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Ferramenta ótica para controlo alimentar: avanços no perfil metabólico das aminas biogénicas e validação de um biossensor ótico

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Optical biosensor for food inspection: Advances in biogenic amines metabolic profiling and chemiluminescence biosensor validation

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Optical biosensor for food inspection: Advances in biogenic amines metabolic profiling and chemiluminescence biosensor validation

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Abstract

During the previous decade, biosensors emerge as a very versatile methodology, in part because they allow the use of nanomaterials, functionalization with different compounds, and immobilization of enzymes, microorganisms, antibodies, and nucleic acid. Therefore, the main objective of this master thesis was to advance in food security comprehension through the development of an optical biosensor and to evaluate the metabolic pathways of biogenic amines in food products. The strategy used was based on a new and highly sensitive methodology that included the development of an optical biosensor specific for hydrogen peroxide detection. The sensing methodology behind the developed optical biosensor is based on the detection of a luminescence signal from the chemical reaction within a solid cellulose membrane with a round shape of 10 millimeters in diameter. The biosensor optimization was conducted on an experimental level (e.g., cellulose membrane chemical constituents' optimization, enzymatic reaction parameters, range of H_2O_2 detection, time of chemiluminescence and enzymatic reaction, and physical aspects of the biosensor) until the final prototype was established. A linear response was attained for pure water from 0.0001% to 0.001 %w/w of H_2O_2, with a limit of detection of $2.0x10^{-5}$% w/w. A coefficient of determination, R^2, greater than 0.99 was achieved, with a relative standard deviation (RSD) not exceeding 10%. The optimized biosensor was successfully applied to milk samples adulterated with H_2O_2, where higher response levels were obtained for free fat milk. In the analyzed milk samples, the lowest concentration detected was 0.001%w/w for raw milk and 0.002% w/w for skim milk, semi-skimmed milk and whole milk. Concentrations of H_2O_2 of 0.001% w/w to 0.005% were detected and the method was calibrated for different types of milk, proving that limit of detection and linearity range of the proposed method are suitable for the analysis of milk samples *in-situ*, which can add value to the food fraud department. A preliminary study of BAs determination by GC-MS was carried out. This allowed the detection of four biogenic amines [histamine (HIS), putrescine (PUT), cadaverine (CAD) and tyramine (TYR)], which can be further applied to its determinations in real samples, for biosensor validation purposes. Since these compounds must be derivatized previously, the silanization method was the chosen method for amines derivatization. Finally, the physico-chemical characteristics and microbiological quality of chicken breast meat were evaluated. Samples stored over 12 days at 8ºC, had significant effects on the physico-chemical characteristics and microbiological quality of meat samples. Microbial counts increased instantaneously, increasing pH values and reducing the color parameters (lightness, yellowness, and redness), indicating breast chicken deterioration. From that study, it was concluded that the evolution of microbial content in chicken meat was coherent with both pH and color determinations, that are dependent on microorganisms grow. The information gathered during this work timeline was useful for the construction of the Food quality chart. This last can be used to renovate and upgrade the existing BAI index, once it gathers and dispose of the information on a new form. On the other hand, it is a practical and user-friendly tool that can be used in the industrial context.

Keywords: Food security, biogenic amines, milk adulteration, shelf-life, biosensors, gas chromatography-mass spectrometry

Resumo

Na última década, os biossensores emergiram como uma metodologia muito versátil, em parte porque permitem o uso de nanomateriais, a funcionalização com diferentes compostos e a imobilização de enzimas, microrganismos, anticorpos e ácidos nucleicos. O principal objetivo desta dissertação de mestrado foi avançar na compreensão sobre o tema da segurança alimentar através do desenvolvimento de um biossensor ótico e avaliar a produção metabólica de aminas biogénicas em produtos alimentares. A estratégia utilizada foi baseada numa nova metodologia que incluiu o desenvolvimento de um biossensor ótico específico para a deteção de peróxido de hidrogénio. A metodologia de deteção por trás do desenvolvimento do biossensor ótico baseia-se na deteção de um sinal de quimiluminescência provocado por uma reação química que ocorre dentro de uma membrana de celulose sólida com uma forma redonda de 10 milímetros de diâmetro. O desenvolvimento do biossensor ótico consistiu na otimização de parâmetros experimentais (p.ex., constituintes químicos da membrana de celulose, reação enzimática, limite de deteção de H_2O_2, tempos da reação de quimiluminescência e enzimática e aspetos físicos) até a obtenção do protótipo final. Obteve-se uma resposta linear para a água pura de 0.0001% a 0.001% w/w de, com um limite de deteção de $2.0x10^{-5}$ % w/w H_2O_2. O coeficiente de determinação, R^2, superior a 0.99, com um desvio padrão relativo (RSD) menor que 10%. O biossensor otimizado foi aplicado com sucesso em amostras de leite adulteradas com H_2O_2, onde os níveis mais elevados de resposta foram obtidos para o leite magro. Nas amostras de leite analisadas, a menor concentração de H_2O_2 detetada foi de 0.001% w/w para o leite cru e 0.002% w/w para o leite magro, meio-gordo e gordo. Foram detetadas concentrações de H_2O_2 de 0.001% w/w a 0.005%, tendo este método sido calibrado para os diferentes tipos de leite, comprovando que tanto o limite de deteção como a faixa de linearidade do método proposto são adequados para a análise de amostras de leite *in-situ*, o que pode representar uma mais-valia ao departamento de controlo alimentar. Um estudo preliminar da determinação de BAs por GC-MS foi ainda realizado. Este permitiu a deteção de quatro aminas biogênicas [histamina (HIS), putrescina (PUT), cadaverina (CAD) e tiramina (TYR)], que podem ser posteriormente aplicadas na determinação de BAs em amostras reais, para fins de validação do biossensor proposto. Como estes compostos devem ser derivados previamente, o método de silanização foi o método escolhido para a derivatização das aminas. Por fim, foram avaliadas as características físico-químicas, assim como a qualidade microbiológica da carne de peito de frango. As amostras armazenadas por mais de 12 dias a 8ºC, tiveram efeitos significativos nas características físico-químicas e na qualidade microbiológica. As contagens microbianas aumentaram, aumentando igualmente os valores de pH e reduzindo os parâmetros de cor (luminosidade, amarelecimento e vermelhidão), indicando deterioração do peito de frango ao longo do tempo. A partir desse estudo, concluiu-se que a evolução do crescimento dos microorganismos na carne de frango foi coerente com as determinações de pH e cor, o que prova a sua dependência. Esta informação adicional foi ainda útil para a construção da tabela para determinação da qualidade dos alimentos. Esta última pode ser utilizada para renovar e atualizar o índice BAI existente, uma vez que reúne e dispõe as informações de uma forma inovativa. Por outro lado, é uma ferramenta prática e fácil de usar que pode ser usada em contexto industrial.

Palavras-passe: Segurança alimentar, aminas biogénicas, adulteração do leite, prazo de validade, biossensores, cromatografia gasosa acopolada à espectrometria de massas

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2. Helena Vasconcelos, José M. M. M. de Almeida, **Ana Matias**, Cristina Saraiva, Pedro A.S. Jorge, and Luís C. C. Coelho. Detection of biogenic amines in several foods with different sample treatments: An overview. *Trends in Food Science & Technology*, **2021**, 113: 86-96. https://doi.org/10.1016/j.tifs.2021.04.043.
3. Helena Vasconcelosa, **Ana Matias**, João Mendes, João Araújo, Bernardo Dias, Paulo Santos, José M. M. M. de Almeida and Luís C. C. Coelho. Optical biosensor for the detection of hydrogen peroxide in milk. **Under submission**.

Posters and oral communications

1. Ana Matias, José M. M. M. de Almeida, Helena Vasconcelos, Cristina Saraiva, P.A.S. Jorge, Luís Coelho. Optic sensor for BAs determination, poster communication, 22º Physics national conference and 30º Iberian meeting for the teaching of Physics, 2nd to 5th September **2020**.
2. Helena Vasconcelos, Ana Matias, Luís C. C. Coelho, Pedro Jorge, Cristina Saraiva, João Mendes, João Araújo, Bernardo Dias, Paulo Santos, José M. M. M. Almeida. Optical biosensor for the detection of hydrogen peroxide in milk, oral communication, 1st International Electronic Conference on Chemical Sensors and Analytical Chemistry, 1st to 15th of June **2021**.
3. Ana Matias, José M. M. M. de Almeida, Helena Vasconcelos, Cristina Saraiva, P.A.S. Jorge, Luís Coelho. Sensors to support a sustainable analysis: hydrogen peroxide detection using a membrane based on hydroxyethyl cellulose, oral communication, 4th Doctoral Congress in Engineering, 28th to 29th of June **2021**.

ABREVIATTIONS AND ACRONYMS

General abbreviations

AOx	Amine oxidases
BA	Biogenic amine
BAI	Biogenic amine index
BS	Biosensor
CAD	Cadaverine
CE	Capillary electrophoresis
DAO	Diamine oxidase
DI-SPME	Direct immersion Solid phase microextraction
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
GC	Capillary Gas chromatography
GC-MS	Gas chromatography mass-spectrometry
HIS	Histamine
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer
LAB	Acid lactic bacteria
LOD	Limit of detection
LOQ	Limit of quantitation
MAO	Monoamine oxidase
MS	Mass spectrometry
NEMI	National Environmental Methods Index
PAO	Polyamine oxidase
PCR	Polymerase chain reaction
PEA	Phenylethylamine
PUT	Putrescine
QI	Quality Index
SPD	Spermidine
SPM	Spermine
TRY	Tryptamine
TVC	Total vial counts
TYR	Tyramine

AIMS AND OUTLINES OF THIS THESIS

Context

Biogenic amines (BAs) are low-molecular compounds that can be found in a vast range of food products (both animal and plant origin), as well as in fermented foods [1]. In recent years, has been a crescent awareness towards food safety, followed by an increase in food regulations [2], strongly justified by the current lifestyle and market globalization [3]. Since their presence in higher concentrations can trigger neurotransmission disorders, headaches, nausea, palpitations, allergic responses, among other symptoms, BAs detection has become a matter of social concern [4], [5]. Keywords such as knowledge, innovation, and ingenuity are the most important approaches to address a particular problem. In this context, it's crucial for the consumers to be ensured that food is not contaminated at the distribution process [6].

Since BAs content is associated with the growth of spoiling decarboxylating microorganisms [6], they are used as a quality index in foods (e.g., meat, fish, beverages and, fermented products) as a means of determining whether the product is fresh or deteriorated [3]. BAs are produced mainly by bacteria through the action of decarboxylases. This class of enzymes act specifically on the amino acids and remove their carboxyl group, with the formation of the correspondent amine and CO_2 [6]. Several factors may condition BAs production, such as raw material, microorganisms, processing, and the conservation conditions [3]. One of the focus of the American Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) is HIS higher concentrations in foods. This BA is present in fish, namely tuna, mackerel, bonito, bluefish, among others. Their consumption is associated with "histamine poisoning" explained by the presence of high levels of free HIS in their muscle tissue [3]. Besides HIS, the most important BAs present in foods include TYR, PUT,CAD, and PEA, which are derived from the respective amino acids, histidine, tyrosine, ornithine, lysine, and phenylalanine [7].

Taking into account the importance of BAs and their toxic role in human health, numerous methods to detect and quantify these nitrogenous compounds have been extensively published [8]. The most commonly used method for deterioration detection of food products has been the organoleptic method. Particularly, for BAs, organoleptic differences were split into good (Class l; 0-1), borderline (Class 2; 1-10), and decomposed (Class 3; above 10) for fish samples. The resulting sum of these values is called the organoleptic score. This method is very subjective since it's based on a rate of agreement/disagreement from different people [9]. Currently, conventional analytical methods such as chromatographic techniques, namely high-performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE), are the most commonly used methods for BAs quantification. Those are sensitive and selective for some BAs, however, lack of repeatability is reported when applied to food matrices [8]. Biosensors (BS) emerge in an era of great biosecurity and biosafety awareness. These are innovative bio-tools, that comprise a biological element (e.g., enzymes, microorganisms, antibodies, and nucleic acids) that recognize specific target analytes. Afterwards, they are amplified, processed, and converted into a digital format [10]. BS's can be used in direct or indirect format. In the first case, the target analyte is detected directly while opposite in the indirect assay, an additional reaction must occur [11]. Apart from biological element, transducers are an important element in the BS since they convert biological signal into a digital signal. Transducing methods reported include electrochemical, optical, colorimetric, and piezo-electric.

The complexity of BAs matrices requires a high throughput and sensitivity methodology to in-depth quantify and characterize their profile. BS's can overcome some problems when compared

with chromatography analysis. Hence, the BS construction and study may help unveil the complex systems, such as food products and subsequently increase data information about BAs.

Aims of this thesis

The main objective of this master thesis is to advance in enzymatic biosensors comprehension through biogenic amines metabolic profiling. In this context, different specific objectives were drawn:

- To contribute to the development of an enzymatic biosensor which consists of a comprehensive characterization of hydrogen peroxide, one of the byproducts of the enzymatic reaction with biogenic amines;
- To calibrate the biosensor using hydrogen peroxide as a direct simulation of the one that is present in enzymatic reactions medium; and also understand its biological formation via oxidation-reduction reactions;
- To use the developed optical biosensor to detect hydrogen peroxide in adulterated milk samples;
- To develop a methodology that can detect biogenic amines in poultry meat matrices based on an enzymatic biosensor; complementary a conventional technique such as gas chromatography mass-spectrometry will also be employed.
- To characterize biogenic amines and their affinity with the newly enzymatic biosensor;
- To provide a correlation between the biogenic amine content, microbial flora, and sensory changes in meat matrices; portions of meat will be chosen and examined from a microbiology perspective.

The present thesis is divided into six fundamentals chapters as follow:

Chapter 1 presents a review of the most relevant literature data of the biogenic amines structural synthesis in food products; followed by methodologies and techniques for their detection, including the conventional techniques and greener processes, with particular emphasis on biosensors.

Chapter 2 show the research conducted on the development of the enzymatic biosensor based on chemiluminescence that allows hydrogen peroxide detection. The strategy is to use Diamine Oxidase, an oxidoreductase enzyme, to oxidase biogenic amine substrate and consequently liberate hydrogen peroxide to the reaction medium. This compound is readily detected by the biosensor at different concentrations.

Chapter 3 contribute to the characterization of the biosensor in real samples, namely milk. For this purpose, different types of milk (with different fat content) from different places, were selected as case-study samples and adulterated with hydrogen peroxide solution.

Chapter 4 establish the biosensor experimental parameters in the biogenic amines determination, taking advantages of the gas chromatography mass-spectrometry equipment as a confirmation and comparison method, where a special emphasis will be put into the idea of a "green analytical chemistry"; experimental parameters of the gas chromatography mass-spectrometry (i.e., derivatization time and temperature, oven temperature and, concentrations of biogenic amines used) are also included.

Chapter 5 will discuss the concentrations of biogenic amines commonly present in chicken breast meat and compare them with their sensorial and microbial analysis content.

Chapter 6 summit the main conclusions of this master thesis, as well as some highlights into future research in the biosensors area.

This master thesis was organized in five distinctive stages over the course of nine months, as illustrated in A.

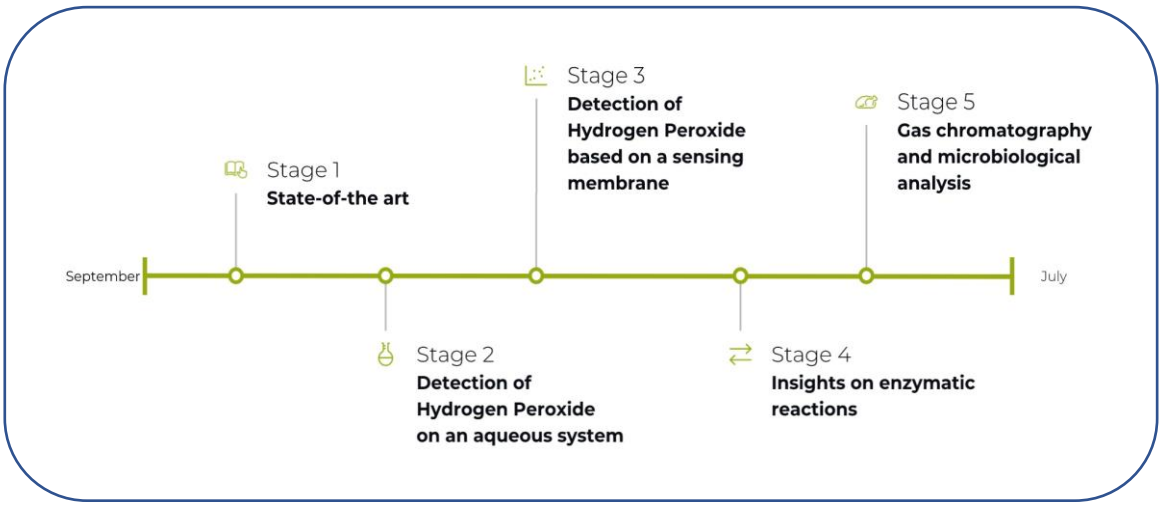


Figure A.: Master thesis workflow: from the state of art to the biosensor characterization, enzymatic reactions, and gas chromatography.

Chapter 1. Introduction

1.1. Structure and biosynthesis of biogenic amines

Biogenic amines (BAs) are organic nitrogen compounds that can be found in animals, plants, microorganisms, and humans. They are mainly formed by decarboxylation of amino acids [3], [7], because of specific microorganisms metabolism (e.g., bacteria, yeasts, and moulds). Formation of the BAs and CO_2 occurs from the enzymatic reaction catalysed by pyridoxal phosphate to decarboxylate the amino acid. Besides the enzymatic decarboxylation route, BAs can also be formed by amination and transamination of aldehydes and ketones in the living cells [12]. Foods in general have a complex matrix that increases the difficulty of analyses and by result, can interfere with the outcome [13]. Decarboxylation of free amino acids that occurs by microbial enzymatic activity, is the main route for BAs formation and is the reason why fermented food products are reported to have an overall higher content of these compounds [14]. This can be explained by the two biochemical pathways, either by the production of exogenous enzymes that convert amino acids into BAs or due to the decarboxylation reaction that can naturally occur in the tissues by the action of endogenous enzymes [15].

The structure of BAs depends on the amino acid, thus based on chemical structure they can be (i) aromatic and heterocyclic as HIS, TYR, and PEA and (ii) aliphatic di-, tri-, and polyamines as PUT, CAD, SPM, SPD, and AGM [10], [12].

Amines as PUT and CAD are two aliphatic diamines formed by the microbial degradation of ornithine and lysine, respectively, and together are known to enhance HIS toxicity [6]. Moreover, aminopropylation reaction of CAD and PUT generates SPM and SPD by arginine decarboxylase enzyme [16]. It is important to mention that BAs such as PUT, CAD and their respective polyamines, SPM and SPD, are well known to form carcinogenic nitrosamines, by nitrite reaction [3], [10], [17].

TYR, a tyrosine amino acid-derived by decarboxylation is considered the most toxic of the BAs. This BA is related to a poising well-known case of intoxication, that is “cheese reaction”, associated to cheese consumption and can leave to severe headaches [18]. Many factors that can influence BAs concentration in foods. TYR presence in cheese at higher concentrations can be explained by the fermentation process that undergoes cheese products since lactic acid bacteria (LAB) can boost BAs [6].

HIS is a heterocyclic diamine mainly formed from its precursor histidine. It is the most frequent food intoxication since it can affect cardiovascular, gastrointestinal, and respiratory systems. This BA is present mostly in fish from the *Scombridae* and *Scomberesocidae* families at high levels [6].

Figure 1.1 illustrates the metabolic pathways for the formations of BAs through two main routes, that are decarboxylation of aminoacids and other alternative pathways.

Contrarily to their formation, BAs can be metabolized by oxidation and acetylation reactions enhanced by both monoamine oxidase (MAO; EC 1.4.3.4) or diamine oxidase (DAO; EC 1.4.3.6) [19]. These enzymes present an important role on controlling the BAs content in human, plant and animal cells, since they can be used as degraders [20]. The products of DAO degradation are aldehyde, ammonia, and hydrogen peroxide [19].

