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

## 1. INTRODUCTION

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

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

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

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

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

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

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

## 2. MATERIAL AND METHODS

### *2.1 Beer samples*

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

### *2.2 Beer Sample treatment*

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

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

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

136 *2.3. Reagents*

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

141 *2.4. Filtration optimization*

142 In order to elucidate the effect that filtration caused on the concentrations of indolic
143 compounds, most usual filters were tested such as nylon and polytetrafluoroethylene.
144 Additionally, cellulose acetate filters were also included since they are used to perform
145 the quenching step for extracellular matrices fermented with yeast (Smart et al., 2010).
146 For the sake of comparison, three different stock solutions (LOQ, 1.5 x LOQ and 3x LOQ)

147 of the 9 indolic compounds were prepared. Values based on the LOQ concentrations
148 (Table 1 of supplementary material) previously described by Fernández-Cruz et al. 2016.
149 The stock solutions were filtered with the different membrane materials and were dried
150 in a vacuum concentrator (HyperVACLITE, GYOZEN, Korea) at 30º C, 2000 rpm.
151 Pellets were subsequently rediluted in dark HPLC vials with 500 µL solution of 10%
152 methanol:water with formic acid (0.1 %) prior to UHPLC/HRMS analysis (Fernández-
153 Cruz et al., 2016) Stock solutions at LOQ, 1.5x LOQ and 3x LOQ values no filtrated were
154 also analysed as control. Results are expressed as a rate between concentration of samples
155 after use of different filter materials (PTFE, nylon and cellulose acetate) and samples with
156 no filtration step.

157 *2.5. Stability of samples and storage conditions*

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

165 *2.6. Wort alcoholic fermentation*

166 The wort was prepared by mixing a liophylized commercial blonde barley malt extract (8
167 EBC, *La tienda del cervecero,* La Palma, Cartagena, Murcia) with sterile water up to a
168 density of 1050 g/cm$^3$. Subsequently, wort was brought to boil for 5 minutes and cooled
169 down in order to add hop up to reach 12 IBUs (isomerized hop extract 6%, *La tienda del*
170 *cervecero,* La Palma, Cartagena, Murcia). Four fermentation experiments were conducted

as follows: 750 mL of wort was placed 1L-bottles with an initial concentration of $10^6$ cel/mL of the different *Saccharomyces cerevisiae* yeast Abbaye and Diamond were purchased by Lallemand (Bayern, Germany); SafLager and SafAle were supplied by Fermentis (Marcq-en-Baroeul, France). Each yeast was provided as active dried yeast (ADY), being rehydrated before inoculation, following the manufacturer's instructions. Fermentations were carried out at 22ºC for ale type (Abbaye, SafAle) and at 16ºC for lager type yeast (Diamond, SafLager). An initial sample of the inoculum was taken before inoculation, and then, sampling was performed daily for seven days until the end of alcoholic fermentation. A volume enough to reach $2x10^9$ cells was taken from bottles and centrifuged at room temperature for 5 minutes at 7600 rpm to separate supernatant from cell pellet. The former was stored at -80º C until UHPLC/HRMS analysis.

*2.7. Quenching and intracellular metabolites extraction*

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

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

196 *2.8. UHPLC/HRMS analysis*

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

207 *2.9. Statistical analysis*

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

214 **3. RESULTS AND DISCUSSION**

215 *3.1. Optimization of filtration procedure*

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

concentrations tested of the indolic compounds, being LOQ the lowest and 3x LOQ the highest, according to values depicted in Table 1 of supplementary material.

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

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

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

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

*3.2. Stability of indolic compounds in beer samples*

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

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

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

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

(Fernández-Cruz et al., 2017). Minor compounds such as TRY and L-TRP EE were stable until day 7 (Figure 2 H, 2I) with a sharp drop at day 15. The complexity of food matrices makes necessary to study the matrix effect on stability during storage conditions. From these results, a storage time for samples not exceeding 7 days shall be recommended while temperature at -20ºC seems to be adequate to preserve most of the indolic compounds under study.

*3.3. Alcoholic fermentation of wort*

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

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

of all yeast (Figure 4A). It seems that Ale type yeast (Abbaye, SafAle) takes a significant higher amount of L-TRP than Lager type during the first step of fermentation (day 1-3).

