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Analytical miniaturization for the control of biogenic amines in craft beer

Dissertation of the Master's Degree in Quality Control

Work developed under the supervision of

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Abstract

Craft beers are part of an expanding market; however, health authorities do not have specific laws to control the quality and food safety of this beverage. Biogenic amines are an important indicator of bacterial contamination and spoilage in foods, so their presence must be monitored. Different analytical techniques are used to determine these amines, mostly based on HPLC coupled to spectrophotometric detection. However, these analytical methodologies present disadvantages such as high cost, need of derivatization, and generation of toxic components. To overcome these limitations the present work proposed the combination of an HPLC-potentiometric system for the simultaneous determination of ten BAs in five different samples of craft beer. The technique showed good linearity with R^2 ranging from 0.9890 to 0.9992. LOD and LOQ are differentiated according to the molecular weight of each BA, having their values between 9.3 to 60.7 µg L^{-1} and 31.1 to 202.3 µg L^{-1}, respectively. The precision was evaluated by repeatability and intermediate precision, attained RSD% lower than 15%. The analyzed samples varied in total content and type of BAs found. The Imperial Stout showed the lowest content - 2.7 ± 0.2 mg L^{-1}, while and the Session IPA the highest - 11.6 ± 0.5 mg L^{-1}

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Keywords: biogenic amines, potentiometric, craft beers

Resumo

As cervejas artesanais fazem parte de um mercado em expansão, porém, as autoridades sanitárias não possuem leis específicas para controlar a qualidade e segurança alimentar dessa bebida. As aminas biogênicas são um importante indicador de contaminação bacteriana e deterioração em alimentos, portanto, sua presença deve ser monitorada. Diferentes técnicas analíticas são utilizadas para determinar essas aminas, principalmente baseadas em HPLC acoplado à deteção espectrofotométrica. Porém, essas metodologias analíticas apresentam desvantagens como alto custo, necessidade de derivatização e geração de componentes tóxicos. Para superar essas limitações, o presente trabalho propôs a combinação de um sistema HPLC-potenciométrico para a determinação simultânea de dez BAs em cinco diferentes amostras de cerveja artesanal. A técnica apresentou boa linearidade com R^2 variando de 0,9890 a 0,9992. LOD e LOQ são diferenciados de acordo com o peso molecular de cada BA, tendo seus valores entre 9,3 a 60,7 µg L-1 e 31,1 a 202,3 µg L-1, respetivamente. A precisão foi avaliada pela repetibilidade e precisão intermediária, atingindo RSD% menor que 15%. As amostras analisadas variaram em conteúdo total e tipo de BA encontrados. A Imperial Stout apresentou o menor teor - 2,7 ± 0,2 mg L^{-1}, enquanto a Sessão IPA o maior - 11,6 ± 0,5 mg L^{-1}

Palavras-chave: aminas biogénicas, potenciometria, cervejas artesanais.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACN – Acetonitrile

ALDH – Aldehyde dehydrogenase

BAs – Biogenic Amines

CAD – Cadaverine

CB6 – Cucurbit[6]uril hydrate

DAO – Diamine oxidase

ETH – Ethylamine

EFSA – European Food Safety Authority

EU – Europe Union

EC – Europe Comission

FL – Fluorescence

GMP – Good Manufacturing Practices

HACCP – Hazard Analysis and Critical Control Points

HIS – Histamine

HNMT – Histamine-N-metyltransferase

HPLC – High performance liquid chromatography

IPA – India PaLe Ale

ISEs – ion-selective electrodes

LOD – Limit of Detectetion

LOQ – Limit of Quantification

MAO – Monoamine oxidase

MET – Methylamine

MWCNTs – Nanotubes

MS – Mass Spectrometry

mV – millivolt

PHE – Phenylethylamine

PVC – Polyvinyl Chloride

PUT – Putrescine

QC – Quality Control

RSD – Relative standard deviation

SBS – butane-1-sulfonic acid sodium salt

SPM – Spermine

SPMD – Spermidine

TCBP – Potassium tetrakis(p-chlorophenyl) Borate

THF – Tetrahydrofuran

TYR – Tyramine

TRYP – Tryptamine

UV – Ultraviolet

o-NPOE – o-nitrophenyloctyl ether

1. Introduction

In general, a product having quality is as suitable for its purpose as concomitantly safe [1]. In the food industry, quality control (QC) is essential because while its purpose as product is more or less intuitively evident a relevant point to be pursued is food safety [2]. Public health authorities indicate compliance with certain guidelines and regulations, such as Good Manufacturing Practices (GMP), as a set of measures to be implemented and which will ensure production within the necessary quality standards [1, 3]. Also noteworthy, the Hazard Analysis and Critical Control Points (HACCP), is a guideline to identify and prevent riskiness in food industries [4]. In 1993 the Council of Europe approved the directive EE/93-43, which was intended to ensure the implementation of the HACCP for all EU food companies until 1995 [5]. The improvement and strict compliance with QC rewards the company with a competitive advantage in the whole market by increasing its credibility near the consumer [6].

Beverage manufacturing was also included regarding these recommendations, albeit some contaminants are generated during the production phases [7]. Therefore, guided by the regulatory guidelines, the QC procedures, particularly the analytical methodologies used in the laboratory need to use robust and fast, environmentally clean, and as affordable as possible since its management is grounded on marginal costs [8].

1.1. Beers

The precise origin of beer or its history is unclear due to the lack of a sufficient amount of archaeological records. On the other hand, alcoholic beverages made from fruits, honey, and cereals such as oats, barley, and wheat, have already at different times been also labelled as beer thus bringing more haziness to the point [9]. It is more or less consensual that it was discovered by chance when grains underwent natural fermentation, giving rise to the beer [10-12]. It is also known that this beverage integrated local habits, religious practices and rituals. The geographic provenience is ascribed to middle east, to Mesopotamian people like Egyptians which are considered the first beer producers [12]. Currently, some of the other mentioned beverages, classified in the past as corresponding to the same drink, are nowadays equivalent to distilled spirits and wines [9]. A written record with the brewing process was found in Mesopotamia, consisting of a poem intended for goddess *Ninkasi*. In it, ingredients like aromatic herbs, cereals, honey, and even wine could be added to the formula for enrichment of taste properties [9].

