Food Science and Technology

Origins, Biological Importance and Human Health Implications

Biogenic Amines (BA)

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BIOGENIC AMINES (BA)

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NOTICE TO THE READER

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CONTENTS

PREFACE

Biogenic amines are nitrogenous organic bases of low molecular weight with biological functions in animals, plants, microorganisms and humans. Their formation in food is either the result of endogenous amino acids decarboxylase activity in raw food material or the breakdown of free amino acids due to the action of decarboxylase enzymes of microbial origin. Consumption of food containing excessive amounts of these amines can have toxicological implications due to their psychoactive and/or vasoactive properties. Therefore, the control of biogenic amines accumulation in foods during processing and storage is a challenge for the food industry. This book reviews origins, biological importance and human health implications of biogenic amines.

Chapter One focus on the occurrence of BAs in different foodstuffs and in the correspondent challenges of their analysis. In Chapter Two the Authors provide an overview and a critical discussion of sample preparation techniques to determine BAs in biological specimens, with special emphasis on microextraction techniques. The advantages and some limitations, as well as how they compare to previously used extraction techniques, are also addressed herein. Chapter Three focuses on the relation of occurrence of dietary biogenic amines and associated adverse health effects following consumption of implicated food. In Chapter Four the Authors focus on some food products and the relationship between the

presence of BA according to their origin, inducing factors and distinctive characteristics of the technological processes that could control BA production in animal foods, plant foods and beverages. Chapter Five tackles the most important aspects of BA formation during manufacture of alcoholic beverages. The significance of BAs is also described from the perspective of microbiological and technological factors. The Authors of Chapter Six critically review the latest knowledge, described in studies carried out throughout the world, about the occurrence and the food safety issues of BAs in two selected animal origin food sources, that is, fish and dairy products. Moreover, different technological strategies adopted for the prevention of BA accumulation in the above mentioned food products, for safety purposes, are evaluated. Chapter Seven briefly summarizes current knowledge on biogenic amines content in dry-cured and fermented meats and collects data on the factors affecting their formation. The methods for aminogenesis control in dry-cured meats are also described. The aim of Chapter Eight is to identify the origin, biological importance, and human health implications of the serotonin (5-HydroxyTryptamine [5-HT]) in pain. Finally, effects of tyramine (TA) and octopamine (OA) on behaviors and physiology in social insects have been introduced in Chapter Nine.

Chapter 1 - Biogenic amines (BAs) are naturally occurring nitrogenous organic compounds of low molecular weight organic bases with aliphatic, aromatic or heterocyclic structures. They are generally formed through microbiological activity during food processing and storage, and can be found in a wide variety of foods, particularly fermented foods and beverages as cheese, wine, beer, in addition to fishery products and meat. At low concentrations, BAs participate in the regulation of several physiological functions, but when present at high concentrations, they may cause several health problems in consumers, as headaches, hypo- or hypertension, nausea and cardiac palpitations, especially to sensitive persons. Therefore, the control of BAs levels in different food products is an important issue for food safety monitoring policies. Due to their low levels in complex food matrices, the analysis of BAs is not an easy task and several methods for their separation, identification, and determination have been described during the last decades. Overall, the chromatographic

approach is the most popular, although the recent trends points to the development of sensors able to measure BAs in food matrices without involving the laborious and complex laboratorial sample analysis methodologies. In this review we will essentially focus on the occurrence of BAs in different foodstuffs and in the correspondent challenges of their analysis.

Chapter 2 - Biogenic amines (BAs) and their metabolites are important neurotransmitters that are released by adrenal glands and sympathetic nervous system. The simultaneous determination of BAs in body fluids is, nowadays, widely used in clinical diagnosis and monitoring to provide valuable information regarding physiological status and metabolic disorders. These neurotransmitters are low molecular weight endogenous polar chemical substances, hence the development of bioanalytical methods for their quantitation is critical. However, the sensitivity and selectivity of these methods are limited by matrix interferences, low abundance and potential instability of the BAs. An appropriate sample preparation step, such as purification, derivatization (if necessary) and extraction is usually required before instrument analysis in order to clean-up interferences and concentrate the analytes. The most common sample clean-up techniques applied in the analysis of BAs are protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE). These are quite efficient but are known for being time consuming as well as the great amounts of biological sample and organic solvents required. Following the mentioned drawbacks, several authors have dedicated their research to miniaturized techniques, and these are gradually replacing the traditional approaches, although assuring similar efficiency. Several miniaturized techniques have been reported and successfully applied to determine BAs in biological samples, among them, micro-solid-phase extraction (μSPE), cloud point extraction (CPE), microextraction in packed syringe (MEPS), solid phase microextraction (SPME), molecularly imprinted polymers (MIP), and, more recently, single-drop microextraction (SDME), micro-total analysis system (μ-TAS), and dispersive liquid-liquid microextraction (DLLME). These have been described as great alternatives, due to their simple, rapid and environmentally friendly

procedure and relative ease of online coupling to chromatographic systems allowing process automation. This chapter will provide an overview and a critical discussion of sample preparation techniques to determine BAs in biological specimens, with special emphasis on microextraction techniques. The advantages and some limitations, as well as how they compare to previously used extraction techniques, will also be addressed herein.

Chapter 3 - Biogenic amines occur naturally at low concentrations in food and raw materials used for food production. They have important physiological functions in humans, animals, plants and microorganisms. Elevated dietary concentrations of biogenic amines are the result of bacterial enzymatic activities. In combination with proteolytic activities particularly in protein rich raw materials and foods, very high concentrations can be formed. Notably, recurrent food poisoning cases are most often reported for fish and products thereof with high histamine concentrations for many years. Besides acute toxic effects, mutagenic potential of biogenic amines by formation of N- and C-nitroso compounds such as nitrosamines has been described. Biogenic amines are temperature stable during food processing and measures to degrade already formed amines in food are of limited effectiveness. Technological measures to prevent a formation and accumulation of biogenic amines in food including good hygiene and manufacturing practices are well known. This comprises the whole food chain until consumption. The primary production stage is important for optimal raw materials quality including for example appropriate fish harvest practices and strict continuous temperature control. This mini review focuses on the relation of occurrence of dietary biogenic amines and associated adverse health effects following consumption of implicated food.

Chapter 4 - Biogenic amines (BA) or biologically active amines are low molecular weight nitrogenous compounds formed from amino acids by decarboxylation, or by amination and transamination of aldehydes and ketones. Due to their precursor amino acids structure and origin, are classified as either aliphatic diamines, aromatic biogenic amines or natural polyamines. Biogenic amines in foods have relevance from both safety and

quality standpoints. They may be found in a wide range of foods containing proteins or free amino acids including meat, dairy and fish products, vegetables, fruits, wine, beer, nuts and chocolate. The biogenic amines most commonly found in foods are cadaverine, putrescine, tyramine, histamine, tryptamine, β-phenylethylamine, agmatine, spermine, and spermidine, the two latter being endogenous amines. Additionally, octopamine and dopamine have been reported in meat and fish products. In non-fermented foods the presence of cadaverine, putrescine, and tyramine, are undesired and can be used as indication for microbial spoilage. On the other hand, in fermented foods, several lactic acid microorganisms involved in the fermentation process, particularly enterococci could produce biogenic amines. Although BA could have an active role at the neurological level as neurotransmitters, their presence in food, particularly of histamine and tyramine are recognized as potential hazards causing intoxication and intolerance symptoms in healthy people. Furthermore, BA are potential precursors for the formation of carcinogenic nitrosamines. This review will focus on some food products and the relationship between the presence of BA according to their origin, inducing factors and distinctive characteristics of the technological processes that could control BA production in: I) animal foods, such as meat and meat products, including dry-fermented sausages, fish and seafood products, and dairy products, such as cheeses; II) plant foods, like vegetables, with emphasis on table olives; III) beverages, such as wines, beers and liqueurs.

Chapter 5 - Biogenic amines (BAs) found in foods have a negative impact on human health and food quality. Foods such as fish, fish products, and fermented foodstuffs, as well as alcoholic beverages (for example, wine, cider, and beer) contain high levels of BAs. The presence of BAs in alcoholic beverages has traditionally been treated as an indicator of undesired microbial activity. Relatively high levels of BAs have also been reported to indicate deterioration of beverages and/or their defective manufacture. Their toxicity has led to the general agreement that they should not be allowed to accumulate in alcoholic beverages. Biogenic amines can be produced by both Gram-positive and Gram-negative bacteria and fungi (yeast and molds). In alcoholic drinks such as wine,

cider and beer, BA formation not only requires the presence of precursors and microorganisms responsible for decarboxylation, but is also influenced by several factors such as pH, the levels of ethanol and sulfur anhydride (SO_2) as well as raw material quality, and fermentation and technological conditions. Today it is well known that Lactic Acid Bacteria (LAB) such as *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Oenococcus* species are the main microorganisms implicated in BA production in alcoholic beverages. The synthesis of BAs by LAB depends on the activity of the enzymes amino acid decarboxylases. For this reason, the genes encoding those enzymes, i.e., histamine decarboxylase (*hdc*), tyramine decarboxylase (*tdc*), and ornithine decarboxylase (*odc*), have become the main targets of PCR methods for detection of bacteria involved in BA production. In alcoholic beverages, more than 20 amines have been identified, and their total concentration has been reported to range from a few mg/l to about 130 mg/l (Ordóñez et al., 2016). The most common BAs in alcoholic drinks are histamine, tyramine, putrescine, cadaverine, and β-phenylethylamine, which are products of decarboxylation of histidine, tyrosine, ornithine, lysine, and β-phenylalanine, respectively. Generally, in alcoholic beverages, the toxic dose is considered to be between 8 and 20 mg/l for histamine, 25 and 40 mg/l for tyramine, whereas as little as 3 mg/l of β-phenylethylamine can cause negative physiological effects (Soufleros et al., 1998). The most common symptoms of excessive BA intake are headaches, heart palpitations, vomiting, and allergic-like adverse responses. This chapter tackles the most important aspects of BA formation during manufacture of alcoholic beverages. The significance of BAs is also described from the perspective of microbiological and technological factors.

Chapter 6 - Biogenic amines (BA) are natural amines that belong to trace compound substances, which show physiological significance in low doses, while they can have deleterious effects on human health in high concentrations. In addition, these substances can also be considered as a freshness index for fish and several other foods. Different bacteria, yeasts and molds produce BAs, through the decarboxylation of the corresponding amino acids, as a stress response mechanism to low pH conditions. The BA

contents in different types of food usually increase due to microbial spoilage during spontaneous or controlled microbial fermentation, and this increase could create several food safety problems. After a brief introduction, this chapter will critically review the latest knowledge, described in studies carried out throughout the world, about the occurrence and the food safety issues of BAs in two selected animal origin food sources, that is, fish and dairy products. Moreover, different technological strategies adopted for the prevention of BA accumulation in the above mentioned food products, for safety purposes, will be evaluated.

Chapter 7 - Dry-cured and fermented meats, constitute one of the food products in which considerable amounts of biogenic amines can be found as a consequence of the use of poor quality raw materials, contamination and inappropriate conditions during processing and storage. Additionally, the microorganisms responsible for the fermentation process may contribute to biogenic amines accumulation. Consumption of foods containing large amounts of these amines can have toxicological consequences due to their psychoactive and vasoactive properties. Therefore, the control of biogenic amines accumulation in dry-cured and fermented meats is a challenge for the meat industry. Undesired accumulation of biogenic amines in meat products requires the availability of precursors (i.e., free amino acids), the presence of microorganisms with amino acid decarboxylases and favorable conditions that allow bacterial growth, decarboxylase synthesis and activity. The capability to form biogenic amines is generally considered to be strain specific rather than species dependent. Dry-cured and fermented meats represent a significant source of proteins. The protein breakdown products, peptides and amino acids, represent precursors for amine formation. The most prevalent biogenic amines in dry-cured and fermented meats are histamine, tyramine, cadaverine, putrescine, products of the decarboxylation of histidine, tyrosine, lysine and ornithine, respectively. The use of selected starter culture without amino decarboxylase activity is a fundamental technological measure to control aminogenesis during dry-cured and fermented meat processing. The inability of the culture to form biogenic amines but also its ability to grow well at the temperature intended for

processing of the product and competitiveness in suppressing the growth of wild amine producing microflora should be taken into consideration in the selection of starter cultures. Secondary control measures to prevent biogenic amines formation in dry-cured and fermented meat products or to reduce their levels also need to be employed. Such approaches may include irradiation, controlled atmosphere packaging, or hydrostatic pressures. This review briefly summarizes current knowledge on biogenic amines content in dry-cured and fermented meats and collects data on the factors affecting their formation. The methods for aminogenesis control in dry-cured meats are also described.

Chapter 8 - The aim of this chapter is to identify the origin, biological importance, and human health implications of the serotonin (5-HydroxyTryptamine [5-HT]) in pain. 5-HT is a monoamine that is widely distributed both in the periphery and in the Central Nervous System (CNS). Serotonin neurons at the CNS level are confined to the brainstem and are located in the raphe nuclei. Serotonin 5-HT is synthesized from the amino acid L-tryptophan (from the diet) by sequential hydroxylation and decarboxylation. It is stored in presynaptic vesicles and released from nerve terminals during neuronal firing. 5-HT is involved in numerous physiological and behavioral disorders, such as major depression, anxiety, schizophrenia, mania, autism, and obesity, and it recently has been proposed as an essential component in the modulation of pain. However, this neurotransmitter is related with a number of physiological processes, such as cardiovascular function, gastric motility, kidney function, etc. Based on pharmacological, structural, and transductional characteristics, the 5-HT receptor family is divided into seven subfamilies ($5\text{-}HT_1$–$5\text{-}HT_7$), comprising 15 receptor subtypes, each of these corresponding to distinct genes. Descending 5-HT pathways exert an inhibitory (descending inhibition) or facilitatory (descending facilitation) influence on the spinal processing of nociceptive information, depending on acute or chronic pain states and the type of receptor acted upon. The exact nature of the receptors involved in 5-HT modulation of pain in the CNS remains to be elucidated. However, studies have revealed the presence of at least three families of 5-HT receptors in the spinal cord ($5\text{-}HT_1$, $5\text{-}HT_2$, and $5\text{-}HT_3$), with varying

affinity for 5-HT, and recently, the 5-HT_7 receptor, which is also excitatory, has been postulated as being linked with, among other things, circadian rhythms, thermoregulation, and migraine. Several lines of evidence have implicated a role for this serotonin in the human health implications of pain; however, it is important to bear in mind the understanding of how pain is modulated by serotonergic receptors in the CNS in terms of the following: the distribution of the different serotonergic receptors in the raphe-spinal pathway; the dose of agonists or antagonists to the 5-HT receptors, the administration route of agonists or antagonists to the 5-HT receptors, and the type and duration of pain.

Chapter 9 - Neuronal effects of biogenic amines including dopamine and serotonin are conserved in vertebrates and invertebrates. Tyramine (TA) and octopamine (OA) are trace monoamines in vertebrates but can be major functional monoamines in invertebrates and act on neurons in the central and peripheral nervous systems and other cells in the peripheral tissues. In insects, TA is synthesized from a common precursor tyrosine and metabolized into OA. Receptors of TA and OA have been characterized independently in several species. A TA receptor (TYR1-R) decreases intracellular second messenger (cyclic adenosine monophosphate: cAMP) levels, whereas OA receptors increase the cAMP levels. Thus, these amines cause antagonistic responses in the signaling of target cells. Although it remains to be determined whether the antagonistic responses of cell signaling between TA and OA can lead to antagonistic behavioral responses or not, evidence suggesting antagonistic behavioral responses mediated by TA and OA have been reported in social insects. In flight behaviors, TA inhibits flight activities and flight motivation, whereas OA accelerates it. In honeybee males, the TA levels in the brain decrease as they age, but the OA levels conversely increase and OA application can promote mating flight activities and its motivation. A similar trend in these levels is seen in honey bee workers: higher levels of TA in younger individuals inhibit flight for foraging, whereas higher levels of OA in older individuals promote foraging. In phototactic responses in honey bees, TA enhances light responsiveness, whereas OA reduces the responsiveness. Other effects of TA or OA on aggression, cooperation and reproduction

have been reported in honey bees, bumble bees, ants, and termites. Some of these are non-antagonistic effects by TA or OA. Thus, evidence for the antagonistic relationships between TA and OA are commonly observed in social insects and gradually accumulate, but particular effects by TA or OA are also present, which might contribute to orchestral effects with several biogenic amines on certain behaviors. Effects of TA and OA on behaviors and physiology in social insects have been introduced in this chapter.

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Chapter 1

BIOGENIC AMINES IN FOOD: OCCURRENCE AND ANALYTICAL CHALLENGES FOR THEIR ANALYSIS

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ABSTRACT

Biogenic amines (BAs) are naturally occurring nitrogenous organic compounds of low molecular weight organic bases with aliphatic, aromatic or heterocyclic structures. They are generally formed through

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microbiological activity during food processing and storage, and can be found in a wide variety of foods, particularly fermented foods and beverages as cheese, wine, beer, in addition to fishery products and meat. At low concentrations, BAs participate in the regulation of several physiological functions, but when present at high concentrations, they may cause several health problems in consumers, as headaches, hypo- or hypertension, nausea and cardiac palpitations, especially to sensitive persons. Therefore, the control of BAs levels in different food products is an important issue for food safety monitoring policies. Due to their low levels in complex food matrices, the analysis of BAs is not an easy task and several methods for their separation, identification, and determination have been described during the last decades. Overall, the chromatographic approaches are the most popular, although the recent trends points to the development of sensors able to measure BAs in food matrices without involving the laborious and complex laboratorial sample analysis methodologies. In this review we will essentially focus on the occurrence of BAs in different foodstuffs and in the correspondent challenges of their analysis.

Keywords: biogenic amines, foodstuffs, derivatization, extraction, analysis, liquid chromatography

ABBREVIATIONS

AGM -	agmatine
AQC -	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
BAs -	biogenic amines
BCl -	benzoyl chloride
CNBF -	4-chloro-3,5-dinitrobenzotrifluoride
CAD -	cadaverine
CE -	capillary electrophoresis
cITP-CZE -	capillary isotachophoresis coupled to capillary zone electrophoresis
DbsCl -	dabsyl chloride
DnsCl -	dansyl chloride
DMA -	dimethylmethylamine
DMETA -	dimethylethylamine

ETA - ethylamine
ETAN - ethanolamine
ECF - ethylchloroformate
FLD - fluorescence detection
FMOC - fluorenylmethoxycarbonyl chloride
GC - gas chromatography
HEX - 1,6-hexamethylenediamine
HIS - histamine
HPLC - high-performance liquid chromatography
ISO - isopentylamine
ITP - isotachophoretic analysis
LLE - liquid-liquid extraction
MA - methylamine
MASC - 10-methyl-acridone-2-sulfonyl chloride
MECC - micellar electrokinetic capillary chromatography
META - methylethylamine
NQS - 1,2-naphthoquinone-4-sulfonate
OCP - octopamine
OPA - ortho-phthalaldehyde
PHE - phenylethylamine
PUT - putrescine
PVPP - poly(vinylpolypyrrolidone)
SALLE - salting-out assisted liquid-liquid extraction
SER - serotonin
SERA - seramine
SLE - solid-liquid extraction
SPE - solid-phase extraction
SPD - spermidine
SPM - spermine
TRYP - tryptamine
TYR - tyramine

1. BIOGENIC AMINES (BAS)

1.1. Definition

Biogenic amines (BAs) constitute a group of basic nitrogen compounds that are important to cell's metabolism and viability. These compounds can be naturally synthetized by certain plants, although their major source is the amino acids decarboxylation mediated by different microorganisms.

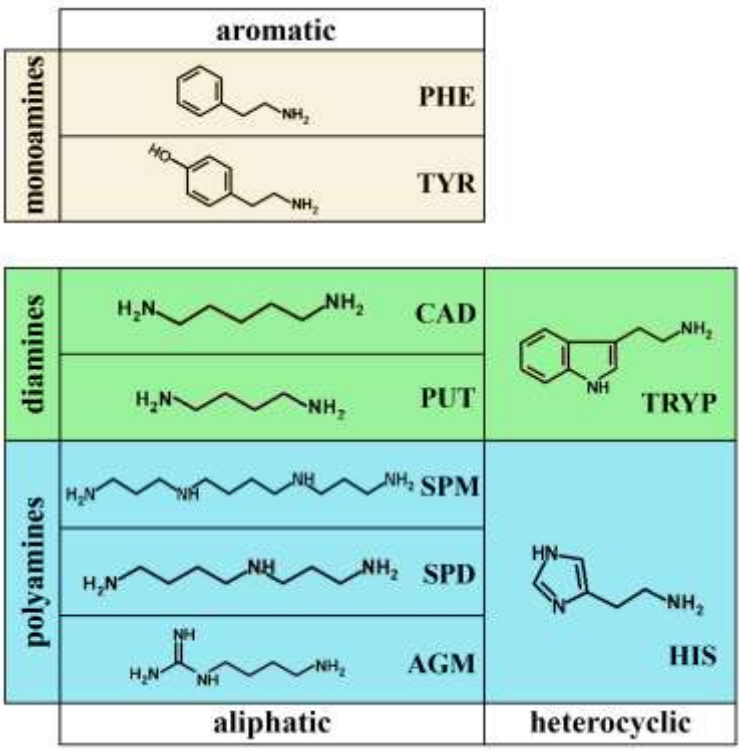

Figure 1. BAs classification according to their structure and number of amine groups. Legend: AGM - agmatine, CAD - cadaverine, HIS - histamine, PHE - phenylethylamine, PUT - putrescine, SPD - spermidine, SPM - spermine, TRYP - tryptamine, TYR - tyramine.

BAs are usually classified according to their chemical structure, as aliphatic (as putrescine - PUT, cadaverine - CAD, agmatine - AGM, spermine - SPM and spermidine - SPD), aromatic (as tyramine - TYR and phenylethylamine - PHE), or heterocyclic (as histamine - HIS and tryptamine - TRYP), or also considering the number of amines, as monoamines (as PHE and TYR), diamines (as CAD and PUT), or polyamines (as SPD and SPM) (Reviewed in (Plonka, 2015) and (Mohammed et al., 2016)) (Figure 1).

1.2. Occurrence in Food Matrices

BAs are formed and degraded during the normal cell metabolism of certain animals, plants and microorganisms. In vegetables, for instance, BAs participate in the fruit development, response to stress and secondary metabolites synthesis. However, BAs can be also generated exogenously under certain circumstances, namely the presence of free precursors amino acids, microorganisms with appropriate catabolic pathways and a suitable environment to the decarboxylation activity (Kantaria & Gokani, 2011). These conditions can be met during food processing, particularly of matrices of animal origin, as fish and meat and their derivatives, which are naturally rich in free amino acids and so susceptible to BAs contamination. Moreover, soon after cells death, a high number of proteolytic enzymes become active and BAs levels can increase several orders of magnitude, creating important food safety concerns (Cardozo et al., 2013). Therefore, the levels of BAs in food matrices are inversely correlated with the freshness and quality of those products, being BAs almost absent or residual in fresh foods. There are, however, some foods in which BAs are naturally present in significant levels, as fish, cheese, meat, eggs and fermented foods (Cardozo et al., 2013). Overall, the BAs most often found in foodstuffs are HIS, TYR, PHE, TRYP, PUT and CAD (Cardozo et al., 2013). Table 1 present indicative ranges of BAs usually found in different foodstuffs.

To prevent BAs contamination, food industry usually employs methods to inhibit microbial growth and reduce decarboxylase activity, as refrigeration (low temperature) or pasteurization (fast treatment with high temperatures to kill the species responsible for BAs formation). However, some bacteria produce BAs even at temperatures lower than 5°C and so additional measures to temperature control are emerging, as the application of hydrostatic pressures, irradiation, packaging, use of additives and preservatives, as well as the use of organisms that do not produce BAs, in the case of fermented products (Naila et al., 2010).

Table 1. BAs presence in different foodstuffs

Food type	BAs identified	Range	Reference
Fish	HIS	10^{-6}-10^{-2} M	Basozabal et al., 2014
Cheese	HIS, TYR, PUT, TRYP, CAD, PHE, SPD, SPM, ETA	0.5-5 mM 0.002-8 mg/L	Redruello et al., 2013 Esatbeyoglu et al., 2016
Meat	METH, AGM, TRYP, PHE, PUT, CAD, HIS, TYR, SPD, SPM	50-250 μM 0.01-15 mg/L	Yang et al., 2014 Sirocchi et al., 2014
Eggs	PUT, CAD, PHE, SPD, SPM, HIS, TYR	0.7-22.4 mg/kg	de Figueiredo et al., 2015
Fermented foods	TRYP, PUT, CAD, HIS, PHE, TYR, SPD, SPM	0.02-10 3.5-200 (μg/ml)	Jia et al., 2011 Gong et al., 2014
Wine	HIS, META, ETA, TYR, DMETA, PHE, ISO, SPM, PUT, CAD, SPD, AGM, TRYP, SERA, ETAN	0.1-100 0.03-1.7 0.01-7.20 (mg/L)	Wang et al., 2014 Ramos et al., 2014 Tuberoso et al., 2015

1.3. Biological Effects

BAs are essential endogenous components that have important metabolic roles in living cells. HIS, TYR and serotonin (SER), for instance, are involved in nervous system functions and control of blood pressure, while the polyamines PUT, CAD, SPM, and SPD are important in the synthesis of proteins, RNA and DNA and are involved in cell signaling, growth and proliferation. Mammals have a relatively efficient detoxification system capable of metabolizing the normal daily intake of

BAs, so their consumption in low concentrations in the normal diet is not dangerous (Özdestan & Üren, 2009; Naila et al., 2010). However, when ingested in excess, BAs have several toxic effects, that can range from reactions of intolerance, to intoxication or even poisoning. The severity of clinical symptoms depends on the amount and variety of BAs ingested, individual susceptibility and the level of detoxification activity in the gut (Ladero et al., 2010). The lighter symptoms include nausea, sweating, rashes, slight variations in blood pressure and mild headache. If symptoms become more severe, including diarrhea, nausea, facial flushing, red rash, respiratory distress, heart palpitation, oral burning, hypo- or hypertension and migraine, then some intoxication is already affecting the organism (Ladero et al., 2010; Yoon et al., 2015). In exceptional cases of BAs poisoning, severe effects will occur, causing damage to different organs, including heart and the central nervous system (Ladero et al., 2010). The BAs that cause most concerns are HIS, TYR and PUT, being able of causing food poisoning (Esatbeyoglu et al., 2016). HIS and TYR poisoning have similar effects in the organism, causing an allergen-type reaction characterized by difficulty in breathing, itching, rash, vomiting, fever, and headaches (Naila et al., 2010), while PUT itself is not toxic, but potentiates the toxicity of HIS and TYR by affecting their clearance from the body (Ladero et al., 2010). Scombroid food poisoning is eventually the most often reported type of BAs intoxication. It is mainly caused by excessive intake of HIS from spoilage fish, particularly those from the *Scombridae* and *Scomberesocidae* families, as tuna, bonito and mackerel.

2. Methodologies Used in Quantification of Food Biogenic Amines

2.1. Sample Preparation

The analysis of BAs in food matrices requires a suitable experimental layout which generically involves a sample pretreatment, then the

extraction and derivatization steps, whose order is often reversed, and finally the quantification of the BAs present in the sample (Figure 2).

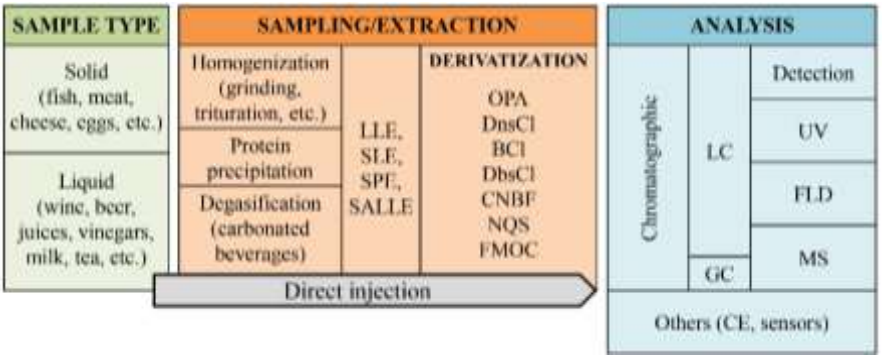

Figure 2. Overview of the generic experimental layout involved in BAs analysis in different food matrices. Legend: AQC - 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, BCl - benzoyl chloride, CE - capillary electrophoresis, CNBF - 4-chloro-3,5-dinitrobenzotrifluoride, DbsCl - dabsyl chloride, DnsCl - dansyl chloride, FLD - fluorescence detection, FMOC - fluorenylmethoxycarbonyl chloride, GC - gas chromatography, LC - liquid chromatography, LLE - liquid-liquid extraction, NQS - 1,2-naphthoquinone-4-sulfonate, OPA - ortho-phthalaldehyde, SALLE - salting-out assisted liquid-liquid extraction, SLE - solid-liquid extraction, SPE - solid-phase extraction.

This procedure, however, is highly dependent on the nature and complexity of the food samples. In solid matrices, as fish and meat, samples need to be triturated (and eventually added a solvent) to obtain homogeneous moistures (Sirocchi et al., 2014; Pinto et al., 2016). Then, BAs extraction can proceed using different approaches, usually liquid-liquid extraction (LLE), solid-liquid extraction (SLE), solid-phase extraction (SPE) and salting-out assisted liquid-liquid extraction (SALLE), or alternatively a simple precipitation and centrifugation step, as illustrated in Figure 2. For liquid matrices, the major challenges to overcome are the degasification of the carbonated beverages, as beers, which is usually performed in a ultrasonic bath (Jastrzębska et al., 2014) and the interference of phenolic compounds in red wines. In this case, wine samples are often filtrated with certain resins (as poly (vinylpolypyrrolidone - PVPP), that retain these compounds (Daniel et al.,

2015). In other cases, the wines samples are simply diluted and analyzed directly without any further sample treatment (Ramos et al., 2014). In Table 2 are presented selected examples of methodologies used to quantify BAs in different food samples.

2.2. Chromatographic Approaches

2.2.1. Derivatization Procedures

From an analytical point of view, a fast, simple and reliable methodology for simultaneous determination of BAs in food matrices is highly desirable (Brückner et al., 2012; Ramos et al., 2014). This is crucial for the identification and quantification of these molecules in different samples and particularly the control of their presence in human diet. Different methodologies have been proposed to analyze BAs, being high-performance liquid chromatography (HPLC) (Romero et al., 2000; Innocente et al., 2007; Ramos et al., 2014) and gas chromatography (GC) (Cunha et al., 2011) the most often used. The major challenge in BAs analysis is their detection since most of them lack a chromophore group and for that reason do not show a satisfactory fluorescence or absorption in UV-visible range. To overcome this, a common strategy is the derivatization of the amino groups with different reagents to increase the sensitivity and specificity of the detection (Jia et al., 2011; Ramos et al., 2014; Preti et al., 2015). Additionally, this modification also alters BAs chromatographic properties (e.g., reduce the polarity for reversed phase separations) (Innocente et al., 2007; Hernández-Cassou & Saurina, 2011). Regardless the nature of the agent used in the derivatization, it must be ensured that the factors affecting the reaction are carefully controlled (e.g., pH, temperature, etc.), the derivatization agent is available in excess in order to complete the reaction in a wide range of concentrations for the selected BAs and the chromophore molecule formed is stable, at least during the time of analysis (Romero et al., 2000; Romero et al., 2001).

Table 2. Different methodologies used in BAs extraction from foodstuffs

Matrix	Sample treatment	Extraction	Derivatization	Analytical methodology	BAs analyzed	LOD	Reference
Fish	Grinding	SLE	DnsCl	LC-UV	CAD, HIS, PHE, PUT, TRYP	0.14-0.50 μg mL^{-1}	Pinto et al., 2016
		LLE	BCl	MECC	CAD, HIS, PHE, PUT, SPD, SPM, TRYP	-	Su et al., 2000
		SLE (US)	-	Screen-printed carbon electrodes sensor	PUT, CAD, TRYP, SPD, SPM, HIS, TYR	0.18-0.40 (μM)	Alonso-Lomillo et al., 2010
Capelin fish meal	TCA	SLE	OPA	LC-FLD	TYR, HIST, PHE, PUT, CAD, TRYP, AGM, SPD, SPM	-	Ruiz-Capillas et al., 2015
Meat	TCA	SPE	-	LC/MS	AGM, CAD, HIS, PUT, PHE, SER, SPD, SPM, TRYP, TYR.	0.03-0.1 mg L^{-1}	Sirocchi et al., 2014
	TCA	SLE	OPA/DnsCl	LC-UV	HIST, TYR, TRYP, PUT, PHE, CAD, SPD, SPM	0.0012-0.0046 ng	Smela et al., 2003
Meat and Cheese	MeOH	SLE	ECF	GC/MS, optical sensor	HIS, PUT, SPD, TYR.	0.165 (μg mL^{-1})	Khairy et al., 2016
Cheese and anchovy	-	SLE (US)	-	Amperometric biosensor	PUT, CAD, HIS	0.33-50 (μM)	Carelli et al., 2007
Cheese	Grinding	SLE	DnsCl	LC-UV	CAD, HIS, PHE, PUT, SPD, SPM, TRYP, TYR.	-	Torracca et al., 2015

Matrix	Sample treatment	Extraction	Derivatization	Analytical methodology	BAs analyzed	LOD	Reference
Eggs	Yolk and albumin separation	SLE	DnsCl	LC-UV	PHE, CAD, HIS, PUT, SPM, SPD, TYR	0.2-0.3 mg kg^{-1}	de Figueiredo et al., 2015
Wine	-	SALLE	DnsCl	LC-FLD	CAD, DMA, EA, HIS, ISO, MA, PHE, PUT, SPD, SPM,	0.005-0.028 (mg L^{-1})	Ramos et al., 2014
	Degasifi-cation	LLE	CNBF	LC-UV	DIEA, HIS, PHE, TRYP, TYR.	0.02-0.03 (mg L^{-1})	Piasta et al., 2014
	-	LLE	BCl	LC-UV	AGM, CAD, HIS, MA, PHE, PUT, SPD, SPM, TRYP, TYR.	0.2-2.5 (mg L^{-1})	Özdestan & Üren, 2009
	-	-	OPA	LC-FLD	HIST, MH, MET, EA, TYR, TRYP, PHE, PUT, CAD	0.03-0.12 (mg L^{-1})	Soleas et al., 1999
	-	dilution	-	cITP-CZE-UV	HIS, TYR, PHE	0.33-0.35 (mg L^{-1})	Ginterová et al., 2012
	-	dilution	NQS	CE	AGM, PHE, CAD, ETA, HIS, TYR, PUT, SER, TRYP.	0.02-0.91 (mg L^{-1})	García-Villar et al., 2006
Rice wine	-	SLE	MASC	LC/MS	CAD, HEX, HIS, PHE, PUT, TRYP, TYR.	0.15-0.23 (μM)	Cai et al., 2016
Wine and juice fruits	-	LLE	DnsCl	LC-UV/FLD	AGM, PHE, CAD, EA, HIS, MA, PUT, SER, SPD, SPM, TYR.	0.002-0.023 (mg L^{-1})	Preti et al., 2015

Table 2. (Continued)

Matrix	Sample treatment	Extraction	Derivatization	Analytical methodology	BAs analyzed	LOD	Reference
Wines and cider	Degasifi-cation	-	OPA	LC-FLD	AGM, CAD, DOP, HIST, OCT, PHE, PUT, SPM, SPD, TYR, TRYP	0.03-0.06 (mg L^{-1})	Vidal-Carou et al., 2003
Wine and fish	-	-	SLE	Solid-phase imprinted nanoparticles sensor	HIS	$1.12x10^{-6}$ (M)	Basozabal et al., 2014
Wine and beer	Degasifi-cation	-	DnsCl	LC-UV/ITP	CAD, HIS, PHE, PUT, SPD, SPM, TRYP, TYR.	0.2-1.4 (mg L^{-1})	Jastrzębska et al., 2014
	PVPP filtration	-	-	CE-MS/MS	HIS, TYR, PUT, CAD, SPM, SPD, TRY, PHE, URO	1-2 (μg L^{-1})	Daniel et al., 2015
Vinegars	-	SPE	AQC	LC-FLD	AGM, CAD, HIS, MA, PHE, PUT, SPD, SPM, TYR.	7-26 (μg mL^{-1})	Ordóñez et al., 2013
Cocoa products	-	SLE SPE	DnsCl	LC-UV	CAD, HIS, PHE, PUT, SER, SPM, SPD, TYR.	0.02-0.04 (μg mL^{-1})	Restuccia et al., 2015

Matrix	Sample treatment	Extraction	Derivatization	Analytical methodology	BAs analyzed	LOD	Reference
Tea	Infusion	SLE	FMOC	LC-FLD	CAD, HIS, OCP, PHE, PUT, SPD, SPM, TYR	-	Brückner et al., 2012
Donkey Milk	Protein precipitat.	LLE	DnsCl	LC/MS	CAD, HIS, PHE, PUT, SPD, SPM, TRYP, TYR.	0.5-15 (μg L^{-1})	La Torre et al., 2010

Legend: AGM - agmatine, AQC - 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, BCl - benzoyl chloride, CAD - cadaverine, CNBF - 4-chloro-3,5-dinitrobenzotrifluoride, DbsCl - dabsyl chloride, DMA - dimethylmethylamine, DMETH - dimethylethylamine, ETA - ethylamine, ECF - ethylchloroformate, FLD - fluorescence detection, FMOC - fluorenylmethoxycarbonyl chloride, HEX - 1,6-hexamethylenediamine, HIS - histamine, ISO - isopentylamine, ITP - isotachophoretic analysis, LLE - liquid-liquid extraction, MA - methylamine, MASC - 10-methyl-acridone-2-sulfonyl chloride, METH - methylethylamine, NQS - 1,2-naphthoquinone-4-sulfonate, OCP - octopamine, OPA - ortho-phthalaldehyde, PHE - phenylethylamine, PUT - putrescine, PVPP - poly(vinylpolypyrrolidone), SALLE - salting-out assisted liquid-liquid extraction, SER - serotonin, SLE - solid-liquid extraction, SPD - spermidine, SPE - solid-phase extraction, SPM - spermine, TCA - trichloroacetic acid extraction, TRYP - tryptamine, TYR - tyramine, UV - ultraviolet detection.

In the literature, several derivatization reagents have been successfully used in BAs analysis in different food matrices, as shown in Table 2. Nowadays, dansyl chloride (DnsCl) is possibly the most popular derivatization reagent for BAs. DnsCl is able to react with primary and secondary amino groups and even with tertiary amines, in extreme conditions, providing very stable dansylamines and dansylamides derivatives (Innocente et al., 2007; Hernández-Cassou & Saurina, 2011). The identification and quantification of these derivatives in different food matrices was the goal of many studies in the last years. However, the dansylation may require high temperatures (more than 40°C) and be a time-consuming process (taking more than 20 min just for derivatization reaction) (Erim, 2013). In recent years, different modifications have been proposed to the original protocol of dansylation to improve this methodology (Innocente et al., 2007; Jiang et al., 2011; Erim, 2013). Jiang et al. (2011), for instance, reported the use of ionic liquids as a media for the derivatization of BAs in wine samples. This modification allowed to complete the derivatization reaction at room temperature in 20 min, considerably improving the cost and time of analysis of BAs in wines (Jiang et al., 2011). Despite of the popularity of DnsCl derivatization, many parameters that affect the efficiency of this reaction, as pH, temperature or concentration of the derivatization reagent, were never been properly studied and there are many opportunities for further optimization that can still can be addressed. The derivatization of BAs using O-phthalaldehyde (OPA) is also very popular. The greatest advantage of using this reagent is the speed of the reaction (Hanczkó & Molnár-Perl, 2003; Notou et al., 2014). In fact, according Hanczkó and Molnár-Perl (2003) for some selected BAs, OPA derivatization can be a very fast reaction, being complete between 1.5 and 7 min. However, the OPA reagents are self-fluorescent and self-UV absorbent, and for this reason blank measurements are always necessary (Hanczkó & Molnár-Perl, 2003). Moreover, OPA only reacts with primary amines and compounds that have the CH_2-NH_2 moiety in their initial structure, result in more than one OPA derivative that has to be considered to obtain reliable data (Hanczkó &

Molnár-Perl, 2003). Beyond, OPA and DnsCl, several other derivatization reagents have been used in BAs analysis, as indicated in Table 2.

2.3. Other Methodologies

Different forms of capillary electrophoresis (CE) have been reported in the literature for the determination of BAs, particularly in wines and beers (García-Villar et al., 2006; Ginterová et al., 2012; Daniel et al., 2015). Overall these approaches, exemplified in Table 2, can be a good alternative to chromatographic analysis, being fast and reliable. Ginterová et al. (2012), for instance, reported a methodology using capillary isotachophoresis coupled to capillary zone electrophoresis (cITP-CZE), not requiring derivatization, to screen BAs in red wines that allowed a good analytical performance in the analysis of HIS, TYR and PHE (0.35, 0.37 and 0.33 mg L-1). In recent years, alternative methodologies using different sensors architectures and chemistries have been proposed for BAs detection and quantification. These solutions are mostly targeted to detect specific BAs using custom designed sensors. Their use in analytical chemistry is particularly interesting as sensors have the potential to allow faster and real-time analysis using disposable lab-on-chips systems that do not require specialized instrumentation or complex laboratory facilities or procedures. However, these approaches are still in their infancy and their application to BAs analysis need further developments before being considered a real alternative to conventional methodologies. Nevertheless, it is noteworthy to refer the work from Alonso-Lomillo et al. (2010), where the authors covalently immobilized monoamine and diamine oxidases coupled with horseradish peroxidase into screen-printed carbon electrodes to quantify different BAs. Using these disposable biosensors, it was possible to measure seven different BAs in fish samples with LODs in the 0.18-0.40 µM range (Table 2). In a similar approach, Carelli et al. (2007) developed an amperometric biosensor also using the diamino oxidase, this time entrapped onto an electrosynthesized bilayer film, to quantify PUT, CAD and HIS. The sensitive biosensor could reach low detection limits

(0.33-050 µM) and was successfully applied to cheese and anchovy samples. In another report, Basozabal et al. (2014), reported a promising potentiometric sensor based on molecularly imprinted nanoparticles for HIS quantification. This approach has the great advantage of allowing fast, specific and label-free quantification of HIS, while achieving excellent analytical performance, with a LOD of 1.12 µM of HIS in wine and fish samples.

3. FINAL REMARKS

Human population exponential growth is already requiring major efforts to obtain more food using natural resources that are not available in the same proportion. This will create enormous challenges in the control of the quality and safety of food chain supplies worldwide. In this aspect, the control of BAs levels in foodstuffs will require more expedite methodologies than the currently used, which often involve laborious and cumbersome protocols. Furthermore, the control of BAs presence in food continues to rely essentially in HIS to which are defined maximum admissible levels. However, other BAs have been already shown to interfere with human metabolism and so more extensive studies are necessary to define safety levels for those compounds. This will be crucial to include the quantification of more BAs in the assessment of food safety and consequently contribute to improve its quality and protect human health.

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Chapter 2

THE ROLE OF MINIATURIZED TECHNIQUES IN THE DETERMINATION OF BIOGENIC AMINES IN BIOLOGICAL SPECIMENS

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ABSTRACT

Biogenic amines (BAs) and their metabolites are important neurotransmitters that are released by adrenal glands and sympathetic nervous system. The simultaneous determination of BAs in body fluids is, nowadays, widely used in clinical diagnosis and monitoring to provide valuable information regarding physiological status and metabolic disorders.

These neurotransmitters are low molecular weight endogenous polar chemical substances, hence the development of bioanalytical methods for their quantitation is critical. However, the sensitivity and selectivity of these methods are limited by matrix interferences, low abundance and potential instability of the BAs.

An appropriate sample preparation step, such as purification, derivatization (if necessary) and extraction is usually required before instrument analysis in order to clean-up interferences and concentrate the analytes.

The most common sample clean-up techniques applied in the analysis of BAs are protein precipitation (PPT), liquid–liquid extraction (LLE), and solid-phase extraction (SPE). These are quite efficient but are known for being time consuming as well as the great amounts of biological sample and organic solvents required.

Following the mentioned drawbacks, several authors have dedicated their research to miniaturized techniques, and these are gradually replacing the traditional approaches, although assuring similar efficiency. Several miniaturized techniques have been reported and successfully applied to determine BAs in biological samples, among them, micro-solid-phase extraction (μSPE), cloud point extraction (CPE), microextraction in packed syringe (MEPS), solid phase microextraction (SPME), molecularly imprinted polymers (MIP), and, more recently, single-drop microextraction (SDME), micro-total analysis system (μ-TAS), and dispersive liquid-liquid microextraction (DLLME). These have been described as great alternatives, due to their simple, rapid and environmentally friendly procedure and relative ease of online coupling to chromatographic systems allowing process automation.

This chapter will provide an overview and a critical discussion of sample preparation techniques to determine BAs in biological specimens, with special emphasis on microextraction techniques. The advantages and some limitations, as well as how they compare to previously used extraction techniques, will also be addressed herein.

Keywords: biogenic amines, microextraction techniques, biological specimens

1. INTRODUCTION

In the present days, both analytical and separation methods can solve practically all kinds of complex analytes and matrices. Analytical methods include usually several processes such as sampling (collection of the samples), sample preparation (separation from the matrix, concentration, fractionation and, if necessary, derivatization), separation, detection and data analysis (Smith, 2003; Mitra, 2004; Vas & Vekey, 2004).

Surveys show that sample preparation can account for more than two thirds of the effort of the typical analytical chemist and the time spent in analysis (Kataoka, 2003; Smith, 2003; Vas & Vekey, 2004). This is necessary because in most cases analytical instruments cannot handle directly sample matrices, and some reports state that the entire analytical process can be useless when an unsuitable sample preparation method has been employed before analysis (Vas & Vekey 2004).

Biological fluids such as blood, plasma and urine are much more complex than many other matrices due to the presence of proteins, salts and various organic and inorganic compounds with similar chemistry to the analytes of interest (Altun and Abdel-Rehim 2008). These samples can also be problematic due to the irreversible adsorption of proteins in the analytical stationary phase, resulting in a substantial loss of column efficiency and an increase in back-pressure (Nováková & Vlčková 2009).

The main concept involved in sample preparation is converting a real matrix sample into a format that is suitable for subsequent analysis by means of an analytical technique, usually involving some kind of separation. This can be achieved by employing a wide range of techniques, many of which have changed little over the last 100 years, but maintaining a common list of aims:

- To remove potential interferences from the sample (for either the separation or detection stages), thereby increasing the method's selectivity;
- To increase the concentration of the analyte and hence the sensitivity of the assay;

- To convert the analyte into a more suitable form for separation and/or detection (if needed);
- To provide a robust and reproducible method that is independent of variations in the sample matrix (Smith, 2003).

As a result, the following features are important in carrying out an efficient sample preparation:

- Minimal sample loss and a good yield of the recovered analyte;
- Coexisting components removed efficiently;
- Problems do not occur in the chromatography system;
- The procedure can be carried out conveniently and quickly;
- Low cost per analysis (Kataoka, 2003).

The measurement of biogenic amines (BAs) including catecholamines (CAs) in biological samples remains a current analytical challenge, in spite of the great diversity of methodologies that have been developed throughout the years (Saracino et al., 2015).

The literature on methods of separation of biogenic amines in body fluids is extensive, and the specimens usually used for are blood and urine. Occasionally, biogenic amines have been isolated from the cerebrospinal fluid (Płonka, 2015).

Besides that, it is often necessary to develop procedures for sample preparation of different body fluids for the comprehensive analysis of BAs, as the concentrations of BAs depend on body fluid analyzed. For instance, the amount of dopamine in the urine of a healthy person is up to 10 times greater than the amount of norepinephrine, while the total plasma catecholamine content is 10 times higher than the content of norepinephrine. (Płonka, 2015).

The effect of the initial preparation of biological material on the content of selected drugs depends on the type of sample and the chemical nature of the analytes. Depending on the pKa of the compound, the sample may be acidified or buffer may be added to bring the pH to a value that

ensures analyte stability. This allows the stability of the analytes to be maintained, even for a period of 3 days (Płonka, 2015).

Conventional sample clean-up techniques, such as protein precipitation (PPT), liquid-liquid extraction (LLE), and solid-phase extraction (SPE), developed for analysis of BAs, tend to be time consuming and require relatively high amounts of sample and organic solvents, which may be unreasonable in routine laboratory practice since simplicity, miniaturization of the procedures and low costs are desired (Konieczna et al., 2016b).

Recently, a variety of new microextraction techniques, including solid phase microextraction (SPME) and microextraction by packed sorbent (MEPS) are gradually replacing the traditional approaches, providing simple, rapid, and environmentally friendly methods for the quantification of endogenous compounds in different kinds of human samples (Konieczna et al., 2016b).

2. CLASSIC SAMPLE PREPARATION TECHNIQUES

The study of sample preparation may be traced back to the very beginning of analytical chemistry when complex samples were first analysed. Following the rapid development of analytical techniques in the post-World War II era, increasing demands were placed on sample quality because the samples collected from natural environment, living bodies and many other sources were of very complex nature, and their subsequent analysis was undoubtedly difficult or even impossible without any pretreatment (Chen et al., 2008).

The oldest and most basic sample preparation method is extraction, in which the analyst aims at isolating the analyte of interest from a sample matrix using a solvent, with an optimum yield and selectivity, so that as few potential interfering species as possible are carried through to the analytical separation stage (Smith, 2003). This process almost inevitably changes the interactions of compounds with their concrete chemical environment. These interactions are determined by the physical and

chemical properties of both analytes and matrices, and they affect the applicability of different sample-preparation techniques and analytical methods (Pavlović et al., 2007). Most of these classic sample preparation techniques involved liquid partition or adsorptive extraction. Liquid partitioning means the transfer and distribution of soluble analytes in a liquid-containing the phase system (Chen et al., 2008). The solvents may be organic liquids, supercritical fluids and superheated liquids or the extraction liquid may be bonded to a support material (Smith, 2003). In adsorptive extraction methods the analytes are trapped onto immobilized phases, and then can be either eluted by an appropriate solvent or thermally desorbed (Chen et al., 2008). Selectivity can be improved by modifying the extraction temperature and pressure, by choosing the extraction solvent, by using pH modifications or additives, such as ion-pair reagents (Smith, 2003). Conventional techniques, such as protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are still widely accepted and used for routine determination of BAs in several biological specimens, hence of great importance to review the chosen conditions for their application, as well as pitfalls associated with these procedures.

2.1. Protein Precipitation (PPT)

Proteins which are present in a large amount in biological specimens can be irreversibly adsorbed onto the chromatographic support, which might cause deterioration of separation efficiency and a rapid column clogging (Souverain et al., 2004).

