHPLC-HRMS analysis.

191 *2.8. UHPLC-HRMS analysis*

192 All the samples were analysed in triplicate by a UHPLC Dionex Ultimate 3000 system
193 (ThermoScientific, San Jose, USA) coupled to a Thermo Scientific Q-Exactive^{TM} hybrid
194 quadrupole-orbitrap mass spectrometer (Bremen, Germany) with a previously validated
195 method according to Fernández-Cruz et al. (2016, 2017). A target analysis was performed
196 in positive mode using a heated electrospray ionization source (HESI) with identical mass
197 spectrometry parameters described by the authors. UHPLC-HRMS system was controlled
198 by the Chromeleon^{TM} Software (v.7.1, Thermo Fisher Scientific, Bremen, Germany).
199 Data analysis was performed using the TraceFinder^{TM} Software (v.3.1) and the
200 Xcalibur^{TM} Software (v.3.0.63) both purchased by Thermo Fisher Scientific (Bremen,
201 Germany).

202 *2.9. Statistical analysis*

203 Statistical differences through ANOVA test were performed using InfoStat Software
204 (version 2018, Centro de Transferencia InfoStat, FCA, Universidad Nacional de Córdoba,
205 Argentina, http://www.infostat.com.ar/). Concentration (ng/mL) was set at the dependant
206 variable, being filter material or fermentation/storage time the classification variables in
207 the variance analysis with the LSD Fisher comparing method. Significance degree was
208 set as follows: $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

209 **3. RESULTS AND DISCUSSION**

210 *3.1. Optimization of filtration procedure*

211 Three different filter types (NY, CA and PTFE) were tested to evaluate their performance
212 on the determination of the indolic compounds under study. Figure 1 displays the ratios
213 obtained for the non-filtered solution versus the filtered solutions of the three

214 concentrations tested of the indolic compounds, being LOQ the lowest and 3x LOQ the
215 highest, according to values depicted in Table 1 of supplementary material.
216 CA filters retained most of the indolic compounds under study (Figure 1). Focusing on
217 the lowest concentration (LOQ) under study, CA filters significantly retained TRY
218 (100%), 5-HT (99%), TOL (98%), L-TRP EE (97%), MEL (82%), 3-IAA (76%), NA5-
219 HT (61%) and L-TRP (46%), being 5-HTRP the only compound unaffected. Moreover,
220 similar results were obtained when the highest concentration (3x LOQ) was tested.
221 Results highlight that the use of CA filters could underestimate actual concentrations of
222 most of the indolic compounds. Although there are no published studies using CA filters
223 for L-TRP derivates analysis, these filters have been used in metabolomic studies related
224 with yeast (Smart et al., 2010). Since MEL metabolites are expected to be present in
225 concentrations close to LOQ, it would be highly recommended to use filters more suitable
226 for these compounds.

227 Concerning NY filters, they retain compounds such as L-TRP, NA5-HT, TOL and MEL
228 in samples (29, 46, 47 and 63% of retention, respectively). It is important to pinpoint
229 these results, since NY filters are commonly used prior to the analysis by HPLC-MS/MS
230 in studies involving MEL in food samples, such as grape skin (Iriti et al., 2006) and wine
231 (Stege et al., 2010). On the other hand, NY filters also retained TOL and 3-IAA, the main
232 L-TRP metabolites, derived from the Ehrlich pathway. Thus, NY filters are not
233 recommended to analyse L-TRP metabolites in fermented beverages.

234 Compared with the other materials, PTFE filters retained a lesser amount of indolic
235 compounds such as LTRP and L-TRP EE (58 and 30 % of retention, respectively), at the
236 lower concentrations. However, the concentration of these compounds is usually much
237 higher than the concentration tested (Table 1 of supplementary material) (Fernández-Cruz
238 et al., 2017; Jia, Kang, Park, Lee, & Kwon, 2011). These filters were previously used to

239 study MEL in and wines (14-130 ng/mL) (Rodriguez-Naranjo et al., 2011). Therefore,
240 PTFE filters seems to be suitable to study indolic compounds derived from the amino
241 acid L-TRP in beer samples.

242 *3.2. Stability of indolic compounds in beer samples*

243 The stability assays involved different storage temperatures (4 ºC, -20 ºC, -80 ºC) and
244 time (7, 15 and 30 days). Due to the expected low concentrations of some indolic
245 compounds, beer samples (a standard pilsner) were enriched with indolic standards (200
246 ng/mL). Results are displayed in Figure 2.