Currently, beer is the most popular and expend alcoholic beverage in the world [13]. It is recognized for its wine-like antioxidant potential [14]. Food regulatory in Portugal defines beer as a beverage obtained by alcoholic fermentation made by yeasts of the genus

Saccharomyces (*Saccharomyces cerevisiae, Saccharomyces carlsbergensis*), using essentially water, cereals, barley and hops as raw materials. The Normative Ordinance nº 1/96, rank the beers into alcohol-free, special, low alcohol, extra, lactic fermented, bottle-fermented, and regular [15, 16]. Beers can differ in the alcoholic content, type of fermentation and primitive extract. These ingredients are submitted to a process to form the beer body [17].

The production of beers starts from a blend of raw materials, each one with its own role and relevance:

Water – The ingredient in the largest quantity in the final product, requiring strict quality control through treatments and analysis of microbiological parameters, pH, turbidity, hardness, among others [17].

Malt – It consists of germinated cereal grains, the most used in brewing is malted barley, obtained from a controlled germination process, the malting. Dry grains are allowed to germinate by immersion in cold water at controlled temperature for a controlled period of time, after which the process is halted. The meanwhile produced amylases catalyze the cleavage of the starches in the seeds into mono-, di- saccharides, maltotriose and maltodextrines. Other cereals such as wheat, rice, corn, and oats can also be malt adjuncts. Yeast growth is aided by cereals, which are sources of amino acids [17-19].

Yeast – Yeasts are responsible for the fermentation step, wherein ethanol and carbon dioxide are produced. The flavor and aroma of beer are mostly dependent on the type of yeast used. For instance, *Saccharomyces cerevisiae* and *Saccharomyces uvarum* are used to produce *ale* and *lager* beer, respectively [19, 20].

Hop - *Humulus Lupulus* is a specie of the genus *Humulus* used in brewing. It is a plant of the Cannabaceae family, which possess female and male flowers. The female flowers are used in beer production as they are sources of lupulin. Such glands contain essential oils, resins, and polyphenols that contribute to characteristics such as aroma, bitterness, the stability of the beer foam, among others. Hops can also be used for its bacteriostatic function in beers, in addition to having antioxidant potential [17, 21].

The combination of different adjuvants gives raise to different beer types. The quality of raw materials is very important parameter for the general quality of the final product and so microbiological and physical-chemical analyses are routinely performed [17]. In general brewing process involves the following steps - malt milling, mashing, filtration, wort boiling, yeast addition, fermentation, maturation, clarification, and packing [20, 22].

The beer market is continuously changing regarding offered products and consumers preference. One of those relevant changes regards the consumption of craft beers [13]. The origin of craft beers relies mainly on local production in micro-breweries and

selling in brew-pubs, which allows smalls producers to commercialize their products [11]. Mass-produced beers differ from craft beers in many respects; the most perceived by consumers are their organoleptic characteristics, due to more limited variation in ingredients and adjuvants. In addition, the production process of craft beers does not require filtration and pasteurization as the industrialized ones do [23, 24]. While the large industries aim to please a greater number of people, producing a simpler beer, microbreweries aim to reach specific and demanding consumers who seek beverages with unique characteristics. That's why these beers further differ in color, amount of malt, barley and other features [11, 25].

Craft beers have a wide variety, but some of the main types are exemplified in the following:

India Pale Ale (IPA) - The production uses water with a high mineral content and high amount of hops. The result is a beer that possess a characteristic bitterness, and an average amount of malt. IPA are categorized into American style, English style, double or imperial IPA. Imperial IPA has higher alcohol content. *Imperial Stout* - is characterized by the dark color due to the roasted malt, it also has a high amount of hops to balance the malt flavor, and should not be too bitter. It also has a high alcohol content and may have fruity adjuvants. *Red Ale* - Reddish or copper beers, barley can be lightly roasted, has low levels of hops, and usually has a fruity flavor and caramel malt. *Wheat Ale* - made using wheat malt, paler in color, generally low in hops, can be made with or without yeast [26, 27].

Pilsener lager – Beers from this type have pale color, high density, high concentration of malt, having a fruity taste characterized by the type of fermentation. *American style lager* - clear golden color, the adjuvants used are generally cereals such as corn and rice. It has a low amount of malt and an average amount of hops [26].

The type of brew implemented in the beer production determines whether it will be of the lager or ale type. Lager types are low fermented beers by yeast *Saccharomyces uvarum*, at temperatures between 7º and 15ºC, for 7 to 10 days. Ale types are high fermented beers, resorting to the *Saccharomyces cerevisiae* yeast, within the temperature of 18º to 22ºC, and maturation time from 3 to 5 days [17].

Fermentation is a noteworthy processing technique of several highly appreciated foods, which simultaneously provides preservation. Dating back to 7000 BC, fermentation by microorganisms such as yeasts has been widely used to produce other food products like bread and wine [28, 29]. It further provides natural preservation by the generation of lactic acid or alcohol. Meanwhile, microorganisms modify food by changing its functional and sensory properties [30]. However, this process does not guarantee immediate safety. Contaminants such as highly toxic mycotoxins can be present in even after undergoing this process [28-31] . Moreover, microorganisms responsible for fermentation can produce

potentially toxic compounds such as ethyl carbamate and large amounts of biogenic amines (BAs), which will be considered in the next section [32].

1.2. Biogenic Amines (BAs)

Biogenic amines (BAs) are organic nitrogenous compounds with low molecular weight formed by amination and transamination of aldehydes and ketones, or after decarboxylation of amino acids through substrate-specific enzymes [33]. The name of each BA derives from the amino acid giving rise to it after decarboxylation [34]. The most common are histamine (HIS), tyramine (TYR), cadaverine (CAD), phenylethylamine (PHE), putrescine (PUT), agmatine (AGM), tryptamine (TRYP), serotonin (SER), spermidine (SPM), and spermine (SPMD) [35]. They are synthesized in several foods, animals or plants, by microorganisms through specific enzyme catalysis during their processing [36]. One of the main factors that cause the formation of BAs in foods is the quality and composition of raw materials, as they can contain both microorganisms expressing decarboxylation ability and their substrates [32]. In addition to pointing out hygiene problems in production and microbiological activity, factors such as freshness and food spoilage can be indicated by the presence of BAs. [37, 38].

