Cláudia Coelho de Ávila Sarmento Espalha

Bachelor of Science in Biomedical Engineering

Direct Detection of Biogenic Amines from Fish

Dissertation to obtain the Master Degree in
Biomedical Engineering

Advisor: PhD. Prof. Valentina Vassilenko, Assistant Professor, FCT/UNL

Advisor: Prof. Mário Diniz, Assistant Professor, FCT/UNL

Júri:

Presidente:	Doutor Jorge Alexandre de Carvalho Silva, Professor Auxiliar da Universidade NOVA de Lisboa - Faculdade de Ciências e Tecnologia
Arguentes:	Doutora Maria Paulina Estorninho Neves da Mata, Professora Auxiliar da Faculdade de Ciências Médicas da Universidade NOVA de Lisboa
Vogais:	Doutora Valentina Borissovna Vassilenko, Professora Auxiliar da Universidade NOVA de Lisboa - Faculdade de Ciências e Tecnologia, orientadora

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September, 2018

Direct Detection of Biogenic Amines from Fish

Acknowledgments

To my advisor Valentina Vassilenko, for transmitted knowledge and demonstrated patience and dedication.

To my advisor Mário Diniz for the availability, commitment, motivation and dedication towards all my work. For providing me with the necessary resources for the development of this thesis.

To my laboratory colleagues for the availability and transmitted knowledge. Particularly to Jorge Fernandes and Paulo Santos for all the help and support I received for the preparation of this work.

To my true friend Maria for always being ready to help me and always encourage me to reach my goals. For the tireless support and for helping me get through the difficult times along this journey. And especially for the fun times during our laboratory nights.

Finally, I thank my sister and my mom for their endless love, support and encouragement. To my sister for all the moments of happiness we shared and for her ability to make me laugh under any occasion. And to my mom, to whom I dedicate this thesis, for all the support, dedication and for always believing in me.

Abstract

The biogenic amines content in fish products has been widely studied due to their potential toxicity and possible indication of the spoilage degree of food. One particular amine, histamine, is behind several outbreaks of food poisoning, particularly in fish products. However, histamine alone appears to be insufficient to cause intoxications, being putrescine and cadaverine, potentiators agents that contribute to its toxicity.

Several methodologies have been studied and developed for determination of biogenic amines in food products. Ion Mobility Spectrometry coupled with Multi Capillary Columns or Gas Chromatography offers a higher sensitivity and selectivity on complex biological matrices analysis, providing the monitoring of trace levels of volatile compounds.

The aim of this thesis is to evaluate Gas Chromatography coupled with Ion Mobility Spectrometry as a tool for monitoring non-volatile amines emission from fish tissues matrices, allowing the detection and establishment of specific patterns of biogenic amines.

Samples of histamine dihydrochloride, putrescine, cadaverine, tyramine, tryptamine, spermine, spermidine and phenethylamine were analysed. Samples of atlantic bonito, atlantic horse mackerel and sardine were collected and analysed over time of four days to allow an assessment of fish spoilage. With the exception of histamine, it was possible to obtain an identifier pattern for all analysed amines. The presence of some amines was also observed in the fish samples spectra.

Keywords: Ion Mobility Spectrometry; Gas Chromatography; Biogenic Amine; Fish Spoilage; Volatile Organic Compounds; Detection

Resumo

O conteúdo de aminas biogénicas tem sido amplamente estudado no pescado devido à sua potencial toxicidade e possível indicação do grau de deterioração dos alimentos. Uma amina particular, a histamina, encontra-se na causa de vários casos de intoxicação alimentar, particularmente em produtos derivados do peixe. No entanto, a histamina isolada parece ser insuficiente para causar intoxicações, sendo a putrescina e a cadaverina, agentes potenciadores que contribuem para a sua toxicidade.

Diversas metodologias foram estudadas e desenvolvidas para a determinação de aminas biogénicas em produtos alimentares. A Espectrometria de Mobilidade Iónica acoplada a Colunas Multicapilares ou a Cromatografia Gasosa oferece uma maior sensibilidade e seletividade na análise de matrizes biológicas complexas, fornecendo a monitorização de níveis residuais de compostos voláteis.

O objetivo desta tese é avaliar a Cromatografia Gasosa acoplada à Espectrometria de Mobilidade Iónica como ferramenta de monitorização da emissão de aminas não voláteis a partir de matrizes de tecidos de peixes, permitindo a deteção e o estabelecimento de padrões específicos de aminas biogénicas.

Amostras de dicloridrato de histamina, putrescina, cadaverina, tiramina, triptamina, espermina, espermidina e feniletilamina foram analisadas. Amostras de sarda, carapau e sardinha foram recolhidas e analisadas ao longo do tempo para permitir uma avaliação da degradação dos peixes. Com exceção da histamina, foi possível obter um padrão de identificação para todas as aminas analisadas. A presença de algumas aminas foi também observada nos espectros das amostras de peixes.

Palavras-chave: Espectrometria de Mobilidade Iónica; Cromatografia Gasosa; Amina Biogénica; Deterioração de Peixe; Compostos Orgânicos Voláteis; Deteção

Contents

List of Figures

List of Tables

Abbreviations

BA	Biogenic Amine
CAD	Cadaverine
CE	Capillary Electrophoresis
DAO	Diamine Oxidase
DMA	Dimethylamine
FIA	Flow Injection Analysis
GC	Gas Chromatography
GC-IMS	Gas Chromatography coupled with Ion Mobility Spectrometry
GC-MS	Gas Chromatography coupled with Mass Spectrometry
HDC	Histidine Decarboxylase
HFP	Histamine Fish Poisoning
HIS	Histamine
HMT	Histamine N-methyltransferase
HPLC	High-performance Liquid Chromatography
HRP	Horseradish Peroxidase
IMS	Ion Mobility Spectrometry
LAB	Lactic Acid Bacteria
LAV	Laboratory Analytical Viewer

MAO	Monoamine Oxidase
MCC	Multi Capillary Column
MCC-IMS	Multi Capillary Column coupled with Ion Mobility Spectrometry
NDMA	Nitrosodimethylamine
PEA	Phenethylamine
PUT	Putrescine
RIP	Reactant Ion Peak
SPD	Spermidine
SPM	Spermine
TLC	Thin Layer Chromatography
TRP	Tryptamine
TYR	Tyramine
UPLC	Ultra Performance Liquid Chromatography
VOC	Volatile Organic Compound
TLC	Thin Layer Chromatography
TMA	Trimethylamine

Chapter 1

Introduction

In the last decades the demand for foods of animal origin has been growing, forcing the suppliers to implement improved measures of control to guarantee the quality and safety of the consumer. The presence of biogenic amines (BAs) in food is of great concern to the food industry as, given the potential risk to health, there is a growing demand from consumers and control authorities to reduce the permitted limits of BAs in food and beverages.

To reduce risks to consumer's health, the control of these compounds in food matrices has moved the scientific community to develop increasingly analytical techniques that enable them to be unambiguously identified and quantified and, therefore to enable the correct evaluation of the risk factors correlated with the intake of the product.

BAs are non-volatile amines formed through the decarboxylation of amino acids and are present in food, originating in food processing or storage, or naturally.

Many BAs have been found in fish, however only histamine, cadaverine, and putrescine have been found to be significant in fish safety and quality control. Usually the concentrations of BAs increase during spoilage, as a result of microbial concentrations and deterioration of sensory quality. Although important in many physiological functions, BAs' consumption in high concentrations should be avoided given the toxicological problems that may arise [1].

Despite the reported association between histamine and scombroid food poisoning, histamine alone is insufficient to cause toxicity. Other BAs such as putrescine and cadaverine also contribute to potentiate histamine toxicity. Cadaverine however also plays a role on the index of the initial stage of fish decomposition [2].

Several methodologies, such as differential culture media, specific enzyme methods and liquid chromatography, have been developed for the detection of bacteria

producing BAs to study some of their formation pathways [3]. Along with the development of these techniques, the separation and quantification of BAs has been studied and reported. Their analysis in foods is usually performed by chromatographic techniques. However, the analytical determination of these amines is not simple due to the enormous complexity of the matrices to be analysed.

Objectives

The main objective of this work was to validate the experimental method and protocol to analyse the volatile organic compounds (VOCs) of biological origin. More precisely BAs present in fish. This protocol was previously developed in the project "3Qs for quality - Development of new devices and techniques for seafood quality assessment", PTDC/MAR-BIO/6044/2014, financed by Foundation for Science and Technology of Portugal.

For this purpose, the analytical technique of Ion Mobility Spectrometry coupled with Gas Chromatography (GC-IMS) was applied. This technique is a promising technology for the analysis of VOCs from biological matrices in very low concentrations by direct sampling and without the use of any chemicals. To collect samples, the direct acquisition cell developed in NMT, S.A. was tested and all important parameters were modified and optimized.

Chapter 2

Biogenic Amines

2.1 General outline of biogenic amines

Biogenic amines are nitrogenous organic bases characterized by the presence of at least one amino group in their molecular structure, which can be formed and degraded during the normal metabolism of microorganisms, animals and plants. Hence, are usually produced by the decarboxylation of amino acids, having two possible biochemical pathways. BAs can be either catalysed by exogenous enzymes produced by several different microorganisms under favourable conditions or by endogenous amino acid decarboxylases that naturally occur in animal or vegetable cells [4].

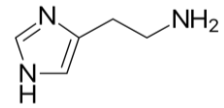
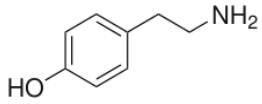
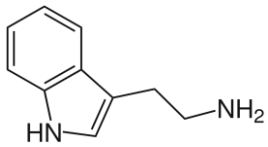
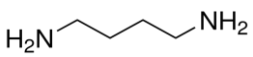
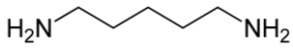
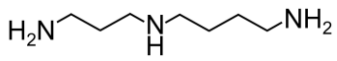
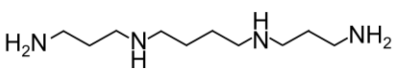
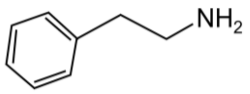
Being amines, they can be derived from ammonia, NH_3, in which the three hydrogen atoms can be replaced by one, two or three radicals, resulting, respectively, in primary, secondary or tertiary amines. The carboxylic group, characteristic of amino acids, is absent in amines so that they behave as cations when in physiological pH values [5]. BAs can be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenethylamine) or heterocyclic (histamine, tryptamine) [6].

These amines, naturally found in the human body, have various biological functions. For example, monoamines such as catecholamine (adrenaline and dopamine) and serotonin act as chemical neurotransmitters [7]. The heterocyclic amine histamine and tertiary amine trimethylamine also have biological functions in regulating the immune response [8]. The polyamines, cyclic or linear organic compounds having two or more primary amino groups (putrescine, spermidine and spermine), play an essential role in the growth, regeneration and metabolism of all the human body organs by being involved in the synthesis of DNA, RNA and proteins [9][10]. In fresh food products, such as vegetables, milk, fruits and fish, natural polyamines are the main amines present having a physiological role related with cell growth and proliferation [11].

The BAs histamine, tyramine, tryptamine, putrescine and cadaverine play an important role in metabolic activity and growth of living cells. They are mainly formed from the bacterial decarboxylation of precursor amino acids, respectively from the free amino acids histidine, tyrosine, tryptophan, ornithine and lysine [12]. Tyramine, putrescine and cadaverine can also be used as indicators of the degree of food spoilage and biological decomposition [6].

In food, the most biologically active amines and the relative precursors are histamine, tyramine, tryptamine, putrescine, cadaverine and phenethylamine, and are known as exogenous BAs (Table 2.1 and Figure 2.1). Natural polyamines spermidine and spermine represent the physiologically natural endogenous amines, being associated not with the microbial decarboxylase activity as expected but with the incorporation of aminopropyl groups into their precursor putrescine. Both exogenous BAs and polyamines are present in several types of foods with a variable range of concentrations [11].

Table 2.1: Most important exogenous amines and natural polyamines, and some characteristics.

BA/Natural polyamine	Precursor	Chemical structure	Molecular Formula	Molecular Weight/g mol^{-1}
Histamine	Histidine		$C_5H_9N_3$	111.148
Tyramine	Tyrosine		$C_8H_{11}NO$	137.182
Tryptamine	Tryptophan		$C_{10}H_{12}N_2$	160.216
Putrescine	Ornithine		$C_4H_{12}N_2$	88.154
Cadaverine	Lysine		$C_5H_{14}N_2$	102.181
Spermidine	Putrescine		$C_7H_{19}N_3$	145.250
Spermine	Putrescine		$C_{10}H_{26}N_4$	202.346
Phenethylamine	Phenylalanine		$C_8H_{11}N$	121.180

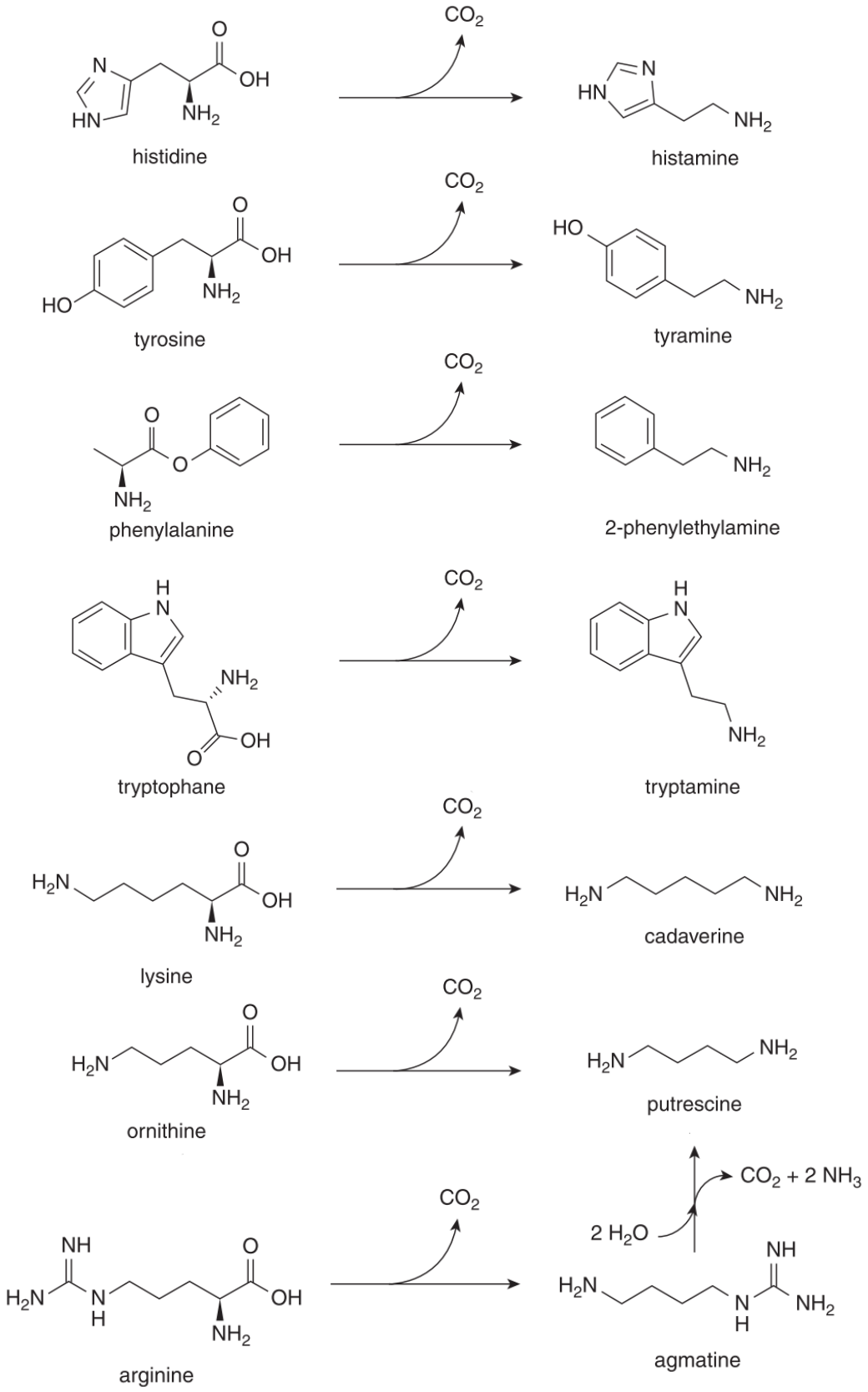


Figure 2.1: Most important biogenic amines in foods and their relative precursors [19]**:** Putrescine can be either obtained from the direct decarboxylation of ornithine or through the agmatine deiminase pathway, which is the result of arginine decarboxylation.

2.2 Biogenic amines production by microorganisms

Biogenic amines can be found in a variety of foods, beverages and fermented foods such as fish and fish products, meat and meat products, eggs, cheeses, fermented vegetables, fruits, beers and wines, and are mainly formed by microbial decarboxylation of amino acids and transamination of aldehyde and ketones [6].

Amines and related compounds are constantly present in food either naturally or as a product of storage or processing. Their concentrations generally increase during spoilage, which can be associated with increases in microbial concentrations and therefore deterioration of sensorial quality. However, BAs can also be produced by the decarboxylase activity of certain microorganisms, which will influence the type and amount of BAs formed in each type of food [1]. Amino acid decarboxylases have been found in species of the genera *Bacillus*, *Pseudomonas*, *Photobacterium*, as well as in genera of the family Enterobacteriaceae, such as *Citrobacter, Klebsiella, Escherichia, Proteus, Salmonella* and *Shigell,* and Micrococcaceae, such as *Staphylococcus*, *Micrococcus* and *Kocuria*. Lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Enterococcus*, *Carnobacterium, Pediococcus*, *Lactococcus* and *Leuconostoc* are also able to decarboxylate amino acids, being producers of tyramine, phenethylamine, cadaverine and putrescine [13]. Tyramine is also associated with Gram-positive microorganisms where species belonging to the genus *Enterococcus* are recognized as the most efficient tyramine producers [11].

The production of histamine, being one of the most biological active amines and thus with the major impact in food's spoilage, is of highest interest. Histamine formation results generally from microbial growth and proliferation of histamine-producing bacteria, being the family Enterobacteriaceae reported as the most significant. Other microorganisms such as *Clostridium perfringens*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Acinetobacter lowffi*, *Plesiomonas shigelloides*, *Pseudomonas* spp., *Aeromonas* spp., *Photobacterium* spp., *Stenotrophomonas maltophilia*, *Morganella morganii*, *Klebsiella pneumoniae*, *Raoultella planticola* and *Hafnia alvei* also play an active role as histamine producers [14][15][16].

In food products obtained by fermentation processes, such as wine and cheese, the presence of BAs may be produced in the own food matrix, resulted of the activity of microorganisms involved in the processes or, alternatively, be produced as a result of the activity of opportunistic microorganisms able to grow and proliferate under the conditions where the maturation process occurs [17].

2.3 Factors influencing the biogenic amines formation in foods

Several intrinsic and extrinsic factors have a significant role in the formation of BAs. In food products, the content of BAs depends strongly on the nature of the product, the availability of free amino acids, the environmental conditions and the microorganisms present.

Being BAs mainly formed by microbial decarboxylation of amino acids and the corresponding amino acids precursors, essentially three conditions are necessary: favourable conditions for bacterial growth, decarboxylase synthesis and decarboxylase activity; presence of decarboxylase-positive microorganisms; and availability of free amino acid, not necessarily leading to amine formation [18].

Free amino acids can either occur in foods or be formed through proteolysis, a process characterized by the breakdown of proteins by proteolytic enzymes, if partial into peptides, and complete, into amino acids. Hence, microorganisms with high proteolytic enzyme activity increase the risk of BAs production in foods, by increasing the availability of free amino acids [18].

BAs content and accumulation in food may vary depending on various factors such as temperature, pH, salt (NaCl) concentration, oxygen supply and redox potential, water activity (a_w), storage time, preservatives, and preservation processes. However, the first four have the major impact affecting microbial activities in foods by influencing the metabolisms of microbial cells and the activity of decarboxylase enzymes [11].

Temperature

Each decarboxylating microorganism and microbial species have an ideal temperature that leads to optimum growth values, and hence increasing cell metabolism and proliferation. Consequently, also the production and accumulation of BAs is verified. However, the optimum conditions for cell growth and proliferation do not necessarily match the conditions for BAs production [19].

A temperature between 20°C and 37°C is ideal for the growth of most decarboxylating microorganism and decreased temperature stops their growth [18]. For example, a strain characteristic of a tyramine producer of *Enterococcus faecalis* was inoculated in model system to determinate the activity of decarboxylase enzymes and the tyramine amount. After 24h of incubation, while the maximum tyramine amount was found at

20°C, the optimum activity of the decarboxylating enzyme was detected at 37°C. Such facts indicate that under the severe cells conditions the tyrosine decarboxylase is more active and thus requires additional energy to induce tyrosine production under stress conditions [20]. Other example involves a study conducted on histidine decarboxylase of a *Streptococcus thermophilus* which reached its maximum activity at 40°C, while the BA accumulation was insignificant at 20°C and at 25°C within the incubation period applied [21].

Regarding raw material such as milk and cheese, thermal treatments can be applied to help eliminate the wild decarboxylating microbiota. While LAB are more resilient and require additional thermal treatments, the Gram-negative BA producers (enterobacteria and pseudomonads) are quickly inactivated by temperatures higher than 60°C. Hence, typically cheeses made from pasteurized milk are characterized by presenting a lower BA amount [21].

pH

The decarboxylase activity is usually induced by the presence of precursor amino acids and at an acidic (low) pH, being the optimum pH between 4.0 and 5.5 [18]. Therefore, the higher the content of precursors in foods, the more susceptible they are to BA accumulation, and a decrease of pH leads to an increasing decarboxylase activity of bacteria. However, although an acidic pH may contribute to the bacterial decarboxylase activity, an acidification in food fermentation can prevent contaminant bacteria and consequently the formation of biogenic amines [11][22].

For example, in two tyrosine decarboxylases from strains of *Enterococcus faecalis* and *Enterococcus faecium* the optimum pH value for the decarboxylase activity was 5.5 and 6, respectively [23]. Other study reports that the histidine decarboxylase of a strain of *Streptococcus thermophilus* reached its optimum pH at 4.5 [24].

On fruits and vegetables, microbial contaminations result in additional increases in the amount of BAs. In most fruits, the low pH restricts the microbial association to acid-tolerant microorganisms, such as LAB and fungi [4].

Salt concentration

In foods, increasing salt concentrations contribute significantly to the reduction of the metabolic activities of decarboxylating microorganisms and therefore decrease the production and accumulation of BAs. However, the amount of NaCl used for a better control of BAs production is often too high [21][24].

The tyramine production was studied in *Enterococcus faecium* and *Enterococcus faecalis* strains in buffered systems containing tyrosine. While the enzymatic activity of *Enterococcus faecium* strain remained constant independently of salt concentration, in *Enterococcus faecalis* the same activity did not change significantly until an increase of NaCl concentration up to 15% [20].

In fermented sausages inoculated with an *Enterococcus faecalis* strain, even in the presence of low salt amounts, the production of cadaverine and putrescine by enterobacteria was limited, resulting in a reduced accumulation of BAs. Also, an increase in salt concentrations reduced the concentration of tyramine and phenethylamine produced by enterococci [25][26].

Several reports stablished that the presence of NaCl strongly inhibits the amine-forming microorganisms, however studies on specific decarboxylase activities report opposing effects: while NaCl inhibits histidine decarboxylase, tyrosine decarboxylase activity is activated under salt concentrations [6][27].

Oxygen supply and redox potential

The presence of oxygen plays a significant role on BAs biosynthesis. Under anaerobic conditions, *Enterobacter cloacae* only produces about half the quantity of putrescine obtained under aerobic conditions and *Klebsiella pneumoniae* reduces significantly the amount of cadaverine produced obtaining however the ability to synthesize putrescine [4].

The redox potential also appears to affect BAs production by some microorganisms [27]. Studies reported that histamine formation is also affected by the oxygen available, where reducing the redox potential of the medium contributes to the production of histamine. Histidine decarboxylase activity of *Proteus morganii* appears to be inactivated or destroyed in the presence of oxygen and is inhibited in atmospheres of 80% CO_2 [28][29].

Usually, the production of BAs in food can be controlled by a strict use of good hygiene in both handling and processing of raw materials with the inhibition of spoiling microorganisms [6]. Specifically, fish decomposition can be avoided by correct handling practices such as icing or rapid immersion in water chilled to -1°C followed by nonstop frozen storage [30].

2.4 Toxicology of biogenic amines

The consumption of food containing high amounts of some BAs can have toxicological effects leading to several health issues due to toxicity, making it critical to monitor these compounds in foods [31].

Amino oxidases are the enzyme responsible for BAs detoxification, inducible in the presence of mono or diamines. The enzymes monoamine oxidase (MAO) and diamine oxidase (DAO) play a significant role in the detoxification process [6].

The mammals own an efficient detoxification system in the intestinal tract capable to metabolize the normal daily ingestion of BAs. In humans, under normal conditions, exogenous amines, absorbed from food and produced by the intestinal bacteria, are quickly converted in degradation products physiologically inactive by the action or conjugation of amine oxidases. However, in allergic individuals, in the presence of amino oxidases (MAO and DAO) inhibitors or upon intake of high amounts of BAs in food, the detoxification process is interrupted, and biogenic amines accumulate in the organism, entering the bloodstream and hence, causing the body intoxication [6][32].

The toxicological levels of BAs are not easily established as they depend on several factors such as individual allergy, intake with food, presence of other amines and consumption of MAO inhibiting drugs and alcohol [6][21].

Based on histamine amounts found in food products involved in histamine poisoning, levels above 500-1000 mg/kg of food are considered potentially hazardous to human health. However, some studies have suggested for histamine a legal upper limit of 100mg/kg of food and 2 mg/l of alcoholic beverage. Tyramine values of 100-800 mg/kg and phenethylamine values of 30 mg/kg have also been reported as toxic for the ingestion of a normal portion of food (≈200 g) [4][33].

In general, levels above 1000 mg/kg (amine/food) and intakes greater than 40 mg of BAs per meal are considered potentially dangerous for human health. However, it is

important to be aware that not all amines are equally toxic, being histamine, tyramine and phenethylamine the BAs with higher levels of toxicity [6][32].

2.4.1 Histamine Toxicity

The most serious foodborne intoxications caused by BAs are usually related with high concentrations of histamine. Several outbreaks of histamine poisoning have occurred after ingesting different food products characterized by containing high levels of histamine such as fish and fish products, dairy products, meat and meat products, and alcoholic beverages such as wine and beer [32].

The main cause of the occurrence of histamine intoxication after food intake seems to lie in the inhibition of the enzymes responsible for histamine inactivation at the digestive tract, specifically DAO [17]. The gastro-intestinal tract is occupied of enzymes capable of metabolizing the resulting histamine from food intake, preventing its absorption in its intact form and consequently blocking its entry into the circulatory chain. Histamine is formed by decarboxylation of L-histidine, catalysed by histidine decarboxylase (HDC), and is primarily metabolized by two major enzymes (Figure 2.1): the above-mentioned DAO which converts the compound by deamination into imidazole acetaldehyde and histamine N-methyltransferase (HMT), which consequently converts histamine to methylhistamine, a compound that is subsequently metabolized by MAO with the formation of 1,4-methylimidazolacetic acid [34][35][36][37].

Occasionally the detoxification process is inhibited, due to the presence of inhibitors of histamine-metabolizing enzymes, also known as potentiators, that allow unmetabolized histamine to be absorbed into the intestine. These inhibitors have been identified as putrescine and cadaverine, or as pharmacologic agents, such as isoniazid. However, the BAs tyramine, tryptamine, and phenethylamine can also act as potentiators. While tryptamine is DAO inhibitor, tyramine can inhibit MAO. Phenethylamine inhibits DAO and HMT. The occurrence of such inhibitors might explain why food products such as spoiled fish or aged cheese are more toxic than histamine itself in aqueous solution [38].