247 L-TRP and 5-HTRP (Figure 2A, 2B) were stable at the different temperatures, with no
248 significant changes until day 15 when the concentration of both compounds decreased.
249 No remarkable differences were appreciated at different temperatures. 5-HT and NA5-
250 HT (Figure 2C, 2D) decreased their concentration in all the temperatures of storage being
251 significantly affected from day 7.

252 As can be seen in Figure 2E, MEL content was unalterable during storage time, regardless
253 temperature tested. Previously, its stability in aqueous solutions was reported elsewhere.
254 It seems that temperature is not a main factor affecting stability, since MEL was stable in
255 both room and cooling temperature for a long time period (6 months) at 100 mg/L
256 (Cavallo & Hassan, 1995).

257 Two compounds derived from the Ehrlich pathway, 3-IAA and TOL, were affected after
258 15 days of storage (Figure 2F, 2G), when their concentration declined regardless of the
259 storage temperature. Previous data have demonstrated that 3-IAA can be affected by the
260 presence of other molecules such as proteins present in wine, being conjugated with them
261 and consequently decreasing its concentration (Hoenicke, Simat, Steinhart, Köhler, &
262 Schwab, 2001). TOL is in fact accumulated during the fermentation process of SM

263 (Fernández-Cruz et al., 2017). Minor compounds such as TRY and L-TRP EE were stable
264 until day 7 (Figure 2 H, 2I) with a sharp drop at day 15. The complexity of food matrices
265 makes necessary to study the matrix effect on stability during storage conditions. From
266 these results, a storage time for samples not exceeding 7 days shall be recommended while
267 temperature at -20ºC seems to be adequate to preserve most of the indolic compounds
268 under study. Further studies should cover the stability of these compounds at room
269 temperature to evaluate possible changes along storage in supermarket shelves.

270 *3.3. Alcoholic fermentation of wort*

271 Wort alcoholic fermentations involved the use of four different *S. cerevisiae* yeast in
272 either bottom (Diamond, SafLager) or top wort fermentation (Abbaye, SafAle). Both
273 extracellular and intracellular content were analysed (Figure 3 and 4). Figure 3 shows that
274 compounds such as L-TRP, 5-HTRP, 5-HT, NA5-HT and 3-IAA were present in the
275 initial wort. Hop solution, an additional component during brewing process, did not
276 contain indolic compounds (data not shown).

277 The initial concentration of L-TRP in wort was 10658 ng/mL. L-TRP was significantly
278 consumed by yeast at day 2 of the alcoholic fermentation (Figure 3A), showing a decline
279 between 53.2 % (Saf Ale) and 99.69% (Diamond). Previously, it has been reported that
280 a 31% of L-TRP is consumed by yeast during brewing process (Lei et al., 2013). These
281 results contracts with the assumed concept that L-TRP is not a preferred nitrogen source
282 by yeast during alcoholic fermentation (Beltran et al, 2004). Each beer type follows a
283 wort production process that causes modifications in wort composition. This fact could
284 explain the differences of L-TRP and derived compounds found in literature (de Carvalho,
285 Mathias, Pereira-Netto, & de Carvalho-Marques, 2018). Additionally, yeast strain and
286 temperature could also produce variations in L-TRP consumption (Marconi, Rossi,
287 Galgano, Sileoni, & Perreti, 2016). It seems that Ale type yeast (Abbaye, SafAle) takes a

288 significant higher amount of L-TRP than Lager type during the first step of fermentation
289 (day 1-3) (Figure 4A).

290 5-HTRP is the first intermediate of the MEL synthesis pathway by yeast. It is present in
291 the initial wort at low concentration (0.03 ng/mL) and, for the first time, in 3 out of 4
292 yeast during the first steps of wort fermentation (days 1-2) (Figure 3B). Low
293 concentrations of 5-HTRP were found in SM fermented with commercial *S. cerevisiae*
294 strains after 7 days (1.78-3.46 ng/mL) (Fernández-Cruz et al., 2017) and it was also
295 quantified in natural must of different grape varieties at low concentrations (<0.5 ng/mL)
296 (Fernández-Cruz et al., 2019a). The hydroxylation activity required to convert L-TRP
297 into 5-HTRP in yeast seems to be scarce or null (Muñiz Calvo, Bisquert, Fernandez-Cruz,
298 García-Parrilla, & Guillamón, 2019), which could explain the lack of intracellular 5-
299 HTRP (Figure 4B). However, it was identified in other *S. cerevisiae* (QA23, P24) and
300 non-*Saccharomyces* strains (*Torulaspora delbrueckii*) along alcoholic fermentation of
301 SM (Fernández-Cruz, et al., 2019a). Thus, yeast strain and fermentation substrate could
302 produce important differences in 5-HTRP occurrence.