In beers, BAs can be formed in the brewing process or along storage [39-41]. The appearance of BAs in the raw materials can be due to flaws in the GMP, in other words, did not comply with HACCP standards [42]. The amount of BAs depends on the amines found in the raw materials, as well those formed during brewing process, like tyramine and tryptamine. Others are naturally found in malt, hops and barley [41]. Eventually, the formation of BAs can be prevented, or reduced, through control of physical-chemical parameters such as pH, humidity and temperature or with the addition of chemical or natural preservatives like salt and sugar [43]. Decarboxylation is also a physiological mechanism of bacteria used to regulate pH. Although the optimum pH is different for each microorganism, pH values between 4.0 to 5.5 generally determines an increase of bacteria's activity [44]. Temperature too, is linked to bacterial activity and is considered an important factor in food quality. Temperatures between 25º and 30ºC promote bacterial growth and hence an enhanced decarboxylation activity leading to BAs production, while the adoption of lower temperatures minimizes its accumulation [45]. BAs are also influenced by the content of free amino acids and additives presents in the matrix, like sugar or sodium sulphide, which can increase or reduce the activity of the microorganisms, affecting directly the production of BAs [46].

Regarding bacteria, the species *Lactobacillus hilgardii, Lactobacillus brevis, Lactobacillus plantarum*, are described as tyramine producers in fermented beverages [47]. *Photobacterium phosphoreum* and *Morganella psychrotolerans* can be BAs producers,

even at temperatures below 5ºC [42]. BAs can also indicative of the presence of fecal contamination of water by enteric bacteria like *Escherichia coli*, *Proteus*, *Klebsiella*, or *Citrobacter* [44].

Biogenic amines are bioactive in the body, therefore they have pathways of metabolism through enzymes : monoamine oxidase (MAO), diamine oxidase (DAO), and histamine-N-metyltransferase (HNMT) the most common enzymes which accept BAs as their substrate and are therefore responsible for its catabolism [48]. Thus, the toxic effects of BAs can be increased by the suppression of such metabolic pathways, usually through consumption of tobacco, ethanol and antidepressants [42]. For instance, high consumption of alcoholic drinks, leads to high amounts of the alcohol metabolite acetaldehyde which competes with histamine respectively for the aldehyde oxidase and aldehyde dehydrogenase (ALDH) enzymes [49]. In turn, tobacco consumption decreases the effectiveness of MAO enzyme function by 60% (several smokers may have complete inhibition of monoamine oxidase) or by the use of MAO inhibitors, like antidepressants [50]. MAO is present in the body in two different isoforms: MAO-A and MAO-B, with its main functions: monoamines metabolization and converting phenylethylamine and tyramine into neurotransmitters. MAO-A is present in almost the entire gastrointestinal tract, while MAO-B is mostly present in brain [50, 51]. Therefore inhibition of BAs metabolic pathways increases toxicological responses, such as hypertensive, digestive, respiratory, vasoactive, and psychoactive effects [52, 53].

Histamine is the best known BA, and the one that causes most food poisoning. High intake of this BA can cause scombrioid syndrome, which presents symptoms such as intense headache, urticaria, diarrhea, vomiting, ventral pain, and may even cause respiratory failure [42, 54]. People with genetic DAO deficiency are more vulnerable to migraine attacks caused by His [34]. As DAO is most responsible for metabolizing histamine in the body, factors that affect its functioning can lead to histamine intolerance, this condition can generate toxic symptoms even with the consumption of low amounts of histamine [42].

Increase in the putrescine and cadaverine fraction in the body enhances histamine toxicity and absorption, in addition to increasing the intracellular content of polyamines (spermine and spermidine). This accumulation has carcinogenic potential and can generate tumors such as colorectal [51, 55, 56]. Moreover, some studies demonstrate the potential of these polyamines, which have putrescine as a precursor [48], to form carcinogenic nitrosamines (N-nitric compounds) after reaction with nitrite salts, a common preservative in smoked foods [57].

From the context stated above, it can be concluded why the determination of BAs is a critical parameter for food quality control and public health risk assessment. The European

Food Safety Authority (EFSA) – published a “Scientific Opinion on risk-based control of biogenic amine formation in fermented foods” which describes the relevance of determining BAs in fermented foods and beverages [42].

Different analytical techniques can be used for this determination, being HPLC the most used [58]. Currently, chromatographic methods are applied in different types of laboratories, being one of the most important separation techniques [59]. Chromatographic methods are often used for BAs separation and quantification [37]. One of the most accurate techniques is HPLC [60]. HPLC using a reversed-phase C18 column is even considered the reference method by the European Union (Regulation (EC) Nº 2073/2005), for histamine determination in seafood and fish [16, 42]. EFSA recognizes HPLC as the only highly sensitive method with the ability to accurately identify all BAs in fermented foods [42].

HPLC can be combined with different detectors like UV-visible, fluorescence (FL), mass spectrometry (MS) and electrochemical [61]. However, the most used detectors have some disadvantages. MS requires expensive equipment operated by experts, whereas UV and FL require derivatization with hazardous chemicals, which consumes larger amounts of time and generates toxic compounds. Alternatively, electroanalytical detection seems to be an efficient option for this analysis. The combination of HPLC with potentiometric detectors provides good ability to characterize in a single run different samples with analytes in very different concentration intervals, but it is not a frequently used methodology [62]. This methodology show advantages in being simple, fast, accurate, inexpensive, and eco-friendly [58]. In the next section a closer view of this methodology is provided.

1.3. Potentiometry

The first potentiometric method appeared in 1889 when Walther Nernst developed the Nernst equation. In his thesis entitled "The electromotive efficiency of ions", he related the cell potential with the concentration of the species in solution. Over the years, potentiometric techniques were applied in several determinations like the endpoint of acid-base or redox titrations, and the so common determination of hydrogen ions, pH, in every laboratory [63].

Potentiometric measurements are carried out through the potential difference between a reference electrode and the indicator electrode, both immersed in the electrolyte sample solution. These measurements are executed under static conditions, without passing a significant current [64, 65]. While the reference electrode keeps its potential constant, the potential of the indicator electrode should change with the amount of analyte in the sample solution. Indicator electrodes can be grouped into metallic electrodes and ion-selective electrodes (ISEs). Metallic electrodes consist of a pure metal wire, for example,

copper, which reacts with the solution and can donate or receive electrons, therefore can directly or indirectly detect anions or cations [66-68]. Whereas ISEs based on a selective membrane respond to a specific ion in the presence of other ions [69].

The selectivity and sensitivity of an ISE can be modulated regarding the main analyte detection. The use of ISE has the advantage of cost, speed, and simplicity, in addition, factors such as turbidity and sample color do not cause interference in the measurements. Generally speaking, ISEs transform the ionic activity of the sample into an electrical potential [68, 70, 71].