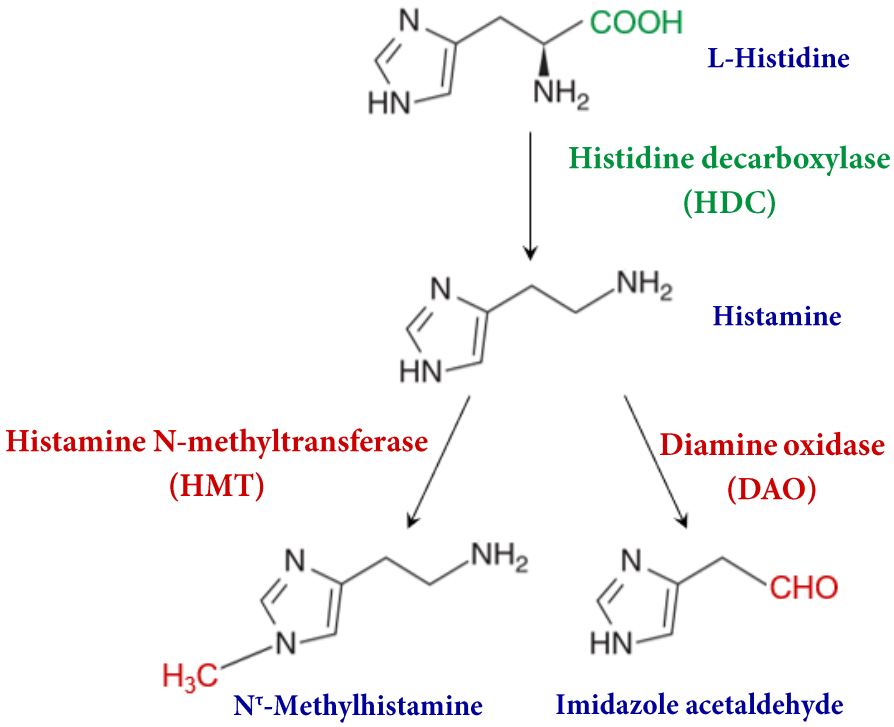


Figure 2.2: Formation and inactivation of histamine. Histamine is formed by decarboxylation of L-Histidine catalysed by HDC and can be inactivated by two major routes: methylation of the imidazole ring, catalysed by HMT, or oxidative deamination of the primary amino group, catalysed by DAO. Adapted from [34].

Histamine is considered the most toxic amine in food, exerting its toxicity through three types of receptors (H_1, H_2 and H_3) present in cellular membranes. This amine acts primarily on the cardiovascular system. Through the dilatation of peripheral blood vessels, high levels of histamine can cause hypotension, urticaria, flushing, and headache. Symptoms of histamine poisoning are in some cases similar to allergy symptoms including difficult breathing, vomiting, rash, itching, fever and hypertension [39][40]. In general, histamine intoxication has a relatively short incubation period, minutes to few hours after ingestion [41].

2.4.2 Tyramine Toxicity

The amines tyramine, tryptamine and phenethylamine are essentially vasoactive amines, causing peripheral vasoconstriction and an increase in the cardiac output, resulting in increased blood pressure [32].

Tyramine is physiologically metabolized by MAO and derived from the amino acid tyrosine. Although not as toxic as histamine, tyramine can have toxicological

implications when reacting with monoamine oxidase inhibitor (MAOI) leading to hypertensive crisis, also known as tyramine pressor response. The consumption of foods with high histamine content, such as fish and fish products, also contribute to this response. Although fresh fish contains low or no concentration of tyramine, high levels can be found in spoiled or fermented fish. Hypertensive crisis is characterized by an increased systolic blood pressure of 30 mmHg or more. It is believed that the cause for vasoconstriction and increased heart rate and blood pressure is the displacement of norepinephrine from neuronal storage vesicles due to the acute tyramine ingestion [42]. Norepinephrine, also called noradrenaline, is a catecholamine that functions predominantly from the ends of sympathetic nerve fibers and acts to increase the rate and force of contraction of the heart, resulting in increased systemic blood pressure and coronary artery blood flow [43].

For adults, dietary tyramine levels of 100–800 mg/kg have been suggested as satisfactory, while higher than 1080 mg/kg is considered toxic [12]. Ingestions of 60 mg/kg of tyramine can cause migraine headache for patients receiving MAOI drugs, while 100–250 mg/kg will originate a hypertensive crisis [6].

2.4.3 Nitrosamines

Although much less toxic than histamine and tyramine, some BAs such as putrescine, cadaverine, spermine and spermidine, can react with nitrite to form nitrosamines and produce carcinogenic compounds [4]. Nitrosamines usually result from the interaction of secondary and tertiary amines with nitrite and nitrogen oxides during the storage and conservation of foods [44].

Studies have reported that secondary amines such as dimethylamine (DMA) and tertiary amines such as trimethylamine (TMA) appear to be implicated in nitrosamine formation in fish and fish products [45]. It has been widely reported the production of nitrosodimethylamine (NDMA) due to reactions of DMA with nitrite in products such as salted, smoked, fermented, and canned fish [46][47].

NDMA was first studied in the 1930s as an industrial solvent due to its miscibility in both hexane and water, and anticorrosion properties. Shortly after the exposure, the two research chemists who were investigating the compound properties were poisoned, presenting symptoms of liver failure. Nearly 7 weeks after exposure, the most acutely poisoned individual died of liver necrosis. In the late 1950s, an incident in Norway

involving foods contaminated with nitrosamines was reported. Domestic animals fed with fish meals preserved with nitrite were suffering from severe liver disorders. NDMA was later stated as the cause and it was suggested a possible reaction of the nitrite with DMA and TMA present in fish which resulted in NDMA production [44].

Concentrations of nitrosamines have been reported in several types of food. Amounts of nitrosamines of DMA, diethylamine, pyrrolidine and piperidine were found in fried foods. Under heating conditions, the BAs putrescine and cadaverine are converted to pyrrolidine and piperidine, respectively, from which N-nitroso-pyrrolidine and N-nitrosopiperidine are produced. Hence, several food processes, such as salting and smoking, appear to induce nitrosamines formation, while cooking (frying) reinforces their formation [32].

Secondary amines such as agmatine and the polyamines spermine and spermidine contribute to the formation of carcinogenic nitrosamines, which occur widely in fish, meat and vegetable products.

2.5 Biogenic amines in fish and fishery products

The predominant factors in the production of BAs, after purchasing the fish, are the duration and temperature of storage. The temperature affects not only the formation of free amino acids *post mortem*, but also their fast decarboxylation. Thus, long storage periods at high temperatures induce the production of BAs, ammonia and other resulting compounds of fish degradation [10]. Other factors such as microflora and muscle type also influence the presence of BAs in fish [48].

During decomposition of fishery products, especially during storage at elevated temperatures (48°C), various amounts of selected BAs are produced, depending on the fish species. Histamine, tyramine, putrescine and cadaverine are the most common BAs found in seafood associated with spoilage, in which histamine and tyramine are the most biologically active [39].

Most bacterial food pathogens from fishery products are aerobic and gram-negative bacteria and belong to the genera *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella*, *Aeromonadaceae* (*Aeromonas* spp.), *Flavobacterium* and the Vibrionaceae families (*Vibrio* and *Photobacterium*). The aforementioned microorganisms are the most common in fish, part of the typical fish flora, and are abundantly present in aquatic environments [2].

Upon death, the defence mechanisms of fish are no longer preventing bacterial growth in the muscle tissue, and histamine forming bacteria may start to grow, resulting in the production of histidine and a subsequent increase of histamine levels above the allowable maximum level (set to 50 ppm by Food and Drug Administration) [2][49]. Therefore, during spoilage, certain bacteria produce decarboxylase enzymes, which act on free histidine in the fish muscle to form histamine. For instance, although *Shewanella putrefaciens* and *Pseudomonas aeruginosa* are the prominent spoilage bacteria of fresh fish, the mesophilic Vibrionaceae dominates at ambient temperature (25°C) and fresh water. However, in polluted waters, the dominant spoilage bacteria family is the Enterobacteriaceae [50].

Although many BAs have been found in fish produced *post-mortem*, such as histamine, putrescine, cadaverine, spermidine and spermine, only the trio, histamine, cadaverine and putrescine, has been acknowledged as significant concerns regarding fish safety and quality, being histamine used as marker for the degradation of fish (Table 2.2). Trimethylamine, also identified during fish spoilage, is one of the main volatile amines produced by spoilage bacteria and is responsible by the characteristic fishy odour [49][51].

Table 2.2: Levels (mean value, mg/kg) of BAs in raw sardine and light cured red horse mackerel. Adapted from [2].

Fish	Temperature/ time	Histamine	Cadaverine	Putrescine	Spermidine	Spermine	Tyramine
Sardine	4°C/0 days	19.5	3.9	13.4	1.2	0.0	0.0
	4°C/15 days	203.0	100.4	114.0	7.6	2.9	16.3
Light cured horse mackerel	-	21.3	244.4	64.5	0.2	0.0	62.8

Usually, newly caught fish have low BAs levels. Studies have reported concentrations of cadaverine between 0.116-1.036mg per 100g in high-quality rockfish, salmon steaks and shrimp, and concentrations of putrescine ranging from 0.136 to 0.63mg per 100g in high-quality lobster tail, salmon and shrimp. Also, high-quality tuna reported cadaverine and putrescine concentrations between 0.024-0.532 and 0-0.184 mg per 100 g, respectively [48].

Although some studies do not observe obvious differences in BAs levels in muscle from different parts of the fish, some have reported higher levels of spermidine in dark muscle and high levels of amines in the intestinal walls [52].

The levels of histidine and histamine spoilage were studied in 21 aquatic species. The conclusions reported that there was a higher histamine production in the red-muscled species, such as tuna and mackerel, than in white-muscled species, such as rockfish. Also, higher concentrations of histidine were found in white when comparing with red muscle [48].

2.5.1 Histamine Fish Poisoning

Histamine fish poisoning (HFP), also known as scombroid fish poisoning, was first described in 1799 as a chemical intoxication caused by the consumption of bacterially contaminated or spoiled fish [53]. This intoxication is usually associated with large amounts of histamine in spoiled fish, levels equal to or greater than 50 mg/100g of fish, and possibly other BAs [39].

The term scombroid is derived from the Scomberesocidae and Scombridae families, which includes several fish species such as bonito, mackerel and tuna. Although histamine formation is mainly associated with the Scombridae family, certain types of non-scombroid fish are also implicated in fish poisoning, such as anchovies, herring, sardines and salmon. These fish species are characterized by a high level of free histidine in muscle tissues [54][55]. Table 2.5.1.1 lists fish species that have been associated with HFP or elevated levels of free histidine as well as the respective mean annual global production and levels of free histidine.

Although histamine is the main toxin involved in HFP, exogenous histamine from spoiled fish can be potentiated by the presence of other BAs such as cadaverine, putrescine and tyramine. In scombroid species and other marine fish containing abundant endogenous histidine, histamine is manly formed by microbial action and not by endogenous histidine decarboxylase activity as expected [56]. The histidine can be catabolized by two metabolic pathways in the fish muscle: degradation via the urocanic acid pathway or through decarboxylation to form histamine. The first is the main pathway under normal physiological conditions, but in *post mortem* conditions and in the case of bacterial contamination, decarboxylation may become the most significant path [6][57].

Table 2.3: Scientific names, free histidine levels and mean annual production levels for some fish associated with HFP or high free histidine levels. Adapted from [42].

Market name	Scientific name		Histidine levels (mg/kg)	Mean annual production (tonne, 2006–2010)
	Family	Genus and species		
Anchovy	Engraulidae			
European		*Engraulis encrasicholus*	6 210	534 483
South African		*Engraulis capensis*		209 250
Japanese		*Engraulis japonicus*	4 810	1 287 215
		Stolephorus spp.		279 139
Bonito	Scombridae			
Dogtooth Tuna		*Gymnosarda unicolor*		669
Plain		*Orcynopsis unicolor*		759
		Sarda spp.		62 215
Herring	Clupeidae			
Herring, Atlantic		*Clupea harengus*	1 230-2 950	2 356 990
Herring, Araucanian		*Clupea bentincki*		624 528
Herring, Pacific		*Clupea pallasii pallasii*		306 839
Mackerel	Scombridae			
Mackerel, Atlantic		*Scomber scombrus*	2 000-4 500	664 231
Mackerel, Chub		*Scomber japonicus*	1 063-8 020	1 767 202
Mackerel, Blue		*Scomber australasicus*	2600	10 364
Mackerel, Horse		*Trachurus japonicus*	172-3 680	278 404
Pilchard or Sardine	Clupeidae			
Sardine, European		*Sardina pilchardus*	2 888	1 101 842
Pilchard, Japanese or South American or Californian		*Sardinops sagax*	1 227-7 626	837 504

Market name	Scientific name		Histidine levels (mg/kg)	Mean annual production (tonne, 2006–2010)
	Family	Genus and species		
Salmon	Salmonidae			
Atlantic Salmon		*Salmo salar*	130–300	
Coho Salmon		*Oncorhynchus kisutch*	219–970	
Cherry Salmon		*Oncorhynchus masou*	387–2 362	
Pink Salmon		*Oncorhynchus gorbusvha*	408–1 557	
Tuna (Small)	Scombridae			
Bonito		*Auxis thazard*	4 330–10 100	
Skipjack		*Katsuwonus pelamis*	13 400–20 000	2 529 408
Longtail Tuna		*Thunnus tonggol*	11 540	239 661
Tuna (Large)	Scombridae			
Yellowfin		*Thunnus albacares*	2 123–12 200	1 113 954
Southern Bluefin		*Thunnus maccoyi*	6 670	
Big-eye Tuna		*Thunnus obesus*	7 450	412 616
Pacific Bluefin Tuna		*Thunnus orientalis*	6 850–7 110	

The increase in histamine production is also related to poor conservation conditions after fish capture, particularly when the fish is not conserved at low temperature, allowing the bacterial metabolism of histidine into histamine [52][58].

Some of the bacteria associated with histamine production are halotolerant (salt tolerant) or halophilic (salt loving). Other bacteria are more capable of forming histamine at an elevated acidity (low pH). As a result, histamine formation is possible through processes such as brining, salting, smoking, drying and fermenting. Refrigeration may contribute to the inhibition of histamine production during these processes [59].

Scombroid poisoning is a foodborne poisoning with symptoms and treatment similar to allergies associated with seafood, being antihistamine treatment the optimal method of therapy for HFP. The clinical manifestations of HFP arise quickly, from 5

minutes to 2 hours after the ingestion of the contaminated fish with scombroid toxins. Although symptoms may persist from 8 hours to several days, long-term sequelae are not known (Table 2.4). Thus, HFP is not considered to be fatal [42].

Although there are similarities regarding some symptoms caused by HFP and histamine intolerance, there are differences. Unlike histamine intolerance, in HFP other toxic decomposition products or components unique to fish may be involved in addition to histamine [55]. Furthermore, HFP may occur in individuals with a normal histamine degradation capacity and not only in histamine intolerant individuals [42].

Table 2.4: Common symptoms of scombroid fish poisoning [42]**.**

Type	Symptoms
Cardiovascular	Flushing, rash (urticaria), hypotension, headache, tachycardia
Gastrointestinal	Abdominal cramps, diarrhoea, vomiting
Neurological	Pain, itching
Other	Oral burning sensation, peppery taste, nausea, swelling of tongue

According to data from the period of 1998 to 2002 from the United States Centers for Disease Control and Prevention (CDC), 463 cases and no deaths were reported [60]. According to data from 1998 to 2008 from the Japanese Ministry of Health, Labour and Welfare, there were 1577 cases reported, 89 incidents and no deaths [42].

2.5.2 Histamine Regulatory Guidelines for Fish

Based on HFP cases reports, certain guidelines needed to be established regarding the acceptable histamine content of fish. Thus, essentially four thresholds have been suggested: <50 mg/kg for normal and safe consumption; 50–200 mg/kg, mishandled and possibly toxic, 200–1000 mg/kg, unsatisfactory and probably toxic; and >1000 mg/kg toxic and unsafe for human consumption [54].

According to a Compliance Policy Guide of Food and Drug Administration (FDA), quality fish should have a histamine content lower than 10 mg/kg; between 20-50 mg/kg indicates significant deterioration, and equal to or greater than 50 mg/kg is a conclusive evidence of decomposition [61]. In the United States, according to FDA, fish is considered spoiled as soon as histamine levels reach the limit of 500 mg/kg [59].

Some BAs present in food such as putrescine and cadaverine inhibit histamine-detoxifying enzyme. Hence, there is a discrepancy in the toxic level of histamine in food due to the absence or presence of these. The European Community has proposed that the average content of histamine should not surpass 10-20 mg/100g of fish [6]. For regulation purposes, in fish and fish products, a limit of 300 mg/kg was stablished for the sum of histamine, putrescine and cadaverine [32].

In the European Union, under Commission Regulation (EC) No 1441/2007 of 5 December 2007, was stablished that the average histamine content should be between 100-200 mg/kg for 9 samples per batch, and only two samples can present values within this range. These limits apply to fish species associated with a high histidine content, such as the families Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae and Scombresosidae [62].

Regarding fishery products which have undergone enzyme maturation treatment in brine, manufactured from the same fish species above listed, the same regulation established a range between 200-400 mg/kg for nine samples, where only two samples can present values within this range [62].

The same regulation states that the results will be satisfactory if the mean of the samples is lower than the minimum of the established limit interval. Lastly, none of the samples can exceed the maximum allowed value in histamine content. The results obtained will be unsatisfactory if the mean value exceeds the maximum limit; if more than the previous stablished samples have histamine levels within the permitted range of values; or if one or more samples have histamine levels above the maximum limit [62].

Chapter 3

Determination of Biogenic Amines

In the past few decades, the identification of BAs has been and remains one of the greatest challenges in food analysis. The development of analytical techniques for examining and understanding the degradation of BAs is extremely important due to their potential toxicity and their possible use as food quality markers.

3.1 Quantitative analysis for biogenic amines

Several fluorometric techniques have been developed to analyse and measure histamine and with their evolution new procedures were studied and tested. However the detection and quantitative analysis of bioactive amines is not simple for several reasons: mainly because of their greater solubility in water than in the often-used organic solvents (as a result of the strong polar character of the compounds); the simultaneous presence of structurally different amines in the same extract; the extreme complexity of the matrix sample; the low concentrations of these compounds in the samples; the potential presence of interfering compounds, such as various amino acids with structural similarities to amines; the absence of intrinsic properties of the compounds that enable their detection directly by physicochemical methods of current application (spectrophotometric, fluorometric or electrochemical methods) [63][64].

To overcome these issues many methods have been developed based on amine extraction and derivatization followed by separation and quantification. Recently, extraction and derivatization processes are performed simultaneously, however, these processes are undesirable due to possible errors and losses in the analyte concentration. The next step involves the quantitative analysis of BAs which is often performed by chromatographic methods: thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE) and high-performance liquid chromatography

(HPLC). Due to the high number and variety of methods there is a need for methods better suited to high-speed screening [64].

3.1.1 High Performance Liquid Chromatography

Among these methods, HPLC is by far the mostly frequently reported technique for BAs separation and quantification. Over the years several different HPLC methods have been studied and proposed. After the amines extraction a derivatization is required which can be performed before or after the column separation, being the principal derivatives used dansylchloride and o-phthaldialdehyde (OPA). Afterwards the detection is done by fluorimetry. However not all HPLC methods require derivatization, using instead an ionic chromatography followed by an electrochemical detection or a diode array detector [65].

Due to low volatility and lack of chromophores, the HPLC method with Reversed-Phase separation makes use of detection schemes based on pre-column or post-column derivatization to produce strong chromophores [2][63].

Compared to GC and CE, HPLC offers disadvantages on the lack of efficiency due to low diffusion coefficients in liquid phase, causing slower diffusions of analytes in the stationary phase [66].

3.1.2 Capillary Electrophoresis

CE is the second most commonly performed technique applied to the determination of BAs in food and, despite of its low sensitivity, has numerous advantages. Among them, it's a rapid and reliable technique, being able to screen a large number of samples in a short period of time [18]. CE is a technique based on free zone electrophoresis in buffer-filled capillaries. The detection is then performed by ultraviolet when the amines are directly separated or fluorescence detectors when the amines are derivatized (using OPA) [65]. In conjunction with pulsed amperometric detection, CE does not need any derivatization procedure which makes it an alternative to reverse phase columns methods. Compared with HPLC, CE can obtain a superior response in terms of limit detection using small sample volumes [67].

3.1.3 Gas Chromatography and Thin Layer Chromatography

Amines are difficult to analyse by GC due to their lack of volatility and their interaction with the GC column which consequently, leads to significant tailing and poor reproducibility. However, GC methods can be cheaper and simpler to apply than HPLC equipment in some cases [49]. It is a reproducible and fast method where one extract analysis requires only 10 minutes without derivatization [65]. Although TLC is relatively simple and requires no special equipment, published methods take an excessive time for analysis and/or inaccuracy of the obtained results (semi-quantitative) [63].

3.1.4 Ultra Performance Liquid Chromatography

Since 2004, a new generation of stationary phases has emerged, capable of withstanding pressures (over 1000 bar), which when integrated into LC systems, the system is called Ultra Performance Liquid Chromatography (UPLC). UPLC systems, when compared to conventional HPLC equipment, are considered advantageous because of their ability for faster analysis with better efficiency, reduced solvents consumption and a better peaks resolution, without detriment to efficiency [68][69].

Along with chromatographic methods, also biochemical assays and immunoassays are used in determination of BAs and have the advantage of being cheap, fast, simple to perform and can be used outside the laboratory. However, semi-quantitative results might be obtained and are often associated with a lack of specificity [10].

Despite the wide range of analytical methodologies already published and applied, new methodologies are continuously being developed with the goal of achieving even higher levels in the double stranded quality of results versus ease and speed of execution.

3.2 Semi-quantitative and quantitative analysis for histamine

In the past decade new methods have been developed for the analysis and quantification of histamine. The first methods of evaluation of histamine were biological methods which measured parameters included the amount of contraction of a histamine

sensitive organ [65]. However, the analysis of this amine suffered an evolution in the applied methods for simpler and more convenient techniques such as colorimetry, TLC, enzymatic and immunoenzymatic methods, and flow injection analysis (FIA).

3.2.1 Colorimetry

To identify quickly and at low cost the presence of histamine in food, several methods of colorimetry have been developed and applied. However, until 2004 the colorimetric assays then reported involved numerous steps such as extraction processes and the use of chromatographic purification of histamine and further coupling with diazonium salts [65]. The new colorimetric assay proposed has a simpler extraction procedure involving an interaction with the reaction of imidazole, a heterocyclic organic compound, which produces a quantitative colour reagent. From there, it is possible to establish a reference colour scale (Figure 3.1) for a rapid estimation of histamine in food, particularly on detection of histamine toxicity levels in fish [70]. This method has a quantitation limit of 10 mg/kg [65].

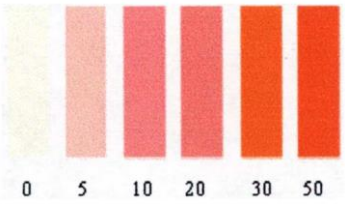


Figure 3.1: Reference colour scale for histamine (concentrations in µg/ml) [64].

3.2.2 Thin Layer Chromatography methods

TLC is considered a rapid method, with a migration time of only 2 hours allowing the analysis up to 8 samples per plate at a time, where two plates can be simultaneously placed in a migration container. This method does not require expensive material, being only needed a basic equipment for extraction, chromatographic plates, migration container and a densitometer for a quantitative determination [65].

Histamine extraction can be made by trichloroacetic acid, methanol or an aqueous fraction of the press juice from canned fish [65][71]. The next step involves migration and separation performed at a suitable stationary phase, with certain solvents. These can either be methanol with ammonia or chloroform, methanol and ammonia, used in the proportions (20:1) and (2:2:1), respectively [72]. Afterwards, to reveal the histamine,

certain reagents can be used such as ninhydrin, fluorescamine and o-diacetylbenzene [73]. Through comparison of the colour intensity of the revealed histamine in the samples with histamine standards run at the same time on the chromatographic plate, a semi-quantitative detection is obtained. Histamine detection can also be quantitative with the use of a densitometer [65]. The detection threshold using TLC is of 100 mg/kg [74].

3.2.3 Enzymatic methods

This technique is based on the deamination of histamine. In the presence of oxygen, DAO deaminates histamine to form imidazole acetaldehyde, with a simultaneous production of hydrogen peroxide (H_2O_2) and ammonia (NH_3). The released hydrogen peroxide is then used to convert a reduced dye to its oxidized form with a colour development associated, through the action of the enzyme horseradish peroxidase (HRP) [75][76]. This reaction is the oxidation of leuco crystal violet, a colourless compound, to crystal violet, a purple compound [77].

$$\text{Histamine} + O_2 \xrightarrow{\text{DAO}} H_2O_2 + NH_3 + \text{imidazole acetaldehyde} \quad (3.1)$$

$$H_2O_2 + \text{reduced dye} \xrightarrow{\text{HRP}} H_2O + \text{oxidized dye} \quad (3.2)$$

The colour intensity is proportional to the amount of histamine and can be evaluated visually (qualitative detection) or by spectrometry (quantitative detection) [65]. The detection limit is 0.5 mg/kg and quantification limit is 1.5 mg/kg [74].

Several enzyme biosensors have been developed to simplify and reduce the duration of the analytical measurements [78][79][80][81]. Also, a solid phase assay (test strip) was developed, based on the coupling of DAO to a peroxidase/dye system [76][82].

Although enzymatic assays are rapid methods with an incubation time ranging from 20 minutes to 2 hours, allowing the simultaneous analysis of several samples, the DAO enzyme can react with other BAs such as agmatine, putrescine and cadaverine when present at high levels [65]. Studies have also reported that these methods tend to overestimate histamine content at levels < 10 mg/kg [83].

Enzymatic methods can be used as histamine screening tests on large numbers of fish samples [77].

3.2.4 Immunoenzymatic methods

Several immunoassays using monoclonal antibodies have been developed for histamine assessment in biological fluids. However, the application of such tests in food products is not easy due to lack of specificity and inadequate sensitivity. Thus, an immunoassay using antibodies was developed for histamine determination in food. Anti-histamine monoclonal antibodies were produced and used to perform a competitive inhibition ELISA (Figure 3.2) [84].

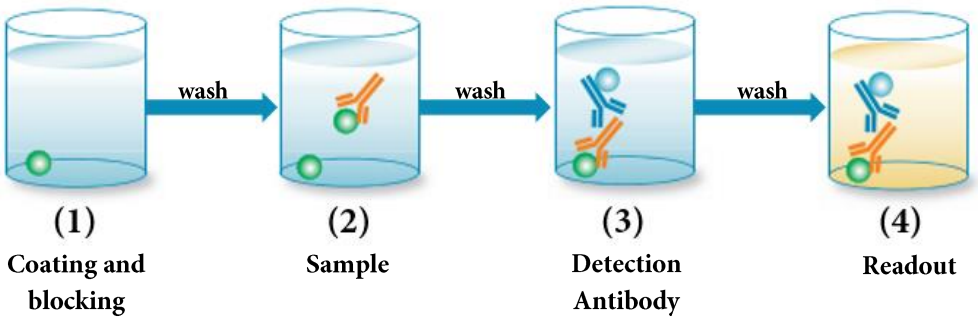


Figure 3.2: Summary of key steps in competitive ELISA. (1) Control antigen is absorbed onto well and a buffer containing an unrelated protein is used to block free sites in the wells; (2) Sample and detection antibody mix is added to wells; (3) Addition of enzyme conjugated with secondary detection antibody; (4) Substrate is catalysed by enzyme to generate coloured readout. The colour intensity is inversely proportional to the antigen amount in the sample. Adapted from [85].