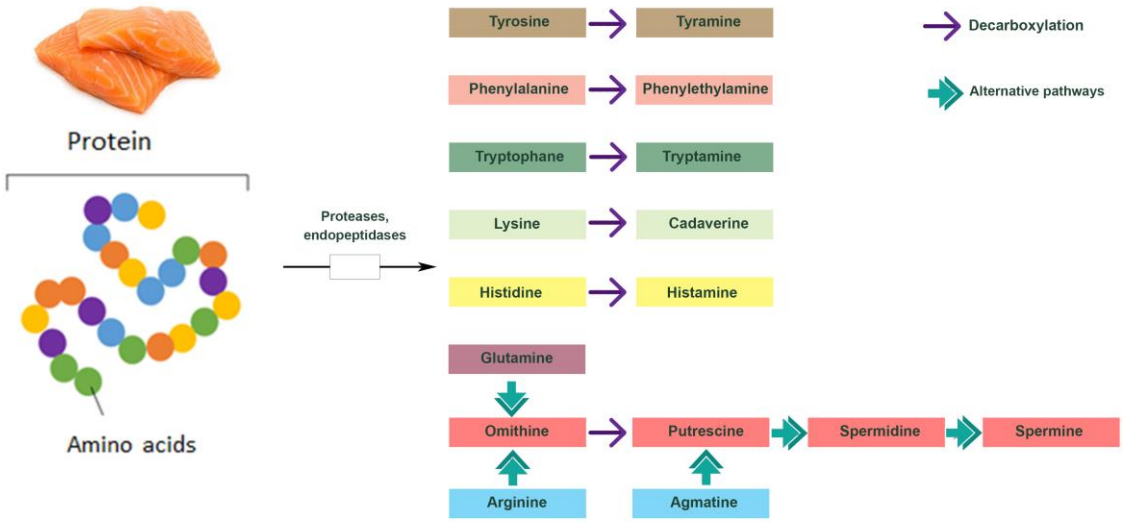


Figure 1.1.: Metabolic pathways for the formation of biogenic amines. They were divided into two main routes: via decarboxylation and alternative pathways. Adapted from [57].

As different challenges may be exposed regarding BAs toxicity, at lower concentrations these compounds can play a role in membrane stabilization, immune functions, while preventing chronic diseases and participate in nucleic acids and protein synthesis [12], [21]. Actually, HIS is considered an important mediator of the immune system and the fundamental biomolecule for a health gastric function [21]. Besides, it is known that this BA is released by cells in response to an injury and allergic and inflammatory reactions, by inducing contraction of smooth muscle and dilation of capillaries [22]. This is particular important for asthma patients, and it can be used as a routine diagnosis of asthmatic response [16] or as a biological treatment [23]. Moreover, PUT, SPD, and SPM can participate in cell division, fruit development, flowering, stress response, and senescence [7] while this last two can prevent protein and nucleic acid cells damage from oxidation with hydrogen peroxide, generated from metabolic reactions during proliferation of living cells [16].

1.2. Detection of biogenic amines in food

Different types of food products represent a diversity of their nanostructure and microorganism composition [14]. According to the ScienceDirect database [24], by searching the keywords: “biogenic amines”; “determination” and “foods”, followed by a refinement, a significant number of papers dealing with BA determination in food (1614) have been published since 2010. In the light of the contrary, a more reduced number, 1261 results, appeared when the publication date is before 2010.

This topic is divided into four sections which will comprise the detection of BAs in several food matrices, as well as aspects related to their taxonomy. First with fish and fish products, meat and meat products, alcoholic beverages, and other food related products.

1.2.1. Fish and fish products

Fish is a well-known source of nutrients, vitamins, salt minerals, and polyunsaturated fatty acids [25], making it a preference for the human diet. However, they are very susceptible to post-mortem modifications, resulting in the formation of spoilage compounds, namely BAs [25]. The most toxic BA detected in fish is HIS [26], where it can be found in large quantities in fish from the family *Scombridae* [27]. Since HIS is the precursor of HIS, the concentration of this free amino acid in fish is determinant [28]. Thus, of all the BAs, HIS is the most studied with a concentration limit well defined, as a result of its toxic pattern [25]. This legal concentration of HIS in seafood products was established by FDA and should not exceed 100 $mg.Kg^{-1}$ (100 ppm) [28]. Moreover, they also reported that 50 ppm concentration of HIS detected in one fish product is a sign of decomposition, whether organoleptic properties are visible or not. Despite HIS importance, PUT is always detected in fish products [29]–[32]. Regarding this, it is suggested that PUT can be used individually as a marker for food quality [31]. The concentrations ($mg.kg^{-1}$ or L^{-1}) of the PUT in raw fish are summarized in Table 1.1. In general, higher values of PUT are found in raw fish samples.

Table 1.1.: Concentrations values obtained for PUT in fish samples.

Analyte	Sample	Scientific name	Temperature/ time	Concentration ($mg.kg^{-1}$)	Ref.
PUT	Raw fish	*Gadus morhua*	30 ºC for 2 days	663.46	[30]
		Thunnini	30 ºC for 2 days	811.4	[30]
		Carassius auratu	"Fresh"	40.07	[31]
		Scomber scombrus	22 ºC for 1 days	350	[32]
			30ºC for 2 days	7050.06	[30]
		Sardina pilchardus	22ºC for 1 day	420	[32]
		Ctenopharyngodon idellus	"Fresh"	11.79	[31]

Certain Scombridae fishes such as tuna, mackerel, mahi-mahi, bluefish, bonito, skipjack, saury sardine are related to HIS formation [22]. However, non-scombroid fish like sardine, anchovy, western Australian salmon, cape yellowtail, among others, are also reported to be associated with scombroid poisoning [28]. For fermented fish products fermentation parameters (e.g., glucose content, pH, and water activity) could prevent or reduce the BAs formation in fermented fish, however, the need for a quality routine screening of BAs in the fermented fish should be accounted to ensure the safety of the consumers [33].

Meanwhile, to control the freshness of fish products, estimation of the BAs concentration should be a priority. Considering that they are not heat sensitive, the high concentrations in fishery products are commonly justified by the microbial contamination, which can be prevented by good hygienic practices [28], [33].

1.2.2. Meat and meat products

International Agency for Research on Cancer (IARC) have gattered enough evidence that red meat may be carcinogenic [34], therefore, representing a matter of public health concern. BAs are included in a wide group of chemical compounds, as a consequence of the interaction between the constituents of meat and process technology factors like drying, heating, fermentation, or cooking. Regarding BAs content in the processed meats, evidence is particularly negative and it includes cooked, cured, and derived meat products [35], [36]. Since the main precursors of BAs formation are the free amino acids available in meat, they will enhance the activity of endogenous meat enzymes. Consequently, the decrease of pH during the fermentation process will promote proteins to denature. Meanwhile, microorganisms responsible for fermentation can also contribute to BAs accumulation [36]. TYR, CAD, PUT, and HIS are the most common BAs present in meat and meat products [36]. The concentrations ($mg.kg^{-1}$ or L^{-1}) of the PUT in raw meat are summarized in Table 1.2.

Table 1.2 .: Concentrations values obtained for PUT in meat samples.

Analyte	Sample	Scientific name	Temperature/ time	Concentration ($mg.kg^{-1}$)	Ref.
PUT	Raw meat	*Bos taurus*	4ºC for 12 days	10.4	[37]
		Gallus domesticus	2.9ºC -5.4 ºC for 12 days	2.2 to 7.4	[38]
			4ºC for 15 days	20.4	[39]
			2ºC for 10 days	52.0	[40]
			4ºC for17 days	409.6	[41]
		Longuissimus dorsi	2ºC for 1 day	0.33	[42]
			-20 ºC for 15 days	11.2	[43]
			5ºC for 15 days	18.9	[43]
			2ºC for 10 days	14.5	[40]
			“Fresh”	9	[44]
		Meleagris	4ºC for 7 days	3.2	[45]
			2ºC for 10 days	68.7	[40]

Levels of BAs in ‘‘red meat’’ and ‘‘white meat’’ can be related to spoilage and sometimes to their protein degradation, under appropriate conditions [4]. Additionally, in white meat such as chicken, BAs concentration is generally higher than the ones present in red meat [40].

BAscontent and consequently their relation to food deterioration, it is somehow undefined. For this purpose, some quality markers have been established, which may include one or multiples amines [46]. Quality index (QI) and biogenic amine index (BAI) have been applied to BAs assessment. QI is the is the sum of HIS, PUT and CAD divided by the sum of spermine and spermidine plus 1. For the BAI index, a sum of TYR, HIS, PUT and CAD must be used [47]. Generally, QI is not considered due to the fact that both SPM and SPD are found to be at constant levels [48]. Additionally, TYR alone can also be used as a quality marker for vacuum-packed

beef and cooked ham, as well as SPD and SPM. Moreover, other proposed quality markers are based on the use of CAD and TYR concentrations to assess beef and chicken deterioration during storage [40].

Like fish, meat and meat-related products accurate assessment of shelf life is important to prevent food waste while providing a basis for regulations to control the quality of meat. Apart from the safety concerns, and from the consumer's point of view, the reduction of biogenic amine accumulation in foods should progress without a noticeable change in the sensorial experience. Thus, come up with a new formula that rearranges both processing and formulation in a way that doesn't induce changes in aroma while keeping the product "natural" may be a hard task. Recently, research has been conducted towards the exploitation of autochthonous microbiota to control the accumulation of BA in fermented meat products while retaining their sensory traits. Besides, another approach using lactic acid can delay BAs production since it inhibits decarboxylase-positive bacteria development [49].

1.2.3. Beer and other alcoholic beverages

Alcoholic beverages include beers, liqueurs, gin, rum, brandy, whiskey, and ciders [50]. Beer is one of the most popular alcoholic beverages in the world thanks to hop (*Humulus lupulus* L.), an essential ingredient that provides bitterness and aroma to beer [51]. At the time of the brewing process, beer malt undergoes fermentation process. Thus, microorganisms such as *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* with wild yeast and lactic acid bacteria are always present. HIS and TYR can be found in beer. These BAs presence is associated with microbial contamination during brewing; however, they can also appear naturally from the raw materials or hop Ciders, fermented apple juice, can also contain indigenous yeast and LAB, whereas some of those strains are known to be BA producers [52].

Wine is an alcoholic fermented and its consumption dates back more than 7000 years ago [53]. Since this product is attractive for human consumption, BAs' higher concentration is currently an issue [14]. The occurrence of BAs in wine is dependent on the time of wine contact with grape skin, amino acid content at the various stages and, the time of wine contact with yeast. Furthermore, some abiotic factors like the type and degree of ripeness of the grapes, the climate and soil of the viticulture area, as well as the vinification techniques can influence BAs presence [54]. More than 15 amines were already identified in wines and their concentrations will depend on two main sources, that is raw materials and fermentation processes [52], [54]. Of those 15, HIS, TYR, and PUT are the most prominent BAs found in wine, yet CAD, phenylethyl-amine, isoamylamine can also be present. Just like fermentation products, it is possible that BAs accumulation in the grapes, and consequently in the wine, occurs [54]. PUT, CAD, and HIS levels were found to be higher in instant pot red wines from 2012 to 2016 available on the Portuguese market, when compared with the early years [55].

Besides the extended consumption worldwide of beer and other alcoholic beverages, BAs' toxic effects as well the factors that can influence their levels in alcoholic beverages is of higher importance for consumer safety.

1.2.4. Other food products

Milk is a fermented beverage that has always been in the human diet and it is expected to increase over the next few years. Dairy's adequate medium is propitious for the growth of microorganisms, due to its rich and balanced chemical composition. By the time of the fermentation process, BAs are produced by yeast and some bacterial strains, the most important ones being LAB. Generally, BAs are produced by microbial dairy contaminants of different origins, but the technological microbiota used in the fermentation and/or ripening of dairy products (e.g., LAB, yeasts, and moulds) can also contribute [56]. Some Gram-negative bacteria such as *Escherichia coli, Hafnia alvei, Klebsiella pneumoniae, Morganella morganii, Pseudomonas*, or *Serratia spp*., are present in milk. These last are capable of decarboxylate free amino acid and form BAs like HIS, PUT, or CAD [57]. After the coagulation of casein protein from milk, cheese is formed. Since the concentration of BA in milk is generally low, cheese is the most monitored dairy product. In part because they can rich concentrations higher than 1000 $mg.kg^{-1}$. This fermented product is one of the most important exogenous sources of BA. TYR, HIS, CAD, PUT, and TRY are the main BAs determined in cheese. Some important factors will change concentration and type of BA present in cheeses, namely the type of milk used for cheese making; the use of heat treatment in milk, section of the cheese, ripening conditions, post ripening processing, packaging, storage time and temperature, and also the microbiota responsible for cheese-making. Yeats, commonly present in dairy products, can contribute to changes in the organoleptic characteristics. Moreover, they act as spoilage organisms, thus, increasing BAs formation in cheese [57].

Besides the extended consumption worldwide of dairy, fresh fruits, vegetables and their products like juices and sauces are also present in the daily dietary habits. Due to endogenous components and uncontrolled microbial enzymatic activity, fresh food and vegetables can accumulate BAs [58]. At this time about 22 BAs were already detected in this group of vegetables, juice, and fermented related products. The most detected BAs for this group are TYR, PUT, CAD, HIS, SPM, and SPD [58]. Furthermore, the highest concentration of BAs was found in fresh fruits instead of juices, possibly as a result of sanitary conditions in prep juices.

For different types of food products, whether they are originally from an animal or a plant, BAs type and concentrations are found to be unpredictable. Many factors can influence it, such as downstream processing, availability and type of amino acids present, conditions and storage time and temperature, type of packaging, and the presence of positive decarboxylase microorganisms.

1.3. Legislation of biogenic amines in food and beverages

The consumers' acceptance is dependent on the specific organoleptic characteristics of food items, which comprises gustative, visual and flavour perceptions. One of the majors concerns towards this emerging subject, that are BAs, is to establish a range of concentrations that can limit their toxicity towards human's health. There are two main factors to account, such as quality and health implications of BAs presence in food and beverages. Despite the efforts that are being made to control BAs production, specific legislation only covers HIS in fishery products [59]. At the time of this writing thesis, no other criteria have been settled for other BAs or other food products. In Europe, the European Commission Regulations (2073/2005, 144/2007, 365/2010) has set foods legislation for HIS in specific fish species during their shelf-life, namely *Scombridae, Clupeidae, Eugraulidae, Coryphenidae, Pomatomidae*, and *Scomberesocidae.*

Briefly, the law criterions determine a sampling plan comprising nine units, two of the units may be between 100 and 200 $mg.kg^{-1}$ of HIS, and none above the limit of 200 $mg.kg^{-1}$. This regulation also applies to fishery products which have undergone enzyme maturation where two of the units may be between 200 and 400 $mg.kg^{-1}$ of HIS, and none above the limit of 400 $mg.kg^{-1}$. The FDA has also suggested that HIS concentration in fish should be less than 500 $mg.kg^{-1}$ correspond to health dangers [60]. Codex Alimentarius and Food Standards Australia New Zealand (FSANZ) have also given special attention to BAs contamination, establishing a limit of 100 $mg.kg^{-1}$ of HIS in fish and fish products of the mentioned species [61].

As legislation on BAs in foods appears insufficient, it is generally accepted that they should not be allowed to accumulate in food products. This difficulty to establish an agreement towards the maximum limit for ingested BA is justified by the fact that their toxic effect strongly depends on the BA typology, the presence of modulating compounds and the efficiency of each person's detoxification system [60]. Since BAs can have harmful effects on health it is of considerable importance to study and evaluate the BA profile so they can be included in future regulations.

1.4. Microbiologic biogenic amines analysis and microflora associated

Since spoilage perception is somehow subjective, several chemical indicators have also been proposed to assess meat quality including volatile bases, nucleotide breakdown, volatile acidity, and the levels of BAs [41]. As previously mentioned, there are specific strains of microbiota responsible for the production of BAs. Specific genes for decarboxylase enzymes can be transmitted between bacteria sharing a common environment by horizontal transfer which increases the risk of intoxication [49]. BAs can be formed by both gram-negative and positive bacteria. Enterobacteriaceae (gram-negative) and certain lactobacilli (acid lactic bacteria) (e.g., *Lactobacillus buchneri*), pediococci, and enterococci (gram-positive) are bacterial species particularly active in the formation of BAs. Among those, Enterobacteriaceae is the most common bacterial species present in chicken meat [48], while for fish mesophilic enteric and marine bacteria are the major producers of HIS [62].

The presence of bacterial spoilage in meat products depends on multifactorial conditions during the rearing, slaughtering, and processing of animals [48]. For example, biological analysis performed on cheese have reported differences in the microbiological profile of the samples of the same region, thus, suggesting that different degrees of hygienic quality of raw materials, as well as different handling and cheesemaking practices, can actively interfere in the growth and activity of certain groups of microorganisms involved in biogenic amine synthesis. Moreover, and apart from the organoleptic parameters, physicochemical ones such as pH and low salt content seem to favour biogenic amine-positive microflora. This is relevant to the food industry since both of these environmental factors can easily be modulated, to control the growth of undesirable microorganisms [63].

1.5. Methods for detection of biogenic amines

Concentration level monitoring is important to control the synthesis or degradation of BA. Thus, sensitive methods are necessary to be able to observe significant changes and consequently assure food security.

This topic is divided into three main sections, the first section will comprise the summary of the conventional methods used in BAs determination; the second section will describe the gas chromatography mass-spectrometer analytical method alone and when applied to BAs determination; and the third and last section will focus on BS's, on an exploratory overview, including a description of the typical BS assay format used, its general structure, and the biorecognition elements used.

1.5.1. Biogenic amines analysis in food

Regarding food analysis, BAs identification remains one of the greatest challenges. This can be explained by several reasons, among them, the strong polar character of these compounds, which makes them less soluble in organic solvents; the complexity of the matrix sample; variable concentration range that can go from very low to very high; the presence of potentially interfering compounds; absence of intrinsic properties of the compounds, which could enable their detection by usual physicochemical methods (e.g., spectrophotometric, fluorometric or electrochemical methods); and the presence of several BAs at the same time [52].

Analytical methods are known to be sensitive, selective for most BAs, however, some of those present lack of repeatability when applied to the challenging matrices of food [52]. After the extraction and derivatization, when required, the determination of BAs is most commonly performed by the following chromatographic techniques, namely high-performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE) [52]. CE is a recent technique that can overcome thermal instability while providing more rapid separations than LC methods, as well as good resolutions. This newly CE technique has already been applied for the separation of BAs in environmental samples, foods, and beverages, with the downside that derivatization of the amines is necessary to enable detection [64]. GC method is not so often applied for the determination of BAs, and it should be improved in combination with derivative technology [64]. Mass spectrometry (MS) coupled with GC or liquid chromatography (LC) methods are suggested to be the most adequate to detect very low concentrations of metabolites, along with the advantage they can bring in terms of high sensitivity and specific structural information [17]. Other detection techniques such as UV, MS, conductometric, indirect UV, electrochemical, immunoassay, enzymatic, and PCR processes have been successfully applied to BAs detection [65]. BS's can solve some of the problems above mentioned, since they are, highly sensitives, low cost, simple to operate with, and can provide continuous dynamic monitoring [64].

Nowadays, choosing what method to use in BAs quantification, some factors should be taken into consideration, like reduced time of analysis, being able to use a lower concentration of derivatization reagent or non, and increased sensitivity.

1.5.2. GC-MS applied to biogenic amines determination

Gas chromatography and mass spectrometry (GC-MS) techniques were first reported at the end of the 1950s, and since then a rapid increase in its application in all areas of organic analysis began. GC-MS is at the mature level being an indispensable routine procedure [66]. Nevertheless, this analytical method continues to develop [67].

Sample preparation should precede GC-MS analysis, once most analytes need to be concentrated or separated from the sample matrix. Simplifying this process is a current trend, therefore leaning towards the automated instrumental technique limiting the error due to manual work. Some things

to consider when choosing the sample preparation protocol include differences in the concentration of the various samples, differences between the volatility of the analytes and the matrix, and the varying chemical nature of the substances. In the particular case of BAs, their complex structure and low concentration levels may cause some interferences which can be overcome by selection of an adequate pre-treatment of samples Generally, BAs are subjected to extraction and derivatization processes (due to their low volatility) followed by separation and quantification, while for simpler matrices, they can be directly assessed [68].

Gas chromatography (GC) is today the most important analytical method in organic chemical analysis justified by the significant improvements that have been accomplished, driven by the demand for analytical tools able to analyse targets from complex matrices. For example, the crescent development of new stationary phases of GC columns (e.g., fused silica) which have provided chemical stability, durability, and characterization of a broad range of compounds [67]. Moreover, improvements in hardware and software have been carried out, more specifically the development of data algorithms for data processing [69]. Briefly, the chromatography system proceeds to the separation of analytes in a sample through partitioning between two phases, stationary and mobile phase. The mobile phase is in gaseous state (e.g., helium, hydrogen, or nitrogen) and in contact with the stationary phase [67]. Interaction of the analyte with stationary phase is what dictates their sequential elution. In respect of detectors coupled to GC, the MS detector is the most used. Mass spectrometry as the detection method gives the most meaningful data by providing a direct determination of the structure of the molecule or fragments. The results of mass spectrometry are one of the means to confirm identity of the element under scope. The complete integration of MS and GC into a single system enabled its widespread use in the analytics field [66]. For instance, the quantification of BAs in Polish wines was investigated using direct immersion solid-phase microextraction (DI-SPME) in association with GC-MS. Using this technique, the authors reported the determination of 16 BAs in Polish wines. Of those, low levels were quantified, therefore suggesting that high-quality vinification practices were used by the producers [70].

