AUTOMATED CHROMATOGRAPHIC METHOD WITH FLUORESCENT DETECTION TO DETERMINE BIOGENIC AMINES AND AMINO ACIDS. APPLICATION TO CRAFT BEER BREWING PROCESS

Mónica PALOMINO-VASCO*, María Isabel ACEDO-VALENZUELA, María Isabel RODRÍGUEZ-CÁCERES, Nielene MORA-DIEZ

Department of Analytical Chemistry and Research Institute on Water, Climate Change and Sustainability (IACYS), University of Extremadura, 06006 Badajoz, Spain

* **Corresponding author:** monicapv@unex.es (Mónica Palomino-Vasco)

Abstract: The combined determination of biogenic amines and amino acids is a challenge for food scientists. In this research, a new methodology for the automatic on-line precolumn derivatization and determination of 8 biogenic amines and 9 precursor amino acids by Ultra-High Performance Liquid Chromatography with fluorescent detection has been developed. The method derivatized the analytes with *o*-phthaldialdehyde and achieved the separation of the 17 derivatives in less than 15 minutes, obtaining good quality parameters (limits of detection varied between 7.00 and 210 μg L^{-1}, and RSD intraday ranged between 1.5 and 6.0 %). The optimization of the derivatization procedure has been carried out employing an experimental design and the Surface Response Methodology. The method has been validated and applied to wine and beer, obtaining good recuperation percentages (72.3 – 138.4%). Also, samples collected during the fermentation of a craft beer, as well as a bottled sample of the same batch, have been analyzed, to monitor the changes in the profile of biogenic amines and amino acids.

Keywords: biogenic amines, amino acids, automatization, UHPLC-FD, Response Surface Methodology, fermented beverages

1. Introduction

Biogenic amines (BAs) are nitrogenous compounds that have an important role in the human metabolism, as they are involved in different processes (i.e. neurotransmission, regulation of blood pressure, synthesis of DNA, RNA and proteins, or regulation of body temperature). BAs naturally occur in protein-rich foodstuffs as fish, meat, vegetables or fruits, by the enzymatic decarboxylation of amino acids (AAs). Fermented products (dairy products, beer and wine) could also have high levels of BAs, due to the action of microorganisms. Conversely, spoiled foods and products manufactured under poor hygienic conditions display high concentrations of some BAs, especially histamine, tyramine, putrescine, and cadaverine. Hence, these compounds are an excellent indicator of the food quality [1,2].

Although their importance in the metabolism, high levels of BAs may be harmful, so the human body has mechanisms to cope with them. However, poisoning can occur when large amounts of BAs are consumed or when the ability to metabolize them is diminished by individual problems, medication or alcohol consumption [1–3]. Among the more important symptoms, it can be pointed migraine and hypertensive crisis [3]. Histamine is the most dangerous one, as it can cause psychoactive, vasoactive, cutaneous and gastrointestinal effects. Also, tyramine is another relevant BA that may increase blood pressure and cardiac frequency, and phenylethylamine may act as a powerful migraine inductor [1]. On the other hand, putrescine and cadaverine are not themselves toxic, but they can increase the toxicity of histamine, tyramine and phenylethylamine, because they interfere in detoxification reactions. Furthermore, these two BAs can also produce negative effects on sensory quality of foods, giving them putrefaction or rotting flesh flavour, respectively [2].

For this reason, it is necessary to develop fast and reliable methods for the determination of BAs in foods, to control their levels, try to reduce them and ensure compliance with legislation [1,4]. Thus, histamine in fishery products, for example, should not exceed 200 mg kg^{-1}, or 400 mg kg^{-1} in the case of products matured in brine [5]. In fermented beverages as wine, limits have not been drawn up, but several

countries (Switzerland, Austria, Germany, Holland, Belgium and France) have stablished their own recommendations for the histamine content, that ranged between 2 and 10 mg L^{-1} [2].

Due to AAs are the precursors of BAs, there is a particular interest for food scientists for their common determination, to obtain information about nutritional and hygienic quality, as well as enable the monitoring of manufacturing processes [6]. However, the simultaneous determination of AAs and BAs is complicated, due to the variety of structures (Figure 1) and the absence of good spectroscopic or fluorescent properties [4,6,7].

The determination of these analytes has been carried out employing different techniques, such as liquid chromatography, capillary electrophoresis or gas chromatography [1,8]. These separative techniques are coupled to different detectors as DAD [3,4,6,9–12], FD [7,13–17] or MS/MS [18–25], being the latter the most sensitive and reproducible, but also the most expensive, so it is not as accessible equipment as the other two detectors. Also, this type of detector is normally employed to determine AAs and BAs in biological samples as urine o brain tissue, due to the low concentration of the analytes in these types of samples.

Among these techniques, liquid chromatography is the preferred one. The use of Ultra-High Performance Liquid Chromatography (UHPLC) improves elution times, as well as the sensitivity and selectivity of the methods. It is considered UHPLC when columns with a particle size smaller than 2 μm are used, that withstand pressures up to 600 bar [4,6].

Sentellas et al. in 2016 [1] and Papageorgiou et al. in 2018 [8] reviewed the developed methods for the determination of BAs in food samples, finding several methods that employed UHPLC. Other three methods have been found that determine BAs in foodstuffs using UHPLC between 2017 and 2019 [4,11,24]. A pre- or post-column derivatization is usually necessary (employing dansyl chloride, *o*-phthalaldehyde or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as derivatization reagents). Reverse phase is the usual mode employed, and mobile phases are formed by a mixture of acidic water

or buffer (ammonium formate/acetate or sodium acetate) and an organic solvent (ACN or MeOH, sometimes acidified). Time of analysis ranged between 5 and 25 minutes, and mass, visible, ultraviolet and fluorescent detection have been employed. The developed methods have been applied to very different matrices, for example, fermented food (wine, cheese, soy sauce or beer), anchovy, seafood and tuna. On the other hand, some methods have been found that determine amines with electrochemical detection, but the analytes are amines with neurotransmitter functions, and that are found in brain dialysis samples [26–30].

The determination of AAs by UHPLC usually has been focused on biological samples (serum, urine, blood…) and their importance as biomarkers of different diseases such as schizophrenia, or Alzheimer's disease [18,25]. There are also methods that determine AAs in foods (black onion, fermented foods and beverages, tea, etc.), usually in conjunction with other analytes (biogenic amines, ammonium ions, phenolic compounds or total antioxidant activity) [3,4,6,12,19,31]. In these methods, AAs are usually determined by pre-column derivatization with ultraviolet detection. Some derivatization reactions take long times and it is not possible to couple them to the chromatographic system.

The on-line derivatization procedures offer several advantages over off-line ones, among which it can be highlighted [7,32]:

- All derivatized products are injected into the system, so there are no problems of dilution of the derivatized products, as it could happen on the off-line derivatization.
- Sample handling is minimized, as well as the associated errors.
- Automated derivatization procedures tend to provide better reproducibility than manual ones.
- In on-line pre-column derivatization, sample treatment (i.e. extraction or clean-up) is often also integrated in the chromatographic process, and can be automatically performed.

On-line pre-column derivatization must satisfy some conditions, such as good stability and compatibility of the derivatizing reagent with the mobile phase; no precipitation or gas generated during

97 the derivatization reaction; and good solubility of the derivatized products into the mobile phase. This
98 type of derivatization is achieved by the incorporation of the derivatizing reagent into the flow scheme
99 of the chromatographic system, either in the mobile phase or through the use of an automatic injector
100 [32].