More recently, numerous ELISA assays have been developed by companies which produce commercial kits for qualitative and/or quantitative analysis of histamine. Such analysis follows the successive steps: 1) Histamine extraction from the fish sample using an acidic solution or water followed by a filtration; 2) Reagents (which differ according to the kit) are added to form a histamine conjugate; 3) Competitive binding of histamine conjugate to the antibodies coated onto reaction tubes wells during the incubation time, followed by a washing; 4) Enzymatic reaction that causes a colour change, being the reaction duration dependent on the test used; 5) Quantitative results can be determined directly using a standard curve and qualitative results can be visually obtained by comparison with standard colour charts [65].

Immunoenzymatic methods can be performed in fish quality control laboratories as histamine screening test, being able to separate products food products according to the histamine level threshold of 50 mg/kg [86].

3.2.5 Flow Injection Analysis

FIA is an analytical technique based on the injection of a liquid sample into a moving, continuous carrier stream of a suitable liquid. The sample zone is then transported into a detector, responsible for recording the changes in absorbance, electrode potential, or other physical parameters resulted from the sample passage through the flow cell (Figure 3.3). A calibration curve is then produced to quantify the analyte in study [65][87].

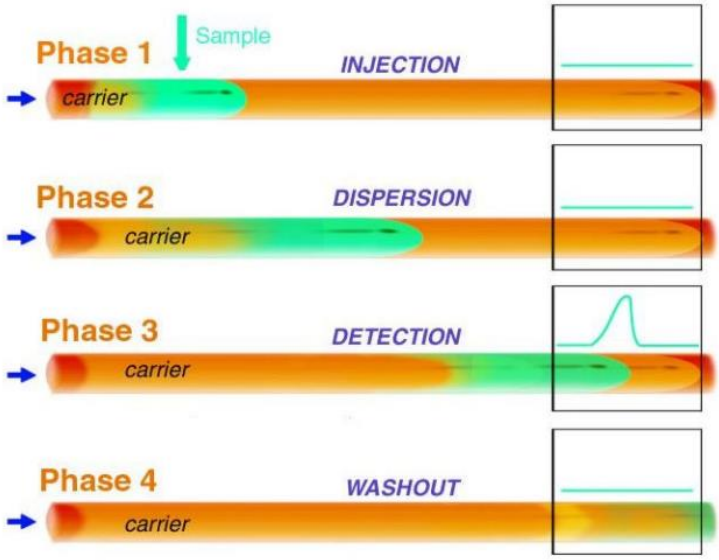


Figure 3.3: The four phases of FIA [87].

The most rapid method for selective determination of histamine is based on FIA and is capable of screening 60 sample extracts/hour [88].

FIA methods using a fluorometric detection were developed to provide a fast screening of fish samples for histamine determination [89]. Although no sample preparation is needed, FIA require a cautious selection of reagent concentrations and pumps control in order to assure specificity for the histamine derivative [90]. More recent FIA procedures have been developed for this screening, having a detection limit of 0.8 mg/kg and quantitation limit of 2.4 mg/kg [91].

Some reports have proposed FIA systems with amperometric biosensors based on enzyme reactors for freshness monitoring in fish samples [92][93][94].

Chapter 4

Ion Mobility Spectrometry

4.1 Working Principles

In the last decade new and innovative ion mobility spectrometry (IMS) applications have emerged and have been used in quality control of toxic contaminants, beverages and food products. Among these are assessment of food freshness or the degree of spoilage of food products and detection of pathogenic micro-organisms or toxins such as the determination of volatile organic compounds of fish spoilage.

Being used for detection, identification and monitoring of trace levels of chemical compounds in different matrices, IMS is an analytical technique of ion separation based on the differences of ion mobilities in a drift tube with a defined electric field. This separation is based on the specific drift times, that ionized compounds take to travel a fixed distance under an electric field (E) [95]. When reaching the equilibrium, the ions move with a constant drift velocity (v_d) proportional to the electric field and in the same direction. Due to the relation between these two components it is possible to extract an independent component for each ion, ion mobility K (Equation 4.1) [96].

$$K = \frac{v_d}{E} = \frac{L}{t_d E} = \frac{L^2}{t_d U} \tag{4.1}$$

Drift velocity is expressed in $\mathrm{cm^2 s^{-1}}$ and the electric field is in $\mathrm{V cm^{-1}}$, hence the ion mobility K is expressed in $\mathrm{cm^2 V^{-1} s^{-1}}$. L is the length of the drift tube, expressed in cm, t_d the drift time expressed in s and U the drift potential difference in V.

Ion mobility depends on the collision frequency, being susceptible to both pressure and temperature. Therefore, by normalizing to the standard pressure and temperature we get the reduced ion mobility K_0 (Equation 4.2).

$$K_0 = K\left(\frac{P}{P_0}\right)\left(\frac{T_0}{T}\right) \tag{4.2}$$

P and T represent the values of pressure and temperature during the experiment and in standard conditions $P_0 = 760$ Torr and $T_0 = 273.15$ K. Thus the reduced ion mobility K_0 is a substance's specific value, being independent of instrumental or environmental parameters [96].

The classic ion mobility spectrometer consists of a segmented drift tube, constructed of alternating insulating and conducting rings, along which an electric field is applied (Figure 4.1). As the ions travel through the tube, they interact with a drift gas, mainly air at atmospheric pressure or pure nitrogen. The slow collisions with the drift gas molecules are more frequent for sterically demanding structures. Therefore, the drift time of each substance is determined by its geometric structure and ion mass in the drift. Thus, ions are separated not only according to their mass but also due to their size, spatial structure and internal charge distribution. The resulting ion current is measured by an electrometer as a function of time [95][97].

IMS technology has the advantage of being easily coupled to other analytical devices such as gas chromatograph or liquid chromatography for pre-separation of complex sample matrices. IMS systems are equipped with gas chromatographic columns, providing compound selectivity. IMS is an extremely sensitive technique that can analyse a multiplicity of compounds at low concentrations with detection limits typically in the low ppb-range or even ppt-range [97].

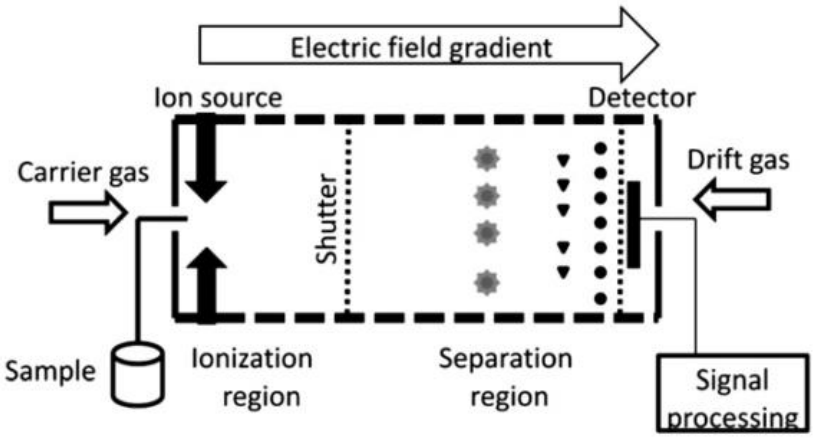


Figure 4.1: Schematic of a classic ion mobility spectrometer. Vapours from the sample are carried into the ionization region and the ions are transported by the electric field through the shutter into the separation region. Ions with different mobility values reach the detector at different times. Adapted from [95].

4.2 Ion Formation

The detection by IMS is based on the difference in the drift time of the product ions through the drift region of an ion mobility spectrometer. The product ions are formed when the compounds of the sample mixture, after being introduced into the instrument, are chemically ionized by means of interactions with the reactant ions. Thus, the reactant ions determinate the ionization outcomes [98].

The formation of ions, the ionization efficiency and therefore the quantitative measure of the analyte depend on the ionization technique applied. Once formed, each ion will move towards the detector at its own velocity with a constant electric field in the drift tube (Figure 4.2.1) [99].

The sources most frequently used in IMS are radioactive due to their stability and own source of power. Although radioactive sources often need special permits and licensing procedures, not to mention the regulatory and safety issues implied. Thus, alternative ionization sources are desired and have been studied to apply in IMS. Non-radioactive sources are however less stable and require additional power. Examples include electron gun sources, corona discharge and photoionization [100].

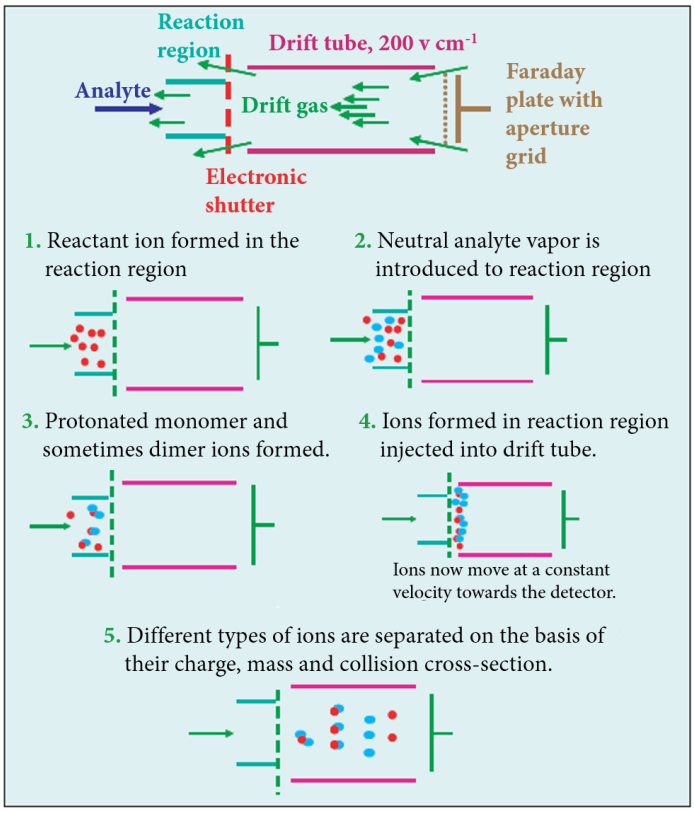


Figure 4.2: Schematic diagram showing the components and the ionization process of an IMS. Adapted from [99].

4.2.1 Formation of Reactant Ions

The ions created in an IMS depend on the ionization technique applied during the measurements, being the radioactive source ^{63}Ni the most commonly used. ^{63}Ni is a beta (electron) emitter and is known by its reliability, efficiency and long lifetime. Other radioactive ion sources used are ^{241}Am and ^{3}H (Tritium) [98][100].

Ions and further free electrons of lower energy are formed, due to the collision of the electrons with air molecules at ambient pressure. The high energy ^{63}Ni electrons will then hit and ionize the nitrogen in the air and will continue the ionization until they lose their kinetic energy [100].

$$N_2 + e^- \longrightarrow N_2^+ + 2e^- \tag{4.3}$$

In positive polarity, ions are formed through a chain of reactions, which initiates with the ion N_2^+ and ends with the formation of three possible positive reactant ion species, $(H_2O)_nH^+$, $(H_2O)_nNO^+$ and $(H_2O)_nNH_4^+$ (Equation 4.4 to 4.9). However, $(H_2O)_nH^+$ tends to predominate under N_2 drift gas flow rates of 600 to 1400 mL/min [101][102].

$$N_2^+ + 2N_2 \longrightarrow N_4^+ + N_2 \tag{4.4}$$

$$N_4^+ + H_2O \longrightarrow 2N_2 + H_2O^+ \tag{4.5}$$

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + OH \tag{4.6}$$

$$H_3O^+ + H_2O + N_2 \longrightarrow (H_2O)_2H^+ + N_2 \tag{4.7}$$

$$(H_2O)_2H^+ + H_2O + N_2 \longrightarrow (H_2O)_3H^+ + N_2 \tag{4.8}$$

$$\vdots$$

$$(H_2O)_{n-1}H^+ + H_2O + N_2 \longrightarrow (H_2O)_nH^+ + N_2 \tag{4.9}$$

The number of water molecules n is a function of the gas temperature and partial pressure of water in the gas [102]. This number depends upon the humidity of the drift gas in the IMS. Typically, humidity is kept at very dry conditions (a few ppm), forming reactant ions with 2 or 3 water molecules, $n = 2$ or $n = 3$ respectively [100].

Enough reactant ions are formed and consequently available for chemical ionization of the analytes. However, the ionization efficiency can differ between analytes, being some ionized more efficiently with negative reactant ions [98].

In negative polarity, ions are formed by thermalized or low energy electrons. When the surrounding gas is air, the high energy electrons emitted by ^{63}Ni are thermalized at ambient pressure, being subsequently attached to oxygen. Through collisions, the electrons will interact with the surrounding gas and lead to the formation of negative reactant ions containing oxygen and water clusters [100]:

$$O_2 + e^- \longrightarrow O_2^- \tag{4.10}$$

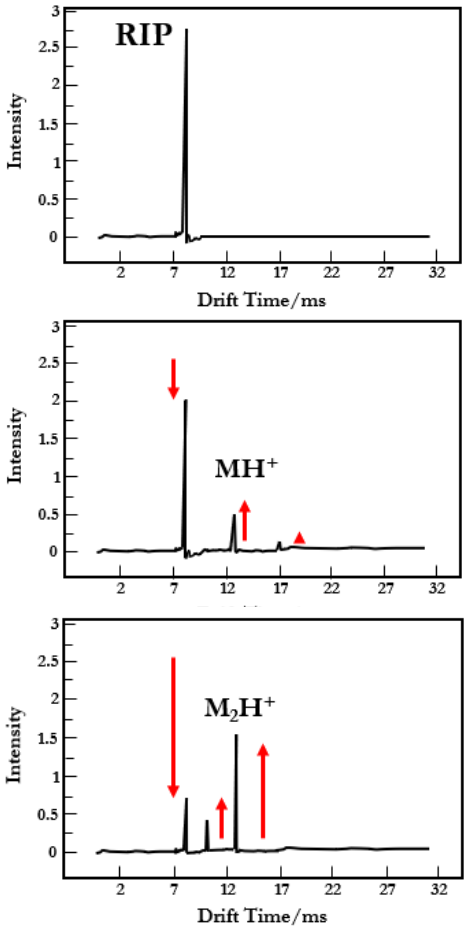
$$H_2O + O_2^- \longrightarrow O_2^-(H_2O) \tag{4.11}$$

$$H_2O + O_2^-(H_2O) \longrightarrow O_2^-(H_2O)_2 \tag{4.12}$$

$$\vdots$$

$$H_2O + O_2^-(H_2O)_{n-1} \longrightarrow O_2^-(H_2O)_n \tag{4.13}$$

The reactant ions yield a peak in the spectrum, known as the reactant ion peak or RIP. RIP is a constant feature in IMS spectra even in the absence of any sample, representing the total of all reactant ions available described as $(H_2O)_nH^+$.



Without analyte:

residual water $\xrightarrow{\beta^-} H^+(H_2O)_n$ | reactant species (**RIP**)

evolution of reactant proton species by interaction with radiation

With analyte:

$H^+(H_2O)_n + M \xrightarrow{\beta^-} MH^+(H_2O)_n + H_2O$ | analyte ion

soft chemical ionization by proton transfer

High analyte load:

$H^+(H_2O)_n + 2M \xrightarrow{\beta^-} M_2H^+(H_2O)_n + H_2O$ | analyte ion

high analyte concentration due to atmospheric pressure

Figure 4.3: IMS Predominant Ionization (positive polarization). Adapted from [97].

As the product ions are produced, the number of available reactant ions in the reaction region decrease, expressed in the decrease of RIP intensity and consequent increase in the intensity of the analyte related peak. Thus, at a given retention time t_r the detection of an analyte peak reduces the RIP intensity at the same t_r (Figure 4.3) [103].

When ions such as $(H_2O)_nNH_4^+$ are formed, their peaks also appear in mobility spectra as small ammonia cluster peaks before the RIP, often named as pre-RIP [104].

4.2.2 Formation of Product Ions

Once formed, the reactant ions will interact with the unknown molecules to produce product ions. Positive reactant ions will produce product ions through collisions with hydrated protons, with the formation of a cluster ion stabilized through the displacement of adducted water [105]. The formation of product ions depends on the proton affinity of the analyte, being the probability of the charge transfer higher when the proton affinity of the analyte is greater than the reactant ion [100]. Being M the molecules to be ionized, the reaction leading to the formation of product ions in positive polarity is as followed [105]:

$$M + H^+(H_2O)_n \longleftrightarrow MH^+(H_2O)_n \longleftrightarrow MH^+(H_2O)_{n-x} + xH_2O \quad (4.14)$$

Sample neutral + Reactant ion — Cluster ion — Product ion (Protonated monomer) + Water

A second product ion can be formed due to the increase of the analyte M concentration in the reaction region, where a further sample neutral attaches to the protonated monomer, displacing a water molecule and producing a proton bound dimer (Figure 4.4) [105]:

$$MH^+(H_2O)_n + M \longleftrightarrow M_2H^+(H_2O)_{n-x} + xH_2O \quad (4.15)$$

Protonated monomer + Sample — Proton bound dimer + water

Also, proton-bound trimers and tetramers can be produced in the gas saturated region of the ion source. These ions have short lifetimes and are hardly observed in ion mobility spectra at ambient temperatures or above.

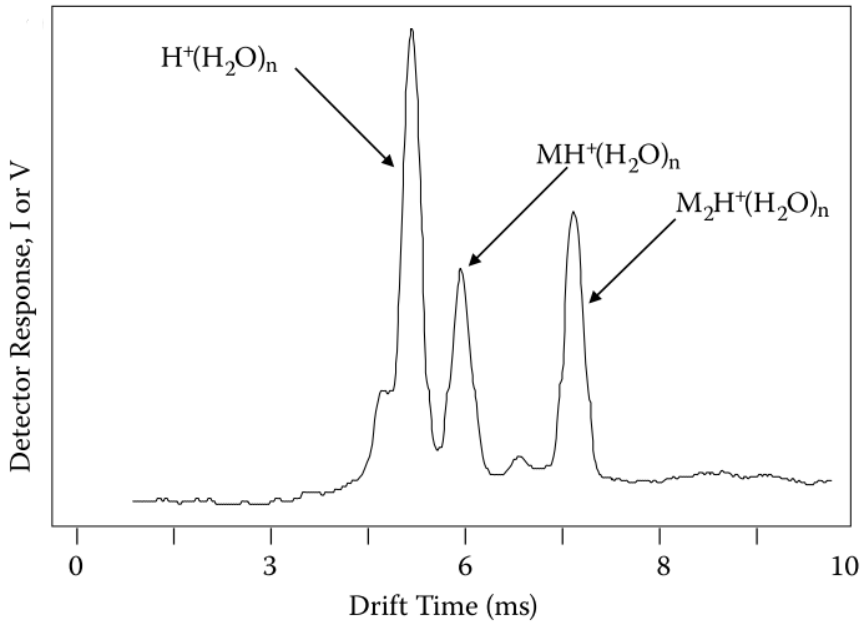


Figure 4.4: A positive polarity mobility spectrum for 2-pentanone in air. The reactant ion peak (RIP) is visible at 4.45 ms. The protonated monomer and the proton-bound dimer appear at 5.075 and 6.225 ms, respectively. Adapted from [105].

Similarly to positive reactant ions, negative reactant ions form hydrated adduct ions between the oxygen anion and the analyte (Equation 4.16). Similarly, to positive reactant ions, also the cluster ion can be stabilized to a negative product ion, by displacement of a water molecule [105].

$$\underset{\text{Sample}}{M} + \underset{\text{Negative reactant ion}}{O_2^-(H_2O)_n} \longleftrightarrow \underset{\text{Cluster ion}}{MO_2^-(H_2O)_n} \longleftrightarrow \underset{\text{Product ion}}{MO_2^-(H_2O)_{n-x}} + \underset{\text{Water}}{xH_2O} \qquad (4.16)$$

Other reactions can also be involved such as charge transfer and proton abstraction. The latter consists essentially in proton transfer to the reactant ion, forming an $(M-H)^-$ ion (Equation 4.17) [105].

$$M + O_2 \longrightarrow (M-H)^- + HO_2 \qquad (4.17)$$

The proton affinity of water (the reactant ion in study) is 691kJ/mol (=7.162eV), allowing the ionization by proton transfer of all molecule with higher proton affinity, typically given for all heteroatom-organic compounds such as alkenes, alcohols, ethers, aldehydes, ketones, carboxylic acids, esters and amines [97][106].

Some compounds can be more susceptible to reactions with ions of the appropriate polarity, depending on the analyte chemical structure. The proton affinity also related to this property also affects the selectivity in ion mobility spectra. It is important to beware that in complex sample mixtures only the molecules with the highest proton affinities will allow their detection. Aromatic amines and phosphorous compounds have the highest proton affinities [100][105].

4.3 Gas Chromatography and Multi Capillary Column

For a more efficient analysis of VOCs, IMS technology is often coupled with gas chromatography (GC) or multi capillary column (MCC), involving a pre-separation of the complex sample mixtures before their entry in the drift tube.

MCC is essentially an altered GC, with the same methodology: while GC consists in only one capillary column, MCC involves several capillaries, as the name suggests (Table 4.1). A capillary column is a very thin tube with a stationary phase coating the interior surface. Due to the distance travelled by the analyte, these columns provide a high sensitivity and separation efficiency.

Table 4.1: Typical characteristics of single and multi capillary column. Adapted from [107].

Parameter	Capillary column	Multi Capillary column
Length, m	30	0.2 - 1
Number of capillaries	1	1000
Pressure, bar	0.1 - 1	2 - 10
Sample capacity, µg	0.1 – 0.5	2 - 3
Optimal flow rate, mL/min	1 – 2	20 – 300

Generally, GC is a method based on the separation and detection of compounds in a mixture. Two phases are essential for this technique: a mobile phase and a stationary phase. The mobile phase, also known as carrier gas, is comprised of an inert gas, which can be helium, argon or nitrogen. The stationary phase is a packed column in which the mobile phase will flow, distributing the solutes between the two phases. Thus, the

sample mixture is separated into its individual compounds. This separation is based on the interaction strengths of the compounds with the stationary phase: with a stronger interaction, the compound will interact longer with the stationary phase, having a slower migration through the column (longer retention time) [108].

Several factors can affect the separation of the components: vapor pressure, the polarity of both components and stationary phase on column, column temperature, carrier gas flow rate, column length and amount of material injected [108].

4.4 Gas Chromatography coupled with Ion Mobility Spectrometry

Being the IMS coupled with GC separation, a 3-dimensional chromatogram is produced, where the x axis represents the drift time of IMS in milliseconds (ms), the y axis corresponds to the time regarding GC separation (retention time) given in seconds (s) and the z axis represents the detector response, related to the amount of ions detected for each compound (Figure 4.5). This 3-dimensional chromatogram can be converted into a 2-dimensional representation by transforming the z-axis data into a color scale for each detected signal. The higher the detector response the more intense the corresponding detection signals [109]. Through the chromatograms it is possible to obtain a qualitative and a quantitative analysis.

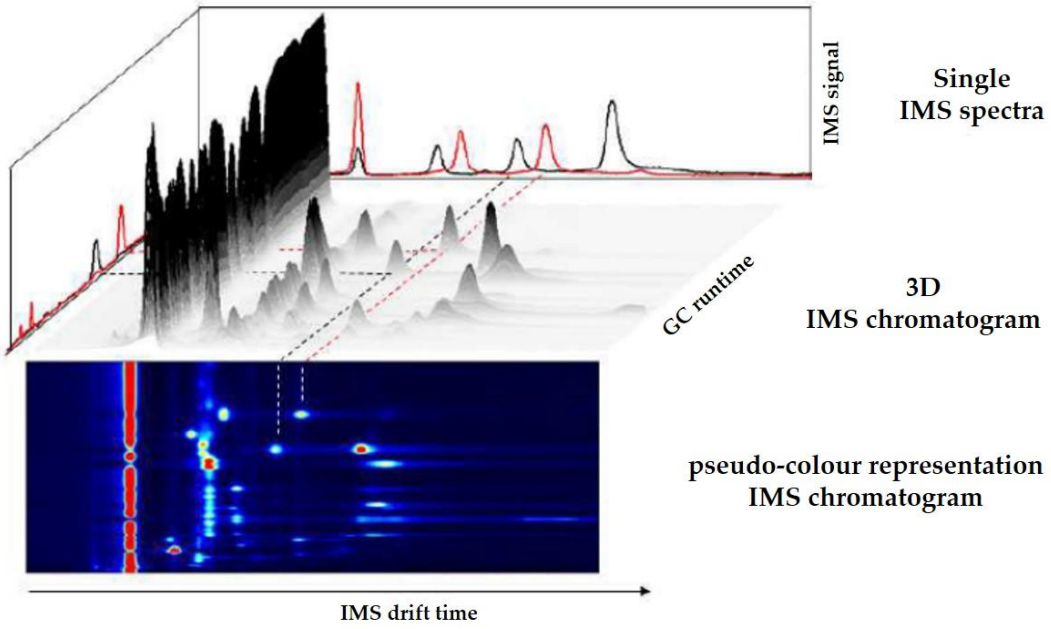


Figure 4.5: 3-dimensional and 2-dimensional chromatogram obtained by GC-IMS. Adapted from [109].

The data obtained using the GC-IMS device is analysed with the Laboratory Analytical Viewer (LAV) software.

LAV software

Developed by G.A.S., the LAV software allows the 2-dimensional and 3-dimensional graphical displaying and processing of measurement data acquired in GC-IMS. By employing plug-in modules, it provides several functionalities, such as the Topographic view, the Reporter and Gallery plugins and the Analytics functions.

In the centre of the topographical view a two-dimensional plot of a part of the currently selected single measurement is displayed (Figure 4.6). The sample value range to be displayed is mapped to one of various colour schemes. The standard colour scheme includes the colours black, blue, magenta, white, orange and red. Sample values above the set value range are displayed in red, while sample values below the set value range are displayed in black.

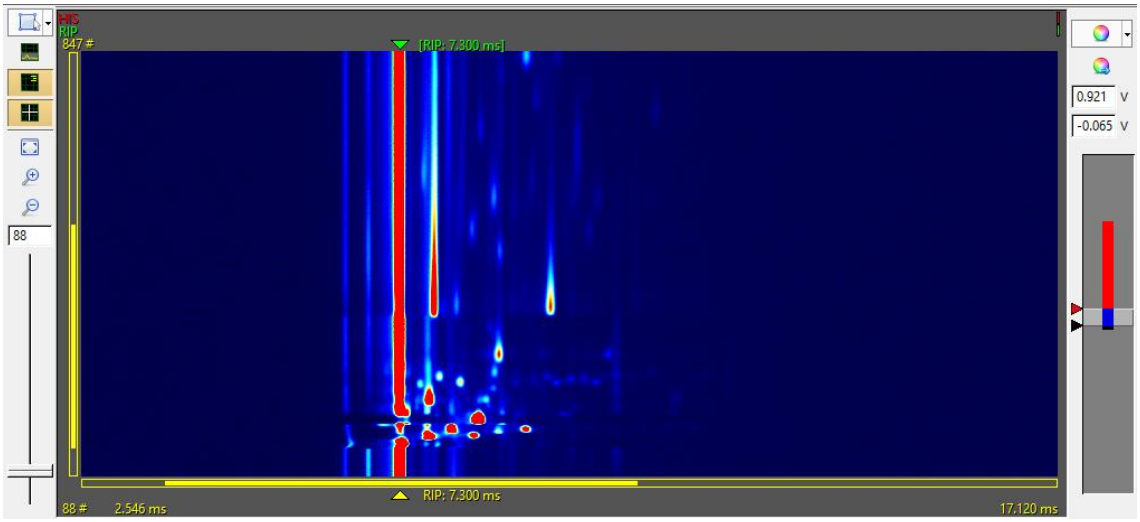


Figure 4.6: The Topographic view of the LAV software. A typical spectrum of a biogenic amine is shown.