Mass spectrometry data can be acquired and processed by several approaches as follow:

- Full-scan mode (scan using m/z range)
- Single ion monitoring mode (SIM- data acquisition with m/z diagnostic ions)
- Ion extraction chromatography mode (IET- data processing with m/z diagnostic ions)

Analyte quantification is estimated by the peak area determination performed by GC-MS analysis since it is proportional to the analyte concentration in the gas phase [66]. This can be achieved by the construction of calibration curves within a range of concentrations. Regarding the analyte's quantification, a second step such as confirmation of identity must be performed. A confirmatory method is defined as a method that provides full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest [66]. A valuable resource inherent to MS detector is the readily available mass spectra database, being the most important strategy for analytes identification. Additionally, it is also common to add a standard (e.g., internal, or external with similar characteristics as the analyte) as a reinforcement in the confirmation identity step.Figure 1.2. shows a schematic representation of the configuration of GC system for BAs determination.

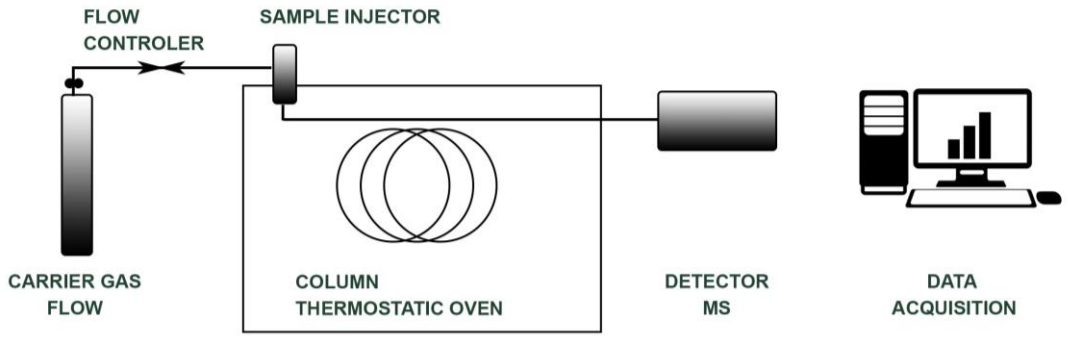


Figure 1.2.: Schematic illustration of one-dimensional gas chromatographic system coupled with mass spectrometry detector.

Although, GC technique is not popular in the case of BA determination and even though these methods required a quite complex procedure, it is suggested that they provide an accurate identification and enable the quantification of more BAs in comparison to the typical LC methods [71]. A reproducible, reliable method must be developed to make advantageous the use of GC for amine-containing compounds analysis.

1.5.3. Biosensors applied to biogenic amines determination

- *Biosensor main characteristics*

To control foodstuff composition and detect chemical or biological contaminants methods should be selective, sensitive, and reliable [72].

Biosensors (BS) are the newly analytical methods for environmental control and food safety. They are analytical devices used to quantify the target molecule in a sample. The biosensing process includes three main components, namely, a bioreceptor (formed by a biological element), a transducer, and a processor [10]. Bioreceptor elements can be enzymes, microorganisms, antibodies, or nucleic acids. Based on the bio-recognition element they are classified into three main receptors, according to their specific interactions: biocatalytic receptors, which are based on catalytic reactions, affinity receptors based on substrate specificity, and hybrid receptors based on complementary sequences of DNA or RNA [73]. Generally, a molecular recognition happens between the recognition element and the target compound, which generates a physiochemical or biological signal followed by its conversion into a measurable quantity by the transducer. Signals can be displayed in the form of optical (e.g., colorimetric, fluorescence, chemiluminescence, and surface plasmon resonance) or electrical (e.g., voltammetry, impedance, and capacitance) or any other preferred format [74]. The transducer is considered the most important component of BS's since it transforms the resulting biochemical signal into a quantifiable electronic signal, proportional to the analyte concentration. Additionally, the choice of transducers (e.g., optical, electrochemical, and gravimetric) will be dependent on the type of signals emitted by the bioreceptor [73].

Analyte detection is performed on BS's either as a direct or indirect format. In the direct assay, the target analyte is bound by its biorecognition element, which is detected directly. The biorecognition element is immobilized on the surface of a transducer while the sample containing the analyte and other components is incubated with the BS. Afterward, the analyte specifically binds to the bio-recognition element and a signal is produced by the transducer. It is important to

mention upon incubation, no signal is obtained since no interaction between the bio-recognition element and the other components occurs. In the light of contrast, in the indirect assay, it must occur an additional reaction to detect the binding of analyte and biorecognition element. This additional reaction can either be competitive or non-competitive. In this case, for the competitive reaction, a competitive molecule is added to the mixture to compete with the analyte for the binding with the biorecognition element. The signal generated is indirectly proportional to the concentration of analyte present in the sample. When the analyte is not present, all the sites of the biorecognition elements will be saturated by the competitor molecule. For indirect assays, data software analysis can compensate by producing low signals for low concentrations and high signals for high analyte concentrations [11].

Selectivity, sensitivity, reproducibility, and reusability are the main characteristics that should be embraced by the BS. They must be able to signal a positive result, by means of interactions with the target analyte [75]. Furthermore, if indirect format is used, they can avoid the false positive since labels used in direct format can interfere with the complex matrix of food products [11]. For instance, the specific reaction between an enzyme or an antibody with the appropriate target can be used to enhance BS selectivity. The ability to respond to small fluctuations in analyte concentrations it is what can be considered a sensitive analytical tool. The upper and lower limit of detection (LOD and LOQ) of the analyte concentration is commonly measured [75]. However, it is suggested that LOD should be defined by the risk assessment analyses, for the purpose of regulating the detection limit on BS's [11]. Reproducibility is another important characteristic that defines a BS. BS should be able to give the same response to a target analyte when several BS's are produced massively [75]. Therefore, it should be reported reproducible variability between each BS. Another important feature is BS reusability. Disposable BS's are more popular, considering that it avoids deterioration of their elements in complex matrices. Besides that, the use of the BS multiple times will increase the overall analysis time because regeneration time [75].

In an academic research context, BS's are extensively studied, however, their commercialization has been limited by several factors, namely complexity of impedance detection, stability of biomolecule immobilization, smaller analytes, and susceptibility to nonspecific absorption [74]. In such a matter, research should be focusing on overcoming these limitations to move forwards towards commercialization and overcome food poisoning worldwide.

- *Mechanism involved in enzymatic detection of biogenic amines*

A bioreceptor or biological recognition element is a biological element that is sensitive enough to recognize the analyte. The specificity inherent to the biological element is crucial to annihilate any possible interference in complex matrices.

In 1962, the first enzymatic BS was presented. Ever since, enzyme-based BS's developed in an apace [76]. Enzymes are very efficient biocatalysts since they can specifically bind to their substrates and catalyse their transformation. Enzymes reactions are based on the lock and key principle; thus, they can detect the targeted analyte from a sample matrix. This specific relation between enzymes and their substrate is crucial to lower the detection limits [77]. Enzymes either devours an electroactive reactant or produces electroactive species during catalysis, in which the analyte can be measured directly by its production or depletion. In the case of BAs, amine oxidase (AOx) is a class of enzymes that are the most widely used enzyme-based BS's quantifying BAs reported in literature. They can be classified as MAO (deamination of one amine group), DAO

(deamination of a maximum of two amine groups), and PAO (deamination of more than one amine group). Polyamine oxidase (PAO) and DAO are known to catalyse the oxidation deamination of amines in corresponding aldehydes, in the presence of molecular oxygen (as an electron acceptor) with the production of ammonia and hydrogen peroxide [78]. DAO (EC 1.4.3.6) is a homodimer of 60–105 kDa, containing tightly bound copper and 6-hydroxy dopa, a carbonyl co-factor. This enzyme exhibits higher affinity (e.g., they are most active) towards diamines like PUT and CAD, but it can measure the total BA content. DAO occur actively in different amount in all living beings, although in plants they are most active during the germination period. DAO from pea (*Pisum sativum*) seedlings is the most extensively studied and well-characterized enzyme [79], however commercial DAO is frequently from porcine liver. To overcome interferences and lack of sensitivity of enzyme-based BS's, mediators like

Horseradish peroxidase (HRP) can be combined with enzymatic BS's as a dual system to determine BAs [80]. The heme group from HRP promotes direct electron transfer from the redox centre to the conduction sites available on transducers [79].

BAs can be quantified either by oxygen consumption or the hydrogen peroxide production [78]. Since hydrogen peroxide is a side product of amines deamination reaction by amine oxidase, it can be used in indirect detection of BAs concentrations. Optical detection commonly relies on a change of the optical properties due to the presence of the analyte, that could be in form of shifting of the absorption/emission spectra, generation of light by reaction or the alteration of frequency/phase [81]. Chemical BS's with immobilized or solid-state reagents have been used for the determination of hydrogen peroxide in real samples, most of them based on chemiluminescence with luminol as reagent [82]–[84]. Chemiluminescence BS's when applied to BAs quantification are based on the idea that the intensity of the light is equal to the concentration of the hydrogen peroxide. By contrast, the concentration of hydrogen peroxide corresponds to the concentration of BAs present in the sample [84].

Despite their many advantages, enzymes are sensitive to pH, temperature, ionic strength, among other factors, which makes them unstable when incorporated in BS's. For example, free enzymes are unable to fulfil the rising demands of industrial applications mostly due to their low specification, low stability of enzyme preparation, and low activity in the organic phase. Also, they are difficult to recover from the product and lose their catalytic activity after one use [79]. Research over recent years has been given a special focus to enzyme immobilization. Immobilization of enzymes onto a suitable carrier can provide much higher stability. Moreover, they can be recovered from the medium a reused at least ten times. Natural polymers such as cellulose have been successfully used for immobilizing many enzymes due to their characteristics, which include better compatibility, non-toxic nature, high chemical reactivity, and low cost [79].

Regarding major accomplishments owing to BS's main characteristics, the lack of stability along with the high costs remains an unsolved challenge. Some goals that should be pursued in the future include improving the stability of enzyme; integrating them in simple, cheap, and portable systems; sample preparation modules and biosensing systems integrated on the same platform and developing multianalyte detection.

- *Biosensor for a sustainable food detection*

With its fast rate of growth, the population increasing demands of food represents a challenge in food safety. Globalization along with industrialization have triggered agriculture, resulting in an

intensive exploration of natural resources. Moreover, higher temperatures, a result of climate changes, are predicted to modify food safety risks during production, storage, and distribution, resulting in more complex food chains. This lack of keeping track of food is associated with food outbreaks [85].

Innovation and research activities are intrinsically connected to improve the quality of life and work for the welfare of the society. A good quality of life is understanded as easy medical diagnostics facilities, better control of diseases, drug and food quality control and safety, environmental monitoring, and pollution control [86]. Sustainable development in food domain, not only is key to sustaining life and promoting good health but also means supporting national economy [85]. For that reason, BS's are in the front line of being sensitive, fast, continuous, and reliable in controlling important parameters in analytics field.

Methods based on direct measurements of untreated samples are assumed to be the best options in terms of greener analytical chemistry [87]. Thus, miniaturization of portable BS's emerges as an important aspect of future bioelectronics since it may allow on-field screening, handling of low-volume samples, reduction in reagent consumption and waste generation, while providing high-density information storage and increasing sample throughput. The main goal is to have an impact on the environment sustainability but also on economy [86].

Green Analytical Evaluation Tools appears to be the answer for the complex decision-making inside the laboratory. While analytical chemistry develops, numerous methodologies are used, thus, multi-criteria tools such as National Environmental Methods Index (NEMI) and Raynie's tool can be applied when a new methodology or analytic method is employed. For NEMI, the procedure is considered "green" when they meet the following criteria: chemical to be used are not hazardous or listed as persistent, bioaccumulative, or toxic (PBT); pH during the analysis is in the 2–12 range and the amount of waste generated is less than 50 g.

A more recent approach is Raynie's tool, where a pentagram shape is designed to provide information on the greenness of five different categories, namely, health hazard, safety hazard, environmental hazard, energy, and waste amount [88]. NEMI is a much more subjective tool than Raynie's, however, if used complementary, it can help the users by providing criteria for a more informed decision about the chosen method. Figure 1.3 illustrates NEMI's and Raynie's tool applicability on the evaluation of two analytical methods: GC-MS and biosensor.

Currently, concerns on environmental matters are an ongoing trend. Conventional analytical methods use toxic reagents and solvents which have increased to a point at which they became unsustainable from an environmental perspective. Less volume of waste generated, along with the toxicity of reagents employed and the amounts of reagents consumed is considered to be analyst responsibility. With that in mind that, it is expected that in the next years advances in the development of new methodologies, namely in the BS's field, take control of daily laboratory activities [87].

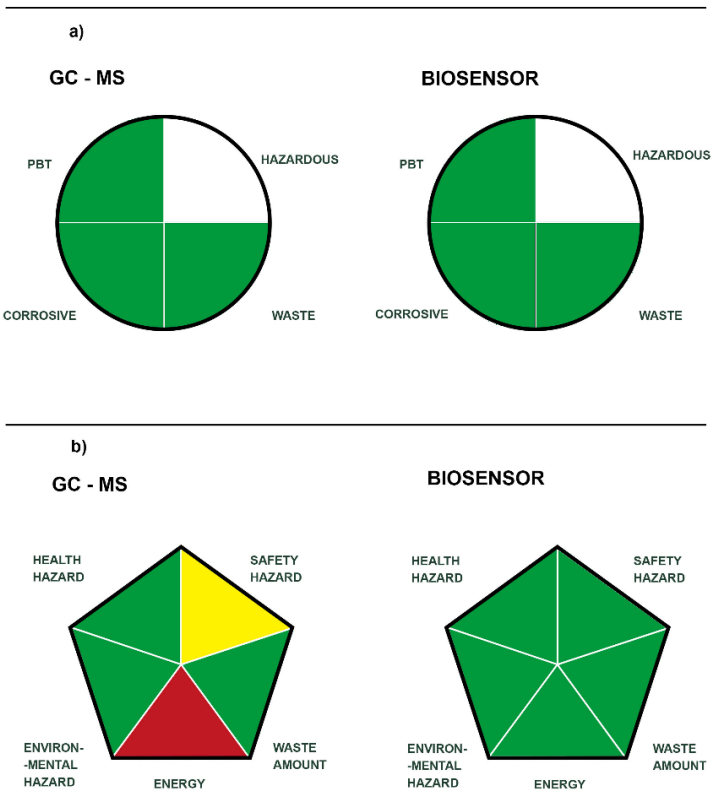


Figure 1.3.: Assessment of the green profile of evaluated analytical methods by (a) NEMI and (b) Raynie's tool.

1.6. Conclusions

It is evident that there is an urge in monitoring food quality, and more importantly, its relation to human health. Food engineering's primary goal is to provide solutions associated with the existing food safety problems. Therefore, coming up with intervention strategies should be the interest of researchers, engineers, as well as people in general. Over the past few years, an increased scientific effort on BAs studies allowed a deeper knowledge about the mechanisms of BAs production. This led to awareness about the possibility of reducing the risks associated with their accumulation in food products (fresh and fermented).

BAs are of particular concern in food hygiene and their content has been widely used as a quality indicator of food products. Different processing and storage conditions of meat, fish, and beverages are the most studied factors involving BAs production. For instance, HIS and TYR seem to be the more relevant BAs, in terms of food security and consumer toxicity Therefore, estimating safety levels for the ingested BAs must be the key to unveil their toxic health effects to consumers. Through this bibliographic revision, it is well-presented that toxicity limits and regulations are scarce. Despite their negative influence on food quality, to date, there are no specific regulations regarding BA's food content, other than HIS in fish and related products. Maybe the solution for this problem will be found in more specific regulations, in order to establish an agreement towards the maximum limit for ingested BAs. As a result, it would be easier for the researcher to choose the analytical method based on the LOD values.

Analytical determination of BAs in food samples is not simple due to the variety of chemical structures that are present in the complex food matrices. As an example, HPLC is one of the most

used techniques to determine these amines due to its high resolution and sensitivity, however, this technique requires sample preparation and are considered to be time-consuming. On the other hand, BS's are described to be much simpler and have been widely applied to BAs determination. Considering the available data, it is clear that BS's are a convenient candidate tool for routine analysis of BAs in all types of food products. LOD and LOQ are significatively low, the analysis is fast and relatively low cost. Nevertheless, it is anticipated that BS's are a green analytical tool since they improve the environmental performance and safety of chemical processes while reducing the risks to human health and the environment.

Concluding, and with respect to the future use of BS's, these will need to meet this new era demands, such as being integrated as sensor networks, providing multi-analyte detection, while being combined with wireless signal for the possibility of easy and remote analysis. In addition. research is needed on the validation of methods for BA analysis and quantification in food matrices which will endorse their commercialization through certified quality standards. This can be achieved by continuous development of reliable BS's, which certainly can provide a better understanding of the overall impacts of BA's presence in our food, and more importantly how can their production be controlled.

Chapter 2. Detection of hydrogen peroxide

2.1. Detection of hydrogen peroxide based on a hydroxyethyl cellulose aqueous solution

A preliminary study for detection of hydrogen peroxide using a hydroxyethyl cellulose was previously developed using chemiluminescence as an indirect method for detection of H_2O_2. Considering the reported methodology, the main goal of this study was to advance in the development of a HEC sensing membrane based on chemiluminescence properties of H_2O_2 when combined with luminol and a catalyst. Experimental tests using a portable acquisition system containing a spectrophotometer were performed. In summary, from this method it was possibly to plot a calibration curve for H_2O_2 standards that ranged from 0.01% w/w to 0.1% w/w.

2.1.1. Materials and methods

The developed membrane solution contains luminol, sodium phosphate, cobalt (II) chloride hexahydrate, sodium lauryl sulphate (SLS), hydroxyethyl cellulose (HEC). The procedure involved adding (0.2 mg, 0.11 mmol/L), sodium phosphate (8.6 mg, 5.25 mmol/L), sodium lauryl sulphate (60 µL, 34.36 mmol/L) and HEC (150 mg, 1.50 %) was added to 10 mL of Milli-Q® water. The mixture was placed on a magnetic stirrer for 30 min and 0.8 mL of membrane solution was injected into a plastic cuvette and then placed into the cuvette holder inside of a small and portable acquisition system specially developed for this work containing a spectrometer (C12880MA from Hamamatsu) that collected part of the light emitted.

Samples consisted of H_2O_2 standard solutions with a concentration of 0.01% w/w to 0.1% w/w, prepared by diluting a standard 30 % w/w solution of H_2O_2. To all sample solutions was added cobalt hydroxide (100 µL, 5.0 mmol/L).

2.1.2. Data processing

The sample solution (0.8 mL) was added to the plastic cuvette placed inside the spectrometer and the emission spectra were acquired being recording at every 100 ms and the total light detected was integrated up to 50 min. The same procedure (Figure 2.1) was repeated to all H_2O_2 concentrations.

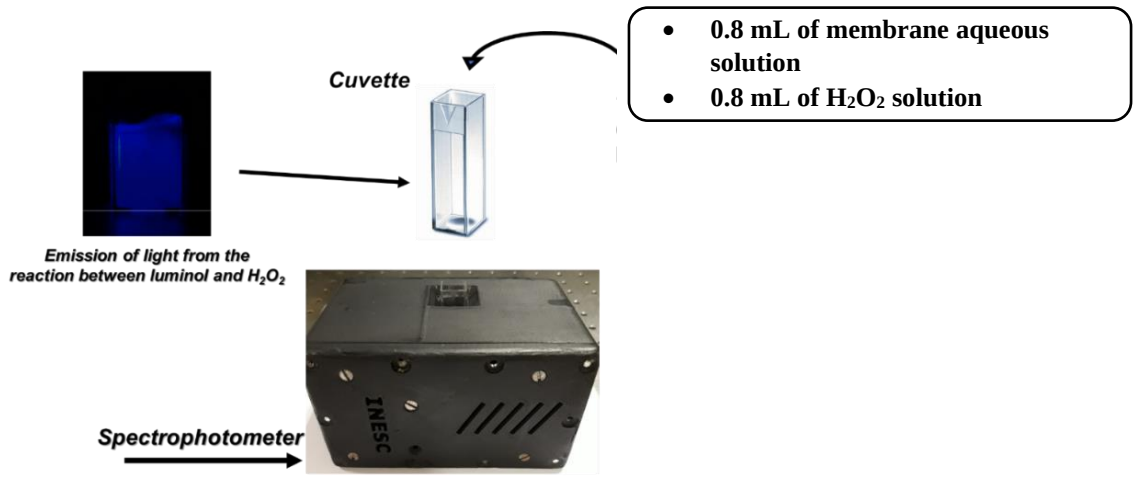
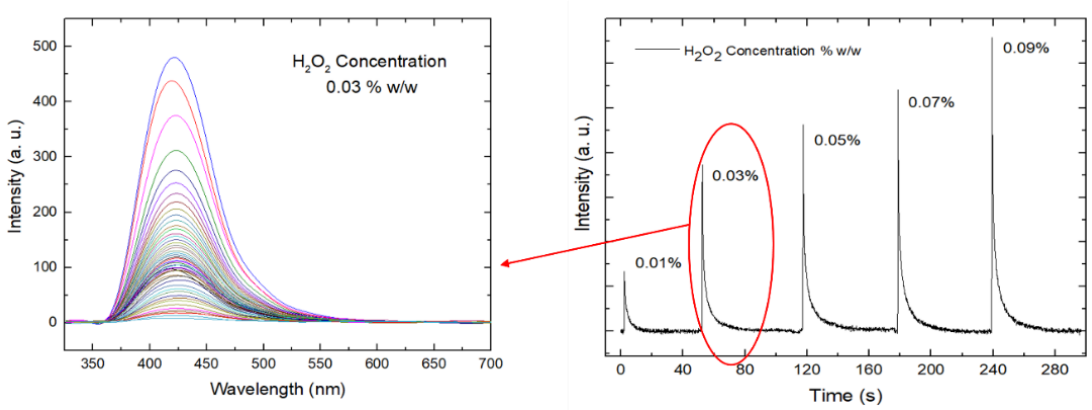


Figure 2.1.: Workflow representation of chemiluminescence reaction on an aqueous system. Luminol and H_2O_2 light emission was detected by the spectrophotometer as illustrated.