101 To the best of our knowledge, there are no methods that use on-line derivatization to determine
102 BAs and AAs together, although some methods determining BAs or AAs separately, using on-line
103 reactions, have been published, for example, Hyötyläinen et al. (2001) [15]; Zacharis et al. (2006) [7]
104 and Peng et al. (2008) [16]. All these methods agree in using *o*-phthalaldehyde as a derivatizing agent,
105 because the reaction is immediate, to determine 9 BAs in wine of different countries, 14 AAs in
106 pharmaceutical products, or histamine in different foods, respectively.

107 In this research, a new on-line automatic pre-column derivatization reaction coupled to UHPLC
108 with fluorescence detection is presented for the simultaneous quantification of 8 BAs and 9 AAs.
109 Derivatization and chromatographic conditions have been optimized, employing experimental design
110 and the Response Surface Methodology for the reaction optimization. In these conditions, all the analytes
111 are determined in 15 minutes, obtaining good figures of merit. The method has been applied to wine and
112 beer samples.

113

114 **2. Materials and Methods**

115 ***2.1. Chemicals***

116 Eight BAs and nine AAs were determined in this study: putrescine (PUT; Sigma), agmatine (AGM;
117 Alfa Aesar), cadaverine (CAD; Aldrich), ethanolamine (ETA; Merck), histamine (HIM; Sigma),
118 tyramine (TYM; Sigma), tryptamine (TRY; Aldrich), 2-phenylethylamine (PEA; Aldrich), glutamic acid
119 (GLU; Sigma), serine (SER; Sigma), histidine (HIS; Fluka), arginine (ARG; Sigma-Aldrich), glycine
120 (GLY; Panreac), tyrosine (TYR; Merck), lysine (LYS; Aldrich), tryptophan (TRP; Panreac), and
121 phenylalanine (PHE; Aldrich-Chemie). Octylamine (OCT; Fluka) was used as internal standard (IS).

BAs stock solutions of 5000 mg L^{-1} (31 – 82 mM, according to the molecular weight of each analyte) and AAs stock solutions of 10000 mg L^{-1} (49 – 133 mM, according to the molecular weight of each analyte) were prepared in diluted HCl (0.1 M; Panreac) and stored refrigerated at darkness. Working analyte solutions were prepared daily by mixing all the analytes and diluting them in 0.1 M HCl to obtain the appropriate concentrations needed.

Weekly, a boric acid/sodium borate buffer (pH 10.50; 0.6 M) was prepared using boric acid and NaOH, purchased from Merck and Panreac, respectively. Then, derivatization reagent was prepared as follows: 1.6 mL of a dilution of *o*-phthalaldehyde (OPA; 2.98 mM in MeOH; purchased from Aldrich and Panreac, respectively) and 1.2 mL of 2-mercaptoethanol (2-ME; Sigma-Aldrich) were added to a 5.0 mL volumetric flask, which was filled up to the mark with the aforementioned boric acid/sodium borate buffer. The derivatization reagent was filtered before its use employing 0.22 μm membrane nylon filters (Teknokroma).

For the chromatographic separation, acetonitrile UHPLC-grade (Sigma-Aldrich), methanol UHPLC-grade (Panreac), and a TRIS buffer were used as mobile phase. TRIS buffer was prepared by dilution in ultrapure water of the adequate amounts of Trizma® base (2-amino-2-(hydroxymethyl)-1,3-propanediol; Sigma) and tris(hydroxymethyl)aminomethane hydrochloride (Acros) to obtain a concentration of 0.10 M and a pH of 8.30. Before its use, mobile phase was filtered (0.22 μm membrane nylon filter; Teknokroma), and ultrasonicated.

The wines and beer analysed were obtained from local markets and were kept refrigerated and in darkness until their analysis. The beer samples collected during the craft beer brewing process were donated by a beer brewery company (Extremadura, Spain), and frozen at -20°C until their analysis. Also, a bottled beer of the same batch was obtained and analysed 5 months after the bottling.

2.2. Instrumentation and software

Experiments were carried out in an Agilent Model 1260 Infinity High Performance Liquid Chromatograph (Agilent Technologies) equipped with an online degasser, quaternary pump (G1311B), column oven compartment (G1316A), autosampler (G1329B), UV-Vis diode-array detector (G1315D) and fluorescence detector (G1321B). The ChemStation software was used to treat data and control the instrument. For the separation of the derivatives a Zorbax Eclipse Plus-C18 analytical column (100 x 4.6 mm; 1.8 µm; Agilent Technologies) was employed.

Calibration curves and analytical figures of merit were calculated using ACOC software (programmed in MATLAB code) [33]. Experimental design and Surface Response Methodology were carried out employing The Unscrambler v9.7 (CAMO Software).

2.3. Online and automated pre-column derivatization reaction

Derivatization reagent was prepared as aforementioned (section 2.1). Then, 0.4 mL of a mixture of AAs and BAs standards solutions (prepared from the mixture of the appropriate volumes of stock solution of each analyte and its dilution in HCl, 0.1 M, to obtain concentrations ranged between 0.40 – 49.0 µM and 0.16 – 7.8 µM, for AAs and BAs respectively, in the reaction mixture) and 0.4 mL of internal standard (OCT; 0.039 mM) were mixed and diluted to a total volume of 5.0 mL with HCl 0.1 M.

The derivatization reaction was fully automated by means of an injector programme, which mixed the reagents by drawing them sequentially into the injection seat. Then, the mixture was injected into the column to separate the derivatives. The steps of the automatic injection, as well as the concentrations of analytes, IS and derivatizing reagent in the final mixture, are summarized in Table 1. The optimization of the reaction parameters was performed by using the Surface Response Methodology.

2.4. Calibration curve

The calibration curves were stablished by means of the internal standard method. Standards were prepared following the methodology explained in section 2.3. Once obtained the chromatograms under the optimized conditions, they were processed using the ChemStation package.

2.5. UHPLC-FD method

The mobile phase employed was composed by a TRIS buffer (pH 8.30; 0.10 M) (eluent A), ACN (eluent B) and MeOH (eluent C).

The elution of the derivatives was performed using a gradient mode, consisting in four linear steps summarized in Table 2. The flow rate was fixed at 1.0 mL min^{-1}, and the column was thermostated at 50°C. The eluate was fluorimetrically monitored (356/445 nm), using the peak area/IS area ratio as analytical signal.

2.6. Analysis of real samples

Wine and beer samples were sonicated and filtered (0.22 μm, nylon) before its analysis. Samples were analysed by means of the standard addition method, combined with internal standard method. To prepare the samples, 0.4 mL internal standard (OCT; 0.039 mM), 0.2 mL wine/beer (diluted when necessary) and 0.2 mL of a mixture of AAs and BAs standard solutions were put into a 5.0 mL volumetric flask, which was filled up to the mark with HCl 0.1 M.

Then, the procedures explained in sections 2.3. and 2.5. were followed. All the analyses were carried out by triplicate (n = 3). Peak area/IS area ratio obtained measuring the fluorescence at 356/445 nm was used as analytical signal.

At the end of the working day, the column was cleaned by the injection of 100 μL of MeOH and going through the column first ultrapure water for 40 minutes and then ACN:MeOH (50:50) for 40 minutes.

3. Results and discussion

3.1. Optimization of chromatographic conditions

For the chromatographic separation of the analytes, a previously optimised chromatographic method for the separation of BAs (unpublished data) was firstly used, in which the mobile phase consisted of ACN and TRIS buffer, and the stationary phase was an Eclipse Plus C18 column (100 x 4.6 mm; 1.8 μm). As more analytes (the precursor AAs) were introduced, the derivatives overlapped, so this method had to be modified. The concentration of the TRIS buffer was slightly increased from 0.08 to 0.10 M to improve its buffering capacity, maintaining its pH (8.30). Also, attempts were made to modify flow and gradient, but it was found that it was necessary to modify the selectivity of the mobile phase to separate some derivatives, so the flow was maintained at 1.0 mL min^{-1} and a new solvent (MeOH) was introduced.