Regarding the determination of biogenic amines in foods using potentiometric sensors, different works have been published in the literature, Poels and Nagels (2001) performed the potentiometric detection of different amines (9 industrial alkylamines, choline and the neurotransmitter acetylcholine) besides the BAs putrescine and cadaverine. In order to achieve the intended performance the inclusion calix[6]arene-hexaethylacetate compound was added to the electrode membrane [72]. Amorim et. al (2010) developed a histamine-selective electrode with a different compound, α-cyclodextrin, as the recognition element (ionophore) to evaluate the histamine content in fresh fish [73]. Basozabal et. al.(2014) used a potentiometric transducer containing molecularly imprinted nanoparticles (MIN) for the rapid identification of histamine in fish and wine samples [74]. Minamiki and Kurita (2019) carried out a potentiometric detection to determine the content of histamine and cadaverine in water samples, using a self-assembled monolayer (SAM) of benzoic acid prepared on a gold indicator electrode [75]. Draz et. al. (2020) performed a trial to determine food spoilage in 4 different cheese samples using tyramine as a marker. They resorted to a glassy carbon electrode coated with a membrane made with 4 ion exchangers (tetraphenylborate, ammonium reineckate, sodium phos-photungstate tribasic hydrate and phosphomolybdic acid) [76].

With exception of the Nagels et. al. (2001) [72] proposal, none of remaining sensors allowed the simultaneous determination of different BAs. To overcome this drawback, a new potentiometric sensor based on CB[6] was assembled in a home-made microfluidic flow-cell and coupled to ion-pair chromatography for the determination of 9 BAs in tomato samples performed by Gil et. al. (2021) [77]. The authors also carried out a study for the determination of 10 BAs in alcoholic beverages (beer and wine) [78]. Based on the described methodology by Gil et. al. (2021), the main goal of this work was to evaluate the figures of merit of this methodology when the BA profile of different types of craft beer samples was envisaged and try to relate them with the beer recipe.

2. Objectives

Craft beers are part of a growing market, their consumption has increased over the years, however there is no specific legislation that regulates their quality and safety parameters. BAs are one of the most recurrent contaminants in fermented beverages. The main goal of this work is to identify and quantify biogenic amines by using a miniaturized amine selective electrode coupled to a chromatographic systems.

3. Materials and Methods

3.1. Reagents

Mobile phase: acetic acid (CH_3COOH from Sigma-Aldrich, ref.33209), ultrapure water with conductivity < 0.055 µS, acetonitrile (ACN from Merck, ref. 1.0 0 029250 0P), butane-1-sulfonic acid sodium salt (SBS, ref. 1,183,030,025 from Sigma-Aldrich).

Indicator electrode membrane: cucurbit[6]uril hydrate (ref. 94,544 from Sigma-Aldrich), tetrahydrofuran (THF, ref. 186,562 from Sigma-Aldrich), high molecular weight polyvinyl chloride (PVC, ref. 81,392 from Sigma-Aldrich), potassium tetrakis (p-chlorophenyl) borate (TCPB from Fluka, ref. 60,591), o-nitrophenyloctyl ether (o-NPOE, ref. 365,130 from Sigma-Aldrich), multi-walled carbon nanotubes (MWCNTs, ref.659258-2G from Sigma-Aldrich).

BA: methylamine hydrochloride (Met, ≥98%, ref. M0505), ethylamine hydrochloride (Eth, 98%, ref. 232,831), putrescine dihydrochloride (Put, ≥97%, P5780), cadaverine dihydrochloride (Cad, 95%, D22606), histamine dihydrochloride (His, ≥99%, H7250), spermidine trihydrochloride (Spmd, ≥98%, ref. S2501), spermine tetrahydrochloride (Spm, ≥99%, ref. 85,610), tyramine hydrochloride (Tyr, ≥98%, ref. T2879), 2-phenylethylamine hydrochloride (Phe, ≥98%, ref. P6513) and tryptamine hydrochloride (Tryp, ≥99%, ref. 246,557) were obtained from Sigma-Aldrich.

Sample preparation: ammonium hydroxide solution (NH4OH, ref. 320,145) and methanol (MetOH from Fisher Chemical, ref. 1.06035.2500P).

3.2. Stock solutions of amines

BAs stock solutions were prepared at the concentration of 1.0×10^{-2} mol L^{-1} (methylamine, ethylamine, putrescine, cadaverine, histamine, spermidine, spermine, tyramine, phenylethylamine, and tryptamine) in 1.0×10^{-2} mol L^{-1} of CH_3COOH. They were stored in the fridge at 5ºC. Seven standard solutions were prepared from the stock solutions, which were diluted in concentrations of 0.1, 0.3, 1, 3, 10, 30, and 100 µmol L^{-1}.

3.3. Samples

Five different types of german craft beers were acquired from the local market (Session IPA, Imperial IPA, Imperial Stout, Wheat Pale Ale, and Red Ale). The samples description provided by the brew manufacture is summarized in Table1. First, 50mL of beer samples were degassed in the ultrasound for 20 minutes and filtered through 0.45 µm nylon membranes (Millipore). afterwards, 1.0 mL of each sample was clean-up using Water Oasis PRIME MCX cartridges (3cc, 60mg, ref. 186.0008.918) coupled in a MilliporeSigma™ Supelco™ Visiprep™ SPE Vacuum Manifold, ***(figure 1)*** [79], according to Zhang et. al. (2017) [79] and adapted by Gil et. al. (2021) [78].

To recover the compounds, the elution was performed three times with 1.0 mL of a solution of MetOH: NH_4OH (95:5, v/v). Afterward, the samples were dried in a nitrogen flow and stored at -20ºC. The solid residue was resuspended in 1.0 mL of mobile phase (eluent A) andfiltered through 0.22 µm nylon filters before the injection into the chromatographic system.

Figure 1.*Samples in solid phase extraction*

Table 1. *Description of the samples according to the beer manufacturer.*

Type of beer	Alcohol degree (% vol.)	Amount of sugar in the must (Plato)	Color	Components	Observation

Session IPA	4,9	11.5°	Golden Yellow	Water, barley malt, hop, yeast, and hop extract.	Greater amount of hops
Imperial IPA	7,8	16.8°	Amber	Water, barley malt, hop, yeast, and hop extract.	Greater amount of hops
German Red	7,9	16.5°	Red	Water, barley malt, and hop extract.	Fruity flavor and aroma
Imperial Stout	7,5	16.5°	Black	Water, barley malt, and hop extract.	Roasted malt
Wheat Pale Ale	5,6	12,7°	Red Gold	Water, wheat malt, barley malt, hops, hop extract, and yeast	Wheat malt

3.4. Chromatography

An HPLC system with a Waters 600 solvent delivery pump (Waters Liquid Chromatography) equipped with a Rheodyne 7725i six-port external sample injector (IDEX Health & Science, LLC) was used ***(Figure 2)***. The chromatographic column used was C18 Luna® Omega 5 µm Polar LC column 150 x 4.6 mm (Phenomenex).