2.1.3. Results and discussion

Preliminary results have shown that H_2O_2 detection is possible using an optic method, on an aqueous system. Low concentrations ranging from 0.01% w/w to 0.1% w/w were easily detected. Moreover, it was established a correlation between H_2O_2 concentration and the light emission intensity (Figure 2.2), as reported in the literature. A good linearity range was obtained; however, some difficulties were encountered. When liquid solution was injected into the liquid phase membrane, the light flash was found to be dependent on the user, i.e., dependent on the injection. Thus, reproducibility of the results among replicas was poor.



(a) (b)

Figure 2.2.: (a) Time evolution of reaction for H_2O_2 concentration of 0.03% w/w and (b) Time evolution of reaction for H_2O_2 concentration of 0.03% w/w.

2.1.4. Conclusions

Overall, this study clearly illustrated the potential use of chemiluminescence as method to determine H_2O_2 on a range of concentrations. The potential use of this method for the development of a biosensor was validated, however, will only be attainable under optimized conditions with instrumental acquisition and data processing.

2.2. Detection of hydrogen peroxide based on a hydroxyethyl cellulose: optimization of the sensing membrane

Hydrogen peroxide can be produced via enzymatic reactions, by the two-electron reduction of molecular oxygen to H_2O_2 [89]. This study presents a complete characterization of the optic sensor, based on H_2O_2 detection by chemiluminescence reaction. From the bottle solution of H_2O_2, it was possible to replicate the enzymatic release H_2O_2 signal, as well as the sensor sensitivity. This work allowed the development of an optimized HEC sensing membrane for the H_2O_2 detection. The expression of different patterns of light emission from the previous study (in liquid) was evaluated. With the membrane in a solid form, it was a lot more stable when stored, with higher reproducibility between samples. After the development of the sensing membrane, two calibration curves were plotted, first with a spectrophotometer and then with the final optical detector. LOD and LOQ values were obtained from this characterization.

2.2.1. Materials and methods

The solid membrane was developed with luminol, sodium phosphate, cobalt (II) chloride hexahydrate, sodium lauryl sulphate, hydrogen peroxide (30%) and hydroxyethyl cellulose (Sigma Aldrich, Germany), ethylenediamine tetraacetic acid (EDTA). The procedure established by Miklicanin and Valzacchi [90] was refined to establish the experiment protocols.

In a first stage luminol (0.2 mg, 0.11 mmol/L), sodium phosphate (8.6 mg, 5.25 mmol/L), sodium lauryl sulphate (60µL, 34.36 mmol/L) cobalt hydroxide (100µL, 5.0 mmol/L), EDTA (1 µL, 10 µM/L) hydroxyethyl cellulose (150 mg, 1.50%) was added to 10 ml of Milli-Q® water. The mixture was placed on a magnetic stirrer for 30 minutes. Sample solutions were prepared from 30% H_2O_2 and the respective standards were used 0.0001% w/w to 0.001 % w/w and 0.001% w/w to 0.01% w/w.

Figure 2.3 illustrates the workflow evolution of the sensing membrane. For the initial protocol, 1 ml was dispensed into a home-made Teflon carrier. The membranes were dried in an oven at 70ºC for 4 h then cooled and stored in a desiccator with vacuum. After drying, the membrane was placed inside the cuvette, and light emission was measured by adding 800 µL of sample solution. For the optimized and final methodology, individual 3D printed cups were used and 500 µL of membrane solution was added and dried for 2 hours (T=70ºC). After that, another 500 µL of membrane solution was added and it was left for 2 more hours in incubation. As opposite to the previous protocol, and after the incubation time, the solid sensing membrane was directly placed onto the membrane holder above the detector. Prior that step, 500 µL of the sample solution was added to membrane and acquisition of the signal started.

Sample solutions were prepared from 30% H_2O_2 and the respective standards were used 0.0001% w/w to 0.001 % w/w and 0.001% w/w to 0.01% w/w.

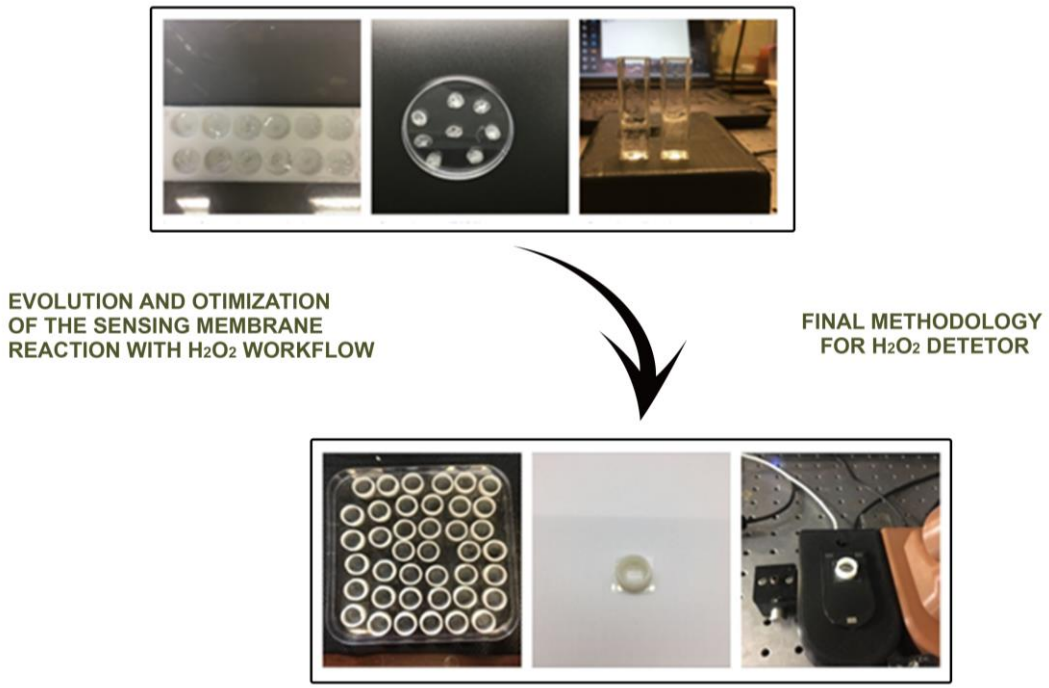


Figure 2.3.: Sensing membrane reaction with H_2O_2 workflow evolution, based on the initial protocol.

2.2.2. Data processing

For this study, a solid sensing membrane was used for all the experiments, to which was added H_2O_2 standard solutions. The first protocol used involved using a dry membrane and placed it into the cuvette, in tiny bits. For the second and final protocol, the sensing membranes procedure involved its direct use and further placement into the membrane holder inside the detector prototype.

From the data processing point a view, the upgrade from the liquid state to solid state, led to differences in spectra signaling. When compared with the previous emission spectra, section 1.2., the narrow pics that only last a few seconds were replaced by well-defined polynomial shape, that could last for hours. Hydration of the solid membrane contributed to a longer and stable chemiluminescent signal. This way, by the integral summatory it was possible to construct two calibration curves for H_2O_2 standard concentrations.

2.2.3. Results and discussion

For the determination of H_2O_2 in low concentrations, the sensor signal should be as high as possible. Therefore, in order to optimize the signal, variations were made in the constitution of the membrane. Only one constituent was varied at a time, keeping the remaining constituents unchanged. Tests were carried out with the variation of the concentration of luminol and sodium sulphate, based on studies presented by [90].

To promote easy membrane handling, the previously tested liquid membrane solution should be used in a solid form, thus improving its stability, manipulation and further storage. However, when all the membrane components were mixed and the humidity is reduced 100%, no light was ever obtained.

First tests were performed by adding the main interferent ($CoCl_2$) separately from the membrane solution. The primary goal in this step was to add cobalt (initially added to the samples) into the sensing membrane, so that it would always be available to catalyse the reaction. This way, by adding H_2O_2 standards, light emission was achieved successfully. In order to use a single solid sensing membrane where all its constituents were present without extra-steps, optimization was performed by adding ethylenediaminetetraacetic acid (EDTA) to the final membrane mix. EDTA is a well-known metal-chelating agent that removes its undesirable effects of these ions by the formation of covalent bonds, a process that consequently forms a complex [91]. This way, EDTA would form a bond with $CoCl_2$ and prevent $CoCl_2$ by interacting with components of the membrane, mainly luminol. Using lower EDTA concentrations it is possible to reduce $CoCl_2$ interferences but still letting it catalyse the chemiluminescent reaction [83]. First, EDTA concentrations ranging from 10 to 100 μmol/L were tested with H_2O_2 standards (0.01% w/w and 0.08% w/w). The interferent component (CoCl2) was introduced to the mixed solution along with EDTA addition right before the measurements were taken. From the results obtained it was possible to see that the optimal EDTA concentration is around 10-20 μM / L (which is equivalent to a concentration of 10^{-5} mol/L), as described by the authors [83]. The next step was to introduce all the components into the membrane to see if light emission would be observed. An almost linear correlation (R^2= 0.989) was obtained for the studied H_2O_2 concentrations, therefore proving the model adjustment where the light emission intensity is proportional to the H_2O_2 concentration, as it would be expected.

To study the effect of the luminol concentration on the chemiluminescent signal obtained, luminol concentration's levels were optimized, and ranged between 0.11 mmol/L to 1.4 mmol/L. Results are presented in Figure 2.4. From the image's observation, it is clear that the increase of luminol concentration led to a decrease in light emission intensity for both of the H_2O_2 standards used. Contrarily to what would be expected, by increasing the amount of the reagent luminol added to the membrane mix, less signal was obtained. These results were justified in a way that no other membrane constituent was altered, thus, the proportional concentrations were not adjusted which led to the decrease of the signal (for 0.11 mmol/L of luminol). For that reason, a concentration of 0.11 mmol/L was chosen to be the best option, not only based on this result but also taking into account the literature available.

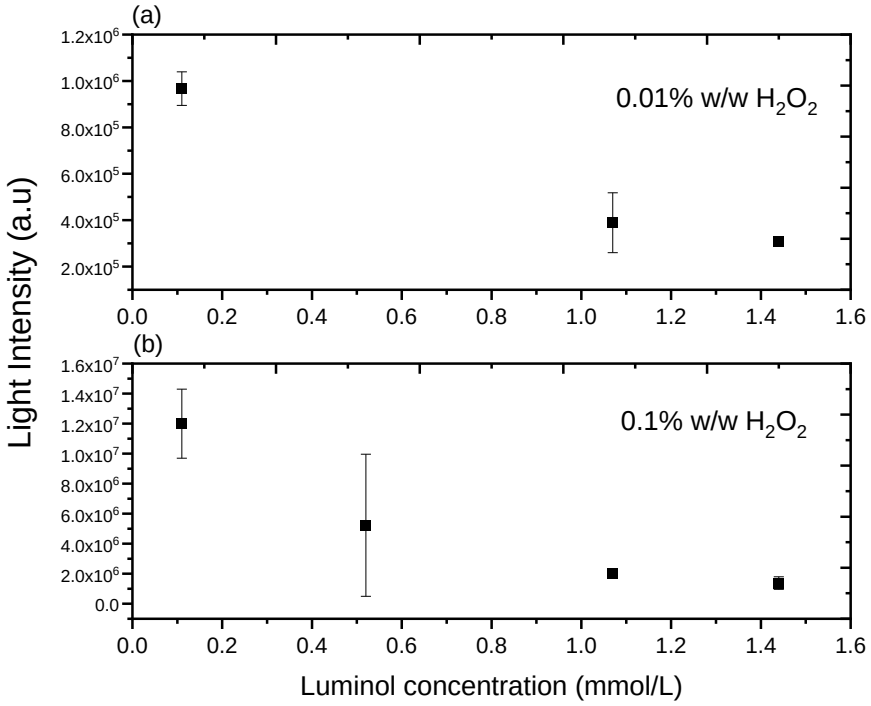


Figure 2.4.: Variation of light emission with different luminol concentrations (mmol / L) added to the sensing membrane using (a) 0.01% w /w H_2O_2 and (b) 0.1% w / w H_2O_2.

Based on article Tahirović et al., 2007 the sodium phosphate concentration, was increased to 50 mmol/L (initial 5.25 mmol / L) and sodium lauryl sulphate to 34.68 mmol/L (initial 5 mmol). With these changes a brittle and opaque membrane was obtained, in which no signal was obtained. In terms of the previously optimized membrane emission signal, duration was longer than the expected based on the previous results. As an example, by adding a 0.01% w/w H_2O_2 standard solution, the signal went on for hours, as the opposite to 30 minutes duration on average. This results maybe be due to the significant increase in SLS (that lowers the surface tension between liquid-liquid solution or, when added to the membrane, liquid-solid interactions) which could contribute to the increased signal duration. Since increasing signal durations was not advantageous, and the membrane structure was greatly altered, the initial concentrations of these constituents (luminol 0.11 mmol/L and 60 µL of SLS, 34.36 mmol/L) were maintained.

To further improve reproducibility among different samples, another strategy of optimization was proposed. Reverse pipetting technique it's a widely used technique of dispensing a measured quantity of liquid by means of air displacement pipette [92]. Particularly, the membrane solution mixture is a viscous solution (similar to hydrogel solution) which sometimes contributed to

inequal measurements of membrane solution into the 3D printed cups, which by consequence led to bad reproducibility. When applied to our membrane solution this technique has proven to be ideal to improve our results. Considering the reproducible results, reverse pipetting was chosen as a technique for membrane preparation.

Energy-dispersive X-ray spectroscopy (EDS) was also performed as a elementary analysis or chemical characterization of the membrane sample. Scanning electron microscope (SEM) images were taken to the optimized sensing membrane and are presented in Figure 2.5.

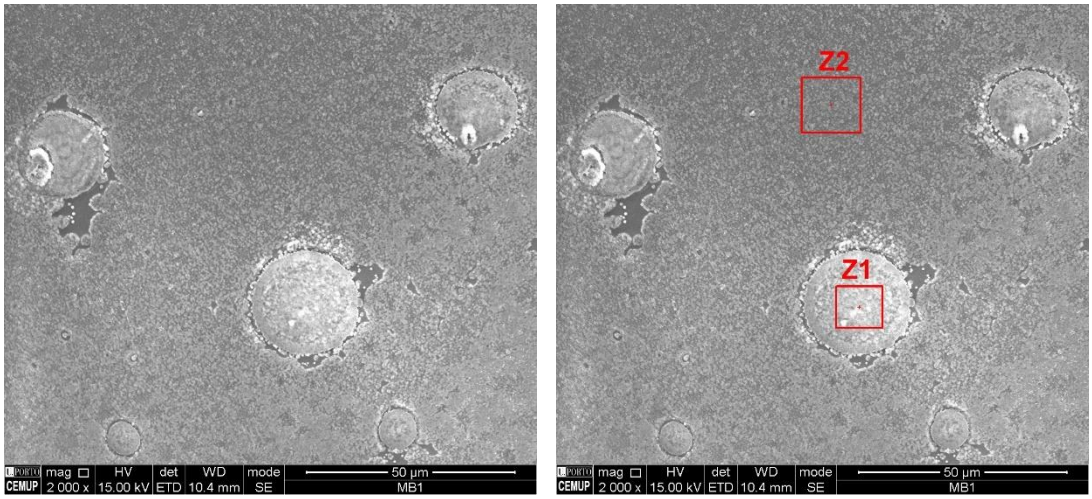


Figure 2.5.: SEM images of the HEC sensing membrane.

Optimization of the sensing membrane was first performed using an optical detector (detector 1). Aiming for the increase in sensitive, needed for enzymatic released H_2O_2 detetction, the gain was upgraded. By doing so, sensitivity was higher than the previous tests. A newly and optimized detector (detector 2) was used for the calibration curves construction. Thus, two calibration curves were constructed using the following H_2O_2 concentrations: 0.001% w/w to 0.01% w/w and 0.0001% w/w to 0.001% w/w. Results are presented bellow in Figure 2.6. For higher concentrations of H_2O_2 the linearity is good (R^2 =0.996), with % RSD not exceeding 10% for the detector 2. LOD and LOQ based on signal to noise ratio of 3 and 10 ranged from $1.2x10^{-4}$ and $3.39x10^{-4}$ % w/w, respectively. For lower concentrations of H_2O_2, the linearity is also good (R^2 =0.998, with % RSD not exceeding 10% for the detector 2, except for the lower concentration measured. The LOD and LOQ based on signal to noise ratio of 3 and 10 ranged from $2.0x10^{-5}$ and $6.8x10^{-5}$ % w/w, respectively. Distilled water was used as a negative result for H_2O_2 presence.

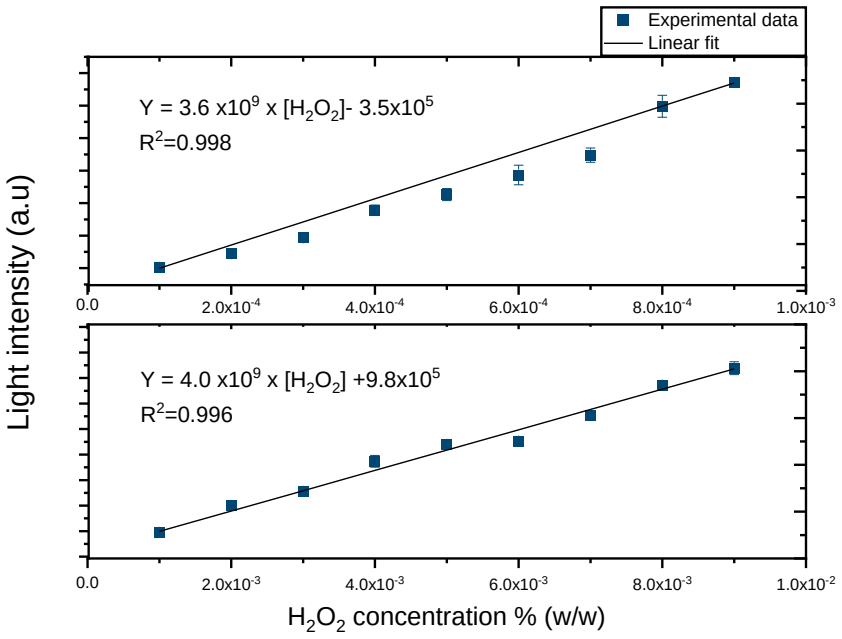


Figure 2.6.: Calibration curves for a concentration range of (a) 0.001% w/w to 0.01% w/w and (b) 0.0001% w/w to 0.001% w/w H_2O_2 using the dectetor 2.

In comparison, detector 2 can largely overcome detector 1 in terms of signal amplification, proven by their higher slope (Figure 2.7).

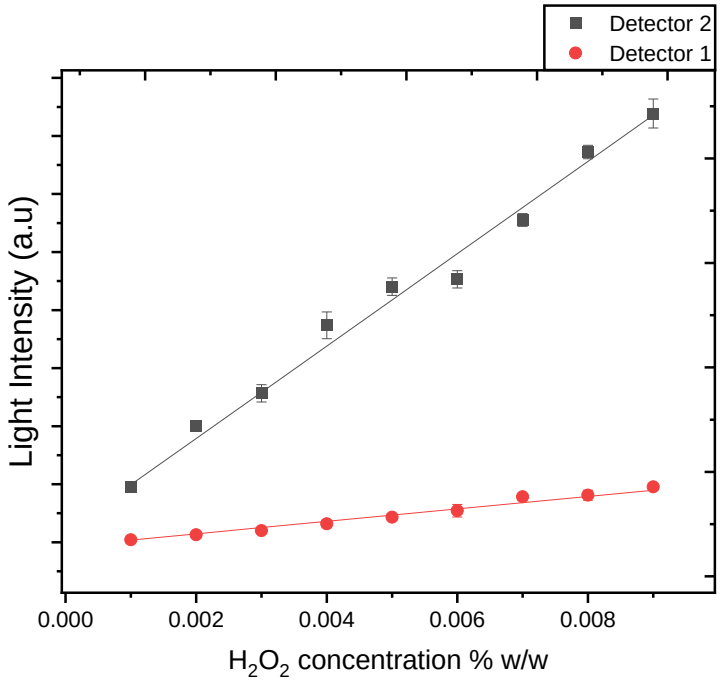


Figure 2.7.: Comparison between the sensitivity of the two detectors based on the intensity of the signal measured for the same H_2O_2 concentrations.