At first, the percentages of the aqueous phase were maintained and the percentages of the organic phase were modified, increasing the percentage of MeOH. Then, gradient steps were also modified, until obtaining the gradient showed on Table 2. In these conditions, the chromatogram showed in Figure 2 was obtained, and it can be seen that all the analytes are determined in less than 15 minutes.

3.2. Optimization of the online and automated derivatization reaction

The literature was reviewed [7,16,17] and some previous studies were performed to fix the initial conditions of the derivatization reaction. With this in mind, the following characteristics were established:

- Sandwich injection (derivatizing agent + sample + derivatizing agent).
- Fixed sample volume (5 μL). Sample was prepared with the corresponding standards/real sample and the internal standard.

- Derivatizing reagent prepared from OPA, 2-ME and boric acid/sodium borate buffer.

Also, some tests were carried out to verify which type of injection (draw sequentially or into seat) and which speed gave the best results, obtaining that the reaction reached a greater extension when the reagents were drawn sequentially and all the steps were made at default speed (90 µL min^{-1}).

Once these previous studies were performed, it was decided to carry out an experimental design for the others parameters optimization. A central composite design was employed, with three variables and two replicas for each experiment (except the central point, which was performed four times), obtaining a total of 32 experiments . The variables introduced in the experiment were mix times (between 0 and 60), volume of derivatizing reagent to be injected (between 0.5 and 8.0 µL) and waiting time before injecting the mixture (between 0 and 5 minutes).

The optimum parameters were selected employing the Surface Response Methodology (SRM). The SRM searches for the optimum value of the variables that result in the maximum value of the response function that defines the system under study. The difficulty lies in designing the appropriate response function. For this study, the chosen response function (Equation 1) relates positively the sum of areas (parameter used for quantification) and the product of the peaks resolutions that appear the closest in the chromatogram (zone from 6.0 to 7.5 minutes), and negatively the total time of analysis (parameters used for peaks separation). A statistical ANOVA analysis gives the goodness of the response surface obtained from this response function, through the adjustment coefficient (R^2), and the lack of fit, whose statistic *p* greater than 0.05 indicates that the model fits well.

This function fixed 2.0 µL of derivatizing reagent as the optimum volume, and, by setting this condition, two possible optimal conditions were obtained: A) 10 mix and 5 minutes of waiting time; or B) 60 mix and 1 minute of waiting time, as it can be seen in Figure 3.

$$F = \frac{\sum A \cdot \pi R_s}{t}$$

Equation 1. *Response function employed for the obtention of the optimum parameters, where "A" is area, "R_s" is the resolution between peaks and "t" is total time of analysis.*

Both possibilities were compared, obtaining very similar chromatograms, with equal signals and the same total analysis time. The decision was made on the basis of the best separation between AGM and CAD, which were better resolved with the second option.

Thus, the optimal parameters for automatic and on-line derivatization of the biogenic amines and the amino acids were 2.0 µL of derivatizing reagent (4.0 µL in total, because it is injected in sandwich mode), 60 mix and 1 minute of waiting before injection, as it can be seen in Table 1.

3.3. Evaluation of the method performance

Under the optimal experimental conditions, the linearity and detection and quantification limits were studied, employing a mixture of BAs and AAs standards, which were injected in triplicate. The precision was calculated inter- and intra-day, employing two different levels of analyte concentrations (low and high).

The developed automated on-line derivatization reaction coupled to UHPLC-FD method presented good linearities (98.48 – 99.45%) with determination coefficients (R^2) which ranged between 0.9970 and 0.9996. Precision was evaluated as relative standard deviations (RSD) of six and ten repeated measurements, for inter- and intra-day, respectively. These values are reported in Table 3, together with the detection limits (calculated by the Long and Winefordner method [34]) and the quantification limits (calculated as 3.3 times the detection limit of Long and Winefordner). Detection limits (LOD) ranged from 7.00 to 206 µg L^{-1}, while quantification limits (LOQ) varied between 22.0 and 681 µg L^{-1}.

In previous studies, a manual off-line pre-column method was established (data not published), but it was only applied to the determination of biogenic amines. Even so, the quality parameters obtained in both cases have been compared (Table S1 – Supplementary Material), finding that, in general, the determination coefficient (R^2), the detection limits (obtained by the Long- Winefordner method) and the

analytical sensitivity have been improved by using the automatic on-line methodology. Furthermore, the intraday relative standard deviation (RSD) has improved, thus improving the precision of the method.

In addition, it should be kept in mind that the automatic on-line method double the number of analytes determined, and that the possibility of performing derivatization in the equipment allows for increased working hours and facilitates the analysis of samples.

3.4. Application of the method to real samples of wine and beer

The proposed method was validated in beer and wine samples, employing a series of test samples that were spiked with known concentrations of the analytes. For this purpose, in a topaz vial, aliquots of solutions containing 200 µL of sample (wine diluted 1:2 and beer without dilution), 200 µL of addition standard and 400 µL of internal standard (OCT, 0.039 mM) and filled up to 5.0 mL with HCl 0.1 M; and aliquots of derivatizing reagent prepared from 1.6 mL OPA (2.98 mM) and 1.2 mL 2-ME, filled up to 5.0 mL with boric acid/sodium borate buffer (pH 10.5; 0.6 M), were placed. The concentrations of the analytes were then determined by the developed method, using standard addition method combined with internal standard. In Table 4, average recoveries of a beer, a white wine and a red wine sample are shown, which varied between 72.3 and 138.4%, showing a satisfactory agreement between the analytes concentrations taken and found.

On the other hand, in order to know if matrix effect was present, the calibration curve equations obtained by internal standard were compared with those obtained using standard addition method (combined with internal standard). The slopes of the curves were significantly different, so standard addition method (combined with internal standard) was chosen for the determination.

Found concentrations of the different analytes are shown on Table 4. As it can be seen, GLY, TYR, PUT, ETA, TRP and TYM appear in all samples, although in quite variable concentrations; while TRY and PEA do not appear. For blonde beer, the AAs in highest concentration are TYR and HIS, while

289 PUT is the main BA found. On the other hand, there are big differences between the two types of wine.
290 Red wine presents average concentrations of all compounds, with TYR and ETA being the main AA and
291 BA, respectively. Meanwhile, white wine has much higher concentrations of TYR and ARG. HIS and
292 TRP also appear in high concentrations and, with regard to BAs, they present lower concentrations than
293 red wine or beer. In all cases, the total AAs concentration (74.0 - 505.2 mg L^{-1}) is much higher than that
294 found for BAs (18.3 - 42.6 mg L^{-1}). The red wine is the one that presents less AAs, and the white wine
295 the one that presents more, having 3.5 times the concentration that presents the blonde beer. In the case
296 of the BAs, the red wine presents greater concentration, and the white wine is the sample that present
297 less.

298 ***3.5. Application of the method to beer samples collected during craft beer brewing process***

299 The main stages of the craft brewing process are: malting, grinding and maceration, cooking and
300 cooling, fermentation and bottling and maturation. During the fermentation, the yeast is added to the
301 cooked must in the fermentor, and the enzymes transform the sugars into alcohol and mark the profile of
302 the beer. This process occurs at a temperature of 20°C, and usually lasts between 7 and 10 days. During
303 this process, the must has to be protected from the presence of oxygen. Finally, during bottling and
304 maturation beer is kept for a while at a cool (16 - 20°C) and dry place to stabilize the flavor and aromas
305 obtained.