They also can interfere with the analytical determination. In order to determine analytes present in biological samples, it is often necessary to disrupt the protein–drug binding so that the total amount of the analyte can be extracted for the analysis. Precipitation is useful as it can be used to denature the protein, destroying its drug binding ability depending on the binding mechanism (Polson et al., 2003).

Protein precipitation (PPT) procedures are commonly used for fast sample clean-up, and considered easy to handle, with possible application to a wide range of analytes. (Polson et al., 2003; Souverain et al., 2004).

PPT techniques can be used as sample cleanup procedures as well as a pre-treatment, coupled to other extraction techniques since the PPT extracts may not be clean enough and still capable of generating matrix effects. After PPT, the isolation of proteins is generally performed by centrifugation or filtration. Then, a small amount of the supernatant can be directly injected with or without pH adjustment or concentrated by solvent evaporation (Souverain et al., 2004).

The different protein precipitation techniques (organic solvent, acid, salt and metal ion) have different mechanisms of action (Polson et al., 2003), since protein solubility results from polar interactions with the aqueous solvent, ionic interactions with salts and repulsive electrostatic forces between like charged molecules (Polson et al., 2003).

The modes of protein precipitation can be summarized as below:

- An organic solvent such as methanol, ethanol, acetonitrile or acetone leads to a decrease of the solution dielectric constant, which increases the attraction between charged molecules and facilitates electrostatic protein interactions, inducing protein precipitation (Souverain et al., 2004; Polson et al., 2003);
- Organic solvents also displace the ordered water molecules around the hydrophobic regions on the protein surface. Hydrophobic interactions between proteins are minimized as a result of the surrounding organic solvent, while electrostatic interactions become predominant and lead to protein aggregation (Polson et al., 2003);
- At a pH value lower than the protein isoelectric point, acidic reagents interact with the positively charged amine group of proteins to form an insoluble salt (Souverain et al., 2004);
- The addition of a salt such as ammonium sulphate induces, at high concentration, a salting-out effect and proteins precipitate from solution (Souverain et al., 2004). The salt ions become hydrated

and the available water molecules decrease, drawing the water away from the protein hydrophobic surface regions which in turn results in aggregation of protein molecules via protein–protein hydrophobic interactions (Polson et al., 2003).

However, this fast and simple method removes proteins with limited efficiency, and still results in fast column deterioration. Precipitation with organic solvents makes it difficult to match the solvent strength with mobile phase, and the use of strong acids is incompatible with pH labile analytes. Subsequent removal of precipitated proteins by high-speed centrifugation or filtration is difficult to automate. In addition, precipitation gives relatively high background of impurities in the chromatogram. The interfering peaks present a problem for trace analysis (Gilar et al., 2001). Nevertheless, this procedure was and still is referenced in many publications, and applied routinely in many laboratories for BAs determination. Table 1 reviews its application for the purpose associated with several analytical equipments. Acetonitrile and perchloric acid seem to be the most frequent precipitation agents, although methanol and trichloroacetic acid (TCA) are also mentioned. The limits of detection (LOD) and quantification (LOQ), as well as the recoveries achieved with the cited methods are also reported when possible.

2.2. Liquid-Liquid Extraction (LLE)

Liquid–liquid extraction (LLE) has been recognized for many years as a powerful technique in the chemistry laboratory and often been a favored choice from process engineers for the development of separation processes (Hanson, 2013; Huddleston et al., 1998).

The choice of the extraction solvent, its polarity, and different distribution coefficient, Kd, for different analytes offers better selectivity in sample preparation than simple protein precipitation, particularly for non-polar analytes (Gilar et al., 2001).

In LLE both phases are liquids, usually one of aqueous nature and the other organic. The phases must be immiscible, and the extraction can be accomplished if the analyte has favorable solubility in the organic solvent (Mitra, 2004).

The analytes will distribute between aqueous and organic layers according to their relative solubility in each solvent and according to the Nernst distribution law, where Kd is equal to the analyte ratio in each phase at equilibrium (Mitra, 2004).

Multiple extractions may be required to recover sufficient amount of the analyte from the aqueous phase. Neutral compounds can have substantial values of Kd. However, organic compounds that form hydrogen bonds with water are partially soluble in water, or are ionogenic (weak acid or bases), and may have lower distribution coefficients and/or pH-dependent distribution coefficients. Additionally, the sample matrix itself (i.e., blood, plasma or urine) may contain impurities that shift the value of the distribution coefficient relative to that observed in purified water (Mitra, 2004).

The principle of repeated extractions demonstrates that:

- The amount of analyte extracted depends on the value of the distribution coefficient;
- The amount of analyte extracted depends on the ratio of the volumes of the two phases used;
- More analyte is extracted with multiple portions of extracting solvent than with a single portion of an equivalent volume of the extracting phase;
- Recovery is independent of the concentration of the analyte in the original aqueous sample (Mitra, 2004).

Salting out of the mixture and in-vial derivatization of the analytes can also be used to improve the extraction efficiency (Ramos, 2012).

However, the employment of an organic solvent and an aqueous solution as the two immiscible phases and the increasing emphasis on the adoption of clean manufacturing processes and environmentally benign

technologies may make such processes seem increasingly anachronistic because of their high usage of toxic, flammable, volatile organic compounds (VOCs). The costs of solvents are high and their safe engineering attracts significant capital costs over and above simple containment. Disposal of spent solvents and diluents will also have increasing costs through the impact of environmental protection regulations (Huddleston et al., 1998).

In addition, LLE is a laborious and relatively slow process, and organic non-polar solvents must be evaporated prior to sample injection in chromatographic systems. Evaporation at elevated temperatures (typically several mL of extract) is often time consuming, and cannot be easily used for volatile and semi-volatile analytes. Also, LLE is of limited use for highly polar analytes, due to unfavorable Kd.(Gilar et al., 2001).

LLE has also several limitations, such as formation of emulsions, low/variable recovery, poor selectivity and high matrix effects. Another limitation is the extraction of a wide variety of compounds with varying liposolubilities, for example, extraction of hydrophilic/water soluble compounds from the matrix. For that reason, efforts have been made recently to improve the limitations associated to LLE and to scale down the dimensions of the extraction system (Kole et al., 2011). This approach has been used to develop modern and miniaturized extraction techniques that have nowadays achieved a different level of success and acceptance by analysts (Ramos, 2012).

Table 2 reviews several LLE procedures to determine BAs in urine and plasma, the most common biological specimens used. The solvent selection, as well as the volume used, is very important regarding the recoveries of the BAs obtained. Solvents like diethyl ether or ethyl acetate present great recoveries in the mentioned procedures, but others like acetonitrile, chloroform and heptane are also reported. Additionally, the extraction time and phases separation parameters can be observed in the same table.

Table 1. Application of protein precipitation for BAs determination in biological samples

BAs	Sample	Volume (mL)	Precipitation solvent	Precipitation solvent volume (mL)	Sepa-ration	Time (min)	Analytical Instrumentation	LOD	LOQ	Recoveries (%)	Reference
CAD, PUT, SPD, SPM	Plasma	0.25	10% $HClO_4$	0.15	1500 rpm	3	HPLC/Q-TOF MS	<0.1 ng/mL	<0.4 ng/mL	>76.76	Liu et al., 2012
MN, NMN	Plasma	0.5	10% TCA -30ºC	0.25	16000 g	10	LC-MS/MS	n.a	<12.6 pg/mL	>86.4	He & Kozak 2012
E, NE	Plasma	0.05	Acetonitrile	0.2	n.a	5	UPLC-MS/MS	0.025 ng/mL	0.05 ng/mL	n.a	Ji et al., 2010
CAD, DA, E MN, NE, NMN, PUT, SPD, SPM	Plasma; Urine	0.1	2M $HClO_4$	0.03	13 000g	12	HPLC-FLD	<1.26 pmol	<4.12 pmol	>69	Lozanov et al., 2007
L-Dopa, DA, DOPAC, 3OMD	Plasma	0.5	1.2 M $HClO_4$	0.3	1250 g	7	HPLC-ECD	n.a	<5 ng/mL	>77	Karimi et al., 2006
TYR	Urine	5	Acetonitrile	n.a	Disposable filter	n.a	HPLC-FLD	n.a	1 nmol/ mL	97.8 ± 3.3	Yoshida et al., 2005
E, MN, DOPA, DA, NE, DOPAC, HVA, 3MT, NMN, DOMA, VMA, 5HT, 5HIAA	Urine	1	0.2 M $HClO_4$	0.3	20000 g	15	HPLC-ECD	0.81-2.49 pg	>1 pg	>67.9	Cao & Hoshino 1998
L-Dopa	Plasma	1	60 mM TCA	0.3	5000 g	10	HPLC-ECD	0.2 µg/L	n.a	101 ± 2.7	Rizzo et al., 1996
L-Dopa	Plasma	0.5	99.7% CH_3OH	2.5	700 g	10	HPLC-UV	n.a	n.a	n.a	Hatori et al., 1996

3MT (3-Methoxytyramine), 3OMD (3-O-methyldopa), 5HIAA (5-Hydroxyindole-3-acetic acid), 5HT (5-Hydroxytryptamine), CAD (cadaverine), DA (dopamine), DOMA (3,4-dihydroxymandelic acid) DOPAC (3,4-dihydroxyphenyl acetic acid), E (epinephrine), HPLC-ECD (high-performance liquid chromatography – electrochemical detection), HPLC-FLD (high-performance liquid chromatography - fluorescence detection), HPLC/Q-TOF MS (high-performance liquid chromatography coupled with Q-time of flight mass spectrometry), HPLC-UV (high-performance liquid chromatography – ultraviolet detection), HVA (homovanillic acid), L-Dopa (levodopa), LC-MS/MS (Liquid chromatography - tandem mass spectrometer), MN (metanephrine), n.a (not available),NE (norepinephrine), NMN (normetanephrine), PUT (putrescine), SPD (spermidine), SPM (spermine), TCA (trichloroacetic acid), TYR (tyramine), UPLC-MS/MS (ultra-performance liquid chromatography - tandem mass spectrometer), VMA (4-hydroxy-3-methoxymandelic acid).

Table 2. Application of liquid-liquid extraction for BAs determination in biological samples

BAs	Sample	Extraction Solvent	Extraction Solvent volume (mL)	Extrac-tion	Sepa-ration	Time	Analytical Instrumentation	LOD	LOQ	Recovery (%)	Reference
E, NE, MN, NMN, DA	Urine (1 mL)	Ethyl acetate	1.5	vortex 60 sec	200 g	5 min	LC-MS/MS	< 3.6 µg/mL	11.1 µg/mL	>92.9	Diniz et al., 2015
HVA, VMA, DOPAC.	Urine (1 mL)	Diethyl ether	1	hand shaken 10 min	3000 rpm	10 min	MEKC - DAD	0.1 µg/mL	0.5 µg/mL	>96.1	Miękus et al., 2015
PUT, CAD, SPM, SPD	Urine (0.3 mL)	Dichloromethane	0.4	n.a	13 000 g	5 min	LC–ITMS/MS	< 3 ng/mL	<9.42 ng/mL	>83.7	Ibarra et al., 2015
PUT, CAD, SPM, SPD	Plasma (0.25 mL)	Diethyl ether	n.a	n.a	n.a	n.a	HPLC-QTOF-MS	< 0.1 ng/mL	<0.2 µg/mL	>76.76	Liu et al., 2012
MEL, NAS	Plasma (0.1 mL)	water/ acetonitrile (4:1)	0.1	n.a	10 000 g	2 h	LC-MS/MS	n.a	<11.7 pg/mL	>71	Carter et al., 2012
E, NE, DA	Urine (0.3 mL)	TOAB in heptane	0.9	vortex 3 min	15 000 g	3 min	LC-MS/MS	n.a	<10 µg/L	71 ± 12	Kushnir et al., 2002
5HIAA	Urine (0.025 mL)	$CHCl_3$ + n-amyl alcohol + C_2H_5 COOH	0.5	vortex 30 sec	spin	20 sec	HPLC-FLD	n.a	2 mg/ L	97.2	Shihabi & Hinsdale, 2000
E, NE, MN, NMN, DA	Urine (0.1 mL)	Chloroform	2 x 0.8	horizontal shaker 200 rpm 5 min	500 g	2 min	HPLC-FLD	375 fmol	1250 fmol	n.a	Chan et al., 2000b

BAs	Sample	Extraction Solvent	Extraction Solvent volume (mL)	Extrac-tion	Sepa-ration	Time	Analytical Instrumentation	LOD	LOQ	Recovery (%)	Reference
E, NE	Plasma (0.5 mL)	1. heptane mixture	1	vortex 30 sec	1200 g	5 min	HPLC-ECD	0.1 fmol	n.a	n.a	Forster & Macdonald, 1999
		2.1 Octanol	0.38	vortex 30 sec	n.a	30 sec					
		2.2 Acetic acid	0.02								
DA, E, NE	Urine (0.5 mL)	C_7H_{16} + 1% $CH_3(CH_2)7OH$ + 0.25% TOAB	5	90 sec	1800 g	3 min	HPLC--ECD	<0.07 pmol/ injection	n.a	99.3 ± 1.98	Hollenbach et al., 1998

5HIAA (5-Hydroxyindole-3-acetic acid), CAD (cadaverine), DA (dopamine), E (epinephrine), HPLC-ECD (high-performance liquid chromatography – electrochemical detection), HPLC-FLD (high-performance liquid chromatography - fluorescence detection), HPLC/Q-TOF MS (high-performance liquid chromatography coupled with Q-time of flight mass spectrometry), HVA (homovanillic acid), LC–ITMS/MS (liquid chromatography - ion trap tandem mass spectrometry), LC-MS/MS (liquid chromatography tandem mass spectrometry), MEKC - DAD (modified micellar electrokinetic chromatography-diode array detector), MEL (melatonine), MN (metanephrine), n.a (not available), NAS (N-acetylserotonin), NE (norepinephrine), NMN (normetanephrine), PUT (putrescine), SPD (spermidine), SPM (spermine), TOAB (tetraoctylammonium bromide), VMA (4-hydroxy-3-methoxymandelic acid).

2.3. Solid-phase Extraction (SPE)

There can be no doubt that SPE continues, today, the most commonly applied sample preparation method. SPE was initially considered as a replacement for LLE. Conventional LLE is labor intensive, difficult to automate, and is frequently plagued by practical problems, such as the aforementioned formation of emulsions (Poole, 2003).

For analytical purposes, SPE is usually performed using a small column or cartridge containing an appropriate packing. The most commonly used material for SPE is chemically bonded silica (usually with a C8 or C18 organic group) and polymeric resins such as porous polystyrene (Huck & Bonn 2000). With this technique, the analytes are transferred to the solid phase where they are retained for the duration of the sampling process. The solid phase is then isolated from the sample and the analytes are recovered by elution using a liquid or fluid, or by thermal desorption into the gas phase (Poole, 2003).

The experimental procedure consists of five steps:

1. Sorbent activation by passing through it an appropriate solvent that conditions the surface of the solid;
2. Removal of the activation solvent by a liquid similar in composition to the sample matrix;
3. Sample application; the analytes will be retained by the sorbent (sorption or retention step);
4. Removal of interfering compounds retained in step 3 with a solvent which does not remove the analytes (washing step);
5. Elution of the analytes from the adsorbent with an appropriate solvent or solvents (desorption or elution step) and collecting them for later analysis (Berrueta et al., 1995).

The different mechanisms of retention or elution are due to intermolecular forces between the analyte, the active sites on the surface of the adsorbent and the liquid phase or matrix. These mechanisms are identical to those involved in column liquid chromatography and have been

discussed in most of the general textbooks on liquid chromatography (Berrueta et al., 1995).

Chemically bonded silica and porous polystyrene have several pitfalls for their use in SPE. Although silica itself is hydrophilic and instable at alkaline pH, the hydrocarbon chains make the surface hydrophobic, leading to poor surface contact with predominantly aqueous solutions. Porous polystyrene resins also have a hydrophobic surface (Huck & Bonn, 2000). Pretreatment of SPE materials with an activating solvent such as methanol, acetone or acetonitrile must be used to obtain better surface contact with the aqueous solution being extracted (Huck & Bonn, 2000).

During the last years a series of different polymer-based materials for the extraction of acidic, neutral or basic compounds from different sample matrices has been developed. In general polymer phases have the advantage over bonded silicas that they can be used over the entire pH range and the disadvantage that the conditioning of the cartridge is more time consuming (Huck & Bonn, 2000).

SPE can be performed off-line, the sample preparation being separated from the subsequent chromatographic analysis, or on-line by direct connection to the chromatographic system (Hennion, 1999). However, the most usual approach is to use SPE in an off-line procedure: analytes are desorbed from the sorbent with a small volume of organic solvent and an aliquot of the extract is subsequently analyzed, which is still a drawback, and has a persistent risk of contamination (Prosen & Zupančič-Kralj, 1999).

Table 3 presents data on the SPE sorbents and procedures applied to pre-concentrate several BAs. The analytical procedures, LODs, LOQs and recovery values obtained are also included in this table. Selection of the appropriate deposit allowed the simultaneous separation of several amines of similar compounds while removing a rich and complex matrix (plasma, serum or urine) (Płonka, 2015).

The samples were commonly purified on extraction columns with octa- and octadecylsilane and diol, on alumina as well as ion-exchange copolymer phases.

Catecholamines (CAs) are biogenic amines characterized by an amino group in the alkalic side chain together with a phenolic catechol group (i.e., hydroxyl groups at 3- and 4-positions) (de Jong et al., 2010). They are usually purified from human plasma by adsorption on alumina or on boric gel or by extraction with organic solvents. However, these methods are not sensitive enough and furthermore they do not often provide high extraction yields (Unceta et al., 2001). The SPE procedure with alumina has also the disadvantage of being laborious with a relatively low extraction yield (Raggi et al., 2003). Silica-based packing materials in SPE appear to yield poorer recoveries of CAs than those of polymer-based resins (Vuorensola & Sirén, 2000; Talwar et al., 2002). The use of PBA-based extraction specific for catechol-containing compounds has been reported to be more effective than the commonly used SPE techniques as cation-exchange and activated alumina (de Jong et al., 2010).

PBA or rather the negatively charged diphenylborate, forms a complex with the vicinal hydroxyl groups of catecholamines in alkaline media. The complex is strongly retained on the polymer- and silica-based cartridge material, and elution of the analytes is carried out with acidic solutions. Immobilized PBA column is chosen for several analytical methods, the advantage being that it is quite specific for catecholamines, and also that it can be easily incorporated in an automated system to reduce analysis time and to ease the procedure (Nikolajsen & Hansen, 2001).

Smedes et al., (Smedes et al., 1982) have developed an alternative extraction involving diphenylboric acid (DPBA) complexation with the diol of the catecholamine, followed by ion-pair formation with tetramethylammonium bromide (TMABr), resulting on the neutralization of the charge, hence helping the extraction of the complex into an organic solvent. The affinity of catecholamines to DPBA in alkaline medium improved their retention on solid-phase extraction (SPE) reverse-phase cartridges. The presence of an ion-pairing reagent (TMABr) increases the retention on C18 sorbents. After removing all interferences from the clinical sample, the complex is directly degraded on the SPE cartridge using the acidic elution solution (Unceta et al., 2001).

Among the commercially available cationic exchange phases, some authors choose to assess only those which give exclusively ionic interactions and select an adsorbent based on silica particles functionalized with carboxypropylic groups (Isolute CBA). The short carbon chain assures that non-ionic interactions are practically negligible, and the carboxylic groups allows for the suppression of ionization at low pH values (Raggi et al., 2003).

Alumina extraction has not been as popular, which may have been due to catecholamine instability due to alkaline solutions, especially when using alumina procedures, usually requiring mildly alkaline conditions. This fact and because alumina needs chemical activation (which can be hard to reproduce) may be part of the reasons why alumina extraction has not been used more extensively (Nikolajsen & Hansen 2001).

CAs are metabolized to metanephrines which include metanephrine (MN) and normetanephrine (NMN) (He et al., 2011). Although CAs and metanephrines have very similar structures, their chemical behaviors, namely interactions with various SPE sorbents, differ significantly. CAs contain a catechol moiety, whereas in the metanephrines, position three of the benzene ring is methoxylated, which produces significant differences in hydrophobicity and pKa values between these two groups of compounds. For the SPE of CAs, alumina or alumina followed by a cation exchanger have usually been used. Metanephrines are very polar, and therefore most frequently extracted using a cation exchanger or a combination of cation and anion exchangers. A few papers have reported simultaneous extraction and determination of CAs and metanephrines in urine and/or plasma, most of these employing on- or off-line strong cation exchangers to extract all analytes (Peterson et al., 2002).

In addition, extraction methods based on hydrophobic interactions might not be the ideal choice due to the high polarity of neurotransmitters. Thus, molecularly imprinted polymers (MIPs) have been also used as solid-phase extraction sorbents (Claude et al., 2011).

Table 3. Application of solid-phase extraction for BAs determination in biological samples

BAs	Sample	SPE Cartridge	Conditioning	Load	Wash	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Ref
L-DOPA, DA, E, NE, NMN, DOPAC, 5HT, HVA, VMA	Urine (2 mL)	Oasis MCX	CH_3OH, H_2O	0.1 M HCl added, heated (90 °C)	0.1 M HCl	CH_3OH, NH_4OH: CH_3OH (5:95, v/v)	GC-MS	<6.24 ng/mL	<17.84 ng/mL	>87.9	Park et al., 2013
MT, NME, DA, NE, E	Urine (2 mL)	MMIP	Acetic acid (1%); PBS (pH 8).	PBS (pH 8) added	n.a	Acetic acid (1%)	CE-DAD	<0.06 µM	<0.21 µM	>92	Bouri et al., 2012
NE, E	Plasma (0.5 mL)	Alumina B 96-well	ACN; 0.2% ammonia; 0.5 M Tris–EDTA buffer (pH 8.0)	0.8 mL of 0.5 M Tris–EDTA buffer (pH 8.0) added	0.5 M Tris–EDTA buffer (pH 8.0); H_2O	ACN:H_2O (60:40, v/v) containing 2.5% formic acid	LC-MS/MS	0.5 pg/mL	<20 pg/mL	n.a	Zhang et al., 2012
E, NE, DA	Urine (1 mL)	Bond Elut PBA	80:20 ACN–H2O(v/v) containing 1% formic acid; 50 mM phosphate buffer (pH 10)	phosphate buffer (pH 8.5) added	ACN–10 mM phosphate buffer pH 8.5 (50:50 v/v)	ACN–water (80:20 v/v) containing 1% formic acid	HILIC-ECD	<25 pg/inj	n.a	>87	Kumar et al., 2011
MN, NMN	Plasma (0.2 mL)	WCX 96-well	n.a	n.a	H2O, MeOH, 0.1% formic acid in acetonitrile	2% formic acid in acetonitrile	LC-MS/ MS	n.a	0.1 nmol/ L	>102.8	Petteys et al., 2012

BAs	**Sample**	**SPE Cartridge**	**Conditioning**	**Load**	**Wash**	**Elution**	**Analytical Instrumen-tation**	**LOD**	**LOQ**	**Recovery (%)**	**Ref**
MN, NMN	Urine (1 mL)	Bond Elut Plexa	n.a	n.a	n.a	n.a	LC-MS/ MS		<0.3 µmol/ L	n.a	Gabler et al., 2011
DA, 3-MT, 5-HT	Urine (2 mL)	MIP	ACN; acetic acid–ammonia buffer (pH 7, 25mM)	acetic acid-ammonia buffer added	Acetic acid-ammonia buffer; ACN	4 mL MeOH–AcOH (99/1, v/v)	CE-UV	n.a	<10 nmol/L	>40	Claude et al., 2011
MN, NMN	Urine (0.25 mL)	PWCX	CH_3OH, H_2O	sodium phosphate buffer, pH 7 added	H_2O, 70% CH_3OH	2% formic acid in 5% methanol	LC-MS/ MS	2.5 nmol/L	10 nmol/ L	> 98	Clark & Frank 2011
MN, NMN	Plasma (0.5 mL)	IP	ACN; 0.1% PFHA in water	0.5 mL DPB buffer added	0.1% PFHA in water	60% ACN in water (v/v)	LC-MS/ MS	n.a	<18 pg/ mL	>90.5	He et al., 2011
E, NE, DA	Urine (1 mL)	Bond Elut PBA	n.a	200 mM ammonium chloride buffer (pH 8) added	MeOH: H2O (9:1), MeOH: H2O (1:9)	100 mM ammonium formate pH 3	LC-MS/ MS	<15.8 nmol/L	<16 nmol/L	n.a	de Jong et al., 2010
E, NE, DA, NMN, MN	Urine (0.5 mL)	Bond Elut Plexa	CH3OH; Ammonium buffer (pH 8.5)	n.a	0.2 M Ammonium buffer with 5% CH3OH	200mM formic acid	LC-MS/ MS	<0.005 µmol/L	n.a	>63	Whiting, 2009

Table 3. (Continued)

BAs	Sample	SPE Cartridge	Conditioning	Load	Wash	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Ref
E,MN, NMN, DA, 5HT, 5HIAA, HVA	Urine (1 mL)	C18	CH3OH, H2O + 0.005 M HCl (pH 4.2)	n.a	n.a	H2O + 0.005 m HCl (pH 4.2), CH3OH	HPLC-FLD	<10 ng/mL	<30 ng/mL	>74	Baranowska & Płonka 2008
E, NE, DA	Urine (1 mL)	XAD (Polymeric resin)	CH_3OH, H_2O (pH 8.5)	n.a	n.a	6 M Acetic acid	HPLC - ECD	<46 nmol/L	n.a	>86	Lee et al., 2007
MN, NMN	Plasma (0.5 mL)	Oasis WCX	ACN; H_2O	H_2O added	H_2O; ACN: H_2O (9:1)	10 mM ammonium formate pH 3	LC-MS/ MS	<0.04 nmol/L	< 0.06 nmol/L	74.5 - 99.6	de Jong et al., 2007
E, NE, DA, L-Dopa	Urine (1.5 mL)	C18	CH_3OH, 0.2 M NH_4Cl–NH_4OH, (pH 8.5).	acidified with 6.0 M hydrochloric acid	0.2 M NH_4Cl–NH_4OH, (pH 8.5); 20% CH_3OH, 0.2 M NH4Cl–NH4OH, (pH 8.5);	1M acetic acid	HPLC-CL	<0.34 µg/L	<1.1 µg/L	n.a	Nalewajko et al., 2007
NE, E, DA	Urine (0.02 mL)	CAT-PBA	n.a	mobile phase A (0.2 M diammonium - hydrogenphosphate, 3.72 g/ l EDTA,	n.a	mobile phase B (50 mM potassium dihydrogen phosphate, 2.5 mM sodium octylsulfonate,	HPLC-ECD	n.a	<5 µg/L	>95	Rozet et al., 2006

BAs	Sample	SPE Cartridge	Conditioning	Load	Wash	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Ref
				CH_3OH, 95:5, v/v, pH 8.7)		0.1 g/l EDTA and ACN, 96.5:3.5, v/v, adjusted with phosphoric acid to pH 3.5)					
NE, E, DA	Plasma (0.5 mL)	C30	CH_3OH H_2O, DPB Buffer	DPB buffer added	washing buffer (pH 8.5); H_2O	0.5 Mobile phase	HPLC-ECD	<40 pg/mL	<100 pg/mL	>87	Machida et al., 2006
DA, E, NE, NMN, MN, DOPAC, 5HT, 5HIAA, HVA, VMA	Urine (2 mL)	Al_2O_3	CH_3OH, borate buffer (pH 9)	Adjusted to pH 8.6 2M NaOH	H_2O	1 M CH_3COOH	HPLC-FLD/ECD	<10 ng/mL	<25 ng/mL	>75	Zydroń et al., 2005
5HIAA, HVA, DA, VMA	Urine (1 mL)	Oasis, IST 101 mixed mode	CH_3OH, H_2O	n.a	CH_3OH:H_2O (5:95, v/v)	CH_3OH	CE-UV	0.05 µg/mL	0.1 µg/mL	n.a	Sirén et al., 2004
NE, E, DA	Plasma (0.5 mL)	Oasis HLB	CH_3OH, H_2O, Buffer	1 M hydrochloric acid and H_2O added	Buffer A; Buffer B-CH_3OH (1:1)	0.5 Mobile phase	HPLC-ECD	0.06 ng/mL	<0.12 ng/mL	>85	Sabbioni et al., 2004
DA, MN, NMN, 3MT	Urine (1 mL)	Oasis HLB	CH_3OH, Buffer	phosphate buffer (0.5 mol/l, pH 7.0) added	H_2O	CH_3OH	HPLC-ECD	<0.2 µmol/L	n.a	>13	Vuorensola et al., 2003

Table 3. (Continued)

BAs	Sample	SPE Cartridge	Conditioning	Load	Wash	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Ref
NE, E, DA	Plasma (0.5 mL)	Isolute-CBA	CH_3OH, H_2O, 20 mM Phosphate Buffer (pH 7.4)	H_2O added	H_2O; perchloric acid	0.2 M perchloric acid	HPLC-ECD	0.03 ng/mL	0.06 ng/mL	96 - 105	Raggi et al., 2003
NE, E, DA, NM, NMN, 3-MT	Urine	Oasis MCX (2 mL)	CH3OH, H2O	0.2 M EDTA and 0.5 M ascorbic acid added	0.1 M HCl; CH3OH	5% ammonium hydroxide in methanol	CE-TOFMS	<0.3 µmol/ L	n.a	>75	Peterson et al., 2002
NE, E, DA	Urine (5 mL)	Isolute MF C18	CH3OH; 0.2 M NH4Cl NH3 (pH 8.5) washing buffer containing 0.05% (w/v) EDTA	2.M NH4Cl NH3 (pH 8.5) buffer added	washing buffer; washing buffer–methanol (70:30 v/v)	6 M CH3COOH	HPLC -FLD	<0.7 nmol/L	n.a	99.3 - 106.3	Fotopoulou & Ioannou, 2002
DA, NE, E	Urine (1 mL)	C18	CH_3OH; 0.2 M NH_4Cl–NH_4OH (pH 8.5)	Buffer (pH 8.5) added	0.2 M NH_4Cl–NH_4OH (pH 8.5); 20 % MeOH in 0.2 M NH_4Cl–NH_4OH (pH 8.5)	1 M Acetic acid	HPLC -ECD	<30 nmol/L	<65 nmol/L	>85	Talwar et al., 2002

BAs	Sample	SPE Cartridge	Conditioning	Load	Wash	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Ref
DA, E, NE, NMN, MN, DOPAC, 5HT, 5HIAA, HVA, VMA	Urine (1 mL)	C18	CH_3OH, NH_4Cl + 0.5% EDTA (pH 8.5)	adjusted to pH 8.5 with 2 M NaOH	0.2 M NH_4Cl: CH_3OH (80:20, v/v, pH 8.5)	0.08 M CH3COOH	HPLC	n.a	n.a	>66.3	Baranowska & Zydron, 2002
E, NE, DA, VMA, DOPAC, HVA	Plasma (n.a)	Oasis HLB	CH3OH, H2O	Desproteinized	CH3OH, H2O (5:95)	CH3OH	HPLC -ECD	<0.05 µg/L	<0.09 µg/L	>64	Unceta et al., 2001
NE, E, DA, NM, NMN	Urine (5 mL)	Bio-Rex 70 cation-exchange resin	n.a	1 M NaOH added	H2O	4M formic acid	LC- MS	<5 ng/ mL	<15 ng/ mL	75-83	Chan & Ho, 2000
NE, E, DA	Plasma (0.02 mL)	IC-SP S	n.a	lithium phosphate buffer (0.2 M, pH 5.8) added	H2O: ACN (60%, v/v).	potassium chloride (0.6 M)–ACN (2 : 3, v/v)	HPLC-CL	<42.6 fmol/m L	n.a	96	Ragab et al., 2000
DA, 3-MT, NM, NMN	Urine (1 mL)	Oasis HLB	CH3OH; 0.5 M phosphate buffer (pH 7.0)	0.5 M phosphate buffer (pH 7.0) added	H2O	CH3OH	CE-UV	<0.7 µM	<0.87 µM	96.4 - 124.4	Vuorensola & Sirén, 2000
NE, E, DA, NM, NMN	Urine (5 mL)	ion-exchange resin	n.a	Na2EDTA, Na2S2O5, adjusted to pH 6.5 with 1 M NaOH	H2O	4 M HCOOH	HPLC-ECD	12 nmol/L	40 nmol/L	>69	Chan et., 2000a

Table 3. (Continued)

BAs	Sample	SPE Cartridge	Conditioning	Load	Wash	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Ref
DA, NE, E	Plasma (0.5 mL)	Oasis HLB	CH_3OH, H_2O; Buffer	Buffer A added	Buffer A; Buffer B-MeOH (1:1)	0.5 Mobile phase	HPLC -ECD	12 ng/L	40 ng/L	>92	Raggi et al., 1999
DA, NE, E	Urine (n.a)	ion exchange resin	CH_3OH, H_2O, 0.2 M ammonium buffer (pH 8.5 + 0.05 % EDTA; CH_3OH:0.2 M ammonium buffer (pH 8.5)	6M HCl at pH 1–3, 0.5% EDTA + 0.1% diphenylborate	CH_3OH:0.2 M ammonium buffer (pH 8.5)	50 mM KH_2PO_4 + 1 mM $C_7H_{15}O_3SNa$ + 0.07 mM EDTA: CH_3OH: CH_3CN (100:8:15, v/v/v) at pH 3.2 1.5M H_3PO_4	HPLC -ECD	<3µg/L	n.a	>97	Pastoris et al., 1995
DA, NE, E	Plasma (0.05 mL), Urine (0.01 mL)	IC-SP S	n.a	0.2 M phosphate buffer (pH 5.8)	n.a	n.a	HPLC-CL	150–450 amol per 100-µl injection	n.a	>98	Ragab et al., 1994

3MT (3-Methoxytyramine), 5HIAA (5-Hydroxyindole-3-acetic acid), 5HT (5-Hydroxytryptamine), CE-DAD (capillary electrophoresis - diode array detector), CE-TOFMS (capillary electrophoresis–time-of-flight mass spectrometry), CE-UV (capillary electrophoresis – ultraviolet detection), DA (dopamine), DOPAC (3,4-dihydroxyphenyl acetic acid), E (epinephrine), GC-MS (gas chromatography – mass spectrometry), HILIC-ECD (hydrophilic interaction chromatography with electrochemical detection), HPLC-CL (high-performance liquid chromatography with chemiluminescence detection), HPLC-ECD (high-performance liquid chromatography – electrochemical detection), HPLC-FLD (high-performance liquid chromatography - fluorescence detection), HVA (homovanillic acid), IP (Ion-paring), L-Dopa (levodopa), LC-MS (liquid chromatography - mass spectrometric), LC-MS/MS (liquid chromatography tandem mass spectrometry), MCX (mixed-mode cation-exchange), MIP (molecularly imprinted polymer), MMIP (magnetic molecularly imprinted polymer), MN (metanephrine), MT (3-methoxytyramine hydrochloride), n.a (not available), NE (norepinephrine), NME (normetanephrine hydrochloride), NMN (normetanephrine), PBA (phenylboronic acid cartridges), VMA (4-hydroxy-3-methoxymandelic acid), WCX (weak cation exchange).

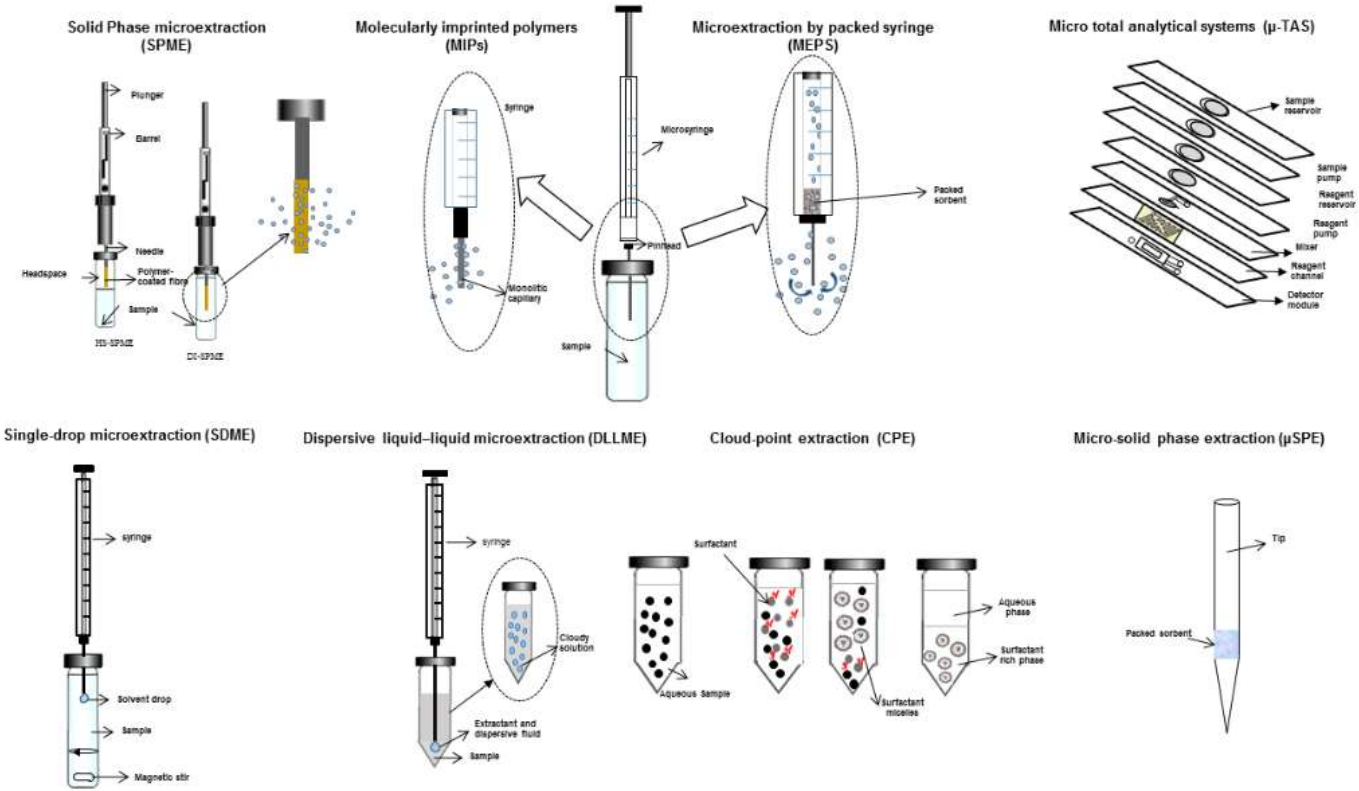

Figure 1. Microextraction procedures to determine biological amines in human biological specimens.

The use of MIPs as sorbents in SPE has led to a SPE protocol known as molecularly imprinted solid-phase extraction (MISPE). Compared to natural receptors, MIPs not only demonstrate comparable molecular selectivities, but they are also more robust and reusable, and less expensive to prepare. However, some drawbacks to MISPE have restricted its widespread application. MISPE is normally used in cartridge mode, which often results in a tedious column packing procedure, high backpressure and a low flow rate. In order to avoid these disadvantages several attempts have been made to develop an on-line MISPE system, or to prepare a monolithic column coupled to a chromatographic system. Providing magnetism to the MIP and then using magnetic separation is another promising alternative (Bouri et al., 2012).

3. MICROEXTRACTION TECHNIQUES (METS)

PPT, LLE and SPE have been conventionally used as sample preparation techniques, however sample preparation has always been at the forefront of research and investigation for newer and more effective ways of extracting the analyte from the biomatrix (Kole et al., 2011). The initial sample extraction procedures, mostly based on LLE or SPE, were developed in an academic environment and were not suitable for the clinical environment because of a number of issues related to organic solvents usage and waste management, the high sample volume required to obtain a satisfactory analytical performance and the low automation and throughput possibilities (Pereira et al., 2014).

The ideal situation would be the complete elimination of the sample preparation step from the analytical process. However, despite the current degree of development of the analytical instrumentation used for final determination, in most instances this is not feasible (Ramos, 2012). In recent times, to cope up with demand for improved selectivity and sensitivity and regulations, technological innovations in material science and robotics and in-depth understanding of biomatrices have paved a path for many new strategies in sample preparation (Pereira et al., 2014;

Barroso et al., 2012). Concepts like miniaturization, integration and simplification became key concepts that have already proved to effectively contribute to solve some of the drawbacks of conventional sample preparation methods and that, in some studies involving size-limited samples, can probably be considered the best, if not the only, analytical alternatives (Ramos, 2012).

The fast evolution in the miniaturization of the most common analytical procedures, particularly in the chromatographic separation, raised the analytical limits higher than ever before and opened new possibilities in several fields, namely in medical diagnosis (Pereira et al., 2014).

This required more efficient sample preparation techniques, and a large number of different approaches to sample extraction are nowadays available. These techniques are generically designated by METs (microextraction techniques) (Pereira et al., 2014).

Although many traditional sample preparation methods are still in use the METs trends in recent years have been towards:

- The ability to use smaller initial sample sizes even for trace analyses;
- Greater specificity or greater selectivity in extraction;
- Increased potential for automation or for on-line methods reducing manual operations;
- More environmentally friendly approaches (green chemistry) with less waste and the use of small volumes or no organic solvents (Smith, 2003).

The use of alternative microextraction techniques for sample preparation reduces the number of errors that commonly result from multi-stage procedures, and limits the negative impact on the environment and the health of analytical chemists performing laboratory work. The reduction of the amount of organic solvents employed during the extraction process translates into lowered utilization costs of waste treatment and spent solvents, which in turn allows the cost reduction of analytical

procedures as well as saving money on the purchase of high purity solvents (Spietelun et al., 2014; Barroso et al., 2012).

Regarding BAs determination in biological specimens, this trend is no different, hence the justification of a small review on the available METs developed and applied to identify and quantify BAs in human samples. Figure 1 summarize the several METs use to determine BASs in human biological specimens.

3.1. Liquid-Phase Microextraction (LPME)

In the present days, there have been several publications on the miniaturization of LLE in analytical chemistry. The major ideas behind this were to facilitate automation and effectively to reduce the consumption of organic solvents (Rasmussen & Pedersen-Bjergaard, 2004).

Liquid-phase microextraction (LPME) as an alternative miniaturized sample-preparation approach, emerged in the mid-to-late 1990s (Rezaee et al., 2010). LPME is a solvent-minimized sample-pretreatment version of LLE, in which only several μL of solvent are required to concentrate analytes from various samples rather than tens or even hundreds of mL needed in traditional LLE (Sarafraz-Yazdi & Amiri, 2010). It also overcomes many disadvantages of solid-phase microextraction (SPME) (e.g., independence of a commercial supplier and sample carryover or cross-contamination) (Rezaee et al., 2010).

In LPME, extraction normally takes place into a small amount of a water-immiscible solvent (acceptor phase) from an aqueous sample containing analytes (donor phase) (Sarafraz-Yazdi & Amiri, 2010). Two main categories have been of interest regarding BAs pre-concentration:

- Dispersive liquid–liquid microextraction (DLLME);
- Single-drop microextraction (SDME).

3.1.1. Dispersive Liquid-Liquid Microextraction (DLLME)

Dispersive liquid-liquid microextraction (DLLME) is a modern and attractive sample pretreatment technique, developed in 2006 by Rezaee et al., (Rezaee et al., 2006; Konieczna et al., 2016). The apparatus involved in the DLLME operation procedure just includes a microsyringe, a centrifuge and some conical test tubes. There is no need for magnetic stirrer, magnets for agitation, hollow fiber and no trouble in the possibility of extraction drop dislodgment (Hu et al., 2013). DLLME is generally based on a ternary component solvent system, in which extraction and disperser solvents are rapidly introduced into the aqueous sample to form a cloudy solution. The extraction time is very short, since the equilibrium is quickly achieved, due to the extensive surface contact between the droplets of the extraction solvent and the sample (Herrera-Herrera et al., 2010). After centrifugation, the droplets of extraction solvent enriched with the analyte are located at the bottom of test tube and are collected after centrifugation for the appropriate analysis (Konieczna et al., 2016).

The factors that affect DLLME efficiency are as follows:

- Suitable extracting solvent;
- Suitable disperser solvent;
- Volume of extracting solvent;
- Volume of disperser solvent (Rezaee et al., 2010).

DLLME presents some advantages, such as simplicity of operation, speed, low sample consumption, low cost, high recovery and high enrichment factor. Nevertheless, reproducibility and anti-interference ability of DLLME are not very satisfactory, and some problems would be met in the selection of extraction and disperser solvents and also in the transfer of the obtained sediment phase to the subsequent detection instrument, because the key point of such technique is to obtain a stable/reproducible sediment phase between each operation procedure (Hu et al., 2013).

The extraction solvent in DLLME should have special characteristics, such as higher density than water, high efficiency in the extraction of target

analytes and low solubility in water. Halogenated hydrocarbons such as chloroform, carbon tetrachloride and carbon disulfide were used most often in this technique. Meanwhile, special attention should be paid to the selection of disperser solvent, that should be miscible in both extraction solvent and aqueous sample and help the extraction solvent distribute homogeneously in the aqueous sample solution. For this purpose, acetone, acetonitrile, methanol and ethanol were often used for the investigation of extraction efficiency (Hu et al., 2013).

The extracting solvent volume has important effect on the preconcentration factor (PF). By increasing the extracting solvent volume, the volume of sediment phase obtained by centrifugation increases, resulting in a decrease on PF. Therefore, the optimal extracting solvent volume should ensure both high PFs and enough volume of the sediment phase for subsequent analysis after centrifugation. The disperser solvent volume also affects extraction efficiency. The suitable volume of disperser solvent for well cloudy solution depends on the volume of both aqueous phase and extracting solvent. In DLLME, the important factors affecting the volume of sediment phase are:

- Solubility of extracting solvent in water;
- Sample solution volume;
- Disperser solvent volume;
- Extracting solvent volume (Rezaee et al., 2010).

Other parameters affecting DLLME also have to be optimized. Two most frequently optimized parameters are the amount of a salt added to the sample (salting-out effect) and the pH of the sample. The latter is particularly important in the extraction of polar analytes. The value of pH is chosen so as to make the analyte less soluble in the aqueous phase (Zgoła-Grześkowiak & Grześkowiak, 2011). Extraction time is another important factor in the extraction procedure, if not the most important. In DLLME, extraction time is defined as the time between the injection of the mixture of disperser and extraction solvents, and centrifugation (Hu et al., 2013).

The danger related to the toxic properties of chlorinated solvents led to the introduction of ionic liquids as the extracting solvents in DLLME. Although these solvents cannot be considered as environmentally friendly, their negligible vapor pressure makes them relatively safe in laboratory experiments. Typically, ionic liquids used in DLLME are heavier than water and are deposited on the bottom of the centrifuge tube. This makes it easier to handle them than long-chained alcohols, hydrocarbons and other solvents less dense than water (Zgoła-Grześkowiak & Grześkowiak, 2011).

Table 4 resumes the successful application of this miniaturized technique to BAs determination in human urine and plasma. Dichloromethane and octanol have been reported as good extraction solvents, while ethanol and acetic acid as disperser solvents.

3.1.2. Single Drop Microextraction (SDME)

The simplest technique belonging to the LPME group is single-drop microextraction (SDME), in which a single microdrop of a water-insoluble solvent suspended at the tip of a gas chromatography (GC) syringe is either immersed in an aqueous sample or exposed to the Headsapace (HS) of a sample contained in a vial. Typical extractant and aqueous volumes are 1-8 μL and 1-10 mL, respectively (Ramos, 2012).

At present, there are seven different modes of solvent microextraction that fall under the category of single drop microextraction. They can be classified into either two-phase or three-phase techniques, depending on the number of phases co-existing at equilibrium (Jeannot et al., 2010). And with the large available database, it is possible to identify the important parameters that affect the rates and efficiencies of SDME extractions, these are:

- Analyte properties (volatility, polarity and ionization);
- Extraction solvent properties;
- Extraction solvent purity;
- Syringe;
- Drop volume;
- Agitation;
- Ionic strength (salting out effect);

- Temperature;
- Sample volume and headspace volume;
- Automation (Jeannot et al., 2010).

Although SDME is an equilibrium technique, it allows enrichment factors as high as 300 with extraction times as short as 1-15 min. Stirring of the sample (up to ca. 600 rpm to prevent drop dislodgment), salting-out, application of temperature and analyte derivatization (to reduce its polarity or increase its volatility) are common practices that, in general, contribute to increase the extraction efficiency and reduce the analysis time (Barroso et al., 2012; Ramos, 2012).

As commonly occurs in these techniques, a suitable solvent is very important to achieve good selectivity and improving extraction efficiency. The extraction solvent has to meet the following requirements: low water solubility (except in headspace mode), good drop stability and good ability in solubilizing the target analytes. The organic drop volume of several micro liters is more commonly used to ensure the formation of a stable/reproducible microdrop and allow fast stirring speeds, albeit with possible loss of analytical sensitivity (Hu et al., 2013).

Transport of analyte molecules from aqueous sample solution to the microdrop is generally limited by slow diffusion rates of the analyte molecules in the condensed (aqueous and/or organic) phases. The samples are normally agitated to increase the amount of convective mixing or interfacial contact area, and therefore reduce the diffusion distance. The time required to reach equilibrium in SDME can be anywhere from seconds to hours, depending on the degree of agitation, phase volumes, interfacial contact area and equilibrium distribution constant. Thus, to avoid excessive analysis times, SDME is often performed under non-equilibrium (kinetically controlled) conditions (Jeannot et al., 2010).

The pH value of the sample solution should also be optimized to ensure the formation of the chelates and their successful extraction into the organic phase. The adjustment of pH can enhance extraction, as dissociation equilibrium is affected together with the solubility of the acidic/basic target analytes. It should be pointed out that pH optimization is

the key point in the process of three-phase mode of SDME (Hu et al., 2013).

The addition of salt to the sample might also affect the extraction process, however, the presence of salt was also found to restrict the extraction of some organic analytes. It was assumed that apart from the salting-out effect, the presence of salt caused a second effect and changed the physical properties of the extraction film, thus reducing the diffusion rates of the analytes into the drop (Hu et al., 2013).

The use of SDME together with chromatography for trace determination of organics has been reported. SDME requires extremely simple laboratory equipments and only 1-3 μL of organic solvents, which is compatible with the sample preparation volume required for interface with analytical instrumentation as GC-MS or MALDI-MS (Wu & Lin, 2006). The possibility to perform it manually or (semi)-automatically using an autosampler, and the feasibility of obtaining ready-to-analyse extracts have probably been additional factors contributing to the rapid development and acceptation of this environmentally friendly technique in different research fields (Ramos, 2012). It is inexpensive, and since very little solvent is used, there is minimal exposure to toxic organic solvents. However, this method presents a few disadvantages:

- fast stirring would tend to break up the organic drop;
- air bubble formation;
- extraction is time-consuming and equilibrium cannot be attained after a long time in most cases (Rezaee et al., 2010).