An ultrasensitive and rapid methodology was developed for the detection of H_2O_2 based on a chemiluminescent reaction. Low limits of detection were achieved, thus demonstrating the

applicability of the essay to real samples exhibiting the required sensitivity for the analytical determination of BAs in biological samples.

2.3. Biosensor final prototype

For a straight and rapid spectrophotometric H_2O_2 detection, a detection module was build up containing a highly sensitive detection system with a photodiode (model S8746-01 Hamamatsu Japan), a dedicated amplification system with variable gain and an embed controlling unit. The sensitive optoelectronic system was isolated inside of a custom-made 3D printed case allowing the easy replacement of the sensing membrane and allowing the sample pipetting preventing the detection of the ambient light. Preparation module includes sample preparation prior to the analysis, which can contain smaller pre-treatments, while sample detection module gathers all the biosensor compartments. Since the enzyme requires controlled temperatures for the reaction to happen, a preparation module containing a small oven and stirring was constructed, as illustrated in Figure 2.8.

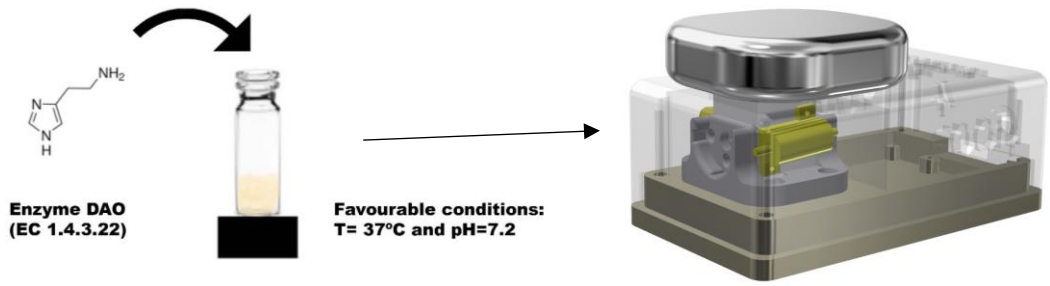


Figure 2.8.: Schematic representation of the analyte sample prepration module of the optical biosensor.

The detection module contains the highly sensitive photo detector, raspberry-pi and a user-friendly graphical interface (GUI) (Figure 2.9).

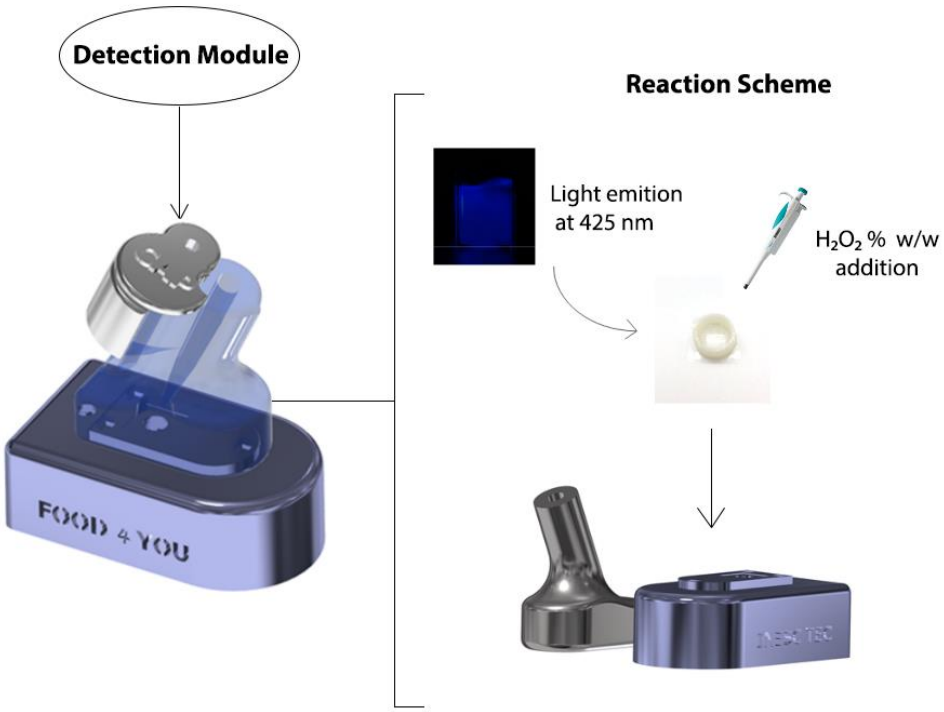


Figure 2.9.: Schematic representation of the analyte detection module, including the chemiluminescence reaction scheme.

(a) (b)

Figure 2.10.: (a) First design of the prototype and (b) Prototye field case photographicdesigned for *in-situ* analysis of food contaminants.

All of the biosensor's modules are integrated, which enables real time monitorization of the chemical reaction, including information on final H_2O_2 concentration (w/w%) allowing a visual evaluation of the sample. Considering that a portable analytical system is essential for a successful

on-site analysis, efforts were dedicated to device improvement and optimization focusing on portability features that included small size, low cost, single shot membranes, and the ability to measure samples with minimal sample preparation without solvents. Figure 2.11 ilustrastes a schematic representation of the of the complete biosensor procedure.

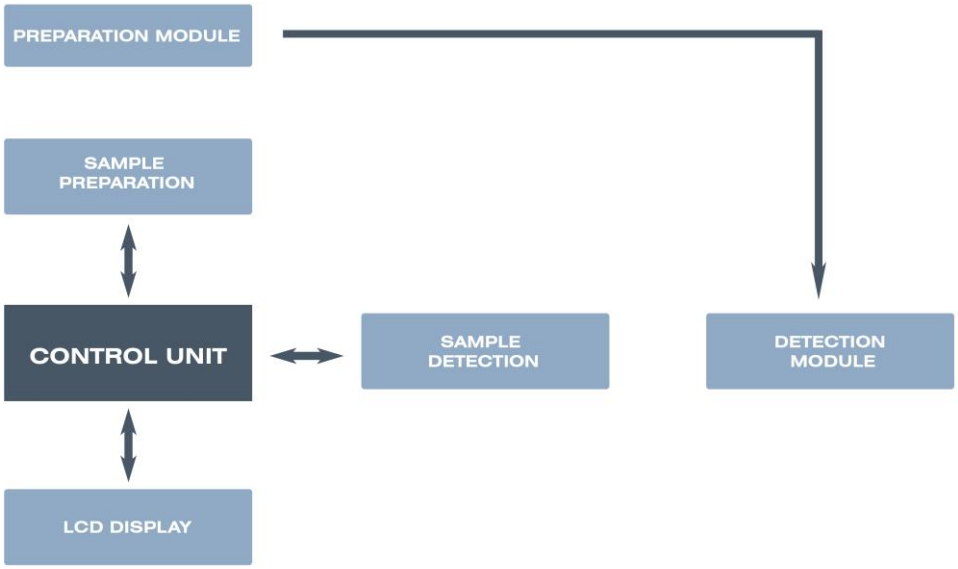


Figure 2.11.: Schematic representation of the biosensor procedure.

2.3.1. Conclusions

The determination of H_2O_2 was performed by the developed sensor. It was important to do the membrane optimization and all the procedures it evolved once it is the main component to be integrated in the final biosensor. Thus, the upgrade of the sensing membrane from the Teflon to the individual 3D printed cups improved our results significantly. The addition of EDTA to the membrane solution, based on literature findings, allowed the transition from liquid to solid sensing membranes for the first time. Also, reverse pipetting significantly contributed to the reproducibility between membranes, thus solving one more problem. However, from the literature available some concerns that are intrinsically related to the H_2O_2 chemistry were raised. Since lower concentrations of H_2O_2 are relatively unstable (H_2O_2 stored solution decomposes at a specific rate) [93]–[95], when the enzymatic reaction releases this compound at some specific conditions it could be unstable and readily decomposed into water and oxygen after a few hours.

Globally, the final sensing membrane was fully functional, stable when stored for days (at least 15 days) and the duration of the signal increased, due to its hydration by the liquid sample. Besides overcoming the existential problems, when a comparation is made over other commercially available analytical methods (e.g., DR1900 and H_2O_2 detection strips) the developed method was sensible and distinguishable enough. Furthermore, it can detect traces of H_2O_2 (concentration level that is almost water - 0.0001% w/w H_2O_2). For distilled water no signal was observed.

This study allowed a full characterization of H_2O_2 detection, thus comprising an in-depth chemical knowledge about these compounds in the tested conditions. Nevertheless, as H_2O_2 is present in different matrices, further studies should be performed using real samples. Therefore, it will be possible to better understand the matrix effects, i.e., possible interferences, on the chemiluminescent signal.

2.4. Insights on enzymatic reactions with biogenic amines taking advantage of the hydrogen peroxide sensor

Enzymes are very efficient biocatalysts since they can specifically bind to their substrates and catalyse their transformation. Polyamine oxidase (PAO) and diamine oxidase (DAO) are known to catalyse the deamination of amines in corresponding aldehydes, in the presence of molecular oxygen (as an electron acceptor) with the production of ammonia and hydrogen peroxide [78].

The following reaction [96] is an example of PUT oxidation using DAO as a catalyst, resulting in the production of 1 mole of H_2O_2 per mole of PUT:

$$PUT + H2O + O2 \rightarrow* 4 - Aminobutan\ aldehyde + NH3 + H2O2 \quad \textbf{Equation 1}$$

*by the action of DAO

The main goal of this study was to give the first steps into BAs oxidation via enzyme reactions. Indirectly, enzymatic released H_2O_2 can be detected, and its concentration is proportional to BAs concentration on a real sample. Time is always variable since 1 unit of DAO is the amount that will oxidize 1.0 μmole of PUT per hour at pH 7.2 at 37ºC. Therefore, a time limit was never established for this protocol. An exploratory application of DAO enzyme in BAs oxidation was performed combining PUT and DAO in the controlled environment. In summary, the obtained results were inconclusive but emphasized the potential use of this enzymatic reaction on the BAs determination by the developed biosensor, therefore allowing an in-depth study of BAs presence in real samples.

2.4.1. Methodology

DAO enzyme was applied to H_2O_2 determination by oxidation of BAs. The scheme of the diagram of the analyte detection performed with DAO was divided into two main stages, which includes the enzymatic process and membrane injection with prior data processing. Materials, as well as the methods used were described in detail in the following sections.

- *Diamine oxidase (DAO) enzymatic essay*

Enzymatic solutions containing the appropriate amount were prepared by mixing DAO powder with solid PUT in phosphate buffer (pH=7.4). Both sodium phosphate buffer, with prior adjustment of pH, and PBS were used for this study. The most important parameter for the enzymatic reaction to happen, is that the buffer should have a pH of 7.2. However, the use of sodium phosphate as a buffer and further pH adjustment with HCL could lead to changes in the medium environment. Thus, PBS was carefully chosen as the selected buffer.

To determine the mass of enzyme required, the following equation was used:

$$C = A\,x\,\frac{m}{V} \qquad \textbf{Equation 2}$$

where c is the wanted concentration of enzyme (Units/mL), A the specific activity of the enzyme, m is the mass (mg) and v is the final volume (mL).

Enzymatic essay of DAO involved two main stages, which were preparation of the buffer solution and the enzymatic solution to be incubated. Thus, to prepare PBS buffer 1 tablet of the PBS was dissolved in 200 mL of distilled water, as instructed by the manufacturer. The strategy of DAO's final protocol was to use a fixed time of reaction (1 hour) and a variable DAO concentration depending on the concentration standard of PUT used. Hence, a starting concentration of 500 mg/L of PUT was prepared in the 250 mL of PBS buffer (pH=7.4). From that concentration, standards ranging from 25 to 500 ppm were prepared from that primary solution. To a 5 mL vial containing 1 mL of PUT in PBS it was added the correspondent amount of DAO, in the solid form, needed to degrade that concentration of PUT in one-hour incubation. After incubation of the sample for one hour, 0.5 mL of sample solution was injected into the HEC sensing membrane, previously optimized and used for the detection of enzymatic release H_2O_2.

- *Commercial hydrogen peroxide (H_2O_2) test kit*

Depending on the concentration, H_2O_2 will oxidize metallic molybdenum and under certain conditions may form "superoxides" with the dissolved molybdenum salts. The H_2O_2 test kit was purchased from HACH and used with DR1900 equipment, that gives absorbance measurements based on a colorimetric assay. Thus, for this analyses, it was used: 5 mL of Solution A (LCW58: 4% sulphuric acid (CAS: 7664-93-9), 94% water (CAS: 7732-18-5) and 2% of sodium molybdate (CAS: 10102-40-6)); glass vials (LCW 96) and syringe and syringe filters. Samples consisted in 0.2 mL of H_2O_2 solution (0.01% to 1% w/w H_2O_2) from 30% w/w H_2O_2 for the calibration curve and DAO solution with PUT addition (concentration equivalent to 0.01% to 0.1% H_2O_2) for the enzymatic measurements. Absorbance was read three times.

2.4.2. Data processing

For this study, previously optimized sensing membranes were used. All the experiments were performed by adding DAO powder to the PUT solution. The first protocol involved injecting DAO solution and PUT solution separately to the membrane. Signal was recorded right after adding the PUT solution to the sensing membrane that already contains DAO diluted in PBS. A much more practical protocol was later developed to eliminate one of the steps, where the amount of the sample to be injected by a micropipette was fixed at 500 µL, and DAO and PUT were mixed together. Here, signal acquisition starts once the enzymatic solution is added to the sensing membrane. Schematic representation of the workflow data processing of both spectrometer and dectetors are illustrated in Figure 2.12 and Figure 2.13.

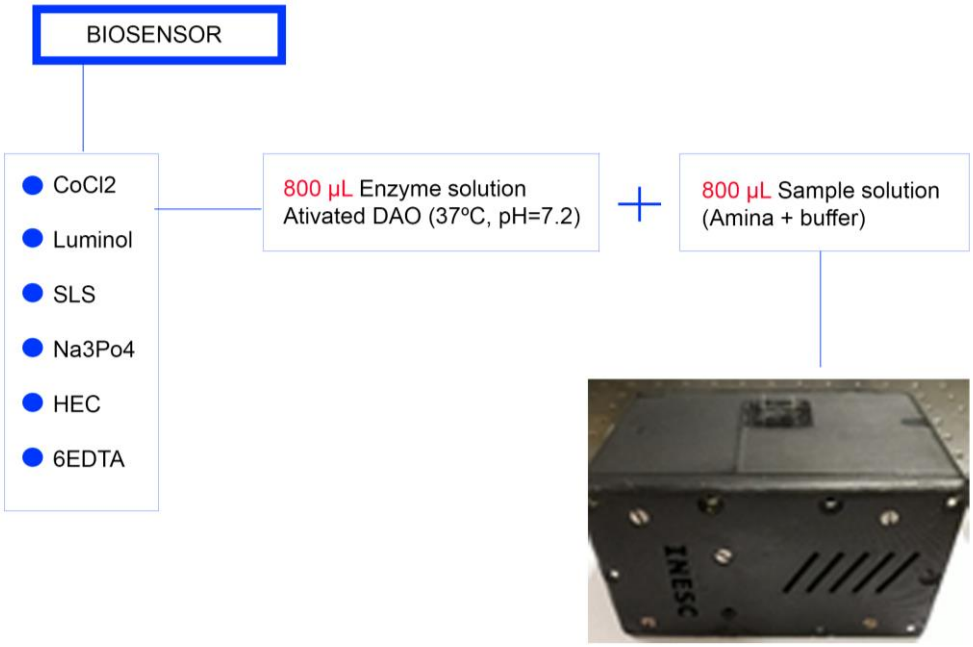


Figure 2.12.: Schematic representation of the workflow data processing using spectrometer

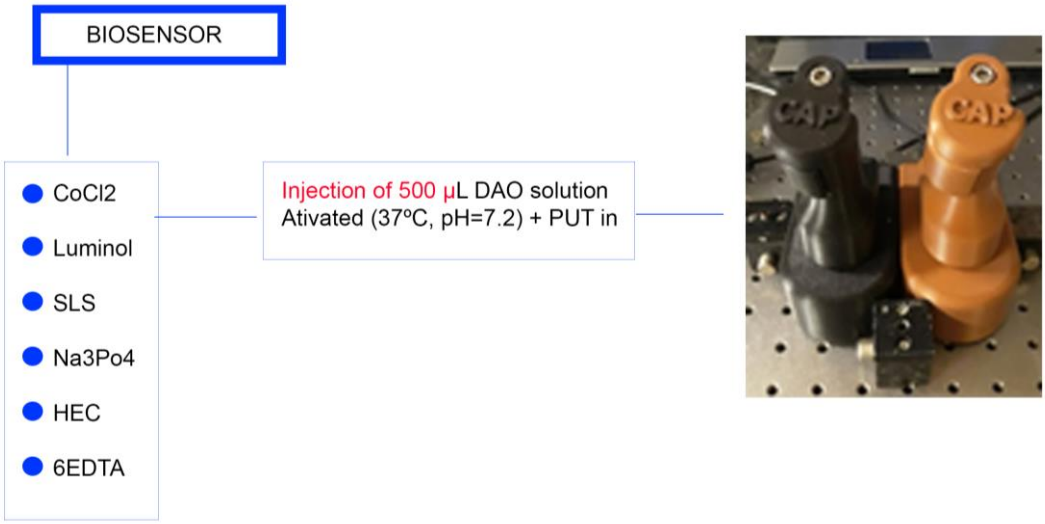


Figure 2.13.: Schematic representation of the workflow data processing using detector 1 and 2, respectively.

2.4.3. Results and discussion

Concentrations levels of BAs found in food products normally do not exceed 100 mg.kg^{-1}. Taking into consideration, that many factors can affect BAs content in food products, mostly fish and meat, a literature review was done on this matter. Since PUT is the most used BA for DAO assays, was choosen to be the reference. The selected range of BAs concentration (10-500 ppm) was ideal for a routine BAs analysis. Besides, for lower concentrations of BAs the use of enzymes is minimal.

Different parameters can contribute to enzymatic kinetics, which will be closely related to the reaction rate. Thus, the most important factors affecting enzymatic reactions were previously studied (Table 2.1). Activating the enzyme is a crucial step since the rise in the temperature will increase the activity of most enzymes by 50% to 100% [97]. Therefore, 15 minutes were found to be a good compromise concerning the ones found in the literature. Each enzyme has a temperature range in which a maximal rate of reaction is achieved. The optimum temperature of 37ºC was found to be consensual between authors, which was also reported by enzyme manufacturer Sigma. In the same way that every enzyme has an optimum temperature, each enzyme also has an optimum pH. DAO enzyme can undergo pH ranging from 6.0 to 7.2, however, optimum pH was chosen as 7.2 based on the manufacturer's essay.

The first enzymatic trials were performed using optimized HEC sensing membranes along with the spectrophotometer. Here, the blank (an empty cuvette) was always recorded before the sample injection. From the literature, five concentrations of PUT (1 to 5 mg/L) were set, as well as a fixed enzyme concentration of 10 mg/L, based on [90]. After injection of 0.4 mL of enzyme solution and 0.8 of PUT solution to the sensing membrane displaced inside the cuvette, the signal was recorded. Since spectrometer software was not fully developed at this point, the differential of the given signal was always increasing instead of stabilizing. The results given were considered not correct, thus it will not be mentioned.

Table 2.1.: Sum up of the reference values for some methodologies used in DAO enzymatic assays.

Temperature (ºC)	Activation time (min)	Enzyme concentration	Buffer
37	30	3.0 to 6.0 units/ml (1.4.3.22)	100 mM Sodium Phosphate Buffer, pH 7.2
38	-	-	100 mM Potassium phosphate buffer, pH 7.0
37	30	1 to 20 mg (porcine liver)	0.05M Sodium phosphate buffer solution, pH 7.2
37	15	27.5 units, 250 mg (porcine kidney)	0.1 M Potassium phosphate buffer, pH 7.2
37	10 a 30	-	-

Aiming for the use of a reference concentration of H_2O_2, in which was known for sure that would be detectable by the spectrophotometer, the amount of PUT needed to enzymatically release the same H_2O_2 concentration was stoichiometrically calculated. By setting a PUT concentration of 525 mg/L (equivalent to 0.01% w/w H_2O_2), to study enzyme kinetics (at 37ºC, pH=7.2), samples of this reaction were taken every hour. Here DAO concentration (0.336g in 10 mL of buffer), based on [20], was increased substantially once 10 mg/L would not be enough to degrade PUT in just a few hours.