306 Samples analysed during this research were donated by a craft brewery (Extremadura, Spain),
307 which prepares different types of beer. In this case, samples were from a blonde beer with type Ale
308 fermentation. Samples were collected during the 9 days that the fermentation of the beer lasted, and kept
309 frozen until their analysis. Also, a sample of the finished bottled beer was analysed. Samples were
310 filtered, sonicated and diluted, when necessary, before their analysis, which was carried out by means of
311 the standard addition method (combined to internal standard method), due to the existence of matrix
312 effect.

Table 5 shows the concentrations of AAs and BAs found during the fermentation and in the bottled beer, and it can be noticed that the components profile varied over time. Regarding AAs, first day it can be observed a must very rich in AAs, in which all AAs are present except for GLY, with a total concentration of 390.0 mg L^{-1}, being TYR the main AA present (161 mg L^{-1}). The following days, total concentration of AAs sharply decreased to 2.0 mg L^{-1} in the fifth day. It is important to note that SER disappeared the second day to form GLY, which appeared from that moment on. Other AAs (as GLU, ARG, TYR, LYS, TRP and PHE) also decreased their concentration until not detected in some point. After the fifth day, the concentrations of AAs increased over time. The AAs that increased their concentrations were HIS, ARG, GLY, TYR, LYS and TRP, while GLU, SER and PHE remained undetected. The total AAs concentration on the last day before bottling was 127.0 mg L^{-1}, approximately one third of the initial concentration of the must, with TYR remaining the main AA present (62 mg L^{-1}). In bottled beer, the AAs content increased by approximately 50% to 234.0 mg L^{-1}. Important changes include the disappearance of ARG and the appearance of SER, as well as the sharp increase in the concentration of HIS, TYR and TRP. GLY and LYS also increase their concentration, but much more slightly.

On the other hand, concerning the BAs, only PUT, AGM and ETA appear, being the rest undetected during all the fermentation process. Their total concentration in the first day must was of 15.6 mg L^{-1}, being much lower than that obtained for AAs (specifically, 25 times less). Their concentrations suffer minor changes during time, but the fifth day their concentration reached its lowest valor (6.6 mg L^{-1}), as in AAs concentration; however, it was the only time when BAs concentration was higher than AAs concentration. The following days, total BAs concentration increased, reaching 20.7 mg L^{-1} the day before the bottling. The BA in higher concentration during all the fermentation process was the AGM, which is the only BA that comes from the malt. Finally, in bottled beer, the content of BAs suffered a great increase, reaching a concentration 4 times greater (81.0 mg L^{-1}). Only PUT, ETA and AGM continued to appear, although TYM was detected (without being quantifiable). The main increase

corresponded to the concentration of AGM, which increased approximately 80% with respect to the last day of fermentation. This increase may be influenced by the disappearance of ARG.

It could be observed that, although having precursors of all BAs, most of them do not appear, which could mean that the microorganisms responsible for the decarboxylation of AAs would be not present in the analysed must.

4. Conclusions

In this work, a new method for the automatic on-line pre-column derivatization of 8 biogenic amines and 9 precursor amino acids has been optimized through experimental design and Surface Response Methodology. The obtained derivatives were chromatographically separated by UHPLC with fluorescent detection, using reverse phase and a simple mobile phase. Under these conditions, the 17 derivatives and the internal standard were separated in less than 15 minutes, obtaining a fast method with good linearity and sensibility (determination coefficients between 0.9970 and 0.9996 and LODs between 7.00 and 206 $\mu g\ L^{-1}$), easy to implement in a quality laboratory.

The method has been validated and applied to three types of fermented beverages (blonde beer, red wine and white wine), obtaining very different profiles of biogenic amines and amino acids. Some compounds appear in all samples (GLY, TYR, PUT, ETA, TRP and TYM), while TRY and PEA are not present in any of them. Samples collected during the fermentation process of a blonde Ale craft beer were also analysed employing the developed method, obtaining a BAs and AAs profile that changed over time, and in which, although a lot of precursor AAs were present in the must, only three BAs appeared (being one of them AGM, which naturally occurs in the malt), so, apparently, the microorganisms responsible for decarboxylation of AAs were not present in the must. This is an indication of the good microbiological quality of the analysed must. In addition, it has also been found that the profile of AAs and BAs varies during the maturation and bottling process, as the concentrations of AAs and BAs found in a finished beer from the same batch indicate 2 times more AAs, as well as 4 times more BAs.

With regard to changes in the characteristics of craft beer from bottling to consumption, the bibliography includes a study [35] on the loss of qualities referring to one of the components of hops, recommending maintaining the beer at cool temperature and consumption before three months. In this way, it could also be interesting to monitor changes in the concentrations of AAs and BAs from bottling and throughout their useful life, using the proposed method.

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References

[1] S. Sentellas, O. Núñez, J. Saurina, Recent advances in the determination of biogenic amines in food samples by (U)HPLC, J. Agric. Food Chem. 64 (2016) 7667–7678. doi:10.1021/acs.jafc.6b02789.

[2] M. V Moreno-Arribas, C. Polo, Wine chemistry and biochemistry, Springer New York, 2008. doi:10.1007/978-0-387-74118-5.

[3] B. Redruello, V. Ladero, I. Cuesta, J.R. Álvarez-Buylla, M.C. Martín, M. Fernández, M.A. Alvarez, A fast, reliable, ultra high performance liquid chromatography method for the simultaneous determination of amino acids, biogenic amines and ammonium ions in cheese, using diethyl ethoxymethylenemalonate as a derivatising agent, Food Chem. 139 (2013) 1029–1035. doi:10.1016/j.foodchem.2013.01.071.

[4] B. Redruello, V. Ladero, B. del Rio, M. Fernández, M.C. Martin, M.A. Alvarez, A UHPLC method for the simultaneous analysis of biogenic amines, amino acids and ammonium ions in beer, Food Chem. 217 (2017) 117–124. doi:10.1016/j.foodchem.2016.08.040.

[5] European Commision, Commission regulation (EC) No 1441/2007, Off. J. Eur. Union. 322 (2007) 2–29.

[6] G. Fiechter, G. Sivec, H.K. Mayer, Application of UHPLC for the simultaneous analysis of free amino acids and biogenic amines in ripened acid-curd cheeses, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 927 (2013) 191–200. doi:10.1016/j.jchromb.2012.12.006.

[7] C.K. Zacharis, G.A. Theodoridis, A.N. Voulgaropoulos, Coupling of sequential injection with liquid chromatography for the automated derivatization and on-line determination of amino acids, Talanta. 69 (2006) 841–847. doi:10.1016/j.talanta.2005.11.028.

[8] M. Papageorgiou, D. Lambropoulou, C. Morrison, E. Kłodzińska, J. Namieśnik, J. Płotka-Wasylka, Literature update of analytical methods for biogenic amines determination in food and beverages, TrAC Trends Anal. Chem. 98 (2018) 128–142. doi:10.1016/j.trac.2017.11.001.

[9] A. Pineda, J. Carrasco, C. Peña-Farfal, K. Henríquez-Aedo, M. Aranda, Preliminary evaluation of biogenic amines content in Chilean young varietal wines by HPLC, Food Control. 23 (2012) 251–257. doi:10.1016/j.foodcont.2011.07.025.

[10] E. Dadáková, M. Křížek, T. Pelikánová, Determination of biogenic amines in foods using ultra-performance liquid chromatography (UPLC), Food Chem. 116 (2009) 365–370. doi:10.1016/j.foodchem.2009.02.018.