Wu and Lin (Wu & Lin, 2006), applied SDME to determine dopamine in urine by atmospheric pressure matrix-assisted laser desorption/ ionization mass spectrometry (AP-MALDI-MS). Using 2 μL drop of octanol (extraction solvent) it was possible to detect 40 ppm of dopamine in 20 mL of biological sample.

According with the authors, the optimum conditions were: stirring rate, 240 rpm; extraction time, 5 min; and sample pH value, 8.

Table 4. Application of dispersive liquid-liquid microextraction for BAs determination in biological samples

BAs	Sample	Extraction Solvent	Disperser solvent	Shaken extraction	Separation	Time	Analytical Instrumentation	LOD	LOQ	Recovery (%)	Reference
E, 5-HT, 3-MT, 5-HIAA, VMA, DA, NE, L-Dopa, HVA	Urine (1 mL)	Dichloromethane	Ethanol	0.1 min	4500 rpm	3 min	LC–HILIC–MS	5 - 10 ng/mL	10 - 20 ng/mL	>99	Konieczna et al., 2016
E, NE, DA, 5-HT, L-Dopa	Urine (1 mL)	Dichloromethane	Ethanol	10 min	4000 g	4 min	MEKC-UV	0.15 ug/mL	0.5 ug/mL	>92	Miękus et al., 2016
NE, E	Plasma (0.5 mL)	Octanol	Acetic acid	30 sec	n.a	30 sec	HPLC - ECD	0.1 fmol/inj	n.a	n.a	Forster & Macdonald 1999

3MT (3-Methoxytyramine), 5HIAA (5-Hydroxyindole-3-acetic acid), 5HT (5-Hydroxytryptamine), DA (dopamine), E (epinephrine), HILIC-MS (hydrophilic interaction chromatography with mass spectometry), HPLC-ECD (high-performance liquid chromatography – electrochemical detection), HVA (homovanillic acid), L-Dopa (levodopa), MEKC - UV (modified micellar electrokinetic chromatography-ultraviolet detector), n.a (not available), NE (norepinephrine), VMA (4-hydroxy-3-methoxymandelic acid).

3.2. Cloud-Point Extraction (CPE)

Cloud-point extraction (CPE) uses surfactants for extraction of materials. Surfactants for extraction have been known to human beings for long for their capability to enhance the solubility of hydrophobic material (Rezaee et al., 2010).

Aqueous solutions of some surfactants are used in micellar extraction (ME) and CPE. In ME, the selective separations can be achieved owing to the fact that the micellar aggregates have a size that prevents them from crossing certain ultrafiltration membranes. The aqueous micellar solutions of some surfactants exhibit the cloud point, or turbidity, phenomenon that happens when the solution is heated or cooled above or below a certain temperature. The temperature at which this phenomenon occurs is known as the cloud point temperature. This methodology is known as CPE or micelle-mediated extraction (Carabias-Martínez et al., 2000).

Regardless of their shape or size, surfactant aggregates orientate their hydrocarbon tail towards the center of the formation, creating a non-polar core. Hydrophobic and covalent compounds initially present in the aqueous solution are favorably partitioned in the non-polar microenvironment. The whole process resembles the traditional LLE, the only difference being that the "organic" phase is generated within the aqueous phase, converting a previously homogeneous solution to heterogeneous one by simply gathering its previously scattered hydrophobic suspensions (Paradkar & Williams 1994; Paleologos et al., 2005). When the conditions of the solution, such as temperature and pressure, are modified appropriately, phase separation occurs for the aqueous micellar solution (Paleologos et al., 2005).

The use of micellar systems such as CPE has attracted considerable attention in the last few years mainly because it is in agreement with the "green chemistry" principles (Bezerra et al., 2005).

The advantages of CPE include:

- The capability to concentrate a plethora of analytes with almost quantitative recoveries;

- The preconcentration factors obtained are comparable or even higher than those obtained with other extraction schemes, and they can also be modified on demand by varying the amount of surfactant;
- Commercial surfactants are environmentally friendly and cost effective, and the amounts used for effective extraction are minimal compared to the amounts of organic solvents used in conventional extraction;
- The mild conditions applied in CPE techniques allow for design of preconcentration schemes targeted at thermally sensitive analytes, such as molecules of biological and environmental interest;
- The surfactant-rich phase is compatible with most mobile phases used in hydrodynamic analytical systems (Paleologos et al., 2005).

Despite many benefits of using CPE, the choices of the surfactants may be problematic when analysis is carried out using some instruments such as GC and HPLC. In addition, the use of anionic surfactants as effective extractants in CPE separation often requires salts and pH adjustments. Pressure and temperature also affect CPE, and therefore it is very important to optimize them in order to obtain good recoveries of the analytes (Rezaee et al., 2006).

Handling the micellar phase is a critical feature for every CPE procedure, especially in terms of reproducibility and credibility of the results. Usually, after phases are separated by centrifugation, the test tubes are cooled in an ice bath, thus increasing the viscosity of the micelles, which adhere to the bottom of the vial, so removal of water is achieved by simple inversion of the vial (Paleologos et al., 2005).

Davletbaeva et. al, (2016) developed an automated method for the fluorometric determination of epinephrine in human urine. The epinephrine derivatization product 1-methyl-1H-pyrrolo [2,3-b] pheazin-3-ol (MPP) was mixed with the nonionic surfactant Triton X-114 in a ratio 10:0.5 (v/v) in a glass vial. The sample in the glass vial was immersed in a cooling bath and equilibrated under periodic agitation. In this work the cloud points were determined by cooling. The initial temperature was 30°C. Then the

temperature was decreased at a rate of 0.5°C/min. The calculated limit of detection was $3x10^{-12}$ mol/L.

3.3. Microextraction by Packed Syringe (MEPS)

MEPS was invented and developed at AstraZeneca, Södertälje, Sweden, and is considered a miniaturized SPE with extended logic to handling biological fluids, hence the small operating volumes reduce the size of sample required (Abdel-Rehim, 2011).

In MEPS, the solid sorbent is either integrated in the barrel of a gas-tight syringe or in a specific vessel located between the needle and the barrel as a cartridge. Plasma, urine or any other aqueous sample is aspirated through the syringe; this step is usually performed manually, but can be autosampler-assisted. By percolating the sample through the solid bed, the analytes are captured by the solid sorbent. Then, to remove the proteins and other interfering compounds, the sorbent is washed with water. The final step is the elution of the analyte with an organic solvent (Moein et al., 2015).

The sorbent, 1-4 mg is either inserted into one syringe (100–250 L) barrel as a plug or between the needle and the barrel as a cartridge. The cartridge bed can be packed or coated to provide selective and suitable sampling conditions. Any sorbent material such as silica based (C2, C8, C18), strong cation exchanger (SCX) using sulfonic acid bonded silica, restricted access material (RAM), HILIC, carbon, polystyrene-divinylbenzene copolymer (PS-DVB) or molecular imprinted polymers (MIPs) can be used (Abdel-Rehim 2010, 2011).

This extraction technique can be fully automated, and the sample processing, extraction and injection steps can be performed online using the same syringe for GC or LC analyses without any modifications (Abdel-Rehim, 2010).

MEPS provides flexibility in different parameters such as washing solution, elution solution and type of sorbent materials (Abdel-Rehim, 2010). Still, a tutorial regarding MEPS recommends dilution of samples

(dilution, 1:5 for plasma, urine and 1:25 for blood) to reduce their viscosity and prevent coagulation and blockage in MEPS cartridge and to achieve low back-pressure. The speed of sample aspiration through the sorbent should not exceed 20 µL/s for better interaction between it and the analyte leading to greater extraction recoveries that can also be increased by increasing the number of MEPS loading cycles (1-10 times draw-eject) (Abdel-Rehim, 2011; Barroso et al., 2012).

This approach for sample preparation is very promising for many reasons: it is easy to use, it is rapid, reduces solvent and sample volumes, and the cost of analysis is minimal compared to conventional solid-phase extraction (Abdel-Rehim, 2010). Nevertheless, just like aforementioned techniques, it's still important to optimize the procedure. The main MEPS-factors that affect performance are:

- Type of sorbent - Important to achieve acceptable clean-up and recovery;
- Conditioning of the sorbent - this step activates the sorbent and ensures reproducible retention of the analytes. For practical reasons, the conditioning solution is the same as used for the washing step;
- Application of the sample (loading) - slowly (20 µL/s) drawn into the syringe, to obtain good percolation of the sample through the adsorbent bed. The sample may be pumped up and down several times, if necessary;
- Washing the sorbent - done at a speed of 50 µL/s. The washing step can be repeated if necessary. This may, however, have a negative effect on recovery;
- Eluting the analytes - because a small amount of sorbent is used, desorption can be achieved with a relatively small amount of elution solvent;
- Cleaning of the sorbent - normally rinsed with 250 µL elution solvent followed by 250 µL washing solvent. In critical cases, rinsing is done up to five times. This is, of course, to minimize carry-over (Blomberg, 2009; Barroso et al., 2012).

Its application on a wide range of BAs determination has been very successful, reporting great recoveries when few microliters of urine or plasma are used. Table 5 resumes the conditions for the pre-concentration, including sorbent type and procedure steps.

3.4. Solid-phase Microextraction (SPME)

Solid-phase microextraction (SPME) was also developed to address the need for rapid sample preparation both in the laboratory and on-site (Pawliszyn, 2002). The method was first reported by Arthur and Pawliszyn in 1990 (Arthur & Pawliszyn, 1990) and is now widely accepted, with a constantly rising number of applications highlighting the advantages on preparation time, solvent purchase, disposal costs and the possibility of improvement of detection limits (Prosen & Zupančič-Kralj, 1999; Kataoka et al., 2000).

The process involves the performance of two basic steps: partition of analytes between the extraction phase and the sample matrix and desorption of concentrated extracts into an analytical instrument (Risticevic et al., 2009).

The relatively simple device looks like a modified syringe consisting of a fibre holder and a fibre assembly, the latter containing a 1-2 cm long retractable SPME fibre (Vas & Vekey, 2004).

Commonly, a fused silica fibre is coated with a stationary phase. The fibre is exposed to an aqueous or gaseous sample until equilibrium is established between the analyte in the sample and on the fibre. Analyte desorption from the fibre coating can be performed by heating the fibre in the injection port of a GC or GC-MS, or by loading solvent into the desorption chamber of the SPME-HPLC interface, and then the analytes are transferred directly to the column for analysis (Kataoka et al., 2000). The choice of a specific coating is usually dependent on the chemical structure of the analytes to be extracted. The needed selectivity is based on polarity and volatility differences among molecules. In addition to commercial coatings, “custom made” fibres have been developed for the

extraction of specific analytes. However they can also be used to extract more polar compounds after optimizing extraction conditions, such as pH, salt concentration and temperature (de Fatima Alpendurada, 2000; Barroso et al., 2012).

Two types of fibre SPME techniques can be used to extract analytes: headspace (HS)-SPME and direct immersion (DI)-SPME. In HS-SPME, the fibre is exposed in the vapor phase above a gaseous, liquid or solid sample, while in DI-SPME the fibre is directly immersed in liquid samples. Agitation of the sample is usually necessary and is often carried out with a small stirring bar to increase the rate of equilibration (Kataoka et al., 2000).

As the extraction time is dependent on the partition coefficient of the analyte and on the samples' agitation, in order to extract the maximum amount of analyte, the equilibrium time has to be reached, which can be considered too long and impractical for many compounds (Kataoka et al., 2000).

Sample temperature has a double impact: at higher temperatures, diffusion coefficients in water are higher and the extraction time is shorter, but the partition coefficients are also lower. Heating of the sample is therefore not recommended and ambient temperature is applied for the direct SPME (Kataoka et al., 2000; Ulrich, 2000; Barroso et al., 2012). The pH of the sample is of crucial importance for slightly acidic or basic compounds. Extraction is more effective if these compounds are kept undissociated, but care must be taken regarding the fibres: for example, the polydimethylsiloxane phase is not resistant under pH values below 4 or above 10 (Kataoka et al., 2000). Addition of a soluble salt into the sample to promote a salting-out effect is expected to increase the ionic strength of the solution. However, this effect is less clear than the pH effect, and it can be important to compensate for a variable salt concentration of biological samples, make organic compounds less soluble, and the partition coefficients several times higher (Kataoka et al., 2000; Ulrich, 2000). Commonly added salts are NaCl or Na2SO4, and it is not recommended adding more than about 10% of NaCl, for example (Kataoka et al., 2000;

Ulrich, 2000). A drawback might be a loss of fibre selectivity (Kataoka et al., 2000).

Desorption time should be as short as possible, and carryover effects must be excluded. Thus, the highest temperature without coating damage associated and the smallest diameter of the injector insert should be applied (Ulrich, 2000).

However, two important recent variants have been developed, in-tube SPME and stir bar sorptive extraction (SBSE); nevertheless, fibre extraction is the most widespread SPME approach and its device continues to be the most widely used format (Risticevic et al., 2009; Vas & Vekey, 2004). Table 6 reviews publications where SPME was applied for the determination of BAs, including type of fibre type and optimized conditions.

3.5. Micro-Solid Phase Extraction (µSPE)

Another miniaturized form of SPE is the µSPE, also commonly known as micropipette tip-based SPE or disposable pipette extraction (DPX). DPX is based on the same principles and presents some similar steps to the traditional SPE. SPE is based on the separation promoted by affinity as well as DPX which is a solid-phase extraction device that uses loosely contained sorbent inside a pipette tip fitted with a screen. This device provides faster extraction times because conditioning steps are not required and differs from other SPE devices because the extraction efficiency is not based on flow rates as in most SPE devices, but instead is based on the equilibration time following the mixing of the sample solutions with the sorbent (Ellison et al., 2009; Bordin et al., 2016). The interferents are concentrated on the sorbent and a clean extract is dispensed, thus reducing the need for solvent evaporation (Barroso et al., 2012; Fernandes et al., 2014).

µ-SPE tips are constructed principally as automated pipette tips.

Table 5. Application of microextraction by packed syringe for BAs determination in biological samples

BAs	Sample	MEPS Bin	Conditioning	Load	Wash	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Reference
DA, E, NE, 5-HT, L-DOPA, 3MT, HVA, DOPAC, 5-HIAA, VMA	Urine (50 µL); Plasma (100 µL)	4 mg APS	3 X 100 µL methanol; 3 X 100 µL water	8 X	2 x 50 µL 0,1% formic acid	3 x 50 µL 0,1% formic acid in methanol	LC–MS	2 - 5 ng/mL	10 - 20 ng/mL	>84	Konieczna et al., 2016
NE, E, DA,	Urine (10 µL); Plasma (150 µL)	4 mg C18	3 X 100 µL methanol; 3 X 100 µL water	12 X	100 µL Buffer; 25 µL water:methanol (50:50; v/v)	100 µL (Plasma) or 200 µL (Urine) of mixture (2.5:97.5, v/v) of methanol and an aqueous solution of 30.0 mM citric acid and 0.5 mM OSA, adjusted to pH 2.92	HPLC-ECD	0.03 ng/ mL	0.1 ng/ mL	>85	Saracino et al., 2015
5-HT, DA, NE, E	Urine (500 µL)	4 mg C18	3 X 250 µL methanol; 3 X 250µL water	8 X	n.a	2 x 100 µL methanol	HPLC-ED	2 - 20 ng/mL	5 - 50 ng/mL	>91	Oppolzer et al., 2013
5-HT, DA	Urine (30 µL)	1 mg C8	n.a	4 X	100 µL Water	50 µL 0.1% formic acid in methanol	LC–MS/MS	1 ng/mL	50 ng/mL	50	El-Beqqali et al., 2007

3MT (3-Methoxytyramine), 5HIAA (5-Hydroxyindole-3-acetic acid), 5HT (5-Hydroxytryptamine), DA (dopamine), DOPAC (3,4-dihydroxyphenyl acetic acid), E (epinephrine), HPLC-ECD (high-performance liquid chromatography – electrochemical detection), HVA (homovanillic acid), LC-MS (liquid chromatography - mass spectrometry), LC-MS/MS (liquid chromatography tandem mass spectrometry), n.a (not available), NE (norepinephrine), NMN (normetanephrine), VMA (4-hydroxy-3-methoxymandelic acid).

Table 6. Application of solid-phase microextraction for BAs determination in biological samples

BAs	Sample	Fibre	pH	Salt	Time	Tempe-rature	Desorption	Analytical Instrumentation	LOD	LOQ	Recovery (%)	Reference
DA, 5-HT, NE	Urine (0.6mL)	85 µm polyacrylate (PA) fiber in immersion mode	n.a	n.a	45 min	room	into the injector set at 300◦C for 15 min	GC-MS/MS	0.38 – 13.5 ng/ mL	0.74 - 21.3 ng/ mL	n.a	Naccarato et al., 2014
HVA, VMA, 5-HIAA	Urine (0.05 mL)	85 µm polyacrylate (PA) fiber in immersion mode	n.a	NaCl	25.8 min	room	into the injector set at 290◦C for 10 min	GC-MS/MS	0.046 - 24.3 ng/ mL	0.063 - 49.6 ng/mL	n.a	Monteleone et al., 2013
DA, 5-HT	Urine (20 mL)	poly(VPBA-co-EDMA) in tube mode	9.0	Sodium meta-bisulfite (6 mM)	in tube at the rate of 100 µL/min. 10 min	n.a	formic acid	LC -MS/MS	1.2 ng/mL	5 ng/ mL	85.6	He et al., 2010
DA, E, NE	Urine Serum (3 mL)	1:6 (MAA: EDMA)	7.0	n.a	60 min	n.a	immersing the fiber in 180 µL of acetone: acetic acid (7:3, V/V) for 15 min	CE–UV	4.8 -7.1 nmol/L	n.a	>85%	Zhang et al., 2012

5HIAA (5-Hydroxyindole-3-acetic acid), 5HT (5-Hydroxytryptamine), CE-UV (capillary electrophoresis – ultraviolet detection), DA (dopamine), E (epinephrine), EDMA (Ethylene dimethacrylate), GC-MS/MS (gas chromatography – tandem mass spectrometry), HVA (homovanillic acid), LC-MS/MS (liquid chromatography tandem mass spectrometry), MAA (methacrylic acid), n.a (not available), NE (norepinephrine), VMA (4-hydroxy-3-methoxymandelic acid).

Compared to the traditional 96-well SPE block, the μ-SPE tips merely require the repetition of the aspirate and dispense functions of an automated liquid handler. The advantages of this are two-fold, it is simple to perform and there are multiple interactions of the sample with the extraction medium.

The small bed volume of the μ-SPE tips allows for efficient release of analytes from sorbent with minimal elution volumes.(Shen et al., 2006).

Other advantages of this technique are the elimination of a vacuum manifold which enables true walk-away automation and the fact that it can perform exhaustive extractions simply by passing the sample back and forth over the sorbent bed (Shen et al., 2007).

Several modified sorbents containing both cation-exchange and reversed-phase mechanisms can be applied to facilitate the retention of basic and acidic/neutral analytes, respectively, however this technique is relatively new, the amount of extracting phases commercially available and the high cost compared to the traditional solid phase cartridges represent a limitation to its applicability in routine analysis (Bordin et al., 2016).

Saraji et. al (Saraji & Shahvar, 2016) successfully synthesized aminophenylboronic acid-functionalized magnetic nanoparticles for the selective micro solid-phase extraction of norepinephrine, epinephrine and dopamine in human urine and plasma. The effects of experimental parameters such as the sorbent amount, sample pH, extraction and desorption conditions on the extraction efficiency of the sorbent were investigated. HPLC with fluorescence detection was used and the linearity range of the method was 0.04-10 ng mL−1 for norepinephrine and epinephrine, and 0.06–25 ng mL−1 for dopamine. The recovery of the analytes was in the range of 96.8-97.5% and 86.3-88.1% for urine and plasma samples, respectively.

3.6. Molecularly Imprinted Polymers (MIPs)

One of the tools to create highly selective extraction procedures is the incorporation of biomolecules and biological-like processes in the

methodologies. However, despite of the specificity of these procedures, natural biomolecules usually are expensive and chemically unstable. Synthetic materials such as molecularly imprinted polymers (MIPs) are valid alternatives to these biological matrices (Augusto et al., 2010; Barroso et al., 2012).

MIPs are polymers formed in the presence of a template molecule. Removal of the template from the polymer matrix creates complementary binding sites with affinity and selectivity for the template molecule. They are attractive materials capable of molecular recognition owing to their versatility, ease of preparation, and robust physical and chemical properties (Rushton & Shimizu, 2012).

These materials are obtained by polymerizing functional and cross-linking monomers around a template molecule, leading to a highly cross-linked three-dimensional network polymer. The monomers are chosen considering their ability to interact with the functional groups of the template molecule (Sarafraz-Yazdi & Amiri, 2010).

The concept of molecular imprinting was originally proposed as a possible mechanism for the production of antibodies by living systems. The synthesis of antibodies was based on the use of the aggressor chemical molecules as templates. Due to the high specific nature of the interaction between the molecularly imprinted materials and selectable molecules, they have been employed in several analytical techniques, including as stationary phases for liquid chromatography, capillary electrophoresis and capillary electrochromatography, and on immunoassay determinations. They are especially helpful when selective extraction of analytes is deemed necessary and where commercial sorbents lack selectivity (Augusto et al., 2010).

MIPs can be synthesized following three different imprinting approaches, called non-covalent, covalent and semi-covalent (Caro et al., 2006). The covalent approach involves the formation of reversible covalent bonds between the template and monomers before polymerization. The non-covalent approach is based on the formation of relatively weak non-covalent interactions (i.e., hydrogen bonding, ionic interactions) between template molecule and selected monomers before polymerization. The

non-covalent approach is the most widely used for the preparation of MIPs thanks to its versatility. However, in parallel, such versatility is also the origin of some of the drawbacks attributed to MIPs. In this sense, the necessity of using a high amount of functional monomer leads to the formation of non-selective binding sites (Tamayo et al., 2007).

In the last decade, one of the most promising technical applications based on the use of MIPs has been molecularly imprinted solid-phase extraction (MISPE). The use of MIPs in SPE is advantageous mainly when a selective extraction must be performed and the commonly used sorbents lack selectivity. Then, MISPE allows not only the analyte to be pre-concentrated but also the other compounds present in the sample matrix to be removed (Caro et al., 2006).

This required selectivity was also provided in SPME, primarily classified as MIP-coated fibers (polymeric membranes) and MIP rodlike fibers (polymeric monoliths) (Zhang et al., 2013).

Compared to natural receptors, MIPs not only demonstrate comparable molecular selectivities but they are also more robust and reusable, and less expensive to prepare. However, some drawbacks to MISPE have restricted its widespread application. MISPE is normally used in cartridge mode, which often results in a tedious column packing procedure, high backpressure and a low flow rate. In order to avoid these disadvantages several attempts have been made to develop an on-line MISPE system, or to prepare a monolithic column coupled to a chromatographic system. Providing magnetism to the MIP and then using magnetic separation is another promising alternative (Bouri et al., 2012).

Although MIP has been shown as important materials for sensing and separation of Bas, few research has been carried out for the extraction of BAs by using MIP fibres (Zhang et al., 2012b).

Luliński et al., (2016) reported a perfect example of an efficient molecularly imprinted solid-phase extraction protocol that was developed for the separation of dopamine from human urine. The authors also presented a new strategy for the selective determination of dopamine in the presence of norepinephrine and epinephrine in human urine. Other applications of MIPS to pre-concentrate BAs are shown on Table 7.

Table 7. Application of molecularly imprinted polymer as sample preparation to determine BAs in biological samples

BAs	Sample	MIP	Association	Loading	Washing	Elution	Analytical Instrumen-tation	LOD	LOQ	Recovery (%)	Reference
DA	Urine (1.5 mL)	MIPD (4-(2-aminoethyl)aniline imprinted polymer)	MISPE	Methanol/water (85:15 v/v)	Methanol/water (85:15 v/v)	0.04 M aq. Ammonium acetate/methanol (70:30 v/v)	HPLC-FLD	0.166 µmol/L	0.50 µmol/L	91.3 - 101	Lulinski et al., 2016
MT, NME, E, NE	Urine (2 mL)	coplymerization of MAA and EDMA, Fe(3)O(4) magnetite	MMIP	acetic acid (1%) PBS (pH 8)	n.a	acetic acid (1%)	CE-CL	0.04-0.06 µM	0.12-0.21 µM	>92	Bouri et al., 2012
DA, NE, E	Urine (3 mL) Serum (3 mL)	coplymerization of MAA and EDMA using E as a template in a capillary mold	MISPME	ACN	ACN	acetone: acetic acid (7:3, V/V)	CE-UV	<0.0074 µmol/mL	n.a	>85	Zhang et al., 2012b
3-MT, 5-HT, DA	Urine (2 mL)	n.a	AFFINIMIP™	acetic acid–ammonia buffer (pH 7, 25 mM)	acetic acid–ammonia buffer; ACN	MeOH–AcOH (99/1, v/v)	CE-UV	n.a	<46 nmol/L	>40	Claude et al., 2011

CE-CL (capillary electrophoresis - chemiluminescence detection), CE-UV (capillary electrophoresis – ultraviolet detection), DA (dopamine), E (epinephrine), EDMA (Ethylene dimethacrylate), HPLC-FLD (high-performance liquid chromatography - fluorescence detection), MAA (methacrylic acid), MIP (molecularly imprinted polymer), MISPE (molecularly imprinted solid phase extraction), MISPME (molecularly imprinted solid phase microextraction), MMIP (magnetic, molecularly imprinted polymer), MT (3-methoxytyramine hydrochloride), n.a (not available), NME (normetanephrine hydrochloride).

3.7. Micro Total Analytical Systems (μ-TAS)

As the present chapter reports, current trends in analytical chemistry are highly focused on improvement in the quality of analytical results, introduction of new technological developments with analytical use and, especially, miniaturization, simplification and automation of the whole analytical procedure, bearing in mind that the final objective is developing micro total analysis systems (Herrera-Herrera et al., 2010).

Micro total analysis systems (μ-TAS), or lab-on-a-chip designs, have been widely used to perform chemical and biochemical analyses and are also recognized as powerful tools for genomics, proteomics, and metabolomics (Tsukagoshi et al., 2005). μ-TAS devices offer highly efficient platforms for simultaneous analysis of a large number of biologically important molecules. These miniaturized and integrated devices are of great interest in the fields of analytical and bio-analytical chemistry (Cakal et al., 2010).

Chip-based devices offer attractive features, such as the potential of fabrication of highly multiplexed systems with zero-dead volume interconnections, automation, mass-production. On-line integration of sample treatment into a μ-TAS still represents one of the remaining obstacles towards achieving fully miniaturized systems. The variation in samples to be analyzed and the need for the compatibility of the pretreatment technique with the analytical device to which it is coupled, are the reasons for the obstacles (Altun et al., 2015).

In all cases, for ideal analytical measurements, these (micro) systems can be characterized by:

- Portability and self-operation, in order to avoid or to reduce the sampling step and allow the possibility of performing field tests;
- Ease of system maintenance and long lifetimes, or reusability at low price alternatively;
- Self-calibration incorporated in the system;
- Possibility of incorporating quality-control activities, in order to assure a reliable response (Ríos et al., 2013).

Ideally, TAS performs all the component stages of a complete analysis in an integrated, automated fashion. These stages can include sampling, sample pre-treatment, chemical reactions, analytical separations, analyte detection, product isolation and data analysis (Ríos et al., 2013). However, there are a number of issues requiring careful consideration when developing microsystems: the difficulty of surface engineering and exact control of fluids in microchannels, detection limits, sample preparation, increased integration, and the question of whether chips are to be reused. These issues need to be properly addressed for μ-TAS devices to be used in a wide range of applications. With all these efforts, the features of μ-TAS - integration of all laboratory equipment functions on a single chip, the commercialization of truly hand-held, easy-to-use, disposable, analytical μ-TAS instruments - are expected to be met in the near future (Lee & Lee, 2004).

Cakal et al., (2010) describe the application of μ-TAS in the analysis of catecholamines in which the preconcentration, separation, and detection steps were integrated in a single microchip. Catecholamine analysis, both microchip electrophoresis and integrated processing, evaluated a variety of channel lengths and designs. Separation conditions for catecholamines were examined first in a microchip electrophoresis device, then chambers for preparation of monolithic disks were incorporated into the microchips at the injection and separation channel intersection for total sample processing (Cakal et al., 2010).

4. DERIVATIZATION

Bioanalysis of BAs requires highly selective and sensitive analytical techniques due to the susceptibility to oxidation processes and to the very polar character of these compounds (Konieczna et al., 2016).

For some classes of compounds, such as amines and compounds with hydroxyl moieties, a derivatization reaction is required before gas chromatographic analysis to increase volatility, reduce polarity and also to

improve chromatographic behavior of these compounds (Naccarato et al., 2014).

The derivatization of aliphatic amines is usually based on the use of commercially available reagents such as pentafluorobenzaldehyde or pentafluorobenzoyl chloride, N-hydroxylsuccinimidyl phenylacetate, N-hydroxylsuccinimidyl phenylacetate, N-succinimidyl benzoate, hexamethyldisilazane (HMDS) and N-methyl-bis (heptafluorobutyramide) (MBHFBA). Some studies proved that also alkyl chloroformates are strong and rapid derivatizing reagents for amino groups (Naccarato et al., 2014).

It is well known that HMDS reagent is an effective TMS reagent for selective reactions on hydroxyl and carboxylic groups, and it does not react with amine groups. MBHFBA is then applied for the selective derivatization of the amine groups. Using these reagents, both acidic and basic biogenic compounds are successfully derivatized without any side products (Park et al., 2013).

The formation of the corresponding alkoxycarbonyl compounds is affected by the type and amount of alkylchloroformate and the amount of pyridine and can occur directly in aqueous media without the requirement of heating, thus simplifying sample pretreatment and derivatization procedures, therefore improving batch repeatability. Moreover, it is compatible with the use of solid phase microextraction (SPME) that allows the extraction of analytes directly to the aqueous phase (Naccarato et al., 2014; Monteleone et al., 2013).

Although underivatized CAs can be readily separated by either high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), detection by ultraviolet-visible (UV-Vis) absorbance does not have sufficient sensitivity or selectivity for their quantitative analysis in biological matrices where concentrations are often of the order of 10 nM or less (Zhu et al., 2003).

Among many methods, high-performance liquid chromatography method coupled to fluorescence detection (HPLC-FLD) has been mainly employed in the determination of low concentrations of CAs in biological samples. Although CAs show their own fluorescence (FL) (Wu & Lin, 2006; Liu et al., 2011), it is relatively weak and cannot be used for

sensitive assays of biological samples. CAs possessing o-dihydroxy and amino groups can react with meso-1,2-diphenylethylenediamine (DPE), trihydroxyindole (THI), O-phthalaldehyde (OPA) or fluorescamine, increasing sensitivity for their detection (Liu et al., 2011; Chan et al., 2000b).

The main chemistries for pre- and post-column fluorescence derivatization of CAs are based on:

- on the reactivity of their primary amines towards fluorescing reagents such as fluorescamine or OPA;
- or on the oxidation of their catecholic group followed either by a base-catalyzed tautomerization to form highly fluorescent THI derivatives;
- or by reacting the oxidation product with ethylenediamine or DPE (Fotopoulou & Ioannou, 2002).

DPE increases the sensitivity of the CAs, however, the reaction products are only stable for 30 min after the reaction. When THI is used, the HPLC requires rather complicated instrumentation for the postcolumn derivatization and cannot be applied to DA. Derivatization with OPA and fluorescamine increases the sensitivity for NE and DA, but E is not measured because only primary amines are derivatized by this reaction (Chan et al., 2000b). A significant increase in the sensitivity of the methods using fluorescent derivatives of catecholamines with DPE was achieved by utilizing post-column peroxyoxalate chemiluminescence detection (Fotopoulou & Ioannou, 2002).

Aromatic glycinonitriles (AGNs) and aromatic methylamines (AMAs) react with CAs to give highly fluorescent derivatives. The reaction of their reagents with CAs proceeded in neutral and slightly alkaline media under mild conditions in the presence of ammonium molybdate and sodium periodate for AGNs and potassium hexacyanoferrate (III) for AMAs. 2-phenylglycinonitrile (PGN) and benzylamine (BA) are found to be good fluorescence derivatization reagents in terms of sensitivity and selectivity (Nohta et al., 1997).

9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) is commonly used as an amino group protector in organic synthesis and a derivatizing agent for amino acid analysis. The advantages of using FMOC-Cl as a derivatizing agent are the following: reaction is straightforward, rapid and can be performed at ambient temperature; the reaction products are stable at room temperature and, more importantly, FMOC-Cl can react with both primary and secondary amines (Chan et al., 2000b).

In recent years, activated esters with succinimidic residues, used as fluorescent derivatizing reagents, have been described in the analysis of amino acids and peptides by HPLC. As example, hydroxysuccinimidyl-3-indolylacetate (SIIA) was synthetized and applied by Wang et. al in 1999 (Wang et al., 1999). These reagents react with primary and secondary amino functions easily and specifically and the excess reagents are hydrolyzed to the corresponding carboxylic acids shortly after amine derivatization (Wang et al., 2000).

The derivatization reagent 4-(1-pyrene)butanoic acid N-hydroxy-succinimide ester was shown to react with primary and secondary amino moieties of polyamine molecules. The resulting polypyrene-labeled derivatives of polyamines provided intramolecular excimer fluorescence at the wavelength region of 440-520 nm, which was shifted markedly to the higher emission wavelengths as compared to the wavelengths of the non-derivatized pyrene monomers themselves and monopyrene-labeled monoamines (360-420 nm). This chemistry allowed analysing selectively polyamines even in the complex samples containing monoaminergic compounds. More recently, it was found that 4-(1-pyrene)butanoyl chloride (PBC) reacts not only with polyamines, but also phenol compounds such as bisphenols (Yoshida et al., 2005).

Chemical derivatization of amine groups on amino acids with differential isotopic dimethyl labeling of N-terminal peptides with d(0)- and d(2)-, or d(0), 12C- and d(2), 13C-formaldehyde combined with MS analysis has also shown to enhance amino acid detection (Ji et al., 2010).

Table 8 resumes the most common derivatizing agents applied to determine BAs in urine and plasma. The derivatizing conditions (i.e., temperature, time and concentration) are also summarized.

Table 8. Derivatizing agents and conditions for BAs determination in biological samples

BAs	Sample	Derivatizing Agent	Concentration	Volume	Temperature	Time	Analytical Instrumentation	Reference
SPD, SPM, PUT, CAD	Urine	FNBT	n.a	200 µL	60ºC	20 min	LC-ITMS/MS	Ibarra et al., 2015
DA, NE, 5-HT	Urine	Propyl chloroformate	n.a	200 µL	n.a	15 min	GC-MS/MS	Naccarato et al., 2014
HVA, VMA,5-HIIA	Urine	Ethyl chloroformate	n.a	18.75 µL	n.a	1 min	GC-MS/MS	Monteleone et al., 2013
DOPAC, 5HT, HVA, VMA, 5-HIIA	Urine	MTBSTFA	n.a	20 µL	90ºC	30 min	GC-MS	Nguyen et al., 2013
L-DOPA, DA, E, NE, NMN, DOPAC, 5HT, HVA, VMA	Urine	HMDS	n.a	40 µL	40ºC	10 min	GC-MS	Park et al., 2013
		MBHFBA	n.a	20 µL	80ºC	5 min		
NE, E	Plasma	D4-acetaldehyde	n.a	180 µL	37ºC	60 min	LC-MS/MS	Zhang et al., 2012a
DA, NE, E, L-DOPA	Urine	DPE	0.1 M	60 µL	30 ºC	20 min	HPLC-FLD	Liu et al., 2011
DA, NE, E, 5-HT, MN	Urine	PFOEI	100 mM	200 µL	60ºC	10 min	HPLC-FLD	Sakaguchi et al., 2011
DA, E, NE	Urine	FITC	n.a	n.a	room	12 h	CE-ICFO-LED-IFD	Diao et al., 2011
NE, E	Plasma	D4-acetaldehyde	20%	200 µL	37ºC	25 min	LC-MS/MS	Ji et al., 2010
SPD, SPM, PUT, CAD	Plasma	SIFA	2 mM	100 µL	45ºC	30 min	HPLC-FLD	Deng et al., 2008
DA, NE	Urine	NBD-Cl	40 mM	100 µL	60 º C	20 min	MEKC-LIF	Zhou et al., 2007
DA, NE, E, MN, NMN	Plasma, urine	FMOC-Cl	5 mM	150 µL	40ºC	10 min	HPLC-FLD	Lozanov et al., 2007

Table 8. (Continued)

BAs	Sample	Derivatizing Agent	Concentration	Volume	Temperature	Time	Analytical Instrumentation	Reference
DA, NE, E, 3MT, MN, NMN, 5-HT	Urine	PBC	5 mM	200 µL	60ºC	60 min	LC-FLD	Yoshida et al., 2007
DA, NE, E, 5-HT	Urine	SAMF	10−3M	30 µL	30ºC	7 min	MEKC-LIF	Cao, 2007
DA, 5-HT, NMN, NE	Urine	OPA	10 mM	100 µL	room	10-15 min	LC-FRET	Yoshitake et al., 2006
DA	Urine	NDA	10−3 M	100 µL	room	20 min	MEKC-LED	Huang & Lin, 2005
5-HT, 5-HIIA	Urine	Benzylamine	500 mM	12 µL	n.a	n.a	HPLC-FLD	Yoshitake et al., 2004
5-HT	Urine	FITC	10^{-2}M	400 µL	45ºC	2 h	CE-LIF	Román et al., 2004
DA, NE, E, MN, NMN	Plasma, Urine	NBD-F	50 mM	10 µL	50ºC	5 min	HPLC-FLD	Zhu et al., 2003
E, DA, IP	n.a	6-AMP	2 mM	100 µL	50ºC	40 min	HPLC-CL	Yakabe et al., 2002
NE, E, DA	Urine	SIFA	5 mM	1000 µL	45ºC	20 min	HPLC-FLD	Wang et al., 2000
E, NE, MN, NMN, DA	Urine	FMOC-Cl	3,75 mg/mL	200 µL	24ºC	15 min	LC- MS	Chan et al., 2000a
NE, E, DA	Plasma	m-CED	50 mM	200 µL	37ºC	25 min	HPLC-CL	Ragab et al., 2000
NE, E, DA	Plasma	SIIA	10 mM	1000 µL	60ºC	15 min	HPLC-FLD	Wang et al., 1999
5-HT, 5-HIIA	Urine	DPE	n.a	n.a	room	n.a	HPLC-FLD	Kai et al., 1998
NE, E, DA, IP	Urine	PGN and BA	0.75 M	100 µL	37ºC	40 min	HPLC-FLD	Nohta et al., 1997
DA, NE, E	Plasma, Urine	1,2-diphenylethylenediamine	n.a	n.a	n.a	n.a	HPLC-CL	Ragab et al., 1994
DA, NE, E	Urine; Plasma	MOED	0.1 M	50 µL	37ºC	40 min	HPLC-FLD	Umegae et al., 1990

BAs	Sample	Derivatizing Agent	Concentration	Volume	Temperature	Time	Analytical Instrumentation	Reference
NE, E, DA, NMN, MN, NMN, L-Dopa	Urine; Plasma	1,2-diphenylethylenediamine	n.a	n.a	n.a	n.a	HPLC-FLD	Nohta et al., 1989
NE, E, DA	Plasma	1,2-diphenylethylenediamine	n.a	n.a	n.a	n.a	HPLC-FLD	Mitsui et al., 1985

3MT (3-Methoxytyramine), 5HIAA (5-Hydroxyindole-3-acetic acid), 5HT (5-Hydroxytryptamine), 6-AMP (6-Aminomethylphthalhydrazide), BA (benzylamine), CAD (cadaverine), CE-ICFO-LED-IFD (capillary electrophoresis - in-column fiber-optic light-emitting diode-induced fluorescence detection), CE-LIF (capillary electrophoresis – laser induced fluorescence), DA (dopamine), DPE (meso-1,2-diphenylethylenediamine), E (epinephrine), FITC (fluorescein isothiocyanate), FMOC-CL (9-fluorenylmethyloxycarbonyl chloride), FNBT (4-fluoro-3-nitrobenzenotrifluoride), GC-MS (gas chromatography –mass spectrometry), GC-MS/MS (gas chromatography – tandem mass spectrometry), HPLC-CL (high-performance liquid chromatography with chemiluminescence detection), HPLC-FLD (high-performance liquid chromatography - fluorescence detection), HVA (homovanillic acid), IP (Isoprenaline), L-Dopa (levodopa), m-CED (1,2-bis(3-chlorophenyl)ethylene diamine), LC-FRET (liquid chromatography - intramolecular fluorescence resonance energy transfer detection), LC–ITMS/MS (liquid chromatography - ion trap tandem mass spectrometry), LC -MS (liquid chromatography - mass spectrometry), LC-MS/MS (liquid chromatography tandem mass spectrometry), MEKC-LED (modified micellar eletrokinetic chromatography - violet light emitting diode), MEKC - LIF (modified micellar electrokinetic chromatography-laser induced fluorescence), MN (metanephrine), MOED (meso-1,2-Bis(4-methoxyphenyl)ethylenediamine), MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide), n.a (not available), NBD-CL (4-chloro-7-nitro-2,1,3-benzoxadiazole), NBD-F (4-Fluoro-7-nitro-2,1,3-benzoxadiazole), NDA (aphthalene-2,3-dicarboxaldehyde), NE (norepinephrine), NMN (normetanephrine), PBC (4-(1-pyrene)butanoyl chloride), PFOEI ((2-(perfluorooctyl)ethyl isocyanate), PGN (2-phenylglycinonitrile), PUT (putrescine), SIIA (N-Hydroxysuccinimidyl-3-indolylacetate), SIFA (N-hydroxy-succinimidyl fluorescein-O-acetate), SPD (spermidine), SPM (spermine), VMA (4-hydroxy-3-methoxymandelic acid).

CONCLUSION AND FUTURE PERSPECTIVES

This chapter intended to review one of the most critical steps in analytical procedures regarding biogenic amines determination in human samples. Nowadays, classic extraction techniques are still widely applied to pre-concentrate these amines. However, the many efforts carried out during the last decades to improve this situation have resulted in the development of new and simpler analytical approaches. The field of clinical analysis has undoubtedly taken benefit from the use of microextraction techniques. The great advantages of these new techniques are related with the ability to use smaller initial sample sizes even for trace analyses, greater specificity or selectivity in extraction, possible automation or on-line coupling reducing manual operations and the use of small volumes or no organic solvents at all, which implies the reduction of analysis cost as well as the environmental pollution. The different chemical properties between biogenic amines will influence the choice of the chemical or physical support applied on their extraction. Factors such as proper solvent, solvent volume, pH, salt addiction, etc., usually require a previous optimization in order to improve the recoveries of these analytes. This improvement will result in the determination of trace amounts in biological specimens, something very characteristic of biogenic amines, such as cathecolamines.

The application of MIPs appear to be a good future trend for sample preparation due to the greater selectivity associated, which is important for the unambiguous identification of the analytes. Other microextraction techniques such as hollow-fiber liquid-phase microextraction, in-tube SPME, thin-film microextraction or stir bar sorptive extraction may be excellent options to apply in this field.

Finally, a promising procedure that could be used in this field is the in vivo sampling using SPME; however, this technique is much more demanding than conventional SPME, since the used devices must be biocompatible and also sterilizable.

Furthermore, there is no doubt that the association of miniaturized techniques with highly sensitive analytical instrumentation such as

Orbitrap, HR-MS/MS or QTOF will allow the detection of minimum levels of biological amines, and this will be extremely valuable in clinical scenarios.

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Chapter 3

OCCURRENCE AND HEALTH IMPLICATIONS OF DIETARY BIOGENIC AMINES

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ABSTRACT

Biogenic amines occur naturally at low concentrations in food and raw materials used for food production. They have important physiological functions in humans, animals, plants and microorganisms. Elevated dietary concentrations of biogenic amines are the result of bacterial enzymatic activities. In combination with proteolytic activities particularly in protein rich raw materials and foods, very high concentrations can be formed. Notably, recurrent food poisoning cases are most often reported for fish and products thereof with high histamine concentrations for many years. Besides acute toxic effects, mutagenic potential of biogenic amines by formation of N- and C-nitroso compounds such as nitrosamines has been described. Biogenic amines are

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temperature stable during food processing and measures to degrade already formed amines in food are of limited effectiveness. Technological measures to prevent a formation and accumulation of biogenic amines in food including good hygiene and manufacturing practices are well known. This comprises the whole food chain until consumption. The primary production stage is important for optimal raw materials quality including for example appropriate fish harvest practices and strict continuous temperature control. This mini review focuses on the relation of occurrence of dietary biogenic amines and associated adverse health effects following consumption of implicated food.

Keywords: biogenic amines, food, histamine, tyramine, health, risk, occurrence

1. INTRODUCTION

Biogenic amines are low molecular weight organic basis with biological activity and important physiological functions in humans, animals, plants and microorganisms. They are sources of nitrogen and precursors for the synthesis of hormones, alkaloids, nucleic acids and proteins. They are formed and degraded as a result of normal metabolic activity mainly by enzymatic decarboxylation of amino acids (Guggenheim, 1951; Smith, 1981; Wortberg & Woller, 1982; Askar & Treptow, 1986; Taylor, 1990; Karovicová & Kohajdová, 2005). Monoamine and diamine oxidases play a major role in amine degradation in the human body. Biogenic amines do not usually represent health hazards to individuals unless large amounts are digested, or the natural mechanism for catabolism is inhibited (Malone, 1986; Halász et al., 1994; Shalaby, 1996; Silla Santos, 1996; Bodmer et al., 1999; McCabe-Sellers et al., 2006).

Important biogenic amines occurring in food are heterocyclic amines such as histamine originating from histidine and tryptamine from tryptophan; aromatic amines as tyramine are formed by decarboxylation from tyrosine and β-phenylethylamine from phenylalanine or aliphatic amines as putrescine formed from ornithine or arginine, and cadaverine

from lysine (Joosten, 1988; ten Brink et al., 1990; Shalaby, 1996; Sarkadi, 2009).

The biogenesis of amines in food depends on the availability of precursor compounds such as free amino acids and favorable environmental conditions such as pH and temperature. Proteolysis in food increases the pool of precursor components, mainly available amino acids for a potential biogenic amine formation. Provided that decarboxylase-positive microorganisms are present in sufficient numbers under optimal enzymatic conditions high biogenic amine concentrations can accumulate (Leuschner et al., 1998; ten Brink et al., 1990; Torrani et al., 2008).

High levels of biogenic amines are reported in the context of microbiological spoilage of foods or in fermented foods (Maga, 1978; Smith, 1981; Pfannhauser & Pechanek, 1984; Askar & Treptow, 1986; Stratton et al., 1991; Nout, 1994; Bodmer et al., 1999; Sarkadi, 2009). Biogenic amine formation is described as a protective mechanism for bacteria against acidic environments (Arena & Manca de Nadra, 2001). Amino acid decarboxylases may be present in gram-positive and gram-negative bacteria and in yeasts (Ryser et al., 1984; Joosten & Northold, 1987; Suzuki et al., 1991; Straub et al., 1995; Caruso et al., 2002; Suzuki & Fausto, 2003; Takahashi et al., 2003; Coton & Coton, 2005). Bacterial species of many genera are able of decarboxylating one or more amino acids, wherebythe amine forming potential of bacteria is strain dependent, highly variable among strains and needs to be confirmed case by case (Edwards et al., 1985; Landete et al., 2008).

Prevention of an accumulation of biogenic amines is the most effective means to control their dietary occurrence. This can be achieved mainly through the implementation of good hygiene and manufacturing practices along the whole food chain including its raw materials (Bodmer et al., 1999; Bover-Cid et al., 2001; 2009; Visciano et al., 2012; Bacellar Ribas Rodriguez et al., 2014). Since biogenic amines are heat stable, the use of enzymes, such as diamine oxidase (DAO) degrading biogenic amines, and/or the use of bacteria that possess these enzymes, are potential means to degrade already formed biogenic amines. However, the enzymatic conditions in food e.g., availability of oxygen, pH and temperature are

often not favorable to enable significant biogenic amine degradation of already formed amines (Leuschner et al., 1998; Leuschner & Hammes, 1998a,b; Fadda et al., 2001; Naila et al., 2010; Guarcello et al., 2016).

2. Elevated Biogenic Amine Concentrations in Food

Natural occurrence of biogenic amines in fresh and processed foods of good hygienic quality is generally low (Yoshinaga & Frank, 1982; Bardocz et al., 1993; Maijala et al., 1993; Bodmer et al., 1999; Novella-Rodríguez et al., 2002). They were therefore used as spoilage indicators however this may not be representative in meat, cheese and other in particular fermented food products (Mietz & Karmas, 1977; 1978; Karmas, 1981; Slemr, 1981; Edwards et al., 1983; Vidal-Carou et al., 1990).

High concentrations can be formed and biogenic amines can accumulate as a consequence of poor hygiene during processing and storage under conditions that favor uncontrolled bacterial growth in particular in proteinaceous foods where proteolysis and amino acid decarboxylation could go hand in hand. The occurrence of biogenic amines as process-induced toxicants in fermented food such as dairy products, meat and meat products, fish, wine, beer and sauerkraut has been reported for a long time (Ferencik, 1970; Pechanek et al., 1980; Smith, 1981; Pechanek et al., 1983; Sarkadi, 2009). Biogenic amines are very heat stable. Histamine was inactivated by heating at 116°C for 90 minutes (Rice et al., 1976).

Foods containing proteins and their metabolites such as peptides and amino acids provide a pool of precursor compounds for biogenic amine formation which can increase by proteolytic microbial or biochemical activities. Uncontrolled increased microbial activities including growth may occur in cases were appropriate manufacturing and storage conditions are not respected and could lead to spoilage and an associated biogenic amine formation. The presence of biogenic amines above a certain level is

considered indicative of undesired microbial activity and maybe an indicator of microbial spoilage (Silla Santos, 1996). However, the presence of biogenic amines in food does not necessarily correlate with the degree of microbial spoilage because spoilage microorganisms may not be responsible for biogenic amines formation (ten Brink et al., 1990).

Microbial activities and growth during food processing are in many foods essential and desired for the final product properties. Fermented food products undergo a fermentation process as part of their manufacturing process and can be derived from a wide range of raw materials such as meat, milk, fruits or vegetables. The fermentation can be performed by spontaneous, uncharacterized microorganisms or by added defined and selected starter cultures specifically developed and selected for a certain product. Amino acid decarboxylase activities may or may not be present amongst strains used as starter cultures. It is therefore recommended to include the absence of this enzymatic activity as a selection criterion for starter cultures (Landete et al., 2008; Torrani et al., 2008).