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

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

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

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

328 MEL has been found in fermented beverages, being yeast metabolism highly important
329 for its production; it is synthesized from serotonin, with L-TRP as its main precursor
330 (Muñiz-Calvo et al., 2019). MEL was quantified only at day 2 at very low levels (0.22
331 ng/mL) in the strain Abbaye (Figure 3E). MEL production by different *S. cerevisiae* and
332 non-*Saccharomyces* yeast strains also reached the highest amounts at day 2 (0.98-2.24
333 ng/mL) in synthetic must (Fernández-Cruz et al., 2017). MEL has been determined by
334 means of ELISA in the different beer production steps reaching the higher concentration
335 (333 pg/mL) in the second fermentation step in bottle, after sugar was added (Garcia-
336 Moreno, et al., 2013). Although so far it is clear that MEL is synthesized during alcoholic
337 fermentation, there is not a sound reproducibility between available data, making difficult
338 to ascertain when and why is effectively produced. Some authors suggest that MEL acts
339 as a radical scavenger, what may diminish its concentration in the medium (Tan,
340 Manchester, Esteban-Zubero, Zhou, & Reiter, 2015), but its precise function in yeast has
341 to be unveiled. In the intracellular medium, MEL was quantified in 3 out of 4 yeast strains

342 (Figure 4E), in days 1-3. SafLager was the strain that synthesized the highest MEL
343 concentration (0.08 ng/mL). Formerly, Sprenger, Hardeland, Fuhrberg, and Han (1999)
344 reported intracellular MEL in *S.cerevisiae*. However, in a recent study developed by our
345 group with both *Saccharomyces* and non-*Saccharomyces* strains MEL was only
346 quantified in the intracellular compartment of non-*Saccharomyces* strains along the whole
347 alcoholic fermentation, with values between 0.13-0.30 ng/mL (Fernandez-Cruz, et al.,
348 2019b). From these data, we can confirm that MEL is synthesised by brewing yeast
349 between days 2 and 3 in agreement with wine yeast.

350 3-IAA and TOL are considered the most usual L-TRP metabolites derived from the
351 Ehrlich pathway of alcoholic fermentation. As far as we are concerned, this is the first
352 time that 3-IAA is determined in wort being present from the initial wort (Figure 3F). It
353 is significantly enriched at days 2-3 (23-34 ng/mL) and diminishes its concentration at
354 the end of alcoholic fermentation (day 7) (9.4-46.6 %) for Diamond, SafLager and
355 Abbaye strains, although the final concentrations are always higher than the initial ones.
356 Only SafAle maintained constant the 3-IAA concentration. In natural grape must
357 fermentations, 3-IAA was synthesized at similar concentrations (10-75 ng/mL)
358 (Fernández-Cruz et al., 2019a; Mihaljević Žulj et al., 2015). However, in SM it was
359 quantified in a larger extent (100-500 ng/mL) (Fernández-Cruz et al., 2017). This suggest
360 that *S.cerevisiae* is able to produce 3-IAA in substrates with different composition (grape
361 must vs wort), although selected strain is also determinant for the 3-IAA synthesis.
362 Nevertheless, this compound is consider as a precursor of 2-aminoacetophenone, an off
363 flavour in wine (Simat, Hoenicke, Gessner, & Christoph, 2004) but its role in yeast is not
364 clear. Its decrease during the alcoholic fermentation may be caused by the bond of 3-IAA
365 to amino acids or peptides that yeasts use for their metabolism after a convenient
366 intracellular hydrolysis (Simat et al., 2004). In the intracellular medium, 3-IAA was

reported in all the strains from the inoculum (Figure 4F), with no changes along alcoholic fermentation with the exception of the lager type yeast, which increased the uptake of 3-IAA at the end (1.27 ng/mL). Previously, when different yeast strains such us *S. cerevisiae* (P24, QA23) and non-*Saccharomyces* (*H. uvarum*, *T. delbrueckii*) where used, changes in 3-IAA uptake at the end of alcoholic fermentation were only significant in two strains (QA23, *T. delbrueckii*) (Fernandez-Cruz et al., 2019b). This data reinforce the importance of strains selection to produce 3-IAA.

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

Minor compounds such as TRY and L-TRP EE could also be quantified along the alcoholic fermentation. TRY is a biogenic amine, seldom found in beers and formed by decarboxylation of free amino acids or by amination/transamination of aldehydes and ketones, by the action of microorganisms, mainly bacteria (Pradenas, Galarce-Bustos,