The mobile phase was based on the eluent A (5 mmol L^{-1} of SBS in 1.0 x 10^{-2} mol L^{-1} of aqueous CH_3COOH) and eluent B (1.0 x 10^{-3} mol L^{-1} of aqueous CH_3COOH: ACN, 90:10, v/v). The injection volume was 100 µL and the flow rate was 1.2 mL min^{-1}. The separation was performed under controlled room temperature (± 2ºC). The gradient program is well described in ***Table 2***.

Table 2. *HPLC flow gradient.*

Time (minutes)	A%	B%
0 – 5.0	100	0
5.0 – 10.0	0	100
10.0 – 17.0	0	100

17.0 – 17.5	100	0

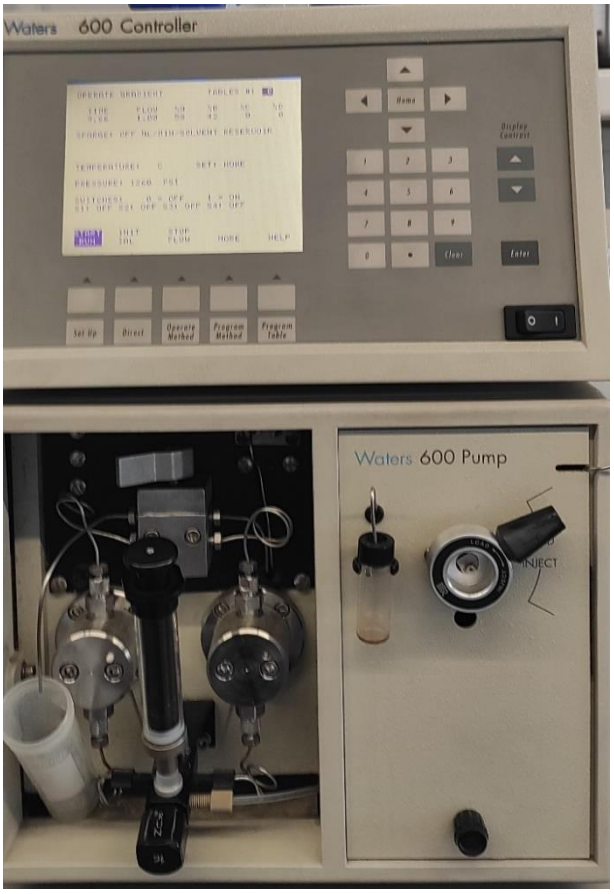

Figure 2.*HPLC system Waters*

3.5. Potentiometric detector

The potentiometric flow-cell consisted of an acrylic cube with four different holes into which the indicator electrode, the commercial reference electrode (Ag/AgCl model 6.0727.000, Metrohm, Switzerland), the inlet, and outlet pipe flow were coupled. The electrodes were connected to 6-Channel Precision Electrochemistry EMF Interface (LawsonLabs, Inc. USA). The flow cell can be observed in ***Figure 3*** (*1*-inflow; *2*-indicator electrode; *3*- reference electrode; *4* - outlet).

The indicator electrode was prepared according to the methodology followed by Gil et. al. (2021) [(78)]. Briefly, a conductive graphite substrate was prepared and inserted. in a polytetrafluoroethylene (PTFE) rod (1.6 mm I.D. x 4.0 mm O.D. x 15 mm length), while a copper wire was inserted from the other ending to establish the electrical connection. The electrodes were left to dry over 24h at room temperature.. After cleaning, the conductive surface was modified with an amine-selective membrane. It was prepared by accurately weighing amounts of CB[6] as an ionophore, TCPB as a lipophilic additive, o-NPOE as a plasticizer, multi-walled carbon nanotubes as electronic conductors, and PVC as a polymer

matrix. 300 mg of the mixture were dissolved in 3.0 mL of THF *(**Table 3**)*. 5 µL of the membrane cocktail was drop-casted onto the electrode surface, four times, with an interval of 20 minutes between each application for solvent evaporation. The miniaturized electrode with the amine-selective membrane is displayed in ***figure 4.***

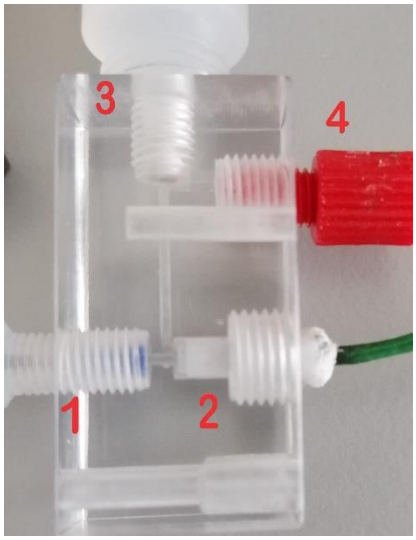

Figure 3. *Potentiometric flow-cell assembled with the indicator and reference electrodes.*

Table 3*. Membrane components of the indicator electrode*

Component	Function	% (w/w)
PVC	Polymer	30.0
THF	Solvent	-
CB6	Ionophore	1.0
TCPB	Lipophylic additive	0.3
o-NPOE	Plasticizer	66.7
MWCNTs	Electronic conductor	2.0

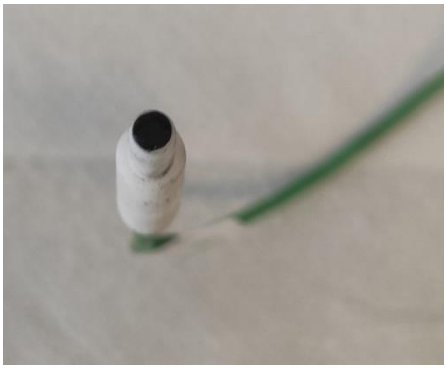

Figure 4.Indicator electrode with membrane

4. Results and Discussion

Several methodologies based on chromatography can be used for the simultaneous analysis of different BA, for quality control purposes. However, the choice of a simple, fast, and reproducible methodology is the main goal. This work aims to explore the application of potentiometry in food analyses, by using ISE coupled to a chromatographic system in the determination of 10 BA in beer.