Histamine and tyramine concentrations of up to five g/kg and three g/kg, respectively were reported in fish and fish products and are of health concern (Sinell, 1978; Pechanek et al., 1983). Cheeses in particular when spoiled, aged and ripened often have high concentrations of biogenic amines (Edwards & Sandine, 1981). Histamine concentrations of up to 2.6 g/kg and for tyramine of 2.17 g/kg with mean concentrations of 398 mg/kg histamine and of 290 mg/kg tyramine were reported (Sinell, 1978; Pechanek et al., 1983; Anskar & Treptow, 1986). In dry retail sausages histamine concentrations of 654 mg/kg dry matter and tyramine concentrations of 1506 mg/kg dry matter were found (Vandekerckhove, 1977; Bauer et al., 1989). Yeast extracts were also associated with high concentrations of histamine and tyramine of up to 2830 mg/kg and 2100 mg/kg, respectively (Blackwell et al., 1969; Anskar & Treptow, 1986; Smith, 1981).

Cheese is a proteinaceous fermented food with a risk to allow for microbial amine production. The amount of biogenic amines formed depends on a variety of factors such as the microflora, bacterial density, synergistic effects between microorganisms, level of proteolysis

(availability of substrate), pH, salt-in moisture level, ripening and storage temperatures. Biogenic amine formation may only reach hazardous levels under favorable enzymatic conditions and therefore cheeses with comparable microbiological profiles may differ enormously in their biogenic amine concentration and distribution (Cousin, 1982; Valsamaki et al., 2000; Novella-Rodríguez et al., 2003; Burdychova & Komprda, 2007; Komprda et al., 2007; Ladero et al., 2009; Linares et al., 2012). An increase of free amino acids from 546 mg/kg at day one of ripening to 3978 mg/kg after 120 days was described which increases available precursors for a potential biogenic amine formation (Leuschner et al., 1999; Valsamaki et al., 2000).

Control options for maintaining low biogenic amine contents in cheese are the use of good hygienic quality raw materials, good manufacturing practice and selected starter strains which do not have amino acid decarboxylase activities. In particular during undefined fermentation processes an early detection of bacterial amine formation would enable to initiate technological control measures (Fernández et al., 2006; Pintado et al., 2008; Elsanhoty et al., 2009; Lupo & Mozola, 2011). For processing of raw milk cheeses are the above precautionary measures particularly important (Fernández-García et al., 2000; Barbano et al., 2006).

In conclusion, an early detection of biogenic amine producing bacteria is important in the food industry (Hungerford, 2010; Bacellar Ribas Rodriguez et al., 2014; Ordóñez et al., 2016). In this respect, fast and reliable molecular methods have been developed for determination of biogenic amine producing bacteria on a routine basis (Landete et al., 2007; Torriani et al., 2008; Ferrario et al., 2012).

3. Toxic Effects Caused by Biogenic Amines

Most biogenic amines are pharmacologically active. Dietary biogenic amines are usually metabolized in the intestinal tract however adverse effects may occur under situations where the amine metabolizing activity of the body is over saturated due to an ingestion of high doses of amines or

where the metabolic activity is reduced due to the presence of specific inhibitors (Askar & Treptow, 1986; Taylor, 1986; Götz et al., 1996; Joosten and Nuñez, 1996).

Histamine is a powerful biologically active compound and can directly stimulate the heart, cause extravascular smooth muscle to contract or relax, stimulate both sensory and motor neurons, and control gastric acid secretion. It is therefore not surprising that histamine poisoning is often manifested by a wide range of symptoms. Characteristic symptoms affecting the cutaneous (i.e., skin) system include rash, urticaria, edema, and localized inflammation. Gastrointestinal involvement is characterized by nausea, vomiting, diarrhea, and abdominal cramps. Other symptoms include hypotension, headache, palpitations, tingling, flushing, and burning sensations in the mouth (Smith, 1981; Taylor et al., 1982; Sattler & Lorenz, 1987; Stratton et al., 1991; Sumner et al., 2004; Maintz & Novak, 2007). However, an involvement of histamine in nitrosamine formation and therefore a mutagenic potential has also been described (Karovicová and Kohajdová, 2005; Bulushi et al., 2009).

Maijala et al. (1996) reviewed foodborne intoxications caused by biogenic amines in Finland during 1983 to 1995. Symptoms reported for histamine as causative agent were primarily flushing and nausea followed by lip and tongue swelling, vomiting, headache and diarrhea. Histamine in foods is not necessarily hazardous. A detoxification system exists in the intestinal tract to metabolize ingested histamine to non-toxic products. It is composed of two distinct enzymes: diamine oxidase and histamine-N-methyl transferase (Shalaby, 1996). Notably, within the gastrointestinal tract, histamine is present at relatively high concentrations, particularly during inflammatory processes. However, its relative protective or pathogenic effects on inflammatory processes within the gut are still poorly defined (Smolinska et al., 2014). According to Sattler et al. (1988) approximately 20% of the European population regularly use DAO-inhibiting drugs and therefore have a high risk of developing food-induced histaminosis.

Histamine fish poisoning (HFP) is associated with consumption of scombroid fish (families *Scombridae* and *Scomberosocidae*), however also

non-scombroid fish caused also identical symptoms as did cheese and wine (Stratton et al. 1991; Bulushi et al., 2009). Lehane and Olley (2000) revisited histamine food poisoning and outlined that although there is compelling evidence to implicate histamine as the causative agent in HFP, there is not a straightforward dose-response relationship, as spoiled fish containing histamine seems to be more toxic than the equivalent amount of pure histamine dose orally. Nevertheless, there is still strong evidence that histamine is the main hazard (Sumner et al., 2002).

Besides histamine, tyramine has been implicated in adverse reactions involving headache and hypertensive crisis in patients taking monoamine oxidase inhibitors (Blackwell, 1963; Joosten, 1988; Stratton et al., 1991). Pharmacologically, phenylethylamine and tryptamine resemble tyramine which is also a neuro-sympathomimetic amine (Franzen & Eysell, 1969; Edwards & Sandine, 1981). Tyramine, phenylethylamine and tryptamine are vasoactive amines and their effects leads to increases in blood pressure by constricting the vascular system, increasing the heart rate and force of contraction of the heart (Smith, 1981; Til et al., 1997; McCabe-Sellers et al., 2006). Under normal situations, monoamines including tyramine, tryptamine, phenylethylamine, dopamine and serotonin are not hazardous to most individuals, because they are detoxified by a multiple enzyme system known as monoamine oxidase (MAO). However, if these mechanisms are impaired, the most typical symptoms of these intoxications are severe headache, or even migraine attacks and hypertension (Hanigton, 1970; Hanigton, 1974; Smith, 1981; Taylor, 1990). An adverse health condition is the ‘cheese reaction’, usually caused by high levels of tyramine in cheese (ten Brink et al., 1990; Smith, 1981).

Tyramine has been proposed as a major mutagen precursor in soya sauce and one of its nitrosated products has been shown to induce tumors in rats (Ochiai et al., 1984; Fuijta et al., 1987; González-Jiménez et al., 2017).

Tryptamine acts as a neuromodulator and vasoactive agent in the human body. Dose-response data on dietary tryptamine are scarce and neither a toxicological threshold value nor tolerable levels in foods have been established so far. Dose-response characteristics and toxicological

effects of tryptamine as well as tryptamine contents in food were reviewed, dietary exposure of Austrian consumers estimated, and risk-based maximum tolerable limits for food categories calculated. A dose without effect of eight mg per kg body weight and day was derived from literature data. In conclusion, dietary intake of tryptamine should not cause adverse health effects in healthy individuals. The assessment did not take into account the combined effects of simultaneously ingested biogenic amines, and increased susceptibility to tryptamine, for example due to reduced monoamine oxidase activity (Wüst et al., 2017).

Both putrescine and cadaverine have less pharmacological activity than the aromatic amines. Only after ingestion of very large amounts of putrescine and cadaverine are toxic effects observed. Intoxication symptoms reported are hypotension, bradycardia, dyspnoea, lockjaw and paresis of the extremities. The most important consequence of these compounds in food is probably the potentiation of the toxicity of other amines (Bjeldanes et al., 1978; Taylor, 1986; Joosten, 1988).

Finally, it should be mentioned that secondary amines such as putrescine and cadaverine can react with nitrite to form potentially mutagenic and carcinogenic nitrosamines and that this would be another reason to prevent accumulation of biogenic amines in food products (Askar & Treptow, 1986; Pfundstein et al., 1991; Bulushi et al., 2009).

4. Adverse Health Effects from Dietary Biogenic Amines

Histamine ingestion of around 100 mg per servings was reported to cause adverse effects (Lüthy and Schlatter, 1983; Askar & Treptow, 1986; Wöhrl et al., 2004). Lower histamine concentrations of 12 mg/kg in tuna products, of 26 mg/kg in herring sauce and of 70 mg/kg in cheese were implicated in histamine outbreaks in Finland (Maijala et al., 1996). An outbreak of histamine poisoning associated with consumption of Swiss cheese revealed average histamine levels of 187 mg/100 g (Taylor et al.,

1983). Factors such as resorption, degradation and additional factors affect the manifestation of adverse reactions following ingestion of dietary histamine (Rauscher-Gabernig et al., 2007). Tolerable histamine concentrations of 400 and 500 mg/kg for cheese and fermented sausage, respectively and for fish species other than those already regulated by the EU, the 'm/M' limits of 100 and 200 mg/kg were suggested (Rauscher-Gabernig et al., 2009). Potential toxic effects after ingestion of more than 100 mg (approx. 1 mmol) histamine or more than 400 mg (approx. 3 mmol) tyramine and as little as 3 to 4 mg (approx. 0.03 mmol) phenylethylamine may precipitate migraine attacks in susceptible persons (Joosten, 1988).

Despite all uncertainties, histamine levels above 500-1000 mg/kg food are considered potentially dangerous to human health based on concentrations found in food products involved in histamine poisoning (Taylor, 1983). Upper limits of 2 mg/l alcoholic beverages and threshold values of 100-800 mg/kg food for tyramine and of 30 mg/kg for phenylethylamine were suggested (ten Brink et al., 1990; Shalaby, 1996).

Beside tyramine, is histamine in cheeses certainly as well of concern as concentrations of up to 400 mg/g or 1300 (mg/kg) in relation to histamine poisoning outbreaks were reported (ten Brink et al., 1990; Stratton et al., 1991). In Dutch cheeses concentrations of up to 10 mmol/kg histamine, up to eight mmol/kg tyramine, up to seven mmol/kg putrescine and up to six mmol/kg cadaverine were reported (Joosten, 1988). However, not only cheese but also other types of food such as pickled herring and dry sausage could have these side effects of the use of MAO inhibitors (Joosten, 1988).

The European Food Safety Authority (EFSA) conducted a qualitative risk assessment of biogenic amines in fermented food. Data from the scientific literature, European Union related surveys, reports, a dedicated call for occurrence data via the EFSA website and already available food consumption data were considered. Based on limited published information, no adverse health effects were observed after exposure to the following BA levels in food (per person per meal): 50 mg histamine for healthy individuals, but below detectable limits for those with histamine intolerance, 600 mg tyramine for healthy individuals not taking

monoamine oxidase inhibitor (MAOI) drugs, but 50 mg for those taking third generation MAOI drugs or 6 mg for those taking classical MAOI drugs. For putrescine and cadaverine, the information was insufficient in that respect (EFSA, 2011).

A risk assessment regarding histamine and tyramine related to the consumption of dry fermented sausage by the Spanish population concluded that the risk of suffering hypertensive crisis or histamine intoxication by the healthy population due to tyramine or histamine intake, respectively, exclusively from dry fermented sausages, can be considered negligible. However, for individuals under treatment with MAOI drugs, the probability to surpass the safe threshold dose of 6 mg/meal was estimated at 34%. For patients with histamine intolerance, even the presence of this amine in food is not tolerable and it could be estimated that 7000 individuals per million could be at risk to suffer the related symptoms after consuming dry fermented sausages (Latorre-Moratella et al., 2017).

Tyramine is one of the most relevant vasoactive ("pressor") amines present in foods. Risk-based tolerable levels of this compound for certain food commodities were reported. Dose-response data indicated that the "no observed adverse effect level" (NOAEL) for healthy individuals is 200 mg per single oral administration. Based on this NOAEL and Austrian food consumption data, maximum tolerable levels for foods (high consumption scenario: female user, 60 kg body mass, 95 percentile) are 1 g per kg for cheese; 2 g per kg for fermented/raw cured meats; 950 mg per kg for fish (raw or processed) and 800 mg per kg for sauerkraut. Literature data indicated that, at least for cheese, these limits may be exceeded in practice. Under medication with monoaminooxidases inhibitors, a "safe-side" NOAEL of 5 mg per meal can be assumed, which may easily be exceeded even under "normal" nutrition conditions requiring some degree of consumer awareness (Paulsen et al., 2012).

5. Official Controls and Monitoring

Commission Regulation (EC) 2073/2005 and its amendments lays down food safety criteria for histamine in fishery products from fish

species associated with a high amount of histidine between 100 mg/kg (m) and 200 mg/kg (M) (n = 9, c = 2) and for fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine between 200 mg/kg (m) and 400 mg/kg (M) (n = 9, c = 2) (European Commission, 2005; 2008). Regulation (EC) No 853/2004 provides for fishery products a possibility to lay down freshness criteria and limits with regard to histamine and places the responsibility on food business operators to ensure that the limits with regard to histamine are not exceeded in the context of health standards for these products (European Union, 2004). Recently, the method for determining histamine in fish as mandated by Regulation (EC) No 2073/2005 was verified (Stroka et al., 2014).

The Rapid Alert System for Food and Feed (RASFF) of the European Commission is a daily updated information exchange database to share information regarding food safety concerns amongst its members (Rapid Alert System for Food and Feed, 2017). Such concerns are trigger in the case of histamine where the legal safety criteria are not met and found to be exceeded. RASFF notification between 2011 and 2016 were reviewed and summarized. Histamine is included in RASFF as a 'biocontaminant'. The methodology for data collection from the RASFF database was performed according to Leuschner et al., (2010).

In total there were 227 histamine notifications and 224 of them related to fish and products thereof with tuna as the most often implicated species (Table 1).

Table 1. Reported histamine notifications from 2011 to 2016 in RASFF

	2011	2012	2013	2014	2015	2016	Total
Fish and products	33	40	47	33	33	38	224
(of which were tuna	14	21	30	17	21	16	119)
Anchovy sauces	0	1	0	0	0	0	1
Yeast extract	0	0	1	0	0	0	1
Cheddar cheese	0	1	0	0	0	0	1
Total	33	42	48	33	33	38	227

The levels of biogenic amines in foods can vary greatly. Biogenic amine concentrations are variable within food and results depend on the sampling regime (Food and Drug Administration (FDA), 2011). After fish, cheese is the most implicated food item associated with biogenic amine food poisoning and reported outbreaks (Stratton et al., 1991; Leuschner et al., 2010). In the latter study, highest histamine concentrations of 5 g/kg in a tuna in oil product and of 1.87 g/kg in Swiss cheese were reported. In 2012, histamine concentrations of 1.2 g/kg cheddar cheese and of 1.4 g/kg anchovies were related to food poisonings and of 0.29 g/kg yeast extract in 2013. Reported histamine concentrations in RASFF of notifications from 2011 until the end of 2016 were summarized in Table 2.

Notably, 14 percent of notifications had histamine concentrations above 2 g/kg and for six percent only a qualitative indication was provided without quantification of highest levels whereby a further four percent did not report any concentrations. Therefore, for 10 percent of the notification, actual histamine concentrations were not reported.

Concerning 31 histamine notifications with concentrations above 2 g/kg, the highest concentration of 6.86 g/kg in thawed raw tuna related to a food poisoning outbreak.

Table 2. Histamine concentrations reported in RASFF for fish (2011 to 2016)

	Fish and products thereof	(product category)
Histamine concentrations (mg histamine/kg fish)	Notifications (n)	Percent (%)
≤200	11	5
>200 and ≤500	75	33
>500 and ≤1000	51	23
>1000 and ≤2000	33	15
>2000	32	14
Not reported	8	4
Reported as '>'*	14	6
Total	224	100

*[Reported as >100 mg/kg (2), as >200 mg/kg (2), as >250 mg/kg (1), as >350 mg/kg (2), as >500 mg/kg (2), as >800 mg/kg (3), as >1920 mg/kg (2)]

Concentrations of 5.3 g/kg, 5.1 g/kg in frozen raw and of 5.6 g/kg in chilled raw tuna were notified. Eight notifications were between 4-5 g/kg (defrosted tuna (2), frozen tuna (1), in chilled tuna (1), chilled vacuum packed tuna (1), chilled smoked tuna (1), anchovies in oil (2)), eight between 3 - 4 g/kg (frozen whole tuna (1), vacuum packed defrozen tuna (1), frozen tuna steaks (1), frozen tuna yellowfin tuna (1), chilled vacuum packed yellowfin tuna (1), chilled tuna fillets (2), anchovy fillets in oil (1)), nine between 2 - 3 g/kg (frozen mackerel (1), chilled mackerel fillets (1), frozen yellowfin tuna slices (1), defrosted tuna slices (1), tuna loins (1), canned tuna in olive oil (1), chilled tuna steaks (1), chilled tuna (1), chilled tuna loins (1)) and histamine levels for three notification were only qualified with being higher as 2.5 g/kg (chilled tuna (1), mackerel (1), canned anchovies (1)).

6. Food Poisoning and Reported Outbreaks

Reliable statistics do not exist because poisoning incidents are often not reported because of a mild nature of the illness, lack of adequate systems for reporting this foodborne diseases, or misdiagnosis of histamine poisoning with an allergic reaction. Food poisoning can also occur in conjunction with other potentiating factors such as drugs, alcohol and gastrointestinal diseases (Sarkadi, 2009).

In the past, outbreaks were mainly reported for histamine intoxications. Between 1976 and 1986, 258 incidents of suspected scombrotoxic fish poisoning were reported in Britain. Between 1987 and 1996 fresh tuna and canned tuna contaminated after processing became increasingly associated with incidents of scombrotoxic fish poisoning and 405 cases of suspected incidents were investigated with histamine concentrations of 50 - 1000 mg/kg (Bartholomew et al., 1987; Scoging, 1998). In Finland, between 1983 and 1994, 26 outbreaks involving 73 cases of suspected foodborne amine intoxications were reported whereby 17 outbreaks were attributed to histamine in tuna products with concentrations between 12 – 3360 mg/kg (Maijala et al., 1996). In Australia, 32 cases of seafood-borne illness were

reported between 1999 and 2000. Ten outbreaks causing 28 cases were attributed to histamine (Sumner et al., 2002).

From 2011 to 2016, in RASFF for the control type ‘food poisoning’ 282 notifications were reported of which 49 concerned fish and tuna (44) in particular. Three food poisonings occurred in 2011 with histamine concentrations of 480 - 3100 mg/kg tuna. In 2012, nine cases (eight were tuna) with concentrations between 253 - 5300 mg/kg. In 2013, ten reports (seven for tuna) with concentrations between 724 - 4375 mg/kg; in 2014 four outbreaks with 865 - 3700 mg histamine per kg tuna and 15 cases in 2015 (780 - 4700 mg/kg), whereby in 2016 seven of eight cases concerned tuna (400 - 5593 mg/kg). One RASFF notification related to anchovy sauce with histamine concentrations between 276 - 294 mg/kg, one to yeast extract with 900 - 1400 mg/kg and one food poisoning case was reported for cheddar with 1227 mg histamine per kg cheese. RASFF introduced the control types ‘food poisoning’ in 2008. During the years 2008 and 2010 the number of cases ranged from five to 12 which was similar to the cases reported thereafter until 2016 (Leuschner et al., 2010).

CONCLUSION AND OUTLOOK

In a wide range of foods mainly in fish, meat and fermented products as cheese and dry sausages high concentrations of biogenic amines are reported for many years. Most often histamine in fish is linked to food poisonings some of which are very sever including hospitalizations. This indicates that the food safety criteria in the European legislation for fish and products thereof remain essential.

Biogenic amines are often heterogeneously distributed in food and it is challenging to quantify their occurrence. The application of a variability factor to account for this uncertainty could be considered. A representative and statistically sound surveillance and monitoring system with regular, standardized and defined sampling and analysis along the whole food chain would provide more meaningful occurrence data.

Since it seems that highest concentrations are most often reported in fish, it needs to be established as to why such health threatening histamine concentrations can accumulate. Possibly fish harvest practices and subsequent temperature control needs to be considered.

Currently, acute tolerable intakes are considered in relation to potential adverse health effects caused by biogenic amines. However, since some biogenic amines are reported to be involved in mutagenic and cancerogenic pathways there seems to be a need to establish as to whether irreversible effects may occur following dietary biogenic amine ingestion considering mutagenic and/or cancerogenic human health endpoints. Chronic toxicological reference values in addition to acute reference values may need to be established in the future.

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Chapter 4

BIOGENIC AMINES IN FOOD: PRESENCE AND CONTROL MEASURES

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ABSTRACT

Biogenic amines (BA) or biologically active amines are low molecular weight nitrogenous compounds formed from amino acids by decarboxylation, or by amination and transamination of aldehydes and ketones. Due to their precursor amino acids structure and origin, BA are classified as either aliphatic diamines, aromatic biogenic amines or natural polyamines.

Biogenic amines in foods have relevance from both safety and quality standpoints. They may be found in a wide range of foods containing proteins or free amino acids including meat, dairy and fish products, vegetables, fruits, wine, beer, nuts and chocolate.

The biogenic amines most commonly found in foods are cadaverine, putrescine, tyramine, histamine, tryptamine, β-phenylethylamine, agmatine, spermine and spermidine, the two latter being endogenous amines. Additionally, octopamine and dopamine have been reported in meat and fish products.

In non-fermented foods the presence of cadaverine, putrescine and tyramine, are undesired and can be used as indication for microbial spoilage. On the other hand, in fermented foods, several lactic acid microorganisms involved in the fermentation process, particularly enterococci could produce biogenic amines.

Although BA could have an active role at the neurological level as neurotransmitters, their presence in food, particularly of histamine and tyramine are recognised as potential hazards causing intoxication and intolerance symptoms in healthy people. Furthermore, BA are potential precursors for the formation of carcinogenic nitrosamines.

This review will focus on some food products and the relationship between the presence of BA according to their origin, inducing factors and distinctive characteristics of the technological processes that could control BA production in: I) animal foods, such as meat and meat products, including dry-fermented sausages, fish and seafood products and dairy products, such as cheeses; II) plant foods, like vegetables, with emphasis on table olives; III) beverages, such as wines, beers and liqueurs.

Keywords: biogenic amines, presence in food, control measures

1. INTRODUCTION

The presence of biogenic amines (BA) in foods is a very important issue from both safety and quality standpoints. They may be found in a wide range of foods containing proteins and/or free amino acids including meat, dairy and fish products, vegetables, fruits, wine, beer, nuts and chocolate.

The BA most present in foods are cadaverine, putrescine, tyramine, histamine, tryptamine, β-phenylethylamine, agmatine, spermine and spermidine, the two latter being endogenous amines.

Since they naturally occur in organisms, spermine and spermidine are generally the most abundant amines in fresh foods, such as fruits, vegetables, milk, meat and fish products. Food products of vegetable origin are richer in spermidine, while spermine can be found in larger amounts in animal food products (Bover-Cid et al., 2014). In non-fermented foods the presence of cadaverine, putrescine and tyramine, are undesired and can be used as indication for microbial spoilage (Bover-Cid et al., 2001c). On the other hand, exogenous amines are generally more abundant in processed or stored foods, because they result from the microbial activity characteristic for these type of foods.

The quantification of BA in food samples has been performed mainly by chromatographic techniques, namely classical reversed-phase High Performance Liquid Chromatography (HPLC) (Bedia Erim, 2013; Onal et al., 2013). Several authors have developed specific methods to detect and quantify BA in different food matrices in the last years (Hornero-Méndez & Garrido-Fernández, 1997; Karovičová & Kohajdová, 2005; Roseiro et al., 2006; Cunha et al., 2011; EFSA, 2011; Almeida et al., 2012; Laranjo et al., 2016; Laranjo et al., 2017).

The current review will focus on some food products and the relationship between the presence of BA according to their origin, inducing factors and distinctive characteristics of the technological processes that could control BA production on: I) animal foods, such as meat and meat products, including dry-fermented sausages, fish and seafood products and dairy products, such as cheeses; II) plant foods, like vegetables, with

emphasis on table olives; and III) beverages, such as wines, beers and liqueurs.

2. Origin and Formation of Biogenic Amines

Biogenic amines or biologically active amines are low molecular weight nitrogenous compounds formed from amino acids by decarboxylation, or by amination and transamination of aldehydes and ketones.

According to their origin they may be considered either natural or endogenous (spermine and spermidine), when occurring naturally in the human organism, or biogenic or exogenous (histamine, serotonin, tyramine, β-phenylethylamine, tryptamine, putrescine, cadaverine and agmatine), which result from the activity of microorganisms (Bover-Cid et al., 2014).

Given the number of amine groups present they may be either monoamines (tyramine and β-phenylethylamine), diamines (histamine, serotonin, tryptamine, putrescine and cadaverine) or polyamines (spermine, spermidine and agmatine) (Smith, 1981).

Due to their precursor amino acids structure, amines are classified as either aliphatic (putrescine, cadaverine, spermine and spermidine), aromatic (tyramine and β-phenylethylamine) or heterocyclic (histamine and tryptamine) (Silla Santos, 1996).

Concerning their action in the human body, amines can be divided into vasoactive (tyramine, tryptamine, β-phenylethylamine, isoamylamine, histamine and serotonin) and psychoactive (norepinephrine, serotonin and dopamine) (ten Brink et al., 1990).

The most important amines that have been described associated with food products are: spermine, spermidine, cadaverine, putrescine, tyramine, histamine, tryptamine, β-phenylethylamine and agmatine (Bover-Cid et al., 2014; Suzzi & Torriani, 2015).

The synthesis of biogenic amines in food and beverages may be performed by bacterial enzymes that promote the decarboxylation of amino acids.

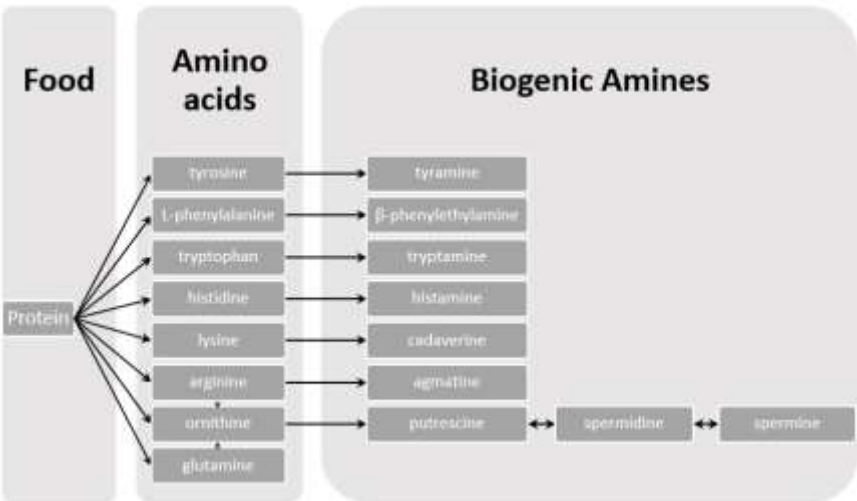

Figure 1. Most important biogenic amines present in food products and their origin amino acids. Diagram adapted from (Bover-Cid et al., 2014).

3. Microorganisms Related to the Formation of Biogenic Amines

Amine decarboxylases are synthesised by microorganisms present in foods (Halász et al., 1994), but not all. Although decarboxylation enzymes are not widely present in bacterial enzymatic systems, species of several genera, such as *Bacillus*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Salmonella*, *Shigella* and *Photobacterium* do have them. Food-fermenting lactic acid bacteria (LAB) are generally non-toxic and non-pathogenic. However, some strains of *Lactobacillus*, *Pediococcus* and *Enterococcus* are also able to produce BA by decarboxylation of one or more amino acids (Suzzi, 2003). In fact, some lactobacilli strains (*Lactobacillus buchneri*, *L. alimentarius*, *L. plantarum*, *L. curvatus*, *L. farciminis*, *L.*

bavaricus, *L. homohiochii*, *L. reuteri* and *L. sakei*) exhibit BA production (Bover-Cid et al., 2001a). The main bacterial groups responsible for the formation of BA are enterobacteria, LAB (particularly enterococci), staphylococci and bacilli (Table 1).

The pre-requirements necessary for BA formation are assured by amino acid availability, presence of microorganisms' decarboxylase positive and factors that allow microorganisms development and synthesis and decarboxylase activity (Silla Santos, 1996). All factors that could influence microorganism grow will affect the production of BA, such as temperature, pH, a_w, redox potential and oxygen (O_2) availability (Halász et al., 1994; Masson et al., 1996). All foods are made up of hundreds of naturally occurring compounds that can have varying effects on us, depending on how much we eat and how sensitive we are. The quantification of BA on food is important because could be related with their freshness but also because some BA could have toxicological effects.

Table 1. Microorganisms with known decarboxylase activity

Bacterial group	Species	Biogenic amines produced	Reference
Enterobacteria	*Citrobacter freundii*	histamine, cadaverine, putrescine	Bover-Cid & Holzapfel, 1999; Durlu-Özkaya et al., 2001
	Enterobacter cloacae	histamine, cadaverine, putrescine	Bover-Cid & Holzapfel, 1999
	Escherichia coli	tryptamine, tyramine, phenylethylamine, histamine, cadaverine, putrescine	Durlu-Özkaya et al., 2001)
	E. fergusonni	tyramine, histamine, cadaverine, putrescine	Durlu-Özkaya et al., 2001

Bacterial group	Species	Biogenic amines produced	Reference
	Hafnia alvei	histamine, cadaverine, putrescine	Durlu-Özkaya et al., 2001
	Morganella psychrotolerans	histamine	Emborg et al., 2006
	Proteus mirabilis	tryptamine, tyramine, histamine, cadaverine, putrescine	Durlu-Özkaya et al., 2001
	Serratia liquefaciens	histamine, cadaverine, putrescine	Bover-Cid & Holzapfel, 1999
	S. marcescens	histamine, cadaverine, putrescine	Bover-Cid & Holzapfel, 1999
Lactic Acid Bacteria	*Carnobacterium divergens*	tyramine	Bover-Cid & Holzapfel, 1999
	C. gallinarum	tyramine	Bover-Cid & Holzapfel, 1999
	C. piscicola	tyramine	Bover-Cid & Holzapfel, 1999
	Enterococcus durans	tyramine	Bover-Cid & Holzapfel, 1999; Bover-Cid et al., 2001a)
	E. faecalis	tyramine, phenylethylamine	Bover-Cid & Holzapfel, 1999; Bover-Cid et al., 2001a
	E. faecium	tyramine, phenylethylamine	Bover-Cid & Holzapfel, 1999; Bover-Cid et al., 2001a
	Lactobacillus acidophilus	tyramine, histamine	Bover-Cid & Holzapfel, 1999
	L. bavaricus	tyramine	Bover-Cid et al., 2001a

Table 1. (Continued)

Bacterial group	Species	Biogenic amines produced	Reference
	L. brevis	tyramine, putrescine, cadaverine	Bover-Cid et al., 2001a
	L. curvatus	tyramine, phenylethylamine, tryptamine, putrescine, cadaverine	Bover-Cid et al., 2001a
	L. paracasei	tyramine	Bover-Cid et al., 2001a
	L. sakei	tyramine	Bover-Cid et al., 2001a
Staphylococci	*Staphylococcus equorum*	cadaverine, putrescine	Bermudez et al., 2012
	S. epidermidis	cadaverine, putrescine	Bermudez et al., 2012
	S. saprophyticus	cadaverine, putrescine	Bermudez et al., 2012
	S. pasteuri	cadaverine, putrescine	Bermudez et al., 2012
Bacilli	*Bacillus subtilis*	cadaverine, putrescine	Bermudez et al., 2012
	B. amyloliquefaciens	cadaverine, putrescine	Bermudez et al., 2012
Vibrios	*Photobacterium phosphoreum*	histamine	Kanki et al., 2004

4. BIOGENIC AMINES AND HEALTH EFFECTS

BA at low concentration are relevant for several physiological functions, such as body temperature regulation and gastric acid secretion (Coton et al., 2010). Nevertheless, the intake of foods containing large amounts of BA may cause important adverse effects, such as headache, hypotension or hypertension, cardiac palpitations, hot flushes and respiratory discomfort (Anli & Bayram, 2008).

Biogenic amines are physiologically important, because they are neurotransmitters distributed in the brain, where they play a role in emotional behaviour and help in regulating the biological clock. They are transmitted locally or via the blood system. Exogenous amines are directly absorbed from food in the intestine. Alcohol can increase the absorption rate.

Biogenic amines are normally quickly broken down in the body with the help of enzymes such as MAO (monoamine oxidase-A), which render them harmless. Missing, sluggish or blocked enzymes can lead to a build-up of amines in the body. MAO breaks down BA and prevents excessive resorption (Youdim et al., 2006). MAO inhibitors (MAOIs) are also used as medications for the treatment of depression to prevent MAO from breaking down amines important for positive mood (Fiedorowicz & Swartz, 2004).

Histamine and tyramine are the amines with the more severe effects for human health, being most commonly associated with undesirable symptoms in consumers (Stratton et al., 1991; Mariné-Font et al., 1995; Gardini et al., 2016). These BA are considered as the most toxic ones and consequently particularly relevant for food safety (EFSA, 2011). Therefore, legislation is more frequent for histamine and tyramine.

Nuñez et al. (2016) have reported that foods containing more than 500 mg/kg of histamine and 1000 mg/kg tyramine are considered toxic or dangerous to the human health. However, such levels are already much too high for susceptible consumers (Stadnik & Dolatowski, 2010).

The toxicological level of BA depends on individual characteristics and the presence of other amines and is thus very difficult to establish. Nevertheless, a total BA content of maximum 750-900 mg/kg has been proposed (Victor et al., 2010).

Eerola and co-workers (1998) recommended the total content in vasoactive amines (histamine, β-phenylethylamine, tryptamine and tyramine) to be below 200 mg/kg fresh weight in dry sausages.

The determination of the toxicity threshold of BA in individuals is extremely difficult because the toxic dose is very dependent on the efficiency of the detoxification mechanisms (Halász et al., 1994). The toxic

dose depends on the sensitivity of individuals and levels above 100 mg/kg of histamine, 100 to 800 mg/kg for tyramine and 30 mg/kg of β-phenylethylamine in foods can cause poisoning (ten Brink et al., 1990). A recent Joint Food and Agriculture Organisation of the United Nations (FAO)/World Health Organisation (WHO) "Expert Meeting on Public Health Risks of Histamine and Other Biogenic Amines from Fish and Fishery Products" identified 50 mg of histamine as the "no observable adverse effect level" (NOAEL) derived from outbreak studies.

The following guidelines levels for histamine content particularly on fish were suggested, based on a revision about the oral toxicity to humans of histamine and other BA (Shalaby, 1996):

- <50 mg/kg safe for consumption
- 50-200 mg/kg possibly toxic
- 200-1,000 mg/kg probably toxic
- >1,000 mg/kg toxic and unsafe for human consumption

The 'cheese effect' is noticed in people who are taking certain drugs known as MAOIs (monoamine oxidase inhibitors); the enzyme is inhibited and tyramine may accumulate, leading to life-threatening high blood pressure as well as a range of symptoms including headaches, itchy skin rashes, heart palpitations and diarrhea. A number of MAOI patients died from strokes or heart attacks before doctors realised that patients taking MAOIs needed to avoid foods high in tyramine. This is called the 'cheese effect' because it was recognised in the 1960s by a British pharmacist who noticed that his wife developed a headache every time she ate cheese (rich in tyramine), while taking MAOI antidepressants (Sathyanarayana Rao & Yeragani, 2009).

Amines have been associated with migraines and headaches, as well as other symptoms of food intolerance, including irritable bowel symptoms, eczema and depression (Maintz & Novak, 2007). Experience suggests that people who are sensitive to amines need to know a lot of about the history and freshness of their foods and approach all possible amine-containing foods with caution.

Consumption of foods containing high levels of BA can result in health problems, such as rash, headache, nausea, hypotension or hypertension, cardiac palpitations, or even more serious damages namely intracerebral haemorrhage and anaphylactic shock, especially if alcohols or monoamine oxidase inhibitors are simultaneously ingested (Cunha et al., 2017).

Many drugs can contain amines, including cold tablets, decongestants, nasal drops or sprays, some pain relievers, some anesthetics and some antidepressants (Bover-Cid et al., 2014).

5. Foods and the Presence of Biogenic Amines

Biogenic amines may be present in fresh foods in such low amounts that they even remain undetected. However, in fermented foods mainly of animal origin they can be present in high concentrations.

Its presence in foods results in undesirable consequences once they affect food safety. The presence of BA in foods, especially histamine, commonly results from the use of poor quality raw materials, microbial contamination or inappropriate conditions (frequently temperature) during food processing and storage (EFSA, 2011).

Fish, cheese, wine, some meats, some fruits, such as bananas and avocados, some vegetables, some mushrooms, chocolate and fermented foods, such as sauerkraut and soy sauce, are just some of the foods that have been listed as containing varying levels of amines, but basically any protein food can contain amines depending on the way it is handled.

The presence of BA has been studied for a large number of authors in several foods (Table 2), such as meat and meat products (Eerola et al., 1998; Roseiro et al., 2006; Latorre-Moratalla et al., 2008; Roseiro et al., 2010; Latorre-Moratalla et al., 2012; Tasić et al., 2012; Laranjo et al., 2017;), cheese (Calzada et al., 2013), wines (Anli & Bayram, 2008; Cunha et al., 2011), beers (Almeida et al., 2012), liqueurs (Cunha et al., 2017), vegetables (Simon-Sarkadi & Holzapfel, 1994; Kalač et al., 2002) and fermented vegetables, such as table olives and other pickled foods (García-García et al., 2001).

Histamine and tyramine are the most toxic and food safety relevant N-aromatic heterocyclic biogenic amines in foods and beverages

The presence of β-phenylethylamine in chocolate results from the thermal processing of cocoa (Irsfeld et al., 2013).

Fermented sausages and cheeses are the two types of food products with higher contents in BA (EFSA, 2011). Spermine levels are higher than those of spermidine in animal food products (Vidal-Carou et al., 2015).

Tyramine is usually the most frequent and abundant BA in fermented sausages, contrary to histamine, which is rare in such products. Furthermore, physiological polyamines are found in significant levels in fermented sausages (Vidal-Carou et al., 2015).

It is imperative to avoid or to control the presence of deleterious compounds that could negatively affect food quality. Among these compounds BA have become a worldwide concern, where several efforts have been made to reduce its content in fermented food products.

According to the Commission Regulation EC No. 1441 (EC, 2007) the limits for histamine have been established in two food categories: fishery products from fish species associated with a high amount of histidine (100-200 mg/kg) and fishery products, which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine (200-400 mg/kg), both for "Products placed on the market during their shelf-life". Furthermore, the European Food Safety Authority (EFSA) Biological Hazards (BIOHAZ) panel has reported that "the present knowledge and data on toxicity of biogenic amines (BA) individually and in combination(s) are limited: nevertheless, histamine and tyramine are considered as most toxic and particularly relevant for food safety" (EFSA, 2011). They also reported that the available information for putrescine and cadaverine is insufficient to identify concentrations that directly cause acute adverse health effects and/or potentiate the toxic effects of histamine and other BA (EFSA, 2011). Moreover, it is commonly assumed that BA should not accumulate (Spano et al., 2010).

Nitrosamines (NA) are formed by a reaction between nitrates or nitrites and certain amines, mainly cadaverine, putrescine, spermine and

spermidine (De Mey et al., 2015). They can be found in diverse food products, such as processed meats, alcoholic beverages, etc. The NA is a large group of compounds of which the majority is carcinogenic (Bouvard et al., 2015; Herrmann et al., 2015).

Table 2. Biogenic amines present in different foods

Food group	Food	Biogenic amines detected		Reference
		Potential hazards	Spoilage indicators	
Animal foods	Poultry meat	tyramine, histamine	putrescine, cadaverine	Fraqueza et al., 2012
	Pork meat	tyramine, histamine	putrescine, cadaverine	Hernández-Jover et al., 1996
	Beef meat	-	putrescine, cadaverine	Slemr, 1981; Hernández-Jover, et al., 1996
	Finish dry-sausages	histamine, tyramine, tryptamine, β-phenylethylamine	putrescine, cadaverine	Eerola et al., 1998
	Portuguese 'catalão' and 'salsichão'	histamine, tyramine, tryptamine	putrescine, cadaverine	Laranjo et al., 2016
	blood sausages	histamine, tyramine, tryptamine, β-phenylethylamine	putrescine, cadaverine	Laranjo et al., 2017
	Serbian traditional dry-fermented sausages	tyramine, tryptamine, β-phenylethylamine	putrescine, cadaverine	Tasić et al., 2012
	traditional European fermented sausages	histamine, tyramine, tryptamine, β-phenylethylamine	putrescine, cadaverine	Latorre-Moratalla et al., 2008
	Portuguese traditional dry-fermented sausages	histamine, tyramine, tryptamine, β-phenylethylamine	putrescine, cadaverine	Roseiro et al., 2006; Roseiro et al., 2010
	fish	histamine	-	Prester, 2011; Visciano, Schirone, Tofalo, & Suzzi, 2014
	fishery products	histamine	-	Visciano et al., 2014

Table 2. (Continued)

Food group	Food	Biogenic amines detected		Reference
		Potential hazards	Spoilage indicators	
	sardine	histamine, tyramine	putrescine, cadaverine	Özogul & Özogul, 2006
	anchovy	histamine, tyramine	putrescine, cadaverine	Pons-Sánchez-Cascado et al., 2006
	mackerel	histamine, tyramine	putrescine	Prester et al., 2009
	yellowfin tuna	histamine	putrescine	Du et al., 2002
	herring	histamine, tyramine	putrescine, cadaverine	Mackie et al., 1997
	'budu'(fish sauce)	histamine, tyramine, tryptamine	putrescine	Saaid et al., 2009
	'cincalok' (shrimp sauce)	histamine, tyramine	putrescine	Saaid et al., 2009
	milk	histamine	-	Notou et al., 2014
	cheese	histamine, tyramine, tryptamine, β-phenylethylamine	putrescine, cadaverine	Calzada et al., 2013
	Iranian cheeses	histamine, tyramine	putrescine, cadaverine	Razavi Rohani et al., 2013
	Feta cheese	tyramine	putrescine	Valsamaki et al., 2000
	'Otlu peynir' (herby cheese)	histamine, tyramine	cadaverine	Andic et al., 2010
	Egyptian cheeses (Mish, Ras and Blue)	histamine, tyramine	putrescine, cadaverine	El-Zahar, 2014
Vegetable foods	frozen spinach purée	histamine, tyramine, tryptamine	putrescine, cadaverine	Kalač, Švecová, & Pelikánová, 2002
	concentrated tomato pasta	histamine, tyramine, tryptamine	putrescine, cadaverine	Kalač et al., 2002
	ketchup	histamine, tyramine, tryptamine	putrescine, cadaverine	Kalač et al., 2002
	frozen green pea	histamine, tyramine, tryptamine	putrescine, cadaverine	Kalač et al., 2002
	Chinese cabbage	histamine, tyramine	putrescine	Simon-Sarkadi & Holzapfel, 1994
	endive	tyramine	putrescine	Simon-Sarkadi & Holzapfel, 1994

Food group	Food	Biogenic amines detected		Reference
		Potential hazards	Spoilage indicators	
	iceberg, lettuce	tyramine	putrescine	Simon-Sarkadi & Holzapfel, 1994
	radicchio	tyramine	putrescine	Simon-Sarkadi & Holzapfel, 1994
	table olives	tyramine	cadaverine, putrescine	García-García et al., 2001
	pickled capers	histamine, tyramine	putrescine	García-García et al., 2001
	pickled cucumbers	tyramine	putrescine	García-García et al., 2001
	pickled caperberries	histamine, tyramine	cadaverine, putrescine	García-García et al., 2001
	chocolate	β-phenylethylamine	-	Irsfeld et al., 2013
Beverages	Port wines	histamine, tyramine	cadaverine, putrescine	Cunha et al., 2011
	red wine	histamine, tyramine, tryptamine, β-phenylethylamine	cadaverine, putrescine	Anli & Bayram, 2008
	grape juice	histamine, tyramine	cadaverine, putrescine	Cunha et al., 2011
	mango juice	histamine, tryptamine	-	Saaid et al., 2009
	orange juice	histamine, tyramine, tryptamine	putrescine	Saaid et al., 2009
	apple juice	-	putrescine	Saaid et al., 2009
	beers	tyramine	putrescine, cadaverine	Almeida et al., 2012
	liqueurs	histamine	cadaverine, putrescine	Cunha et al., 2017

6. Factors Influencing Biogenic Amine Levels in Different Foods

Formation of biogenic amines can occur during food processing and storage as a result of bacterial activities. Consequently, higher amounts of certain amines may be found in foods as a consequence of the use of poor

quality raw materials, microbial contamination and inappropriate conditions during food processing and microbial contamination and inadequate conditions during storage. There is evidence that as the hygienic quality of the product decreases, the biogenic amine content increases (EFSA, 2011). Several factors may affect BA levels in different foods, namely the content of free amino acids available in the different food matrices, the hygiene level of the raw materials, the hygienic control of the technological process (Naila et al., 2010), characteristics of the technological process (such as fermentation processes, temperature, pH, available sugars, etc.), packaging strategies (modified atmosphere, vacuum, etc.) (Nadon et al., 2001), storage temperature (EFSA, 2011). The production of BA is mainly influenced by temperature; also the oxygen availability and redox potential appear to have a significant effect on their biosynthesis. pH influences the formation of amines, when the growth of bacteria occurs in acidic environment, there is stimulation of the decarboxylase activity, the optimum pH for the activity of these enzymes being between 2.5 and 6.5 (Masson et al., 1996). The presence of carbohydrates, such as glucose, also favour the growth of bacteria and their decarboxylase activity (Silla Santos, 1996). In foods, histamine production decreases below 10°C and almost ends at 5°C due to inhibition of histidine-decarboxylase producing bacteria (Halász et al., 1994). *Proteus morganii*, *P. vulgaris* or *Hafnia* strains do not produce histamine after one month of incubation at 1°C (Halász et al., 1994). However, *Pseudomonas* spp., which could be the dominant spoilage microorganisms in refrigerated meats, mainly produce putrescine, whereas enterobacteria preferentially form cadaverine (Halász et al., 1994; Bover-Cid et al., 2003; Fraqueza et al., 2012).

Spermidine and spermine are naturally present at relatively constant concentrations in pork and fresh beef and meat products, their formation not being attributed to meat spoilage or fermentation processes, while the other amines are not usually found or are present in very low amounts (Zee et al., 1983; Hernández-Jover et al., 1997; Alfaia et al., 2004). Agmatine is a poorly detected polyamine (30% of meat products analysed) and does not occur during meat deterioration or product processing (Hernández-Jover et

al., 1997). The formation of putrescine, histamine, cadaverine and tyramine occurs during meat storage, although it is observed that while spermidine concentration is constant, that of spermine decreases slightly (ten Brink et al., 1990; Hernández-Jover et al., 1997).

In pork and beef meat stored at 4°C for 35 days, it was found that concentrations of putrescine and cadaverine increased, followed by those of tyramine and histamine; however, the amount of these BA did not exceed 150 mg/kg. In putrid meat samples, concentrations above 1,000 mg/kg were found (Bauer et al., 1994). The spermidine content in pork meat was 1 to 16 mg/kg during the storage period, whereas spermine showed values of 20 to 50 mg/kg and did not change its content over time (Bauer, 1995).

The Biogenic Amine Index (BAI) was first proposed for measuring the quality of raw and processed seafood, but nowadays it is mainly used to assess food hygienic quality also for evaluating the quality and freshness of food products (Mietz & Karmas, 1977):

$$BAI = [Cadaverine] + [Putrescine] + [Histamine]/1+ [Spermine] + [Spermidine]$$

A BAI value above 10 is regarded as representing some quality loss (Bunčić et al., 1993). The poultry meat quality and freshness is frequently evaluated only by microbial indicators or by sensorial evaluation. However, chemical indicators could be useful to assess those attributes including the evaluation of the levels of BA (Halász et al., 1994; Dainty, 1996; Balamatsia et al., 2006; Fraqueza et al., 2008). The quantification of BA in meat and meat products is important not only as an indicator of freshness but also from a toxicological point of view. Fraqueza and co-workers (2012) evaluated the effect of a modified atmosphere packaging (MAP), with gas mixtures with carbon dioxide (CO_2) and argon (Ar) or nitrogen (N_2) in different proportions and without O_2, on biogenic amine production in turkey meat stored at 0°C. The authors concluded that in turkey meat under MAP, tyramine was not the best indicator for meat spoilage; it was suggested that freshness/spoilage could be characterised by

using cadaverine or the sum of the amines putrescine, cadaverine and tyramine as an indicator of freshness.

The production of BA analysed in turkey meat increased over time. Cadaverine had the highest concentration in packaged turkey meat during storage time. Histamine was not detected in turkey meat packaged under study MAP conditions or, when present, the levels were below the limit of quantification (1.03 mg/kg) (Fraqueza et al., 2012).

Poultry and pork meat are frequently used in diverse meat products. In meat industry either fresh meat or frozen are used, but the hygienic level of this raw meat is the major factor that influences the presence of BA on processed meat products. If raw meat is highly contaminated with spoilage microbiota the levels of cadaverine, putrescine and tyramine will be higher (Fraqueza et al., 2008; Fraqueza et al., 2012). There is a great diversity of fresh meat preparations, cured cooked meat products and dry-cured or dry-fermented or smoked products. The different types of processing and storage conditions that are applied to meat products can influence the formation of BA. In cured cooked meat products the thermal treatment conditions, temperature and time applied in order to reach a final temperature in the thermal center of the product influences decarboxylase positive bacteria, which in some cases, become inactive at over 65°C, a temperature reached in most cooked products (Ruiz-Capillas & Jimenez-Colmenero, 2004). Most enterobacteria at 65°C during 30 minutes will be inactivated and the formation of BA will be prevented. A thermal regime designed to kill bacteria responsible for histamine and tyramine formation can prevent the subsequent formation of these BA. If cadaverine and putrescine are detected in cured cooked meat products, precaution should be taken. In fact, during heating, cadaverine and putrescine can suffer transformation to pyrrolidine and piperidine, respectively; these could react with the nitrites present in the meat product, forming nitrosamines, highly carcinogenic (Ruiz-Capillas & Jimenez-Colmenero, 2004).

Fermented meat products are mentioned as principal sources of biogenic amines, since fermentation processes generally promote the formation of BA.