No light was obtained for the enzymatic solution of DAO and PUT. To test if the problem would be on the newly optimized membranes, a solution of 0.01% H_2O_2 was injected into the membrane. When the H_2O_2 solution was injected, light emission was obtained, thus excluding problems regarding the sensing membrane.

Theoretically, an increase in the amount of PUT added to the reaction medium would lead to higher amounts of H_2O_2. Therefore, the maximum PUT concentration of 0.15% w/w H_2O_2 was used. The possible interference of DAO coloration was studied using a spectrometer HITACHI U-2010. Spectrometer conditions used for all the enzymatic tests were as follow: absorbance mode, a wavelength of 1095 to 195 nm, with a scan speed of 400 nm/min and with an optic path length of 10 nm. Since a higher amount was being used (0.336 g of DAO in 10 mL of phosphate buffer), it was necessary to dilute our samples (x10). The enzyme diluted in buffer was used as reference, while the "mix solution" contained DAO and PUT. Results obtained are presented in Figure 2.14.

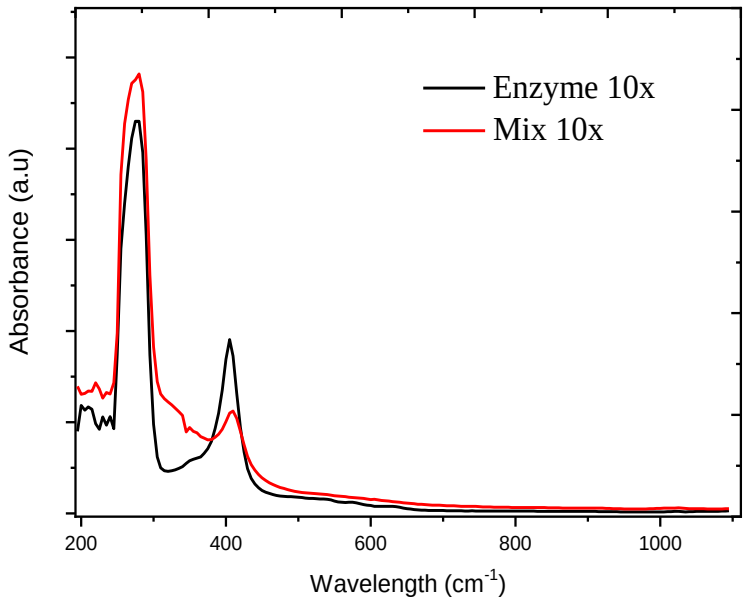


Figure 2.14.: Comparative graphic of the absorption spectrum of the enzyme solution (Enzyme 10x) with or without reaction (Mix 10x).

According to the absorbance spectrum analysis, some differences may be noticeable between the DAO solution that didn't undergo to incubation reaction and DAO solution that did. Considering that the only difference between the two enzymatic solutions is the heat activation and subsequently the enzymatic process, these alterations in the absorbance spectrum can be justified by protein conformational change with the heat increase. The variation of DAO solution absorbance before and after the enzymatic reaction was measured using the spectrometer. PBS was used as a reference. Figure 2.15 shows the results obtained from DAO solution absorbance before and after the enzymatic reaction.

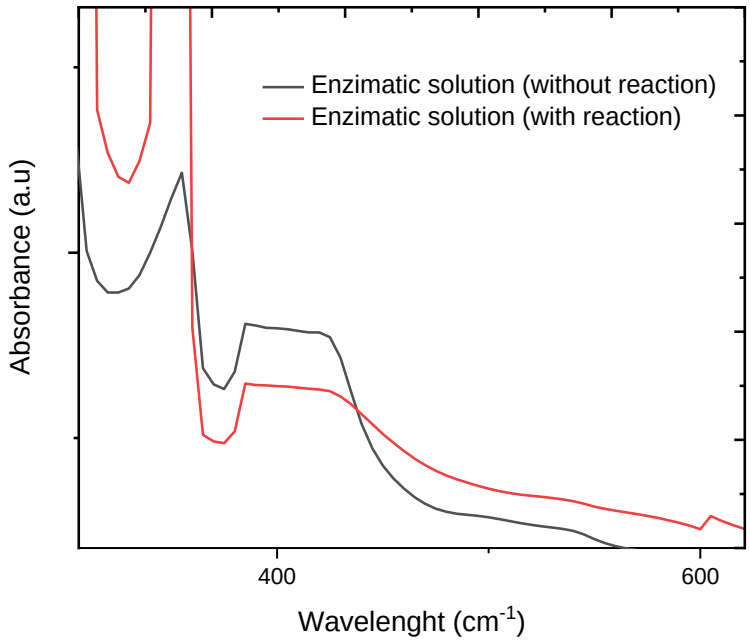


Figure 2.15.: Comparative graphic of the absorption spectrum of the enzyme solution with or without reaction.

From the observation of Figure 2.15, it was observed an alteration of the absorbance spectrum before and after the reaction has occurred. Although there's a diminution of the absorbance at 425 nm, no light was observed and no explainable reason was found. To test whether the drastic increase in absorbance at the wavelengths ranging from 340 to 360 was due to the added PUT or to the enzyme changing its conformation during the activation time, the solution of DAO containing PUT with reaction (Figure 2.16) that was subjected to reaction was tested and stored at 4ºC for a couple of days. The absorbance spectrum was measured after storage to see if it would return to the original one. After storage, there was an accentuated decease of the absorbance spectrum at the wavelength range from 320 to 360 nm, right after the enzymatic reaction occurred. Therefore, it was assumed it to be the conformational change of DAO that happens with the increase of temperature. Although advances were not made from this test it was possible to assume that the enzyme is active during incubation time, which could be further applied to real-time monitorization.

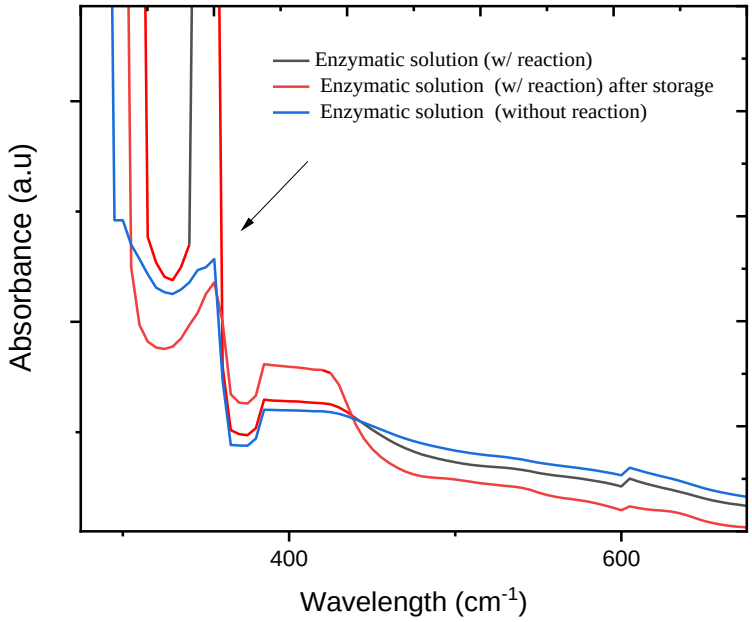


Figure 2.16.: Comparative graphic of the absorption spectrum of the enzyme solution with, with storage and without reaction.

Final tests were performed, with the optimized detector so the lowest H_2O_2 concentration could be reached out. For a straight and rapid evaluation of the enzymatic reactions, without compromising too much enzyme, a new and final protocol was developed. The solid enzyme was directly added to the vial containing 1 mL of PUT and to the time of reaction (set to 1 hour by the unit definition) 15 minutes of enzyme activation was added. The optimized detector (detector 2) was used along with this protocol. After 4 hours of reaction for a fixed enzyme concentration, 0.5 mL of the reaction solution were injected into the sensing membrane, however, no signal was obtained. To test the effect of DAO solution on the signal, on the same membrane 0.01% w/w H_2O_2 solution was added. After that, DAO solution that underwent enzymatic reaction was injected into the sensing membrane already containing 0.01% w/w H_2O_2 (Figure 2.17). When the DAO solution is injected, the signal dropped instantly to its absence in just a few seconds. For the standard H_2O_2 without DAO addition, a 13-minute signal emission is presented, therefore, proving that DAO solution affects chemiluminescent signal negatively.

By comparison, using a ten times lower concentration than the one injected into DAO's reaction solution, a saturation of the measured signal was observed. At first glance and contrarily to when DAO is present, 87% of our initial signal was lost. After increasing PUT concentration to 1500 ppm (higher and exaggerated putrification state), no signal was obteined.

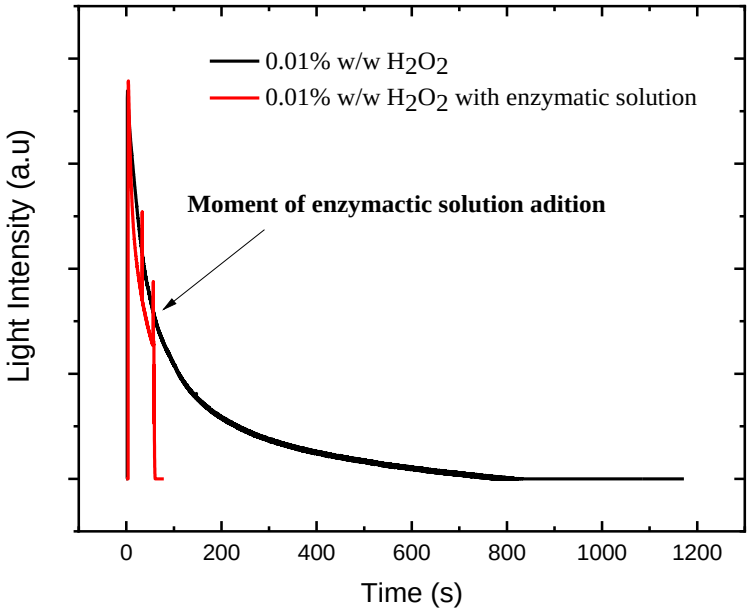


Figure 2.17.: Comparative graphic of the light emission spectra when DAO (pH=7.4) is added to a 0.01% H_2O_2 solution and the real spectra.

Regarding problems with the manufacturer, the product diamine oxidase with CAS - 9001-53-0 - was bought with different physical characteristics, including color. The 1st bottle was white, and the others are brownish. In addition, the chemical-physical properties were not the same: no results were obtained from the 2nd and 3rd bottles when using the same protocol.

To perform a final test, an older enzyme bottle was used. Thus, 0.1g of DAO from the white powder and brown powder were incubated at 37ºC in 3 mL of PBS (pH=7.2), containing 500 ppm of PUT. First, the DAO solution was allowed to react for 1 hour plus 15 minutes of activation, and the signal obtained was measured. Reactional parameters were the same for both solutions. For further evaluation of the time factor, the enzymatic solution was injected into the membrane at different times of incubation, specifically 1 and 4 hours. Results were obtained for white DAO powder only and are presented in Figure 2.18. Triplicates were performed for each injection.

According to the results obtained, it would be predicted that for an enzymatic solution the signal will be proportional to the concentration of H_2O_2 released. However, there was no big difference after incubation for 1h and 4h. For the brown solution, no signal was obtained.

When integrated with the previous calibration curve, the signal corresponds to a concentration of 0.0002% w/w H_2O_2. After 4 hours of reaction, the concentration obtained is very far from what would be theoretically expected (500 ppm of PUT is similar to 0.01% H_2O_2 w/w). Positive results were obtained when an older white powder DAO solution was used, from which it was concluded that enzymatic H_2O_2 was being released.

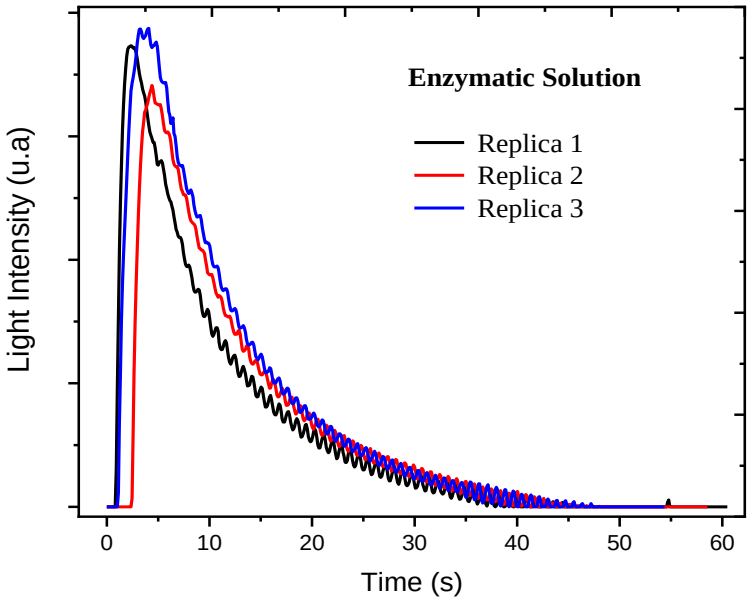


Figure 2.18.: Graphic of the emission spectrum obtained by the DAO reaction after 4 hours of incubation at T = 37ºC and pH = 7.2

Proteins, such as enzymes, tend to aggregate, foam or precipitate when subjected to agitation. Since temperature could not be used to denature DAO, due to the possible loss of H_2O_2, the first strategy to eliminate DAO from the main solution after enzymatic reaction, was using vortex as a disruptor. This process can denature proteins by the formation of two distinctive phases: one containing the enzyme in form of foam or particles and the other the supernatant. The procedure involved denaturation of DAO solution for repeated times (10-40 minutes). After each cycle of the vortex, DAO foam was separated from the liquid phase with the help of a pipette. After 40 minutes, the final solution contained less visible foam.

The variation of DAO solution absorbance over the steps of vortex denaturation was measured using the spectrometer . For the times of 30 to 40 of vortex, a notorious change in the absorbance spectrum was obtained. Thus, indicating the time at which the enzymes started to lose its characteristics. Figure 2.19 compares the difference between time 0 to 40 minutes.

Even though a visible change in the absorbance spectrum was obtained, the denaturation rate was unsatisfactory, which can be related to the presence of stabilizers in the commercial DAO (one molecule of pyridoxal phosphate and one atom of copper for each subunit). Finally, to the denaturized solution (t=40 minute) was added 3.3 µL of H_2O_2 (cf=0.01% w/w), but no light was observed.

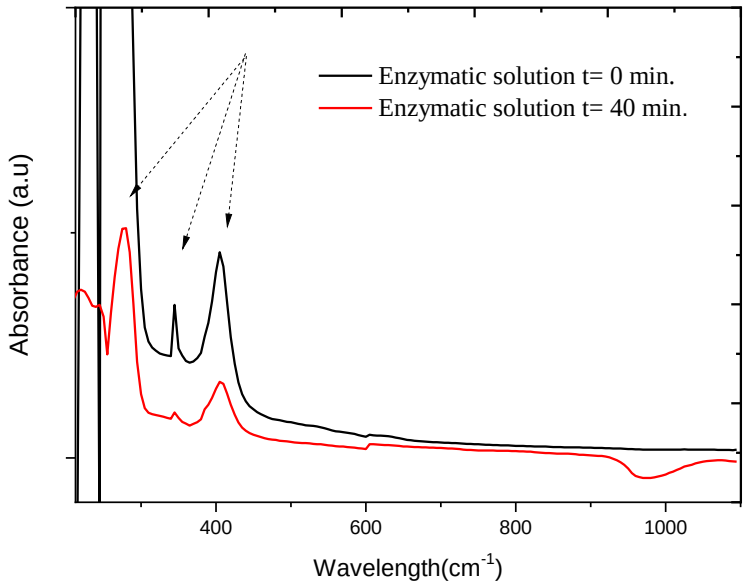


Figure 2.19.: Close up of the comparative graphics of the absorption spectrum of the enzyme solution corresponding to the time t =0 minutes and t= 40 minutes of denaturation.

From this study, it was concluded that it is possible to denature proteins using the vortex, however, it's a tedious, not practical methodology that didn't help solve the problem. Moreover, it was not possible to say for sure if there was still enzyme left on the final sample.

Any protein in its native or "folded" state is operational and in its "folded" structure could be secondary, tertiary, or quaternary structure [98]. The change in pH protocol was developed with the main goal of testing if the abrupt decrease of the pH of the medium can separate DAO from the solution. DAO isoelectric point extracted from the rat intestine is reported to be 6.0 [99], however, for commercial DAO, its most favorable pH is at 7.2. At the isoelectric point, the charge is neutral and therefore the enzyme is stable. When HCL is added and pH drops to values of 2 or 3 there is a decrease in the stability of the enzyme in the native state (electrostatic interactions not favorable), due to the increase of positive charges (H^+) in the medium in which the protein is dissolved [98]. This way, extreme pH and temperature environments alter a protein structure and, consequently, its function.

For this study, a pH of 2-3 was selected as the pH to fully denature DAO. Starting from a solution of DAO and PUT, in the general conditions of pH, HCL (37% v/v) was added drop by drop until a pH of 2-3 was reached. The pH variations were measured by using HACH pH meter equipment with constant stirring. After denaturation, the solutions were filtered with a syringe and a syringe filter (0.2 microns).

To test if DAO absorbance at 425 nm would decrease proportional to the denaturation by the action of decreasing pH, the absorbance spectrum was measured, and the results are shown below in Figure 2.20. From the results obtained, it is concluded that by decreasing pH values from an alkaline environment to an acid one, the absorbance of DAO reduces significantly at 425 nm, region of light emission from chemiluminescence reaction. Specifically, measurements of absorbance from the control sample were dopped from 0.306 to 0.131 right after pH denaturation followed by syringe filtration. The sample of DAO at pH=2.4 before filtration has suffered an

overall increase of the absorbance which can be justified by the presence of particles in suspension (denaturized DAO).

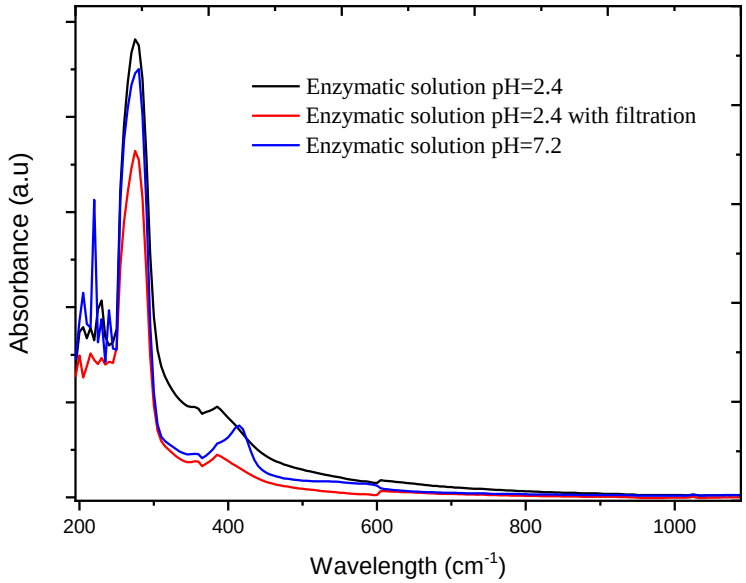


Figure 2.20.: Absorption spectrum of DAO solution at different stages of the pH denaturation.

To the denaturized solution (DAO at pH=2.4) was added 3.3 µL of H_2O_2 (cf=0.01% w/w). This solution (0.8 mL) was injected into the HEC sensing membrane, and the signal previously obtained for 0.01% w/w H_2O_2 alone was compared with the signal of the mentioned solution of DAO. From the observation of the two light spectrums, it was clear that for the denatured DAO after H_2O_2 the signal lasts for a few seconds as the opposite half an hour for the signal of the 0.01% w/w H_2O_2 alone, similar to the previously mentioned results when DAO is present in any form. Overall, the analysis of the absorbance and light emission spectrum allowed to make some assumptions between the solution of H_2O_2 diluted in water and the same H_2O_2 concentration when the solution have DAO in it.

1.4.3.1. Validation with a commercial sensor: H_2O_2 test kit

HACH kit was used as instructed by the manufacturer, which starts with the addition of 5 mL of Solution A to the kit vial, followed by the addition of 0.2 mL of the sample. Firstly, a calibration curve was constructed for two ranges of concentration: 0.01%-0.1% H_2O_2 w/w and 0.1% to 1% w/w H_2O_2. Calibration curves were constructed by measuring absorbance at 440 nm for each concentration of the H_2O_2 solution. Blank was prepared by adding 5.2 mL of Solution A. Figure 2.21 represents a schematic diagram of the workflow used for H_2O_2 determination.