303 5-HT was identified in all the yeast fermentation experiments and in the initial wort (20.5
304 ng/mL) as well. It was apparently consumed throughout the alcoholic fermentation by
305 most of the yeast, especially at day 2 (Figure 3C). Intracellular 5-HT was found at days
306 1-2 but decreased at the end of alcoholic fermentation (<0.01 ng/mL) (Figure 4C).
307 Intracellular 5-HT occurrence was formerly reported in different *S. cerevisiae* strains
308 (QA23, P24), diminishing its concentration along the fermentation (Fernandez-Cruz, et
309 al., 2019b). These data suggest that *S. cerevisiae* is capable of synthesizing 5-HT in
310 alcoholic fermentation of wort.

311 This is the first time NA5-HT is described in wort during alcoholic fermentation. It was
312 identified in the initial wort (0.01 ng/mL) and during fermentation was increasingly

313 produced by all the yeast, reaching final values between 0.08 and 0.16 ng/mL at day 7
314 (Figure 3D). This increase in the medium is produced when 5-HT extracellular levels
315 decrease, suggesting that 5-HT is converted into NA5-HT. NA5-HT has also been
316 described in SM fermented by the yeast *Metschnikowia pulcherrima* (Fernandez-Cruz et
317 al., 2016), in Tempranillo grape must fermented by *S. cerevisiae* QA23 and Red Fruit
318 (Fernández-Cruz et al., 2019a). On the other hand, no NA5-HT was identified in the
319 intracellular compartment (Figure 4D) in any of the fermentations. Interestingly, NA5-
320 HT was reported in the intracellular extract of SM fermented by non-*Saccharomyces*
321 strains (*Hanseniaspora uvarum, T. delbrueckii*) (Fernandez-Cruz, et al., 2019b). Since *S.*
322 *cerevisiae* strains have been used in the present work, it is not surprising that no NA5-HT
323 was found in the intracellular compartment.

324 MEL has been found in fermented beverages, being yeast metabolism highly important
325 for its production; it is synthesized from 5-HT, with L-TRP as its main precursor (Muñiz-
326 Calvo et al., 2019). MEL was quantified only at day 2 at very low levels (0.22 ng/mL) in
327 the strain Abbaye (Figure 3E). MEL production by different *S. cerevisiae* and non-
328 *Saccharomyces* yeast strains also reached the highest amounts at day 2 (0.98-2.24 ng/mL)
329 in SM (Fernández-Cruz et al., 2017). MEL has been determined by means of ELISA in
330 the different beer production steps reaching the higher concentration (333 pg/mL) in the
331 second fermentation step in bottle, after sugar was added (Garcia-Moreno, et al., 2013).
332 Although so far it is clear that MEL is synthesized during alcoholic fermentation, there is
333 not a sound reproducibility between available data, making difficult to ascertain when
334 and why is effectively produced. Some authors suggest that MEL acts as a radical
335 scavenger, what may diminish its concentration in the medium (Tan, Manchester,
336 Esteban-Zubero, Zhou, & Reiter, 2015), but its precise function in yeast has to be
337 unveiled. Recent works reported MEL with a cell signalling role in fermentation process

338 using *S. cerevisiae* (Valera et al., 2019) being quantified in the extracellular medium with
339 a zig-zag trend (Fernandez-Cruz et al., 2019a; Morcillo-Parra, Valera, Beltran, Mas &
340 Torija, 2019). Since MEL has been reported at different concentrations in similar
341 experiments, its quantification is usually uncertain. In the intracellular medium, MEL was
342 quantified in 3 out of 4 yeast strains (Figure 4E), in days 1-3. SafLager was the strain that
343 synthesized the highest MEL concentration (0.08 ng/mL). Formerly, Sprenger,
344 Hardeland, Fuhrberg, and Han (1999) reported intracellular MEL in *S. cerevisiae*.
345 However, in a recent study developed by our group with both *Saccharomyces* and non-
346 *Saccharomyces* strains MEL was only quantified in the intracellular compartment of non-
347 *Saccharomyces* strains along the whole alcoholic fermentation, with values between 0.13-
348 0.30 ng/mL (Fernandez-Cruz, et al., 2019b). Several yeast strains proved different
349 capacities of forming this molecule (Fernández-Cruz et al., 2017). Thus, further studies
350 should be performed to address which brewing yeasts have the capability of synthesizing
351 MEL along fermentation process.