LAB play a recognised role in fermented foods preservation and safety, thus promoting final products microbial stability. Bacterial communities coexist in fermented meat products, allowing microbial diversity. Many factors influence the final microbiota of fermented meat sausages. The selective influence of intrinsic (concentration and availability of nutrients, pH, redox potential, buffering capacity, a_w, meat structure) and extrinsic (temperature, relative humidity and oxygen availability) factors may determine differences in microbial ecosystem raw meat substrate. This initial microbiota will be influenced by technological particularities used in sausage processing and will adapt to this special niche. The addition of ingredients, such as sodium chloride, nitrate and/or nitrite, sugars, wine, condiments (garlic, pepper), as well as particular a_w (0.85-0.92), temperature (12-18°C to 24-30°C), oxygen gradient during ripening and smoking application will select a microbiota able to develop in fermented meat sausages (Fraqueza, 2015).

Among the different genera belonging to the LAB group, those that are frequently found in fermented sausages are *Lactobacillus*, *Enterococcus*, *Pediococcus* and *Leuconostoc*. In some of these genera there are important aminogenic strains that induce the presence of histamine, tyramine, tryptamine, β-phenylethylamine, cadaverine and putrescine in fermented meat sausages (Latorre-Moratalla et al., 2008). The temperature at which fermentation takes place (usually between 7-28°C), influences the formation of BA. Indeed, it has been suggested that temperature could be a very useful parameter for preventing tyramine formation in dry-sausages, chiefly by assuring conditions favourable to the growth of starters (Maijala et al., 1995; Eerola et al., 1998). Some authors have reported higher BA concentrations during ripening than during drying and salting (Maijala & Eerola, 1993; Hernández-Jover et al., 1997). This might be explained by a decrease of a_w that could inhibit BA forming bacteria. Other factors, like sausage diameter in fermented products, have also been reported to affect the formation of BA during ripening (Bover-Cid et al., 1999; Laranjo et al., 2016). These authors found that the concentrations of BA in larger diameter sausages were greater than in sausages of smaller diameter; moreover, the concentration was higher in the central part than in the ends.

As in other foods, as mentioned above, the most important factor influencing the formation and accumulation of BA in fish is undoubtedly temperature. According to European Legislation, fish must be maintained at a temperature approaching that of melting ice as soon possible after harvest, in order to comply with freshness criteria and to avoid the growth of histamine producing and spoilage bacteria (Visciano et al., 2014). All subsequent fish-handling operations (heading, gutting, filleting, cutting, etc.) should also be carried out hygienically on board vessels. Moreover, fresh fishery products must be kept at the abovementioned temperature during storage and transportation, so that food safety is not compromised (Visciano et al., 2014).

In fish products, histamine and other biogenic amines are produced by enzymatic decarboxylation of the corresponding free amino acid induced by specific bacteria. In order to cause BA production in fish, particularly histamine, it is necessary that fish muscles contain free histidine as substrate for histamine formation; that fish and fish products become contaminated with bacteria able to decarboxylate histidine and other amino acids; that fish products characteristics and storage conditions allow the growth of histamine-producing bacteria or BA producing bacteria attaining high concentrations of about 10 million cells per gram or more. Consequently, BA control can be achieved by eliminating one or more of these mentioned factors. The production of BA is a significant hazard in all products based on scombroid fish or all fish containing large amounts of free histidine in their flesh (Visciano et al., 2012).

The kinetics of histamine formation during storage of seafood is sometimes characterised by a long phase with little or no histamine production, followed by a second phase where the concentration can increase rapidly. The first phase corresponds to the time needed for the specific histamine-producing bacteria to reach high concentrations and the length of this phase depends primarily on the initial concentration of these bacteria, their growth rate and temperature. The rate of histamine formation during the second phase corresponds to the activity of high concentrations of histamine-producing bacteria and it is influenced by storage conditions and product characteristics.

Chilling of fish and fish products is highly important to increase the time to formation of critical histamine concentrations. Below 7-10°C, mesophilic and strongly BA producing bacteria do not form toxic concentrations of histamine in fish products. However, the psychrotolerant bacteria *Morganella psychrotolerans* and *Photobacterium phosphoreum* can produce toxic concentrations of histamine at 0-5°C (Okuzumi et al., 1982; Kanki et al., 2004; Emborg et al., 2005; Dalgaard et al., 2006).

Fish and fish products packaged under MAP with reduced-oxygen must be stored and distributed at less than 3.3°C. The distribution under MAP and refrigeration will control toxin formation by *Clostridium botulinum* type E and also histamine formation (FDA, 2011).

The use of salt concentrations above 1-2% NaCl is able to reduce growth of the Gram-negative bacteria and strongly inhibit histamine-producing bacteria. According to Emborg and Dalgaard (2006) for cold-smoked tuna under vacuum packaging could be possible to inhibit the potential histamine formation by *M. psychrotolerans* and *P. phosphoreum* by using 5% water phase salt and a declared shelf-life of 3-4 weeks or less at 5 C for fish under MAP. MAP with gas mixtures containing CO_2 and N_2 it can slightly delay histamine formation when high CO_2 concentrations are used (Emborg et al., 2005).

The use of MAP gas mixtures with high CO_2 levels is also very important in reducing BA content in fish products, since the concentration of histamine decreases with increasing levels of CO_2 (Gardini et al., 2016).

The antimicrobials benzoate and sorbate have also been successfully used to limit BA production during the storage of fishery products (Lapa-Guimarães et al., 2011; Gençcelep et al., 2014).

Generally, total amine contents in milk and dairy products is very low, with the exception of cheese, which being a fermented product can accumulate high levels of BA. Besides, fermented milks and yogurts have been consistently reported to contain little or undetectable levels of BA, probably due to their shorter processing time and shelf-life (Benkerroum, 2016).

Polyamines are naturally present in cow's milk by the following order of importance: spermine, putrescine, spermidine and agmatine (Bardócz et al., 1993).

Different types of cheese have different biogenic amines profile. Spermine and spermidine are commonly present at low levels, while the contents in other amines may vary greatly with cheese type, ripening time or aging and microbiota present. Nevertheless, high contents of histamine and tyramine have been report in various types of cheese (Stratton et al., 1991; Vale & Glória, 1998; Innocente & D'Agostin, 2002; Pinho et al., 2004).

Cheese is rich in protein and provides the ideal environment for the accumulation of BA, with free amino acids available and the presence of contaminant microbiota, as well as starter cultures, in conditions that favour microbial growth and decarboxylase activity, namely pH, temperature, salt concentration, a_w, ripening time and temperature and storage temperature (Stratton et al., 1991). Furthermore, LAB starter cultures used in the manufacture of cheese could be identified as potentially hazardous due to their ability to produce BA (Fernandez-Garcia et al., 2000).

Pasteurisation of raw milk may be a good alternative to decrease the levels of BA usually accumulated in cheese. This thermal treatment not only destroys the milk pathogenic microbiota, but it also accelerates the ripening process, because high pressure promotes proteolysis without increasing the content in BA (Novella-Rodriguez et al., 2000).

The aging process in the manufacture of cheese is also a very important factor that may influence the formation of BA. In mature cheeses made from raw milk, such as Gouda, the detected of BA may be relatively high (EFSA, 2011).

It is very difficult to make cheese free of BA, however the technology must be optimised in order to assure low levels of BA. Cheeses with a good bacteriological quality will contain smaller amounts of BA than cheeses with high counts of non-starter bacteria. Furthermore, measures to prevent or minimise BA levels should be adopted, such as the use of raw materials with good hygienic quality, milk pasteurisation, good hygienic

practices during manufacture, temperature control throughout manufacturing and selection of starter cultures without or with low decarboxylase activity (Novella-Rodríguez et al., 2002).

In vegetable products, changes in the BA profile and contents before harvest have been reported in response to different environmental stresses: water, mineral deficiency, acid, herbicides, ozone-cause damages, osmotic shock, temperature, altitude and chilling injury. All these stress conditions cause an increase in the levels of polyamines, particularly putrescine (Glória, 2005).

Many studies have addressed the relationship between food freshness and BA levels. BA may indeed be good indicators of freshness in fruits and vegetable products. Putrescine contents increase throughout the storage period, although at different levels depending on the type of product (Simon-Sarkadi et al., 1993).

Processed vegetables and fruits usually contain higher levels of BA, when compared to the fresh products, since the addition of ingredients during processing may change the content and BA profile (Kalač et al., 2002).

The adoption of a low fermentation temperature in green olives reduced the accumulation of cadaverine and tyramine, concomitantly with the decrease of the "zapatera" defect (Garcia et al., 2004).

In wine, the production of BA is mainly associated with the activity of LAB (Lonvaud-Funel & Joyeux, 1994). Therefore, the selection of starter cultures used for malolactic fermentation must be carefully planned.

BA are naturally present at low levels in the raw materials, such as malt and barley, used for beer brewing, mainly putrescine, spermidine and agmatine. The use of rice has been reported to reduce BA contents in beer. The formation of tyramine, histamine and cadaverine occurs mainly during the main fermentation (Kalac & Krízek, 2003).

Regarding liqueurs, the content in BA is greatly influenced by their composition, the quality of raw materials and the processing and storage conditions. Thus, homemade products usually have higher contents than commercial liqueurs (Cunha et al., 2017).

7. CONTROL MEASURES

According to the recommendations of the EFSA BIOHAZ panel (2011), the key BA control strategy is focused on preventing the formation of BA at all important points of the food chain. In fact, if histidine and tyramine are identified as potential hazards in any foodstuff production step, different preventive measures for BA control should be implemented. Many of these measures are related with pre-requirements implemented along the food chain and in HACCP plans well implemented in any specific line of food production (I) animal foods, such as meat and meat products, including dry-fermented sausages, fish and seafood products and dairy products, such as cheeses; and II) plant foods, like vegetables, table olives, etc.). The Codex Alimentarius with FAO support has elaborated many codes of good hygiene and good manufacturing (GHP/GMP) practices for different food sectors that have been already disseminated in order to be specifically implemented. It is possible to find codes GHP/GMP for meat and meat products (FAO, 2005), dairy products particularly cheese (FAO, 2011), fish and fish products (Ryder et al., 2014). The planning and implementation of pre-requirements' programs, correctly monitored and verified, are necessary for hazards control and essential elements of HACCP systems development (Henriques et al., 2014; Fraqueza & Barreto, 2015).

Many of the mentioned preventive measures for BA control are based on food preservation technologies, mainly for bacteria control, particularly for those that were pointed in Table 1 as BA producers.

Table 3 sumarises some strategic technologies used on BA control by several authors that are based on classical technologies, such as temperature control, thermal treatment, use of additives, modified atmosphere packaging and other emergent technologies, namely active packaging, biopreservation with the use of starters, irradiation, omics treatment, high hydrostatic pressure.

Conventionally, BA formation in food is prevented, above all by limiting microbial growth through temperature control-chilling and freezing. However, this is not always practical and possible. Hence,

alternative control measures to prevent BA formation in foods or to reduce their levels have to be taken into consideration (Naila et al., 2010).

The main preventive measure to avoid BA formation in fish is a rapid chilling of fish immediately after capture. Generally, fish should be packed in ice or chilled seawater within 12 hours after catch or, in the case of large fishes, such as tuna, chilled to an internal temperature of 10°C or less within 6 hours after capture (Ryder et al., 2014).

The application of high isostatic pressures (HIP) is used as a non-thermal preservation method that inactivates of microorganisms (De Las Rivas et al., 2008), thus inhibiting BA formation. Naturally, the inhibition of BA formation depends on the level of pressure applied. A great advantage of this technology is that food products are able to maintain their organoleptic characteristics, while extending their shelf-life (Patterson, 2005).

The possibility that HIP affect not only bacteria responsible for BA formation, but also their enzymes has not been adequately addressed (Naila et al., 2010).

HIP have been applied to different food products, such as sausages (Latorre-Moratalla et al., 2007; Ruiz-Capillas et al., 2007), fish (Bolton et al., 2009), sauerkraut (Peñas et al., 2010) and cheese (Novella-Rodríguez et al., 2002). HIP-treated food products are already commercially available in the United States, Japan and Europe (e.g., Portugal and Spain).

Irradiation has been used in food industry since the 1950s to ensure food safety and extend shelf-life, without chemical preservatives (Mbarki et al., 2009). Irradiation may control BA formation in food products in two ways: by reducing biogenic amine producing bacteria (Kim et al., 2003) and by radiolysing BA (Mbarki et al., 2009).

In a study based on a model system, significant degradation of spermine, spermidine and putrescine occurred above 5 kGy irradiation (Kim et al., 2004). However, the application to a food system requires further investigation, because the use of a high dosage may affect food sensory quality. According to the WHO (1994), irradiation at 10 kGy is considered safe for any food product.

Table 3. Effective control measures

Control Measure	Minimises/Inhibits formation of BA	Removes formed BA	Food	Reference
use of starter cultures	x		meat and dairy products	Latorre-Moratalla et al., 2012; Linares et al., 2012; Gardini et al., 2016
temperature control	x		fish	Naila et al., 2010
pasteurisation		x	dairy products	Linares et al., 2012
high isostatic pressures (HIP)		x	milk, cheese, meat products	Gardini et al., 2016
technological additives (sugars)	x		meat	Gardini et al., 2016
Packaging (MAP, VP)	x		fish and fish products, meat	Gardini et al., 2016
non-thermal treatments (irradiation, PEF)	x		sausages, juices	Gardini et al., 2016
antimicrobial substances	x		meat products	Gardini et al., 2016
food additives and preservatives	x		meat products, seafood	Naila et al., 2010

MAP-modified atmosphere packaging; VP-vacuum packaging; PEF-pulsed electric fields.

Irradiation has also been used for extending the shelf-life of food products, such as pork and beef meat (Min et al., 2007a), chicken meat (Min et al., 2007b), sausages (Kim et al., 2005), fish (Mbarki et al., 2009), cheese (Rabie et al., 2011) and table olives (Irmak et al., 2017).

Active packaging involves the change of the gases and their concentrations inside the food package. Different gases may be used to

control the environment within the package, namely O_2 and CO_2. This technology may delay the production of BA, due to inhibition of the BA producing microorganisms or their enzymes.

There are several reports on the successful control of BA through packaging: vacuum packaging of salmon (Mbarki et al., 2009), MAP of fish (Emborg et al., 2005; Dalgaard et al., 2006), chicken (Balamatsia et al., 2006; Patsias et al., 2006), sausages (Kim et al., 2005) and active packaging of seer fish (Mohan et al., 2009).

Vacuum packaging further extends the shelf-life of food products compared to air packaging (González-Montalvo et al., 2007). An innovative packaging method was developed (Schirmer et al., 2009) combining organic acids with CO_2 from the headspace dissolving into the product until a vacuum is formed ("CO_2-vacuum packed" products). This was used for example on salmon to effectively inhibit the growth of *Photobacterium phosphorium*, an active histamine former under normal MAP conditions and extend shelf-life (Emborg et al., 2005; Tao et al., 2009).

The use of food additives and preservatives may reduce the formation of BA in food products; however, a great variety of these products are commercially available and their effectiveness and harmlessness needs to be investigated.

Sodium sorbate may limit the formation of BA and 2% sodium hexametaphosphate has been shown to delay histamine production (Shalaby, 1996). Citric acid, succinic acid, D-sorbitol and malic acid inhibited decarboxylase activity and consequently histamine formation in mackerel stored for 10 days at 25°C (Shalaby, 1996). Citric acid used (1%) during pickled cabbage fermentation produced a slight decrease in BA at 6, 8 or 10% salt (Yücel & Üren, 2008). Potassium sorbate has also been found to extend the shelf-life of seafood (Shalini et al., 2001). Sausage containing potassium sorbate and ascorbic acid showed a significant reduction in BA accumulation (Bozkurt & Erkmen, 2004). Sodium nitrite and sodium nitrate decrease or inhibit BA production in sausages (Bozkurt & Erkmen, 2004; Kurt & Zorba, 2009). The addition of sugar may also slightly reduce BA formation (Bover-Cid et al., 2001b). When glycine was

applied to Myeolchi-jeot, a salted and fermented anchovy product, the overall production of BA was severely reduced. BA in other fermented fish products may also be reduced using glycine as a food additive (Mah & Hwang, 2009a). Naturally occurring specific inhibitory substances in spices and additives have also been shown to inhibit BA formation (Komprda et al., 2004). Such substances include curcumin (turmeric), capsaicin (red pepper) and piperine (black pepper) (Wendakoon & Sakaguchi, 1992; Shakila et al., 1995). Components of spices, such as thymol, may inhibit BA formation (Singh et al., 1999). However, thymol, having unpleasant pungent flavour, may not be accepted by consumers as an ingredient for food formulation (Lee et al., 2008). Ginger, garlic, green onion, red pepper, clove and cinnamon have been shown to delay BA production in Myeolchi-jeot (Mah et al., 2009).

On one hand, food additives and preservatives commonly used in food products require further investigation into their effectiveness in hindering BA production. On the other hand, additives that have shown a positive effect on retarding BA formation need to be tested in different food matrixes (Naila et al., 2010).

Biopreservation is the use of natural or controlled microorganisms, their metabolic products or both to preserve food products and extend their shelf-life (Montville & Chikindas, 2007).

Starter cultures used for fermentation purposes may also delay the formation of BA (Bover-Cid et al, 2001a; Bover-Cid et al., 2001c; Špička et al., 2002; Latorre-Moratalla et al., 2007; Mah & Hwang, 2009b). However, these starters must be carefully chosen to ensure that they are either decarboxylase-negative (not able to decarboxylate amino acid into BA) or amine oxidizing (oxidize BA into aldehyde, hydrogen peroxide and ammonia) bacteria (Bover-Cid et al., 2000a; Suzzi, 2003). The safety assessment of the strains used as starter or protective cultures for dry-fermented sausages' production should discard the presence of antibiotic resistance genes (EFSA, 2012, 2013) and should not produce other virulence factors such as BA (EFSA, 2011; Fraqueza & Barreto, 2015).

The effect of starter cultures on controlling BA has been studied in a number of fermented foods: sausages (Bover-Cid et al., 2000a; Bover-Cid

et al., 2000b; Latorre-Moratalla et al., 2007), cheeses (Fernandez-Garcia et al., 2000; Nieto-Arribas et al., 2009), vegetables (Špička et al., 2002; Tamang et al., 2009) and wine (Hernández-Orte et al., 2008).

CONCLUSION

In this review, a general outline on the presence of biogenic amines in food products and available control measures to prevent or minimise them has been given.

Biogenic amines could be present in a wide range of foods rich in proteins or free amino acids, some of them being recognised as potential hazards. The more representative BA in foods are the exogenous amines cadaverine, putrescine, tyramine, histamine, tryptamine, β-phenylethylamine and agmatine. Spermine and spermidine are endogenous BA, the first one more frequent in animal products and the second in vegetable products. Endogenous BA are generally most abundant in fresh foods and exogenous BA in processed or stored foods, resulting from microorganisms with amino acid decarboxylase activity.

Enterobacteria, lactic acid bacteria, staphylococci and bacilli are the main bacterial groups responsible for the production of BA in food. All factors affecting microorganisms grow will also influence biogenic amines production, among them temperature is the most determinant. The ingestion of large amounts of BA cause health effects such as headache, hypotension or hypertension, cardiac palpitations, hot flushes and respiratory discomfort. Histamine and tyramine are BA that more affect human health. According to the Commission Regulation EC No. 1441 (EC, 2007) the limits for histamine have been established in two food categories: fishery products from fish species associated with a high amount of histidine (100-200 mg/kg) and fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine (200-400 mg/kg).

In order to minimise the presence of BA in foods, several preventive measures should be considered such as the hygiene level of the raw

materials, the GHP and GMP of the technological process, the monitoring of critical control points of the technological process, mainly related to storage temperature, the binomial parameters time and temperature of thermal treatment, use of additives preservatives, application of modified atmosphere packaging with bacterial inhibitory gas mixtures, use of starter cultures, omics treatment or high isostatic pressures.

Furthermore, it will be important to establish guidelines regarding safety limits for biogenic amines, other than histamine, for diverse food groups, particularly meat and dairy products, vegetables and beverages.

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Chapter 5

BIOGENIC AMINES IN ALCOHOLIC BEVERAGES

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ABSTRACT

Biogenic amines (BAs) found in foods have a negative impact on human health and food quality. Foods such as fish, fish products, and fermented foodstuffs, as well as alcoholic beverages (for example, wine, cider, and beer) contain high levels of BAs. The presence of BAs in alcoholic beverages has traditionally been treated as an indicator of undesired microbial activity. Relatively high levels of BAs have also been reported to indicate deterioration of beverages and/or their defective manufacture. Their toxicity has led to the general agreement that they should not be allowed to accumulate in alcoholic beverages. Biogenic amines can be produced by both Gram-positive and Gram-negative

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bacteria and fungi (yeast and molds). In alcoholic drinks such as wine, cider and beer, BA formation not only requires the presence of precursors and microorganisms responsible for decarboxylation, but is also influenced by several factors such as pH, the levels of ethanol and sulfur anhydride (SO_2) as well as raw material quality, and fermentation and technological conditions.

Today it is well known that Lactic Acid Bacteria (LAB) such as *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Oenococcus* species are the main microorganisms implicated in BA production in alcoholic beverages. The synthesis of BAs by LAB depends on the activity of the enzymes amino acid decarboxylases. For this reason, the genes encoding those enzymes, i.e., histamine decarboxylase (*hdc*), tyramine decarboxylase (*tdc*), and ornithine decarboxylase (*odc*), have become the main targets of PCR methods for detection of bacteria involved in BA production. In alcoholic beverages, more than 20 amines have been identified, and their total concentration has been reported to range from a few mg/l to about 130 mg/l (Ordóñez et al., 2016). The most common BAs in alcoholic drinks are histamine, tyramine, putrescine, cadaverine, and β-phenylethylamine, which are products of decarboxylation of histidine, tyrosine, ornithine, lysine, and β-phenylalanine, respectively. Generally, in alcoholic beverages, the toxic dose is considered to be between 8 and 20 mg/l for histamine, 25 and 40 mg/l for tyramine, whereas as little as 3 mg/l of β-phenylethylamine can cause negative physiological effects (Soufleros et al., 1998). The most common symptoms of excessive BA intake are headaches, heart palpitations, vomiting, and allergic-like adverse responses.

This chapter tackles the most important aspects of BA formation during manufacture of alcoholic beverages. The significance of BAs is also described from the perspective of microbiological and technological factors.

Keywords: alcoholic beverages, lactic acid bacteria, fermentation

1. INTRODUCTION

Biogenic amines are low molecular weight nitrogen compounds found in animal, plant and bacterial cells (Punakivi et al., 2006). They are simple organic bases with a high activity. They can be formed by the decarboxylation of amino acids or by the amination or transamination of ketones and aldehydes with the participation of bacterial organisms. Some

aromatic amines can also be produced "*in vitro*" by the amination of the corresponding aldehydes (Santos, 1996). Amines produced in living cells in the process of amino acid decarboxylation are called biogenic amines. At the cellular level, biogenic amines such as spermine, spermidine, putrescine, and cadaverine, which are used in protein synthesis, can affect the permeability of cell membranes and DNA replication (Bardocz et al., 1993, Halász et al., 1994). For example, histamine and tyramine are involved in the function of the nervous system and blood pressure control (Lonvaud-Funel, 2001). While biogenic amines are necessary to maintain cell viability, they can also have toxic, allergenic or even carcinogenic effects (Beneduce et al., 2010; Naila et al., 2010). Detection of BAs in food serves as an indication of the presence of undesirable microorganisms and their metabolites (Arena & Manca de Nadra, 2001). High concentrations of certain BAs are associated with deterioration of the quality of food products or indicate faults in production. Being toxic, these compounds should obviously not be present in food. BA formation in foods requires the presence of precursors or appropriate amino acid substrates, the presence of microorganisms with amino acid decarboxylase activity, and favorable conditions for growth (Arena et al., 2008).

Problems related to high concentrations of biogenic amines in foods mainly concern such products as cheese, fermented meat and sausages, and also beer and wine. Alcoholic beverages, despite containing certain health-promoting components such as high contents of phenolic compounds, may cause adverse health effects associated with the intake of biogenic amines (Preti et al., 2016). The most common and most important BAs found in alcoholic drinks are histamine, putrescine, tyramine, and cadaverine. The human body is able to efficiently break down low concentrations of BAs due to the presence of mono- and diaminoxidase enzymes in the intestinal tract. People, however, have individual susceptibility to poisoning by biogenic amines. Importantly, ethanol, beyond its immediate health effects, can directly inhibit the activity of enzymes responsible for the detoxification of biogenic amines (Maynard & Schenker, 1962). It has been observed that alcoholic beverages exacerbate headache in patients suffering from migraine (Peatfield, 1995). In addition, pharmacological

reactions may occur after ingestion of some biogenic amines. The best known of those detrimental reactions are caused by histamine, which can trigger symptoms such as rash, headache, edema, vomiting, hypertension, palpitations, heart problems and diarrhea. Other amines, such as phenylethylamine and tyramine, are thought to be responsible for hypertension and complications associated with vasoconstriction induced by norepinephrine (especially migraine and cerebral hemorrhage). Cadaverine and putrescine, which are not toxic to humans, can potentiate the damage produced by the action of histamine, phenylethylamine and tyramine. In addition, putrescine and cadaverine can produce an undesirable (putrid) off-odor and off-flavor in wine.

2. BIOGENIC AMINES IN WINE

The presence of BAs in wine is inextricably linked to its quality. Several BAs have been detected in wine in amounts ranging from a few milligrams per liter to about 130 mg per liter (Ancín-Azpilicueta et al., 2008). BAs commonly found in wine include histamine, tyramine, and putrescine, the last of which has a toxic effect on people and may negatively change the taste of wine.

The various amines present in wines can be traced to the different stages of vinification. The factors that contribute to the production of amines are the origin and quality of the raw material (grapes), storage conditions, storage time, and the possibility of microbial contamination at each stage of production. (Bover-Cid & Holzapfel, 1999). Grapes are naturally rich in amino acids, which account for 40% of total nitrogen in wine. Grapes themselves already contain some amines, most commonly histamine and tyramine, but also polyamines and volatile amines. The occurrence of cadaverine and putrescine, on the other hand, is indicative of improper raw material storage (Leitão et al., 2005; Ancín-Azpilicueta et al., 2008).

Moreover, the presence of BAs in wine, their type and quantity depend on viticulture conditions such as grape variety, the type and composition of

the soil, and fruit ripeness. For example, the presence of putrescine in wine can be linked to potassium deficiencies in soil (Leitão et al., 2005). The BAs found in the initial must account for one third of the total BA content in the final product. To increase the contents of phenolic and aromatic compounds in wine, grape harvest is delayed, which also causes the accumulation of free amino acids, which are easily converted to biogenic amines (Guo et al., 2015b). The content of BAs in wine and its dependence on grape variety have been investigated in numerous studies. Martinez-Pinilla et al., (2013), for instance, examined five different biogenic amines in seven grape varieties. They found considerable differences in the contents of the different BAs (except agmatine) among the grape varieties studied. This and similar findings suggested that the amine profile could be used as a marker for varietal authentication and estimation of the vintage (Soufleros et al., 2003; Herbert et al., 2005; Martínez-Pinilla et al., 2013). However, biogenic amines do not always correlate with the grape variety that goes into making a wine, because some of those compounds can be used as a source of carbon and nitrogen by several microorganisms.

Many microorganisms, both yeast and bacteria, are capable of processing amino acids to BAs. From the viewpoint of the microbes, this reaction is beneficial since it lowers the acidity of the environment, promoting microbial survival and growth (Lonvaud-Funel, 2001).

During alcoholic fermentation, yeast cells use amino acids as a source of nitrogen for growth, which plays an important role in the formation of aromatic compounds in wine (Caruso et al., 2002). A comparison of the contents of biogenic amines produced by yeasts isolated from grapes and wine showed that *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Candida krusei*, *Kloeckera apiculata*, and *Brettanomyces bruxellensis* were responsible for the highest amounts of these compounds in wine (around 15 mg per liter). *S. cerevisiae* alone produced an average of 12.14 mg per liter. This information is important for the selection of a suitable yeast strain as a starter culture for alcoholic fermentation. Unfortunately, many authors have ignored the significant increase in the amount of biogenic amines due to yeast activity in the production of industrial red wines (Marcobal et al., 2006). There was even a drop in putrescine concentration

during spontaneous and yeast-induced alcoholic fermentation (Ancín-Azpilicueta et al., 2008).

Obviously, the formation of biogenic amines during alcoholic fermentation by yeast is a minor process compared to BA production by lactic acid bacteria. BAs are typically produced as an effect of fermentation of alcoholic beverages by LAB during storage. The presence of BAs in wine is quite inevitable. The final content of BAs is the result of the interaction of microorganisms at the various stages of wine production and storage. In addition to alcoholic fermentation, there is also malolactic fermentation (MLF), during which lactic acid bacteria convert malate to lactate. In the process, malic acid, which contains two carboxyl groups, is transformed into a mild lactic acid containing one such group. The process occurs naturally and causes a decrease in the acidity of wine as well as improving its aroma. The concentration of BAs formed during alcoholic fermentation is low, and increases mainly during malolactic fermentation (Guo et al., 2015a).

For a long time it had been thought that *Pediococcus* spp. was the major bacterium responsible for the production of BA during MLF. Now, it is known that it is mainly *Oenococcus Lactobacillus* and *Leuconostoc* spp. that contribute to the increase in BA in MLF, in particular strains of *Lactobacillus buchneri*, *Lactobacillus brevis*, *Lactobacillus hilgardii*, and *Lactobacillus mali* (Moreno-Arribas et al. 2000, 2003; Martin et al., 2005; Costantini et al., 2006; Landete et al., 2007). Landete et al. (2007) established that *Lb. hilgardii* X1B was capable of converting agmatine to putrescine without urea secretion. In addition, *Lb. hilgardii* X1B was found to be unable to metabolize urea, which means that the formation of ammonia during putrescine synthesis was not dependent on the urease pathway. The post-culture medium contained N-carbamyloputrescine, which indicated that the transformation of agmatine into putrescine was catalyzed by N-carbamoylputrescine hydrolase and agmatine deiminase. These studies were carried out using samples of wine with an ethanol concentration of about 12%. In fermented wine with lower contents of ethanol, in which *Lb hilgardii* occurs naturally, putrescine production may be higher (Arena et al., 2008). This is why it is extremely important that

LAB be removed from wine immediately after MLF (Soufleros et al., 1998). Landete et al. (2007) reported that different strains of *Lactobacillus brevis* synthesized phenylethylamine and tyramine whereas *Lactobacillus mali*, *Pediococcus parvulus*, and *Leuconostoc mesenteroides* only produced histamine. *O. oeni* produced histamine and putrescine. In a study by Sebastian et al. (2011), *Lb. casei, Lb. brevis*, *Pediococcus damnosus* and *P. parvulus* were found to produce histamine alone.

Studies show that the production of biogenic amines may be strain dependent. For example, among the strains of *O. oeni*, there are those that are capable of BA synthesis, and those that do not produce the amines. This and similar findings have encouraged researchers to look for strains that have particular metabolic pathways in which amino acid precursors are converted to BA.

The enzymes involved in these conversions include histidine decarboxylase (HDC), which participates in the formation of histamine, tyrosine decarboxylase (TDC), which catalyzes the formation of tyramine, ornithine decarboxylase (ODC), which is specific for putrescine formation, and agmatine deiminase (AgDI) (Guirard & Snell, 1980; Coton et al., 1998a, 2010; Marcobal et al., 2004).

3. BIOGENIC AMINES IN CIDER

Apple wine, also called cider, is a popular alcoholic beverage in Europe, North America, Australia, and China (Morrissey et al., 2004, Madrera et al., 2008, Ye 2014). Cider is made by fermenting crushed fruit or pressed juice. In cider mills, juice is extracted using vertical mechanical presses with wooden platforms or pneumatic presses. AF and MLF occur spontaneously with the participation of indigenous yeasts and LAB present in the apple must. The routine procedures used in cidermaking such as sulfur dioxide treatment, clarification, and acidity correction are not applied. There are two main types of cider, sparkling and still, depending on the addition of sugars and CO_2 (Picinelli et al., 2000). BAs found in ciders are usually products of ripening and fermentation. Generally, in

alcoholic beverages, the toxic dose is considered to be between 8 and 20 mg/l for histamine, 25 and 40 mg/l for tyramine, whereas as little as 3 mg/l of phenylethylamine can cause negative physiological effects (Soufleros et al., 1998). Amino acids in apple juice are the main nitrogen source for yeasts and bacteria. Many amino acids are intermediates or precursors of some volatile compounds and polyamines (Wolken et al., 2006). The presence of biogenic amines in ciders is believed to result mainly from the microbial decarboxylation of precursor amino acids (ten Brink et al., 1990; Halász et al., 1994). However, two works published in the recent years have reported the presence of BAs in fruit juices, including apple juice (Jastrzębska et al., 2015; Preti et al., 2016) Preti et al. (2016) analyzed the profiles and levels of BAs in apple juices: among the seven amines found, cadaverine was the most abundant BA, followed by putrescine and tyramine. The maximum contents of these amines ranged from 2.3 to 0.67 mg/l, which means that BA content in apple juices does not pose risk to consumer health. In cider, nine amines were identified. Their total concentrations ranged from a few mg/l to about 70 mg/l, depending on the quality of the cider (Table 1).

The most important biogenic amines in cider, both qualitatively and quantitatively, are putrescine, cadaverine, histamine, tyramine, and phenylethylamine, which are products of decarboxylation of ornithine, lysine, tyrosine, histidine, and β-phenyloalanine, respectively. The variability of amine contents in cider could be explained by differences in the cider-making process, time and storage conditions, and raw material quality, as well as possible microbial contamination during the steps of the cider-making process (Garai-Ibabe et al., 2013).

3.1. Microorganisms Involved in the Production of Biogenic Amines in Ciders

During the production of cider, two types of spontaneous fermentation occur, alcoholic and malolactic fermentations . Since the initial juice is not inoculated with starters, cider is fermented by indigenous yeast (AF) and

LAB microbiota (MLF) (Ladero et al., 2011). The latter can produce metabolic energy by using catabolic pathways that convert amino acids into BAs amine-containing compounds referred to as biogenic amines (BA) (Griswold et al., 2006). *Lactobacillus* and *Oenococcus* are the most abundant LAB genera in cider (Coton et al., 2010; Sánchez et al., 2012). Most researchers agree that production and accumulation of BAs by LAB depends on the presence of decarboxylase and deaminase activity and enviromental factors such as temperature, pH, alcohol concentration, etc. (Ladero et al., 2011). Interesingly, BA pathways in LAB have been described to be strain dependent rather than species specific, suggesting that horizontal gene transfer may account for BA production by LAB (Coton & Coton, 2009). Furthemore, the enzymes of the pathways involved in BA production can be encoded by unstable plasmids, and only strains harbouring BA-related plasmids are able to produce BAs (Lucas et al., 2005). Over the past few years, some authors have described the genetic organization of decarboxylase clusters for tyramine (Marcobal et al., 2012), histamine (Landete et al., 2008), putrescine (Wunderlichová et al., 2014), and agmatine deaminase (Lucas et al., 2007) in different species of lactobacilli. Although different LAB species isolated from cider can produce histamine and tyramine, they are the most efficient producers of putrescine (Table 2). Most LAB produce histamine via the histidine decarboxylase (HDC) pathway (Coton et al. 1998b), putrescine via the ornithine (ODC) pathway (Arena & Manca de Nadra 2001), and tyramine via the tyrosine decarboxylase (TDC) pathway (Lucas et al., 2003). PCR-based methods of detection of the genes encoding these enzymes have been designed to identify (and quantify) LAB involved in the synthesis of BA in cider (Ladero et al., 2011). These methods, which consist in detecting microorganisms that have amino acid decarboxylases or agmatine deiminase, can be used to determine the risk of BA spoilage but not the final BA levels (Lucas et al., 2007).

Table 1. Examples of biogenic amine concentrations given in the literature

Amine	Concentration (mg/l)	Analytical methods	References
Wine			
Putrescine	0.84-25.5	HPLC	Moreno-Arribas et al., 2008; Preti et al., 2016
Cadaverine	0.33-9.9	HPLC	Moreno-Arribas et al., 2008; Preti et al., 2016
Histamine	0-5.5	HPLC	Moreno-Arribas et al., 2008; Preti et al., 2016
Tyramine	0.75-34.99	HPLC	Moreno-Arribas et al., 2008; Preti et al., 2016
Phenylethylamine	0-1.75	HPLC	Moreno-Arribas et al., 2008; Preti et al., 2016
Ethylamine	0.9-16.40	HPLC	Preti et al., 2016
Methylamine	0.21-1.63	HPLC	Preti et al., 2016
Cider			
Putrescine	2.47-34	HPLC/RP-HPLC	Garai et al., 2006; Ladero et al., 2011; Garai-Ibabe et al., 2013
Cadaverine	4-34	HPLC/RP-HPLC	Ladero et al., 2011; Garai-Ibabe et al., 2013
Histamine	2.7-16	HPLC/RP-HPLC	Ladero et al., 2011; Garai-Ibabe et al., 2013
Tyramine	1.3-14	HPLC/RP-HPLC	Garai et al., 2006; Ladero et al., 2011; Garai-Ibabe et al., 2013
Phenylethylamine	4.0-6.0	RP-HPLC	Garai-Ibabe et al., 2013
Ethylamine	>1	RP-HPLC	Garai-Ibabe et al., 2013
Methylamine	>1	RP-HPLC	Garai-Ibabe et al., 2013
Beer			
Putrescine	1.3-6.8	GC-MS	Fernandes et al., 2001
Cadaverine	0.1-0.8	GC-MS	Fernandes et al., 2001
Histamine	0.03-0.1	GC-MS	Fernandes et al., 2001
Tyramine	0.1-1.6	GC-MS	Fernandes et al., 2001
Phenylethylamine	0.01-0.05	GC-MS	Fernandes et al., 2001
Ethylamine	0.05-0.3	GC-MS	Fernandes et al., 2001
Methylamine	0.05-0.16	GC-MS	Fernandes et al., 2001
Isoamylamine	0.02-0.03	GC-MS	Fernandes et al., 2001

Table 2. Some of biogenic amines produced by lactic acid bacteria isolated from wine, cider and beer

Species	Histamine	Putrescine	Tyramine	Cadaverine	References
Wine					
Lactobacillus brevis	x		x		Moreno-Arribas et al., 2000; Sebastian et al., 2011
Lactobacillus mali	x				Landete et al., 2007
Lactobacillus mesenteroides	x	x	x		Moreno-Arribas et al., 2003; Landete et al., 2007; Moreno-Arribas et al., 2008
Lactobacillus plantarum			x		Moreno-Arribas et al., 2003
Lactobacillus zeae		x			Moreno-Arribas et al., 2008
Oenococcus oeni	x	x			Coton et al., 1998b; Marcobal et al., 2004; Arena & Manca de Nadra, 2001
Pediococcus damnosus	x				Sebastian et al., 2011
Pediococcus parvulus	x				Landete et al., 2007
Cider					
Lactobacillus brevis		x	x		Ladero et al., 2011; Garai et al., 2006
Lactobacillus casei	x				Ladero et al., 2011; Garai et al., 2006
Lactobacillus collinoides	x	x			Ladero et al., 2011; Coton et al., 2010
Lactobacillus diolivorans	x		x		Costantini et al., 2006; Lucas et al., 2007, Ladero et al., 2011
Lactobacillus hilgardii	x				Ladero et al., 2011

Table 2. (Continued)

Species	Histamine	Putrescine	Tyramine	Cadaverine	References
Lactobacillus mali	x	x	x		Ladero et al., 2011
Lactobacillus paracollinoides		x			Costantini et al., 2006
Lactococcus mesenteroides		x			Garai et al., 2006
Oenococcus oeni	x	x	x		Garai et al., 2006
Sporolactobacillus sp.			x		Coton et al., 2010
Beer					
Lactobacillus backii			x		Geissler et al., 2016
Lactobacillus brevis			x	x	Lorencová et al., 2012, Geissler et al. 2016
Lactobacillus buchneri	x				Kalač and Krizek 2003
Lactobacillus casei/paracasei			x		Lorencová et al., 2012
Lactobacillus lindneri	x				Geissler et al., 2016
Lactobacillus paracasei	x	x		x	Kalač & Krizek, 2003
Lactobacillus plantarum			x		Lorencová et al., 2012
Pediococcus damnosus			x		Kalač & Krizek, 2003
Pediococcus sp			x		Lorencová et al. 2012

4. BIOGENIC AMINES IN BEER

Beer is a low alcoholic beverage produced by controlled fermentation of malt with the addition of water and hops (Buňka et al., 2012). Recently, beer has been reported to be a potential health risk for some consumers due

to its BA content. People who are most exposed to the risk of consuming high levels of biogenic amines are those who consume large quantities of beer (several bottles) in a short period of time (Kalač & Krízek, 2003). The problem may concern whole nations as in many countries, annual beer consumption per capita is about 100 liters, with the Czech Republic leading the way with about 140-170 liters per capita per year.

The biogenic amines found in beer can be divided into two groups. The first group includes the amines naturally occurring in malt beer: putrescine, spermidine, spermine and agmatine. The other group includes mainly histamine, tyramine and cadaverine, which are produced by LAB and are indicative of bacterial contamination during brewing (Izquierdo-Pulido et al., 1996b). Studies on BAs in beers show that agmatine is the most abundant beer BA (concentration of 10 mg/L). As agmatine has minimal negative effects on the human body, it is rarely the main focus of research (Izquierdo-Pulido et al., 1996a; Glória & Izquierdo-Pulido, 1999). The next most prevalent BAs in beer are tyramine (average concentration range 2.2-8.3 mg/l) and putrescine (average concentration range 1.8-8.8 mg/l). Histamine is found at significantly lower concentrations (average concentration range 0.3–24 mg/l), which differ by country, for example, 4.7 mg/l in Swiss beer, 15 mg/l in Danish beer, and 20 mg/l in beer originating from France (Shalaby, 1996). In the light of these data, tyramine has the greatest toxicological significance in cases of high intake of beer. Ingestion of about 6 mg of tyramine in four hours with beer is considered dangerous for patients with hypertension. This effect has been observed for both non-alcoholic and alcoholic beer, however, alcohol can exacerbate the harmfulness of biogenic amines (Tailor et al., 1994). The highest concentrations of harmful tyramine and histamine have been found in Belgian spontaneous fermentation beers and top-fermented beers (Gasarasi et al., 2003).

In raw material, higher concentrations of BAs are found in malt than in hops. Malt is a source of amines such as putrescine, spermidine, spermine, and agmatine, but some of them are lost with spent grain. The highest increase in histamine, tyramine, and cadaverine levels is observed during the main fermentation (Kalač et al., 1997; Gorinstein et al., 1999). Since

the yeast *Saccharomyces cerevisiae* var. *uvarum* does not contribute to the increase in tyramine and histamine and does not affect the level of amines despite several cycles of fermentation, the largest share in the production of amines is attributed to bacteria.

Beer does not provide an environment conducive to bacterial growth. The main obstacles to the multiplication of bacteria in beer are ethanol (from 5% to 10%), high carbon dioxide concentrations (about 0.5% w/v), hops and iso-α-acids, low pH (3.8-4.7), and, above all, an insufficient amount of nutrients. Despite these adverse conditions, spoilage lactic acid bacteria are present in beer. They mainly include microorganisms of the genus *Lactobacillus* and *Pediococcus*, whose metabolites spoil beer causing visible turbidity, acidification, sediment formation, and changes in odor and flavor (Suzuki, 2011). It has been established that biogenic amines in beer are produced by LAB during storage rather than by chemical reaction. In a study conducted by Geissler et al., (2016), all the heterofermentative lactic acid bacteria they investigated produced at least one BA. In addition, Izquierdo-Pulido and others reported that bacteria isolated from beer, which mainly belonged to *Pediococcus* spp., in particular *P. damnosus*, produced tyramine at concentrations ranging from 15 to 25 mg/l at CFU about 1×105 per ml. None of the isolated bacteria belonged to *Lactobacillus* spp. (Izquierdo-Pulido et al., 1996a). In Geissler et al.'s study, *L. lindneri* strains derived from beer metabolized histidine to histamine and *L. brevis* strains transformed tyrosine into tyramine (Geissler et al., 2016).

5. Molecular Methods Used to Detect BA-Producing Bacteria

Today, genetic engineering methods can be used to prevent the formation of BAs in alcoholic beverages by helping wine-makers to choose the right microorganisms (ones that do not produce BAs) for alcoholic and malolactic fermentation. In PCR, specific primers have been designed to

detect the presence of genes encoding the enzymes of BA production pathways. Four primer pairs have been described which can be used to detect the histamine decarboxylase gene (hdc), and two pairs for ornithine decarboxylase (ect) and tyramine decarboxylase (tdc) each. By using the multiplex method, all the aforementioned genes can be detected in one reaction and at the same time using different bacteria. Coton et al. (2010) analyzed 810 strains of lactic acid bacteria for the presence of BA genes in a multiplex reaction. They found that 80% of the strains had at least one of the detected genes, and that seven genera of LAB were able to produce BAs. Landete et al., used molecular methods to test a commercial yeast starter culture contaminated with lactic acid bacteria. They found that LAB identified as *Lactobacillus rossiae* and *Lactobacillus parabuchneri* had *hdc* genes (Landete et al., 2011). Ruiz et al., (2010) studied eight strains of *O. oeni* isolated from Spanish tempranillo wines, which could all be used as starter strains since none of them possessed the *hdc* gene (Ruiz et al., 2010).

Quantitative PCR (QPCR) has also been used to identify and quantify BA-producing LAB. The QPCR method outperforms conventional PCR by using fluorescence techniques to monitor the amount of the amplification product at each stage during the QPCR cycle, and determines the populations of BA-producing bacteria. QPCR for detecting histamine-producing bacteria was designed by Lucas et al., (2008). It detects even single bacteria in one ml of wine and is not impaired by significant amounts of polyphenols and yeast cells. Although the standard curve was made for *Lb. hilgardii*, this method can be used to effectively detect other histamine-producing species. QPCR allows counting of LAB which have the *hdc* gene in a range from 1 to 107 CFU/ml. In Lucas et al.'s experiment, 264 samples of wine in the malolactic fermentation stage were examined, almost all of which contained histamine-producing LAB. Seventy percent of the samples had LAB counts exceeding 103 CFU/ml (Lucas et al., 2008).

6. Effect of Biogenic Amines on Human Health

Biogenic amines, and especially histamine, are neutralized by mucin in the gastrointestinal tract, and more specifically in the intestinal mucosa. If the concentration of histamine is too high, some of it is absorbed by the body. The human body can inactivate histamine by methylation (by methyltransferase activity) or by acetylation (Lehane & Olley, 2000). While histamine methyltransferase has a specific activity, monoaminoxidase (MAO) and diaminoxidases (DAO) have a broader spectrum of action.

Unfortunately, certain drugs, ethanol, and other amines derived from food can inhibit the action of the detoxifying enzymes (Jung & Bieldanes, 1979; Sattler et al., 1985). Taking into account the toxicity of BAs, their amounts ingested with food should be properly estimated. It is important to remember that concurrent consumption of several fermented foods can confer toxic effects even if the single products are considered safe. Frequent consumption of cheeses, fermented meats with large quantities of wine or other alcoholic beverages can be associated with complications such as headache, vomiting, rash and specific allergic symptoms (Sessa et al., 1984). It has also been reported that biogenic amines, especially large amounts of histamine and tyramine, can cause food spoilage. This may be related to the fact that putrescine and cadaverine, which are secondary amines, can form carcinogenic compounds with nitrates. Lyte (2004) investigated the effect of the presence of BAs on the ability of *Escherichia coli* O157:H7 to adhere to intestinal mucosa. The presence of tyramine increased the adhesion of the opportunistic pathogen *E. coli* to the intestine (Lyte, 2004). Intake of high doses of BAs or insufficient detoxification due to individual problems (genetic predisposition or interactions with drugs a person is taking) leads to the entry of BAs into the circulatory system, where the release of epinephrine and norepinephrine results in the secretion of gastric acid, tachycardia, and elevated blood pressure (Beneduce et al., 2010).

CONCLUSION

Foods and drinks that contain proteins and free amino acids are most at risk of the formation of biogenic amines (Smit et al., 2008). Fermented alcoholic beverages sometimes contain a variety of biogenic amines which can cause some health problems. The most common symptoms experienced after consuming large amounts of wine, beer or cider are headaches and flushing, most often in the face and neck (Stratton et al., 1991). These symptoms more closely resemble an allergic reaction than the effects of consumption of alcohol. Furthermore, large amounts of biogenic amines consumed together with ethanol, may lead to synergistic reactions that can cause more severe health effects, particularly in susceptible individuals, e.g., those suffering from diseases such as hypertension (Coton et al., 2010). The amount and variety of biogenic amines are specific to every product and depend on many factors. In wine, the most prevalent and the most toxic amines are putrescine and histamine, while beer contains mostly agmatine, which does not pose a threat to human health, but also high amounts of tyramine. In the case of cider, the most abundant BAs are putrescine and cadaverine. Monitoring of biogenic amines content at the various stages of production of alcoholic beverages, from raw material testing, through fermentation control, to proper storage of alcoholic beverages, is extremely important, as these compounds can be released at each stage (Beneduce et al., 2010). It is important to remember that the main responsibility for the formation of BAs is borne by LAB and that contamination with these bacteria can often be avoided. The ability to produce BAs seems to be a random, strain-dependent feature of microorganisms (both yeasts and bacteria) rather than a common species-specific trait. The final content of biogenic amines is the sum of several factors, so it is extremely important to know how they are formed and what triggers their accumulation at what stage of production of foods and beverages, because this is the only way to prevent unwanted microorganisms from contaminating those products.

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Chapter 6

THE OCCURRENCE OF BIOGENIC AMINES IN FOOD ORIGINATING FROM ANIMALS: CURRENT KNOWLEDGE AND FUTURE PERSPECTIVES

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ABSTRACT

Biogenic amines (BA) are natural amines that belong to trace compound substances, which show physiological significance in low doses, while they can have deleterious effects on human health in high

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concentrations. In addition, these substances can also be considered as a freshness index for fish and several other foods. Different bacteria, yeasts and molds produce BAs, through the decarboxylation of the corresponding amino acids, as a stress response mechanism to low pH conditions. The BA contents in different types of food usually increase due to microbial spoilage during spontaneous or controlled microbial fermentation, and this increase could create several food safety problems. After a brief introduction, this chapter will critically review the latest knowledge, described in studies carried out throughout the world, about the occurrence and the food safety issues of BAs in two selected animal origin food sources, that is, fish and dairy products. Moreover, different technological strategies adopted for the prevention of BA accumulation in the above mentioned food products, for safety purposes, will be evaluated.

Keywords: histamine, microbial spoilage, food safety, seafood, dairy products

1. INTRODUCTION

Biogenic amines (BAs) are low molecular weight organic compounds that can be naturally found in organisms, or in natural metabolic processes due to the fermentation activity of microorganisms. They can be classified as (Sheng et al., 2016):

- Monoamines, e.g., histamine (HIM), tyramine (TYM), tryptamine (TRM) and 2-phenylethylamine (PEA)
- Diamines or polyamines, e.g., putrescine (PUT), cadaverine (CAD) and agmatine (AGM).