[11] H.K. Mayer, G. Fiechter, UHPLC analysis of biogenic amines in different cheese varieties, Food Control. 93 (2018) 9–16. doi:10.1016/j.foodcont.2018.05.040.

[12] G. Fiechter, H.K. Mayer, UPLC analysis of free amino acids in wines: profiling of on-lees aged wines, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 1361–1366. doi:10.1016/j.jchromb.2011.02.005.

[13] M.L. Latorre-Moratalla, J. Bosch-Fusté, T. Lavizzari, S. Bover-Cid, M.T. Veciana-Nogués, M.C. Vidal-Carou, Validation of an ultra high pressure liquid chromatographic method for the determination of biologically active amines in food, J. Chromatogr. A. 1216 (2009) 7715–7720. doi:10.1016/j.chroma.2009.08.072.

[14] H. Wu, G. Li, S. Liu, Z. Ji, Q. Zhang, N. Hu, Y. Suo, J. You, Simultaneous determination of seven biogenic amines in foodstuff samples using one-step fluorescence labeling and dispersive liquid–liquid microextraction followed by HPLC-FLD and method optimization using Response Surface Methodology, Food Anal. Methods. 8 (2015) 685–695. doi:10.1007/s12161-014-9943-2.

[15] T. Hyötyläinen, N. Savola, P. Lehtonen, M.L. Riekkola, Determination of biogenic amines in wine by multidimensional liquid chromatography with online derivatisation, Analyst. 126 (2001) 2124–2127. doi:10.1039/b108189f.

[16] J. Peng, K. Fang, D. Xie, B. Ding, J.-Y. Yin, X. Cui, Y. Zhang, J. Liu, Development of an automated on-line pre-column derivatization procedure for sensitive determination of histamine in food with high-performance liquid chromatography-fluorescence detection, J. Chromatogr. A.

1209 (2008) 70–75. doi:10.1016/j.chroma.2008.09.028.

[17] O. Busto, J. Guasch, F. Borrull, Improvement of a solid-phase extraction method for determining biogenic amines in wines, J. Chromatogr. A. 718 (1995) 309–317.

[18] Z. Li, Y. Xing, X. Guo, Y. Cui, Development of an UPLC–MS/MS method for simultaneous quantitation of 11 D-amino acids in different regions of rat brain: Application to a study on the associations of D-amino acid concentration changes and Alzheimer's disease, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1058 (2017) 40–46. doi:10.1016/j.jchromb.2017.05.011.

[19] J.M. Moreno-Rojas, A. Moreno-Ortega, J.L. Ordóñez, R. Moreno-Rojas, J. Pérez-Aparicio, G. Pereira-Caro, Development and validation of UHPLC-HRMS methodology for the determination of flavonoids, amino acids and organosulfur compounds in black onion, a novel derived product from fresh shallot onions (Allium cepa var. aggregatum), LWT - Food Sci. Technol. 97 (2018) 376–383. doi:10.1016/j.lwt.2018.07.032.

[20] K. Nalazek-Rudnicka, A. Wasik, Development and validation of an LC – MS / MS method for the determination of biogenic amines in wines and beers, Monatshefte Für Chemie - Chem. Mon. 148 (2017) 1685–1696. doi:10.1007/s00706-017-1992-y.

[21] N. Gray, R.S. Plumb, I.D. Wilson, J.K. Nicholson, A validated UPLC-MS / MS assay for the quantification of amino acids and biogenic amines in rat urine, J. Chromatogr. B. 1106–1107 (2019) 50–57. doi:10.1016/j.jchromb.2018.12.028.

[22] D. Zildeana, S. Furtado, F. Brunale, V. De Moura, C. Nunes, B. Faria, L. Dias, L. Jedlicka, E. De Jesus, H. Dominguez, E. Jose, H. Bechara, N.A. Assunc, Profiles of amino acids and biogenic amines in the plasma of Cri-du-Chat patients, J. Pharm. Biomed. Anal. 140 (2017) 137–145. doi:10.1016/j.jpba.2017.03.034.

[23] Z. Xie, P. Lorkiewicz, D.W. Riggs, A. Bhatnagar, S. Srivastava, Comprehensive, robust, and sensitive UPLC-MS/MS analysis of free biogenic monoamines and their metabolites in urine., J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 1099 (2018) 83–91.

doi:10.1016/j.jchromb.2018.09.012.

[24] H. Dong, K. Xiao, Modified QuEChERS combined with ultra high performance liquid chromatography tandem mass spectrometry to determine seven biogenic amines in Chinese traditional condiment soy sauce, Food Chem. 229 (2017) 502–508. doi:10.1016/j.foodchem.2017.02.120.

[25] W.F. Visser, N.M. Verhoeven-Duif, R. Ophoff, S. Bakker, L.W. Klomp, R. Berger, T.J. de Koning, A sensitive and simple ultra-high-performance-liquid chromatography-tandem mass spectrometry based method for the quantification of d-amino acids in body fluids, J. Chromatogr. A. 1218 (2011) 7130–7136. doi:10.1016/j.chroma.2011.07.087.

[26] S. Parrot, P.C. Neuzeret, L. Denoroy, A rapid and sensitive method for the analysis of brain monoamine neurotransmitters using ultra-fast liquid chromatography coupled to electrochemical detection, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 3871–3878. doi:10.1016/j.jchromb.2011.10.038.

[27] B. Ferry, E.P. Gifu, I. Sandu, L. Denoroy, S. Parrot, Analysis of microdialysate monoamines, including noradrenaline, dopamine and serotonin, using capillary ultra-high performance liquid chromatography and electrochemical detection, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 951–952 (2014) 52–57. doi:10.1016/j.jchromb.2014.01.023.

[28] D. Van Dam, Y. Vermeiren, T. Aerts, P.P. De Deyn, Novel and sensitive reversed-phase high-pressure liquid chromatography method with electrochemical detection for the simultaneous and fast determination of eight biogenic amines and metabolites in human brain tissue, J. Chromatogr. A. 1353 (2014) 28–39. doi:10.1016/j.chroma.2014.05.004.

[29] J. Van Schoors, J. Viaene, Y. Van Wanseele, I. Smolders, B. Dejaegher, Y. Vander Heyden, A. Van Eeckhaut, An improved microbore UHPLC method with electrochemical detection for the simultaneous determination of low monoamine levels in in vivo brain microdialysis samples, J. Pharm. Biomed. Anal. 127 (2016) 136–146. doi:10.1016/j.jpba.2016.01.015.

[30] L. Zhang, J. Yang, Y. Luo, J. Shang, X. Jiang, Simultaneous determination of eleven compounds related to metabolism of bioamines in rat cortex and hippocampus by HPLC-ECD with boron-doped diamond working electrode, J. Pharm. Biomed. Anal. 118 (2016) 41–51. doi:10.1016/j.jpba.2015.10.020.

[31] S. Eto, M. Yamaguchi, M. Bounoshita, T. Mizukoshi, H. Miyano, High-throughput comprehensive analysis of D- and L-amino acids using ultra-high performance liquid chromatography with a circular dichroism (CD) detector and its application to food samples, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 3317–3325. doi:10.1016/j.jchromb.2011.07.025.

[32] I.S. Krull, Z. Deyl, H. Lingeman, General strategies and selection of derivatization reactions for liquid chromatography and capillary electrophoresis, J. Chromatogr. B Biomed. Sci. Appl. 659 (1994) 1–17. doi:10.1016/0378-4347(94)00151-0.