352 3-IAA and TOL are considered the most usual L-TRP metabolites derived from the
353 Ehrlich pathway of alcoholic fermentation. As far as we are concerned, this is the first
354 time that 3-IAA is determined in wort being present from the initial wort (Figure 3F). It
355 is significantly enriched at days 2-3 (23-34 ng/mL) and diminishes its concentration at
356 the end of alcoholic fermentation (day 7) (9.4-46.6 %) for Diamond, SafLager and
357 Abbaye strains, although the final concentrations are always higher than the initial ones.
358 Only SafAle maintained constant the 3-IAA concentration. In natural grape must
359 fermentations, 3-IAA was synthesized at similar concentrations (10-75 ng/mL)
360 (Fernández-Cruz et al., 2019a; Mihaljević Žulj et al., 2015). However, in SM it was
361 quantified in a larger extent (100-500 ng/mL) (Fernández-Cruz et al., 2017). This
362 suggests that *S. cerevisiae* is able to produce 3-IAA in substrates with different

363 composition (grape must vs wort), although selected strain is also determinant for the 3-
364 IAA synthesis. Nevertheless, this compound is considered as a precursor of 2-
365 aminoacetophenone, an off flavour in wine (Simat, Hoenicke, Gessner, & Christoph,
366 2004) but its role in yeast is not clear. Its decrease during the alcoholic fermentation may
367 be caused by the bond of 3-IAA to amino acids or peptides that yeasts use for their
368 metabolism after a convenient intracellular hydrolysis (Simat et al., 2004). In the
369 intracellular medium, 3-IAA was reported in all the strains from the inoculum (Figure
370 4F), with no changes along alcoholic fermentation with the exception of the lager type
371 yeast, which increased the uptake of 3-IAA at the end (1.27 ng/mL). Previously, when
372 different yeast strains such us *S. cerevisiae* (P24, QA23) and non-*Saccharomyces* (*H.*
373 *uvarum*, *T. delbrueckii*) where used, changes in 3-IAA uptake at the end of alcoholic
374 fermentation were only significant in two strains (QA23, *T. delbrueckii*) (Fernandez-Cruz
375 et al., 2019b). These data reinforce the importance of strains selection to produce 3-IAA.

376 Concerning TOL, it was by far the most abundant metabolite derived from the L-TRP
377 metabolism along the alcoholic fermentation (Figure 3G). It was not present in the initial
378 wort, but all yeast strains produced it at day 2 (794-1731 ng/mL), except Diamond (at day
379 3), with no changes until the end of alcoholic fermentation. This compound was studied
380 with more extent in natural grape must fermented with *S. cerevisiae* Aroma White,
381 reaching similar concentrations (Fernández-Cruz et al., 2019a) and also in SM, where 10-
382 folds higher values were reached with different yeast strains (Fernández-Cruz et al.,
383 2017). In the intracellular medium, TOL was quantified in the four yeast strains (Figure
384 4G). Formerly, TOL had been described in the intracellular medium of different
385 *Saccharomyces* and non-*Saccharomyces* strains in a wide range (14-7615 ng/mL)
386 (Fernandez-Cruz et al., 2019b). This compound has proved to be a quorum sensing
387 molecule and a kinetic mediator during alcoholic fermentation (Valera et al., 2019).