The HPLC-potentiometric method proposed by Gil [78] was followed and the analytical performance was assessed to verify the working conditions. For that, the linear range and linearity, the limit of detection (LOD), the limit of quantification (LOQ), and the precision were evaluated by the injection of standard solutions covering the concentration range between 0.1 to 100 µmol L^{-1} (*n=8*). Moreover, the chromatographic parameters were also evaluated, such as the retention time (t_r), capacity (k) and selectivity (α) factors, number of theoretical plates (N), peak resolution (R_s), and peak symmetry (As).

4.1. Analytical figures of merit

The analytical figures of merit of the potentiometric detector were assessed by the triplicate injection of standard solutions of BAs covering the concentration range of 0.1 to– 100.0 µmol L^{-1} (*n=8*), which is summarized in ***Table 4***. Firstly, the potentiometric signal (E) in mV (i.e. peak height) was plotted as a function of the logarithmic BA concentration, showing a Nernstian behavior for all amines. The slopes (S) ranged from 10.1 to 38.8 mV dec^{-1} for the monoamines (Met, Eth, Tyr, Phe, and Tryp), 7.6 to 12.2 mV dec^{-1} for diamines (Put, Cad, and His), and 8.9 to 10.1 mV dec^{-1} for polyamines (Spmd and Spm) in the overall linear range between 10.0 to 100.0 µmol L^{-1}. Afterwards, the potentiometric signal (E) was converted in a transformed response $tR = 10^{E/S} - 1$, which was plotted against the BAs concentration [80]. Linear regression lines were obtained for Eth, Put, Cad, His, Spm, Phe, Tryp in the range between 1.0 to 100.0 µmol L^{-1} and for Met, Spmd, and Tyr in the range between 1.0 to 30.0 µmol L^{-1}, with coefficients of determination (R^2) ranging from 0.9890 to 0.9992. As a representative example, typical chromatograms obtained after the injection of a standard mixture of BAs at ascending concentrations are displayed in ***Figure 5***.

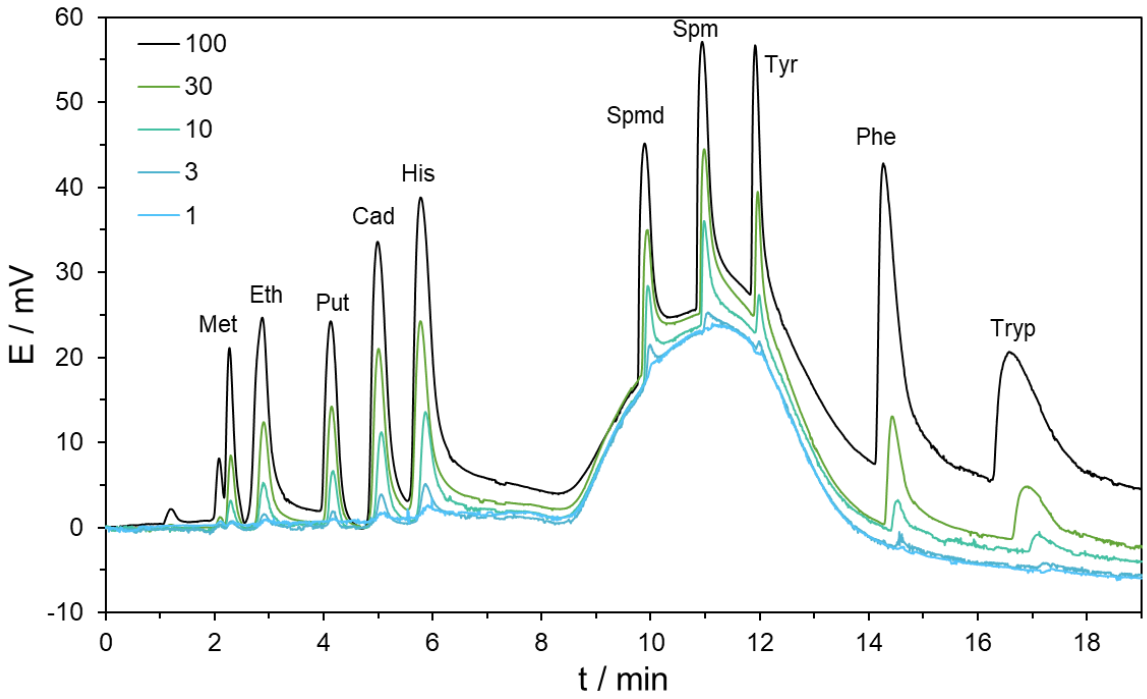

Figure 5. *Chromatogram from BAs standard solutions*

The LOD and LOQ were calculated from signal-to-noise ratios of three and ten, obtaining values of 0.3 and 1.0 µmol L^{-1} for all BAs, respectively (***Table 4***). Attending to the molecular weight of each BAs, the LOD ranged from 9.3 to 60.7 µg L^{-1} and from 31.1 to 202.3 µg L^{-1}, respectively.

Table 4*. Linear range, linearity, linear equation, LOD, and LOQ of the potentiometric detector in HPLC.*

BAs	Linear Range (µmol L^{-1})	Slope	R^2	Intercept	LOD (µg L^{-1})	LOQ (µg L^{-1})
Met	1 – 30	78.9 ± 0.5	0.9915 ± 0.4125	-0.007 ± 0.048	9.3	31.1
Eth	1 – 100	156.6 ± 0.9	0.9898 ± 0.0623	-0.004 ± 0.088	13.5	45.1
Put	1 – 100	179.5 ± 1.4	0.9955 ± 0.5775	0.024 ± 0.135	26.5	88.2
Cad	1 – 100	253.6 ± 2.3	0.9992 ± 1.3114	0.013 ± 0.227	30.7	102.2
His	1 – 100	459.4 ± 10.3	0.9965 ± 1.2810	-0.032 ± 0.227	33.3	111.1
Spm	1 – 100	424.2 ± 27.5	0.9985 ± 2.3502	0.077 ± 0.708	43.6	145.3
Spmd	1 – 30	3454.0 ± 172.3	0.9931 ± 2.5189	-0.777 ± 1.709	60.7	202.3

Tyr	1 – 30	110.1 ± 1.3	0.9890 ± 0.5457	-0.021 ± 0.275	41.2	137.2
Phe	1 – 100	129.3 ± 0.4	0.9947 ± 0.0761	-0.016 ± 0.044	36.4	121.2
Tryp	1 – 100	132.5 ± 11.6	0.9907 ± 0.9840	-0.014 ± 0.067	48.1	160.2