In order to compare multiple spectra, the definition of area sets is necessary. These area sets are denoted rectangular areas in drift time/retention time coordinates. Then, using the Gallery plugin of LAV, the user can compare all defined area sets, allowing the identification and characterization of peaks. Figure 4.7 illustrates the window of Gallery plugin.

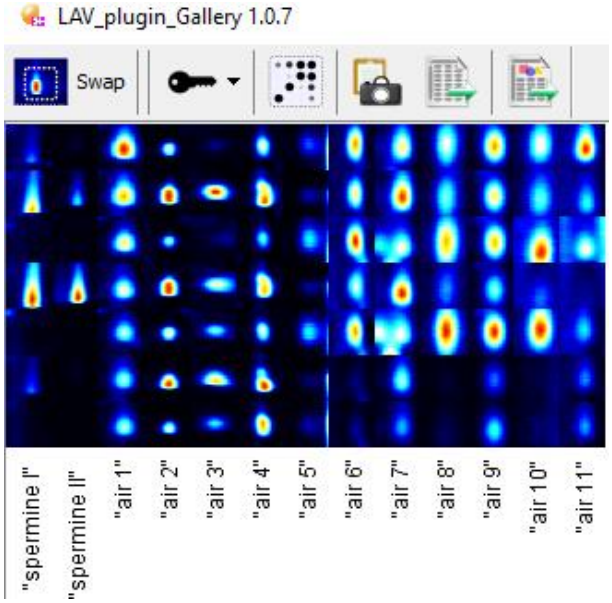


Figure 4.7: Window of the Gallery plugin. In this example, seven spectra are compared for the defined area sets, being the name of each area set placed bellow each column.

The automatic extraction of analyte ion peaks data, such as retention time, drift time, intensity and RIP position, can be obtained through the Analytics functions. Using quantification modules and calibration functions, compounds concentrations can be calculated based on these functions for every IMS measurement which the model is applied to. Lastly, the Reporter plugin allows the comparison between multiple spectra (Figure 4.8). The display of area sets, as well as drift and retention times, is available, being also possible to apply spectra zoom in and zoom out.

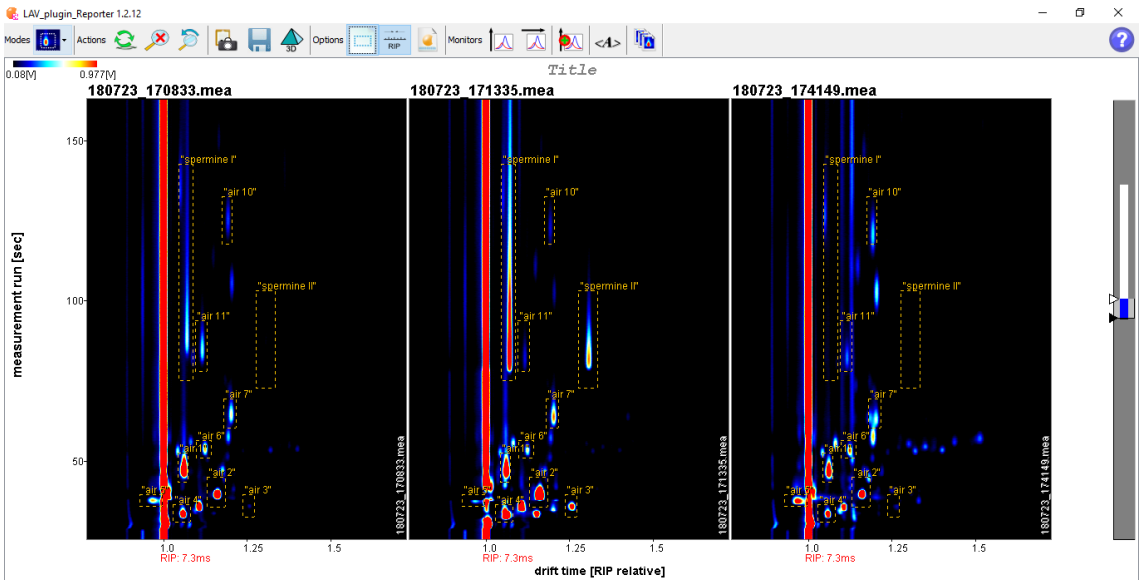


Figure 4.8: Window of the Reporter plugin. In this example, three typical spectra are compared, and the defined area sets are displayed.

Chapter 5

Materials and Methods

5.1 Materials

The materials required for all measurements for three studies conducted are presented in Table 5.1:

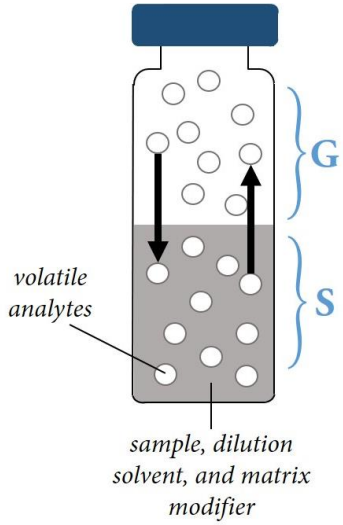
Table 5.1: Materials used in the experimental protocol.

Material	Manufacturer
Multi Capillary Column coupled with Ion Mobility Spectrometry	G. A. S.® GmbH, Dortmund, Germany NMT, Tecnologia, Inovação e Consultoria, S.A.
Gas Chromatography coupled with Ion Mobility Spectrometry	G. A. S.® GmbH, Dortmund, Germany
Nitrogen bottle 9.3 m^3	Air Liquide ALPHAGA®, Lisbon
Analogic Heatblock	VWR®, Radnor, PA
20 mL glass vials with threaded, open-centered magnetic caps and rubber seals	VWR®, Radnor, PA
Teflon tube connected to stainless steel female thread	Bohlender GmbH, Grünsfeld, Germany Swagelok, Solon Ohio
Regular bevel needles 21 G	BD™, Becton, Dickinson and Company, Franklin Lakes, New Jersey
5mL syringes	PIC Solution®, Pikdare S.r.l., Italy
Micropipette 0 - 10 µL and tips	-----
Stainless steel surgical tweezer and micro spatula	
Digital Thermometer and Chronometer	
Power Supply	G. A. S.® GmbH, Dortmund, Germany

5.2 Sample preparation

In this thesis three different studies were conducted: detection of biogenic amines, study of fresh fish decomposition and study of BAs in fish control samples previously measured by other techniques (HPLC and Gas Chromatography coupled with Mass Spectrometry).

The easiest way to introduce the sample into the IMS device is through direct injection by flowing a carrier gas directly into the ionization region. However, when dealing with liquid or solid samples, which is the case, a different sampling methodology is required to vaporize the sample and consequently obtain its gas state for IMS analysis. Syringe injection is the methodology applied in all three studies. This methodology is complemented with static headspace analysis (Figure 5.1) which samples directly the volatile headspace from the container in which the sample is placed (glass vial).



G = Gas phase (headspace)
The gas phase is commonly referred to as the headspace and lies above the condensed sample phase.

S = Sample phase
The sample phase contains the compound(s) of interest and is usually in the form of a liquid or solid in combination with a dilatation solvent or a matrix modifier.

Once the sample phase is introduced into the vial and the vial is sealed, volatile compounds diffuse into the gas phase until the headspace has reached a state of equilibrium as shown by the arrows. The sample is then taken from the headspace.

Figure 5.1: Phases of the headspace vial. Adapted from [110].

Once injected into the sample system of both devices, the MCC-IMS and GC-IMS systems will record all measurements spectra and create a resulting file. Subsequently this file will be processed in the LAV software, capable of providing 2D or 3D representation of spectra.

In order to verify if the observed signals are characteristic of the amines analyzed and are volatile compounds resulting from fish decay, room air measurements were also performed.

5.2.1 Detection of biogenic amines

The first study involves 8 different biogenic amines obtained from Sigma-Aldrich (Darmstadt, Germany): histamine dihydrochloride (HIS), putrescine (PUT), cadaverine (CAD), tyramine (TYR), tryptamine (TRP), spermine (SPM), spermidine (SPD) and phenethylamine (PEA) (Table 5.2). These amines were studied in their pure form (except histamine) to avoid any possible peaks appearance of unknown or undesired compounds in mobility spectra.

Table 5.2: Structural properties of the biogenic amines analysed.

Biogenic Amine	Abbreviation	CAS Number	Molecular Formula	Molecular Weight ($g\ mol^{-1}$)	Density ($g\ cm^{-3}$)
Histamine dihydrochloride	HIS	56–92–8	$C_5H_{11}Cl_2N_3$	184.067	–
Tyramine	TYR	51–67–2	$C_8H_{11}NO$	137.182	1.1±0.1*
Tryptamine	TRP	61–54–1	$C_{10}H_{12}N_2$	160.216	1.2±0.1*
Spermine	SPM	71–44–3	$C_{10}H_{26}N_4$	202.346	0.937
			solid		
			liquid		
Putrescine	PUT	110–60–1	$C_4H_{12}N_2$	88.154	0.873 (25°C)
Cadaverine	CAD	462–94–2	$C_5H_{14}N_2$	102.181	0.877 (25°C)
Spermidine	SPD	124–20–9	$C_7H_{19}N_3$	145.250	0.925 (25°C)
Phenethylamine	PEA	64–04–0	$C_8H_{11}N$	121.183	0.954 (20°C)

*Predicted data generated using the ACD/Labs Percepta Platform - PhysChem Module [111].

While putrescine, spermidine, cadaverine and phenethylamine are in their liquid state, the remaining amines are in solid state. The liquid amines have a distinctive fishy smell, easing the measurement and detection of the amine with the technique applied. The solid amines although having a faint characteristic odor (except histamine) were hardly identified in mobility spectra.

Each solid BA was then weighed and placed in a 20 mL glass vial. Regarding the liquid amines, 1 µL of each amine was removed using a micropipette and set into similar

vials. The vials were immediately sealed and only when the headspace has reached a steady state were the samples withdraw.

All samples were taken with a 5 mL syringe designated for each amine to avoid possible VOCs accumulations and subsequent contaminations of the measurements.

5.2.2 Fresh fish decomposition

The second study conducted involved the purchase and preparation of three different fish species samples: atlantic bonito (*Sarda sarda*), atlantic horse mackerel (*Trachurus trachurus*) and sardine (*Sardina pilchardus*).

Fresh specimens of the fish species atlantic bonito, atlantic horse mackerel and sardine were purchased from a fish market in Almada, Portugal. Gutting and filleting were manually performed before transportation to the laboratory. To avoid any contaminations, only sterile knives were used. The samples were then packed in polystyrene boxes with ice and transported in a thermal isolated bag to the laboratory within two hours after purchase.

Once in the laboratory, the samples were weighed to establish the weight range of all measurements (Table 5.3). Due to the high sensitivity of the device and strong characteristic fish odour, the weight of the fish muscle samples to be analysed should not surpass the mass unit gram, the average weight being 0.1540 g.

Table 5.3: Scientific names and weights of the fish species analysed.

Fish species	**Scientific name**		**Weight (g)**		
	Family	**Genus and species**	**Sample 1**	**Sample 2**	**Sample 3**
Atlantic bonito	Scombridae	*Sarda sarda*	0.1153	0.1630	0.1772
Atlantic horse mackerel	Carangidae	*Trachurus trachurus*	0.1190	0.1822	0.1527
Sardine	Clupeidae	*Sardina pilchardus*	0.1626	0.1425	0.1711

Fish muscle samples were placed into separate vials immediately after weighing. The vials were then sealed and placed in the laboratory exposed to room temperature for 8 days at room temperature to promote degradation of fish muscles samples. Samples headspace was analysed over the first four days. *Sarda sarda* samples headspace was also measured on the 8th day.

5.2.3 Control fish samples

Control samples of the fish species *Sarda sarda* were studied and analysed in GC-IMS. The preparation of these samples was conducted as part of a research conducted on seafood quality assessment by determination of target biogenic amines in fish involving different techniques such as Gas Chromatography coupled with Mass Spectrometry (GC-MS) and HPLC. This research is also inserted in the project 3Qs for quality - Development of new devices and techniques for seafood quality assessment (PTDC/MAR-BIO/6044/2014), the same project as this thesis.

After selection of the fish species, the fish was fileted and exposed to room temperature for 13 days, with a daily samples collection. These samples were then frozen for later analysis at -80°C. Following the periods of exposure, stored samples were homogenized in 75:25 Methanol: 0.4N HCl.

Similarly to fresh fish samples, a small amount of control samples of each day was removed and weighed (Table 5.4). Then the weighed samples were placed into separate sealed vials. Afterwards, samples headspace was measured and analysed.

Table 5.4: Weight of all samples taken from the control samples.

Control fish samples	Day of fish collection	Weight (g)		
		Sample 1	Sample 2	Sample 3
T0	1st	0.1170	0.1263	0.1310
T4	5th	0.1191	0.1275	0.1145
T7	8th	0.1271	0.1223	0.1312
T11	12th	0.1240	0.1267	0.1108
T13	14th	0.1246	0.1406	0.1359

5.3 Analytical Equipment: MCC-IMS and GC-IMS

For the accomplishment of the tests and measurements of this experimental work both MCC-IMS and GC-IMS were used. The devices were manufactured by the G.A.S. Gesellschaft für analytische Sensorsysteme mbH headquartered in Dortmund, Germany. Both systems consist of the IMS coupled to a gas chromatographic column, where MCC-IMS has multiple short parallel capillaries coated with a stationary phase and GC-IMS only presents one long capillary tube (Table 5.5).

The IMS technology coupled with Gas Chromatography provide the detection of VOCs traces by simple directed sample. The main purpose of the GC-IMS is measuring the headspace of these compounds of both solid or liquid samples. Thus, allows the measurement of the odour performance over time, providing identification and quantification of gas odours. The enormous advantages of the device are the speedy availability of the results (within a few minutes), and the friendly-user and very easy menu provided.

The schematic represented in Figure 5.2 shows the principle structure of the gas flow system of the GC-IMS equipment.

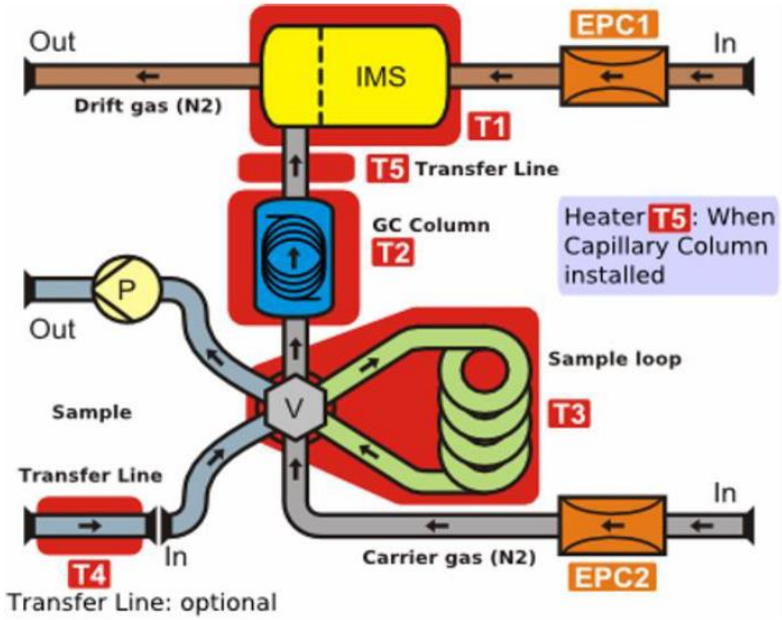


Figure 5.2: Schematic of the GC-IMS equipment. IMS is coupled to a gas chromatography column, having other components associated in the system such as EPC1 and EPC2. These represent electronic pressure control units, used to supply the drift gas and the carrier gas for the column, respectively. Both gases leave the device at the gas out, connected to an exhaust. IMS, GC Column and Sample loop are heated, represented by T1, T2 and T3 [97].

Table 5.5: Detailed specifications of GC-IMS and MCC-IMS.

Parameters	GC–IMS	MCC–IMS
Dimension	450 x 500 x 295 [mm] (Width x Depth x Height)	449 x 495 x 184,5 [mm] (Width x Depth x Height)
Weight	approximately 20 kg	12-15 kg
Drift length	98 mm	98 mm
Electrical field strength	500 V/cm	500 V/cm
Ionisation source	β-radiation source (Tritium ^{3}H)	β-radiation source (Tritium ^{3}H)
Sampling system	6-port-valve; sample loop 1 mL	6-port-valve; sample loop 1 mL
Test gas method	Headspace	Headspace
Gas chromatographic column	General capillary columns: Column length: 30 m Capillaries I.D.: 0.53 mm (I.D. stands for Inside Diameter)	Multi capillary column Film thickness: 0.2 µm Column length: 20 cm Capillaries I.D.: 40 µm Number of capillaries: 1200
Gas	Air	Nitrogen 5.0 (99.999%)
Drift gas pressure	0.752 KPa	–
Carrier gas pressure	63.478 KPa	–
IMS Temperature	70°C	45°C
Column Temperature	40°C	50°C
Valve Temperature	55°C	45°C

In both systems, there are two types of fundamental separations in the detection and analysis of compounds, the first being the chromatographic separation and the second drift separation. Chromatographic separation provides a selective separation of headspace volatile and semi-volatile compounds through the GC or multi capillary column. Once separated, the compounds are introduced into the ionization region of the IMS where they are converted into product ions from interactions between positive or negative reactant ions. In the presence of an electric field, the product ions travel through the drift tube region until their detection according to their charge, mobility and ion cross section.

Figure 5.3: MCC-IMS and GC-IMS devices (left to right). Adapted from [112][113].

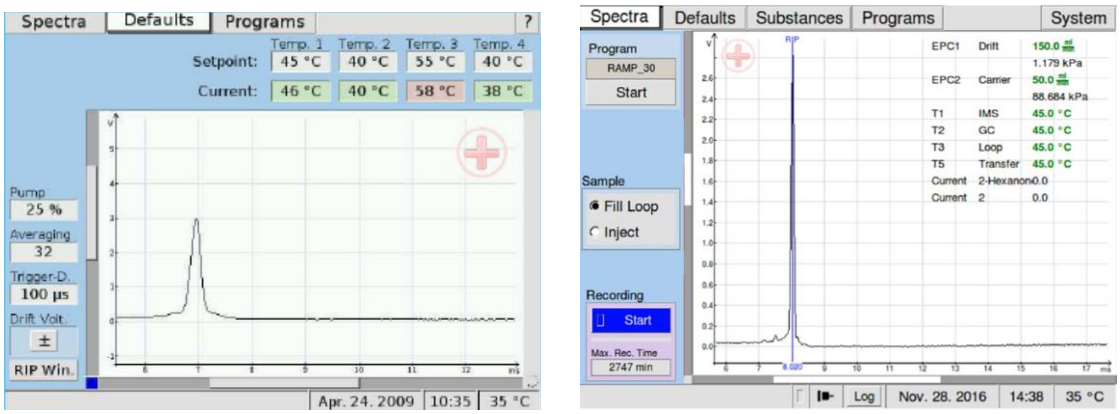


Figure 5.4: Zoom in of the display of the defaults window for MCC-IMS and GC-IMS devices, respectively. Adapted from [97][114].

5.4 Measurement methodology

To carry out the measurements of the samples in both devices, was necessary to produce a protocol that allowed both the detection of the pure BAs and of amines in fish muscle samples through VOCs emission.

The initial approach on the development of a measurement methodology was a trial and error approach, which had satisfactory results regarding the detection of pure amines. However, due to immediate saturation on mobility spectra, measurements of fish samples required an optimization of the method applied.

Several factors such as gas flow rates, headspace's volume, vapor pressure, length of Column, temperature and humidity influence the separation process and thus need to be taken in consideration to achieve a better separation and maximization of sensitivity and selectivity.

During all measurements the room relative humidity of the laboratory air and room temperature were maintained at 50-70% and 22-24°C respectively. All headspace measurements occurred at ambient temperature (23°C).

The headspace temperature is also a factor to take in consideration due to the volatility of organic compounds. This property is specified by the tendency of a substance to vaporize, also known as vapor pressure. Usually the compounds volatility is higher for lower boiling point temperatures, hence the volatility of organic compounds is often defined and classified by their boiling points. The compound will more likely be emitted into the air with a higher volatility (lower boiling point) [115].

However, little information is known regarding vapor pressure and boiling points of biogenic amines. Most values are a prediction based on studies and trials (Table 5.6). Hence all amines studied were measured not only at ambient temperature but also heated to 40°C to test their volatility. To achieve the temperature of 40°C, the vials were placed into the well of the heatblock during a pre-established time (10 minutes) and then connected to the device through a needle attached to the Teflon tube or to a syringe containing the headspace to analyse. Between the measurements, a cleaning program was used to prevent accumulation of amine and remove any contaminates.

Table 5.6: Boiling points and vapour pressure of the analysed BAs.

Biogenic Amine	Boiling Point	Vapour Pressure
HIS	246°C[1]	–
TYR	166°C[1] at 2 mmHg	–
TRP	137°C[2] at 0.15 mmHg	–
SPD	129°C[1] at 14 mmHg	–
SPM	159°C[1] –	–
PUT	158-160°C[2] –	2.33 mmHg at 25°C[1]
CAD	179°C[1] –	1.01 mmHg at 25°C[1]
PEA	195°C[1] –	–

[1]Data from the toxicology database HSDB [115].

[2]Data from Chemical Book, Analytical Standards [116].

5.4.1 Gas flow rates

While drift gas flow is responsible for the separation of ions in the drift region, the carrier gas flow has a minimal contribution on the partitioning process, being simply stated as just a carrier to transport the volatile molecules to the drift region through the column during the partitioning process.

The pure amines cadaverine, putrescine, phenethylamine and spermidine were first measured in the MCC-IMS device. To verify which flow was the most appropriate to measure the amines, three drift gas flows were tested: 150 mL/min and the default value 500 mL/min. Carrier gas flow was also verified, testing 50 mL/min and 100 mL/min. In all spectra shown below, the x-axis represents the drift time relative to the RIP in milliseconds and the y-axis represents the retention time in seconds.

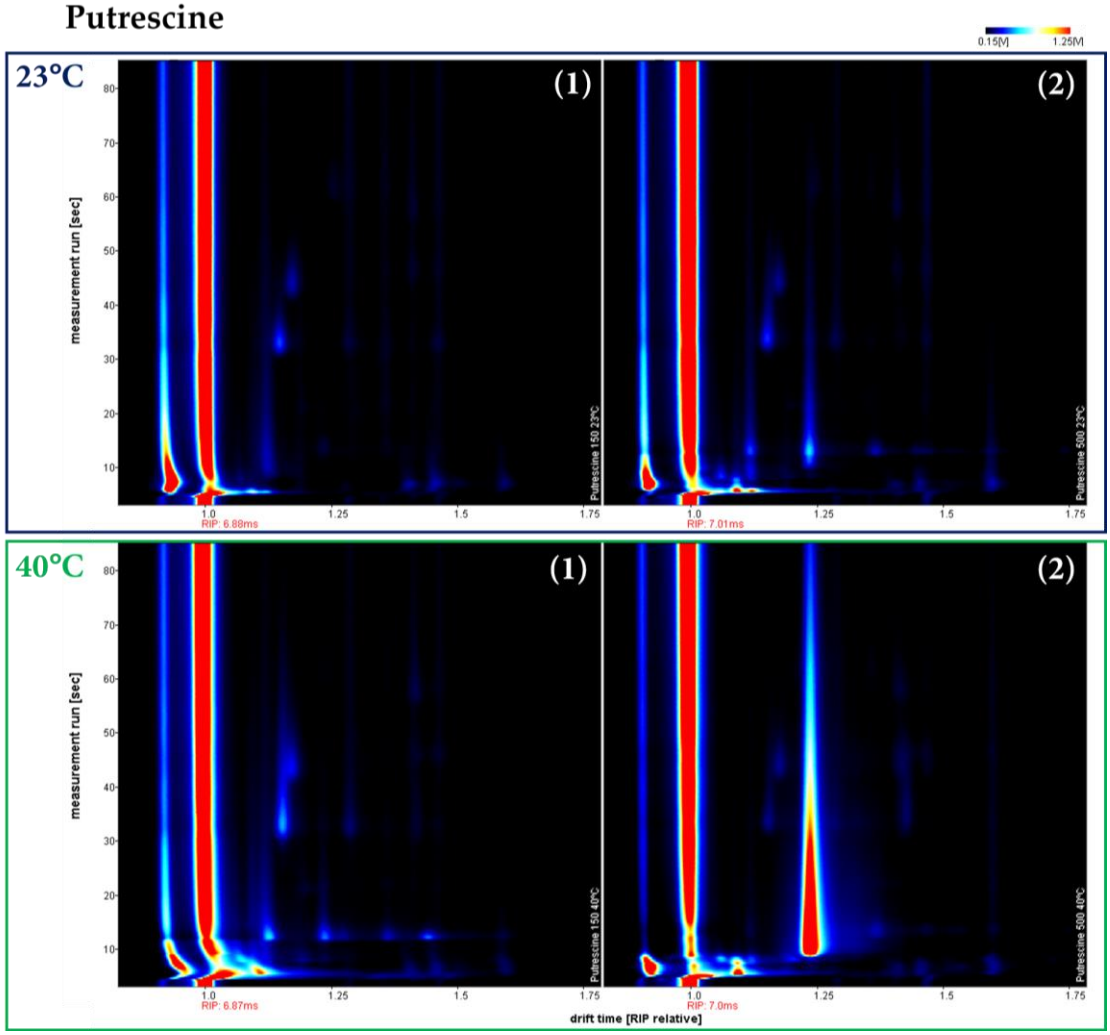


Figure 5.5: Zoomed spectra of putrescine measurements at 23°C and 40°C. Variation of drift gas flow: (1) 150 mL/min and (2) 500 mL/min. Carrier gas flow selected was 50 mL/min. Measurements acquired using MCC-IMS device.