They can also be classified, according to their structures, as aliphatic (PUT, CAD), aromatic (TIR, PEA) or heterocyclic BAs (HIM, TYM) (Papavergou et al., 2012).

Each BA can be generated by decarboxylation from a different amino acid precursor: HIM from histidine, TYM from tyrosine, TRM and melatonin from tryptophan, PEA from 2-phenylalanine, TRM from

tryptophan, PUT from ornithine and CAD from lysine. Moreover, other BAs can be produced from the BAs themselves, e.g., spermine (SPD) and spermidine (SPM), which can be produced from PUT (Pessione & Cirrincione, 2016; Sheng et al., 2016).

The presence of BAs in food can arise from different sources (EFSA, 2011; Custódio et al., 2016):

1) Preformed BAs in raw materials can be found at low levels: PUT, SPD and SPM in meat, offal such as liver, fish, milk and fruit. HIM in offal and in blood;
2) Spoilage products, because spoilage microorganisms may contribute to a great extent to the formation of BAs, and in many cases are a part of the natural environmental and animal flora (gills and gut of fishes e.g.,) (Viscano et al., 2014);
3) Fermentation by Lactic Acid Bacteria (LAB) or other starter bacteria during the production of fermented sausages, cheese, sour dough bread and also during the malolactic fermentation of wine (Khairy et al., 2016; Pessione & Cirrincione, 2016). Close correlations between protein decarboxylation, the pathway for BA formation, and lipid oxidation have recently been found, and it has been demonstrated that the presence of lipid oxidation contributes to the chemical decarboxylation of amino acids (Loizzo et al., 2016). LAB are important producers of BAs, because they use this metabolic pathway to create energy (through a proton gradient) and to alkalinize the environment (the products of this fermentation are acids) (Pessione & Cirrincione, 2016). At present, there is no evidence about the massive formation of BAs from yeast and molds (EFSA, 2011);
4) The possible *in situ* formation of biogenic amines in the gut by microbiota from both the intestine and food, but at the moment this possibility is unclear and has not yet been proven (EFSA, 2011; Wüst et al., 2016).

BAs are involved in many biological processes, and their presence at non-physiological levels in food can therefore be considered a biological hazard (FAO, 2014). Because of their presence in many fundamental reactions, even at low levels, the intoxication symptoms can have/show very different degrees of intensity and, as will be seen later on, it is very difficult to establish what constitutes a toxic dose. Intoxications are frequently misdiagnosed, since they only sometimes require medical attention or adequate diagnostic systems are not available (Visciano et al., 2014; Latorre-Moratalla et al., 2017). BAs can be linked to food intolerance at a moderate level, or intoxication at a high level (Pessione & Cirrincione, 2016). In addition, direct toxicity and toxicity due to the saturation of detoxification mechanisms should be considered for symptoms and toxic doses.

Only HIM and TYR are known to have a direct toxicity effect. Intoxication involving HIM ingestion is usually known as "Histamine Poisoning" or "Scombroid Poisoning." This BA acts on the nervous, gastric and intestinal systems, and can affect blood pressure regulation (Visciano et al., 2014; Zhou et al., 2016). The main symptoms of the intoxication are: reddening of the skin, edemas and rashes, eyelid edema, hypotension and headaches (due to dilatation of the peripheral blood vessels), and to a lesser extent, diarrhea and vomiting (due to contraction of the smooth muscle), cardiac palpitation, and in the most severe cases, even intracerebral hemorrhaging and anaphylactic shock (Papavergou et al., 2012; Khairy et al., 2016; Del Rio et al., 2017).

Another well-known intoxication is the "Cheese Reaction," for which TYM, but also PHE and TRM, are responsible. As for HIS, the symptoms are: severe headaches, migraines, neurological disorders, nausea, vomiting, respiratory disorders and hypertensives crisis (Papavergou et al., 2012; Del Rio et al., 2017). PUT have been also associated with tumoral growth (Rauscher-Gabernig et al., 2017). As far as indirect toxicity is concerned, PUT and CAD can potentiate HIS and TYR intoxication and form nitrosamines, highly carcinogenetic compounds, with nitrite in meat products (Rauscher-Gabernig et al., 2017).

Many authors have linked other BAs, such as SPD and SPM, to food allergies. It has been observed that these BAs are able to increase intestinal permeability, and as a consequence, an increase in food allergens can be assumed. This means a potential facilitation in the induction of food allergies by other substances, in other words allergens (Papavergou et al., 2012). This subject can be of great concern if it is taken into account that SPM and SPD are the only ammines naturally present in fresh meat (Lu et al., 2010; Papavergou et al., 2012; Jastrzębska et al., 2016).

However, BAs do not always affect human health negatively. The decarboxylation of TRM or the metabolism of serotonin Lactic Acid Bacteria (LAB) can synthetize melatonin, a molecule that is involved in sleep and reproductive behavior, immunity, inflammation and carcinogenesis. TRM and PEA are involved in mood control and appetite/satiety balance regulation, but controversial effects, such as insomnia, anxiety and MAO-blocking activity, have also been reported for the latter (Pessione & Cirrincione, 2016).

From the organoleptic point of view, some authors have made a contribution to the development of food aromas (Ranucci et al., 2016).

If we consider the fact that BAs can be produced by decarboxylating spoilage bacteria, another useful application is their quantification as indexes/indices of the hygienic quality of raw material, of the shelf life or the spoilage of a product (Schirone et al., 2011; Singh et al., 2012; Khairy et al., 2016; Efenberger-Szmechtyk et al., 2017). For this purpose, a number of the so-called Biogenic Amine Indexes (BAIs) have been suggested, which may include one or more BAs.

The former was proposed by Metz and Karmas in 1977 and was calculated according to the equation (Stadnik & Dolatowski, 2010):

$$\frac{cHIM+cPUT+cCAD}{1+cSPM+SPD}$$

(c – concentration, $mg \cdot kg^{-1}$).

HIS has been considered as a single BA Index for fish (Khairy et al., 2016), while TYR has been considered as an index for meat and seafood products (He et al., 2016). Several multiple ones e.g.,: HIS+PUT+SPM+SPD or PUT+CAD+HIM+TYR (Zare & Ghazali, 2017) and a sum of TYR+PEA+HIM+TRM have been proposed for ripened meat products (Wüst et al., 2016). All these indexes are useful, but are not always reliable because BAs are present at very different doses. For this reason, it has been proposed to give different weights to HIS and other BAs (Zare & Ghazali, 2017).

Finally, TYR has been proposed, with other substances, for Principal Component Analysis (PCA), as an indicator of irradiation in beef (Efenberger-Szmechtyk et al., 2017).

The European Union (2011) published an extensive review on the risk assessment of BAs, in which it was stated that these substances are still of great concern, and that there is still a lack of information on BAs. The results of this study proved/pointed out that the risk for a healthy population can be assumed negligible, but the possibility of the simultaneous assumption of more than one BA, and the factors that can lower the toxic dose, should also be considered (EFSA, 2011; Wüst et al., 2016). Another factor that can increase toxicity is the synergistic effect among different BAs. A synergistic effect among different BAs is becoming more and more evident (FDA, 2009). This assumption has recently been proved on intestinal cell cultures, and it has been found that HIS induces apoptosis and TYR induces necrosis, while acting in a synergistic way (Del Rio et al., 2017).

There is still a lack of studies on the risk assessment of BAs, as recently pointed out by several authors. Rausher-Gabering et al., studied a risk assessment for HIS in 2009. Wüst et al., (2016) conducted a detailed investigation on the presence of TRM in Austrian food, and they found that even though the presence of BAs alone does not seem to be a reason for health concern, the simultaneous ingestion of other BAs, combined with an increased susceptibility to them, could be dangerous. Latorre-Mortalla et al., (2017) reached the same conclusions for the case of the consumption of Spanish sausages and HIS or TIR intoxication.

Apart from the synergistic effect mentioned above, their potential effect on the human metabolism is influenced to a great extent by many other factors, but in general it is possible to state that an increased sensitivity to BAs is connected to a weakened enzymatic degradation, caused by genetic or acquired impairment of detoxifying systems (Mono Amino Oxidase (MAO), Di Amino Oxidase (DAO), and the HNMT function).

This acquired impairment could be due to several physiological or pathological factors:

- a decrease in the activity of B-type MAO, as has been reported for the premenstrual period (EFSA, 2011);
- food allergies (DAO activity) (EFSA, 2011);
- people suffering from pathologies, such as chronic urticaria, atopic eczema, respiratory and coronary problems, hypertension, and vitamin B6 deficiency, are particularly at risk because of their sensitivity to lower doses of biogenic amines (Maintz & Novak, 2007).

The functionality, and therefore the detoxification systems, of intestinal oxidases can also be reduced by gastro intestinal pathologies, such as inflammatory and neoplastic diseases (Maintz & Novak, 2007).

Till now, there is only weak evidence that shows that bacterial endotoxins produced primarily by gram-negative bacteria, can cause hypersensitivity to histamine or induce histamine release in animals, which in turn can cause endotoxin shock (FDA, 2009). It has been reported that tobacco smoke, and other food components, such as alcohol and its metabolite acetaldehyde, phenols, may reduce MAO levels by as much as 40% (EFSA, 2011; Redruello et al., 2017). Finally, the ingestion of other amines seems to have a great influence on the toxicity levels of HIS and TYM, as it facilitates the transport of histamine through the intestinal wall and increases its toxicity (FDA, 2009).

It is obvious that this status can be transient and reversible. Apart from the acquired factors, it is also possible to observe a genetic impairment of

oxidases. It has been estimated that 1% of the population has histamine intolerance, and 80% of those are middle-aged female patients (EFSA, 2011). In Spain, HIS intolerance raises the cases of HIS intoxication to 7000 cases per million inhabitants (Latorre-Moratalla et al., 2017).

On the other hand, a physiological increase in DAO production (up to 500-fold) has been reported in pregnant woman, which could explain the remissions of food intolerance frequently observed during pregnancy (Maintz & Novak, 2007). All these variables point out that, at the moment, it is not completely clear at which level and to what extent the synergisms occur among all the different BAs (FDA, 2009). It is still difficult to establish a toxic dose for BAs, because very few studies on the dose response, based on alimentary administration, have been developed (Rauscher-Gabernig et al., 2009; Sheng et al., 2016). The "no effect level" for HIS was calculated to correspond to an ingestion that ranged from 6 to 25 mg/meal (Rauscher-Gabernig et al., 2009). EFSA (2011) stated a No Adverse Effect Level (NOAEL) of 50 mg/kg meal. A more detailed classification has recently been proposed that takes into account three different levels of symptoms: a slight poisoning (8-40 mg/kg meal); an intermediate poisoning (40-100 mg/kg meal) and intensive poisoning (100 mg/kg meal or higher) (He et al., 2016). No adverse heath effect has been observed for TYM up to 600 mg/person meal (EFSA, 2011; Jastrzębska et al., 2016) but, if MAO Inhibitor (MAOI) drugs or even third generation MAOI drugs are assumed, the safe limits decrease dramatically to 50 and 6 mg, respectively (EFSA Panel, 2011; He et al., 2016; Iacumin et al., 2017).

Very few data are also available for other BAs. Rausher-Gabernig et al., (2017) reported a safe daily ingestion amount of PUT of 9.8 mg (female adults) and 8.8 mg (male adults), and of 9.8 mg (female adults) and 11.6 mg (male adults) for CAD.

The only BA regulated in Europe is HIS. Even though this BA can be produced in almost all the foods obtained from fermentation processes, the only foods mentioned in the laws are: fishery products from fish with high amounts of HIS, fishery products which have undergone an enzyme maturation treatment in brine (REG CE 2073/2005; REG CE 1441/2007)

and fish sauce produced from the fermentation of fishery products (REG CE 1019/2013). The criteria stated in the Regulations are all "Food Safety Criteria"; this means that these criteria have to be respected in order to avoid pathologies related to ingestion. The sampling plan consists of a number of units of the sample (*n*) and a maximum number of sample units (*c*) that give values between (*m*) and (*M*). The batch is considered satisfactory when:

- all the samples show values below *m*
- all the batches show values below m, and a number of batches, equal to *c*, are between *m* and *M*.
- none of the batches is above *M*

Nine batches have to be sampled for "Fishery products from fish species associated with high amounts of histidine," that is, from species such as Tuna, Scombroid and so on, and the maximum quantity must me less than 100 mg/kg. Only two out of 9 can be between 100 and 200 mg/kg, but none can be over 200 mg/kg. The quantities are doubled for fishery products which have undergone a enzyme maturation treatment in brine (from fish species with high amounts of histidine): 200 mg/kg and 400 mg/kg, respectively. As stated above, a new product has recently been added, through EU regulation 1019/2013 (2013): fish sauce produced by the fermentation of fishery products. The maximum quantity of HIS for this category is 400 mg/kg. In the United States of America, the Food and Drug Administration (2009) also established safety levels, but only for HIS, for Scombroid and Scombroid-like fish, and set this safety level at 50 mg/kg.

At present, the limits for TYR that have been proposed in the EU and in the USA are only recommendations, but they do not have any legal value. These limits are 600 and 100 mg/kg, respectively (EFSA, 2011; Alizadeh et al., 2017). Unfortunately, there is still a lack information about the safe limits of PUT and CAD (EFSA, 2011).

The total content of BAs in food depends to a great extent on several factors, such as the nature of the product, the hygienic quality of the raw

materials, the time of and the temperature during the production and the ripening, the fermentation process by amino acid decarboxylase-positive microorganisms, such as LAB, and many others. Some of them have to be considered carefully as they constitute a crucial part of the production process. This scenario, based on multiple and complex variables, makes it difficult to characterize the influence of each technological factor. At the moment, the most frequently adopted approach is based on (EFSA, 2011):

1. Control of the hygienic quality of the raw materials and production processes, in order to limit contamination
2. Control of the production techniques with the aim of inhibiting and limiting the growth and the decarboxylating activity of microorganisms.

This approach has been proposed as a "low histamine technology." It is based on Good Hygienic Practices (GHP), Good Manufacturing Practices (GMP), Hazard Analysis and Critical Control Point (HACCP) and on the implementation of specific technological measures. The main problem that arises involves conserving all the organoleptic characteristics that the consumer choses as criteria, while achieving a safe product. First used for wine, this approach has since been studied for many other foods (EFSA, 2011).

1.1. Hygienic Quality of Raw Materials and Production Processes

The hygienic quality of raw material can be efficiently implemented through an HACCP process, GHP and GMP, as well as cleaning and disinfection procedures (Ruiz-Capillas & Jimenez Colmenero, 2010). It has been demonstrated that aminogenic bacteria are frequently part of the natural flora of fish, or are in the intestine and gut, and a proper manipulation and practices that efficiently reduce the number of bacteria can therefore be successful in preventing the spread and the growth of

these bacteria. These measures also should adopted for other sources of BAs that produce bacteria, such as the water and salt that are used for brine, spices and other ingredients.

During the storage of fresh commodities (e.g., meat and seafood products), the most effective measure seems to be time/temperature control, probably due to the inhibition of the decarboxylase activity of bacteria (EFSA, 2011).

High temperature treatments can also be used. However, these measures deserve further research before being applied to the industrial food chain, because very little information is available about thermostability of decarboxylating enzymes, and, in addition, pasteurization does not prevent the presence of ripening decarboxylating microbiota (EFSA, 2011).

Even the use of low temperatures, in order to control bacterial growth, should be studied carefully, because psychrotolerant spoilage bacteria are also relevant in relation to the biogenic amine production in fish (Rodrigues et al., 2016). Only freezing temperatures or temperature close to 0°C can inhibit the growth and activity of aminogenic organisms, and they constitute the most effective way of preventing biogenic amine accumulation in raw material (Ruiz-Capillas & Jimenez Colmenero, 2010).

When the use of these low temperatures leads to a deterioration of the quality of the products, alternative non-thermal technologies, such as irradiation and high hydrostatic pressure, can be proposed (Latorre-Moratalla et al., 2007; Vidal-Carou et al., 2007; Ruiz-Capillas & Jimenez Colmenero, 2010). The use of gamma-irradiation to reduce BAs in food does not seem to have been fully demonstrated yet in industrial food production systems (EFSA, 2011).

1.2. Control of Production Techniques

Microbiota plays a fundamental role in the assessment of the formation of BAs for two main reasons: they can decarboxylate AA, and they have shown a strong proteolytic activity, with the production of AA, which in

turn leads to an increase in the availability of precursor free amino acids (EFSA, 2011). For this reason, the control of the fermentation process and the microbiota can be another way of reducing or modulating the formation of BAs. The solution is to create environmental conditions that favor fermentative (non-aminogenic) microorganisms and inhibit undesired (aminogenic) microbiota.

This can be obtained through the aid of technological processing parameters (temperature, time, redox potential, size etc.) and certain ingredients, such as salt, sugar, preservatives, spices) (EFSA, 2011). The problem is that all of these factors can have multiple targets or mechanisms of action, e.g., acidification inhibits the contamination of microflora, but at the same time stimulates decarboxylation processes as a response against unfavorable acidic environments (Vidal-Carou et al., 2007).

Unfortunately, a rule of thumb cannot be defined for each type of product, because each type of food needs specific formulation and processing parameters, which have to be assessed on a case-by-case basis.

Temperature and time have already been discussed, and the conclusions are the same. The effect of technological factors has recently been investigated extensively by Gardini et al., (2016) for LAB. Conditions that result in a reduced redox potential stimulate HIM production (Stadnik & Dolatowski, 2010).

Considering the food size, this aspect has a great influence on the environment, and as consequence on the growth of microbial flora. The salt concentration and water activity are lower in larger diameter sausages than in thinner ones, and it may be for this reason that some BAs, such as TYR and PUT, have been found in larger amounts in salami with the largest diameters (Aniello et al., 2010; Stadnik & Dolatowski, 2010).

The use of additives, such as nitrites, nitrates, sugars and salt, has been proven to be effective in modulating microbial flora, and consequently BA production. Some acids, such as citric acid, succinic acid and malic acid, some sorbate salts (potassium sorbate) and D-sorbitol show inhibiting activity of the decarboxylases in fish, meat and vegetable products, in association with salt (Naila et al., 2010). Sodium sorbate and sodium hexametaphosphate have also been shown to delay histamine production

(Naila et al., 2010). Nitrites are used in the production of salami as a color preservation and antimicrobial additive. This additive has also been proved to be useful for BA formation (Coloretti et al., 2008; Naila et al., 2010; Lu et al., 2010). For example, large amounts of the total BA count have been correlated to a high *Enterobacteriaceae* count and a low nitrite content in Chinese ripened sausages (Lu et al., 2010). The use of sodium chloride in salami could inhibit histamine formation in many ways: by controlling the microbiota count, influencing the composition and causing a progressive disturbance of the microbial membrane decarboxylases (Aniello et al., 2010).

Sugars and additives have also been proved reliable, as essential oils, in the reduction of aminogenesis (Latorre-Moratalla et al., 2010; Naila et al., 2010; Gardini el al., 2016). Certain substances present in spices, such as allicin (garlic) and 6-gingerol (ginger), have positive effects on controlling the formation of BAs. Polyphenols derivated from roses have also been proposed recently for Chinese meat products (Zhang et al., 2017). Unfortunately, some active substances in spices, such as capsaicin (red pepper), piperidine (black pepper) and curcumin (turmeric), are not heat stable, or have unpleasantly strong flavors, such as thymol (thyme and oregano). In addition to the lack of consumer acceptance, another disadvantage is a lack of available knowledge on their effectiveness (Naila et al., 2010).

At the moment, the most reliable approach is the use of selected starter cultures for both large-scale and traditional small productions of fermented foods and beverages (EFSA, 2011; Lorenzo et al., 2017).

These kinds of approaches are quite complicated, because many different criteria should be taken into account such as: the type of product, the technological attitude, competitiveness, influence on organoleptic characteristics. As proved by the risk assessment, the inability to produce biogenic amines should become a usual criteria for starter culture selection. Starter cultures should ideally be free of the potential to form biogenic amines, and the most appropriate approach should once again be on a case by case basis (EFSA, 2011). For example *L. sakei*, *L. curvatus* and *S. xylosus*, have proved to be useful as starter cultures for fermented sausages

(Latorre-Moratalla et al., 2009; Latorre-Moratalla et al., 2010). The inoculation of amine-negative mixed *Pediococcus acidilactici*, *Staphylococcus carnosus* and *Lactobacillus sakei* starters has been proved effective in fish products (Naila et al., 2010).

Starter cultures should be tested in the same processing conditions, and should take account of the technological requirements of each product.

Another possibility is the selection of microbes that can influence the development of decarboxylase flora by acting in different ways (Naila et al., 2010; Stadnik & Dolatowski, 2010; EFSA, 2011; Gardini et al., 2016):

1. They can show a suppression of inhibiting behavior by producing specific substances, such as bacteriocins (for example curvacin A), thus increasing their competitiveness
2. They can act by modifying the factors that influence biogenic amine formation (the availability of precursor amino acids, acidification, etc.)

LAB bacteria, *Tetragenococcus koreensis* and other LAB have been proved to be reliable species, if they find proper conditions (Lorenzo et al., 2017). Studies should also be undertaken on them in order to find the best fermentation parameters. This is crucial to enable them to exert their protective effect so as to prevent BA accumulation.

A natural reservoir for this flora should be the product itself, and it is from this that the research should begin. Several studies, which did not consider the influence of the strain, have proved that indigenous starters are more effective that commercial starter cultures, (Latorre-Moratalla et al., 2010). Mixed starter cultures, not only of LAB, but also those with other species involved in meat fermentation (e.g., staphylococci) can contribute to the control of a wider variety of aminogenic microorganisms (Vidal-Carou et al., 2007; Naila et al., 2010).

In short, many approaches may be followed to control BA formation or to reduce their formation, since their biological production and metabolization pathways are conditioned by several factors. However, the starting point should always be the product and its production process,

because, as stated above, aminogenesis in fermented food is a complex phenomenon and the interaction among all the involved factors is not yet fully understood (EFSA, 2011).

2. FISH PRODUCTS

2.1. Introduction

Shellfish, cephalopods and fish provide a healthy source of high-quality proteins, essential vitamins, minerals and high levels of long chain n-3 polyunsaturated fatty acids, which have been shown to have various health benefits in human nutrition, such as protection against certain cancer and coronary heart diseases. Unfortunately, these benefits may be offset by the decomposition of the fish, and the subsequent formation of chemical contaminants, such as BAs. Although many BAs have been found in fish, only histamine, CAD and PUT have been found to be significant for fish safety and quality determination. Despite the widely reported association between histamine and scombroid food poisoning, histamine alone appears to be insufficient to cause food toxicity. PUT and CAD have been suggested to potentiate histamine toxicity. On the other hand, as far as spoilage is concerned, only CAD has been found to be a useful index of the initial stage of fish decomposition (Al Bulushi et al., 2009).

2.2. Factors That Affect BA Production

Environmental factors, such as the temperature, salt concentration and pH, can influence the formation of BAs in food through the modulation of the microbial activities responsible for the overall metabolism of the decarboxylating cells as well the activity of decarboxylases, as recently pointed out in a review by Gardini et al., (2016).

Amino biogenic bacteria growth is an essential, but not sufficient, condition for BA production. In fact, it has been demonstrated that some

decarboxylase, like histidine decarboxylase in *S. thermophilus* (Tabanelli et al., 2012), and Gram-negative bacteria, such as *Photobacterium phosphoreum*, *Photobacterium damselae*, *M. morganii*, and *R. planticola* (Kanki et al., 2007), can maintain their activity, independently of the integrity of the microbial cells. Different studies have been carried out on seafood, as recently reviewed by Prester (2011), to assess the effect of different environmental factors on the shelf life of these animal origin food products. As far as storage temperatures are concerned, the shelf life of yellowfin tuna (*Thunnus albacares*) has been determined, during storage at 0, 8, and 20°C, through changes in the microbial, chemical, and organoleptic attributes, while its safety has been determined through HIM development. Yellowfin tuna stored at 8 and 20°C reached unacceptable HIM levels after 4 and 1 day, respectively, and therefore became unsafe for human consumption, while HIM development was found to be lower than the Food and Drug Authority (FDA) safety level of 5 mg/100 g fish for storage at 0° for 17 days (Guizani et al., 2005).

In another study on the quality of yellowfin tuna fillets, Du et al., (2002) evaluated the effects of storage at 0, 4, 10, and 22°C for 0, 1, 3, 5, and 9 days, respectively, on the development of some BAs. The samples stored at 4°C for 9 days, 10°C for 5 days, and 22°C for 3 days were rated/judged unacceptable for consumption. Amounts of 832 ppm, 35.8 ppm and 147 ppm, respectively, of HIM, PUT and CAD were detected in fillets stored at 22°C for 3 days. A histamine-producing bacterial population of 7 log10 CFU/g was found, and the decarboxylase-positive bacterial species responsible for histamine production isolated in the tested tuna fillets and identified during storage were *Morganella morganii*, *Enterobacter agglomerans*, *Enterobacter intermedium*, *Pseudomonas fluorescens, Proteus vulgaris* and *Serratia liquefaciens*.

The formation of BAs has been investigated in the brackish water of barramundi fish (*Lates calcarifer*) at 0°C and 4°C over a 15 day storage period. Significant differences were observed in the BA concentrations of barramundi slices stored at 4°C and at 0°C after 3 days of storage. All the amines, except TYM, AGM, TRM and PEA, increased with time during storage at both temperatures. Samples kept at 0°C and 4°C showed HIM

concentrations of 82 mg/kg and 275 mg/kg, respectively, at the end of the storage period. The histamine-forming bacteria in all the samples ranged from 5.4 to 6.1 log CFU/g at 0°C and 4°C, respectively (Bakar et al., 2010).

Anchovy (*Engraulis encrasicholus*) is another seafood that has been studied extensively for BA quantification and formation. Rossano et al., (2006) studied the influence of the time of freezing and the storage temperature on histamine formation, utilizing a fast and reproducible analytical method named capillary zone electrophoresis. The obtained results show that a freezing time longer than 7 hours is important to reduce or stop the rate of histamine formation after thawing and storage at 4°C, while HIM formation is increased greatly at storage temperatures above 4°C.

3. TECHNOLOGICAL FACTORS FOR CONTROLLING STRATEGIES

3.1. Natural and Chemical Additive Treatments

In order to contrast the microbiological deterioration that can generate a wide array of off- flavor compounds in fish products, such as BAs, the use of natural products utilized as food additives has fostered research in order to identify new strategies that have the commercial potential of producing fish-based foods with a longer shelf life and a higher degree of safety (Corbo et al., 2008; Del Nobile et al., 2009).

Křížek et al., (2002) studied the preservation effects of a commercial product based on lactic acid (Purac), at various concentrations, and they monitored the changes in BA content in whole or minced carp (*Cyprinus carpio*) muscle stored at 3 and 15°C. The results showed a clear effect of Purac, even though it cannot counteract the influence of temperature, which has a dominant effect on BA formation. The authors in particular

proposed adopting a PUT concentration as a chemical indicator of carp meat quality.

Potassium sorbate, applied at concentrations of 0, 1, 3 and 5% w/v, is another additive that has been tested to establish the shelf life of pearl mullet (*Chalcalburnus tarichi*) fillets packaged under a vacuum. Microbiological and chemical (pH, thiobarbituric acid-TBA, total volatile base nitrogen-TVB-N, water activity, and BA) analyses were carried out on certain days (0, 3, 6, 9 and 12 days) of storage on fillets kept at 4 ± 1°C. The application of potassium sorbate had a significant effect on the PEA, PUT and TRM contents, as well as on most of the selected microbial parameters. The antimicrobial effect increased as the concentration was increased. The authors concluded that BA values are clearly affected by a potassium sorbate treatment, vacuum packaging and storage temperature. The application of vacuum packaging and potassium sorbate, especially at a 5% level, helps to produce a product with enhanced lasting qualities (Gençcelep et al., 2014).

Lapa-Guimarães et al., (2011) investigated the effects of ripening, temperature and the use of sodium benzoate and citric acid as additives on the quality of ripened cod roe. The BA, volatile base nitrogen (VBN), and trimethylamine (TMA) contents were monitored in ripened cod roe and were compared with the contents in fresh roes. Higher contents of VBN, TMA and BA were found in the ripened roes, regardless of the temperature and additives used during the ripening process. The BA level increased significantly in samples stored at 17°C, without additives, in the ripened cod roe, reaching 126.7 mg/100 g. HIM and CAD reached levels of 8.8 and 0.9 mg/100 g, respectively, in cod roe ripened at 17°C without additives. The addition of sodium benzoate, or citric acid, as a preservative had a significant effect on maintaining the quality of the cod roes, mainly at high ripening temperatures.

Alternative chemical additives have been investigated over the last few years, such as the essential oils of plants, due to their antimicrobial effects. Cai et al., (2015) evaluated the effectiveness of clove, cumin and spearmint oils in maintaining post-mortem quality and extending the shelf life of red drum (*Sciaenops ocellatus*). The microbiological characteristics and BA

content were determined in fresh fish fillets treated with 4 µl/L of each oil at 10°C for 2 h. All the essential oil treatments caused a low BA content, especially HIM, PUT and CAD, which inhibited the microbiological properties throughout the storage periods. The authors therefore concluded that an essential oil treatment might be a suitable application to extend the shelf life and maintain the quality of red drum.

As far as other essential plant oils are concerned, the addition of rosemary oil at three concentrations (0.2%, 1% and 3%) has been evaluated on the BA contents of minced rainbow trout muscle, as were some freshness indicators at 4 ± 1°C after different periods of storage (3 and 9 days). The rosemary oil treatment had a positive effect on the BA content, and led to lower values of PUT, CAD, TYM and HIM. Differences in BA were also found to be due to the storage time, with the exception of SPM, which was not influenced by time (Peiretti et al., 2012).

The effects of extracts from rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) tea were evaluated on vacuum packed sardine (*Sardina pilchardus*) fillets stored at 3 ± 1°C to establish the formation of BAs. Sardine muscle samples showed a lower accumulation of BAs in the rosemary and sage tea groups, due to their phenolic compound antibacterial effects. HIM, PUT and CAD concentrations were reduced significantly in the fish muscle treated with the rosemary and sage tea extracts. After 20 days of storage, the PUT and CAD BA contents in the control group were 100-fold higher than those of the rosemary and sage tea groups (Özogul et al., 2011).

Among the natural substances investigated as potential antimicrobial additives, an important role has been attributed to the polyphenols contained in berry and agricultural by-products. Sampels et al., (2010) carried out a study on the shelf life and quality of herring fillets marinated in a solution of 50 g/L powder of elderberry, cranberry or black currant. All the marinating treatments resulted in a significantly decreased liquid holding ability, coinciding with a lower muscle pH, while increased levels of the BAs (AGM and TYM), carbonyls and ammonium were recorded after the injection of fillets with a 5% salt solution. The authors concluded that the quality and shelf life of the herring fillets marinated in solutions

containing berry powder was enhanced, due to the natural antioxidant supplementation.

Agricultural by-products are rich sources of phenolic compounds, which have antioxidant and antimicrobial effects, and among them, the properties of grape pomace have been investigated in different studies carried out in fish muscle models (Pazos et al., 2005; 2006). Gai et al., (2015) evaluated the effects of red grape pomace ethanolic extracts on the shelf life of minced rainbow trout (*Oncorhynchus mykiss*) muscles. Extracts added to trout patties to obtain final concentrations of 0, 1 and 3% were effective in delaying CAD formation and lipid oxidation in this fish muscle model after 6 days of refrigerated storage. The authors concluded that red grape pomace extract can enhance the quality and shelf life of minced trout and, at the same time, can provide health benefits for consumers, in terms of quality and safety.

3.2. Irradiation and Packaging Treatments

Another way of increasing the safety and shelf life of foods is that of using physical treatments, such as food irradiation, which can reduce microbial growth. The BA content in food could be reduced by means of gamma-irradiation, a physical treatment that is able to delay the initial growth of adventitious microorganisms (Kim et al., 2004).

Křižek et al., (2012) examined the effects of vacuum packaging, followed by high-energy electron beam irradiation, on the shelf life of rainbow trout fillets. Irradiation doses of 0.25, 0.50, 0.75, 1.0 and 2.0 kGy were applied to the samples, and then the control and treated packs were stored at 3.5°C for up to 28, 42, 70 and 98 days. Among the detected BAs, a very good correspondence with the irradiation dose and organoleptic properties was shown for PUT, CAD and TYM. However, the irradiation dose and the time of storage did not affect the SPM and SPD polyamine contents.

In another study, carried out on the shelf-life of chub mackerel (*Scomber japonicus*) fillets, the effects of vacuum packaging, followed by

a gamma irradiation treatment (1.5 kGy), were evaluated during chill storage. The microbial characteristics and chemical (TMA, TBARS, BAs) parameters were analyzed periodically in the control and the treated packs. Vacuum packaging on its own was not sufficient to extend the shelf life of the chub mackerel. The HIM contents reached the defect action levels on the 7th day, and a remarkable presence of mesophiles (3.7 log UFC/g); total coliforms (3.5 log UFC/g); staphylococci (1.9 log UFC/g) was observed in both the control and the vacuum packaged lots, together with the emergence of *Pseudomonas* (1.7 log UFC/g). Gamma irradiation, in combination with vacuum packaging, was found to delay spoilage for 14 days of refrigerated storage. The application of low-dose irradiation (1.5 kGy), on the 3rd day of storage, optimized the sensory quality, as pointed out by the acceptability test. The authors concluded that a low-dose g-irradiation to chub mackerel (1.5 kGy), in combination with vacuum packaging, is recommended to alleviate chemical changes, to extend the shelf life by 7 days and to enhance microbiological quality (4 log reduction) (Mbarki et al., 2009).

A comparative study has been carried out on sea bream (*Sparus aurata*) stored in ice, to establish the effects of different gamma radiation doses (2.5 and 5.0 kGy) on the formation of 12 BAs during storage. The three main amines that formed in the muscle of sea bream were trimethylamine, PUT and CAD, while HIM, TYM and PEA were not detected. The concentration of dopamine, serotonin and SPM did not change significantly for any of the groups during the storage periods, while a minor increase in SPD, AGM and TRM concentrations was detected in irradiated groups on some storage days. The BA content was reduced in a similar manner, except for AGM and TRM, regardless of the radiation dose used (Özogul & Özden, 2013).

The main atmospheric modification technologies used for food preservation are Vacuum Packaging (VP) and Modified Atmosphere Packaging (MAP), both of which play important roles in the selection of spoilage microorganisms and decarboxylating bacteria (Curiel et al., 2011). Therefore, these technologies can affect the qualitative and quantitative formation of BAs. Sivertsvik et al., (2002) highlighted that the application

of MAP to most fishery products improved the shelf life of these products, compared with those packaged under air, but conferred little or no additional shelf life, compared to VP. Another important aspect underlined by the authors is that the benefits of MAP may be lost if the storage temperature is not controlled appropriately. A smaller content of dissolved CO_2 in the product, due to higher temperatures, inevitably leads to a loss of inhibitory effects and can result in possible microbial safety risks.

Křižek et al., (2004) evaluated the effects of storage temperature and packaging method on carp flesh samples stored at 3 and 15°C. The chemical, sensory and microbial qualities were measured in the VP and non-VP treated samples. Seven BAs were determined, and the best correspondence with the sensory and microbial states was found for PUT and CAD. The effect of VP was not evident in samples kept at 15°C, while the shelf life was prolonged by about 4-5 days through the application of VP at 3°C.

Alak et al., (2011) performed a study on Atlantic bonito (*Sarda sarda*) fillets stored at 4°C, where the effect of VP and MAP (100% CO_2) packaging, chitosan film and cling film wrapping was evaluated on the formation of BAs during storage. The levels of CAD, TYM and HIM were significantly different in the four treatments, while PUT, phenylethylamine and TRM did not report the same trend. SPM and SPD BAs were not detected throughout the storage period in any of the experimental conditions. The fillets stored in cling film showed the highest BA contents, and this was followed by the VP, MAP and chitosan film treatments, the latter of which was considered to be the most effective in BA reduction by the authors.

Emborg et al., (2005) studied the effects of VP and MAP on the formation of HIM and other BAs in chilled fresh tuna involved in an outbreak of histamine fish poisoning. The authors utilized challenge tests at 2°C and 10°C in order to evaluate the effect of VP and MAP on the formation of BAs due to psychrotolerant bacteria. The results showed that psychrotolerant *Morganella morganii*-like bacteria, or *Photobacterium phosphoreum*, were responsible for the histamine fish poisoning, and a HIM concentration of >7000 mg/kg was found in the VP tuna, while in

fresh MAP tuna, which showed 60% CO_2/40% N_2, a similar psychrotolerant *M. morganii*-like bacteria formed >5000 mg/kg of HIM after 24 days at 1.7°C. Another interesting point that emerged from the study was that, in the challenge tests, MAP with 40% CO_2/60% O_2 had a strong inhibitory effect on the growth and formation of HIM in both isolated strains. Therefore, the authors concluded that MAP containing ~40% CO_2 and ~ 60% O_2 could be suggested for lean tuna loins instead of VP, in order to reduce the current histamine fish poisoning problems.

Özogul et al., (2002) investigated the application of a rapid high-performance liquid chromatographic (HPLC) method to detect nine BAs in herring stored at 2 ± 2°C and in MAP. The concentrations of amines in the herring kept at 2 ± 2°C increased more rapidly than in the herring stored in MAP. A significant difference in the histamine concentrations was found after 6 days of storage between the herring stored at 2 ± 2°C and those in the MAP, while no differences were recorded during the early stages of the storage period. The between group PUT (after 6 days) and CAD (after 4 days) concentrations were found to be significant different, with lower levels being recorded in the MAP group.

A comparative study was carried out (Özogul & Özogul, 2006) on the effects of packaging on the formation of BAs during the storage of sardines (*Sardina pilchardus*) at 4°C in air, MAP and VP. The maximum levels of PUT (12.2 mg/100g) in the air group was reached at 12 days, while that of CAD (10.0 mg/l00g) was reached at 15 days. The CAD and PUT levels were significantly different, while the SPM and SPD levels did not change much throughout the storage period for any of the experimental conditions. In short, the highest content of BAs was found in sardines stored in air, and this was followed by VP and MAP.

4. DAIRY PRODUCTS

4.1. Introduction

Dairy products are one the most important primary commodities in the diet of human beings, since they have a high nutritional value. Milk is

produced in each and every member state of the European Union (EU), and it represents a very important part of the EU agricultural economy (EU, 2014). Moreover, the consumption of dairy products has risen over the last decade, in particular as far as cheese consumption is concerned. Similarly, cheese production has grown steadily in the United States over the years, and cheese has become a staple part of the American diet.

Therefore, high levels of BAs in cheese may represent a serious threat to consumers' health. The composition of milk encourages the growth of a variety of microorganisms, thus the in situ production of BAs can occur because of its microbial metabolism, which involves the decarboxylation of amino acids by microbial enzymes. Aliphatic amines can be produced by the amination and transamination of aldehydes and ketones (Benkerroum, 2016).

4.2. Incidence of BAs in Dairy Products

The BA content of milk, fermented milks, yogurts and unripened cheeses is usually undetectable, or less than a few tens of milligrams per kg (Linares et al., 2011; Benkerroum, 2016). Among the various dairy products, cheese is the one that is most frequently implicated in BA intoxications, and different studies have shown that BAs commonly occur in cheeses. BAs have been detected in cheeses such as Blue cheese, Cheddar, Emmental, Gouda, Gruyere, Swiss cheese, Ras, Processed cheese and traditional cheeses from cow, ewe and goat milk, and in some cases more than 1000 mg of BA has been detected per kilogram of cheese.

Cheeses associated with BA outbreaks are made from both raw and pasteurized milk (EFSA, 2011), with ripened cheeses being more prone to amine formation. The main BAs that have been reported in cheese are: TYM, PUT, CAD and HIM, but TYM is the BA that is most present, and the term "cheese reaction" was coined to refer to it. In addition, spermidine and SPM, produced by microbial enzymes, have been detected in cheeses. AGM, TRM, PEA and SPD instead do not represent health concerns for dairy products. Different levels of BAs can be detected in different samples

of the same cheese, since the formation of biogenic amines is closely related to the hygienic conditions in which the production occurs. As far as HIM is concerned, No-Observable-Adverse-Effect-Level was observed at 50 mg by the EFSA BIOHAZARD panel, who considered a HIM content in cheese ≤ 200 mg/kg as the safe level, even in the case of high exposure (EFSA 2011), while a study by Rauscher-Gaberning et al., (2009), indicated a maximum concentration of 400 mg/kg in cheese for safe consumption. A dose of 600 mg of TYM was considered safe for healthy individuals, but this dosage should be reduced considerably for people who are taking monoamine oxidase inhibitor (MAOI) drugs. No threshold levels were defined for PUT and CAD.

Short-ripened cheese (<30 d) generally shows a lower BA content than long- term ripened cheese (>30 d); in a study on 20 Southern Italian cheeses, the presence of toxic concentration of BAs was not detected in cheeses ripened for less than 60 days (Guarcello et al., 2015). During ripening, the proteolysis of casein leads to an increase in the free amino acids, some of which can be decarboxylated by bacterial enzymes to produce BAs. Nevertheless, a high degree of proteolysis is not a sufficient requirement for BA production, since the simultaneous presence of decarboxylase bacteria is necessary. It is important to underline that BAs derived from the microbial metabolism are not homogeneously distributed in the cheese, and a concentration gradient generally occurs.

4.3. Factors that Affect BA Production

The presence and the content of BAs in cheese depends to a great extent on different factors related to the hygienic quality of the raw materials, as well as to the production process and storage of the cheese.

The main factors that affect BA production are: the bacterial count in the milk, the preliminary milk treatments, the availability of the amino acid precursor and the cofactor for decarboxylation reactions, starter cultures, chemical-physical factors and the ripening process (duration and environmental conditions) (Novella Rodriguez et al., 2003; Combarros-

Fuertes et al., 2014; Guarcello et al., 2015). Moreover, the presence of BAs in cheese is closely correlated to the section of the cheese (edge/core), the storage time and temperature and the type of packaging. The BA content of cheese reaches a maximum level during ripening, and it can subsequently decrease because of BA degradation (Pinho et al., 2004).

The microorganisms that produce decarboxylases, mainly *Enterobacteriaceae*, *Micrococcaceae* and *Pseudomonas* spp., may be present naturally in the milk, but they can also been found in the starter (whose main function is the production of lactic acid) and non-starter lactic acid bacteria (NSLAB). Decarboxylase activity is strain specific. Tyramine is the main amine formed by LAB, the main tyramine-producing bacteria being enterococci, while Enterobacteriaceae are the main producers of HIM, CAD and PUT (Bover-Cid & Holzapfel, 1999). A capacity for BA formation has also been described for some yeasts (*Debaryomyces hansenii*, *Yarrowia lipolytica*, *Candida intermedia*, *Kluyveromyces marxiamus* var. *lactis* and *Saccharomyces*) (Benkerroum, 2016).

The preliminary treatments of milk, such as the heat treatment, bactofugation or microfiltration, may reduce the risk of BA formation by reducing the bacteria content, and in particular Gram – bacteria, such as *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia* and *Shigella* (Marino et al., 2000). The heat treatment of milk causes a reduction of the BA content in Zamorano cheese of almost 60% (Combarros-Fuerte et al., 2016).

In addition, pasteurization affects the availability of pyridoxal phosphate (the cofactor) since it is heat-sensitive (Ordóñez et al., 1997). The amino acid precursor is available in the matrix, as a consequence of the proteolytic activity of the native milk protease, the proteases originating from the somatic cells and of the adjunct or naturally present microorganisms, but they may also be derived from proteases added with the rennet.

Commercial starters do not usually contain free AA (FAA) decarboxylating bacteria, but natural starters and secondary microbiota can harbor bacteria that possess this activity. Lactic acid bacteria (LAB) belonging to the following genera, can in particular be capable of

decarboxylating amino acid producing BAs: *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Streptococcus* (Burdychova & Komprada, 2007; Russo et al., 2010). Lactobacilli, which are the leading actors during cheese ripening, because of their proteolytic and lipolytic activities, can possess the ability to degrade BAs. Herrero-Fresno et al., (2012) isolated strains of *Lactobacillus casei*, which are able to degrade HIM and TYM, from strains of long-ripened cheeses, and they proved their efficacy in reducing the presence of these BAs in cheese when used as competitive adjunct cultures. Low pH values favor the production of BAs, since FAA decarboxylation is a cell response to acid stress (Marcobal et al., 2006). In addition, moisture (a water activity level of 0.90 - 1.00) and the NaCl content influence BA production to a great extent.

Since the cell content is the most important factor in BA production, the temperature adopted during cheese making and ripening affects the growth kinetics, and consequently the decarboxylase activity. Pinho et al., (2001) suggested TYM and PUT, along with valine and leucine, as indicators of temperature abuse conditions during storage of ripened Azeitão cheese. Buňková et al., (2010) detected higher contents of TYM, PUT and CAD in cheese ripened and stored at 10°C, than those detected in cheeses moved to 5°C after a shorter ripening period at 10°C.

Ripening and storage conditions also affect the distribution of BAs in cheese. In fact, the BA content has been shown to vary in the different parts of the cheese, with the edge showing higher concentrations than the core, and the spatial distribution is not related to the size of the cheese (Buňková et al., 2010). The reason for the non-homogenous level of BAs can be attributed to the different microbiota and LAB count levels in the different layers of the cheese. The washing of the cheese surface of smear-ripened cheeses has been proved to reduce the BA content by more than a hundred-fold, compared to unwashed cheese (Samková et al., 2013).

4.4. Controlling Strategies

Different strategies can be adopted during cheese production to minimize the risk of a high content of BAs in cheese. Moreover, secondary

control measures can be adopted to prevent their formation, or to reduce their content once they have formed.

High levels of HIM, PUT and CAD can be due to a high content of Gram-negative bacteria, mainly Enterobacteriaceae, in the raw milk, as a consequence of poor hygienic conditions during the processing and storage. Therefore, a risk mitigation strategies should start at a farm level, where the adoption of suitable hygiene measures can lead to a reduction in the occurrence of BA-producing microorganisms in milk.

On the other hand, high concentrations of TYM, which is mainly produced by LAB, may be prevented through the use of non-producing or degrading BA starter and adjunct cultures (Linares et al., 2011). Strains belonging to *Brevibacterium linens* (Leuschner & Hammes, 1998a), *Lactobacillus casei* (Herrero-Fresno et al., 2012), *Lb. sakei* and *Lb. curvatus* (Dapkevicius et al., 2000), *Micrococcus varians* (Leuschner & Hammes, 1998b) have been shown to effectively reduce the TYM and HIM contents in experimental cheese. Milk pretreatments, such as microfiltration and bactofugation, contribute to reducing the BA content in cheese by reducing the bacterial count, while pasteurization in addition provides a reduction in the decarboxylation reaction cofactors in milk. Low pH and a high salt content, along with a non-extended proteolysis and low temperatures, create unfavorable environmental conditions for BA production in cheese (Valsamaki et al., 2000). In addition, since BA formation increases with time and for certain storage temperatures, the adoption of strictly controlled storage temperatures, even though they do not influence the maximum level of BAs, can effectively delay their formation in cheese.

The high-pressure processing (HPP) of cheese made from unpasteurized milk has been shown to be a useful way of inactivating microorganisms and enzymes, and consequently of reducing BA formation, without compromising the sensory characteristics over a prolonged ripening and storage period (Calzada et al., 2015). Moreover, any pre-formed BAs could be removed from the cheese by means of an irradiation treatment, which reduces both the microbial load and the BA

content, and is also useful to avoid the overipening of the cheese during storage (Aly et al., 2012).

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Chapter 7

BIOGENIC AMINES IN DRY-CURED AND FERMENTED MEATS

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ABSTRACT

Dry-cured and fermented meats, constitute one of the food products in which considerable amounts of biogenic amines can be found as a consequence of the use of poor quality raw materials, contamination and inappropriate conditions during processing and storage. Additionally, the microorganisms responsible for the fermentation process may contribute to biogenic amines accumulation. Consumption of foods containing large amounts of these amines can have toxicological consequences due to their psychoactive and vasoactive properties. Therefore, the control of biogenic amines accumulation in dry-cured and fermented meats is a challenge for the meat industry.

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Undesired accumulation of biogenic amines in meat products requires the availability of precursors (i.e., free amino acids), the presence of microorganisms with amino acid decarboxylases and favorable conditions that allow bacterial growth, decarboxylase synthesis and activity. The capability to form biogenic amines is generally considered to be strain specific rather than species dependent. Dry-cured and fermented meats represent a significant source of proteins. The protein breakdown products, peptides and amino acids, represent precursors for amine formation.

The most prevalent biogenic amines in dry-cured and fermented meats are histamine, tyramine, cadaverine, putrescine, products of the decarboxylation of histidine, tyrosine, lysine and ornithine, respectively.

The use of selected starter culture without amino decarboxylase activity is a fundamental technological measure to control aminogenesis during dry-cured and fermented meat processing. The inability of the culture to form biogenic amines but also its ability to grow well at the temperature intended for processing of the product and competitiveness in suppressing the growth of wild amine producing microflora should be taken into consideration in the selection of starter cultures. Secondary control measures to prevent biogenic amines formation in dry-cured and fermented meat products or to reduce their levels also need to be employed. Such approaches may include irradiation, controlled atmosphere packaging, or hydrostatic pressures.

This review briefly summarizes current knowledge on biogenic amines content in dry-cured and fermented meats and collects data on the factors affecting their formation. The methods for aminogenesis control in dry-cured meats are also described.

Keywords: biogenic amines, dry-cured meats, fermented meats

1. BIOGENIC AMINES CONTENT IN DRY-CURED AND FERMENTED MEATS

Presence, formation, quantification and control of biogenic amines in dry-cured and fermented meats have demanded considerable attention in the scientific literature of the last decades (Maijala et al., 1995; Eerola et al., 1997; Ruiz-Capillas & Jiménez-Colmenero, 2004; Bover-Cid et al., 2006; Alves et al., 2017). Biogenic amines can be present in this products as a consequence of the use of poor quality raw materials and ingredients,

contamination or inappropriate conditions during processing and storage which favor bacterial growth, decarboxylase synthesis and decarboxylase activity (Jairath et al., 2015). Proteolysis during fermentation and ageing supply abundant free amino acids, the main substrates for decarboxylation activity (Bodmer et al. 1999; Suzzi and Gardini 2003; Karovičová & Kohajdová 2005; Bover-Cid et al., 2006; Latorre-Moratalla et al., 2010a, 2010b). However, the lack of significant correlation between free amino acids content and biogenic amines accumulation proved that, the factors affecting decarboxylase activity could be more important than the precursor amino acids availability (Joosten, 1988; Latorre-Moratalla et al., 2014).