388 Therefore, TOL formation pathway from L-TRP metabolism seems to be favoured by
389 yeast.

390 Minor compounds such as TRY and L-TRP EE could also be quantified along the
391 alcoholic fermentation. TRY is a biogenic amine, seldom found in beers and formed by
392 decarboxylation of free amino acids or by amination/transamination of aldehydes and
393 ketones, by the action of microorganisms, mainly bacteria (Pradenas, Galarce-Bustos,
394 Henríquez-Aedo, Mundaca-Uribe, & Aranda, 2016). In our study TRY was produced by
395 yeast at the extracellular level from day 2-3 (16.5-32.3 ng/mL) (Figure 3H). These values
396 are higher than those reported by the authors in SM (<0.5 ng/mL) after alcoholic
397 fermentation (Fernández-Cruz et al., 2017). In the intracellular content, TRY is present at
398 day 1, before the increase of extracellular TRY (Figure 4H). In fact, tryptamine in yeast
399 is formed via tryptophan decarboxylation as an intermediate step for 5-HT formation
400 (Muñiz-Calvo et al., 2019). On the other hand, L-TRP EE was present along the alcoholic
401 fermentation, being produced at day 2-3 (Figure 3I). This compound is likely formed
402 thanks to the ethanol presence during the alcoholic fermentation (Arapitsas, Guella, &
403 Mattivi, 2018). Previously, the concentration of L-TRP EE has been reported with an
404 increased trend until 17.48 ng/mL in SM (Fernández-Cruz et al., 2016). However, other
405 studies show how L-TRP EE increases its concentration in the early stages of alcoholic
406 fermentation in grape must, but it disappears quickly after 3 days from the inoculation
407 day (Vigentini et al., 2015). The intracellular content of this compound is extremely low
408 (< 0.06 ng/mL) (Figure 4I). L-TRP EE has been also quantified in the intracellular
409 medium of *S. cerevisiae* at day 2-3 of alcoholic fermentation (0.11-0.88 ng/mL)
410 (Fernández et al., 2019b).

411 *3.4. Indolic compounds content in commercial beers*

412 Nineteen commercial beers were analysed by UHPLC-HRMS in order to study the
413 occurrence of different indolic compounds related with L-TRP (Table 2). To the best of
414 our knowledge, this paper reports for the first time the occurrence of 5-HTRP, NA5-HT,
415 3-IAA and L-TRP EE in different commercial beers that were unequivocally identified
416 by HRMS.

417 As expected, beers showed a remarkable content of L-TRP (Table 2), ranging from
418 348.08 ng/mL to 6508.83 ng/mL. Other authors quantified L-TRP in commercial beers
419 ranging 4.8-21500 ng/mL (Jia et al., 2011).

420 Conversely, in our alcoholic fermentation experiments, 5-HTRP was quantified in
421 commercial beers for the first time, in the first days (Table 2). The other MEL pathway
422 intermediate, 5-HT, was also present in all beer samples (0.99-22.35 ng/mL). This is in
423 agreement with previous studies in which it was determined in different beers (3.5-24.2
424 mg/L) by means of a derivatization process and a HPLC-DAD analysis (Kirschbaum,
425 Meier, & Brückner, 1999). This fact could explain the huge difference between the 5-HT
426 found in the literature (3.5-24.2 mg/L) and the values reported in this work. The presence
427 of this compound in a common beverage like beer is fairly interesting since it is able to
428 perform a high inhibition of amyloid β-peptide aggregation, thus evidencing its potential
429 bioactivity as neuroprotective (Hornedo-Ortega et al., 2018).

430 Although it is not a main compound of beer, NA5-HT appears in all commercial brands
431 for the first time (0.02-0.39 ng/mL) (Table 2). MEL content (9.95-29.3 pg/mL) was even
432 lower than that found for NA5-HT. Previously, MEL was reported in beers at higher
433 concentration using UHPLC-HRMS (94.5 ng/mL) (Kocadağlı et al., 2014).

434 The original presence of 3-IAA in beer is noteworthy since its anti-angiogenic effect
435 (IC_{50}= 0.9704 mM) is outstanding (Cerezo, Hornedo-Ortega, Álvarez-Fernández,

436 Troncoso, & García-Parrilla, 2017). Due to the lack of available data about bioavailability
437 of 3-IAA in the gastrointestinal tract, if we use MEL values (19%) (Harpsøe et al., 2015)
438 and the highest 3-IAA concentration (143 ng/mL) we would need to consume 28 bottles
439 of beers to have a bioactive effect. Thus, beers should be considered as an additional
440 source of 3-IAA, but not the main one. Regarding TOL, the concentration range was
441 higher (53-1893 ng/mL) than those previously reported in beers (0.2-2.5 ng/mL)
442 (Bartolomé, Peña-Neira, & Gómez-Cordovés, 2000).

443 TRY was also quantified in commercial beers (2.64-38.41 ng/mL), at low concentration
444 but in the range found in the literature for beer (n.d - 28600 ng/mL) (Pradenas et al., 2016;
445 Nalazek-Rudnicka & Wasik, 2017). Additionally, L-TRP EE, which was quantified
446 during wort alcoholic fermentation, was also described for the first time in commercial
447 beers (0.05-2.57 ng/mL). Likely, the higher level of L-TRP EE of commercial beers with
448 respect to wort alcoholic fermentation levels is in accordance with the higher ethanol
449 content in the final product (Arapitsas et al., 2018).