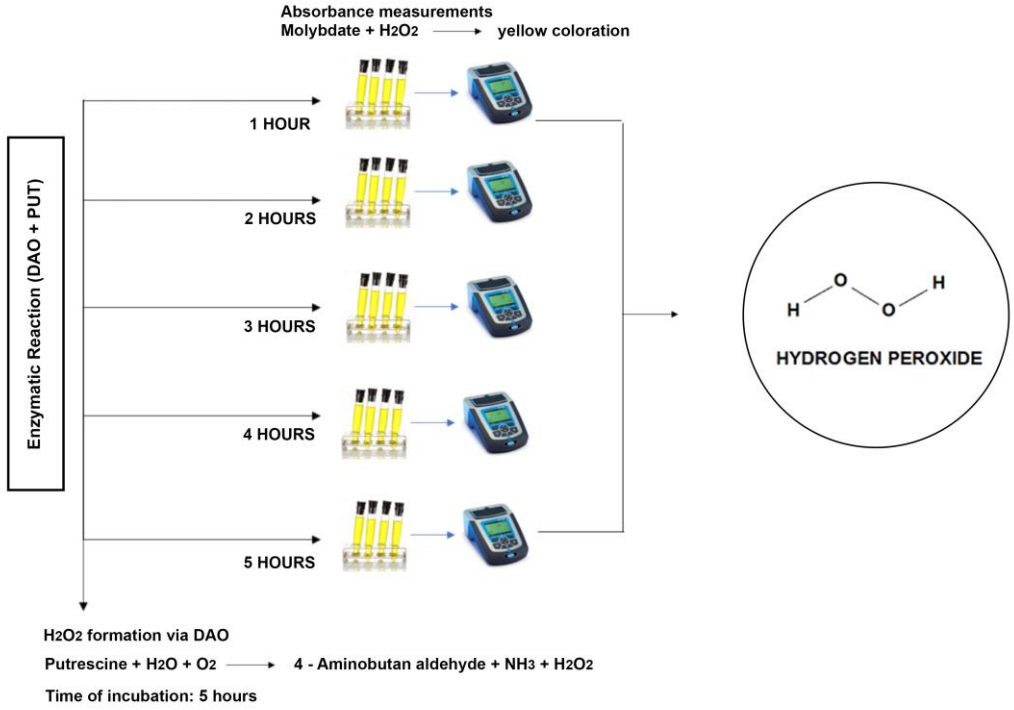


Figure 2.21.: Schematic representation of the workflow including the main stages for H_2O_2 determination: DR1900 protocol and respective results.

Secondly, enzymatic samples containing DAO and PUT (0.2 mL), at different times of incubation (1 to 5 h), were added to Solution A (5.0 mL) and absorbance was measured. Here, DAO and PUT without incubation were used as blank. Considering that Solution A contains sulphuric acid it was expected that DAO would suffer denaturation (Figure 2.22). After denaturation, and to prevent the increase of the signal and consequent false results, syringe filtration (x2) was used to help thesample “clean-up”. After each filtration step, absorbance was measured for each incubation time.

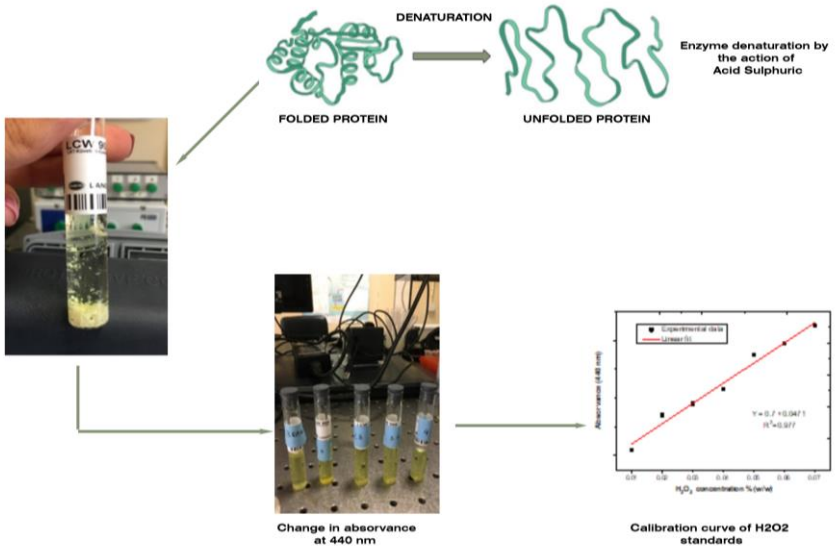


Figure 2.22.: DR1900 sample preparation: moment of enzymatic sample addition for each incubation time.

From the results obtained (Table 2.2) it was not possible to notice an increase in absorbance over the time of incubation.

Table 2.2.: DR1900 test using diamine oxidase enzymatic essay.

Colorimetric test kit	**Absorbance (a.u.)**		
Blank	0.272	0.270	0.263
Sample 1	0.250	0.250	0.251
Sample 2	0.258	0.258	0.258
Sample 3	0.262	0.263	0.263

Two main factors affecting H_2O_2 stability are high temperature and alkaline pH (above 6) [100]. Enzymatic pH of 7.2 could potentially indicate that H_2O_2 is a lot less stable and could be degraded in just a few hours. However, the buffer's pH is considered suitable for the enzymatic reaction. Moreover, low temperatures (37ºC) were used upon incubation. Thus, no conclusions were made from these tests.

- *Mid-infrared spectroscopy of DAO structure and H_2O_2 relation*

Determination of H_2O_2 in an aqueous solution can be performed by mid-infrared spectroscopy (FTIR) [101]. However, the analytical results provided by this technique is often applied to higher concentrations of H_2O_2 (e.g., industrial bleaching products). To test the presence of enzymatic released H_2O_2, FTIR equipment was used, with the following parameters: 64 scans and a range of 40 to 4000 cm^{-1}. Different concentrations of H_2O_2 solutions were prepared (0.01%, 0.1%, 1% and, 30% w/w) and FTIR spectra were measured using distilled water as a reference. H_2O_2 should produce two bands at 3000 to 2500 wavelengths and from 1500 to 1000 [101]. For higher concentrations of H_2O_2 (1% and 30% w/w), the presence of two bands was noticed, however, for the lower concentrations (0.01% and 0.1% w/w) the FTIR spectra were identical to the one

obtained using just distilled water. Enzyme solution after incubation was tested, using FTIR, for the presence of H_2O_2 (Figure 2.23).

According to the results obtained when DAO reaction solution spectra were measured, no bands in H_2O_2 region were observed. A new band around 1000 wavelength has appeared. According to the IR table found in the literature, this band presence is not indicative of the protein structure or amino groups, thus, no conclusions were made. The concentration of H_2O_2 can be measured by FTIR spectrometry, however, this technique is not prepared to detect concentrations found in industrial applications, that range from 0.1% to 10%. Thus, results were found to be inconclusive, since H_2O_2 present in the enzymatic reaction sample would be much lower than the H_2O_2 from the bottle solution.

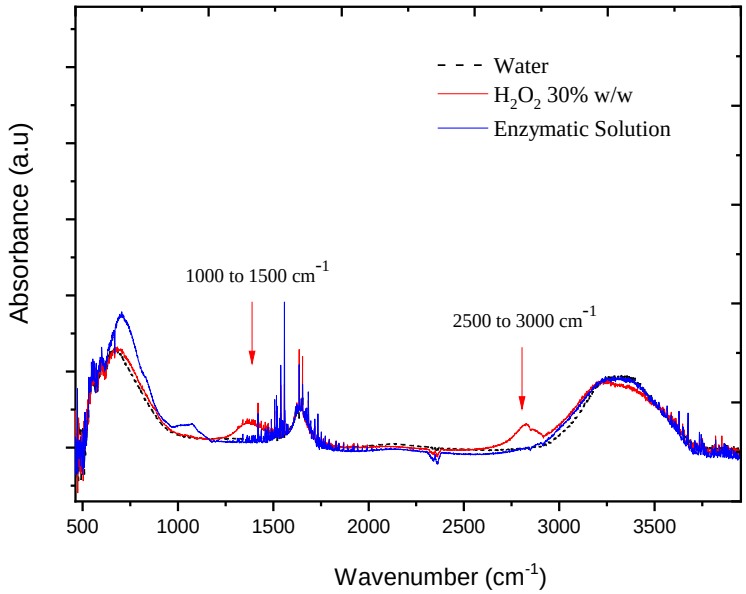


Figure 2.23.: Comparison spectra of water, aqueous hydrogen peroxide solution (30% w/w) and enzymatic solution in the mid-infrared spectral region.

2.4.4. Conclusions

Since the enzymatic reaction is a critical procedure; thus, the optimum kinetics characteristics must be ideal. Keeping in mind that the conditions that promoted the highest enzyme activity, the following parameters were selected: pH=7.2, T=37ºC, with magnetic stirring. The results presented above showed that no signal was obtained when DAO solution was added to the developed membrane, thus, several strategies were considered. In the first place, the buffer was changed, to discard any problem in the reaction medium. Prior to that change, and in an attempt to exclude all the possible reasons for the light emission absence, DAO absorvance was measured. It was possible to observe an overall higher absorbance spectrum when the sample solution contained the enzyme. Moreover, when enzyme solution was added to a H_2O_2 standard it was found to affect the signal acquisition instantly. To test if the color was responsible for the absorvance of the light that was being emitted and could explain its absence, an almost white enzyme powder was used. Experiments included having both enzyme powders (darker and lighter ones) reacting in the same exact conditions. It was concluded that the white enzyme powder has

given positive results for H_2O_2 presence, however, concentration-wise, it bellowed the one's predicted to be enzymatically released. In a last attempt to understand DAO color problems, a commercially available H_2O_2 determination kit was used. From that test, H_2O_2 presence was negative. Since this method have lower sensitivity towards H_2O_2 (min. concentration of 0.1% H_2O_2 w/w) than the final biosensor, tests were considered to be inconclusive.

As a means of removing the enzyme after the reaction was complete, its denaturation was performed by two different means: using vortex and by decreasing the pH (2-3). Using vortex as the disruptor, absorbance decreased significantly, however, was revealed to be a tedious, time-consuming methodology. Moreover, no light emission was observed. On the other hand, denaturation by the rapid decrease in pH seemed to be a much practical method. Light emission was obtained by adding H_2O_2 from the bottle directly to the sample, although no real test was performed afterward. Anyhow, it was concluded that changing pH to induce enzyme denaturation could possibly destroy the sensing membrane since it requires high pH to occur. Moreover, the addition of DAO solution to the membrane makes the almost one-hour signal decrease dramatically until 0 in a matter of seconds. This last, strongly points out that DAO content should be reduced or separated from the solution once the reactions were complete. For this matter, an immobilization process, based on literature, was also proposed. Although promising, commercially available DAO has a very low activity (≥0.05 Unit/mg solid), compared with vegetal DAO [102]. Nitrocellulose material, the chosen immobilization carrier, has a binding capacity of 80 to 100 $\mu g/cm^2$, which meant that it wouldn't be possible to compensate with a higher activity, since a very high amount of enzyme was needed. Thus, the technique was not developed at the time of this master thesis, however, could be further considered as future work.

To conclude, the positive results that were obtained with DAO white powder, previously bought and stored, might explain the decrease of the H_2O_2 signal when dark DAO solution was added to the membrane. Consequently, the attempt of the development of the biosensor was delayed and no further calibration was performed. However, some further tests with this white enzyme must open the possibility to exploit BAs determination by the proposed biosensor.

Chapter 3. Biosensor for the determination of hydrogen peroxide in adulterated milk

Adapted from:

Helena Vasconcelos, Ana Matiasb, João Mende, João Araújo, Bernardo Dias, Paulo Santos, José M. M. M. de Almeida and Luís C. C. Coelho. Optical biosensor for the detection of hydrogen peroxide in milk. **Under submission.**

Milk is one of the most complete foods for humans, containing nutrients including carbohydrates, proteins, fats, minerals, and vitamins [103].
Owing to its rich composition, milk becomes a substrate for the growth of undesirable microorganisms that can easily deteriorate milk. To prevent this from happening, prohibited substances are fraudulently added [104]. Hydrogen peroxide, hypochlorite, formaldehyde, potassium dichromate and salicylic acid are examples of substances used as adulterants that need monitoring and quality control as they are toxic to humans [105].
One of those substances is hydrogen peroxide (H_2O_2), which is widely used in the dairy industry as an antimicrobial agent, thus helping preserve the raw milk in the absence of refrigeration [106]. Despite its conventional use, when added to milk, hydrogen peroxide solution can cause a decrease in the nutritional value of the food, due to the destruction of vitamins A and E, which generates reactive and cytotoxic oxygen species, including hydroxyl radicals, that can initiate oxidation and damage nucleic acids, lipids and proteins. By consequence, when ingested, milk can lead to negative effects on the health of the population, especially in individuals immunocompromised persons [104][106].
In the United States, hydrogen peroxide is used in cheese production in concentrations up to 0.05%, however in other countries its addition is prohibited due to its toxic effects. Peroxide concentration > 0.1% w/w induce cancer in the duodenum of mice and present short-term genotoxicity [105].
Here, it is presented a study for the detection and quantification of H_2O_2 using a chemiluminescence technique. A small low-cost hydroxyethyl cellulose sensitive membrane combined with a high-sensitive photodetector is used to measure H_2O_2 concentrations in semi-fat milk samples. The acquired data showed that fat content in cow's treated milk can interact with chemiluminescent signal, lowering the LOD. Concentrations of H_2O_2 of 0.01% w/w to 0.06% were detected and method was calibrated for four different types of milk. Results have proven that the optical biosensor can be successfully applied to the real-time monitorization of milk for fraud controlling purposes.

3.1. Materials and methods

3.1.1. Materials and reagents

The luminol-based membrane was developed with luminol, sodium phosphate, cobalt (II) chloride hexahydrate, sodium lauryl sulphate (SLS), hydroxyethyl cellulose (HEC) and ethylenediamine tetraacetic acid (EDTA) (Sigma Aldrich, Germany). The procedure established by Miklicanin and Valzacchi (Omanovic-Miklicanin & Valzacchi, 2017) was refined into this protocol. Preparation of all the solutions and dilutions employed deionized water obtained from a Milli-Q system (Millipore), and Class A glassware. Fabrication of the HEC membranes was performed as mentioned in Chapter 2.

3.1.2. Samples

Four different types of milk were used, containing different fat concentration, raw milk (3.7%), skim milk (0.5%), semi-skimmed milk (1.5% a 1.8%) and whole milk (3.5%). Samples of raw milk were obtained directly from local farmers in the region of Trás-os-Montes, Portugal, and were analyzed within 4 hours of milking, while treated milks were obtained from the nearest supermarkets. Milk samples were produced by H_2O_2 spiking, with concentrations ranging from

0.001 to 0.006% w/w. To contaminate the milk samples with H_2O_2 the same procedure as for the calibration samples was followed, but in this case the solvent was milk. A 0.01% H_2O_2 solution was made, i.e., in 10 ml of milk, 3.3 ul of 30% H_2O_2 solution was added. To obtain the different standards ranging from 0.001 to 0.005% w/w, they were obtained by diluting a 0.01% w/w H_2O_2 solution. For the measurement procedure, the membrane was placed directly onto the membrane holder in the detector, especially design to have high sensitivity with different gains, and the light emission was measured by adding 500 µL of sample solution.

3.2. Results and discussion

In this chapter, a study for the detection and quantification of H_2O_2 is presented. The previously reported sensitive membrane combined with the high-sensitive photodetector is used to measure H_2O_2 concentrations from raw to ultra-pasteurized milk samples. The main components of milk include lactose, fat, or proteins, which are expected to interfere with the signal measured. Accordingly, the ability to determine the presence or absence of H_2O_2 adulterant in milk samples was evaluated employing the newly optimized biosensor. Commonly, these tests are mainly qualitative and hard to use once they are dependent on sample pre-treatment and can give false positives. Thus, a new analytical technique for the quantification of H_2O_2 was developed as an upgrade for milk quality control. The use of a compact, sensible, fast, and convenient optical biosensor is the proposed methodology to be customized in terms of sensitivity and LOD for H_2O_2 in milk, widely used as a preservative. Four types of milk (raw, skim, semi-skimmed, and whole milk) were spiked with H_2O_2 concentrations from 0.001 to 0.006% w/w by diluting a standard 30% w/w solution of H_2O_2.

The variation of the fluorescence intensity is presented in is shown in Figure 3.1 to Figure 3.5 for all milk samples, together with the time integral of the decaying fluorescence signal for each H_2O_2 concentrations.

From the data presented in Figure 3.1, it is possible to observe that for the 0.006% of H_2O_2 concentration, the light intensity as well as the reaction time decrease over time. In a contrast, in boiled raw milk the same does not happen. This decrease in intensity verified over time [Figure 3.1(a)] can be explained by the natural presence of the enzyme peroxidase in raw milk. Since raw milk has naturally present enzymes, namely catalase, this represents a stability problem when H_2O_2 is added to raw milk samples. This enzyme has the ability to degrade H_2O_2 (Koksal et al., 2016). For that reason, raw milk samples were previously boiled, in order to perform enzyme denaturation by heat. After enzyme denaturation, signal emission was maintained constant at 25ºC, which helped signal recording and further calibration. To prove this fact, a thermal treatment of raw milk (heated at boiling for 20 min) was carried out, a procedure described in (L. S. Lima et al., 2020). After the heat treatment, it was found that over time the intensity of the reaction remained more stable than before the heat treatment [Figure 3.1 (b)].

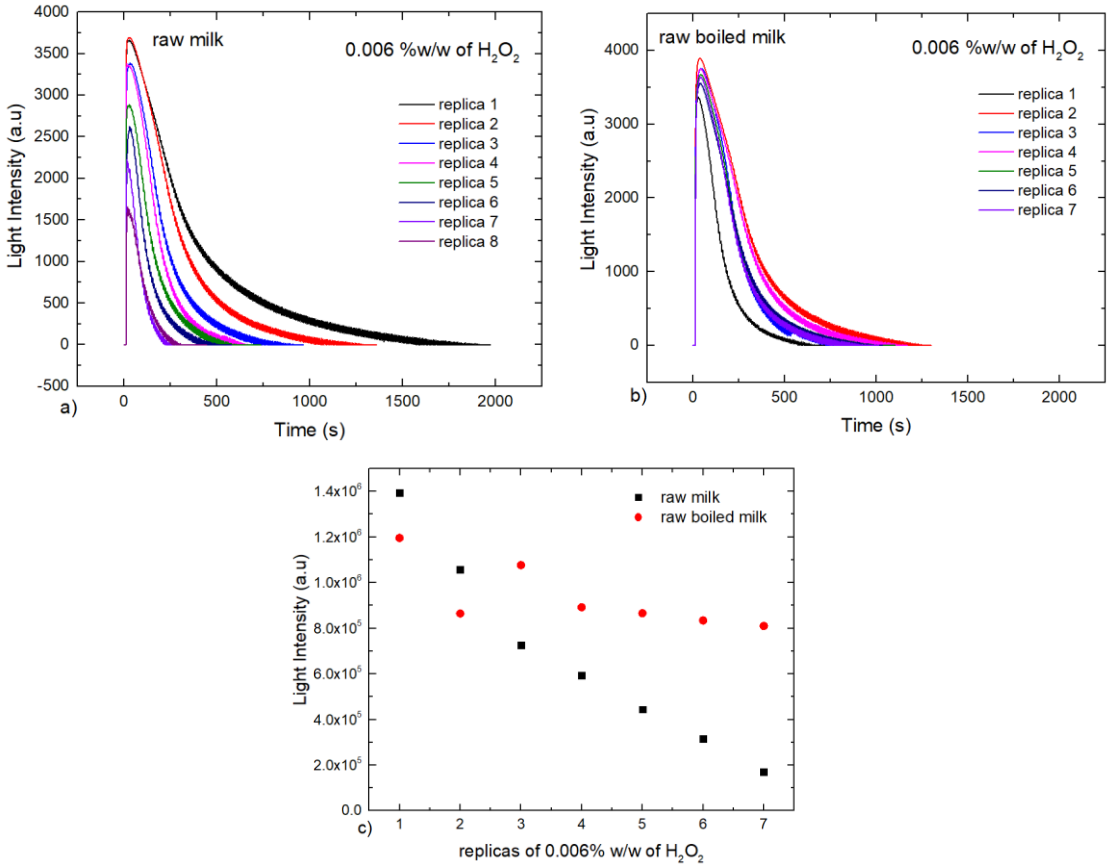


Figure 3.1.: (a) and (b) Variation of the intensity of the light emission for the concentration of 0.006%w/w of H_2O_2 as a function of time in raw milk and boiled raw milk (c) time integral of the decay time for raw milk and boiled raw milk.