Henríquez-Aedo, Mundaca-Uribe, & Aranda, 2016). In our study TRY was produced by yeast at the extracellular level from day 2-3 (16.5-32.3 ng/mL) (Figure 3H).). These values are higher than those reported by the authors in SM (<0.5 ng/mL) after alcoholic fermentation (Fernández-Cruz et al., 2017). In the intracellular content, TRY is present at day 1, before the increase of extracellular TRY (Figure 4H). In fact, tryptamine in yeast is formed via tryptophan decarboxylation as an intermediate step for serotonin formation (Muñiz-Calvo et al., 2019). On the other hand, L-TRP EE was present along the alcoholic fermentation, being produced at day 2-3 (Figure 3I). This compound is likely formed thanks to the ethanol presence during the alcoholic fermentation (Arapitsas, Guella, & Mattivi, 2018). Previously, the concentration of L-TRP EE has been reported with an increased trend until 17.48 ng/mL in SM (Fernández-Cruz et al., 2016). However, other studies show how L-TRP EE increases its concentration in the early stages of alcoholic fermentation in grape must, but it disappears quickly after 3 days from the inoculation day (Vigentini et al., 2015). The intracellular content of this compound is extremely low (< 0.06 ng/mL) (Figure 4I). L-TRP EE has been also quantified in the intracellular medium of *S.cerevisiae* at day 2-3 of alcoholic fermentation (0.11-0.88 ng/mL) (Fernández et al., 2019b).

### *3.4. Indolic compounds content in commercial beers*

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

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

5-HTRP has been unequivocally quantified for the first time in commercial beers (Table 2); in fact, it is present in all beer samples (0.15-1.05 ng/mL). Conversely, in our alcoholic fermentation experiments, 5-HTRP was quantified only in the first days. The other MEL pathway intermediate, 5-HT, was also present in all beer samples (0.99-22.35 ng/mL). This is in agreement with previous studies in which it was determined in different beers (3.5-24.2 mg/L) by means of a derivatization process and a HPLC/DAD analysis (Kirschbaum, Meier, & Brückner, 1999). However, this is the first time that it is determined in commercial beers by means of UHPLC/HRMS, being a more accurate technique especially for identification purposes. This fact could explain the huge difference between the 5-HT found in the literature (3.5-24.2 mg/L) and the values reported in this work. The presence of this compound in a common beverage like beer is fairly interesting since it is able to perform a high inhibition of amyloid β-peptide aggregation, thus evidencing its power as neuroprotective (Hornedo-Ortega et al., 2018).

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

Additionally, this is the first time that 3-IAA is quantified in commercial beers (8-143 ng/mL) (Table 2). Its presence is noteworthy since its anti-angiogenic effect ($IC_{50}$= 0.9704 mM) is outstanding (Cerezo, Hornedo-Ortega, Álvarez-Fernández, Troncoso, & García-Parrilla, 2017). Due to the lack of available data about bioavailability of 3-IAA in

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

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

## Conclusions

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

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**Conflict of interest statement**

The authors declare no conflict of interest

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**Figure captions**:

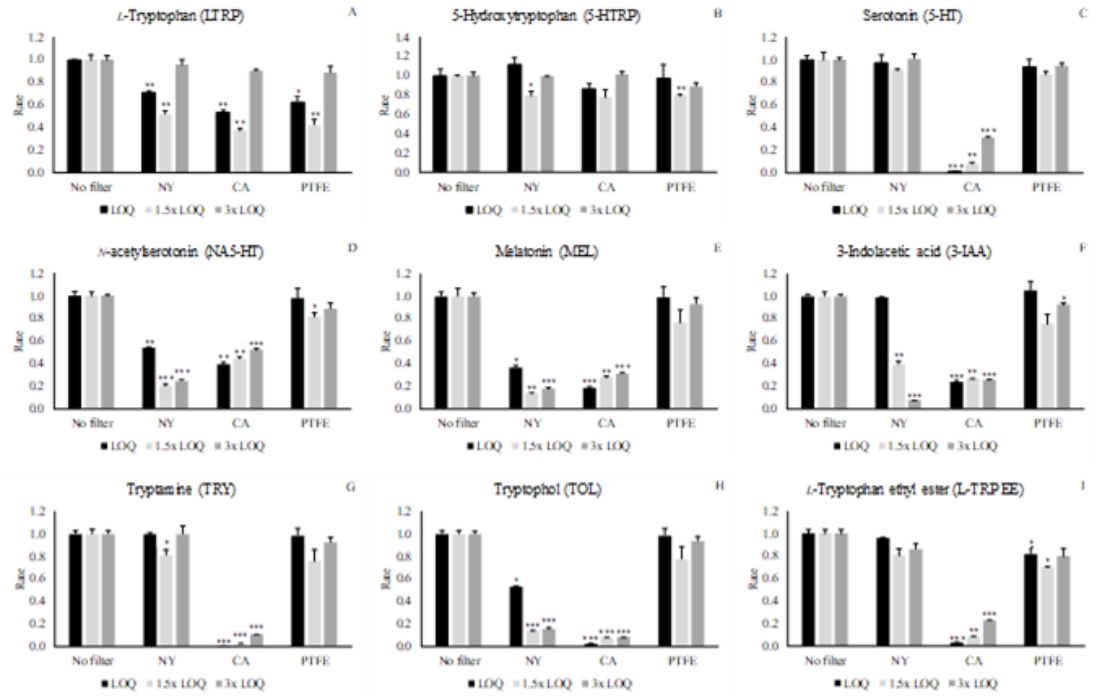
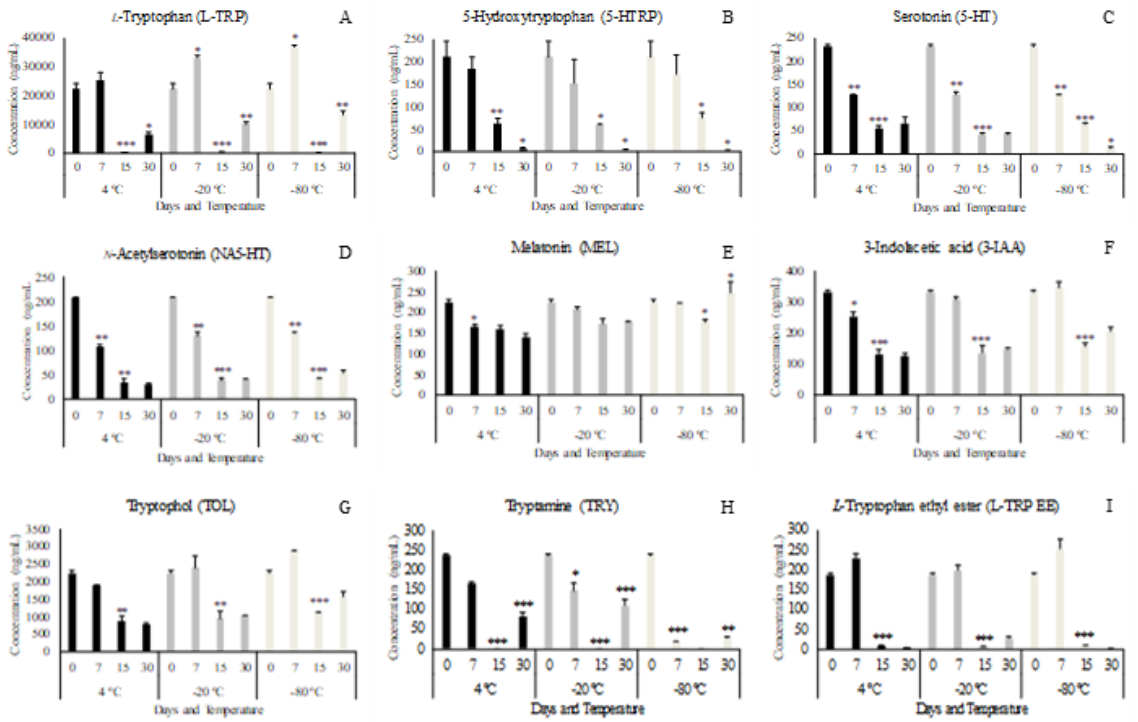



Figure 1. Ratio for the indolic compounds at different LOQ values (LOQ, 1.5 LOQ and 3x LOQ) and filters.

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632 Figure 2. Concentration of different *L*-tryptophan derivates compounds in beer along 30

633 days of storage at different temperatures.