450 **Conclusions**

451 Our results show that sample treatment is an important step to further analyse bioactive
452 compounds of L-TRP metabolism using a UHPLC-HRMS technique, as they are present
453 in low concentrations. Filters of PTFE showed to be the most convenient to diminish
454 possible loss of minor bioactive compounds such as MEL, 5-HT and 3-IAA. In addition,
455 MEL is stable in beer matrix for 30 days at -20ºC. For the first time, it was described the
456 formation of L-TRP yeast metabolites such as 5-HTRP, NA5-HT, 3-IAA and L-TRP EE
457 along wort alcoholic fermentation and also in commercial beers. This fact pinpoints beer
458 as a source of compounds with demonstrated biological activity such as antioxidant, anti-
459 angiogenic and neuroprotective properties. Although concentrations found are not enough

460 to exert a bioactive level as discussed earlier, beer could contribute to the intake of these
461 compounds in the diet.

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472 **Conflict of interest statement**

473 The authors declare no conflict of interest

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625 **Figure captions**:

626 Figure 1. Ratio for the indolic compounds at different LOQ values (LOQ, 1.5 LOQ and
627 3x LOQ) and filters.

628 Figure 2. Concentration of different *L*-tryptophan derivates compounds in beer along 30
629 days of storage at different temperatures.

630 Figure 3. Indolic compounds concentration (ng/mL) in the extracellular medium along
631 the alcoholic fermentation of wort by different yeast strains.

632 Figure 4. Indolic compounds concentration (ng/10^9 cells) in the intracellular compartment
633 of the four yeast strains throughout wort alcoholic fermentation.

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646 Table 1. Characteristics of commercial beers

Code	*Beer type*	*Country of origin*	*Glass bottle type*	*Alcohol content (%)*
AT	Pilsner lager	Spain	Brown glass	4.6
AB	Pilsner lager	Spain	Brown glass	5.9
AM	Pilsner lager	Spain	Brown glass	5.0
B0	Pilsner lager	Spain	Brown glass	0.0
BW	American lager	United States	Brown glass	5.0
CB	Pilsner lager	Denmark	Green glass	5.0
CO	American lager	México	Clear glass	4.6
CC	Pilsner lager	Spain	Brown glass	4.8
CZ	Pilsner lager	Spain	Brown glass	5.9
DP	Flavoured Lager	Belgium	Clear glass	5.9
ED	Pilsner lager	Spain	Brown glass	5.4
EG	Pilsner lager	Spain	Brown glass	5.5
GN	Irish dry stout	Ireland	Black glass	5.0
HK	Pilsner lager	The Netherlands	Green glass	5.0
MA	Pilsner lager	Spain	Brown glass	5.5
MU	Irish red ale lager	Irish	Brown glass	5.0
PL	Weissbier lager	Germany	Brown glass	5.5
SM	Pilsner lager	Spain	Brown glass	5.4
VD	Märzenbier lager	Spain	Brown glass	7.2

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654 Table 2. Concentration (ng/mL) of *L*-Tryptophan (L-TRP) and its metabolites in commercial beers.