The precision of the potentiometric detector was evaluated by the repeatability (i.e. intra-day) and intermediate precision (i.e. inter-day) of the potentiometric signal of each BA. The intra-day was assessed after triplicate injection of a standard mixture of ten BAs at five different concentrations (1, 3, 10, 30, and 100 µmol L^{-1}) and the relative standard deviation (RSD%) was calculated. The values are summarized in ***Table 5*** ranged from 1.5 to 10.7, 0.1 to 1.0, 0.2 to 11.6, 2.6 to 12.3, and 2.7 to 14.8, respectively, which indicates a great precision of the method by the acceptable RSD% obtained for each amine [81]. The inter-day precision was evaluated by the analysis of the potentiometric detector on two independent days, in which the amine-selective electrode was used for the first time after membrane deposition and conditioning. In this case, a triplicate injection of a standard mixture of ten BAs at three concentrations (1, 10, and 100 µmol L^{-1}) was performed, showing RSD% values between 4.0 to 14.4, 4.6 to 14.6 and 3.1 to 14.1, respectively. Moreover, the sensor performance was also evaluated on two consecutive days in a similar basis to assess its lifetime, in which the electrodes remained assembled in the flow-cell overnight. Despite the good repeatability obtained in both day, the RSD% values obtained from the two days ranged 44.0 to 105.0, 3.4 to 99.0, and 50.8 to 69.8 for 1, 10, and 100 µmol L^{-1}, respectively. These findings indicate that the lifetime of the membrane is short due to its constant contact with a flowing stream rich in organic solvents. However, this is not considered an issue because the sensor can be easily prepared as described elsewhere.

Table 5. *Precision of the potentiometric detector in HPLC, expressed as RSD%.*

BAs	**Intra-day (RSD%)**					**Inter-day (RSD%)**		
	Concentration µmol L^{-1}							
	1	3	10	30	100	1	10	100
Met	6.4	0.1	9.3	10.3	14.8	6.3	14.6	14.1
Eth	10.7	0.9	4.6	9.7	11.1	6.1	6.9	10.6
Put	5.0	0.1	4.8	9.4	9.4	13.0	14.0	3.1
Cad	7.0	0.4	4.8	5.5	12.4	14.4	13.3	3.4
His	8.7	1.0	3.6	3.7	9.5	4.0	8.7	6.2
Spm	5.2	0.7	0.2	2.6	9.5	8.7	12.7	12.8

Spmd	3.7	0.1	2.4	9.1	2.7	9.5	6.5	14.7
Tyr	3.5	0.2	9.7	12.3	6.9	9.3	4.6	11.5
Phe	1.5	0.2	11.6	6.8	9.9	12.5	12.6	14.6
Tryp	1.5	0.9	8.2	8.5	6.5	8.0	5.2	10.4

4.2. Chromatographic parameters

The chromatographic retention parameters obtained after the separation, namely the retention time (t_r), peak symmetry (As), number of theoretical plates (N), peak resolution (Rs), retention capacity factor (k), and selectivity (α) are listed for each BA in ***Table 6***.

Table 6. *Chromatographic retention parameters*

	t_r	k	α	N	Rs	As
Met	2.5	1.1	1.5	872.6	1.7	1.2
Eth	3.1	1.6	2.0	1207.2	5.1	1.6
Put	5.1	3.2	1.3	2287.5	2.0	1.6
Cad	6.1	4.1	1.2	1606.3	1.5	1.4
His	7.3	5.0	1.8	1164.8	7.4	1.4
Spmd	12.0	9.0	1.1	11860.3	2.6	1.3
Spm	13.2	10.0	1.1	15351.2	2.4	1.5
Tyr	14.0	10.7	1.2	48190.6	6.2	1.0
Phe	16.0	12.4	1.1	24714.6	3.3	1.3
Tryp	17.9	13.9	not applicable	10040.3	not applicable	1.5

t_R – retention time (min); As – peak symmetry; N – number of theoretical plates; k – capacity factor; α – selectivity factor; Rs – peak resolution.

4.3. Real samples analysis

The analytical performance of the potentiometric detector and the chromatographic parameters showed to be similar to those reported by Gil et. al. (2021) [(78)] being further applied to the analysis of real samples. However, the assessment of the accuracy was not evaluated in this work, though it was already demonstrated by Gil et. al. (2021) [(78)] in different alcoholic beverages, particularly industrial beer samples. Herein, five independent beer samples from the same producer (Imperial IPA, Imperial Stoud, Red Ale, Session IPA, and Wheat Pale Ale) were processed to obtain three replicates of each sample, each one analysed in triplicate. The results are summarized in ***Table 7***.

The relationship between the total amount of BAs founded and the type of craft beer sample is displayed in ***Figure 6***. Higher content of total BAs was found in the Session IPA

(11.6 ± 0.5 mg L^{-1}), which can be ascribed to the high amounts of hops and yeast in its composition. On the other hand, the Imperial IPA (6.5 ± 1.5 mg L^{-1}), the Wheat Pale Ale (5.6 ± 0.5 mg L^{-1}), and the Red Ale (3.9 ± 0.2 mg L^{-1}) beers showed a similar amount of BAs while Imperial Stout beer (2.7 ± 0.2 mg L^{-1}) presented the lowest amount of total BAs. The roasting process of the malt in Imperial Stout beer can decrease the enzymatic activity of the enzymes responsible for the BA production. Nonetheless, the levels found in the analysed beers samples do not entail any risk to the health of consumers, as long as risk factors, such as amine oxidase-inhibiting drugs and alcohol are prevented.

Table 7.*Content of BAs in the samples*

Beer type	BAs identify	Total (mg L^{-1})	RSD (%)
Imperial IPA	Eth, Put, His, Spmd, Spm, Tyr and Tryp	6.5 ± 1.5	14.0
Imperial Stout	Eth, Put, Cad, His, Spmd, Spm, Tyr and Tryp	2.7 ± 0.2	10.3
Red Ale	Eth, Put, His, Spmd, Spm, Tyr and Tryp	3.9 ± 0.2	5.9
Session IPA	Eth, Put, Cad, Spmd, Spm, and Tryp	11.6 ± 0.5	4.8
Wheat Pale Ale	Eth, His, Spmd, Spm, Tyr	5.6 ± 0.5	9.5