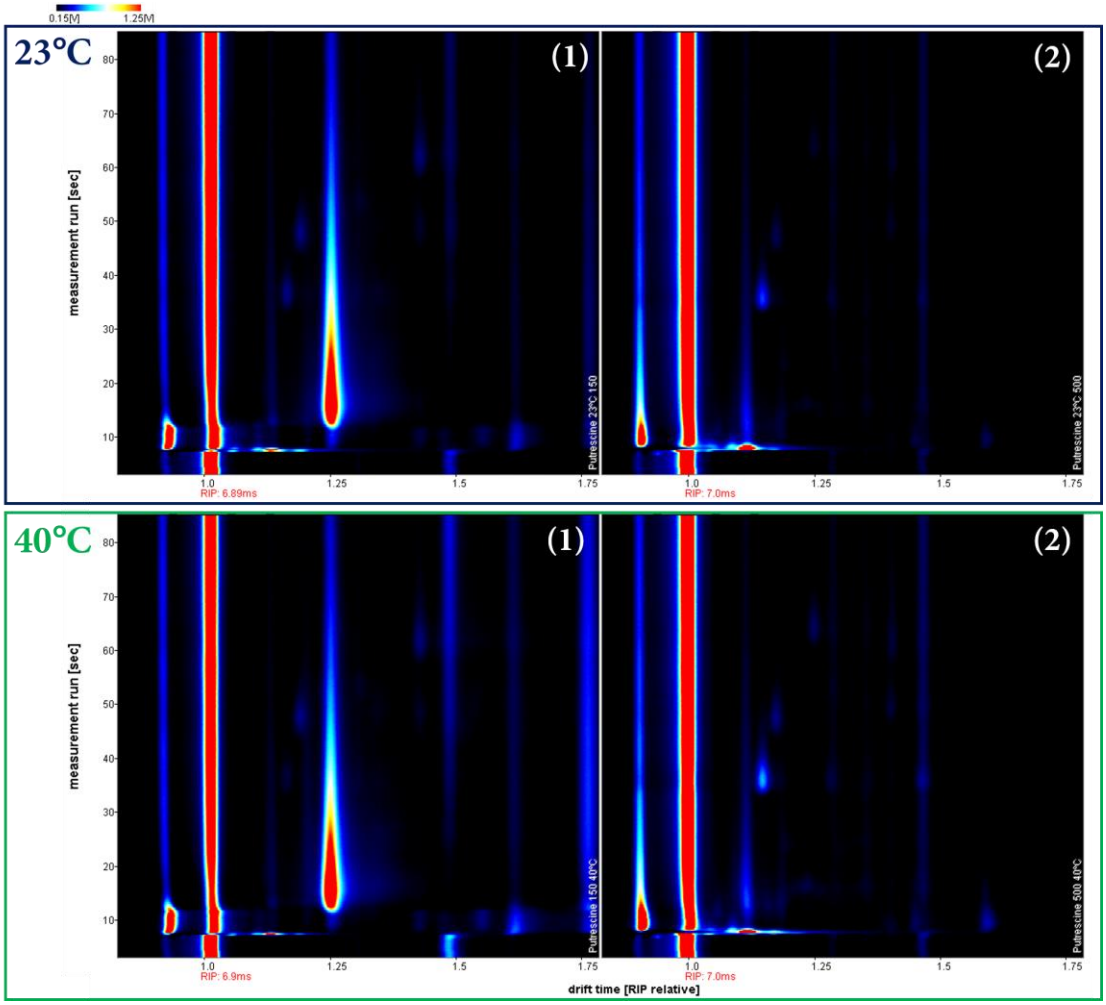


Figure 5.6: Zoomed spectra of putrescine measurements at 23°C and 40°C. Variation of drift gas flow: (1) 150 mL/min and (2) 500 mL/min. Carrier gas flow selected was 100 mL/min. Measurements acquired using MCC-IMS device.

Comparing the Figures 5.5 and 5.6, it was possible to conclude that the drift gas flow of 500 mL/min and carrier gas of flow 50 mL/min (Figure 5.7) show better spectra to identify putrescine' signals and a possible pattern of the amine.

In Figure 5.7, putrescine samples at ambient temperature and 40°C were compared. A signal was observed in both spectra (yellow rectangle) with higher signal intensity at 40°C. Some additional signals (white rectangle) showed intensity increase at 40°C. The signal identified for putrescine, yellow rectangle, has retention time of 12.012 seconds and drift time of 1.236 milliseconds relative to the RIP (drift gas flow 500 mL/min and carrier flow gas 50 mL/min). There was some accumulation of the amine in the column, that can be removed with the flush of the system. Additionally, the signals within the black rectangles were not clearly identified and might be originated from the air constituents when heated to 40°C.

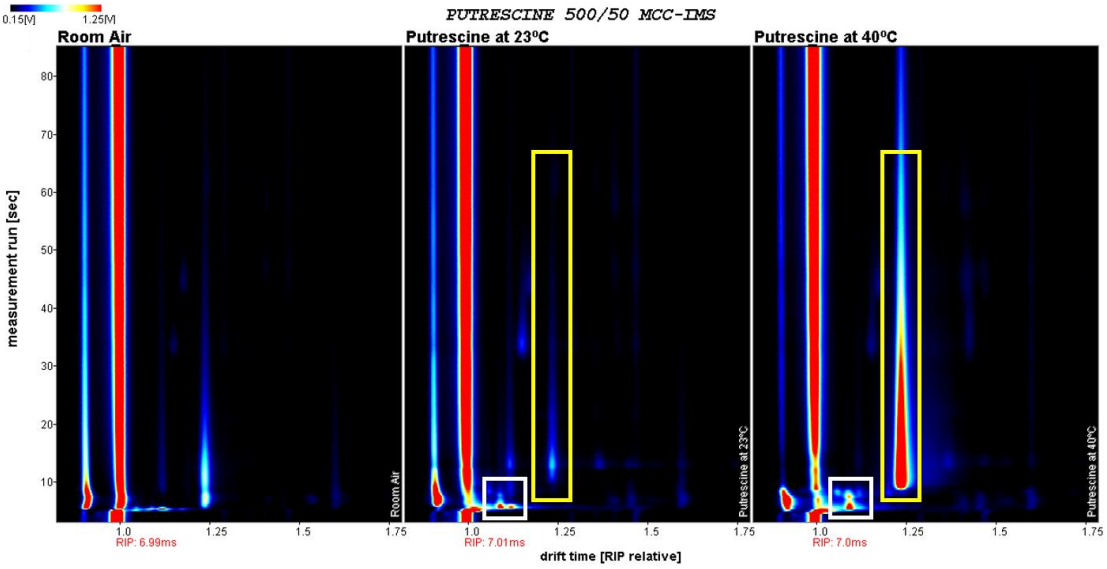


Figure 5.7: Measurements of the room air and pure putrescine, at ambient temperature (23°C) and 40°C for a drift gas flow of 500 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using MCC-IMS device.

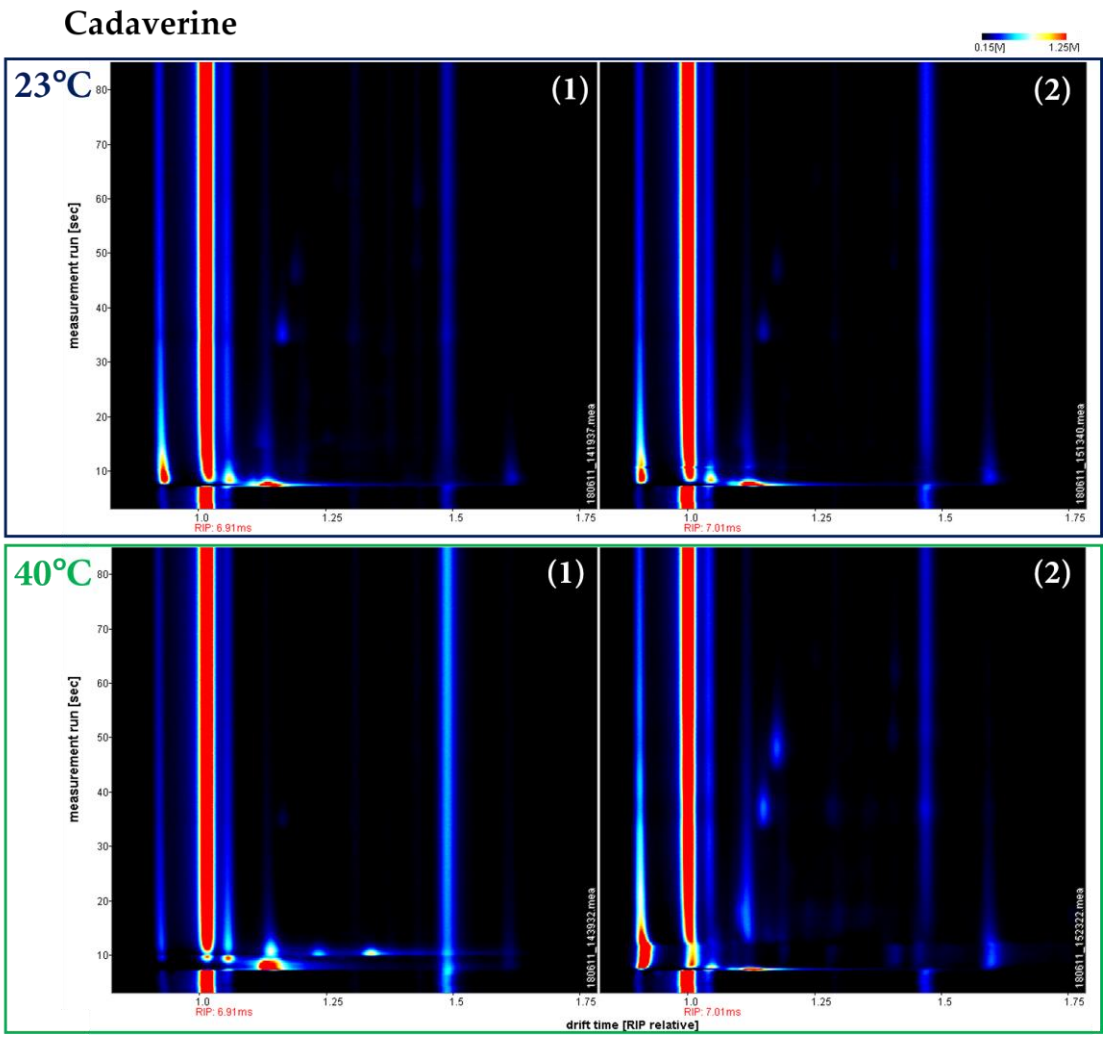


Figure 5.8: Zoomed spectra of cadaverine measurements at 23°C and 40°C. Variation of drift gas flow: (1) 150 mL/min and (2) 500 mL/min. Carrier gas flow selected was 50 mL/min. Measurements acquired using MCC-IMS device.

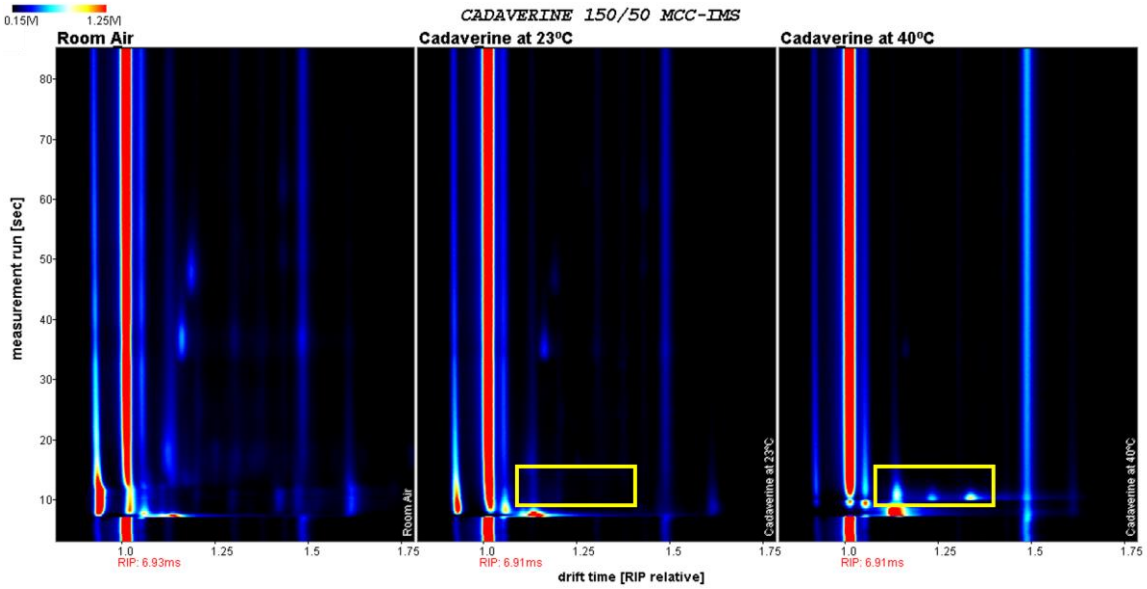


Figure 5.9: Measurements of the room air and pure cadaverine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using MCC-IMS device.

After the observation of the obtained spectra for cadaverine (Figure 5.8), it was verified a better peaks separation with the drift gas flow of 150 mL/min and carrier gas of flow 50 mL/min (Figure 5.9).

The comparation of cadaverine samples at ambient temperature and 40°C is represented on Figure 5.9. Three new signals were observed on the spectrum of cadaverine when heated to 40°C (yellow rectangle). It is safe to assume that these three peaks are specific to cadaverine, since they are not present in room air spectrum. The retention and drift times of the signals identified in yellow rectangle are listed in the Table 5.7.

Similarly to putrescine measurements, cadaverine spectra present some amine accumulation in the column, which can be removed with a flushing program.

Table 5.7: Drift and retention times of the peaks within the yellow rectangle of Figure 5.9.

Drift time relative to RIP (ms)	Retention time (sec)
1.142	11.0
1.237	11.0
1.339	11.0

To guarantee a good separation of peaks in further measurements of BAs on both MCC-IMS and GC-IMS, 150 mL/min and 50 mL/min were chosen for drift gas flow and carrier gas flow respectively.

Phenethylamine

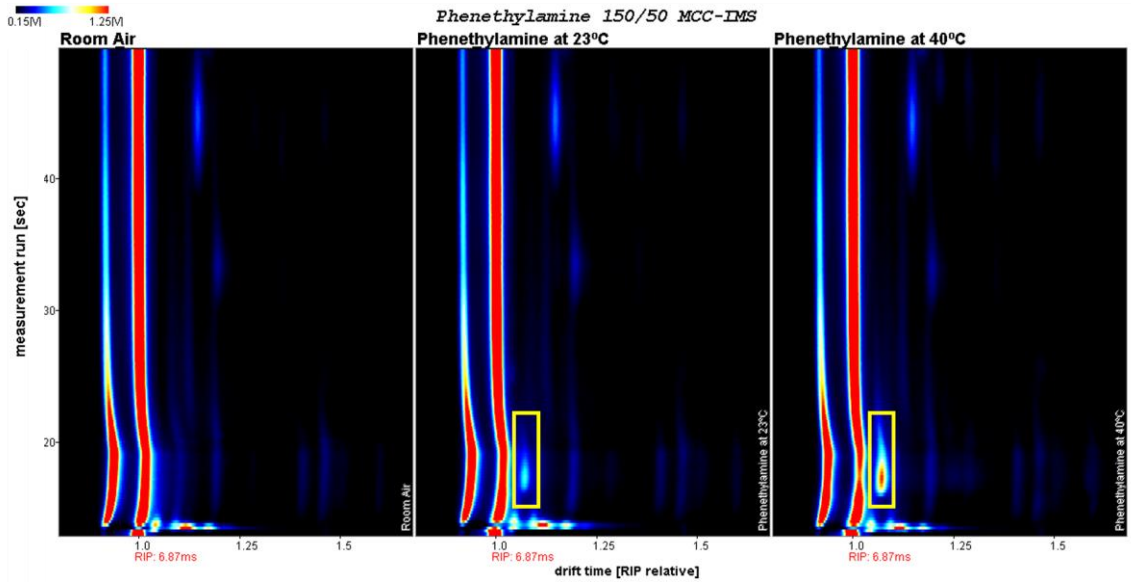


Figure 5.10: Measurements of the room air and pure phenethylamine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using MCC-IMS device.

Samples of phenethylamine at ambient temperature and 40°C were compared in Figure 5.10. In both spectra, was observed a peak with higher signal intensity at 40°C (yellow rectangle). This signal has a retention time of 18.0 seconds and drift time of 1.074 milliseconds relative to the RIP.

Spermidine

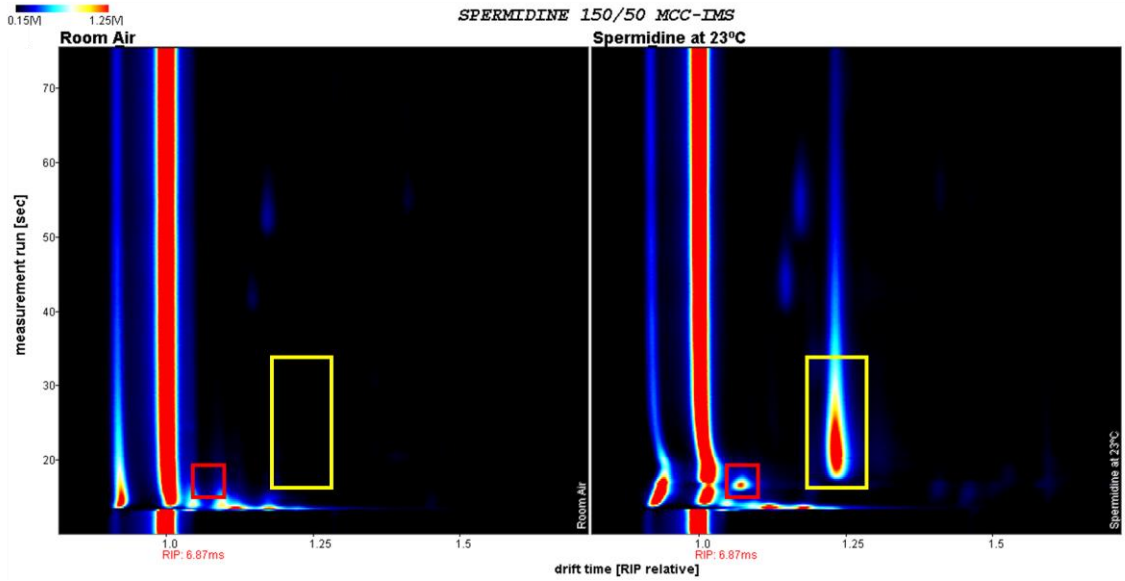


Figure 5.11: Measurements of the room air and pure spermidine, at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using MCC-IMS device.

Due to the limited amount of biogenic amines available, and in order to also measure in GC-IMS device at both temperatures 23°C and 40°C, it was only possible to analyse spermidine at room temperature in the MCC-IMS.

Nevertheless, a peak was observed in the amine spectrum (yellow rectangle) at 23°C with a high signal intensity. This signal then identified for spermidine has a retention time of 21.0 seconds and drift time of 1.234 milliseconds relative to the RIP.

5.4.2 Headspace volume

The headspace volume to analyse for each sample depends on the volatility of the compound, the sample physical stated, the headspace resting time and the sample size.

The higher the volatility of the compound, the quicker will the VOCs emitted saturate the headspace. Such condition is also verified when, with a large sample amount on vial, the headspace is lower than the volume occupied by the sample. In the opposite situation, when the headspace volume is much larger than the sample volume, the time taken by the headspace until it reaches an equilibrium state will be consequently higher and the concentration of the emitted VOCs will be lower. Thus, there is a need of adjustment of both size and amount of sample to obtain better spectra.

The physical state of the sample also influences the ideal headspace volume for its analysis. Headspace sampling is used to analyse and trace levels of volatile compounds in liquids and solids. Solids that are not soluble are the perfect targets for this technique. However, depending on the solid volatility, the sample analysis can require a higher headspace volume, which was verified in some BAs studied in this thesis.

5.4.3 Running time

Due to their low molecular weight, measurements of the biogenic amines were performed with a running time of 5 minutes in both devices.

However, not knowing the specific content of fish tissues samples and due to the different matrix associated with each fish species, running times of 5, 7, 8, 9 and 10 minutes were applied. After spectra observation, it was possible to shorten the running time according to the fish species to a value from which no more significant compounds were detected.

5.4.4 Design of measurement programs

For the analysis of both biogenic amines and fish samples, running programs were created on both devices MCC-IMS and GC-IMS. Each program has values for several parameters: duration of the pump activation, duration of the valve aperture, duration of the record, gas flows and temperatures.

5.4.4.1 Detection of biogenic amines in MCC-IMS

During the trial-and-error approach, three programs were tested in the MCC-IMS, differing only in pump activation and valve opening time. The power of the pump activation essentially depends on the carrier gas flow and the sample volume required to allow it to reach the loop.

The first two programs made use of the pump, being activated with a power of 12%. It is known from the device specifications that the power of pump is set as the percentage of the maximum 1800 mL/min. Thus 12% corresponds to 216 mL/min. The maximum volume which could be removed from a vial is the volume of the vial itself, 20 mL. However, the sample loop has a capacity of 1 mL and thus only that volume will be needed for analysis.

The duration of the valve aperture also depends on the carrier gas flows, since during valve opening the pump will pull a certain headspace volume according to the gas rate. As previously described, two carrier gas flow were tested: 50 mL/min and 100 mL/min. In order to recover all sample volume, 1 mL, the valve needed to be opened for 1.2 seconds and 0.6 seconds. However due to often malfunction of the pump, the duration of the valve aperture applied was of 6 seconds. The resulted programs applied with the pump activated in putrescine and cadaverine samples are presented in Tables 5.8 and 5.9.

In the third program created, instead of pump activation, amines samples headspace was injected through a syringe into the device: headspace of 2ml for both phenethylamine and spermidine. For a better separation of the analyte, a carrier gas flow gas ramp was applied. Additionally, the duration of valve aperture was increased to 10 seconds and the values of the spect parameter were also modified as seen in the program represented in Table 5.10.

Table 5.8: Program used on MCC-IMS measurements for amines samples, using pump activation for a drift gas flow of 500 mL/min and carrier gas flow of 50 mL/min.

Program name: AMINES_500						
Spect	**Time**	**V**	**P**	**R**	**E1**	**E2**
0	0s	|	12%	|	500mL/min	50 mL/min
1	0.231s	|	|	rec	|	|
18	4.158s	open	off	|	|	|
44	10.164s	close	|	|	|	|
1297	5min0s	|	|	stop	|	|
1298	5min0s	|	|	|	|	|

Table 5.9: Program used on MCC-IMS measurements for amines samples, using pump activation for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min.

Program name: AMINES_150						
Spect	**Time**	**V**	**P**	**R**	**E1**	**E2**
0	0s	|	12%	|	150mL/min	50 mL/min
1	0.231s	|	|	rec	|	|
18	4.158s	open	off	|	|	|
44	10.164s	close	|	|	|	|
1297	5min0s	|	|	stop	|	|
1298	5min0s	|	|	|	|	|

Table 5.10: Program used on MCC-IMS measurements for amines samples, using syringe injection methodology.

Program name: AMINES_SER						
Spect	**Time**	**V**	**P**	**R**	**E1**	**E2**
0	0s	|	off	rec	500mL/min	50 mL/min
43	9.933s	open	|		|	|
86	19.866s	close	|	|	|	|
388	1min29s	|	|	|	|	10 mL/min
779	3min0s	|	|	|	|	25 mL/min
1384	5min32s	|	|	stop	|	35 mL/min
1385	5min33s	|	|	|	|	|

5.4.4.2 Detection of biogenic amines and fish analysis in GC-IMS

In GC-IMS, it was necessary to develop a program that, when applied, would allow the detection of pure biogenic amines and VOCs emitted by fish tissues. Thus, it was stablished the application of the syringe injection methodology with specific amine sample headspace volumes (Table 5.11).

Table 5.11: Headspace volumes used for BAs measurements according to each sample.

Amine Sample	Sample headspace volume			
	Ambient temperature (23°C)			Heated to 40°C
HIS	2 mL	4 mL	5 ml	5 ml
TRP	1 mL	2 mL	3 ml	2 ml
TYR	2 mL		3 mL	3 mL
SPM	1 mL		2 mL	2 mL
PUT	0.5 mL		1 mL	1 mL
CAD	1 mL		2 mL	2 mL
PEA	1 mL		2 mL	2 mL
SPD	2 mL			2 mL

Regarding fish samples, the measurements of the first and second days involved a sample headspace of 2 mL while in the third and fourth day a headspace of 1 mL was analyzed, due to considerable spectra saturation.

The running time was also taken in consideration. While due to their low molecular weight, the biogenic amines are detected within 5 minutes, the running time of fish tissue samples is not as linear. The emission of VOCs depends on the fish matrix of each species and thus some compounds can be detected within 5 or more minutes. After several attempts involving more fish samples, two ideal programs for amines and fish samples analysis were developed, with the single difference of the running time, being 5 minutes for BAs and 7 minutes for fish tissues (Table 5.12 and 5.13).

Table 5.12: Program used on GC-IMS measurements, for BAs samples.

Program name: AMINES					
Time	**V**	**R**	**E1**	**E2**	**P1**
00:00, 000	|	rec	150mL/min	50 mL/min	|
00:10, 000	open	|	|	|	|
00:20, 000	close	|	|	|	20%
01:30, 000	|	|	|	10 mL/min	|
03:00, 000	|	|	|	25 mL/min	|
05:00, 020	|	stop	|	35 mL/min	|
05:00, 060					

Table 5.13: Program used on GC-IMS measurements, for fish tissues samples.

Program name: AMINES_FISH					
Time	**V**	**R**	**E1**	**E2**	**P1**
00:00, 000	|	rec	150mL/min	50 mL/min	|
00:10, 000	open	|	|	|	|
00:20, 000	close	|	|	|	20%
01:30, 000	|	|	|	10 mL/min	|
03:00, 000	|	|	|	25 mL/min	|
05:00, 020	|	|	|	35 mL/min	|
07:00, 040	|	stop	|	35 mL/min	|
07:00, 060					

Chapter 6

Results and Discussion

6.1 Detection of biogenic amines

After the development and optimization of the program to measure BAs, all amines samples were analysed in the GC-IMS device at both ambient temperature (23°C) and 40°C.

The spectra obtained are represented below, with yellow rectangle identifying the characteristic peak of each amine, where the x-axis represents the drift time relative to the RIP in milliseconds and the y-axis represents the retention time in seconds (Figures 6.1 to 6.8). Apart from histamine, it was possible to identify one to two signals specific of each amine analysed. The drift position (relative to the RIP) and retention time of all peaks identified are listed in the Table 6.1.

Table 6.1: Drift and retention times of the peaks within the yellow rectangle of Figures 6.1-6.8, being $t_{drift\ position}$ the drift position relative to RIP, t_d the drift time and t_r the retention time.