[33] T. Galeano Diaz, A. Muñoz de la Peña, A. Espinosa-Mansilla, I. Durán Martín-Merás, M.I. Acedo-Valenzuela, F. Cañada Cañada, D. Gónzalez-Gómez, Herramienta estadística para Química Analítica ACOC v2.0, Servicio de Publicaciones de la Universidad de Extremadura, Cáceres, 2007.

[34] G.L. Long, J.D. Winefordner, Limit of Detection: a closer look at the IUPAC definition, Anal. Chem. 55 (1983) 712A–724A. doi:10.1021/ac00258a001.

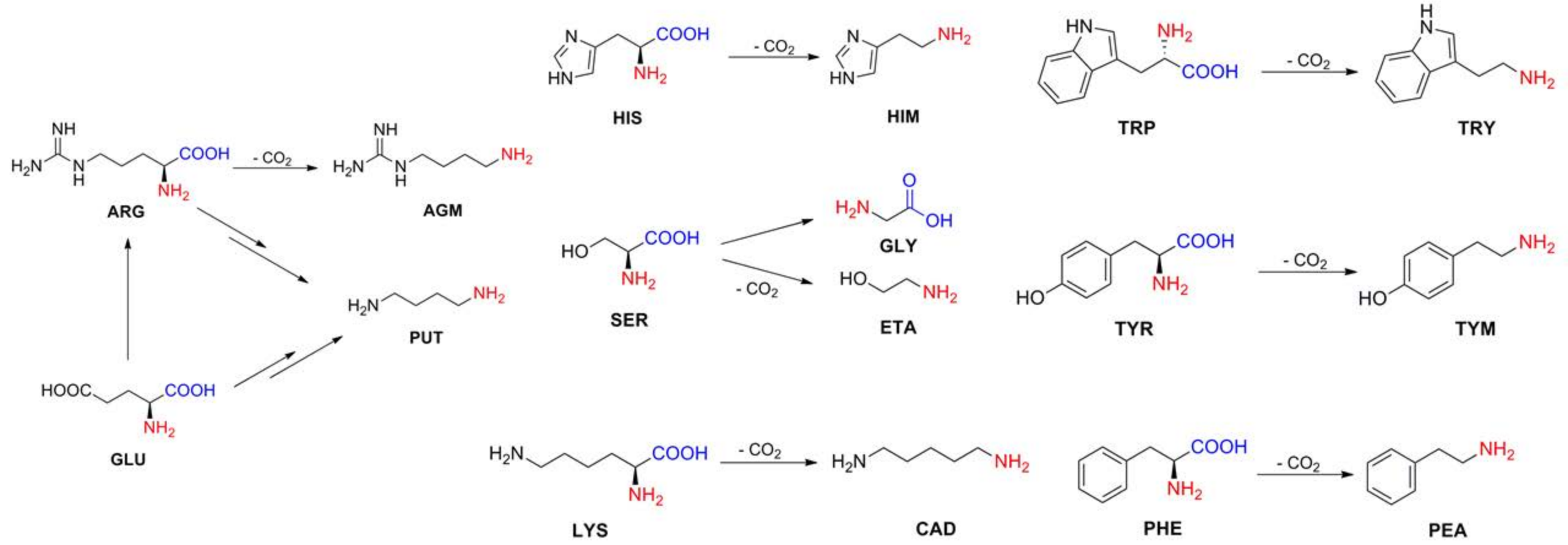
[35] K. Reglitz, N. Lemke, S. Hanke, M. Steinhaus, On the behavior of the important hop odorant 4-mercapto-4-methylpentan-2-one (4MMP) during dry hopping and during storage of dry hopped beer, Brew. Sci. 71 (2018) 96–99. doi.org/10.23763/BRSC18-13STEINHAUS.

Figure captions

Figure 1. Structure of the biogenic amines and precursor amino acids studied during this research. ARG: arginine; AGM: agmatine; GLU: glutamic acid; PUT: putrescine; HIS: histidine; HIM: histamine; SER: serine; ETA: ethanolamine; LYS: lysine; CAD: cadaverine; TRP: tryptophan; TRY: tryptamine; TYR: tyrosine; TYM: tyramine; PHE: phenylalanine; PEA: 2-phenylethylamine.

Figure 2. Chromatogram of the derivatives of biogenic amines and precursor amino acids obtained under optimal conditions, after on-line derivatization and chromatographic separation. For abbreviations, see Figure 1.

Figure 3. Response surface obtained by using the response function (Equation 1), and setting the OPA volume to 2.0 mL.