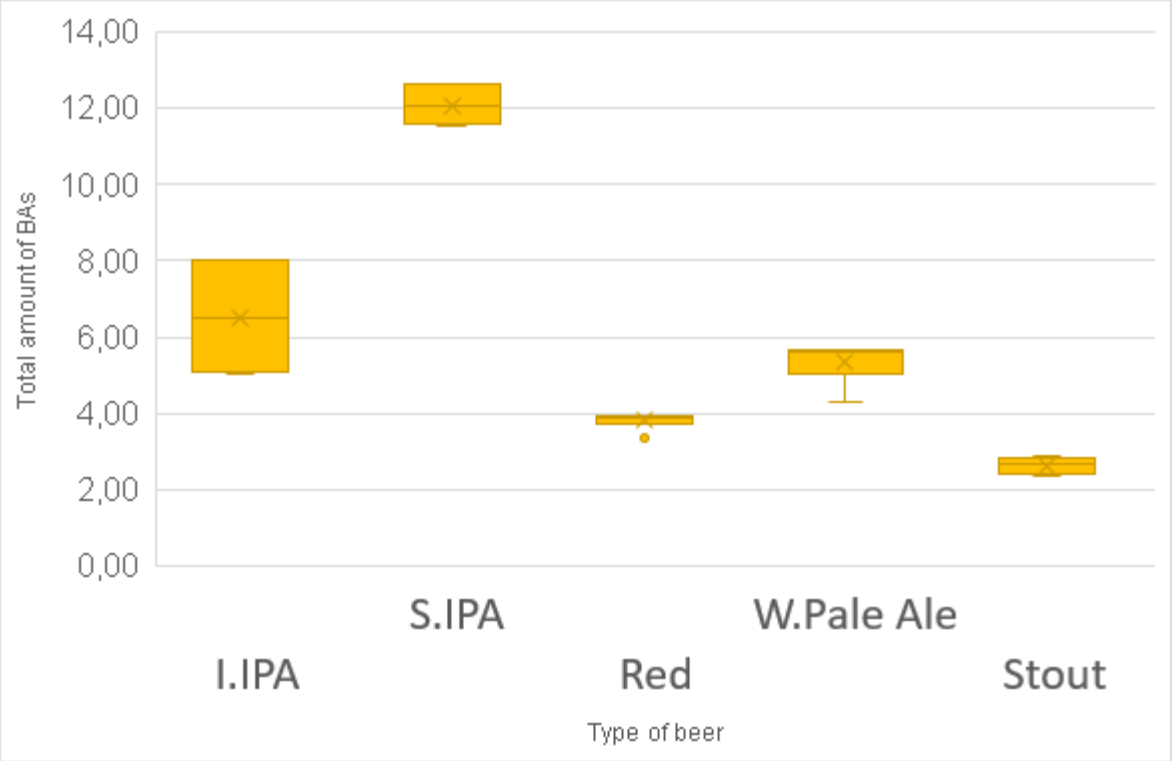

Figure 6*. Boxplot chart of the relationship total amount of BAs (mg/L) vs. the type of beer.*

The individual levels of the BAs founded in the beer samples are summarized in ***Table 8***. All samples showed amounts of tyramine, spermine and spermidine, which may be attributed to their presence in hops and barley malt as already reported by Izquierdo-Pulido (1994), who founded that BA in hops [82].

Imperial IPA and Red Ale present a higher concentration of histamine when compared with the other beer samples. Even being the most toxicological BAs, it content in these beers are still lower than the regulated values [83]. Nonetheless, histamine can interact with other amines and increase their toxic effects [42, 82, 84, 85]. The formation of histamine may occur during the malting process [82]. On the other hand, the study carried out by Izquierdo-Pulido (1996) revealed that the presence of histamine may be indicative of contamination by lactic acid bacteria (LAB) [86]. Almeida, Fernandes & Cunha (2012) also reported the presence of histamine in beer samples as an indicative parameter of the presence of decarboxylative microorganisms [87]. Kalac&Krizek (1997) points out that the amount of histamine and tyramine increases with storage time [88].

Regarding other BAs concentrations, Session IPA showed a higher amount of tryptamine (6.00 mg L^{-1}), also present in most of the samples, though at much lower levels. This finding is similar to those reported by Tang et. al. (2009), in which 11 of the 18 analysed samples of Chinese beers contains tryptamine [89]. The widespread presence of tryptamine may be attributed to the fermentation process, as reported by Ramon-Marquez (2015) [90].

Cadaverine was only found in Imperial Stout and Session IPA, which may be indicative of contamination. According to Lucas et. al. (2005) the presence of *enterobacteriaceae* may lead to the production of cadaverine and putrescine [91]. However, Kalac&Krizek (2003) reported that these BAs may be formed during fermentation [84]. Furthermore, EFSA points out that there is a lack of information about cadaverine and putrescine, which hinders the determination of the critical toxic concentration limits of these BAs. [42].

Ethylamine was founded in all analyzes samples, though its presence in beers does not seem to be directly related to any raw material. Palamand et. al. (2018) points out that excessive amounts ethylamine can change the taste of beer [92] but more studies are required to better understand the origin and its importance in foodstuff. In the case of methylamine and phenylethylamine, they were not detected at significant values in any of the samples. Gasarasi et. al. (2003) reports the appearance of phenylethilamine in spontaneously fermented beers by its presence in barley malt [93].

Table 8*.Individual levels of BAs founded in the craft-beer samples.*

Type of beer	Biogenic Amines (mg L^{-1})									
	Met	**Eth**	**Put**	**Cad**	**His**	**Spmd**	**Spm**	**Tyr**	**Phe**	**Tryp**
Imperial IPA	N.D.	0.339	0.352	N.D.	0.287	1.507	0.310	2.497	N.D.	1.174
Imperial Stout	N.D.	0.102	0.134	0.079	0.036	0.174	0.110	1.718	N.D.	0.430
Red Ale	N.D.	0.288	0.631	N.D.	0.250	0.019	0.274	1.729	N.D.	1.100
Session IPA	N.D.	0.44	0.463	0.310	N.D.	3.377	0.430	1.493	N.D.	6.000
Wheat Pale Ale	N.D.	0.383	N.D.	N.D.	0.086	1.778	0.150	2.977	N.D.	N.D.

N.D. Not detected.

5. Conclusions

The increased consumption of craft beers has been noticed in Europe and other continents. This type differs from mass-produced beers by its composition and manufacturing process. For this reason, this work aims to analyze de BA content of five different craft-beers. Its content was related to the raw material and the manufacturing process. At least in the analyzed samples it was possible to identify different quantities of eight different BAs, showing their relevance in fermented beverages. The HPLC-potentiometric method showed a good sensitivity and robustness for the analysis of beer samples, competing with the UV-Vis and fluorimetric detectors, that resorts to the derivatization as an additional sample preparation step, to improve the detection limit. Moreover, potentiometric detection makes possible a fast, eco-friendly, safe and simple analysis.

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