Biogenic amine	RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	t_r (s)
HIS	7.26	–	–	–
TYR	7.28	1.146	8.338	113
		1.091	7.936	54
TRP	7.27	1.148	8.389	113
		1.093	7.051	54
SPM	7.26	1.070	7.771	80
		1.309	9.506	80
PUT	7.29	1.956	7.706	41
CAD	7.29	1.149	8.361	93
		1.384	10.072	93
SPD	7.30	1.072	7.809	79
		1.309	9.552	79
PEA	7.30	1.071	7.804	84

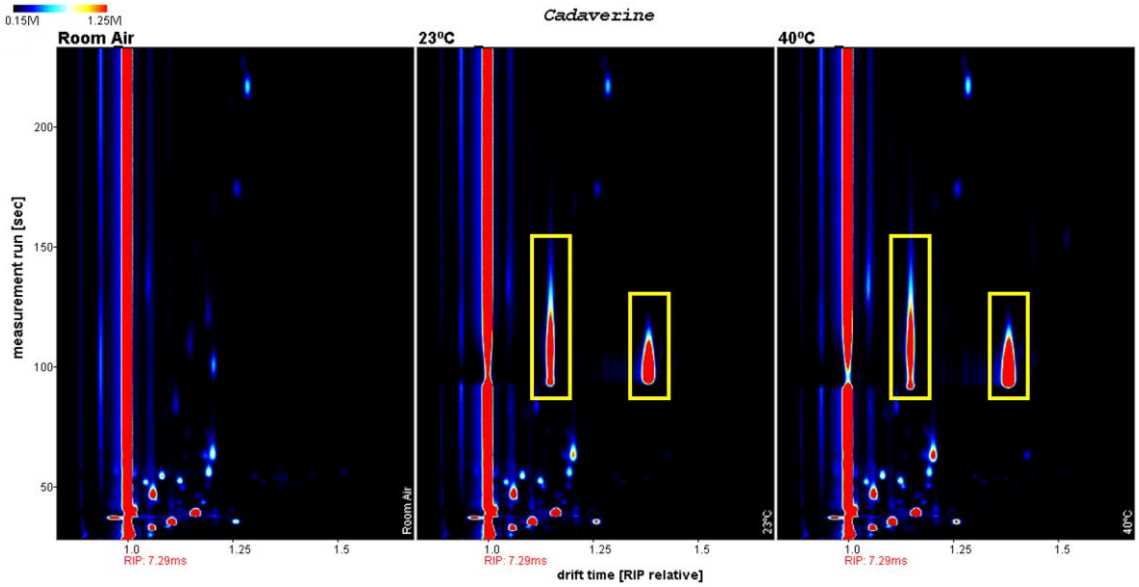


Figure 6.1: Measurements of the room air and cadaverine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

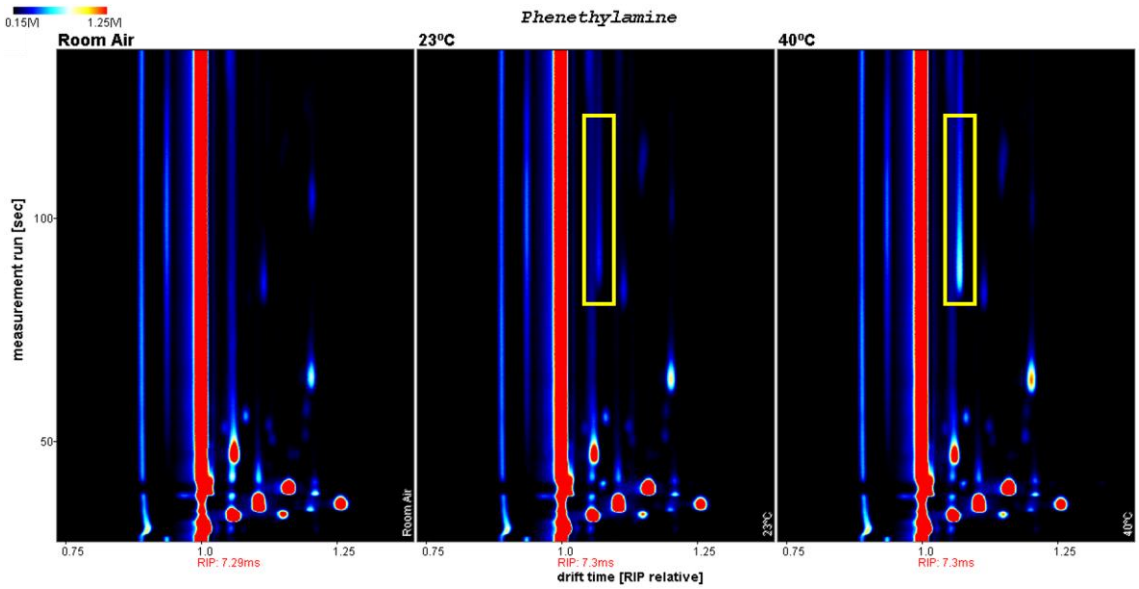


Figure 6.2: Measurements of the room air and phenethylamine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

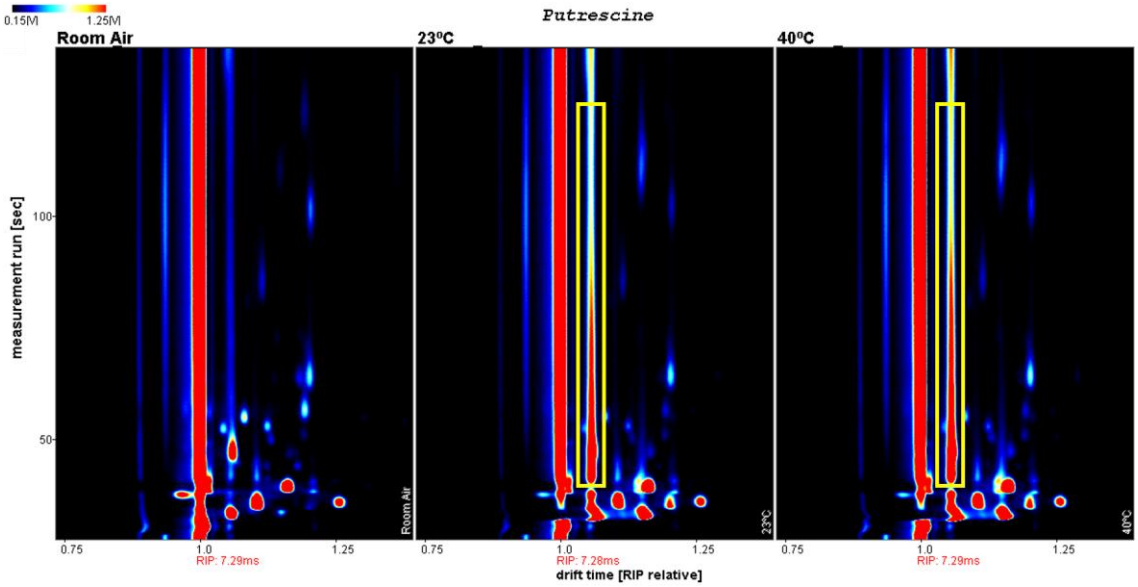


Figure 6.3: Measurements of the room air and putrescine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

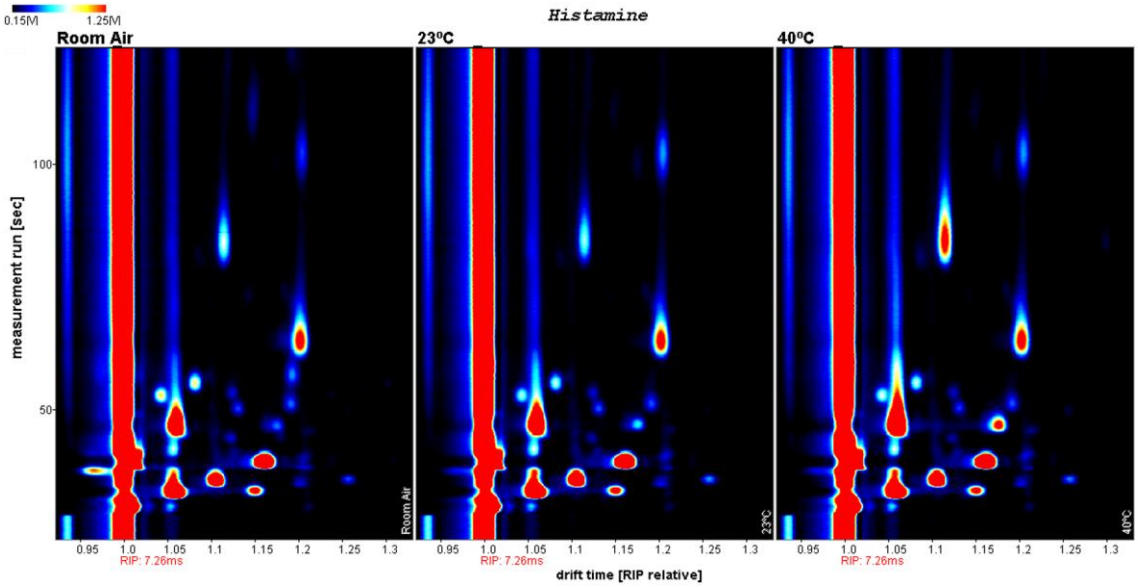


Figure 6.4: Measurements of the room air and histamine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. No differences were observed between room air spectrum and histamine spectrum. Thus, it was not possible to characterize the BA histamine. Measurements acquired using GC-IMS device.

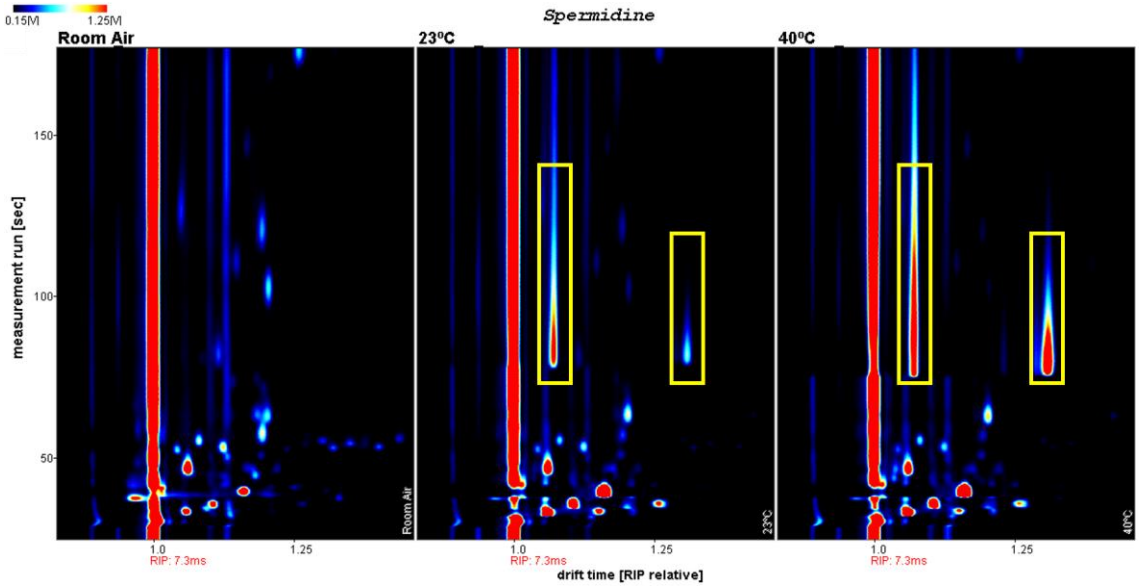


Figure 6.5: Measurements of the room air and spermidine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

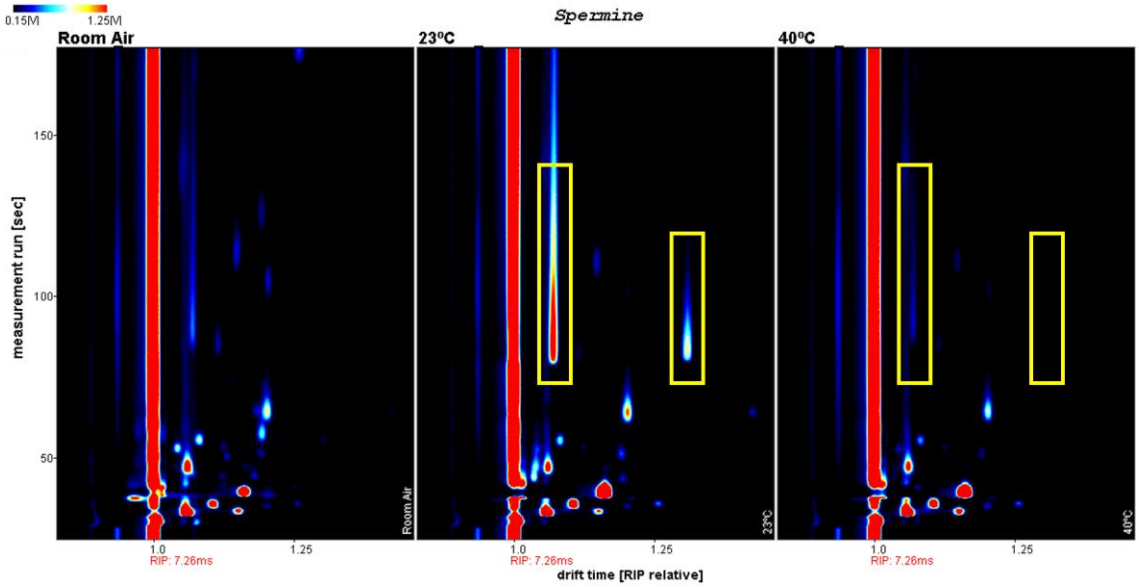


Figure 6.6: Measurements of the room air and spermine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

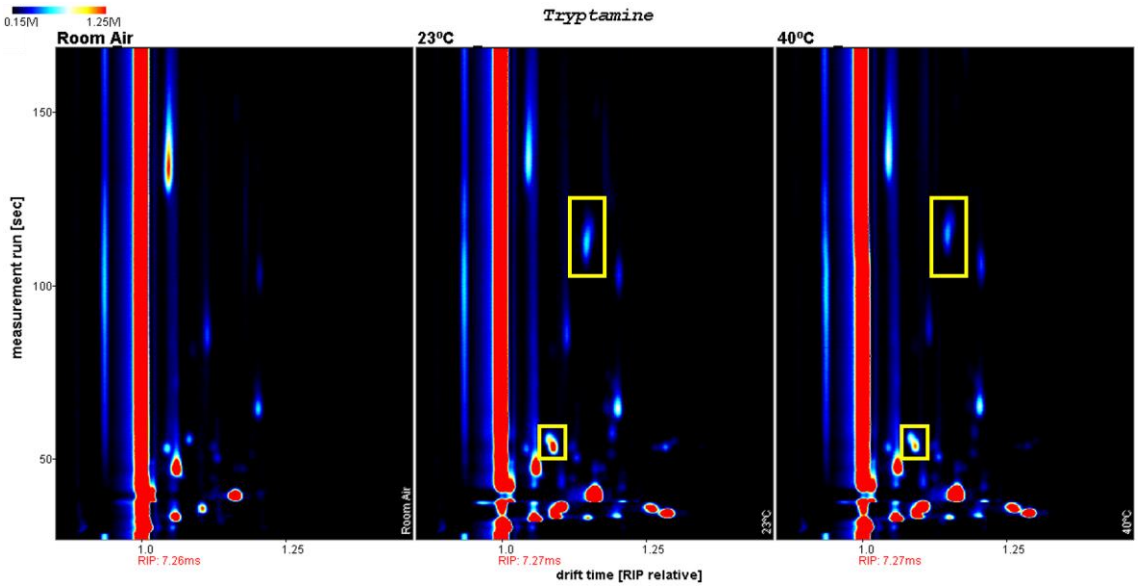


Figure 6.7: Measurements of the room air and tryptamine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

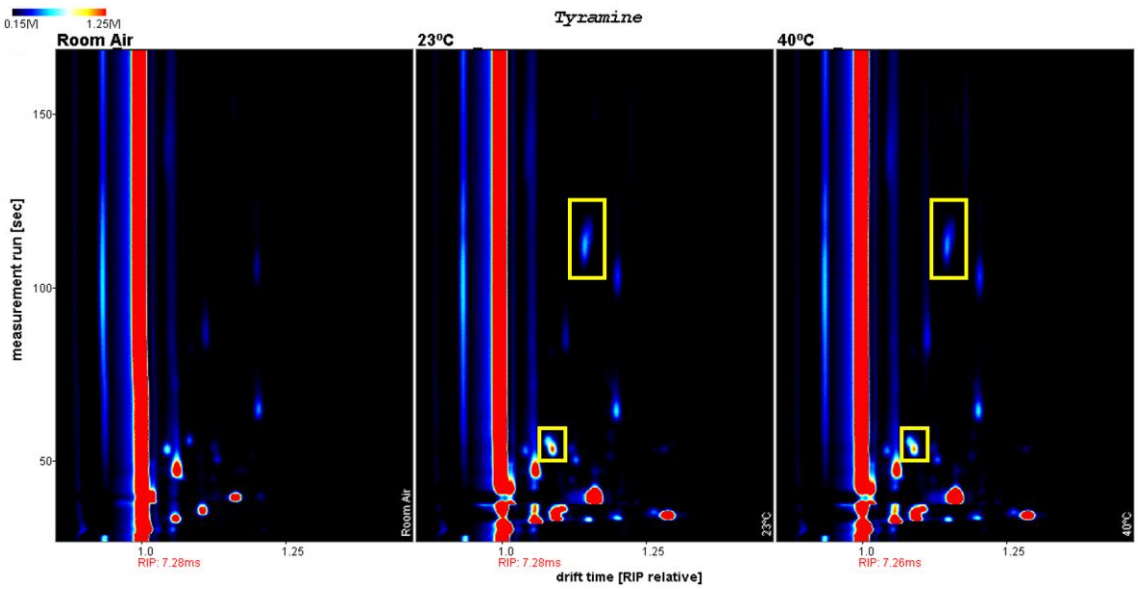


Figure 6.8: Measurements of the room air and tyramine, at ambient temperature (23°C) and 40°C for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

6.1.2 Ion mobility constant: Biogenic amines

Values for reduced ion mobility constant were calculated for the identified peaks of all biogenic amines (Table 6.2). Through the extraction of the retention time, the drift position relative to RIP and the drift time, it is possible to calculate the ion mobility constant and thus characterize each amine, with a substance-specific value. The calculation of the reduced ion mobility constant follows the Equations 4.1 and 4.2, described in Chapter 4:

$$K = \frac{v_d}{E} = \frac{L}{t_d E} = \frac{L^2}{t_d U} \qquad K_0 = K\left(\frac{P}{P_0}\right)\left(\frac{T_0}{T}\right)$$

Where:

		Value	Expressed in
Drift tube length	L	9.8	cm
Drift potential difference	U	5000	V
Drift tube temperature	T	343.16	K
Standard temperature	T_0	276.16	K
Drift tube pressure	P	5.64	Torr
Atmospheric pressure	P_0	760	Torr

Table 6.2: Ion mobility constant for the detected BAs presented in Table 6.1.

Biogenic amine	RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	$K_{,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	$K_{0,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	K_{t_d} ($cm^2V^{-1}s^{-1}$)	K_{0,t_d} ($cm^2V^{-1}s^{-1}$)
TYR	7.28	1.146	8.338	2.3023	0.0138	2.3037	0.0138
		1.091	7.936	2.4184	0.0144	2.4204	0.0145
TRP	7.27	1.148	8.389	2.3014	0.0137	2.2900	0.0137
		1.093	7.051	2.4172	0.0144	2.7241	0.0163
SPM	7.26	1.070	7.771	2.4727	0.0148	2.4718	0.0148
		1.309	9.506	2.0212	0.0121	2.0206	0.0121
PUT	7.29	1.956	7.706	1.3471	0.0080	2.3037	0.0138
CAD	7.29	1.149	8.361	2.2932	0.0137	2.2971	0.0137
		1.384	10.072	1.9038	0.0114	1.9071	0.0114
SPD	7.30	1.072	7.809	2.4545	0.0147	2.4597	0.0147
		1.309	9.552	2.0101	0.0120	2.0109	0.0120
PEA	7.30	1.071	7.804	2.4568	0.0147	2.4613	0.0147

6.2 Fresh fish decomposition

The fish decay takes place through several biological processes, which lead to the production of different metabolic products. Muscle and fat (rancidity) decay and bacterial growth are three key processes of fish spoilage.

Upon death, the microorganisms present in the fish quickly proliferate and through the action decarboxylase enzymes proteolysis takes place, initiating a breakdown of fish muscles. While muscle decay produces monoamine and polyamine by-products, fat decay produces volatiles through lipid oxidation such as aldehydes, ketones, fatty acids and alcohols [117].

The presence of biogenic is currently the most reliable indicator of fish decomposition, due to their association with organoleptic evaluation of fish freshness. Very low molecular weight compounds such as ammonia, DMA and TMA are often present in fish tissues when spoilage takes place. DMA and TMA reduced by trimethylamine oxide (TMAO) and ammonia are products of urea and amino acids decomposition [117][118].

Although ammonia, DMA and TMA are gases at room temperature, they present different boiling points (Table 6.3).

Table 6.3: Molecular weights and boiling points of ammonia, DMA and TMA [118]**.**

Compound	Molecular Weight ($g\ mol^{-1}$)	Boiling Point
Ammonia	17.031	-33°C
DMA	45.085	8°C
TMA	59.112	5°C

In a closed chamber the vapor pressure of a compound is proportional to its concentration in the water part of the fish meat. The ammonia having the lower boiling point is the most volatile of the three compounds and has probably the highest proportional coefficient [118].

Ammonia was identified (green rectangle) in all fish species studied: atlantic bonito, atlantic horse mackerel and sardine. Mackarel presented the higher concentration of all three, which might be explained by its high spoilage rate.

Possible traces of some biogenic amines were found in all fish species, however with both drift and retention times deviations from the peaks identified for pure amine's samples (Table 6.4). Sardine only presented traces of cadaverine, tryptamine and tyramine (Figure 6.11).

Table 6.4: Identification of peaks and drift and retention times for traces found in fish, being $t_{drift\ position}$ the drift position relative to RIP, t_d the drift time and t_r the retention time.

Compound	Peak identified (rectangle colour)	RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	t_r (s)
Ammonia	Green	7.30	0.893	6.512	29
HIS	–	–	–	–	–
TYR **TRP**	White	7.30	1.122	8.198	53
SPM **SPD**	Red	7.30	1.390	10.160	153
PUT	–	–	–	–	–
CAD	Yellow	7.30	1.074 1.288	7.855 9.426	152 152
PEA	Pink	7.30	1.143	8.328	153

On the third day of all measurements, an unpleasant odour took place, indicating the fish decay. Although TMA is the main responsible for the "fishy" odour, other compounds may also contribute [105]. It was then stablished that in the third day after fish purchase, the fish is considered spoiled.

Observing the spectra of the three fish species of each day, mackerel presents the highest content of VOCs. Sardine, although visible more degraded than bonito and mackerel, shows a low content of volatile compounds comparing with mackerel. The continuously fish decay over the days enhance the VOCs emission, which will quickly saturate the headspace, causing a saturation behaviour on all spectra, particularly on the third day after purchase.

Atlantic bonito presents traces of six biogenic amines (Figure 6.9) and with a higher concentration than the amines identified in sardine and mackerel.

Atlantic bonito (*Sarda sarda*)

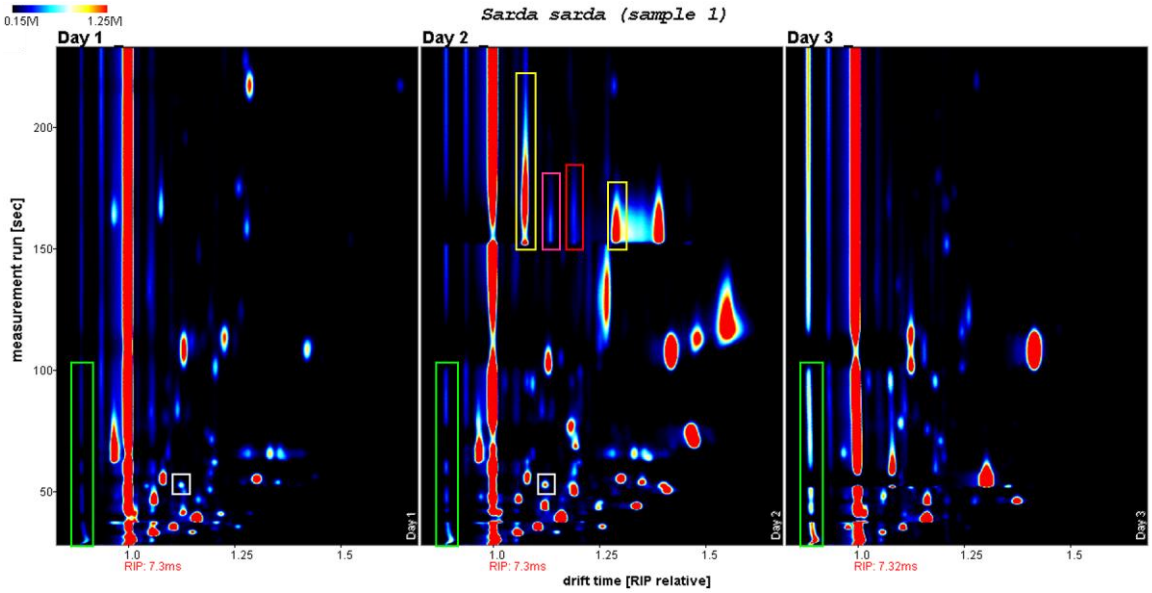


Figure 6.9: Measurements of *Sarda sarda* sample 1, at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Green, yellow, pink, red and white rectangles correspond to ammonia, CAD, PEA, SPD and SPM, and TRP and TYR, respectively. Measurements acquired during the three days after fish purchase, at the same time of day, using GC-IMS device.

Atlantic horse mackerel (*Trachurus trachurus*)

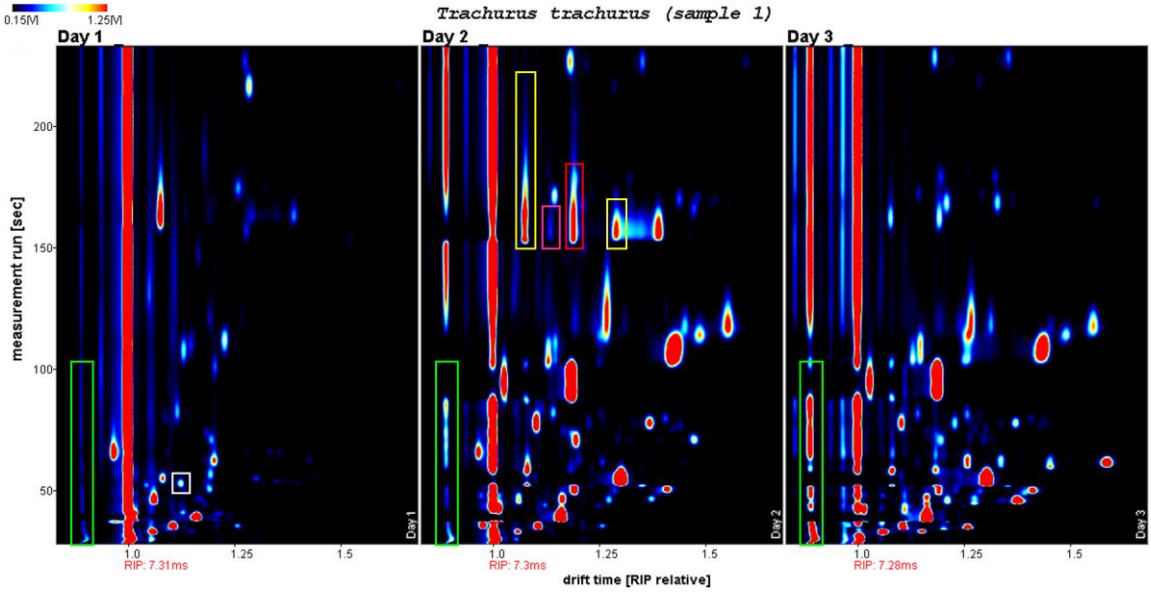


Figure 6.10: Measurements of *Trachurus trachurus* sample 1, at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Green, yellow, pink and red rectangles correspond to ammonia, CAD, PEA and, SPD and SPM, respectively. Measurements acquired during the three days after fish purchase, at the same time of day, using GC-IMS device.

Sardine (*Sardina pilchardus*)

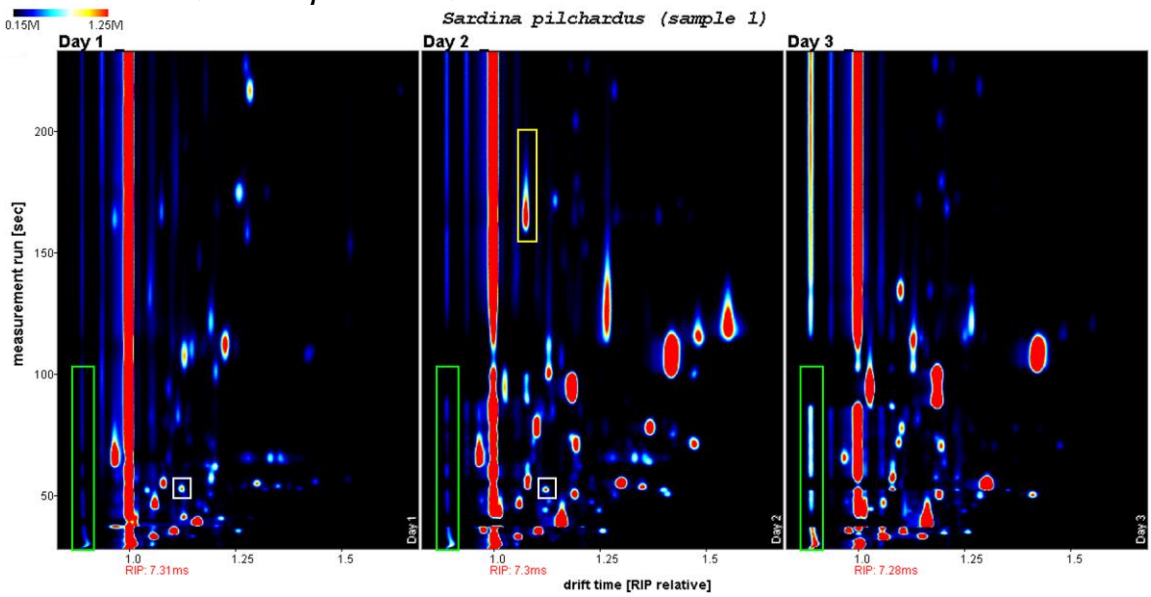


Figure 6.11: Measurements of *Sardina pilchardus* sample 1, at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Green and yellow rectangles correspond to ammonia and CAD, respectively. Measurements acquired during the three days after fish purchase, at the same time of day, using GC-IMS device.