ARG
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AGM
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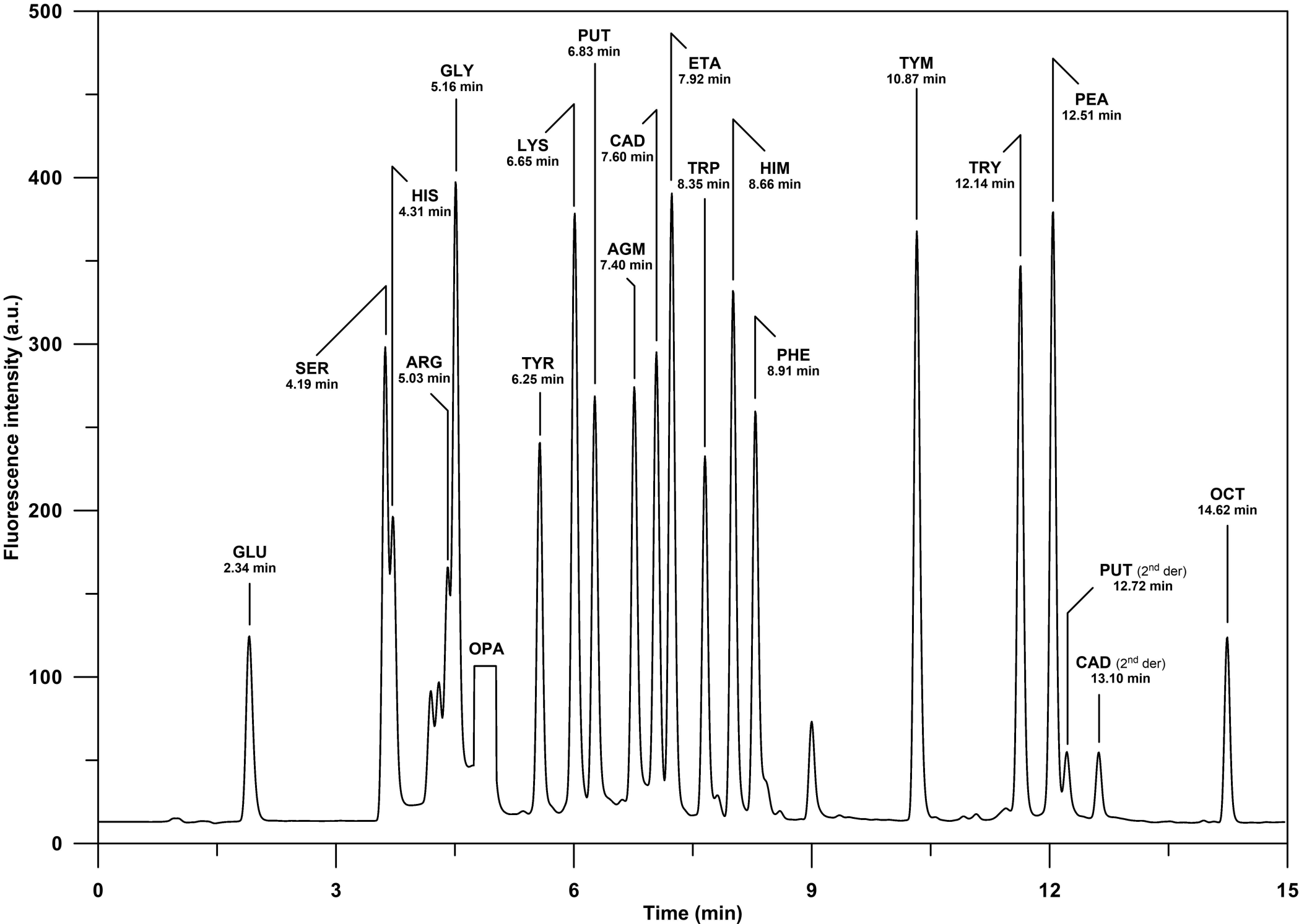
Fluorescence intensity (a.u.)
Time (min)
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100
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3
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12
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GLU
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SER
4.19 min
HIS
4.31 min
ARG
5.03 min
GLY
5.16 min
OPA
TYR
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LYS
6.65 min
PUT
6.83 min
CAD
7.60 min
AGM
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ETA
7.92 min
TRP
8.35 min
HIM
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PHE
8.91 min
TYM
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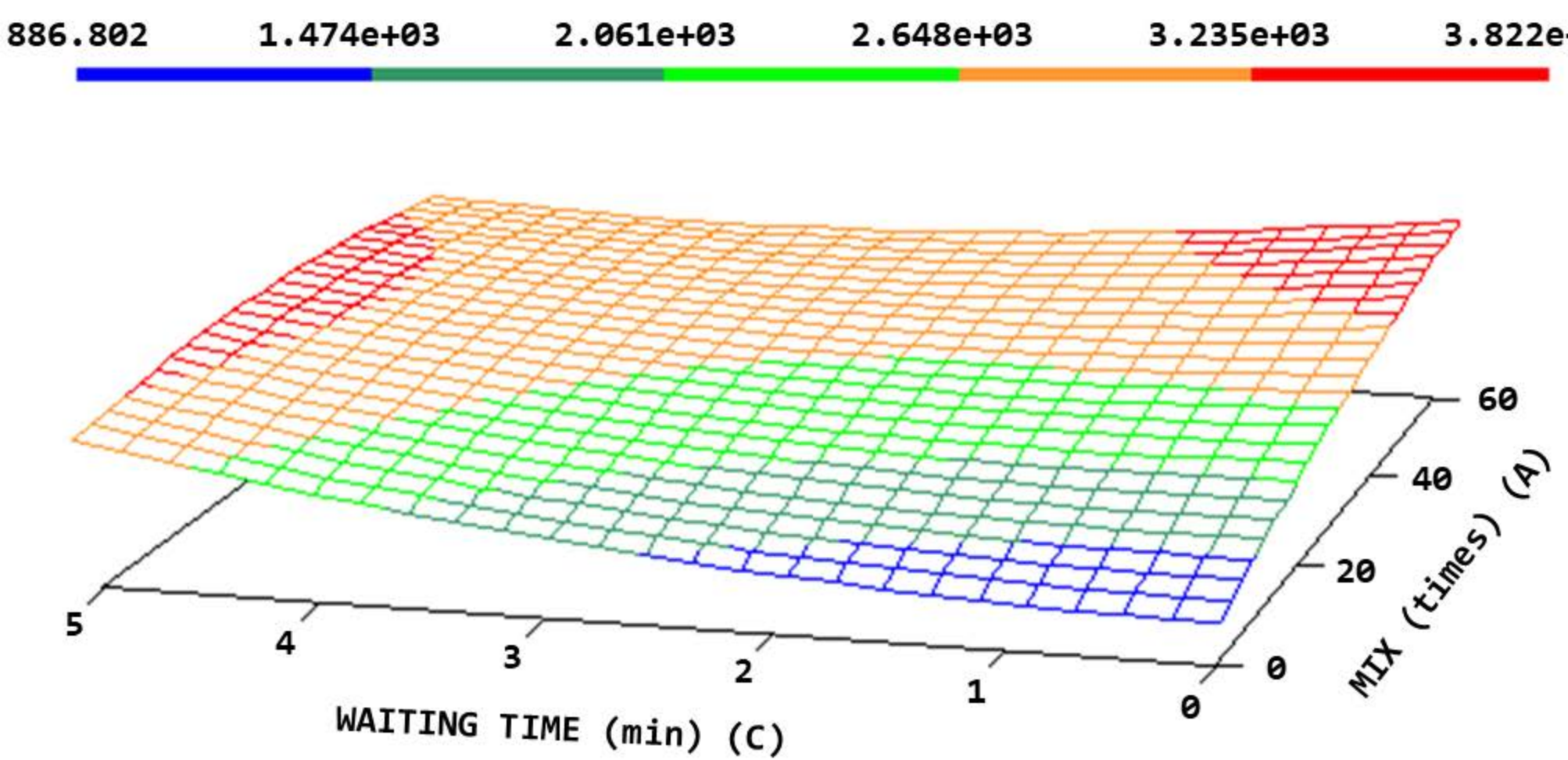

RESULT1, Y-var:*, (X-var = value): OPA volume (B) = 2.00

Table 1. *Injection programme for the on-line pre-column derivatization of the amino acids and the biogenic amines.*

FUNCTION	VOLUME	SUBSTANCE	DETAILS
Draw	2 μL	Derivatization reagent	Default speed (90 μL min^{-1}) Default offset
Wash needle		MeOH-H_2O	2 times
Draw	5 μL	Sample	Default speed (90 μL min^{-1}) Default offset
Wash needle		MeOH-H_2O	2 times
Draw	2 μL	Derivatization reagent	Default speed (90 μL min^{-1}) Default offset
Wash needle		MeOH-H_2O	2 times
Mix	Default volume (9 μL)	Mixture	From air Default speed (90 μL min^{-1}) 60 times
Wait			1 minute
Inject	9 μL	Reaction mixture	[AAs]: 0.40 – 49 μM [BAs]: 0.16 – 7.8 μM [IS]: 1.7 μM [OPA]: 0.42 mM

Table 2. *Elution gradient optimized for the separation of the amino acids and the biogenic amines derivatives.*

TIME **(min)**	**%A** **(TRIS buffer)**	**%B** **(ACN)**	**%C** **(MeOH)**
0	80	6	14
6	50	17.5	32.5
14	0	35	65
15	80	6	14