Biogenic amines can be produced both by Gram-positive and Gram-negative bacteria which are present naturally in meat products, introduced by contamination, or also added to meats as a starter culture. Among Gram-negative bacteria, spoilage microorganisms belonging to enterobacteria and pseudomonas are known as the major producers of cadaverine, histamine and putrescine. The ability to produce biogenic amines is widespread also among Gram-positive bacteria. The decarboxylase activity has been found in strains belonging to the genera *Bacillus* and *Staphylococcus*. However, the attention has been mainly focused on lactic acid bacteria (LAB), which are commonly present in fermented foods (Gardini et al., 2016). Although the aminogenic potential among fermentative bacteria is low (form 10% to 20%), the microorganisms responsible for the fermentation process may also contribute to the accumulation of biogenic amines. Some LAB are amino acid decarboxylase positive and can produce histamine, cadaverine, putrescine, but, in particular, they are the most efficient producers of tyramine (Gardini et al., 2016). Thus, to prevent biogenic amines formation in fermented meats it is not only important to avoid contaminant bacteria, but also to select starter culture bacteria and conditions that reduce the activity of potential amine formers (Latorre-Moratalla et al., 2010b).

In fresh meat the only biogenic amines present at significant levels are spermidine and spermine (Hernández-Jover et al., 1997). High spermine contents, usually between 20 and 60 mg kg^{-1}, are typical for meat and meat

products of warm-blooded animals. Spermidine level in meat hardly exceed 10 mg kg^{-1} (Kalač & Krausová, 2005). Some amines such as cadaverine, putrescine and tyramine can be formed during meat storage (Hernández-Jover et al., 1996; Galgano et al., 2009). It was found that the tyramine concentrations in stored beef is the highest on the meat surface, and that it can be effectively reduced by washing (Kaniou et al., 2001; Paulsen et al., 2006). The most prevalent biogenic amines in dry-cured and fermented meat products are histamine, tyramine, cadaverine, putrescine, products of the decarboxylation of histidine, tyrosine, lysine and ornithine, respectively (Ruiz-Capillas & Jiménez-Colmenero, 2004).

Fermented meat products with comparable microbiological profiles may differ in their biogenic amines content, indicating that the production of these compounds depends on various extrinsic and intrinsic factors, such as temperature, pH, redox potential, NaCl content, the size of the sausage, hygienic conditions of manufacturing practices, and effect of starter cultures (Gardini et al., 2001; Suzzi & Gardini, 2003; Komprda et al., 2004; Latorre-Moratalla et al., 2008; Jairath et al., 2015; Domínguez et al., 2016; Laranjo et al., 2017).

The quality of meat raw materials seems to be one of the main factors affecting biogenic amines formation in dry-cured and fermented meat products. Meat is the natural source of the substrate from which biogenic amines are produced. It also is the largest component of the matrix in which the decarboxylation reactions take place and any conditions altering its characteristics will influence the formation of biogenic amines (Ruiz-Capillas & Jiménez-Colmenero, 2004).

Many authors (Maijala et al., 1995; Eerola et al., 1997; Komprda et al., 2004) observed significant fluctuations in biogenic amines content in dry sausages depending on raw meat hygienic quality. The same raw material can lead to very different amine levels in the final products depending on the presence of decarboxylating microorganisms, either derived from environmental contamination or from starter cultures, and the conditions supporting their growth and activity. The hygienic quality of processing plant is also crucial for the biogenic amines formation, since most contaminant microbial population contributes largely to the occurrence of

certain amines being indicative of improper hygienic conditions (Suzzi & Gardini, 2003; Ruiz-Capillas & Jiménez-Colmenero, 2004; Sun et al., 2016). Putrescine and cadaverine production is frequently found in enterobacteria, and tyramine production is reported in the majority of enterococci (Jairath et al., 2015).

The pH is an important factor for the formation of biogenic amines as pH affects their production by two mechanisms. The optimal pH for bacterial amino acid decarboxylases is usually acidic (Santos, 1996). Decrease of pH results in increasing decarboxylase activity of bacteria as part of their protective mechanism against acidic stress. The loss of a carboxylic group, contributes to the intracellular (and extracellular) pH increase (Gardini et al., 2016). Histidine decarboxylase activity increases in acid media, with an optimum pH range of 4 to 5.5 (Halasz et al., 1994). However, sharp and rapid reduction in pH at the beginning of fermentation is known to inhibit the growth of the amine-positive microorganisms (Maijala et al., 1995). This can explain the low level of tyramine found in Nordic meat products which are generally characterized by low pH (<5) limiting bacterial growth, and, consequently reduces tyrosine decarboxylase activity (Masson et al., 1999). However, this demonstrated mechanism is only true for some particular amines such as the putrescine. No relationship between the tyramine content and pH was found, which suggests that the acidifying activity by the starter cultures did not reduce the tyramine production (Van Ba et al., 2016).

The redox potential of the medium has also been reported to influence biogenic amines production (Halász et al., 1994). Histidine decarboxylase activity seems to be inactivated or destroyed in the presence of oxygen while conditions resulting in a reduced redox potential, stimulate histamine production (Karovičová & Kohajdová, 2005).

Temperature decisively influence biogenic amine formation by bacteria and its production rate increase generally with the increase of temperature (Halász et al., 1994). Higher temperature may favor proteolytic activity which provides substrate for decarboxylase enzymes resulting in increased amine level. Temperature between 20°C and 37°C is optimal for the growth of the most bacteria containing decarboxylases,

decreased temperature stops their growth. Nevertheless, the optimal temperature of production is highly dependent on the species (Karovičová & Kohajdová, 2005).

The inhibitive effect of natural antioxidant and antibacterial substances on biogenic amines formation during manufacture of the dry-cured and fermented meat products has recently been studied. Biogenic amines (putrescine, cadaverine, histamine, tyramine and spermine) content in bacons during dry-curing and storage was significantly reduced by plant polyphenols (green tea polyphenols (GTP) and grape seed extract (GSE)) or α-tocopherol (Wang et al., 2015). Rose polyphenols (RPs) inhibited the biogenic amines formation in sausages. The lowest biogenic amines concentration was observed in the batch supplemented with 3 mg/g RPs (Zhang et al., 2017).

NaCl plays an important role in microbial growth and therefore influences the amino acids decarboxylases activity. According to Suzzi and Gardini (2003) biogenic amines accumulation decreases markedly with the increase of NaCl concentration, which can be attributed to the reduced microbial cell yield in the presence of high NaCl concentration and to a progressive disturb of the membrane located decarboxylase enzymes. However, for intermediate concentration of salt proteolytic activity is higher, pointing out that there is not necessarily a correlation between these two variables. Karovičová and Kohajdová (2005) reported that the presence of NaCl activates tyrosine decarboxylase activity and inhibits histidine decarboxylase activity, which evidence that either inhibiting or stimulating effect of NaCl on the biogenic amines production is strain specific. In the recent study Laranjo et al. (2017) showed that the salt reduction from 6% to 3% in two Portuguese traditional blood dry-cured sausages significantly increased the level of most individual biogenic amines with higher amounts, particularly of cadaverine, histamine and tyramine. The replacement of 40% NaCl by KCl effectively reduced putrescine, cadaverine and histamine contents in dry-cured bacon. The lower histamine content in KCl-treated samples might be attributed to the reduced a_w value by the substitution (Li et al., 2016).

A relationship has also been found between the biogenic amines content and the size of dry fermented sausages since the diameter of the sausage affects the environment in which microorganisms grow. As an example, water activity is usually higher and salt concentration is lower in sausages with a larger diameter. A higher production of certain amines, such as tyramine and putrescine may result from a larger diameter of the sausages. Generally, biogenic amines levels in the bigger diameter sausages were higher than in the thinner sausages and in the central part of the sausages than in the edge (Ruiz-Capillas & Jiménez-Colmenero, 2004; Suzzi & Gardini, 2003).

The concentrations of tyramine, putrescine, cadaverine and histamine normally increase during the processing and storage of meat and meat products, whereas spermidine and spermine content decrease or remain constant (Halász et al., 1994; Bardócz, 1995; Ruiz-Capillas & Jiménez-Colmenero, 2004). The presence of biogenic amines is associated with a relevant growth (>7 log CFU/g) of decarboxylating microorganisms (Gardini et al., 2016). Therefore their amounts and ratios have been proposed as indirect indicators of excessive microbial proliferation (Mietz & Karmas, 1977; Wortberg & Woller, 1982; Hernández-Jover et al., 1996). The usefulness of biogenic amines content as a microbial quality index depend on the nature of the product. Results tend to be more satisfactory in fresh meat and heat-treated products than in fermented products in which they seem to be of very limited use. In this products a low biogenic amine concentration does not always signal good microbiological quality as not all spoilage or starter microorganisms can decarboxylate free amino acids. Even within the same species, not all strains develop the same decarboxylating capacity as this ability is more strain- rather than species-dependent (Durlu-Özkaya et al., 2001). Therefore it is, no simple matter to establish a biogenic amine index that reliably predicts quality for products of this kind (Ruiz-Capillas & Jiménez-Colmenero, 2004).

Determination of the exact toxicity threshold of biogenic amines in dry-cured and fermented meat products is extremely difficult, because their effect does not depend on their presence alone, but is also influenced by other compounds and the individual susceptibility and health status of the

consumer (Halász et al., 1994; Eerola et al., 1997; Gardini et al., 2001; Ruiz-Capillas & Jiménez-Colmenero, 2004). Based on a qualitative risk assessment of biogenic amines, no adverse health effects were observed after exposure (per person per meal) to: 50 mg histamine for healthy individuals, but below detectable limits for those with histamine intolerance; 600 mg tyramine for healthy individuals not taking monoamino oxidase inhibitor (MAOI) drugs; 50 mg tyramine for those taking third generation MAOI drugs or 6 mg for those taking classical MAOI drugs. For putrescine and cadaverine, the information was insufficient in that respect (EFSA, 2011).

Commercially-available dry fermented sausages from Portugal and Serbia show lower levels of cadaverine, putrescine and tyramine when compared with similar products (Alves et al., 2017). Only histamine values slightly exceeded 100 mg/kg in two Portuguese sausages. However, a daily consumption of an average portion of 50 g of both dry fermented sausages will never exceed the threshold safety level of histamine to elicit adverse reactions (EFSA, 2011).

2. Methods of Detection Biogenic Amines in Dry-Cured Meats

Identification and quantitation of biogenic amines in dry-cured and fermented meats is of interest not only because of its potential toxicity but also due to the possibility of using them for quality control of raw materials, intermediates and end products and monitoring fermentation processes (Önal, 2007).

The typical problems encountered in the biogenic amines analysis in dry-cured and fermented meats are the complexity of the sample matrix (e.g., presence of free amino acids), the presence of potentially interfering compounds, the low concentration levels at which the biogenic amines are present in the samples and the occurrence of several biogenic amines simultaneously. The difficulty of the detection and reliable quantification

of biogenic amines may provide insufficient information about their occurrence in meat products (Shalaby 1996; Alberto et al., 2002; Karovičová & Kohajdová, 2005; Önal, 2007).

Several methods have been developed for separation and quantitative determination of biogenic amines in food products (Kovács et al., 1999; Alberto et al., 2002; Karovičová & Kohajdová 2005; Önal et al., 2013). All these methods employ extraction of the amines and their quantitative determination. The extraction of amines from meat matrices represents the crucial step in terms of obtaining adequate recoveries for all amines. For the separation of biogenic amines it is very useful to take advantage of the fact that amines are strong organic bases. For this purpose reagents such as trichloroacetic acid, perchloric acid, hydrochloric acid, methanesulfonic acid, and organic solvents have been suggested for the liquid-liquid extraction (LLE) of amines from food matrices (Shalaby, 1996; Vinci & Antonelli, 2002). The extraction and recovery of biogenic amines is significantly influenced by the degree of saturation of the extracting solution by salts and their pH (Karovičová & Kohajdová, 2005). An adequate alternative to LLE seems to be the solid-phase extraction (SPE) due to the wide availability of selective sorbent materials. This procedure increases the selectivity of the method, provides good recovery for the most important biogenic amines and also very significantly reduces the need to dispose of organic solvents as waste (Moret et al., 1992; Busto et al., 1994).

After extraction, the quantitative determination of biogenic amines is usually performed by means of chromatographic methods: gas chromatography (GC) used for determination of volatile derivatives of the amines, thin-layer chromatography (TLC), and most often applied high-performance liquid chromatography (HPLC) with pre- or post-column derivatisation. Ion-exchange chromatography (IEC) is used for the determination of biogenic amines on an amino acid analyzer. Biochemical assays and capillary electrophoresis (CE) have also been described (Önal, 2007). Chromatographic methods coupled with mass spectrophotometric detection (HPLC-MS) offers higher ability to identify the structure of the

analytes and also higher resolution than conventional methods (Kovács et al., 1999; Önal et al., 2013).

Due to lack of chromophores and low volatility of most biogenic amines, the HPLC procedures involve pre- (carried out before the chromatographic separation) or postcolumn (carried out after the chromatographic separation) derivatization step. The pre-column derivatization technique is used more frequently than the post-column derivatization as it provides more sensitive detection. The derivatization reactions occur via amino group with different chemical reagents such as ninhydrine and *o*-phthaldialdehyde, as a postcolumn derivatization reagent, dansyl and dabsyl chloride, benzoyl chloride, fluoresceine, 9-fluorenylmethyl chloroformate with precolumn derivatization. Dansyl chloride reacting with both primary and secondary amino groups which provide stable derivatives, is the most widely used. For the detection ultraviolet (UV) or fluorescence (FL) detectors are used. FL provides higher sensitivity than UV detection (Karovičová & Kohajdová, 2005; Önal et al., 2013).

Biogenic amines levels of sucuk (Turkish dry fermented sausage) obtained from retail markets and butchers were determined by using HPLC method with diode array detector (DAD) after pre-column derivatization with dansyl chloride. Cadaverine and putrescine were detected as 87% and 93% of the samples, respectively. Spermidine and spermine were detected in ranges from not detected to 10.7 mg/kg and from not detected to 16.4 mg/kg, respectively. Histamine was found to be between 50 and 100 mg/kg as 17% of the samples. Tryptamine was detected in the range of 1.2-82.3 mg/kg. Tyramine contents in all samples were within the acceptable level. Phenylethylamine was found in 17 of the 30 samples and levels in all detected samples were found to be below 25 mg/kg (Gençcelep et al., 2008).

3. METHODS FOR AMINOGENESIS CONTROL IN DRY-CURED MEATS

Biogenic amines accumulation in dry-cured and fermented meat products should be prevented to ensure their safety. The biogenic amines produced are heat stable and, once formed, there is no treatment capable to significantly reduce their concentration (Gardini et al., 2016). In this way, the development of appropriate manufacturing technologies to obtain dry-cured and fermented meats free or nearly free from biogenic amines is one of the current targets of the meat sector. Biogenic amines formation during meat processing can be controlled through inhibiting microbial growth or inhibiting the decarboxylase activity of bacteria (Latorre-Moratalla et al., 2010b).

Hygienic conditions of both raw materials and processing units are one of the key measures that enable the control of the aminogenesis during meat (Halász et al. 1994). However, proper hygiene may not be enough to avoid the development of aminogenic contaminant bacteria and in turn biogenic amines formation and other technological measures must be applied (Latorre-Moratalla et al. 2010b).

As biogenic amine formation is temperature dependent (Shalaby, 1996), their production may be prevented by the inhibition of microbial growth and the reduction of enzyme activity through decreasing the temperature and strict adherence to the cold chain (Naila et al., 2010). However control of biogenic amines formation through temperature alone is not always possible, since some psychrotolerant bacteria produce biogenic amines at temperature below 5°C (Emborg & Dalgaard, 2006).

Therefore, alternative control measures to limit biogenic amines formation or to enhance their degradation in dry-cured and fermented meats need to be taken into account. Such methods may include the use of amine-negative or amine-oxidizing starter cultures, use of enzymes to oxidize amines, use of microbial modeling to assess favorable conditions to delay biogenic amines formation, packaging techniques, high

hydrostatic pressure (HHP), irradiation, and food additives and preservatives (Naila et al. 2010).

Lactic acid bacteria, responsible for the acidification process, together with coagulase-negative staphylococci, related to proteolysis, color formation and aroma development, are the microorganisms widely used in the meat industry as starter cultures (Suzzi & Gardini, 2003; Latorre-Moratalla et al., 2010b). The choice of suitable starter culture plays a fundamental role in preventing the formation of biogenic amines in fermented meat products due to their preventative capacity of the outgrowth of the potential aminogenic endogenous bacteria by acidification (Suzzi & Gardini, 2003; Karovičová & Kohajdová, 2005; Van Ba et al., 2016). Strains of *Lactobacillus sakei* and *Lactobacillus curvatus* are usually employed for sausage elaboration in Europe. However, many strains of the latter produce up to four different biogenic amines. Decarboxylating activity has not been described in connection with *Lactobacillus sakei* strains (Kołożyn-Krajewska and Dolatowski 2009, Vinci and Antonelli 2002). This indicates that *Lactobacillus sakei* may be more suitable than *Lactobacillus curvatus* for use as a starter culture to prevent biogenic amines formation in the sausages (Roig-Sagués & Eerola, 1997). Careful screening for amino acid decarboxylase activity is recommended before selecting starter strains in food industry (Landeta et al., 2013).

The selection of starter cultures with application in the meat fermentation has to be performed at the strain level and take in consideration the various, specific requirements of the fermentation process in the meat system (Roig-Sagués & Eerola 1997). The inability of the culture to form biogenic amines but also its ability to oxidize biogenic amines into ammonia, aldehyde, and hydrogen peroxide should be taken into consideration in the selection of starter cultures (Bover-Cid et al., 2000). Selection of potential starter cultures for fermented sausages production should consider not only the amine-negative properties of the culture but also their ability to grow well at the temperature intended for processing of the product (Suzzi & Gardini, 2003; Lu et al., 2010). Use of starter cultures in the production of dry-cured and fermented meat products

can also affect the formation of biogenic amines indirectly through the competitiveness in suppressing the growth of wild amine producing microflora (Huis in't Veld et al., 1990). A rapid pH drop caused by amine negative starter cultures can largely prevent biogenic amines accumulation in fermented meat products. Moreover, starter cultures able to compete with nonstarter bacteria during the later phase of ripening and during storage can further avoid excessive biogenic amines production (Suzzi & Gardini, 2003). The formation of bacteriocin (e.g., curvacin A) by the strains can increase their competitiveness (Hammes & Hertel, 1996).

Works cited by Latorre-Moratalla et al. (2012) have indicated that the controlled fermentation carried out by the starter cultures containing bacteria that lack decarboxylase activity could be one of the best technological measures to prevent excessive amine accumulation during meat fermentation. However, González-Fernández et al. (2003) and Lu et al. (2010) reviewed some research suggesting the lack of effectiveness of the starter culture to prevent the formation of biogenic amines in fermented meat products. The discrepancies observed could depend on the raw meat microbiological quality and the characteristics of natural microflora, in particular aminepositive nonstarter LAB (Jairath et al., 2015).

An attractive approach for designing functional fermented meat products is the use of probiotic strains of LAB (Työppönen et al., 2003; Stadnik & Dolatowski, 2012; Ojha et al., 2015). Selected strains of *Lactobacillus reuteri* and *Lactobacillus fermentum* identified as a potential probiotic meat starter culture suitable for manufacture of dry-fermented Iberian sausages were considered safe to be used with regard to its low aminogenic potential (Ruiz-Moyano et al., 2009).

Biogenic amines may potentially be degraded by the use of enzymes, such as diamine oxidase (DAO), and the use of bacteria that possess this enzyme. Biogenic amine degrading bacteria include *Micrococcus varians*, *Natrinema gari*, *Brevibacterium linen*, *Vergibacillus sp* SK33, *L. sakei*, *Lactobacillus curvatus*, and *S. xylosus*. Bacteria with amine oxidizing activity could be used as a starter for fermented foods (Naila et al., 2010). However, at this stage, amine degradation seems to be restricted to aerobic microorganisms that are of limited use in fermented meats, which

characteristically constitutes an anaerobic environment (Spano et al., 2010). The effectiveness of the amine oxidizing bacteria and enzymes in degrading biogenic amines needs to be investigated in meat matrices prior to recommending their use (Naila et al., 2010).

Microbial modeling can be used to design conditions to limit biogenic amines productions. Available models for biogenic amine producing bacteria include this developed by Gardini et al. (2008) for *Enterococcus faecalis* EF37 in dry fermented sausage from Northern Italy. According to the mathematical models obtained, sodium chloride concentration was the most determinant factor of the final tyramine and β-phenylethylamine accumulation in the final product. Temperature and glucose had negligible effects on tyramine accumulation. However, apart from many known bacterial species capable of producing biogenic amines already known there are probably others yet to be found, then mathematical modeling to account for all these species would be complicated, time consuming, and laborious (Naila et al. 2010).

The qualitative and quantitative formation of biogenic amines can be affected the packaging system (Loizzo et al., 2016). The production of biogenic amines may be delayed through inhibition of biogenic amines forming bacteria or the enzymes producing biogenic amines by vacuum packaging, active packaging, and modified atmosphere packaging. Reports on the successful control of biogenic amines formation through modified atmosphere packaging been reviewed by Ercan et al. (2013). The success of inhibition of biogenic amines forming bacteria or enzyme activity through packaging depends on concentration of gases used in modified atmosphere packaging, type of microflora present and environmental conditions. However, both anaerobic and aerobic bacteria are capable of producing biogenic amines, so finding a balance that will control microbial growth and enzyme activity may be difficult (Naila et al. 2010). However, both anaerobic and aerobic bacteria are capable of producing biogenic amines, and as well as degrading biogenic amines so finding a balance that will control their growth and enzyme activity may be difficult.

Pathogenic and spoilage microorganisms in foods may be inactivated through high hydrostatic pressure (HHP) which is a nonthermal

preservation method. Inactivation of microorganisms extends shelf life with fewer changes in the original flavor, texture and nutrients compared to conventional technologies (Patterson, 2005). The application of HHP results in a reduction in the number of bacteria which may inhibit biogenic amine formation. Results also suggested that HHP treatment did not reduce the fermentative capacity of the bacterial strains present, while it had a protective effect against non-fermentative diamine (putrescine, cadaverine) producing microflora (Ojha et al., 2015). To control biogenic amine formation in fermented meat products, high pressure application could be feasible before starter culture addition in order to avoid reduction of LAB together with biogenic amines (Ercan et al., 2013).

The application of high hydrostatic pressure (200 MPa for 10 min at 17°C) to meat batter just before sausage fermentation and the inoculation of starter culture, inhibited the growth of *Enterobacteria* and simultaneously strongly inhibited the accumulation of putrescine and cadaverine but hardly any influenced tyramine formation (Latorre-Moratalla et al., 2007).

The high pressure (350 MPa for 15 min) treatment of fermented sausages significantly reduce cadaverine, putrescine, and tyramine levels during 160 days chilled storage (2°C) compared to sausage not treated with high pressure. More research is needed to determine how high pressure affects the enzymes as well as the bacteria that cause biogenic amine formation (Ruiz-Capillas et al., 2007).

Irradiation may be used to control biogenic amine formation in foods by inducing their radiolytic degradation (Mbarki et al., 2009) and by reducing the number of bacteria responsible for their formation (Kim et al. 2003). Limited reports are available demonstrating the application of gamma irradiation for the reduction of major biogenic amines in fermented meats and sausages. Gamma irradiation (5, 10 and 20 kGy) was effective in reducing putrescine, spermidine, spermine and tyramine levels in pepperoni sausage (Kim et al., 2005). Gamma irradiation of dry-cured Chinese Rugao ham with a dose of 5 kGy degraded tyramine, putrescine and spermine. On the other hand, it promoted the formation of spermidine, phenylethylamine, cadaverine and tryptamine (Wei i in., 2009).

Additives and preservatives used in manufacturing dry-cured and fermented meats have shown a positive effect on delaying biogenic amine formation can be inhibit bacterial growth and subsequent biogenic amine formation. Kurt and Zorba (2009) found that the formation of tyramine and cadaverine in sucuk (a dry fermented Turkish sausage) was decreased by increasing nitrite levels (45 to 195 ppm) thus confirming the results of previous studies (Bozkurt & Erkmen, 2004; Gençcelep et al., 2007) that sodium nitrite and sodium nitrate inhibit biogenic amine production.

Sugars (glucose and occasionally lactose or saccharose) are usually included for the manufacture of fermented meat products, though traditional processes may omit this ingredient. The sugar omission in slightly fermented sausages resulted in higher levels of biogenic amines especially tyramine and, to a lesser extent, cadaverine during ripening and storage. However, the addition of sugars has a limit, since the quantity (and type) of sugar added to control biogenic amine should not compromise the 'traditionally' ripened sausages flavor (Bover-Cid et al. 2001a).

The effect of sodium sulphite on biogenic amine production during the ripening of a slightly fermented sausage (*fuet*) of fermented sausages has been studied by Bover-Cid et al. (2001b). The authors aimed to find out whether the inhibitory effect of sulphites on microbial growth was also reflected in lower biogenic amine production during the ripening. Although sausages with sulphite showed lower microbial counts, only cadaverine production was drastically inhibited in sausages without sulphite. Tyramine and putrescine production seemed to be stimulated by the presence of sodium sulphite.

Significant decrease in the levels of histamine and putrescine through a pH decrease in meat has been observed as a result of addition of glucono-delta-lactone (GDL) to meat (Maijala et al., 1993).

Many studies cited by Ercan et al. (2013) and Naila et al. (2010) have shown that herbs and spices components (e.g., allicin, curcumin, capsaicin, piperine, and thymol) contributed to the reduction in biogenic amines formation in foods due to their antimicrobial and antioxidant activities.

There are a few common effective ways suggested for preventing aminogenesis in dry-cured and fermented meat products. Firstly, the high-

quality meat not only with low total counts of contaminant microorganisms but also free of amine producing microorganisms must be used. Contamination prevention by hygienic production conditions as well as strict control of the whole fermentation process is absolutely necessary. The use of specifically selected amine-negative or amine oxidizing starter cultures can additionally suppress the unwanted microbial activity thus preventing the formation of biogenic amines (Stadnik, 2014).

CONCLUSION

Today, consumers increasingly prefer high-quality products that are minimally processed, safe, etc., and the meat industry is, therefore, looking for emerging technologies that can achieve this in processing and storage. There is a clear interest in the study of toxicity of biogenic amines and the factors determining their formation in the context of meat processing conditions and preservation.

There are a few common parameters which can be defined for low-biogenic amines technology. First, high quality raw materials, free of biogenic amines, must be used. Additionally, proper and careful treatment of raw materials until processing is absolutely crucial. Growth and activity of decarboxylasepositive microorganisms must be avoided, and activity of endogenous proteases and amino acid decarboxylases have to be inhibited by technological measures and proper and hygienic production conditions. If microbial transformations are part of the production process, use of carefully selected, decarboxylase negative starter cultures for these fermentations is important, as well as processing conditions which favor the growth of the starter strains.

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Chapter 8

SEROTONIN (5-HT): ORIGINS, BIOLOGICAL IMPORTANCE, AND HUMAN HEALTH IMPLICATIONS IN PAIN

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ABSTRACT

The aim of this chapter is to identify the origin, biological importance, and human health implications of the serotonin (5-HydroxyTryptamine [5-HT]) in pain. 5-HT is a monoamine that is widely distributed both in the periphery and in the Central Nervous System (CNS). Serotonin neurons at the CNS level are confined to the brainstem and are located in the raphe nuclei. Serotonin 5-HT is synthesized from the amino acid L-tryptophan (from the diet) by sequential hydroxylation and decarboxylation. It is stored in presynaptic vesicles and released from nerve terminals during neuronal firing. 5-HT is involved in numerous physiological and behavioral disorders, such as major depression, anxiety, schizophrenia, mania, autism, and obesity, and it recently has been proposed as an essential component in the modulation of pain. However, this neurotransmitter is related with a number of physiological processes, such as cardiovascular function, gastric motility, kidney function, etc. Based on pharmacological, structural, and transductional characteristics, the 5-HT receptor family is divided into seven subfamilies ($5\text{-}HT_1$–$5\text{-}HT_7$), comprising 15 receptor subtypes, each of these corresponding to distinct genes. Descending 5-HT pathways exert an inhibitory (descending inhibition) or facilitatory (descending facilitation) influence on the spinal processing of nociceptive information, depending on acute or chronic pain states and the type of receptor acted upon. The exact nature of the receptors involved in 5-HT modulation of pain in the CNS remains to be elucidated. However, studies have revealed the presence of at least three families of 5-HT receptors in the spinal cord ($5\text{-}HT_1$, $5\text{-}HT_2$, and $5\text{-}HT_3$), with varying affinity for 5-HT, and recently, the $5\text{-}HT_7$ receptor, which is also excitatory, has been postulated as being linked with, among other things, circadian rhythms, thermoregulation, and migraine. Several lines of evidence have implicated a role for this serotonin in the human health implications of pain; however, it is important to bear in mind the understanding of how pain is modulated by serotonergic receptors in the CNS in terms of the following: the distribution of the different serotonergic receptors in the raphe-spinal pathway; the dose of agonists or antagonists to the 5-HT receptors, the administration route of agonists or antagonists to the 5-HT receptors, and the type and duration of pain.

Keywords: serotonin, neurotransmitter, pain

1. INTRODUCTION

Serotonin (5-HydroxyTryptamine, [5-HT]) is a monoamine that is widely distributed in both the periphery and in the Central Nervous System (CNS) and in non-neuronal tissues such as blood, gastrointestinal, and endocrine, to mention a few. It is estimated that 5-HT appeared about 700-800 million years ago in unicellular eukaryotic organisms such as paramecia, rendering it one of the oldest neurotransmitters of evolution. Its receptors are found in a very wide range of organisms, from planaria through insects to man, and its structure is well preserved (Hannon & Hoyer, 2008; Kandel et al., 2012). Vittorio Erspamer was, to our knowledge, the first to describe serotonin in the 1903s; it was isolated from the intestine and presented constricting activity and was denominated "enteramine". It was not until 1948 that serotonin was rediscovered by Page, Rapport, and Green, who isolated it from blood and appropriately named it "serotonin" because it was released from the platelets. Eventually, Rapport purified, crystallized, and characterized serotonin from a great amount of blood, finding that enteramine and serotonin comprised the same molecule and possessed an indole group, as suggested earlier by Erspamer. It was the concluded to call it serotonin. Later, in 1953, Twarog and Page detected serotonin in brain extracts, whereas serotonin was present in three key systems of the body: in platelets; the gastrointestinal system, and in nervous system (Whitaker-Azmitia, 1999; Hannon & Hoyer, 2008).

5-HT does not cross the Blood-Brain Barrier (BBB); thus, its presence in the CNS is limited only to its synthesis in the brain. 5-HT Synthesis is carried out in the nucleus of the raphe and its projections in the brain. The first step in 5-HT synthesis is the facilitated transport of the amino acid tryptophan from the blood to the brain, followed by serotonergic neurons containing the enzyme tryptophan hydroxylase (L-tryptophan-5-monooxygenase), which hydroxylates tryptophan into 5-5-Hidroxy-

Triptophan5-HTP); finally, the enzyme mono-amino- decarboxylase converts 5-HTP into 5-HT. Degradation of 5-HT is carried out by MonoAmine Oxidase (MAO) and, in the pineal gland, by 5-HT N-acetyltransferase, which degrades 5-HT into melatonin (Kandel et al., 2012).

5-HT is involved in numerous physiological and behavioral disorders, such as major depression, anxiety, schizophrenia, mania, autism, obesity, pain, etc. It is stored in presynaptic vesicles and released from nerve terminals during neuronal firing. Serotonin neurons at the CNS level are confined to the brainstem and are located in the raphe nuclei. The neurons project into the majority of the brain, including hippocampus, midbrain, the prefrontal, parietal, and occipital cortical regions, cingulate cortex, thalamus, and cerebellum, whereas 5-HT neurons in caudal raphe nuclei project into cerebellum and spinal (Hedlund & Sutcliffe, 2004). It has been established that descending 5-HT pathways exert an inhibitory (descending inhibition) or facilitatory (descending facilitation) influence on the spinal processing of nociceptive information, depending on acute or chronic pain states and the type of receptor acted upon (Bardin et al., 2000; Jeong et al., 2004; Dogrul et al., 2009). Based on pharmacological, structural, and transductional characteristics, the 5-HT receptor family is divided into seven subfamilies (5-HT_1-5-HT_7), comprising 15 receptor subtypes, each of these with their corresponding distinct genes. The involvement of diverse receptor subtypes in pain neurotransmission remains largely unknown. Indeed, the use of relatively selective agonists and antagonists for these subtypes has led to inconsistent results due to poor selectivity of drugs and to the diversity of experimental conditions (Bardin et al., 2000; Obata et al., 2001; Sasaki et al., 2001; 2006).

The peripheral pronociceptive role of 5-HT is well established to date; in contrast, its action at the spinal cord level and in supraspinal structures appears to be highly variable and remains a matter of debate (Millan, 2002). The exact nature of the receptors involved in 5-HT modulation of pain in the spinal cord continues to be unelucidated. However, studies have revealed the presence of at least three families of 5-HT receptors in the spinal cord (5-HT_1, 5-HT_2, and 5-HT_3), with varying affinity for 5-HT, and

recently, the 5-HT_7 receptor, which is also excitatory and has been linked with, among other things, circadian rhythms, thermoregulation, and migraine has been postulated (Leopoldo et al., 2011). Therefore, successful pain management requires therapeutic strategies directed toward alleviating its affective attributes. The development of these strategies requires an understanding of the neurobiological mechanisms that modulate the affective dimension of pain.

2. SERTONERGIC RECEPTORS IN PAIN MODULATION

2.1. Serotonergic Receptor 5-HT_1 as Modulator of Nociceptive Response

The 5-HT_1 Gi/o-linked G protein-coupled receptor families are divided into A, B, C, D, E, and F subtypes (Peroutka, 1988; Zemlan et al.; 1988; Barnes & Sharp, 1999). 5-HT_1 receptors are present in the grey matter of the whole spinal cord in all of the areas examined. The major class of 5-HT receptor found in the dorsal horn is the 5-HT_1 family (Murphy & Zemlan, 1990; Zemlan & Schwab, 1991). Among the subtypes of 5-HT_1 receptors that potentially contribute to the medullospinal regulation of pain, the rostroventral medial medulla provides the major 5-HT descending pathway to the spinal superficial dorsal horn, which is the initial relay point for nociceptive inputs into the CNS (Millan, 2002). The $5HT_{1A}$ receptor has been the most studied as a modulator of pain and appears to play a modulatory role in nociception. Its large distribution in the CNS renders it possible for the actions of selective agonists to involve various spinal and supraspinal mechanisms that modulate pain processes. 5-HT_{1A} receptor messenger RNA (mRNA) labeling was most pronounced in the olfactory bulb, anterior hippocampal rudiment, septum, hippocampus, entorhinal cortex, interpeduncular nucleus, thalamus, and medullary raphe nuclei, and is widely distributed in the spinal dorsal horn (Wright et al., 1995; Zhang et al., 2001). 5-HT_{1A} receptors appear to be present in primary afferent nociceptive fibers and possess a significant role, in that they are expressed

both in the rostroventromedial medulla as well as in the spinal dorsal horn, and their 5-HT_{1A} receptor density is found in laminae I and II of the spinal cord (Zemlan et al., 1988; Marlier et al., 1991; Laporte et al., 1995). Administration of 5-HT_{1A} receptor agonists in the spinal cord has produced both pro- and antiallodynic effects (Millan, 2002). The widespread presence of 5-HT_{1A} receptors in the spinal cord, in the dorsal and median raphe nuclei, as well as in cortical and limbic areas, suggests a possible involvement of these receptors in emotional states, cognition, and pain modulation (Hensler et al., 1991). Studies in nonhuman primates (Azmitia et al., 1996) and humans indicate that many areas involved in pain mediation or modulation, such as the raphe nucleus, amygdala, cingulate cortex, insula, and prefrontal cortex, have a high density of 5-HT_{1A} receptors (Parsey et al., 2000; Rabiner et al., 2002; Hirvonen et al., 2007; Martikainen et al., 2007). In raphe nuclei, 5-HT_{1A} receptors are located in serotoninergic cell bodies and dendrites and function as somatodendritic autoreceptors (Riad et al., 2000). Among the many types of serotonin receptors, the 5-HT_{1A} receptor appears to be that which plays a significant role in mediating the regulatory effects of pain (Colpaert, 2006; Mico et al., 2006). EI Yassir et al. (1988) and Zemlan et al. (1983) showed that both 5-HT_{1A} and 5-HT_{1B} receptors were implicated in nociception at the dorsal-horn level. Indeed, 5-HT_{1A} receptors appear to mimic the non-selective antinociceptive effects of serotonin, while 5-HT_{1B} receptors mimic the selective effect.

Eide et al. (1990) examined whether the injection of 5-HT_{1A} and 5-HT_{1B} receptor agonists in mice possessed the ability to alter the tail-flick reflex, and whether effects on reflex latency involve changes in tail-skin temperature. These authors found that, in the mouse, both 5-HT_{1A} and 5-HT_{1B} receptor agonists inhibit the nociceptive tail-flick reflex when administered into the spinal subarachnoid space, and that the effect does not depend on changes in tail-skin temperature. Ali et al. (1994) adopted a single route of microinjected intrathecally (i.t.) into anesthetized rats, while the recordings are made from dorsal- horn neurons on application of the drugs in both behavioral and electrophysiological studies and specifically attempted to compare the results. The authors showed that 5-HT increased

nociceptive responses, and it is suggested that this effect is associated with activation of 5-HT_{1A} receptors. Activity at 5-HT_{1B} receptors exerts the effect of suppressing or reducing responsiveness. The increased responsiveness of dorsal-horn neurons to noxious stimulation associated with activity at the 5-HT_{1A} receptors may be associated either with increases in receptive field size, promotion of spinal nocifensive reflexes, or facilitation of rostral transmission to specific brainstem sites. Moreover, the modulatory effects of 5-HT_{1B} receptor activation on wide dynamic range neurons in the spinal cord were studied by Gjerstad et al. (1997); their results demonstrated that stimulation of 5-HT_{1B} receptors may exert both pro- and antinociceptive effects on wide dynamic range neurons in the dorsal horn after repeated electrical stimulation. Likewise, Zhang et al. (2001) reported that the excitability of dorsal-horn neurons and the sensitivity of the neurons to i.t. 5-HT_{1A} and 5-HT_{1B} receptor agonists might increase following the inflammation model. These authors employed intraplantar (i.p.) injection of carrageenan, which is characterized by both a rapid onset and resolution of inflammation, which gives rise to restricted distribution of hyperalgesia. Liu et al. (2002) conducted a study to confirm which type of 5-HT receptor was involved in the descending pathway of antinociception from the brainstem to dorsal horn of the spinal cord in rats. These authors reported that the 5-HT_{1A} receptor, but not the 5-HT_2 or the 5-HT_3 receptor, plays an important role in the descending pathway of antinociception from brainstem to spinal cord in intact rats, in rats with nerve injury, and in rats with inflammation. Hains et al. (2003) carried out a study to characterize the excitability of dorsal-horn neurons to 5-HT, and to 5-HT_{1A} and 5-HT_3 receptor antagonists and agonists. High densities of 5-HT_3 receptors are found in the substantia gelatinosa at all levels of the spinal cord, and the electrophysiologic evidence demonstrates the plasticity of 5-HT systems after spinal cord injury, as well as indicating the importance of 5-HT modulation in the attenuation of ensuing chronic central pain.

Bonnefont et al. (2005) performed a study in an attempt to investigate, according to the nature of the noxious stimulus, the manner in which the blockade of spinal 5-HT_{1A} receptors could influence the antinociceptive

actions of exogenous 5-HT, as well as two analgesics involving endogenous 5-HT, Paracetamol and Venlafaxine. The results of the latter show that stimulation of spinal 5-HT_{1A} receptors could mediate a dual influence on the integration of nociceptive mechanisms, and the stimulation of 5-HT_{1A} receptors utilizing exogenous 5-HT or endogenous 5-HT mobilized by Paracetamol or Venlafaxine can elicit antinociception in the formalin test.

The role of medullary and spinal 5-HT_{1A} receptors in endogenous regulation of neuropathic hypersensitivity was studied by Wei et al. (2006); they concluded that administration of a selective 5-HT_{1A} receptor antagonist, WAY-100635, into the rostroventromedial medulla or systemically, produced selective attenuation of mechanical hypersensitivity in animals with an experimental neuropathy and disinhibited descending pathways, this leading to attenuation of hypersensitivity. Jeong et al. (2012) examined the spinal actions of a range of 5-HT_1 agonists, including Sumatriptan, on acute pain, plus their effect on afferent-evoked synaptic transmission into superficial dorsal-horn neurons. These authors concluded that at the cellular level, 5-HT_{1A}, but not 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptor activation, presynaptically inhibits primary afferent-evoked synaptic transmission into a subpopulation of lamina II superficial dorsal-horn neurons.

Studies in humans reveal results similar to those reported in biomodels. Ferrari et al. (2001) reported the effect of 5-$HT_{1B/1D}$ receptor agonist drugs in patients with acute migraine. The results of 53 clinical studies involving more than 24,000 patients demonstrated that oral administration of 10 mg of Rizatriptan, 80 mg of Eletriptan, and 12.5 mg of Almotriptan provides effectiveness in terms of the inhibition of acute pain caused by migraine. Jelinski et al. (2006) worked with the drug Sumatriptan, a 5-HT_1 receptor agonist, in which their objective was to determine the efficacy of Sumatriptan, 50 mg and 100 mg, for the treatment of migraine pain during 2 hours. The results of 361 patients demonstrated the efficacy of the two doses compared with placebo to relieve migraine pain. On the other hand, Martikainen et al. (2007) evaluated 5-HT_{1A} receptors in pain regulation in healthy humans by comparing the binding [carbonyl-11C] WAY-100635

in different regions of the brain with the subject's response to pain and its modulation; results in 11 patients suggest that 5-HT_{1A} receptors in the brain exert an influence on pain and reflex sympathetic vasoconstriction. Brandes et al. (2007) evaluated the effect of Sumatriptan (a 5-HT_1 agonist drug) in combination with Naproxen in 2,956 patients with acute migraine pain; the investigators concluded that administration of 85 mg of Sumatriptan plus 500 mg of Naproxen orally has favorable effects of the mitigation of pain compared with other monotherapies.

2.2. Serotonergic Receptor 5-HT_2 as Modulator of Nociceptive Response

mRNA for 5-HT_{2A} and 5-HT_{2C} receptor subtypes has been detected in the modulation of pain at the central level. In rat, highest levels of 5-HT_{2A} receptors are found in the frontal cortex and other neocortical areas, claustrum, and olfactory tubercle (Pazos et al., 1985) and exist in brainstem descending pain-modulation pathways, including nucleus raphe magnus, ventrolateral periaqueductal gray, and spinal dorsal horn, reticular formation, central grey, thalamus, cerebral cortex, and limbic structures (Xie et al., 2008; Van Steenwinckel et al., 2009). 5-HT_{2A} receptors in the prefrontal cortex also play an important role in cognitive functions, such as working memory, conditioned avoidance, aversive classical conditioning, and visual discrimination (Kupers et al., 2009). Contrariwise, 5-HT_{2C} receptor mRNA demonstrates widespread distribution in the locus coeruleus, retrorubral area, substantia nigra pars compacta, ventral tegmental area, periaqueductal gray, basal nucleus, parabigeminal nucleus, and laterodorsal tegmental nucleus, while 5-HT_{2C} receptors are involved in the serotoninergic control of catecholaminergic and cholinergic areas.

Abbott et al. (1996) conducted a study in which the main objective was to identify the receptor subtype mediating the synergistic interaction between 5-HT and other inflammatory mediators. These authors found that 5-HT_{2A} antagonists may be effective in peripherally acting analgesic agents and/or in analgesic adjuncts. These analgesic actions would be expected to

be specific to situations in which 5-HT release contributes to the generation of pain; similar results were obtained with the 5-HT_3 agonist. 5-HT_1 and 5-HT_{2C} receptors have attracted interest because they play a role in the control of mood, motor behavior, nociception, and endocrine secretion, are colocalized in individual neurons and, in functional models, can modify each others' actions. To aid in the elucidation of the functional role of peripheral 5-HT_{2A} receptors, Van Steenwinckel et al. (2009) investigated their localization in lumbar dorsal-root ganglia employing immunocytochemistry; they found that the majority of 5-HT_{2A} receptor immunoreactivity in lumbar dorsal-root ganglia is localized in small- and medium-sized cell bodies, presumably nociceptive.

Millan et al. (1997) reported that activation of 5-HT_{2C} receptors enhances 5-HT_{1A} receptor mediation in the tail-flick model in rats, providing concrete support for the concept of interplay between 5-HT_{1A} and 5-HT_{2C} receptors in the expression of their functional actions. Obata et al. (2002) examined the antiallodynic effect of i.t. administered serotonin receptor agonists, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, and 5-HT_3 receptor subtypes in a rat model utilizing spinal nerve ligation; the authors reported that administration of the 5-HT_2 receptor agonist exhibited dose-dependent antiallodynic actions with no associated motor weakness, this suggesting that the 5-HT_2 receptor plays an essential role in spinal suppression of neuropathic pain. Activation of 5-HT_2 receptors in the spinal cord's dorsal horn possesses antiallodynic action. These results are similar to those of Sasaki et al. (2001); these authors determined the subtypes of spinal 5-HT receptors involved in modulating nociceptive transmission in the formalin test in order to achieve better understanding of the pharmacological mechanisms of 5-HT-induced antinociception. Their results revealed that the 5-HT_2 receptor agonist (10, 30, and 100 mg) caused a dose-dependent reduction in the number of flinches of the formalin-injected paw in both phases 1 and 2. Dorsal-horn 5-HT_2 and 5-HT_3 receptors, then, inhibit nociceptive transmission in response to chemical inflammatory stimuli.

Expression of 5-HT_{2A} receptor mRNA in the lumbar spinal dorsal horn, the nucleus of raphe magnus, ventrolateral periaqueductal gray and dorsal raphe nucleus following carrageenan inflammation employing the in

situ hybridization technique was evaluated by Zhang et al. (2001). These authors reported that, 1 h after carrageenan injection, expression of 5-HT_{2A} receptor mRNA was markedly increased in all layers of the ipsilateral dorsal horn. 5-HT_{2A} receptor mRNA was expressed at low-to-moderate levels in lumbar spinal dorsal horn, nucleus of raphe magnus, ventrolateral periaqueductal gray, and dorsal raphe nucleus. Following carrageenan inflammation, expression of 5-HT_{2A} receptor mRNA in ipsilateral dorsal horn, bilateral nucleus of raphe magnus, ventrolateral periaqueductal gray, and dorsal raphe nucleus was significantly increased. Obata et al. (2002) investigated the possible involvement of other associated spinal-receptor systems with respect to the antiallodynic action of a 5-HT_2 receptor agonist; they reported that muscarinic receptors may be involved in the antiallodynic action of i.t. injected 5-HT_2 receptor agonist. Doly et al. (2004) analyzed the distribution of 5-HT_{2A} receptor in rat spinal cord by immunocytochemistry with the use of an antibody directed against an N-terminal sequence of the receptor; these authors found that 5-HT_{2A} receptors were widely distributed in the whole spinal cord, with particularly high expression in motoneuron groups, in the sympathetic preganglionic cell group, and in the dorsal horn. They concluded that 5-HT_{2A} receptor localization is mainly postsynaptic.

Obata et al. (2004) evaluated the antiallodynic actions of the i.t. administration of 5-HT_{2C} receptor agonists MK212, mCPP, and TFMPP in a rat model of neuropathic pain produced by spinal nerve ligation, and concluded that i.t. administration of each 5-HT_{2C} receptor agonist produced antiallodynic effects in a dose-dependent manner. These results suggest that stimulation of spinal 5-HT_{2C} receptors produces antiallodynic effects via a mechanism different from that of 5-HT_{2A} receptors. Nitanda et al. (2005) investigated the possible involvement of the 5-HT_{2A} receptor in pathogeneses of neuropathic pain utilizing chronic constriction injury of the sciatic nerve in rats; their results indicated that 5-HT_{2A} receptor antagonist specifically ameliorated hyperalgesia without affecting the normal nociceptive reaction.

The role of peripheral serotonin $5HT_{2A}$ and $5HT_{1A}$ receptors on the orofacial nocifensive behavioral activities evoked by the injection of

formalin into the masseter muscle was evaluated in rat by Okamoto et al. (2005). These authors reported that local administration of the $5HT_{2A}$ antagonist receptor, Ketanserin, but not the $5HT_{1A}$ antagonist receptor, Propranolol, into rat masseter muscle significantly reduces the orofacial nocifensive behavioral activity evoked by the injection of formalin into the masseter muscle. Wei et al. (2005) examined the effects of i.p. administration of the 5-HT_{2A} receptor antagonist Ketanserin on hyperalgesia, inflammation, and expression of c-fos-like immunoreactivity in spinal cord dorsal horn in the carrageenan model of inflammation. These authors' results showed cellular evidence indicating that peripheral 5-HT_{2A} receptors are involved in nociceptive processing in the CNS and are responsible for the production of neuronal activity at the spinal-cord level in an inflammatory pain model. Additionally, the 5-HT_{2A} receptor and the 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, and 5-HT_7 receptors are also expressed in primary afferent nociceptors. In their study, Sasaki et al. (2006) examined the effect of a selective 5-HT_{2A} receptor antagonist, Sarpogrelate, on hyperalgesia and allodynia induced by thermal injury in rats. They concluded that Sarpogrelate (antagonist) blocks 5-HT_{2A} receptors at primary afferent-fiber terminals in the periphery to inhibit primary thermal hyperalgesia and secondary mechanical allodynia. Dorsal-root ganglion neurons express 5-HT_{2A} receptor mRNA, and stimulation of peripheral 5-HT_{2A} receptors produces thermal hyperalgesia.

Kayser et al. (2007) used mutant mice, which do not express 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, or 5-HT_{3A} receptors, as an indirect approach for further assessment of the respective roles of these receptors in the physiological control of nociceptive responses to a wide range of noxious mechanical and thermal stimuli and to i.p, injection of formalin. 5-HT_{1B} and, to a lesser degree 5-HT_{1A} receptors, mediate endogenous inhibitory control of nociception by 5-HT, whereas 5-HT_{2A} and 5-HT_3 receptors play a role in formalin-induced hyperalgesia in male mice. On comparison with paired wild-type mice, the authors also found that 5-HT_{1A} mutants exhibited increased sensitivity in the hot-plate test. Concomitant decrease of 5-HT-mediated pronociceptive influences can also be postulated as a result of 5-HT_{2A} receptor downregulation.