6.2.2 Ion mobility constant: Biogenic amines in fish

Similarly to the previous subchapter, the reduced ion mobility constant was calculated for the compounds detected and listed in Table 6.5. All values presented are arithmetic averages of values of all fish species samples that presented the correspondent compounds.

Table 6.5: Ion mobility constant for the compounds detected in all fish species (Table 6.4).

Peak	RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	$K_{,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	$K_{0,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	K_{t_d} ($cm^2V^{-1}s^{-1}$)	K_{0,t_d} ($cm^2V^{-1}s^{-1}$)
Ammonia	7.30	0.893	6.512	0.4041	0.0024	2.9500	0.0176
TYR **TRP**	7.30	1.122	8.198	0.3209	0.0019	2.3430	0.0140
SPM **SPD**	7.30	1.390	10.16	0.2590	0.0015	1.8910	0.0113
CAD	7.30	1.074 1.288	7.855 9.426	0.3350 0.2791	0.0020 0.0017	2.4453 2.0378	0.0146 0.0122
PEA	7.30	1.143	8.328	0.3160	0.0019	2.3064	0.0138

Fish species spectra comparation for each day

The Figures 6.12 to 6.15 present two types of signals: room air signals and characteristic fish signals. The signals of room air are identified by orange rectangles, while fish characteristic compounds are within green rectangles.

It is verified a correlation between all three fish species regarding the signals of VOCs emitted. Compounds concentrations may differ due to sample weight differences and distinct fish matrix according to each species. The latter may also explain the deviation of retention times of the peaks.

Over the days, the fish decay is notorious with the emission of more volatile compounds. These accompany the active progression of the "fishy" odour resulted from the fish spoilage. The presence of cadaverine was observed on all fish species, along with other unidentified compounds, at the first two days of fish decomposition. Thus on the second day of fish storage under room temperature conditions, TMA and other volatile amine compounds show an extent increase following the fish decay.

On the fourth day of measurements, the fish is already spoiled and thus the spectra present the same signals with slight intensity differences as those identified on the third day (Figure 6.14 and 6.15).

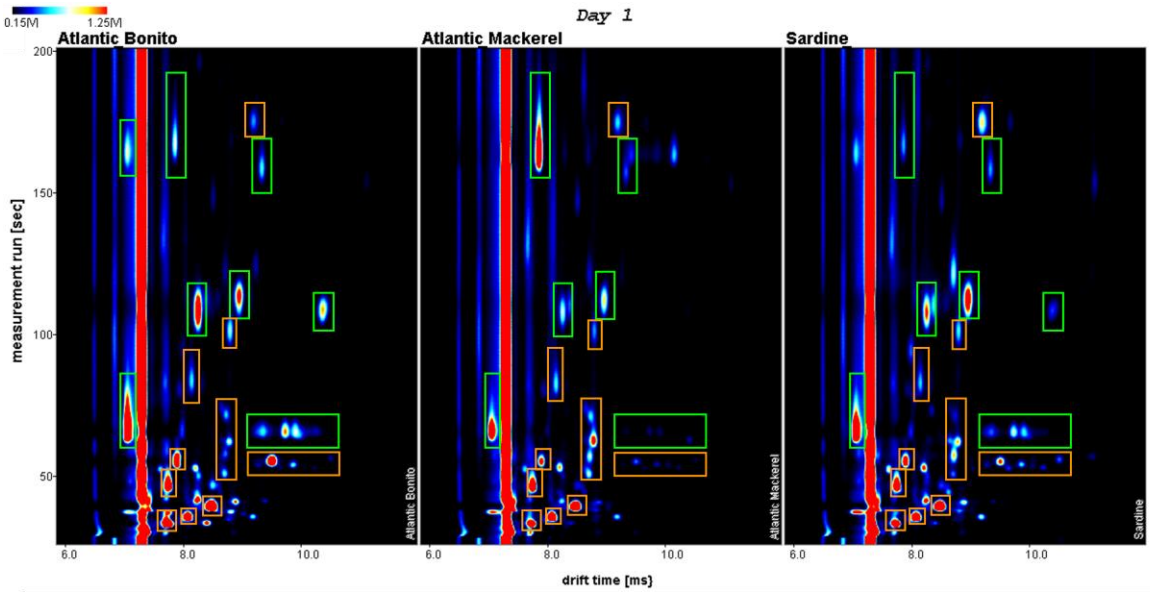


Figure 6.12: First day measurements of all three species (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. The signals of room air are identified by orange rectangles, while fish characteristic compounds are within green rectangles. Measurements acquired using GC-IMS device.

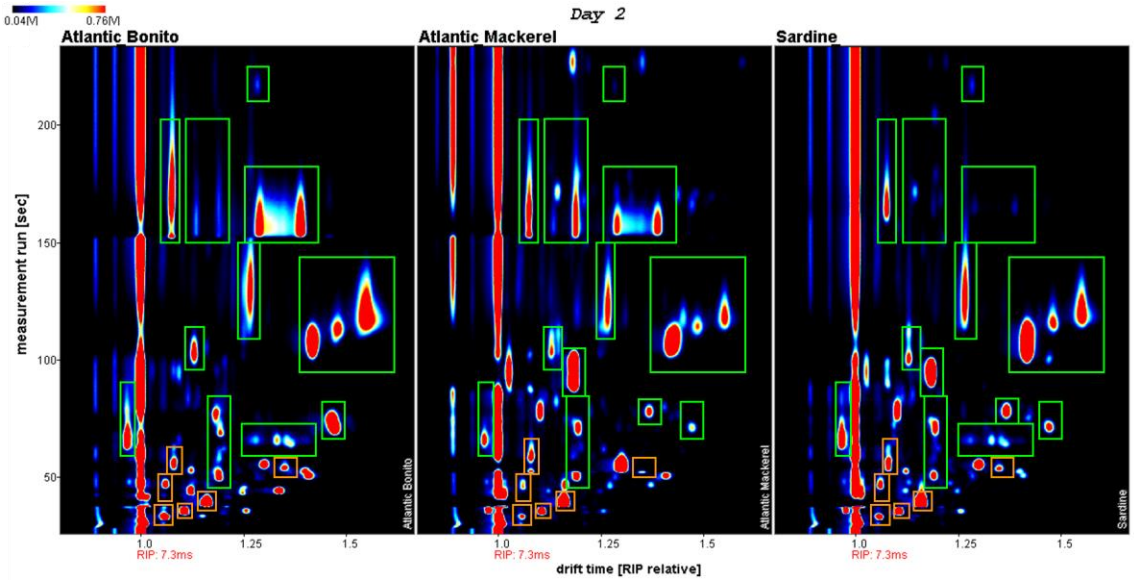


Figure 6.13: Second day measurements of all three species (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. The signals of room air are identified by orange rectangles, while fish characteristic compounds are within green rectangles. Measurements acquired using GC-IMS device.

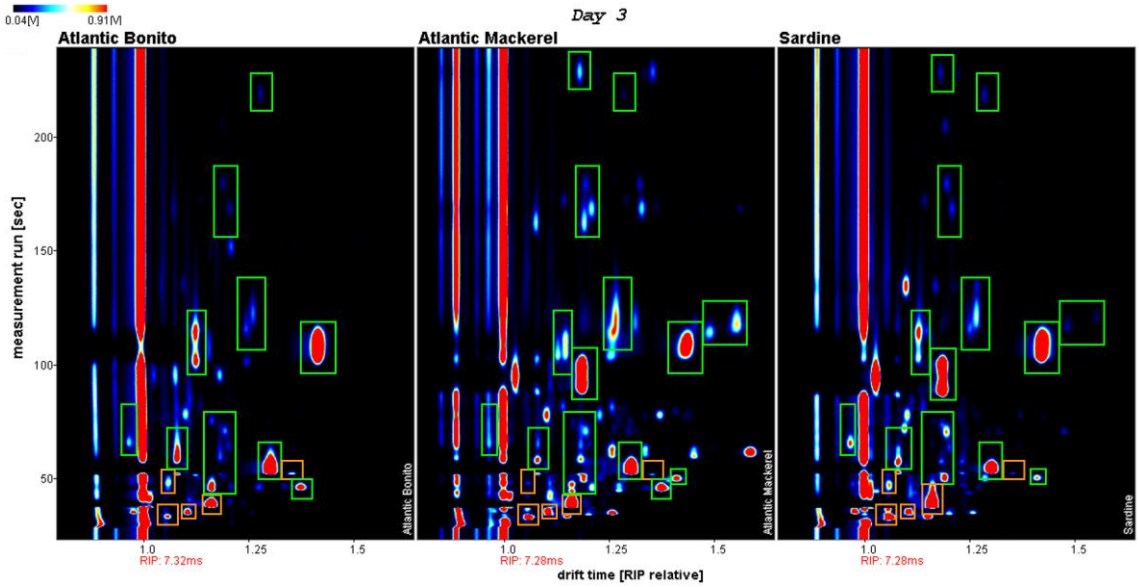


Figure 6.14: Third day measurements of all three species (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. The signals of room air are identified by orange rectangles, while fish characteristic compounds are within green rectangles. Measurements acquired using GC-IMS device.

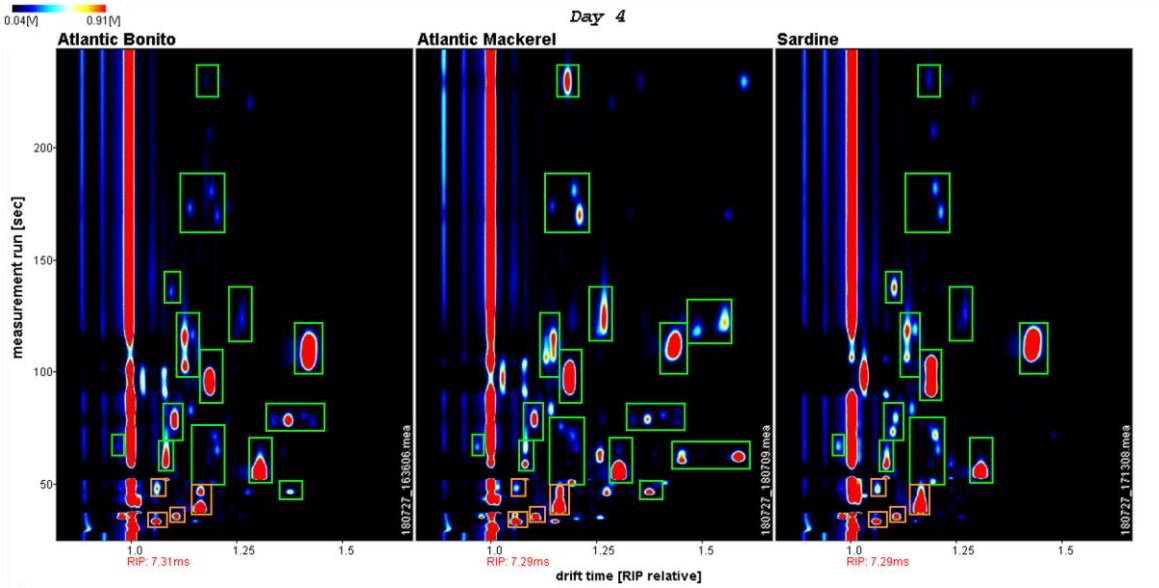


Figure 6.15: Fourth day measurements of all three species (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. The signals of room air are identified by orange rectangles, while fish characteristic compounds are within green rectangles. Measurements acquired using GC-IMS device.

6.3 Fish control samples

Sarda sarda, an Atlantic Scombridae species, was used as model organism to develop a sensory classification scale of seafood quality. Samples were prepared as described in Chapter 5.

Sarda sarda samples were then treated and analysed by GC-IMS, for biogenic amines detection and comparation with fresh fish samples.

It is possible to verify a higher fish decay on the spectra of control fish samples comparing with fresh fish samples (Figure 6.18), which might be due to the long storage time at very low temperatures (−80°C) of control fish samples. However, low storage temperature is considered safe regarding the control of the production of biogenic amines.

First and eighth day of fresh fish samples of *Sarda sarda* and control samples T0 and T7 were compared in Figures 6.18 and 6.19. The common signals between both spectra are identified by green rectangles. The reduced ion mobility constant was calculated for the compounds detected and listed in Tables 6.8 and 6.9. It was not possible to identify any of the BAs previously analysed.

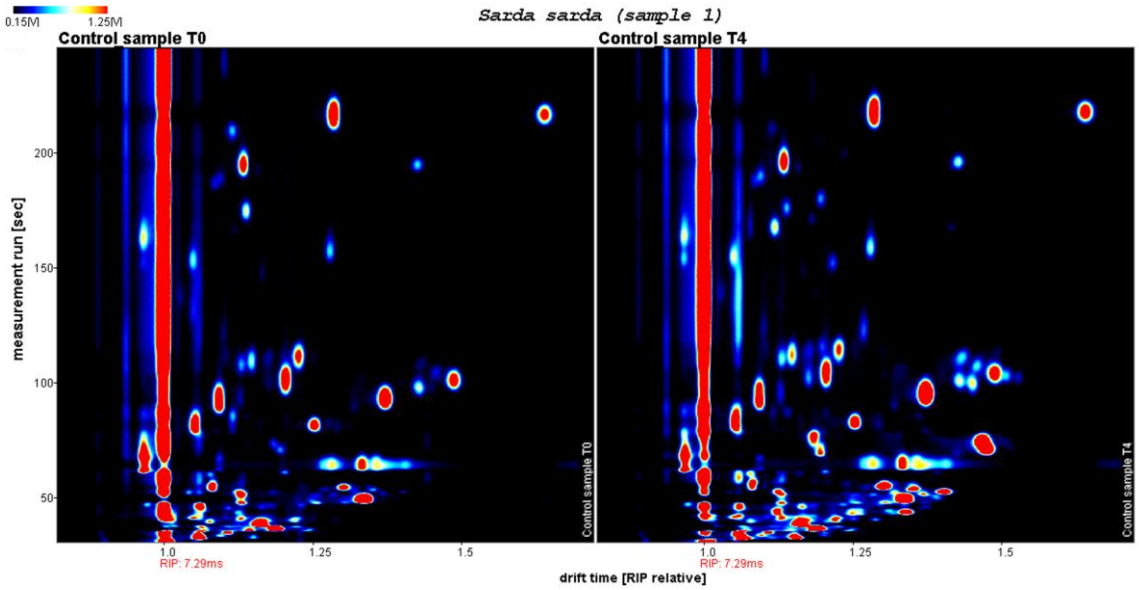


Figure 6.16: Measurements of control samples T0 and T4 of *Sarda sarda* (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

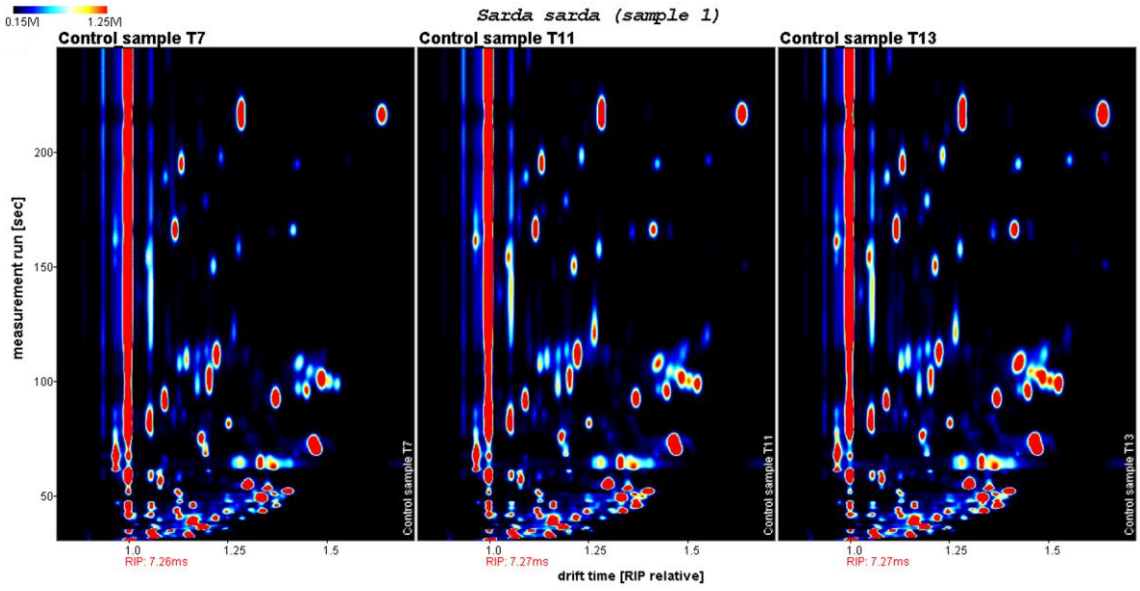


Figure 6.17: Measurements of control samples T7, T11 and T13 of *Sarda sarda* (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. Measurements acquired using GC-IMS device.

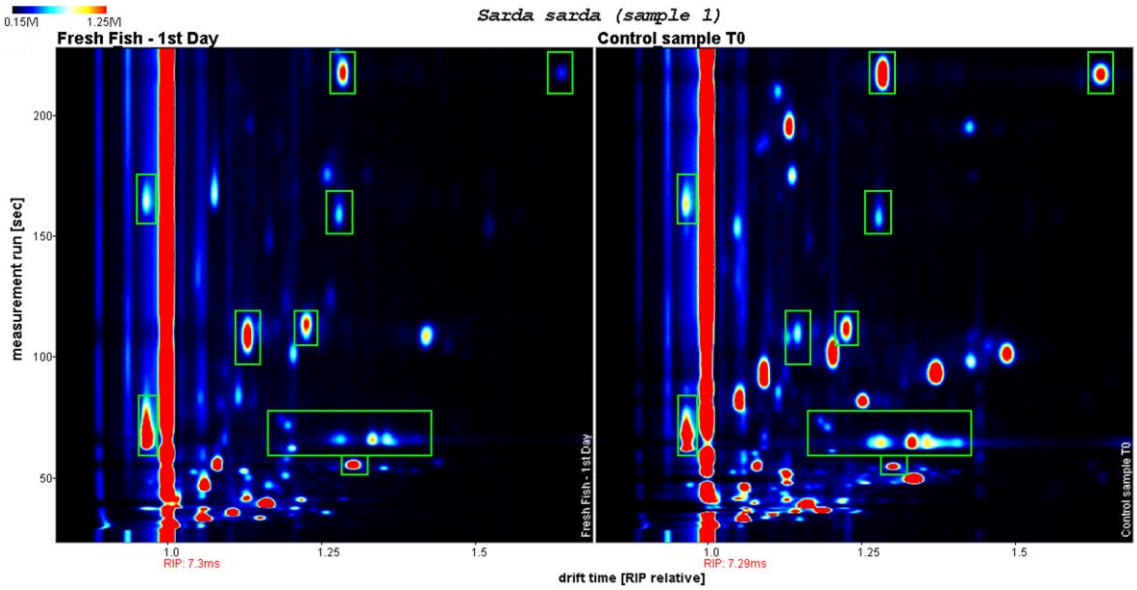


Figure 6.18: Measurements of the first day of fresh fish sample and control samples T0 of *Sarda sarda* (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. The common signals between both spectra are identified by green rectangles. Measurements acquired using GC-IMS device.

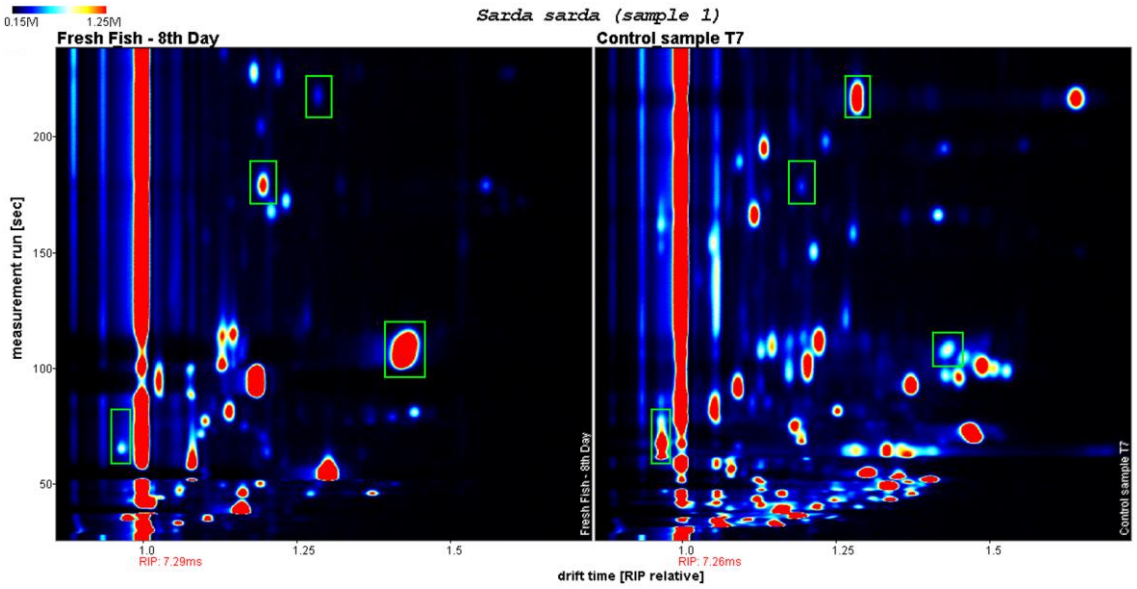


Figure 6.19: Measurements of the eighth day of fresh fish sample and control samples T7 of *Sarda sarda* (sample 1), at ambient temperature (23°C) for a drift gas flow of 150 mL/min and carrier gas flow of 50 mL/min. The common signals between both spectra are identified by green rectangles. Measurements acquired using GC-IMS device.

Table 6.6: Drift and retention times for traces found in Figure 6.18 within green rectangles, being $t_{drift\ position}$ the drift position relative to RIP, t_d the drift time and t_r the retention time.

RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	t_r (s)
7.30	1.203	8.803	63
7.30	1.282	9.352	66
7.30	1.333	9.743	66
7.30	1.358	9.912	66
7.30	1.408	10.281	66
7.30	0.968	7.062	68
7.30	1.197	8.729	72
7.30	1.185	8.655	75
7.30	1.130	8.256	109
7.30	1.227	8.955	114
7.30	1.281	9.337	159
7.30	0.966	7.044	165
7.30	1.286	9.398	218
7.30	1.642	11.966	218

Table 6.7: Drift and retention times for traces found in Figure 6.19 within green rectangles, being $t_{drift\ position}$ the drift position relative to RIP, t_d the drift time and t_r the retention time.

RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	t_r (s)
7.29	0.967	7.055	66
7.29	1.428	10.395	108
7.29	1.196	8.723	180
7.29	1.285	9.387	219

Table 6.8: Ion mobility constant for the common compounds detected in fresh fish sample and control samples T0 (Table 6.6).

RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	$K_{,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	$K_{0,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	K_{t_d} ($cm^2V^{-1}s^{-1}$)	K_{0,t_d} ($cm^2V^{-1}s^{-1}$)
7.30	1.203	8.803	2.1872	0.0137	2.1820	0.0130
7.30	1.282	9.352	2.0524	0.0123	2.0539	0.0123
7.30	1.333	9.743	1.9739	0.0118	1.9715	0.0118
7.30	1.358	9.912	1.9376	0.0116	1.9379	0.0116
7.30	1.408	10.281	1.8688	0.0112	1.8683	0.0112
7.30	0.968	7.062	2.7182	0.0162	2.7199	0.0162
7.30	1.197	8.729	2.1982	0.0131	2.2005	0.0131
7.30	1.185	8.655	2.2204	0.0133	2.2193	0.0133
7.30	1.130	8.256	2.3285	0.0139	2.3266	0.0139
7.30	1.227	8.955	2.1444	0.0128	2.1449	0.0128
7.30	1.281	9.337	2.0540	0.0123	2.0572	0.0123
7.30	0.966	7.044	2.7238	0.0163	2.7269	0.0163
7.30	1.286	9.398	2.0461	0.0122	2.0438	0.0122
7.30	1.642	11.966	1.6025	0.0096	1.6052	0.0096

Table 6.9: Ion mobility constant for the common compounds detected in fresh fish sample and control samples T7 (Table 6.7).

RIP (ms)	$t_{drift\ position}$ (ms)	t_d (ms)	$K_{,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	$K_{0,drift\ position}$ ($cm^2V^{-1}s^{-1}$)	K_{t_d} ($cm^2V^{-1}s^{-1}$)	K_{0,t_d} ($cm^2V^{-1}s^{-1}$)
7.29	0.967	7.055	2.7248	0.0163	2.7226	0.0163
7.29	1.428	10.395	1.8451	0.0110	1.8478	0.0110
7.29	1.196	8.723	2.2030	0.0132	2.2020	0.0132
7.29	1.285	9.387	2.0505	0.0122	2.0462	0.0122

Chapter 7

Conclusions and Future Perspectives

Being volatile amines the most indicative substances of fish spoilage, the principle of this thesis was based on finding markers, specific patterns which are characteristic for a specific type of compounds, particularly biogenic amines, and thus stablishing a correlation between fish matrices and the VOCS emitted by amines.

Through two-dimensional representations of VOCs emitted by both amines and fish tissues, the analysis by GC-IMS allowed the assignment of different patterns of seven of the most important biogenic amines in food products. Chemical fingerprints were obtained for three different fish species, through the analysis of fish decay from the emission of odour compounds. Comparing with MCC-IMS, the GC-IMS technique has proven to have a higher sensitivity for shorter analysis time with an exceptional repeatability, being ideal for monitoring food freshness and decomposition, and thus providing an alternative to other monitoring applications such as electronic noses. Additionally, the discrimination ability of the device can be enhanced with the increase of the current database of identified volatile compounds, through the analysis of a large number of control samples.

Although MCC-IMS provides a high selectivity due to the pre-separation of the samples in the multi capillary column, this technique is not the most appropriate regarding fish tissues analysis, due to the accumulation of VOCs in the sample loop which causes a high saturation in spectra.

Researches on BAs production in fish and fish products have been focused on histamine toxicology having reported a direct implication of the families Scombridae and Scomberesocideae in histamine poisoning. Being responsible for the most serious foodborne intoxications, histamine represents the most important biogenic amine. Its detection did not have satisfactory results most likely due to the low amount of amine available for measurement.

Regarding fish decay, it is important to take in consideration the possible reaction of BAs such as putrescine, cadaverine, spermine and spermidine, with nitrite present in fish tissues, which may lead to nitrosamines production. The presence of nitrosamines and other characteristic fish compounds in fish matrices such as aldehydes, ketones and alcohols can explain the differences in peaks positions of BAs in fish samples spectra.