Beer code	*L-TRP*	*5-HTRP*	*5-HT*	*NA5-HT*	*MEL**	*3-IAA*	*TRY*	*TOL*	*L-TRP EE*
AT	1890.85 ± 20.34	0.28 ± 0.00	6.77 ± 0.10	0.06 ± 0.00	29.2 ± 1.27	82.3 ± 0.09	18.5 ± 0.07	1511 ± 24.0	0.32 ± 0.00
AB	3648.60 ± 36.70	0.20 ± 0.00	3.97 ± 0.10	0.14 ± 0.00	13.5 ± 0.00	66.0 ± 0.95	12.6 ± 0.16	293 ± 5.98	1.02 ± 0.01
AM	3510.67 ± 80.38	0.42 ± 0.02	8.38 ± 0.10	0.26 ± 0.00	23.7 ± 0.57	7.93 ± 0.03	24.2 ± 0.29	117 ± 1.05	0.73 ± 0.01
B0	4163.76 ± 66.63	0.46 ± 0.02	22.35 ± 0.32	0.36 ± 0.00	21.7 ± 0.99	11.7 ± 0.00	2.64 ± 0.06	51.7 ± 1.22	0.05 ± 0.00
BW	3832.94 ± 83.81	0.27 ± 0.01	6.01 ± 0.12	0.15 ± 0.00	15.7 ± 0.71	25.9 ± 0.62	31.1 ± 0.75	104 ± 2.44	1.42 ± 0.01
CB	3621.13 ± 25.86	0.32 ± 0.01	5.43 ± 0.23	0.28 ± 0.01	18.4 ± 2.12	20.1 ± 0.58	23.6 ± 1.26	366 ± 15.7	0.49 ± 0.03
CO	4535.70 ± 67.84	0.30 ± 0.01	1.32 ± 0.01	0.19 ± 0.00	13.5 ± 0.42	25.0 ± 0.61	9.15 ± 0.15	82.2 ± 1.44	0.44 ± 0.01
CC	6078.06 ± 36.93	0.35 ± 0.03	11.48 ± 0.22	0.29 ± 0.00	27.15 ± 0.21	66.5 ± 0.16	29.8 ± 0.05	163 ± 0.38	0.74 ± 0.00
CZ	4108.09 ± 9.42	1.05 ± 0.02	7.14 ± 0.12	0.31 ± 0.01	18.8 ± 1.13	15.4 ± 0.48	36.6 ± 0.49	149 ± 5.26	0.81 ± 0.03
DP	4432.98 ± 398.26	0.33 ± 0.03	1.63 ± 0.17	0.25 ± 0.02	25.6 ± 2.83	143.4 ± 3.11	20.5 ± 1.71	139 ± 8.43	0.49 ± 0.04
ED	3744.20 ± 161.19	0.27 ± 0.01	1.81 ± 0.06	0.11 ± 0.00	10.85 ± 0.78	35.6 ± 0.06	14.8 ± 0.58	58.8 ± 0.99	0.89 ± 0.03
EG	4559.18 ± 75.47	0.22 ± 0.00	7.15 ± 0.07	0.21 ± 0.00	16.3 ± 0.57	63.1 ± 0.22	16.9 ± 0.23	106 ± 1.09	0.47 ± 0.01
GN	4767.53 ± 31.78	0.36 ± 0.00	0.99 ± 0.01	0.22 ± 0.00	17.7 ± 0.71	7.68 ± 0.06	38.1 ± 0.19	81.2 ± 0.01	1.52 ± 0.00
HK	3938.52 ± 10.78	0.83 ± 0.00	8.85 ± 0.02	0.35 ± 0.00	21.15 ± 0.21	7.79 ± 0.01	19.3 ± 0.24	97.3 ± 1.23	0.31 ± 0.00
MA	1102.37 ± 29.52	0.17 ± 0.01	10.64 ± 0.20	0.06 ± 0.00	20.2 ± 0.99	100 ± 0.34	16.5 ± 0.64	1354 ± 22.2	0.61 ± 0.02
MU	5935.79 ± 10.56	0.73 ± 0.01	16.23 ± 0.90	0.21 ± 0.01	19.1 ± 1.84	18.1 ± 0.53	34.6 ± 1.68	182 ± 2.61	0.58 ± 0.03
PL	1624.19 ± 26.50	0.48 ± 0.02	2.09 ± 0.05	0.39 ± 0.00	10.8 ± 0.28	11.8 ± 0.20	31.9 ± 0.08	819 ± 1.58	1.48 ± 0.04
SM	348.08 ± 5.51	0.15 ± 0.01	7.60 ± 0.04	0.02 ± 0.00	27.85 ± 1.34	109 ± 0.31	25.7 ± 0.29	1893 ± 20.6	2.57 ± 0.01
VD	6534.82 ± 378.16	0.34 ± 0.03	2.35 ± 0.00	0.21 ± 0.01	9.95 ± 1.06	40.7 ± 1.24	38.4 ± 2.79	61.4 ± 3.39	2.04 ± 0.16

655 * Concentration expressed in pg/mL.

656 L-TRP: *L*-tryptophan; 5-HTRP: 5-hydroxytryptophan; 5-HT: serotonin; NA5-HT: *N*-acetylserotonin; MEL: melatonin; 3-IAA: 3-indolacetic acid;
657 TRY: tryptamine; TOL: tryptophol; L-TRP EE: L-tryptophan ethyl ester.

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