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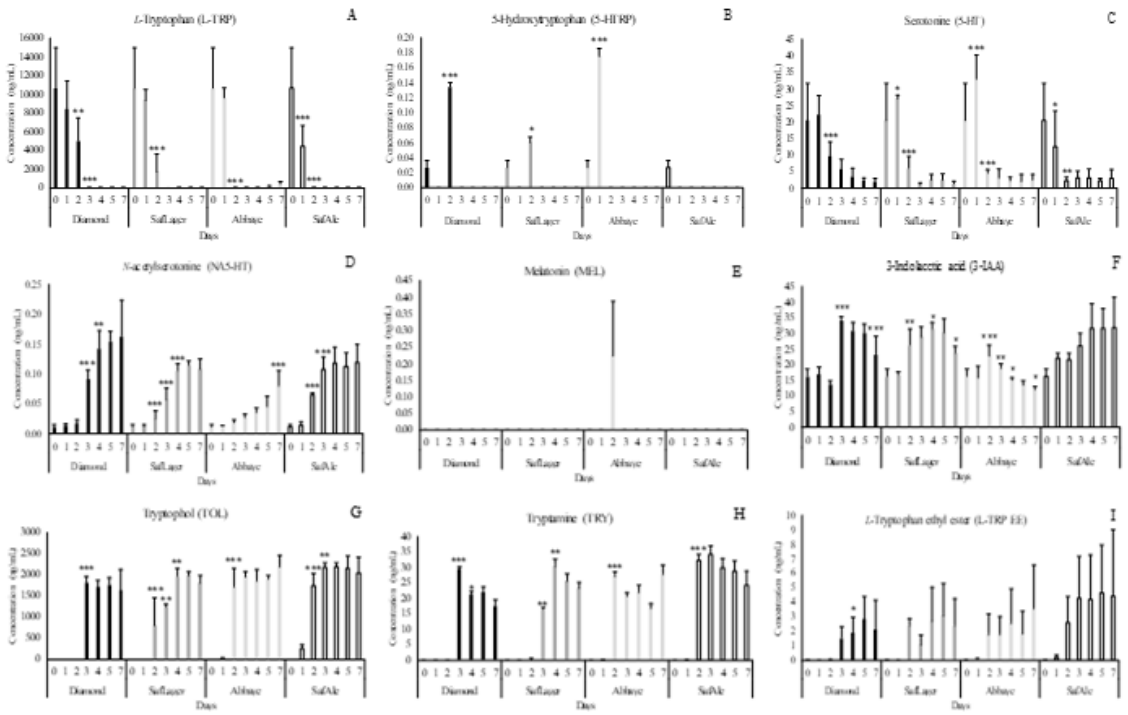


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637 Figure 3. Indolic compounds concentration (ng/mL) in the extracellular medium along

638 the alcoholic fermentation of wort by different yeast strains.

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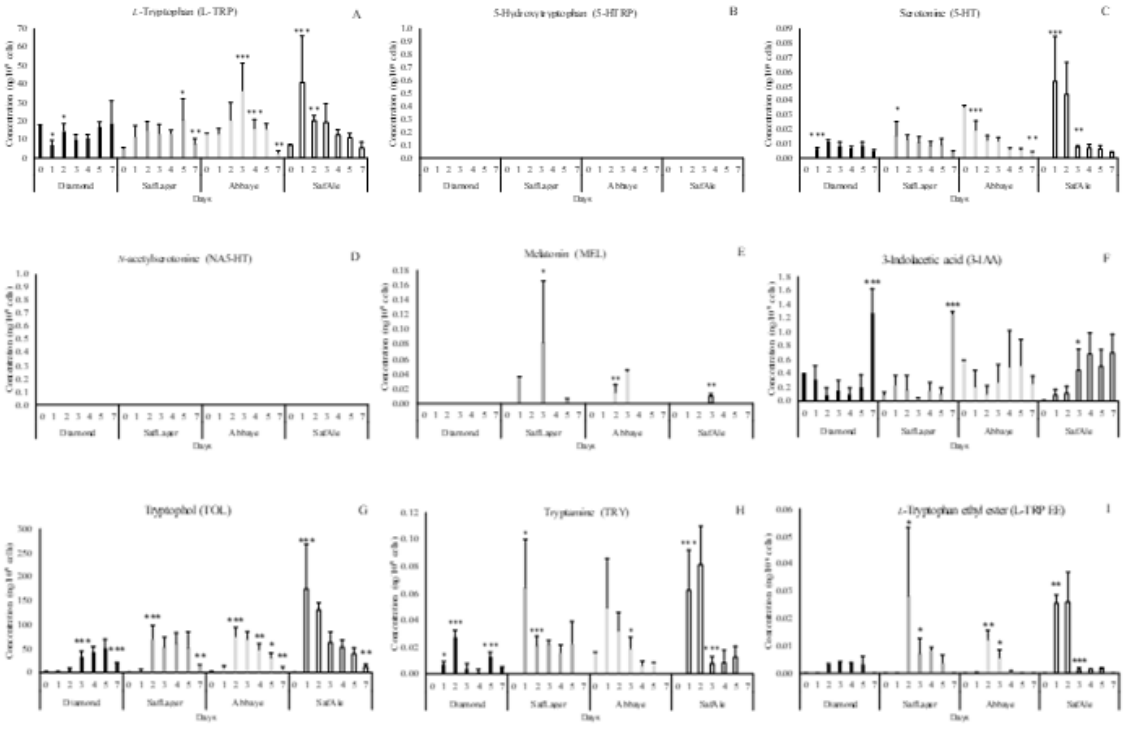


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642 Figure 4. Indolic compounds concentration (ng/$10^9$ cells) in the intracellular compartment

643 of the four yeast strains throughout wort alcoholic fermentation.

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654 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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662 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 |

663 * Concentration expressed in pg/mL.

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