Although reduced ion mobility constants were calculated for pure amines and compounds identified as possible biogenic amines in all fish species, the identification of the volatiles compounds was not possible due to the parameters variation on the techniques applied for the analysis of this type of compounds.

Further research objectives will include the treatment and analysis of higher histamine concentrations in GC-IMS, as well as the validation of once more the detection of the already identified biogenic amines. An expansion of the GC-IMS library with VOCs data will also facilitate volatile amines detection and further identification.

Another approach for the detection of volatile amines would be measuring samples headspace when heated to higher temperatures than 40°C. Reaching the boiling point temperatures of each compound would be ideal to maximize its volatility.

Further experimentation with fish samples can also be accomplished. Instead of the sample preparation method applied in this thesis, fish specimens could be placed into containers with more than two fish (for example, 15 fish in one container) and then tissues samples would be collected daily for GC-IMS analysis. The containers would simulate the conditions of fresh fish in e.g. fish market when placed in pallets for purchase and consumption.

Bibliography

[1] I. Ferreira and O. Pinho, "Biogenic Amines in Portuguese Traditional Foods and Wines," *J. Food Prot.*, vol. 69, no. 9, pp. 2293–2303, 2006.

[2] P. Visciano, M. Schirone, R. Tofalo, and G. Suzzi, "Biogenic amines in raw and processed seafood," *Front. Microbiol.*, vol. 3, p. 10, 2012.

[3] S. Bover-Cid and W. H. Holzapfel, "Improved screening procedure for biogenic amine production by lactic acid bacteria.," *Int. J. Food Microbiol.*, vol. 53, pp. 33–41, 1999.

[4] A. Halász, Á. Baráth, L. Simon-Sarkadi, and W. Holzapfel, "Biogenic amines and their production by microorganisms in food," *Trends Food Sci. Technol.*, vol. 5, no. 2, pp. 42–49, 1994.

[5] J. Horta, A. Monteiro, and P. Ricardo, "Aminoácidos - Verbo," 2000.

[6] M. H. Silla Santos, "Biogenic amines: Their importance in foods," *Int. J. Food Microbiol.*, vol. 29, no. 2–3, pp. 213–231, 1996.

[7] K. Hyland, "Neurochemistry and defects of biogenic amine neurotransmitter metabolism," *J. Inherit. Metab. Dis.*, vol. 22, no. 4, pp. 353–363, 1999.

[8] M. A. Medina, J. L. Urdiales, C. Rodríguez-Caso, F. J. Ramírez, and F. Sánchez-Jiménez, "Biogenic amines and polyamines: similar biochemistry for different physiological missions and biomedical applications.," *Crit. Rev. Biochem. Mol. Biol.*, vol. 38, no. 1, pp. 23–59, 2003.

[9] J. A. O. Ribeiro, "Caraterização e Otimização de Sensores Eletroquímicos para Aminas Biogénicas," 2013.

[10] N. Gouveia, "Desenvolvimento de uma Metodologia Analítica para determinação de Aminas Biogénicas em tunídeos," 2009.

[11] S. Bover-Cid, M. L. Latorre-Moratalla, M. T. Veciana-Nogués, and M. C. Vidal-Carou, "Processing Contaminants: Biogenic Amines," *Encycl. Food Saf.*, vol. 2, pp. 381–391, 2013.

[12] B. ten Brink, C. Damink, H. M. L. J. Joosten, and J. H. J. Huis in't Veld, "Occurrence and formation of biologically active amines in foods," *Int. J. Food Microbiol.*, vol. 11, no. 1, pp. 73–84, 1990.

[13] G. Suzzi and F. Gardini, "Biogenic amines in dry fermented sausages: a review," *Int. J. Food Microbiol.*, vol. 88, no. 1, pp. 41–54, 2003.

[14] S.-H. Kim, B. Ben-Gigirey, and E. Alii, "Histamine and Biogenic Amine Production by Morganella morganii Isolated from Temperature - Abused Albacore," *J. Food Prot.*, vol. 63, no. 2, pp. 244–251, 2000.

[15] B. Kim, B. Y. Byun, and J. H. Mah, "Biogenic amine formation and bacterial contribution in Natto products," *Food Chem.*, vol. 135, no. 3, pp. 2005–2011, 2012.

[16] D. H. Pimentel De Araujo, "Determinação de histamina e outras aminas bioativas e perfil de ácidos graxos de peixes da região amazônica," pp. 1–101, 2013.

[17] J. de O. Fernandes, "Desenvolvimento de metodologias de cromatografia gasosa – espectrometria de massa para a determinação de aminas biogénicas em vinhos do Porto e em mostos," 2001.

[18] J. Karovicova and Z. Kohajdova, "Biogenic Amines in Food," *Chem. Pap.*, vol. 59, no. 1, pp. 70–79, Aug. 2005.

[19] G. Tabanelli, C. Montanari, and F. Gardini, "Biogenic Amines in Food: A Review of Factors Affecting Their Formation," *Ref. Modul. Food Sci.*, pp. 1–7, 2018.

[20] E. Bargossi, F. Gardini, V. Gatto, C. Montanari, S. Torriani, and G. Tabanelli, "The Capability of Tyramine Production and Correlation between Phenotypic and Genetic Characteristics of Enterococcus faecium and Enterococcus faecalis Strains," *Front. Microbiol.*, vol. 6, no. Article 1371, pp. 1–12, 2015.

[21] F. Gardini, Y. Özogul, G. Suzzi, G. Tabanelli, and F. Özogul, "Technological factors affecting biogenic amine content in foods: A review," *Front. Microbiol.*, vol. 7, no. AUG, pp. 1–18, 2016.

[22] J. Stadnik and Z. J. Dolatowski, "Biogenic Amines in Meat and Fermented Meat Products," *Acta Sci. Pol., Technol. Aliment.*, vol. 9, no. 3, pp. 251–263, 2010.

[23] F. Liu *et al.*, "Heterologous Expression and Characterization of Tyrosine Decarboxylase from Enterococcus faecalis R612Z1 and Enterococcus faecium R615Z1," *J. Food Prot.*, vol. 77, no. 4, pp. 592–598, 2014.

[24] G. Tabanelli, S. Torriani, F. Rossi, L. Rizzotti, and F. Gardini, "Effect of Chemico-Physical Parameters on the Histidine Decarboxylase (HdcA) Enzymatic Activity in Streptococcus thermophilus PRI60," *J. Food Sci.*, vol. 00, no. 0, 2012.

[25] S. Bover-Cid *et al.*, "Relationships between microbial population dynamics and putrescine and cadaverine accumulation during dry fermented sausage ripening," *J. Appl. Microbiol.*, vol. 106, no. 4, pp. 1397–1407, 2009.

[26] F. Gardini *et al.*, "Modeling the Aminogenic Potential of Enterococcus faecalis EF37 in Dry Fermented Sausages through Chemical and Molecular Approaches," *Appl. Environ. Microbiol.*, vol. 74, no. 9, pp. 2740–2750, 2008.

[27] N. Zdolec, *Fermented Meat Products: Health Aspects*. CRC Press, 2016.

[28] S. H. Arnold and W. D. Brown, *Histamine toxicity from fish products*, vol. 24, no. C. 1978.

[29] D. A. Watts and W. D. Brown, "Histamine Formation in Abusively Stored Pacific Mackerel: Effect of CO2-Modified Atmosphere," *J. Food Sci.*, vol. 47, no. 4, pp. 1386–1387, 1982.

[30] T. K. Nahla and H. E.-S. M. Farag, "Histamine and Histamine Producing Bacteria in Some Local and Imported Fish and Their Public Health Significance," *Res. J. Agric. Biol. Sci.*, vol. 1, no. 4, pp. 329–336, 2005.

[31] B. Bilgin and H. Gençcelep, "Determination of biogenic amines in fish products,"

Food Sci. Biotechnol., vol. 24, no. 5, pp. 1907–1913, 2015.

[32] A. R. Shalaby, S. Kurt, and O. Zorba, "Significance of biogenic amines to food safety and human health," *Food Res. Int.*, vol. 29, no. 15, pp. 675–690, 1996.

[33] B. ten Brink, C. Damink, H. M. L. J. Joosten, and J. H. J. Huis in 't Veld, "Occurrence and formation of biologically active amines in foods," *Int. J. Food Microbiol.*, vol. 11, pp. 73–84, 1990.

[34] H. G. Schwelberger, F. Ahrens, W. A. Fogel, and F. Sánchez-Jiménez, "Chapter 3: Histamine Metabolism," in *Histamine H4 Receptor: A Novel Drug Target in Immunoregulation and Inflammation*, 2013, pp. 63–101.

[35] T. Biegański, J. Kusche, W. Lorenz, R. Hesterberg, C. D. Stahlknecht, and K. D. Feussner, "Distribution and properties of human intestinal diamine oxidase and its relevance for the histamine catabolism," *Biochim. Biophys. Acta*, vol. 756, no. 2, pp. 196–203, 1983.

[36] J. Sattler, W. Lorenz, K. Kubo, A. Schmal, S. Sauer, and L. Lüben, "Food-induced histaminosis under diamine oxidase (DAO) blockade in pigs: Further evidence of the key role of elevated plasma histamine levels as demonstrated by successful prophylaxis with antihistamines," *Agents Actions*, vol. 27, no. 1/2, pp. 212–214, 1989.

[37] O. Sjaastad and O. V. Sjaastad, "Catabolism of Orally Administered l4C-Histamine in Man," *Acta pharmacol. toxicol.*, vol. 34, pp. 33–45, 1974.

[38] J. E. Stratton, R. W. Hutkins, and S. L. Taylor, "Biogenic Amines in Cheese and other Fermented Foods: A Review," *J. Food Prot.*, vol. 54, no. 6, pp. 460–470, 1991.

[39] L. Lehane and J. Olley, "Histamine fish poisoning revisited," *Int. J. Food Microbiol.*, vol. 58, p. 37, 2000.

[40] M. B. R. Rodriguez, C. da S. Carneiro, M. B. da S. Feijó, C. A. C. Júnior, and S. B. Mano, "Bioactive Amines: Aspects of Quality and Safety in Food," *Food Nutr. Sci.*, vol. 05, no. 02, pp. 138–146, 2014.

[41] K. P. Rai, H. R. Pradhan, B. K. Sharma, and S. K. Rijal, "Histamine in Foods: Its Safety and Human Health Implications," *J. Food Sci. Technol.*, vol. 8, pp. 1–11, 2013.

[42] Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), *Public Health Risk of Histamine and other Biogenic Amines from Fish and Fshery Products*. 2012.

[43] Biomed Limited, "Noradrenaline Infusion - Data Sheet," 2010.

[44] J. H. Hotchkiss, "A review of current literature of N-nitroso compounds in foods," *Adv. Food Res.*, vol. 31, pp. 53–115, 1988.

[45] J. J. Warthesen, R. A. Scanlan, D. D. Bills, and L. M. Libbey, "Formation of Heterocyclic N-Nitrosamines from the Reaction of Nitrite and Selected Primary Diamines and Amino Acids," *J. Agric. Food Chem.*, vol. 23, no. 5, pp. 898–902, 1975.

[46] T. Gough, K. Webb, and R. Coleman, "Estimate of the volatile nitrosamine content of UK food," *Nature*, vol. 272, pp. 161–163, 1978.

[47] E. J. Mitacek *et al.*, "Exposure to N-Nitroso Compounds in a Population of High Liver Cancer Regions in Thailand: Volatile Nitrosamine (VNA) Levels in Thai Food," *Food Chem. Toxicol.*, vol. 37, pp. 297–305, 1999.

[48] W. M. Dabrowski and Z. E. Sikorski, *Toxins in Food*. CRC Press, 2005.

[49] L. Nakovich, "Analysis of Biogenic Amines by GC/FID and GC/MS," p. 70, 2003.

[50] L. Gram and H. H. Huss, "Microbiological spoilage of fish and fish products," *Int. J. Food Microbiol.*, vol. 33, no. 1, pp. 121–137, 1996.

[51] I. Bulushi, S. Poole, H. Deeth, and G. Dykes, "Biogenic Amines in Fish: Roles in Intoxication, Spoilage, and Nitrosamine Formation - A Review," *Crit. Rev. Food Sci. Nutr.*, vol. 49, pp. 369–377, 2009.

[52] M. B. A. Glória, M. A. Daeschel, C. Craven, and K. S. Hilderbrand, "Histamine and Other Biogenic Amines in Albacore Tuna," *J. Aquat. Food Prod. Technol.*, vol. 8, no. 4, pp. 55–69, 1999.

[53] C. Feng, S. Teuber, and M. E. Gershwin, "Histamine (Scombroid) Fish Poisoning: a Comprehensive Review," *Clin. Rev. Allergy Immunol.*, vol. 50, no. 1, pp. 64–69, 2016.

[54] B. A. Bartholomew, P. R. Berry, J. C. Rodhouse, R. J. Gilbert, and C. K. Murray, "Scombrotoxic fish poisoning in Britain: features of over 250 suspected incidents from 1976 to 1986," *Epidemiol. Infect.*, vol. 99, pp. 775–782, 1987.

[55] J. M. Hungerford, "Scombroid poisoning: A review," *Toxicon*, vol. 56, pp. 231–243, 2010.

[56] J. D. Baranowski, A. Brust, and H. A. Frank, "Properties of Its Histidine Decarboxylase System in Resting Cells," *J. Food Biochem.*, vol. 9, no. 2908, pp. 349–360, 1984.

[57] A. Meister, *Biochemistry of the Amino Acids*, vol. II. 1965.

[58] V. Vosikis, A. Papageorgopoulou, V. Economou, S. Frillingos, and C. Papadopoulou, "Survey of the histamine content in fish samples randomly selected from the Greek retail market," *Food Addit. Contam. Part B*, vol. 1, no. 2, pp. 122–129, 2008.

[59] Food and Drug Administration (FDA), "Chapter 7: Scombrotoxin (Histamine) Formation," *Fish Fish. Prod. Hazards Control. Guid.*, pp. 113–152, 2011.

[60] M. Lynch, J. Painter, R. Woodruff, and C. Braden, "Surveillance for Foodborne-Disease Outbreaks — United States, 1998–2002," *Morb. Mortal. Wkly. Report, Surveill. Summ.*, vol. 55, no. 10, pp. 1–42, 2006.

[61] Food and Drug Administration (FDA), "Decomposition and Histamine—Raw, Frozen Tuna and Mahi-Mahi; Canned Tuna; and Related Species," *Fed. Regist.*, vol. 60, no. 149, pp. 39754–39756, 1995.

[62] Commission Regulation (EC), "Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs," *Off. J. Eur. Union*, vol. 322, pp. 12–29, 2007.

[63] A. Önal, "A review: Current analytical methods for the determination of biogenic amines in foods," *Food Chem.*, vol. 103, pp. 1475–1486, 2007.

[64] M. Papageorgiou, D. Lambropoulou, C. Morrison, E. Kłodzińska, J. Namieśnik, and J. Płotka-Wasylka, "Literature update of analytical methods for biogenic amines determination in food and beverages," *TrAC Trends Anal. Chem.*, vol. 98, 2017.

[65] M. Etienne, "Methodology for histamine and biogenic amines analysis," *Ifremer*, no. December, pp. 1–20, 2005.

[66] M. Taleuzzaman, S. Ali, S. Gilani, S. Imam, and A. Hafeez, "Ultra Performance Liquid Chromatography (UPLC) - A Review," *Austin J Anal Pharm Chem.*, vol. 2, no. 6, p. 1056, 2015.

[67] X. Sun, X. Yang, and E. Wang, "Determination of biogenic amines by capillary electrophoresis with pulsed amperometric detection," *J. Chromatogr. A*, vol. 1005, no. 1–2, pp. 189–195, 2003.

[68] M. Liu, Y. Li, G. Chou, X. Cheng, M. Zhang, and Z. Wang, "Extraction and ultra-performance liquid chromatography of hydrophilic and lipophilic bioactive components in a Chinese herb Radix Salviae Miltiorrhizae," *J. Chromatogr. A*, vol. 1157, no. 1–2, pp. 51–55, 2007.

[69] D. T. T. Nguyen *et al.*, "High throughput liquid chromatography with sub-2 μm particles at high pressure and high temperature," *J. Chromatogr. A*, vol. 1167, no. 1, pp. 76–84, 2007.

[70] S. B. Patange, M. K. Mukundan, and K. A. Kumar, "A simple and rapid method for colorimetric determination of histamine in fish flesh," *Food Control*, vol. 16, no. 5, pp. 465–472, 2005.

[71] J. S. Lin, J. D. Baranowski, and H. S. Olcott, "Rapid thin-layer chromatographic-densitometric determination of histamine in tuna," *J. Chromatogr.*, vol. 130, pp. 426–430, 1977.

[72] E. R. Lieber and S. L. Taylor, "Thin-Layer chromatographic screening methods for histamine in tuna fish," *J. Chromatogr.*, vol. 153, pp. 143–152, 1978.

[73] E. R. Lieber and S. L. Taylor, "Comparison of thin-layer chromatographic detection methods for histamine from food extracts," *J. Chromatogr.*, vol. 160, pp. 227–237, 1978.

[74] E. I. López-Sabater, J. J. Rodríguez-Jerez, A. X. Roig-Sagues, and M. T. Mora-Ventura, "Determination of histamine in fish using an enzymic method," *Food Addit. Contam.*, vol. 10, no. 5, pp. 593–602, 1993.

[75] Y. Yamagami, T. Naito, M. Takayanagi, S. Goto, and T. Yashiro, "Colorimetric Assay of Diamine Oxidase Activity with Histamine as the Substrate," *Chem. Pharm. Bull.*, vol. 35, no. 7, pp. 3037–3040, 1987.

[76] M. Hall, D. B. Eldridge, R. D. Saunders, D. L. Fairclough, and R. C. Bateman, "A rapid dipstick test for histamine in tuna," *Food Biotechnol.*, vol. 9, no. 1–2, pp. 39–

57, 1995.

[77] P. A. Lerke, M. N. Porcuna, and H. B. Chin, "Screening Test for Histamine in Fish," *J. Food Sci.*, vol. 48, no. 1, pp. 155–157, 1983.

[78] K. B. Male, P. Bouvrette, J. H. T. Luong, and B. F. Gibbs, "Amperometric Biosensor for Total Histamine, Putrescine and Cadaverine using Diamine Oxidase," *J. Food Sci.*, vol. 61, no. 5, pp. 1012–1016, 1996.

[79] P. Bouvrette, K. B. Male, J. H. T. Luong, and B. F. Gibbs, "Amperometric biosensor for diamine using diamine oxidase purified from porcine kidney," *Enzyme Microb. Technol.*, vol. 20, pp. 32–38, 1997.

[80] I. Frébort, L. Skoupá, and P. Peč, "Amine oxidase-based flow biosensor for the assessment of fish freshness," *Food Control*, vol. 11, pp. 13–18, 2000.

[81] J. Lange and C. Wittmann, "Enzyme sensor array for the determination of biogenic amines in food samples," *Anal. Bioanal. Chem.*, vol. 372, pp. 276–283, 2002.

[82] M. Hall *et al.*, "A Test Strip for Diamines in Tuna," *J. AOAC Int.*, vol. 82, no. 5, pp. 1102–1108, 1999.

[83] B. Ben-Gigirey, C. Craven, and H. An, "Histamine Formation in Albacore Muscle Analyzed by AOAC and Enzymatic Methods," *J. Food Sci.*, vol. 63, no. 2, pp. 210–214, 1998.

[84] D. Serrar, R. Brebant, S. Bruneau, and G. A. Denoyel, "The development of a monoclonal antibody-based ELISA for the determination of histamine in food: application to fishery products and comparison with the HPLC assay," *Food Chem.*, vol. 54, pp. 85–91, 1995.

[85] Bio-Rad, "Chapter 2 - Types of ELISA," *Guide: An Introduction to ELISA*, 2018. [Online]. Available: https://www.bio-rad-antibodies.com/elisa-types-direct-indirect-sandwich-competition-elisa-formats.html. [Accessed: 16-Aug-2018].

[86] Y. V. Wu, K. W. Tudor, B. Paul, and R. R. Rosati, "Histamine Test Kit Comparison," *J. Aquat. Food Prod. Technol.*, vol. 9, no. 2, pp. 5–17, 2000.

[87] T. Kikas, "Introduction to Flow Injection Analysis (FIA) - Determination of Chloride Ion Concentration," *CHEM 3211*, 2014.

[88] J. M. Hungerford, K. D. Walker, M. M. Wekell, J. E. LaRose, and H. R. Throm, "Selective determination of histamine by flow injection analysis," *Anal. Chem.*, vol. 62, no. 18, pp. 1971–1976, Sep. 1990.

[89] J. B. Luten, "An Automated Fluorimetric Method for the Determination of Histamine in Canned Fish Products," *J. Food Sci.*, vol. 46, pp. 958–959, 1981.

[90] J. M. Hungerford, K. D. Walker, M. M. Wekell, J. E. LaRose, and H. R. Throm, "Selective Determination of Histamine by Flow Injection Analysis," *Anal. Chem.*, vol. 62, pp. 1971–1976, 1990.

[91] J. M. Hungerford, T. A. Hollingworth, and M. M. Wekell, "Automated kinetics-enhanced flow-injection method for histamine in regulatory laboratories: rapid screening and suitability requirements," *Anal. Chim. Acta*, vol. 438, pp. 123–129,

2001.

[92] M. A. Carsol and M. Mascini, "Diamine oxidase and putrescine oxidase immobilized reactors in flow injection analysis: a comparison in substrate specificit," vol. 50, pp. 141–148, 1999.

[93] M. Niculescu, C. Nistor, I. Frébott, P. Peč, B. Mattiasson, and E. Csöregi, "Redox Hydrogel-Based Amperometric Bienzyme Electrodes for Fish Freshness Monitoring," *Anal. Chem.*, vol. 72, pp. 1591–1597, 2000.

[94] K. Takagi and S. Shikata, "Flow injection determination of histamine with a histamine dehydrogenase-based electrode," *Anal. Chim. Acta*, vol. 505, pp. 189–193, 2004.

[95] Z. Karpas, "Applications of ion mobility spectrometry (IMS) in the field of foodomics," *Food Res. Int.*, vol. 54, no. 1, pp. 1146–1151, 2013.

[96] V. Gabelica and E. Marklund, "Fundamentals of ion mobility spectrometry," *Curr. Opin. Chem. Biol.*, vol. 42, pp. 51–59, 2018.

[97] G.A.S. Gesellschaft für analtische Sensorsysteme mbH, "User Manual for the GC-IMS," 2016.

[98] M. J. Waltman, "Atmospheric Pressure Chemical Ionization Sources Used in the Detection of Explosives by Ion Mobillity Spectrometry," 2010.

[99] A. B. Kanu and H. H. Hill, "Ion mobility spectrometry: recent developments and novel applications," *LabPlus Int.*, no. April/May, pp. 20–26, 2004.

[100] A. Walte, B. Ungethüm, and W. Münchmeyer, "Measuring Toxic Gases in Low Concentrations with Ion Mobility Spectrometry - Overview of the Technology, its Advantages and Disadvantages," *11. Dresdner Sens. Symp.*, pp. 119–126, 2013.

[101] I. Dzidic, D. I. Carroll, R. N. Stillwell, and E. C. Horning, "Comparison of Positive Ions Formed in Nickel-63 and Corona Discharge Ion Sources Using Nitrogen, Argon, Isobutane, Ammonia and Nitric Oxide as Reagents in Atmospheric Pressure Ionization Mass Spectrometry," *Anal. Chem.*, vol. 48, no. 12, pp. 1763–1768, 1976.

[102] R. H. St. Louis, H. H. Hill, and G. A. Eiceman, "Ion Mobility Spectrometry in Analytical Chemistry," *Crit. Rev. Anal. Chem.*, vol. 21, no. 5, pp. 321–355, 1990.

[103] A. Scheinemann, S. Sielemann, J. Walter, and T. Doll, "Evaluation Strategies for Coupled GC-IMS Measurement including the Systematic Use of Parametrized ANN Training Data," *Open J. Appl. Sci.*, vol. 02, no. 04, pp. 257–266, 2012.

[104] R. Cumeras, E. Figueras, C. E. Davis, J. I. Baumbach, and I. Gràcia, "Review on Ion Mobility Spectrometry. Part 1: Current Instrumentation," *Analyst*, vol. 140, no. 5, pp. 1376–1390, 2015.

[105] G. A. Eiceman, Z. Karpas, and H. H. Hill, *Ion Mobility Spectrometry*, Third Edit. CRC Press, 2014.

[106] C. B. Hariharan, "Implementation of Multi-Capillary Column-Ion Mobility Spectrometry (MCC-IMS) for Medical and Biological Applications," 2012.

[107] P. Belov, M. M. Ulyanova, and V. N. Sidelnikov, "Multicapillary Columns for Chromatography," *American Laboratory*, 2005. [Online]. Available: https://www.americanlaboratory.com/913-Technical-Articles/19158-Multicapillary-Columns-for-Chromatography/. [Accessed: 17-Aug-2018].

[108] A. D. Bacher, "Gas Chromatography Theory," *Gas Chromatography*, 2006. [Online]. Available: http://www.chem.ucla.edu/~bacher/General/30BL/gc/theory.html. [Accessed: 17-Aug-2018].

[109] L. Vera, E. Companioni, A. Meacham, and H. Gygax, "Real Time Monitoring of VOC and Odours Based on GC-IMS at Wastewater Treatment Plants," *Chem. Eng. Trans.*, vol. 54, pp. 79–84, 2016.

[110] R. Parchami, M. Kamalabadi, and N. Alizadeh, "Determination of biogenic amines in canned fish samples using head-space solid phase microextraction based on nanostructured polypyrrole fiber coupled to modified ionization region ion mobility spectrometry," *J. Chromatogr. A*, 2016.

[111] ACD/Labs, "Percepta Predictors," *Software Modules to Predict Physicochemical, ADME, and Toxicity Properties from Structure*, 2018. [Online]. Available: https://www.acdlabs.com/products/percepta/predictors.php. [Accessed: 20-Aug-2018].

[112] A. B. Kanu and H. H. Hill, "Ion mobility spectrometry detection for gas chromatography," *J. Chromatogr. A*, vol. 1177, no. 1, pp. 12–27, 2008.

[113] Imspex Diagnostics Ltd, "BreathSpec: Gas Chromatograph- Ion Mobility Spectrometer for VOC Trace Detection," *BreathSpec for VOC Trace Detection*. [Online]. Available: http://imspex.com/breathspec-for-voc-trace-detection/. [Accessed: 04-Sep-2018].

[114] S. A. NMT, Tecnologia, Inovação e Consultoria, "MCC-IMS, Multi Capillary Column - Ion Mobility Spectrometer."

[115] U. S. E. P. A. EPA, "Technical Overview of Volatile Organic Compounds," *Indoor Air Quality (IAQ)*, 2018. [Online]. Available: https://www.epa.gov/indoor-air-quality-iaq/technical-overview-volatile-organic-compounds#4. [Accessed: 15-Sep-2018].

[116] Chemical Book, "Tryptamine," *Analytical Chemistry*, 2017. [Online]. Available: https://www.chemicalbook.com/ProductChemicalPropertiesCB8192006_EN.htm. [Accessed: 10-Sep-2018].

[117] A. P. Snyder, C. S. Harden, D. M. Davis, D. B. Shoff, and W. M. Maswadeh, "Hand-portable Gas Chromatography-Ion Mobility spectrometer for the determination of the freshness of fish," in *Inorganic and Physical Chemistry*, 1995, pp. 146–166.

[118] L. Y. Chang *et al.*, "One-Minute Fish Freshness Evaluation by Testing the Volatile Amine Gas with an Ultrasensitive Porous-Electrode-Capped Organic Gas Sensor System," *ACS Sensors*, vol. 2, pp. 531–539, 2017.