5-HT_{2A} antagonist receptors might aid in alleviating various types of neuropathic pain. Van Steenwinckel et al. (2009) evaluated the effect of an epidural (e.d.) injection of a 5-HT_{2A} antagonist receptor on the appearance of mechanical allodynia and hyperalgesia in model of peripheral neuropathy. The authors reported significant upregulation of 5-HT_{2A}-receptor immunoreactivity in the lumbar dorsal horn and peripheral nociceptive cells after peripheral neuropathy treatment. The 5-HT_{2A} receptor is involved in wide central sensitization of dorsal-horn neurons and in peripheral sensitization of nociceptive neurons. The effect of a 5-HT_{2A} receptor antagonist on the mechanical hypersensitivity induced by antineoplasic drug in rats comprised a study conducted by Thibault et al. (2008); the authors reported that 5-HT_{2A} receptors also play a pro-nociceptive role in the sensitization of peripheral nociceptors and spinal nociceptive processing. Studies to clarify the mechanism of action of some drugs have been carried out. Xie et al. (2008) reported that Tramadol treatment alters 5-HT_{2A} receptor mRNA in brainstem nuclei and the spinal dorsal horn, which may partially mediate the analgesic effect of Tramadol. Kupers et al. (2009) investigated the role of the 5-HT_{2A} receptors system in pain processing, finding that the 5-HT_{2A} receptor system possesses a role in the processing of tonic heat pain, but not in the processing of short phasic heat pain stimuli. 5-HT_{2A} receptors in discrete brain areas are involved in the regulation of responses to painful tonic stimulation. The correlations observed in the prefrontal and posterior cingulate cortices suggest a possible role in response to cognitive evaluative appreciation and in the emotional processing of pain.

In clinical studies where the objective was to evaluate the effect of 5-HT_2 receptors on pain management, Schreiber et al. (2001) evaluated the effect of Fluoxetine, a 5-HT_{2C} agonist drug, compared with Amitriptyline in the treatment of pain in 40 patients with musculoskeletal disorder. The investigators concluded that there are no significant differences between Fluoxetine and Amitriptyline, both of which reported similar effects on pain inhibition. Moreover, Kanayama et al. (2005) evaluated the administration of 5-$HT_{2A/2B}$ antagonists (Sarpogrelate 300 mg) in 40 patients with pain caused by lumbar-disc herniation; their results

demonstrated the efficacy of Sarpogrelate in the inhibition of pain, which is comparable to that of Non-Steroidal Anti-Inflammatory Drugs (NSAID). Kupers et al. (2009) reported the relationship of 5-HT_{2A} receptors in the modulation of painful stimulus by heat in 21 young patients; they concluded that 5-HT_{2A} receptors in prefrontal areas of the brain are involved in the regulation of tonic pain responses.

2.3. Serotonergic Receptor 5-HT_3 as Modulator of Nociceptive Response

Kilpatrick et al. (1987) found 5-HT_3 binding sites in the brain for the first time, and it is now known that 5-HT_3 participates in a wide variety of functions, notably including modulation of pain. The 5-HT_3 receptor is a ligand-gated ion channel that, when activated, gives rise to rapid, depolarizing responses in neurons (Maricq et al., 1991). Depolarization of both the fibers and cell bodies of vagal afferents occurs in response to 5-HT_3 receptor activation; this activates a variety of second- messenger signaling systems and, through these, indirectly regulates the function of ion channels and is involved in local, presynaptic control of neurotransmitter release (Maricq et al., 1991; Glaum et al., 1992; Miquel et al., 2002). 5-HT_3 receptor-mediated enhancement of 5-HT release has been reported in brain regions, for example, hippocampus, frontal cortex, hypothalamus, and raphe nucleus (van Hooft & Vijverberg 2000). On the other hand, 5-HT_3 receptor expression takes place at sites such as the medial preoptic area, dorsal tegmental nucleus, trochlear nerve nucleus, and facial nerve nucleus, and there is exists hybridization within dorsal-root ganglia and olfactory and somatosensory regions, which is consistent with a role for 5-HT_3 receptors in sensory processing (Tecott et al., 1993). Nayak et al. (1999) reported that 5-HT_3 serotonin receptors are present in isolated terminals of the corpus striatum, hippocampus, amygdala, and cerebellum of rat brain. 5-HT_3 receptors activation produces, among other effects, postsynaptic depolarization of neurons throughout the nucleus tractum stratium and, in addition, affects both spontaneous and evoked

synaptic transmission (Glaum et al., 1992). A significant proportion of $5HT_3$ binding sites (40%) are associated with the terminals of unmyelinated primary afferents, while others are found in intrinsic interneurons.

Comparison of the cellular population containing 5-HT_{3A} and 5-HT_{3B} mRNA subunits demonstrated that the population of peripheral neurons expressing the 5-HT_{3A} subunit is larger than that expressing the 5-HT_{3B} subunit (Morales & Wang, 2002). No functional differences between these alternatively spliced 5-HT_{3A} receptor subunits have been found to date; thus, it appears unlikely that alternative splicing of this subunit contributes to functional 5-HT_3 receptor heterogeneity (van Hooft & Yakel, 2003). Morales et al. (1996) reported the distribution of 5-HT_3 receptors immunoreactivity in rat CNS and found this in the forebrain (isocortex, olfactory regions, hippocampal formation, and amygdala), brainstem (sensory and motor nuclei and nuclei of the reticular formation), and spinal cord (dorsal and ventral horn). 5-HT_3 innervation of the striatum modulates dopaminergic activity. Blandina et al. (1988) indicated that at least part of this interaction occurs by means of the activation of a 5-HT_3 receptor, therefore presenting, to our knowledge, the first direct evidence of a functional role of a 5-HT_3 receptor in brain.

Activation of 5-HT_3 receptors produces a variety of effects, including membrane depolarization and an increase in intracellular Ca^{2+}, modulation of neurotransmitter release, excitation of central and peripheral neurons, and the release of 5-HT from enterochromaffin cells of the small intestine (Dubin et al., 1999). 5-HT_3 receptors may increase spontaneous release by producing localized depolarization of the presynaptic terminal, leading to an increased influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (Glaum et al., 1992). The 5-HT_3 receptors in particular are most closely related with the ACh receptor, and these receptors are present in the CNS and in the Peripheral Nervous System (PNS) (Nayak et al., 1999). Activation of the 5-HT_3 receptor opens the cation channel of the 5-HT_3 receptor, which becomes permeable preferentially to Na^+ and K^+, but also to Ca^{2+} and other cations, including the organic cation guanidinium (Brüss et al., 2000). 5-HT_3 receptors are expressed in GABAergic interneurons; thus, it is likely

that 5-HT_3 receptors are located in GABAergic nerve terminals in the basolateral amygdala, modulating synaptic GABA transmission (Koyama et al., 2000). GABA antagonists block the action of 5-HT_3. Tecott et al. (1993) showed that distribution of the 5-HT_{3A} subunit closely resembles that of 5-HT_3 receptor binding sites; the authors suggest that the scattered distribution of the 5-HT_3 receptor in the forebrain may reflect expression in Gamma Amino Butyric Acid (GABA)ergic interneurons (Chameau & van Hooft, 2006). 5-HT_3 receptors have been suggested as being involved in dopamine release, both in the striatum and in the nucleus accumbens (Chameau & van Hooft 2006). In the spinal cord, 5-HT_3 AllatoStasin-like-immunoreactivity was concentrated in dorsal-horn superficial layers in lamina I/III (Asante & Dickenson, 2010), where high densities of 5-HT_3 receptor binding sites were previously detected (Miquel et al. 2002). In addition to the postsynaptic localization of 5-HT_3 receptors in interneurons, 5-HT_3 receptors can also be found in presynaptic terminals (Chameau & van Hooft, 2006).

Alhaider et al. (1991) noted that 5-HT_3 receptors in intrinsic spinal cord neurons inhibit nociceptive spinal transmission in both behaviorally (in mice) and electrophysiologically (in rats). Glaum et al. (1992) reported that the antinociceptive effects of 5HT in both the tail-flick and hot-plate tests were blocked following i.t. application of the $5HT_3$ receptor antagonist ICS205-930. $5HT_3$ receptors are associated with the sensory endings of primary afferents, and there is good evidence that $5HT_3$ antagonists have antinociceptive actions at this peripheral site (Ali et al., 1996). 5-HT_3 receptors mediated serotonergic control of noxious transmission in the spinal cord. The responses of wide dynamic range (nociceptive) spinal dorsal-horn neurons to subcutaneous (s.c.) injection of formalin and the electrically evoked responses of such neurons following i.p, injection of carrageenan as inflammatory stimulus comprised a study conducted by Green et al. (2000). Their results showed that in normal animals with no inflammation, blockage of the 5-HT_3 receptors exerted no significant effect on the electrically evoked responses of spinal dorsal-horn neurons. Moore et al. (2002) reported that 5-HT, a proinflammatory neurotransmitter, can activate 5-HT_3 receptors to depolarize vagal afferent

neurons, whereas 5-HT_1 and 5-HT_2 receptor subtypes bear highest affinity for the endogenous ligand and are thought to exert overall antinociceptive action, and models of persistent pain have suggested a role for 5-HT_3 receptor activation in pain maintenance (Oatway et al., 2004). The 5HT_3 receptor could contribute to any central plasticity that accompanies this injury state. The hyperalgesia and allodynia manifested after tissue injury involves different peripheral and/or central mechanisms (Rahman et al., 2004). 5-HT facilitates persistent pain-like states via 5-HT_3 receptor activation, most likely due to an increased descending serotonergic drive from higher centers in the brain and, in particular, the rostral ventromedial medulla (Asante & Dickenson, 2010). At the spinal-cord level, blocking 5-HT_3 receptors reversed the increase in hypersensitivity induced by amygdaloid administration of a low dose of glutamate. This finding suggests that spinal 5-HT_3 receptors mediated CeA -induced increase of neuropathic hypersensitivity (Sagalajev et al., 2015).

Studies conducted in humans in relation to 5-HT_3 receptor-dependent pain modulation report similar effects to those studied in biomodels. Couturier et al. (1991) were, to our knowledge, the first to evaluate Granisetron (BRL 43694) as a 5-HT_3 receptor antagonist in the treatment of migraine, but the study had not been completed by 1998. Faris et al. (1998) evaluated the 5-HT_3 antagonist drug (Ondansetron) in patients with bulimia-related pain; the results of these authors concluded that administration of this drug in 14 patients with bulimia nervosa inhibited pain-detection thresholds, such as the appearance of the next bulimic episode. On the other hand, McCleane et al. (2003) evaluated the effect of Ondamsetron in patients with neuropathic pain; the results of their study revealed that administration of 8 mg of this antagonist of 5-HT_3 receptors during 1 week exerted significant effects on the reduction of neuropathic pain. Stratz and Muller (2004) treated two patients with systemic sclerosis and secondary fibromyalgia with 5 mg of Topisetron for 6 weeks in a pilot study; after 2 weeks, the patients exhibited recovery in the mobility of several joints, thus, a clear reduction of pain.

2.4. Serotonergic Receptor 5-HT$_7$ as Modulator of Nociceptive Response

5-HT$_7$ receptors are the most recently described member of the serotonin receptor family. An increasing number of studies have described the distribution of the 5-HT$_7$ receptor protein in rodents employing immunohistochemical techniques (Belenky & Pickard 2001; Bickmeyer et al., 2002; Geurts et al., 2002; Muneoka & Takigawa, 2003). These reports demonstrated that protein distribution is similar to that of mRNA, with highest abundance in thalamus, hypothalamus and hippocampus (Hedlund & Sutcliffe, 2004; Kvachnina et al., 2009). The 5-HT$_{7A}$ isoform predominates, followed by the 5-HT$_{7B}$ splice variant, while the 5-HT$_{7C}$ and the 5-HT$_{7D}$ isoforms are least frequently expressed (Vanhoenacke et al., 2000). The 5-HT$_{7A}$ receptor was the first, to our knowledge, splice variant cloned from human with a predicted length of 445 amino acids; in the spinal cord, 5-HT$_7$ receptors were mainly found in superficial laminae I and II of the dorsal horn, postsynaptically in local interneurons, and presynaptically in peptidergic fibers and in astrocytes (Meuser et al., 2002). Electron microscopic examination of the dorsal horn further revealed three main localizations: postsynaptic localization in peptidergic cell bodies and in numerous dendrites; presynaptic localization in unmyelinated and thin myelinated peptidergic fibers, and in astrocytes (Matthys et al., 2011). The pharmacological profile of 5-HT$_7$ receptors is quite similar to that of the 5-HT$_{1A}$ receptor subtype (Hoyer et al., 1994).

The following five major properties appear to define the 5-HT$_7$ receptor and differentiate it from other 5-HT receptors: limited sequence homology; the presence and location of at least two introns; the existence of an eighth hydrophobic domain; high-affinity binding of 5-HT, and positive coupling to adenylyl cyclase (Ruat et al., 1993). 5-HT$_7$ receptors stimulate cAMP formation by activating adenylyl cyclases via a stimulatory Gs-protein, which also leads to Ras-dependent activation of extracellular signal-regulated kinases (Norum et al., 2003). Activation of 5-HT$_7$ receptors directly stimulates extracellular signal-regulated kinase in hippocampal neurons (Errico et al., 2001), an effect that can be of

importance for hippocampal function and mood regulation (Hedlund & Sutcliffe 2004). 5-HT_7 receptors appear to be associated mainly with limbic brain divisions receiving serotoninergic inputs (e.g., the hippocampus, amygdaloid complex, or mammillary nuclei). This suggests that 5-HT_7 receptors are also involved in sleep induction and hypothermia, learning, mood, and in neuroendocrine or vegetative behaviors, and such observations were confirmed in a mouse strain with a disrupted 5-HT_7 gene (Ruat et al., 1993; Hedlund et al., 2003). Certain behavioral stimuli can trigger the electrical activity of the dorsal raphe nucleus, leading to 5-HT release and subsequent activation of 5-HT_7 receptors in both the dorsal raphe nucleus and medial raphe nucleus, which ultimately results in 5-HT release in the CNS (Matthys et al., 2011).

Rocha-González et al. (2005) carried out a study in which the principal objective was to determine the possible participation of local peripheral and spinal 5-HT_7 receptors in formalin- induced nociception; they reported that electrophysiological, immunohistochemical, and behavioral results suggested a pronociceptive role for the 5-HT_7 receptor in the dorsal horn of the spinal cord. Therefore, microinjection of formalin was preceded by either local or spinal administration of SB-269970 and/or 5-HT and reduced 1% formalin-induced flinching, while local 5-HT or 5-CT dose-dependently increased 0.5% formalin-induced nociceptive behavior, which was significantly reduced by SB-269970. On the other hand, the role of spinal 5-HT_7 receptors in the antinociceptive effects of systemic morphine was a study conducted by Drogul and Seyrek (2006); the authors reported that systemically administered morphine activated descending serotonergic pathways and that 5-HT_7 receptors in the spinal cord play an important role in systemic morphine antinociception. Brenchat et al. (2009) evaluated the potential role of the 5-HT_7 receptor in nociception associated with a sensitizing stimulus in mice. The intrinsic efficacy as activators of human 5-HT_7 receptors and the selectivity of 5-HT_7 receptor agonists utilized were also investigated. The investigators' results showed that 5-HT_7 receptors participate in antinociceptive mechanisms and that 5-HT_7 receptor blockade by i.t. administration of SB-269970 inhibited the antinociceptive effect of systemic morphine in the tail-flick test. The

following year, Brenchat et al. (2010) examined whether 5-HT_7 receptors participate in some modulatory control of nerve injury-evoked mechanical hypersensitivity and thermal hyperalgesia in mice. They found a significant increase of 5-HT_7 immunoreactivity in laminae I–II and III–V of the dorsal horn on the ipsilateral side of the spinal cord 11 days after nerve injury. In the case of 5-HT_7 receptors, a recent study found that systemic administration of 5-HT_7 receptor agonists reduced mechanical hypersensitivity in nerve-injured mice, suggesting that 5- HT7 receptors play an antinociceptive role. Studies suggest that spinal 5-HT_7 receptors may possess a pronociceptive rather than an antinociceptive role (Vanhoenacker et al., 2000). Likewise, systemic and spinal administration of the selective 5-HT_7 receptor antagonist SB-269970 reduced tactile allodynia induced by L5/L6 spinal nerve ligation, and 5-HT_7, receptors play a pronociceptive role in this type of pain. Spinal nerve ligation leads to a reduction in the level of 5-HT_7 receptors (Amaya-Castellanos et al., 2011).

The analgesic effect of morphine co-administered with the selective 5-HT_7 receptor agonist E-55888, the antagonist SB-258719, or both, was evaluated by Brenchat et al. (2011) and these investigators reported that 5-HT_7 receptors in opioid analgesia showed a potential use of 5-HT_7 receptor agonists as adjuvants of opioid analgesia. Systemic administration of a selective 5-HT_7 receptor agonist per se is not sufficient to reproduce the antinociception exerted by opioids in acute thermal nociceptive models. The respective roles of peripheral and spinal 5-HT_7 receptors in the modulation of mechanical hypersensitivity were investigated under two different experimental pain conditions by Brenchat et al., (2012). The authors demonstrated that activation of 5-HT_7 receptors exerts antinociceptive effects at the spinal-cord level and pronociceptive effects at the periphery. Previous study at the light-microscope level reported that 5-HT_7 receptors co-localize with GABA in neurons of the dorsal horn of the spinal cord (Brenchat et al., 2010), and it has been reported that spinal GABAergic interneurons are involved in 5-HT_7 receptor-mediated antinociception. Dogrul et al. (2012) investigated the role of descending serotonergic pathways and spinal 5-HT_7 receptors compared with 5-HT_3

and 5-HT_{2A} receptors in terms of the antinociceptive and antihyperalgesic effects of Paracetamol. They noted that activation of descending serotonergic pathways and spinal 5-HT_7 receptors following systemic administration of Paracetamol produces antinociceptive and antihyperalgesic effects, and that the 5-HT_7 receptor antagonist blocks the antinociceptive and antihyperalgesic effects of systemic Paracetamol, indicating a novel role of spinal 5-HT_7 receptors in the mechanism of Paracetamol. The role of spinal 5-HydroxyTriptamine (5-HT) and 5-$HT_{4/6/7}$ receptors in long-term associated mechanical allodynia and hyperalgesia induced by formalin in rat comprised a study conducted by Godínez-Chaparro et al. (2012). They showed that formalin activates a descending serotonergic system, which releases 5-HT at the spinal cord and contributed to the development and maintenance of associated allodynia and hyperalgesia. Viguier et al. (2012) investigated how 5-HT_7 receptors contribute to neuropathic pain modulation by employing a potent 5-HT_7 receptor antagonist (SB-269970) and/or agonists (MSD-5a, AS-19, E-55888) in rats with unilateral ligations of the sciatic nerve or of the infraorbital nerve; they reported that 5-HT_7 receptors mediated inhibitory control of the underlying neuropathic pain due to excitation of GABAergic interneurons within the dorsal horn. Yang et al. (2014) compared the role of 5-HT_7 receptors and the influence of descending serotonergic modulation between formalin and carrageenan-induced inflammatory pain. They concluded that activation of 5-HT_7 receptors exerted a significant antinociceptive effect on formalin-induced pain, but had no effect on carrageenan-induced pain, indicating differences in the involvement of 5-HT_7 receptors according to pain modality.

CONCLUSION

As we have observed throughout the chapter, several investigations have been carried out involving the importance of serotonergic receptors as modulators of the nociceptive pathway both in animal models as well as in clinical studies, with controversial results. However, researchers have

proposed 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_7 receptors as those involved in modifying the spinal raphe-spinal system in nociceptive modulation. The medulla nucleus of the raphe is a major source of serotonergic descending fibers that terminate in the spinal cord with a direct impact on pain perception. The physiological functions of the spinal cord and the importance of 5-HT receptors on the nociceptive stimulus are distributed in four areas: the first of these is the dorsal lamina of the spinal cord, which corresponds to the relief of the primary afferent neuron that transmits the nociceptive stimulus; the second is the intermediate-cellular cell column from which preganglionic sympathetic neurons originate; the central canal, which could be involved in exchanges with the cerebrospinal fluid, and finally, the ventral lamina, which is involved in motor functions. Serotonin-containing axons that descend from the brainstem terminate in both the ventral lamina and the medial column, as well as in dorsal spinal-cord lamina (Belcher et al., 1978). At the cellular level, serotonin produces pre- and postsynaptic inhibition and excitation in the superficial dorsal lamina of the spinal cord (Grudt et al., 1995; Hori et al., 1996; Travagli & Williams 1996; Ito et al., 2000; Lu & Perl, 2007; Jeong et al., 2012). This may explain the pronociceptive and antinociceptive effects of the administration of agonist and 5-HT receptor antagonists for the treatment of pain.

Different techniques, drugs, and pain models have been employed to locate the distribution of the different serotonergic receptors that are capable of modulating the nociceptive response mediated by the descending system in the spinal cord. The results of these studies demonstrated the presence of 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_7 in different amounts and at different levels. The majority of these studies involve these receptors in the inhibition of pain; however, studies mention the participation of these receptors in hyperalgesia or even maintain the stimulus painful. There have been different pain biomodels that have attempted to explain the involvement of serotonergic receptors in the nociceptive response (tail-flick, hot-plate, formalin test, carrageenan, etc.), and studies reported that the type of pain (acute, inflammatory, neuropathic pain, tonic pain, or chronic pain) entails the involvement of different

serotonergic receptors. Research with agonists or antagonists on the administration of 5-HT receptors revealed that the anti-nociceptive effect depends on the dose of the drug in all of the receptors. Finally, several authors have reported that the involvement of serotonergic receptors depends on the duration of pain, and that the different serotonergic receptors may even participate together to inhibit, excite, or maintain the painful stimulus.

There are several drugs for the treatment of pain: first-line drugs include the opioids. The analgesic effects of opioids are measured through the activation of μ-opioid receptors in the CNS that inhibit afferent nociceptive alterations; however, at present, these drugs appear to have no significant effect on diseases such as neuropathic pain, neuralgia, rheumatoid arthritis, osteoarthritis, etc. The treatment of pain is not an easy task in that it involves the change of different nociceptive relay pathways; however, clarifying the participation of 5-HT receptors in pain modulation on considering the previous points could generate knowledge that could be used for generate new drugs to mitigate pain and to improve the quality of life of patients with this type of disease. We conclude that the 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_7 receptors modulate the nociceptive pathway, but depend on receptor distribution, agonist or antagonist dose, administration route, pain type, and the duration of pain.

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Chapter 9

SOCIAL BEHAVIORS MODULATED BY TYRAMINE AND OCTOPAMINE IN INSECTS

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ABSTRACT

Neuronal effects of biogenic amines including dopamine and serotonin are conserved in vertebrates and invertebrates. Tyramine (TA) and octopamine (OA) are trace monoamines in vertebrates but can be major functional monoamines in invertebrates and act on neurons in the central and peripheral nervous systems and other cells in the peripheral tissues. In insects, TA is synthesized from a common precursor tyrosine and metabolized into OA. Receptors of TA and OA have been characterized independently in several species. A TA receptor (TYR1-R) decreases intracellular second messenger (cyclic adenosine monophosphate: cAMP) levels, whereas OA receptors increase the cAMP levels. Thus, these amines cause antagonistic responses in the signaling of

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target cells. Although it remains to be determined whether the antagonistic responses of cell signaling between TA and OA can lead to antagonistic behavioral responses or not, evidence suggesting antagonistic behavioral responses mediated by TA and OA have been reported in social insects. In flight behaviors, TA inhibits flight activities and flight motivation, whereas OA accelerates it. In honeybee males, the TA levels in the brain decrease as they age, but the OA levels conversely increase and OA application can promote mating flight activities and its motivation. A similar trend in these levels is seen in honey bee workers: higher levels of TA in younger individuals inhibit flight for foraging, whereas higher levels of OA in older individuals promote foraging. In phototactic responses in honey bees, TA enhances light responsiveness, whereas OA reduces the responsiveness. Other effects of TA or OA on aggression, cooperation and reproduction have been reported in honey bees, bumble bees, ants, and termites. Some of these are non-antagonistic effects by TA or OA. Thus, evidence for the antagonistic relationships between TA and OA are commonly observed in social insects and gradually accumulate, but particular effects by TA or OA are also present, which might contribute to orchestral effects with several biogenic amines on certain behaviors.

Keywords: behavior, biogenic amine, brain, insect, nervous system

1. INTRODUCTION

Biogenic amines are neuroactive substances in both vertebrates and invertebrates, and act on central and peripheral nerve tissues and other peripheral tissues including muscle. Large amounts of dopamine, serotonin, and histamine are present and used commonly in the central nervous system (CNS) in both vertebrates and invertebrates. Noradrenaline and adrenaline are synthesized more actively in the nerve tissues in vertebrates than in invertebrates, whereas tyramine (TA) and octopamine (OA) are synthesized more in invertebrates (Evans, 1980; Roeder, 1999; Berry, 2004; Roeder, 2005). This may be derived from the expression of different enzymatic genes for biosynthesis of the particular biogenic amines. Both TA and OA occur in nanomolar quantities in mammals and are considered trace amines, but there is still considerable debate as to their functional roles (Berry, 2004). In the present chapter, I introduce the

properties of TA and OA receptors and their behavioral effects in social insects.

2. Distribution of OA and TA Neurons and Receptors in the CNS

2.1. TA/OA Neurons

Projection of TA and OA secretory neurons within the CNS would help to understand the involvement of TA and OA in particular behaviors. Since TA is a precursor of OA, OA neurons could produce and possess TA. Therefore, if we stained neurons immunohistochemically by using anti-TA and anti-OA antibodies, most of the OA-immunoreactive neurons would also be stained by anti-TA antibodies (Nagaya et al., 2002; Kononenko et al., 2009; Ishikawa et al., 2016). In fact, similar neurons were stained by anti-TA and anti-OA antibodies in the brain and subesophageal ganglion in the damp-wood termite *Hodotermopsis sjostedti* (Ishikawa et al., 2016). However, in the fruit fly *Drosophila melanogaster* and the locust *Schistocerca gregaria*, some TA-like immunoreactive neurons have been found in non-OA-immunoreactive cells (Nagaya et al., 2002; Kononenko et al., 2009; Lange, 2009). By this methodology, a neuron stained by anti-TA antibodies but not by anti-OA antibodies can be defined as a TA neuron. A neuron stained by both anti-TA and anti-OA antibodies is an OA neuron that can secrete both TA and OA. Another determination of TA neurons is by biochemical determination of TA with electric stimulation of the nerve containing candidate TA neurons (Donini & Lange, 2004; da Silva & Lange, 2008). When the nerve containing TA-immunoreactive neurons are stimulated electrically by the electrode, the hemolymph around the nerve ends may contain released TA. The released TA can be detected by high-performance liquid chromatography-electrochemical detection. It is unknown whether the neurons possessing both TA and OA could release the two substances at the same time or not.

It is possible that the release of TA and OA in the same neuron could be controlled by tyramine-β-hydroxylase (TβH) activity. The TA/OA neuron with a lower activity of TβH might produce a larger amount of TA and release mainly TA, whereas the neuron with a higher activity of TβH might produce more OA than TA and release mainly OA. If so, the TβH activity or expression of a TβH gene in a neuron might be important to determine neuronal characteristics of released substances and the roles of the neuron.

There have been several reports on the morphology of monoamine neurons by using immunohistochemistry in the brains of insects (Stevenson & Spörhase-Eichmann, 1995; Burrows, 1996; Bicker, 1999). In social insects, dopamine-immunoreactive and serotonin-immunoreactive neurons were described in not only the honey bee *Apis mellifera* (Schürmann & Klemm, 1984; Schäfer and Rehder, 1989; Schürmann et al., 1989; Bicker, 1999) but also in several species of ants (Hoyer et al., 2005; Seid et al., 2008). However, reports on OA-immunoreactive neurons in the brains of social insects are limited only to the honey bee (Kreissl et al., 1994; Sinakevitch et al., 2005). In the honey bee, 15 clusters of OA-immunoreactive neurons locate in the central brain. These neurons are paired and provide the processes to circumscribed regions of the antennal lobe glomeruli, neuropils of protocerebrum and optic lobes, and subesophageal ganglion (Sinakevitch et al., 2005). In the subesophageal ganglion, OA-immunoreactive neurons are ventral unpaired median neurons (VUM neurons) with somata located ventrally. In other insects, including cockroaches and locusts, the subesophageal ganglion is completely separated from the brain, the presence of both unpaired and paired OA-immunoreactive neurons have been reported in the subesophageal, thoracic and abdominal ganglia (Stevenson & Spörhase-Eichmann, 1995). In these insects, the unpaired OA-immunoreactive neurons are dorsal and ventral unpaired median neurons (DUM and VUM neurons) (Stevenson & Spörhase-Eichmann, 1995; Sinakevitch et al., 2005). DUM and VUM neurons release OA within a ganglion and nerve ends near muscles or peripheral sensory organs, suggesting that OA can act on both neuronal networks in the central and peripheral nervous systems or muscles.

TA-immunoreactive neurons have been reported in the fruit fly *D. melanogaster* (Nagaya et al., 2002), the locusts *S. gregaria* and *Locusta migratoria* (Donini & Lange, 2004; da Silva and Lange, 2008; Kononenko et al., 2009) and the termite *H. sjostedti* (Ishikawa et al., 2016). The double staining of TA-immunoreactive and OA-immunoreactive neurons in the locust show a different distribution of TA and OA within a cell (Kononenko et al., 2009). Both the somata and primary neurites in the ventral nerve cord are double-labeled, as well as varicose profiles in the neuropil, whereas many fibers in the neuropil express only TA-immunoreactive. OA is restricted to more terminal varicose structures (boutons), whereas TA is more evenly distributed, suggesting that TA may be present and more evenly distributed in the cytoplasm, whereas OA may be more concentrated and associated with vesicles. In a whole brain, the distribution of TA-immunoreactive neurons seems to be similar with that of OA-immunoreactive neurons in the locust (Kononenko et al., 2009) and the termite (Ishikawa et al., 2016). In the honey bee, there are no reports on the distribution of TA-immunoreactive neurons, but a quantitative study shows that TA presents in the optic lobes, deutocerebrum, and protocerebrum, and the ratios of the TA amounts in optic lobes and deutocerebrum to a whole brain is larger than the ratios of the dopamine amounts (Sasaki & Nagao, 2002). This finding suggests that TA/OA function may bias more for visual and odor sensory processing than dopamine. In fact, several reports demonstrate that TA/OA can modulate phototaxis (Scheiner et al., 2014) and chemosensory response (Scheiner et al., 2002) in the honey bee.

2.2. TA/OA Receptors

Both TA and OA receptors have been characterized and their genes have been cloned in several insects (Blenau et al., 2000; Blenau & Baumann, 2001; Farooqui, 2012). Originally, two types of OA receptors (OCT-1 and OCT-2) were pharmacologically classified in the locust (Evans, 1981). OCT-1 is associated with an increase in intracellular Ca^{2+}

levels, whereas the OCT-2 is associated with an increase in intracellular cAMP levels. OCT-2 is further divided into three types (OCT-2A, OCT-2B, and OCT-2C). Then, another type of OA/TA receptor (OCT/TYR-R) was identified in *D. melanogaster* (Arakawa et al., 1990; Saudou et al., 1990). This receptor had a higher affinity with TA in comparison to OA and reduces cAMP levels in response to TA binding, leading to the discovery of a TA receptor. At present, two types of OA receptors (OCTα-R and OCTβ-R) and two types of TA receptors (TYR1-R and TYR2-R) are genetically and physiologically classified in insects (Farooqui, 2012) (Table 1).

OCTα-R and OCTβ-R correspond to OCT-1 and OCT-2, respectively. OCTβ-R is further divided into at least four types (OCTβ1-R, OCTβ2-R, OCTβ3-R and OCTβ4-R). In TA receptors, TYR1-R is associated with a decrease in intracellular cAMP levels, whereas TYR2-R is associated with an increase in intracellular Ca^{2+} levels (Table 1). Interestingly, it is known that TYR1-R activation in response to OA is coupled with an increase in intracellular Ca^{2+} release in *D. melanogaster* (Robb et al., 1994; Reale et al., 1997). Homologs of these receptors are found in the genome sequence in the honey bee and other eusocial insects (Beggs et al., 2011). Antagonistic response at intracellular levels between OCTβ-R and TYR1-R might be related to the antagonistic motor outputs. For example, in the larvae of *D. melanogaster*, OA can enhance the excitatory junctional potentials in the dorsal acute muscles which are innervated by OA neurons, whereas TA can reduce the potentials in a dose-dependent manner (10^{-7} to 10^{-5} M) (Kutsukake et al., 2000; Nagaya et al., 2002). A similar antagonistic response of the junctional potentials in the larval body wall muscle by TA and OA has been reported (Ormerod et al., 2013).

Distribution of the OA and TA receptors in social insects have been reported in workers in the honey bee by using *in situ* hybridization with RNA probes or immunohistochemistry with anti-AmOA1 (OCTα-R type) antibodies. In AmOA1, the receptor protein distributes to several cell body clusters throughout the brain and within the brain neuropils including the antennal lobes, the calyces, pedunculus, vertical and medial lobes of the mushroom body, optic lobes, central complex and subesophageal ganglion

(Sinakevitch et al., 2011). In some of the AmOA1-positive cells in the antennal lobes, inhibitory feedback neurons in the mushroom body are double-stained by anti-gamma-aminobutyric acid (GABA) antibodies, suggesting that one effect of OA via AmOA1 in these regions is to modulate inhibitory neurons. These results are consistent with the results of *in situ* hybridization with *Amoa1* RNA probes (Grohmann et al., 2003).

In TA receptor AmTYR1 (TYR1-R type), the brain neurons in the optic lobes, deutocerebrum and intrinsic neurons in mushroom body express *Amtyr1* (Blenau et al., 2000). The expression pattern seems to resemble the pattern of the mRNA of a dopamine D1 receptor (Blenau et al., 1998). It is not known whether the pattern is similar with those of OA receptors or not.

Table 1. Classification of octopamine and tyramine receptors in insects

Class of receptors	Subdivision	Ligands	Intracellular response	Notes
OCTα-R		OA > TA	Ca^{2+} increase, cAMP increase	α-adrenergic-like
OCTβ-R	OCTβ1-R	OA > TA	cAMP increase	β-adrenergic-like
	OCTβ2-R			
	OCTβ3-R			
	OCTβ4-R			
TYR1-R		TA > OA	cAMP decrease	
		OA ≥ TA	Ca^{2+} increase	
TYR2-R		TA	Ca^{2+} increase	

cAMP: cyclic adenosine monophosphate, OA: octopamine, OCT: octopamine receptor, TA: tyramine, TYR: tyramine receptor

3. BEHAVIORAL EFFECTS OF TA AND OA

3.1. Flight Behavior

Flight motor pattern is formed by the combination of the central pattern generators in the thoracic ganglia and sensory feedback from the peripheral sensory organs associated with flight movements (Burrows, 1996). The flight movement is modulated on multiple levels by the OA. Within the CNS, OA acts directly on the flight central pattern generator

and affects motivational states. In the periphery, OA sensitizes sensory receptors, alters muscle contraction kinetics, and enhances flight muscle glycolysis (Roeder, 1999).

In the fruit fly *D. melanogaster*, mutant flies lacking OA (TβH null mutants) can fly. So, OA is not the natural signal for flight initiation, but the mutants show profound differences with respect to flight initiation and flight maintenance compared with wild-type flies (Brembs et al., 2007). The mutant flies not only lack OA but also show an eightfold higher concentration of TA. The flight deficits are rescued by substituting OA or blocking the TA receptors with yohimbine (TA antagonist). In contrast, blocking TA action in flies with normal OA and TA levels does not affect the flight behavior. Therefore, OA is necessary for flight maintenance, and TA acts most likely as an inhibitor, especially for flight initiation at high concentration (Brembs et al., 2007). This report opposes the interpretation that OA and TA simply act antagonistically on the same targets, but it may be explained by dose effects and different sites of action. In the moth *Manduca sexta*, OA and TA exert distinct effects on the flight central pattern generating network (Vierk et al., 2009). In the preparation of isolated ventral nerve cord, OA is sufficient to induce fictive flight, whereas TA increases synaptic drive to depressor motor neurons, the number of depressors spikes during each cycle and decreases the depressor phase (Vierk et al., 2009). Blocking TA receptors does not affect cycle by cycle elevator motor neuron spiking. These results suggest that the roles of OA and TA are not simply antagonistic, rather the concerted interaction of OA and TA might be more important than the concentration of a single amine.

In workers in the honey bee (*A. mellifera*), OA promotes the flight for foraging behavior (Schultz & Robinson, 1999, 2001; Fussnecker et al., 2006). Brain TA levels decrease as they age (Sasaki & Nagao, 2002), whereas OA levels increase contrary (Harris & Woodring, 1995; Wagener-Hulme, 1999). Young workers with high levels of TA in the brain tend to stay in the nest, old workers with high levels of OA go out to forage. In fact, oral application of OA promotes foraging behavior, whereas the application of TA inhibits foraging (Schultz & Robinson, 2001). In more

detailed analyses of locomotor behaviors including walking, grooming, fanning, and flying, the OA-treated bees increase flying, whereas those receiving TA decrease flying (Fussnecker et al., 2006). Thus, the antagonistic effects on flight behaviors by TA and OA are seen in workers in honey bees. In honey bee colonies in the absence of a queen (queenless colonies), the workers have higher TA levels in the brains compared to those in colonies with a queen (Sasaki & Nagao, 2002). The workers in queenless colonies seem to intake tyrosine that is a precursor of both TA and dopamine, from royal jelly-like food, because the consumer of royal jelly (a queen and larvae) is absent in the colony. Oral application of tyrosine to the workers in the queenless colonies can enhance the brain TA and dopamine levels, but not OA levels, and can inhibit foraging so that they stay in the nest to prepare reproduction (Matsuyama et al., 2015). Thus, the flight initiation in honey bee workers can be controlled by TA and OA depending on age. Both TA and OA levels in the brain can be regulated by enzymatic activities of tyrosine hydroxylase (TH) and TβH and the amount of substrate for the enzymes. So, age-dependent TA and OA biosynthesis contribute to switching from tasks in the nest to tasks out of the nest.

An age-dependent decrease of TA and increase of OA are also found in males in the honey bee (Figure 1). Males in the honey bee do not forage but specialize in reproduction. Young immature males have relatively higher TA levels in the brain, whereas mature males at 8 days old have higher OA levels. Injection of OA shortens the duration for initiation of flight and elongates the duration of flight (Mezawa et al., 2013). TA is metabolized into both OA and *N*-acetyltyramine. When TA is released in the brain, the released TA could be inactivated by *N*-acetyltransferase and converted into *N*-acetyltyramine (Sasaki & Nagao, 2002). In the male honey bee, the brain levels of *N*-acetyltyramine decrease with age, most likely as TA. Similar changes of the brain levels of TA and *N*-acetyltyramine implicates that TA is released in the brains of young males that stay in the nest. However, there has been no reports showing the inhibition of flight by TA in the males. Therefore, TA effects on flight in males remain to be determined.

Phototaxis seems to be associated with foraging and mating behaviors in social insects. In honey bees, foraging workers show phototaxis and walk toward a light source. Oral applications of OA reduce responsiveness to light, whereas TA application enhances phototaxis (Scheiner et al., 2014). OA increases the walking times to the light source but does not change walking speed (locomotor activity). TA increases the locomotor activities and results in the enhancement of phototactic response. The targets of OA and TA in the neural circuits may be different, but the antagonistic behavioral outputs are expressed by OA and TA applications. These effects of OA and TA seem to contradict the association between OA and foraging behavior in honey bees. However, a similar OA effect on a reduced phototaxis has been reported in the fruit fly (Dudai et al., 1987). Among foragers in honey bees, the nectar foragers prefer light sources in the experimental setup and go faster to the light than the pollen foragers (Scheiner et al., 2014). Moreover, in the optic lobes, the nectar foragers have lower levels of OA than the pollen foragers. Therefore, the antagonistic response by OA and TA on phototaxis might be functional for switching between nectar and pollen foragers.

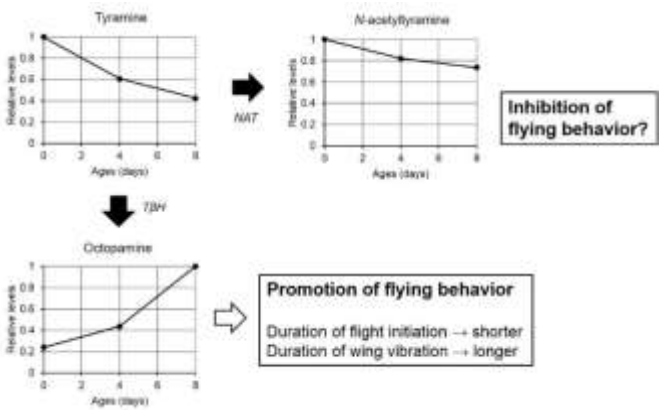

Figure 1. Level changes of tyramine (TA), octopamine (OA), and *N*-acetyltyramine in the brain of males in honey bees. The levels of TA decrease as males are aging, whereas those of OA increase mediated by tyramine-β-hydroxylase (TβH). OA promotes flight behavior for mating. When TA is released into the brain or peripheral tissues, the TA is inactivated by *N*-acetyltransferase (NAT) and converted into *N*-acetyltyramine. Based on data from Mezawa et al. (2013).

3.2. Aggressive and Cooperative Behaviors

In social insects, aggressive behaviors can be categorized into interactions within species (intra-species) and between species (inter-species). The former contains an interaction between individuals from different nests (nestmate recognition) and within a nest (social dominance among reproductive females, etc.). The latter can be further divided into an attack for foraging (predation) and a nest defense from predators. OA is known to elevate aggression levels between conspecific males in general solitary insects including the fruit fly (Baier et al., 2002) and the cricket (Stevenson et al., 2005). These interactions between rival males may correspond to intra-species interactions in social species. In honey bee workers, several OA agonists enhance the aggressive levels during nestmate recognition (Robinson et al., 1999). A similar OA effect for the nestmate recognition has been reported in the ant *Oecophylla smaragdina* (Kamhi et al. 2016) (Table 2). In the ants, OA and serotonin might be involved in the aggressiveness for the interaction of intra- and inter-species (Table 2). The functional differences between OA and serotonin are unknown. There are positive correlations between aggressiveness and OA or serotonin in several species of ants. The effects of OA or serotonin on aggressive behaviors are confirmed in a limited number of species (Table 2). The effects of each amine enhancing aggression level might be context-dependent or be integrated for adjustments of aggression levels. Bioassays to use monoamine receptor drugs or amines remain to be done. OA is also correlated with social dominance between reproductive females in the bumble bee *Bombus terrestris* (Bloch et al., 2000) and the ant *Streblognathus peetersi* (Cuvillier-Hot and Lenoir, 2006).

There are a few reports examining TA effects on aggression in ants (Szczuka et al., 2013). In *Formica polyctena*, TA decreases aggressiveness for colony defense against *Formica fusca*, whereas TA increases aggressiveness for predation against a cricket nymph (Table 2). In this ant species, OA effects on aggressiveness are not detected, and potential effects of dopamine and serotonin on aggression can be detected (Szczuka et al., 2013). Such effects of dopamine and serotonin have been known in

Formica rufa (Kostowski & Tarchalska, 1972; Kostowski et al., 1975; Kostowski & Tarchalska-Krynska, 1975) (Table 2). In the damp-wood termite *H. sjostedti*, the soldiers have higher TA levels in the brain and subesophageal ganglion and OA levels in the brain than workers (pseudergates) (Ishikawa et al., 2016).

TA application promotes the defensive behavior in workers with high aggressiveness against enemy ants, whereas OA application does not enhance aggressiveness. Although TA is a precursor of OA and TA application may potentially increase the OA levels, the enhancement of aggressiveness may be caused by TA, because of the non-effective results by OA application and the similar OA levels in the subesophageal ganglion between soldiers and workers. Thus, there might be TA-specific effects enhancing aggressive behaviors against different species in the termite and the ant (*F. fusca*). General effects of TA on aggression are still unknown.

Cooperative behaviors in social insects can be observed under the condition of low aggressiveness between partners. Therefore, disappearance of cooperation might be associated with OA or serotonin that enhance aggression levels. Furthermore, OA can decrease frequency of trophallaxis (food exchanges) between nestmates in ants (Bouley et al., 2000; Koyama et al., 2015) (Table 2). In *Camponotus fellah*, the application of serotonin does not affect the frequency of trophallaxis, suggesting that OA can act stronger on food exchange than serotonin (Bouley et al., 2000). In *Polyrhachis moesta*, the brain levels of OA are greater in colony queens (higher aggressiveness) than in founding queens (lower aggressiveness) (Koyama et al., 2015), but the serotonin levels do not differ between them (Sasaki et al., unpublished). However, in another ant species, *Veromessor pergandei*, the brain serotonin levels are higher in queens in secondary monogyny established colonies than in founding queens, whereas this relationship is opposite in haplometrosis colonies (Muscedere et al., 2016). Serotonin might enhance aggressiveness after the disappearance of cooperative behaviors in this species. This possibility should be tested by drug application.

Table 2. Correlation and effects of biogenic amines on aggressive and cooperative behaviors in ants

Behavior	Species	Caste	Behaviors	Correlation		Drug application		Reference
				Amine	Trend	Drug	Effect	
Aggression	*Streblognathus peetersi*	Worker	Social rank	OA	Positive	-	-	Cuvillier-Hot & Lenoir (2006)
	Formica japonica	Worker	Predation (inter-species)	OA	Positive	-	-	Aonuma & Watanabe (2012)
	Formica polyctena	Worker	Nest defense (inter-species)	-	-	DA	Increase	Szczuka et al. (2013)
						5HT	Increase	
						TA	Decrease	
			Predation (inter-species)	-	-	DA	Increase	
						5HT	Increase	
						TA	Increase	
	Formica rufa	Worker	Nestmate recognition	5HT	Positive	5HT, 5HTP	Increase	Kostowski & Tarchalska (1972); Kostowski et al. (1975)
						DA, DOPA, DDTC	Increase	Kostowski & Tarchalska-Krynska (1975)
			Nest defense (inter-species)	5HT	Positive	5HT, 5HTP	Decrease	Kostowski & Tarchalska (1972); Kostowski et al. (1975)
	Oecophylla smaragdina	Worker	Nestmate recognition	OA	Positive	OA	Increase	Kamhi et al. (2016)
						Epinastine	Block	
	Veromessor pergandei	Queen	Aggression	5HT	Positive	-	-	Muscedere et al. (2016)

Table 2. (Continued)

Behavior	Species	Caste	Behaviors	Correlation		Drug application		Reference
				Amine	Trend	Drug	Effect	
Cooperation	*Camponotus fellah*	Worker	Trophallaxis	-	-	OA	Decrease	Boulay et al. (2000)
						OA + phentolamine	Block	
	Formica japonica	Worker	Trophallaxis (number, proportion)	OA	Negative	-	-	Wada-Katsumata et al. (2011)
			Trophallaxis (duration)	OA	Positive	-	-	
	Polyrhachis moesta	Queen	Trophallaxis	OA	Negative	OA	Decrease	Koyama et al. (2015)

DA: dopamine, DDTC: diethyldithiocarbamate, DOPA: 3,4-dihydroxyphenylalanine, OA: octopamine, TA: tyramine, 5HT: serotonin, 5HTP: 5-hydroxytryptophan.

3.3. Reproductive Organs

Female reproductive organs in insects are innervated by the nerves from the terminal abdominal ganglion. It has been reported that the muscles of the lateral and common oviducts can be controlled by motor neurons and DUM neurons in solitary insects (Kalogianni & Theophilidis, 1993; Monastirioti, 2003; Donini and Lange, 2004). The muscles of the oviducts contract rhythmically to transport an egg to the posterior region by peristaltic movements. OA and TA released from DUM neurons modulate the rhythmic movement. In the locust L. migratoria, the actions of TA in the oviduct are dependent on dose (Donini and Lange, 2004). TA increases the amplitude of excitatory junctional potentials and decreases cAMP levels at a lower concentration, whereas there is a dose-dependent decrease in spontaneous and induced contractions of oviduct at higher concentrations in a similar manner to that seen for OA (Lange, 2009). The complicated action of TA might be involved in the cyclic guanosine monophosphate (cGMP) second messenger system. The levels of cGMP in the lateral oviducts increase by TA depending on its concentration, but not by OA. To generalize the cGMP system in the TA receptors in the visceral muscle, further studies are required.

In social insects, regulation of the female reproductive system is very important because the regulation is associated with the reproductive division of labor. In queenless colonies of honey bees, workers can develop their ovaries and become reproductive individuals instead of a queen, but they do not mate with males and only lay unfertilized eggs (male eggs). The workers in queenless colonies have higher TA levels in the brains than those in colonies with a queen (Sasaki & Nagao, 2002). Oral application of TA to the workers in queenless colonies promote the development of the ovaries with elevation of the brain dopamine levels and finally become reproductive individuals (Sasaki and Harano, 2007; Salomon et al., 2012). Oral application of tyrosine to the workers in queenless colonies can enhance the brain TA and dopamine levels and development of the ovaries (Matsuyama et al., 2015). The gene expression of a TA receptor Amtyr1 (TYR1-R) in the ovaries in honey bees is

different between workers with developed and undeveloped ovaries, suggesting that TA might directly act on the TA receptor in the ovaries (Thompson et al., 2007). In the biosynthesis of pheromone in the workers of queenless colonies, oral application of TA enhances the production of esters in the Dufour's gland and 9-hydroxy-2-decenoic acid (9HDA, queen component) in the mandibular glands, facilitating worker reproductive dominance (Salomon et al., 2012). OA, on the other hand, did not enhance ester production but increased the production of 10-hydroxy-2-decenoic acid (10HDA, worker major component). OA is known to induce foraging behavior in workers, while increased production of 10HDA characterizes nursing workers. TA induces reproductive division of labor, while OA treatment results in caste differentiation of workers to foragers and nurses (Salomon et al., 2012). Thus, the independent effects of TA and OA can lead to opposite directions for division of labor in honey bee workers. The antagonistic behavioral outputs or opposite direction of division of labor depending on ages or colony conditions caused by the relative substances (TA and OA) in a common metabolic pathway is one of the social systems regulating division of labor.

CONCLUSION

Effects of TA and OA on behaviors and physiology in social insects have been introduced in this chapter. The evidence that suggests antagonistic effects between TA and OA are still limited and need to be accumulated. Especially, studies on TA effects in social insects are just starting and are ongoing. The antagonistic behaviors are not always caused by the same target cell or neural circuit. Some of these might be caused by different muscles, sensory organs or neural circuits, and might not be derived from the antagonistic cell signaling between TA and OA. These antagonistic behavioral responses might be evolutionarily selected under age-dependent or colony condition-dependent metabolisms of TA and OA. If the social regulation systems could be explained by the mediation of TA/OA antagonistic effects, it would be fruitful.

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