Table 3. *Method performance parameters. For abbreviations, see Figure 1.*

	GLU	**SER**	**HIS**	**ARG**	**GLY**	**TYR**	**LYS**	**PUT**	**AGM**	**CAD**	**ETA**	**TRP**	**HIM**	**PHE**	**TYM**	**TRY**	**PEA**
t_R (min)	1.91	3.64	3.73	4.44	4.53	5.59	6.04	6.25	6.75	7.02	7.24	7.67	8.02	8.30	10.33	11.64	12.05
Linear range (mg L^{-1})	0.15 – 10.0	0.075 – 5.0	0.15 – 5.0	0.10 – 1.25	0.03 – 1.25	0.15 – 5.0	0.05 – 2.5	0.03 – 0.625	0.03 – 1.25	0.03 – 0.625	0.01 – 0.50	0.10 – 5.0	0.04 – 1.25	0.10 – 5.0	0.04 – 1.0	0.04 – 1.0	0.01 – 1.0
R^2	0.9983	0.9988	0.9982	0.9980	0.9975	0.9994	0.9996	0.9992	0.9977	0.9981	0.9992	0.9970	0.9984	0.9978	0.9971	0.9996	0.9980
Linearity (%)	98.85	99.02	98.81	98.77	98.60	99.34	99.45	99.23	98.67	98.80	99.24	98.48	98.90	98.71	98.50	99.44	98.75
LOD Long-Winefordner (µg L^{-1})	206.4	79.4	96.4	25.8	29.4	53.3	21.4	8.8	30.1	13.9	6.6	123.2	24.7	104.8	27.6	10.4	22.9
LOQ ($3.3 \cdot LOD_{L\text{-}W}$) (µg L^{-1})	681.1	262.0	318.1	85.1	97.0	175.9	70.6	29.0	99.3	45.9	21.8	406.6	81.5	345.8	91.1	34.3	75.6
Analytical sensitivity (γ^{-1}) (µg L^{-1})	156.4	68.3	82.1	19.3	22.9	45.4	18.6	6.7	22.2	10.5	5.4	105.6	18.2	89.9	19.9	7.5	16.9
RSD intraday (%) (low point)	4.28	3.61	6.03	3.50	4.42	4.59	4.72	3.15	5.59	2.70	4.30	4.28	4.59	4.61	4.83	3.40	4.41
RSD intraday (%) (high point)	2.39	2.38	2.86	4.43	2.04	2.93	2.21	1.73	3.49	3.26	2.55	2.49	1.33	1.92	1.50	1.70	1.55
RSD interday (%) (low point)	5.01	5.90	3.34	8.75	7.64	7.38	8.85	6.19	11.09	12.6	9.32	6.50	7.11	5.81	6.78	8.38	6.89
RSD interday (%) (high point)	3.94	5.51	4.10	9.26	5.75	5.73	6.60	4.08	5.05	3.55	6.57	5.32	6.97	6.89	5.85	9.07	8.19

Table 4. *Biogenic amines and precursor amino acids concentrations found in different samples of fermented beverages (± SD; mg L^{-1}). For abbreviations, see Figure 1.*

	GLU	SER	HIS	ARG	GLY	TYR	LYS	PUT	AGM	CAD	ETA	TRP	HIM	PHE	TYM	TRY	PEA	TOTAL AAs	TOTAL BAs
BLONDE BEER	<0.15	<0.08	33 ± 6	n.d.	15 ± 1	73 ± 9	n.d.	18 ± 2	n.d.	n.d.	7.3 ± 0.9	13 ± 5	6.5 ± 0.8	11 ± 1	4 ± 1	n.d.	n.d.	145.2	35.8
RED WINE	n.d.	n.d.	n.d.	n.d.	10 ± 1	35 ± 9	11 ± 3	16 ± 1	<0.06	<0.03	20 ± 2	18 ± 5	3 ± 1	n.d.	3.5 ± 0.7	n.d.	n.d.	74.0	42.6
WHITE WINE	<0.15	n.d.	47 ± 16	56 ± 10	19 ± 2	320 ± 71	27 ± 3	8 ± 1	n.d.	n.d.	7.3 ± 0.9	27 ± 9	n.d.	9 ± 6	3 ± 1	n.d.	n.d.	505.2	18.3
Recuperation in beer (%)	97.3	97.7	88.5	98.9	91.8	78.7	103.2	107.4	92.8	88.9	111.0	93.8	108.5	99.4	121.9	104.1	102.5	94.0	104.6
Recuperation in red wine (%) *					99.7	117.3	101.7	120.7	96.8	72.3	138.4	88.5	110.7		94.7			101.8	105.6
Recuperation in white wine (%) *	81.3		75.3	82.2	101.8		110.1	129.9			107.8	85.7		94.3	93.2			105.1	110.3

*In wine samples appear the recuperations of the found analytes.

Table 5. *Biogenic amines and precursor amino acids concentrations found in craft beer samples collected during elaboration process (± SD; mg L^{-1}). For abbreviations, see Figure 1.*

	GLU	SER	HIS	ARG	GLY	TYR	LYS	PUT	AGM	CAD	ETA	TRP	HIM	PHE	TYM	TRY	PEA	TOTAL AAs	TOTAL BAs
DAY 1	39 ± 8	18 ± 3	40 ± 4	42 ± 6	n.d.	161 ± 13	32 ± 2	2.3 ± 0.4	8 ± 2	n.d.	2.3 ± 0.3	29 ± 4	n.d.	29 ± 4	3 ± 1	n.d.	n.d.	390.0	15.6
DAY 2	13 ± 7	n.d.	13 ± 4	9 ± 2	7.2 ± 0.6	131 ± 15	3 ± 1	2.4 ± 0.2	8 ± 1	n.d.	2.8 ± 0.2	12 ± 3	n.d.	7 ± 3	n.d.	n.d.	n.d.	195.2	13.2
DAY 3	8 ± 4	n.d.	8 ± 2	n.d.	3.8 ± 0.7	16 ± 3	2 ± 1	2.1 ± 0.3	7.0 ± 0.9	n.d.	2.2 ± 0.4	6 ± 2	n.d.	n.d.	n.d.	n.d.	n.d.	44.2	11.3
DAY 4	n.d.	n.d.	9 ± 4	n.d.	4 ± 1	22 ± 10	2.6 ± 0.9	3.2 ± 0.8	11 ± 2	n.d.	3.4 ± 0.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	37.6	17.6
DAY 5	n.d.	n.d.	n.d.	n.d.	2.0 ± 0.7	n.d.	n.d.	1.3 ± 0.4	4 ± 1	n.d.	1.3 ± 0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.0	6.6
DAY 6	n.d.	n.d.	14 ± 5	n.d.	7 ± 2	30 ± 10	3 ± 1	3.8 ± 0.6	12 ± 2	n.d.	4 ± 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	54.0	19.8
DAY 7	n.d.	n.d.	15 ± 5	n.d.	7 ± 2	20 ± 5	3 ± 1	3.0 ± 0.4	10 ± 2	n.d.	3.0 ± 0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	45.0	16.0
DAY 9	n.d.	n.d.	31 ± 5	6 ± 1	10 ± 1	62 ± 8	7 ± 1	3.8 ± 0.3	12 ± 1	n.d.	4.9 ± 0.4	11 ± 5	n.d.	n.d.	n.d.	n.d.	n.d.	127.0	20.7
BOTTLED BEER	n.d.	14 ± 5	74 ± 18	n.d.	16 ± 6	95 ± 14	10 ± 5	8 ± 2	66 ± 16	n.d.	7 ± 2	25 ± 12	n.d.	<LOQ	<LOQ	n.d.	n.d.	234.0	81.0

Table S1. *Comparation of analytical parameters obtained using the manual off-line method (first row) and the automatic on-line one (second row).*

	PUT	**AGM**	**CAD**	**ETA**	**HIM**	**TYM**	**TRY**	**PEA**
R^2	0.9992	0.9917	0.9980	0.9992	0.9984	0.9971	0.9996	0.9980
	0.9961	0.9990	0.9965	0.9942	0.9988	0.9995	0.9981	0.9991
LOD (µg L^{-1})	8.8	30.1	13.9	6.6	24.7	27.6	10.4	22.9
	22.4	16.3	21.4	10.8	17.6	5.6	11.6	6.3
Analytical sensitivity (γ^{-1}) (µg L^{-1})	6.7	22.2	10.5	5.4	18.2	19.9	7.5	16.9
	16.8	11.7	16.1	8.4	12.6	3.7	7.7	5.2
RSD intraday (%) (low point)	3.15	5.59	2.70	4.30	4.59	4.83	3.40	4.41
	5.24	2.70	4.57	4.24	3.20	4.22	4.40	6.05
RSD intraday (%) (high point)	1.73	3.49	3.26	2.55	1.33	1.50	1.70	1.55
	2.37	1.62	1.44	1.45	1.58	1.90	1.49	1.69