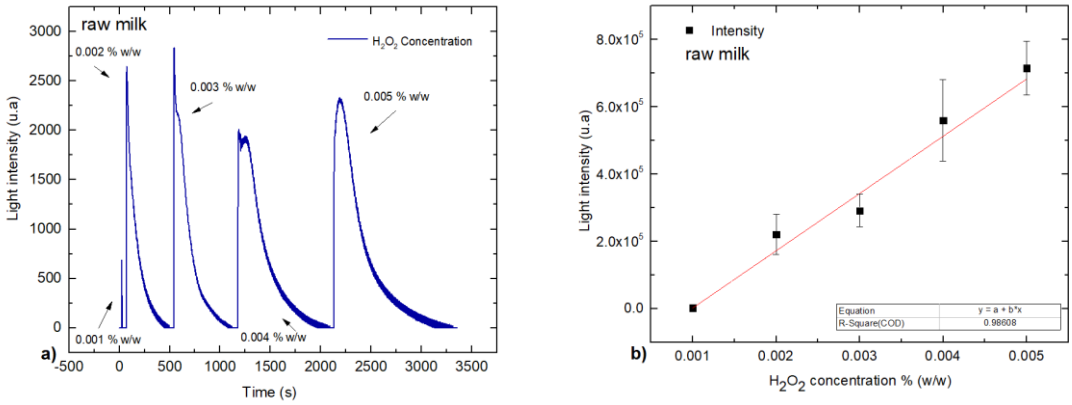


Figure 3.2.: (a) Variation of the intensity of the light emission for the concentrations of 0.001 to 0.005%w/w of H_2O_2 as a function of time in raw milk, and (b) time integral of the decay time for each H_2O_2 concentration for the raw milk.

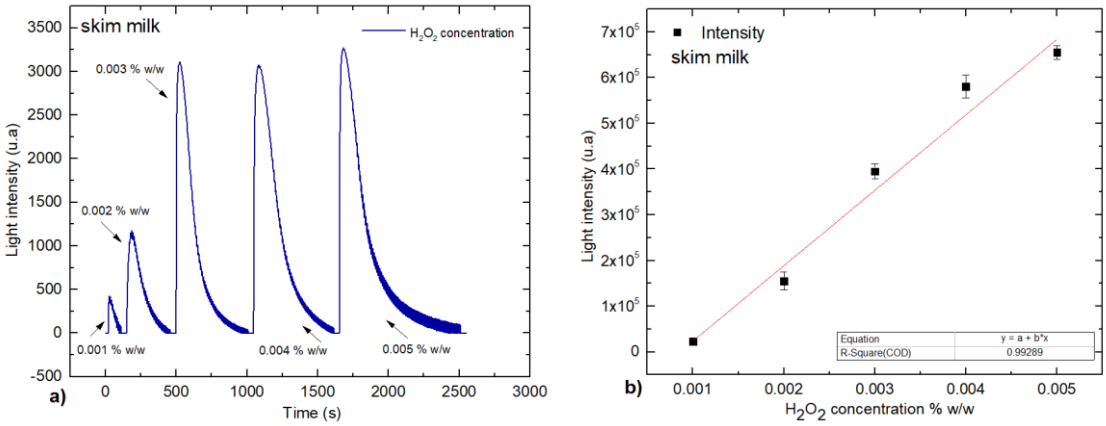


Figure 3.3.: (a) Variation of the intensity of the light emission for the concentrations of 0.001 to 0.005%w/w of H_2O_2 as a function of time in skim milk, and (b) time integral of the decay time for each H_2O_2 concentration for the skim milk.

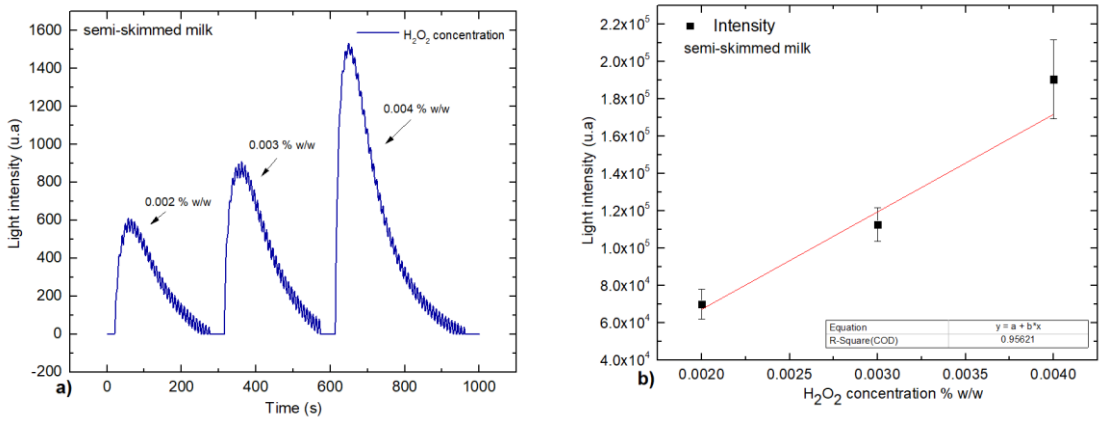


Figure 3.4.: (a) Variation of the intensity of the light emission for the concentrations of 0.002 to 0.004%w/w of H_2O_2 as a function of time in semi-skimmed milk, and (b) time integral of the decay time for each H_2O_2 concentration for the semi-skimmed milk.

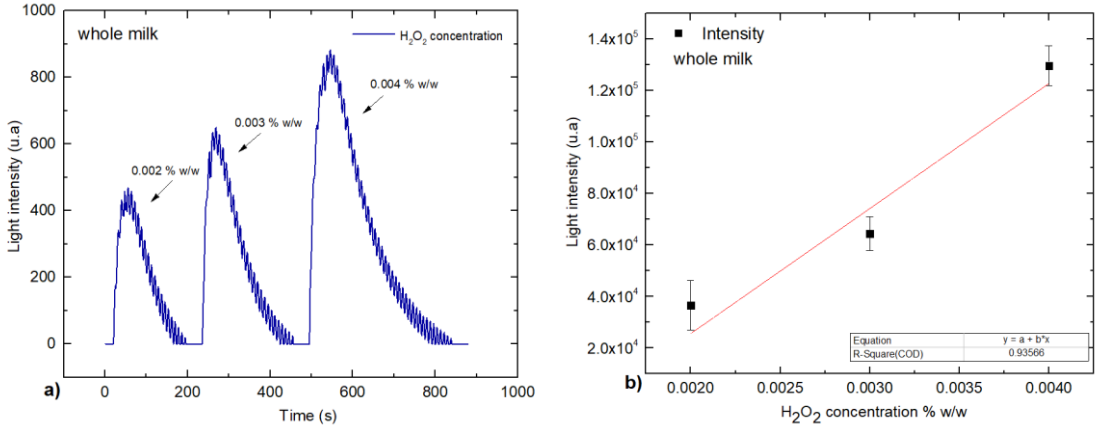


Figure 3.5.: (a) Variation of the intensity of the light emission for the concentrations of 0.002 to 0.004%w/w of H_2O_2 as a function of time in whole milk, and (b) time integral of the decay time for each H_2O_2 concentration for the whole milk.

From the results obtained for raw milk, signal duration increased as the H_2O_2 concentration also increased. This translated into higher light emission, as expected. As for skim milk, the chemiluminescence behavior is similar, with an almost imperceptible higher light intensity values for skim milk. This is explained by the fact that boiling changes fat content in milk due to long-chain fats conversion into short- and medium-chain fats. For semi-skimmed and whole milk samples, the limit of detection was lower mainly due to the higher density induced by fat content, which proves its interference in light emission intensity. Overall, from the data on milks adulterated with H_20_2, it is observed that as the % of milk fat increases, there is a decrease in intensity and reaction time over time. In semi-skimmed milk and whole milk the lowest concentration detected was 0.002% H_2O_2.

Linearity of the method ranged from 0.001% to 0.005% w/w H_2O_2 in raw and skimmed milk, while for semi-skimmed and whole milk samples calibration curves were of the polynomial kind ranging from 0.002% to 0.005% w/w H_2O_2. Taking into consideration that 0.05 % w/w of H_2O_2 is the defined limit for the FDA in milk for cheese production [107], the developed sensor would be suitable for portable determinations of H_2O_2 as a fraud controller in milk samples, within the legal limits of different countries.

The analytical performance of the biosensor was compared with the H_2O_2 biosensors found in the literature (Table 3.1). It can be pointed out that all the biosensors presented here are sensitive enough to detect H_2O_2 in the medium. Among the chemiluminescence biosensors, validations results are comparable with the ones obtained for the prepared biosensor, where it is prepared to classify the analyte adulterant qualitatively and quantitatively in real-time monitoring of milk fraud. Moreover, LOD values are lower than the typical concentration range of H_2O_2 found in milk samples. Noticeably, these biosensor's responses of real samples are consistent and proved that biosensors are great candidates as sensing portable devices, to be used and exploited in the diverse areas of impact. Besides, when compared with other methods available for the determination of H_2O_2 presence in milk, this portable biosensor is an easy and reliable method that ensures the required sensitivity, while offering a low time of analysis and no need for additional laboratory equipment.

Table 3.1.: Method validation and respective reference values from literature.

Linearity		**Validation**	**Precision**	**References**
R^2	Linear range (% w/w)	LOD (µM)	R.S.D (%)	
0.998	$1.0x10^{-4}$ to $1.0x10^{-3}$	6.6	6.6	This work
0.9991	$2.0x10^{-6}$ to $1.6x10^{-4}$	0.26	4.5	Reference method [83]
-	$1.1x10^{-5}$ to 4.0 $x10^{-4}$	1.6	2.4 to 8.0	[108]
0.998	$3.3x10^{-4}$ to $8.1x10^{-3}$	1.6	2.8	[109]
0.997	$6.8x10^{-5}$ to 3.0x 10^{-3}	6.2	2.4	[110]

In all the techniques presented above, they had a greater sensitivity than that obtained in our study, however, some techniques require specialized operators, others need sample treatments, therefore they require the use of larger amounts of reagents, and in some cases the equipment does not it is portable and therefore does not allow *in-situ* analysis.

Figure 3.6 shows the difference in intensity and reaction time for the four samples of milk adulterated with 0.004% H_2O_2.

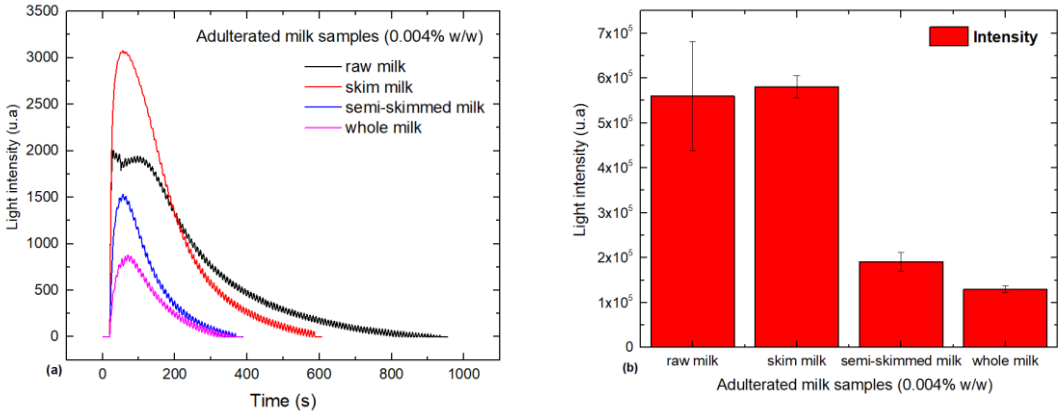


Figure 3.6.: (a) Signal intensity for milk adulterated samples and (b) integral of the decay time for samples of milk adulterated with H_2O_2 (0.004% w/w).

This fact can be explained by the organoleptic characteristics of milk such as its white color, as well as the other milk constituents that include lactose, fat or proteins, which are expected to interfere with the signal measured. There was also a slight difference in signal between skim and whole milk. Skim milk ($5.8x10^5$) showed more reaction intensity than whole milk ($1.3x10^5$) fact also reported by (M. J. A. Lima et al., 2020).
In raw milk, its intensity was expected to be close to that of whole milk, as its fat concentration should be close. However, the composition of milk varies with lactation stage, breed, cow health status, diet and genetic factors (Heck et al., 2009).

3.3. Conclusions

Purity and quality of milk have been at stake, since several adulterants like melamine, urea, formalin, detergents, ammonium sulphate, H_2O_2, among others, are fraudulently added. This adulteration is seen as unacceptable once it can interfere with human wellbeing. H_2O_2, in particular, is commonly added to milk as a "washing solution" to extend milk shelf-life. Due to an economic point of view, these practices continue to rise, thus the detection of these chemical preservatives has become of extreme importance in this sector.

The proposed sensor provided to be a rapid, cost-effective, and environmentally friendly approach for the determination of H_2O_2 as a milk adulterant. This optimized and validated method has a very good linearity range when the sample in its liquid state, where concentrations of H_2O_2 as low as 0.0001% w/w can be detected with good repeatability. As a practical application for this methodology under controlled conditions, adulterated milk samples were analyzed. The acquired data showed that fat content in cow's treated milk can interact with chemiluminescent signals, increasing LOD. Concentrations of H_2O_2 of 0.001 to 0.005% w/w were detected and the method was calibrated for different types of milk, proving that limit of detection and linearity range of the proposed method are suitable for the analysis of milk samples *in-situ*, which can add value to

the food fraud department. Moreover, the reagents required are commonly used in analytical laboratories, inexpensive, and consumed in low amounts (0.5 mL), thus resulting in negligible and non-toxic waste generation. In addition to the mentioned advantageous features, the proposed method validation is comparable to those found in the literature.

Chapter 4. Determination of biogenic amines by gas chromatography mass-spectrometry

Despite the many challenges, BA's determination through gas chromatography is still one of the preferred techniques. Pretreatment always includes a derivatization step to improve volatility of these compounds, while improving peak shape by reducing tailing, increasing sensitivity, selectivity and an induces a better separation [111]. Several reagents of derivatization are commercially available, including the widely used combination of N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and Trimethylchlorosilane (TMCS), for amines derivatization. BSTFA is an effective trimethylsilyl donor with donor strength, that reacts with a wide range of polar compounds to replace hydrogens with a $Si(CH_3)^3$ group. Therefore, it is used to prepare volatile and thermally stable derivatives for gas chromatography and mass spectrometry [112]. Concentrations of TMCS between 1% and 10% can conveniently be prepared along with BSTFA in excess to add to the reaction mixture for a better and faster derivatization process. Since water decomposes both TMS reagents and derivatives, BA's samples must be dried out before this process. Silylation reagents react with active hydrogen atoms lowering the polarity inherent to amines, therefore increasing its volatility. This increase in volatility will enhance the separation of amines by the GC column.

For this purpose, BSTFA + 1% of TMCS combination was used as the chosen derivatization reagents for the determination of four BA's by gas chromatography coupled with mass spectrometry in poultry samples. To calibrate the equipment, a wide set of identifying MS fragments were originated for each amine and quantified by *MassHunter* software by area peaks quantification. In total, four different BAs (PUT, CAD, TYR and HIS) were studied and first three of them were calibrated.

4.1. Materials and methods

The GC-MS equipment comprised an Agilent DB-5MS (30 m × 0.250 mm × 0.10 µm) column installed in an Agilent 7890B coupled with an Agilent 5977B Series MSD. Injection was performed with a 7650A Automatic Liquid Sampler (ALS) equipped in the GC-MS system where 2 mL graduated screw threads vial (Sigma Aldrich, Germany) were used. Conditioning and vacuum processes of the equipment were performed upon use, as instructed by the manufacturer. For the initial GC-MS tests performed for alcohols determination purposes, a set of standard solutions were prepared using 1-propanol (99%, Sigma Aldrich, Germany) or methanol anhydrous (99.8%; Sigma Aldrich, Germany) in acetonitrile anhydrous (99.8%, Sigma Aldrich, Germany). To the biogenic amine's calibration step, four BAs were selected and analyzed, namely cadaverine dihydrochloride (99.9%; Sigma Aldrich, Germany), putrescine dihydrochloride (99.9%; Sigma Aldrich, Germany), tyramine hydrochloride (98%; Sigma Aldrich, Germany) and histamine dihydrochloride (99%; Sigma Aldrich, Germany). All the biogenic amine standard solutions were prepared using a Milli-Q system (Millipore), and Class A glassware. Derivatization process by salinization was carried out with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA 98%; Thermo Fisher Scientific, Karlsruhe), with 1% Chlorotrimethylsilane (TMCS 98%; ThermoFisher, Kandel).

4.1.1. Alcohol's determination

A test was conducted using volatile alcohols and performing a complete analysis for qualification to quantification. For that purpose, methanol and 1-propanol dissolved in acetonitrile were used as samples. The methodology of the preparation of the standard solutions was extracted from

[113] and performed as follows: for the quality analysis, it involved adding 60 µL of sample (either 1-propanol or methanol) diluted in 10 mL of acetonitrile; while for the quantitate analysis 5, 10, 20, 40 and 60 µL of sample (1-propanol) was added to a 10 mL of acetonitrile. For each analysis, 1 mL of the previous standard solutions was added to the appropriate GC-MS sample vials. Acetonitrile was used either as a solvent to wash the needle in-between injections or as a blank solution. Table 4.1 describe the analyte's standard preparation and the respective concentrations (µg/L).

Table 4.1.: Analyte's standard preparation and respective concentrations (µg/L).

Analyte	Standard	Volume (µL)	Dilution volume (mL)	Final Concentration (µg/mL)	Final Concentration (µg/L)
1-propanol	1	5	10	402	0.402
	2	10	10	804	0.804
	3	20	10	1608	1.608
	4	40	10	3216	3.216
	5	60	10	4824	4.824

- *GC-MS conditions*

GC-MS conditions were extracted by [113], with some modifications at the temperature ramp. The run time was set to 10 minutes, with a flow rate of 1.0mL/min. Injector temperature was of 250ºC, and the injection volume was 1.0µL. Oven temperature ramps were set to 30ºC(6min) 60ºC at 10ºC/min (1min). Mass detector source temperature was 230ºC and the quadruple temperature was 150ºC, with no solvent delay. Specific ions were selected as being in the range (ion scan- 29 to105 m/z) of the expected mass spectrum of both alcohols' groups.

4.1.2. Calibration curve

Standard amine mix solutions of 500 ppm were prepared by adding 0.025 g to 50 mL distilled water in a volumetric flask. The 500 ppm solution was diluted using sequential dilution in order to obtain the following solutions: 500, 300, 100, 50, and 25, or 10 ppm. From the previous concentrations, 150 µL was added to GC vials and the sample was left to evaporate for 2h at 70ºC. Derivatization reagent was prepared by adding BSTFA and TMCS in the right proportions (9:1). After removing all the moisture, 300 µL of the derivatization reagent was added to each concentration vial for the derivatization to happen. Vials containing the amine standards were placed in the incubator at 30ºC and left overnight with a cap. Afterward, the derivatives were analyzed in GC-MS with the conditions described below.

4.1.2.1. GC-MS conditions

GC-MS conditions were extracted by [114], with some modifications at the temperature ramp. An overall decrease in the initial ramp temperature was needed since BSTFA+ 1%TMCS reagent gives a noisy spectrum. The run time was set to 25 minutes, with a flow rate of 3.0 mL/min. Injector temperature was of 250ºC, and the injection volume was 1.0µL. Oven temperature ramps

were set to 100ºC (for 1 min), then increasing to 160ºC at 10ºC/min and the final temperature was 280ºC at 25ºC/min Mass detector source temperature was 230ºC and the quadruple temperature was 150ºC, with no solvent delay.

4.2. Results and Discussion

4.2.1. Chromatogram plot analysis

- *Alcohols*

In order to analyze, characterize, and to make quantification of the compounds dissolved in a mixture, 1-propanol and methanol were selected according to their volatile structure as samples. The methodology adapted from a previous study [113] enabled the detection and quantification of the proposed analytes using the GC-MS equipment. Qualitative analysis was carried out by selecting a random concentration of the calibration curve standards, 4824 µg/mL and 4746 µg/mL for 1-Propanol and methanol, respectively. Using those concentrations, a single run was performed for each analyte, where acetonitrile was used as blank sample. The analyte identification was accomplished through two parameters: by mass spectra comparison (NIST library) and by analysis of the analyte's retention time according to the chromatogram principle, which highlights those compounds have a specific time to be eluted based on stationary phase interaction. To avoid overlapping of the compounds based on their closest boiling points, temperature ramps were adapted to our column. The criteria were set based on compound chemical structure, and by comparing the conditions available in the literature for the same analytes using the column (5%-phenyl)-methylpolysiloxane or DB-5MS. Indeed, the retention times of the three peaks (methanol, acetonitrile, and 1-propanol) are illustrated in Figure 4.1. The physicochemical properties of the analyte to be separated dictate their behavior while the elution is happening. Methanol has a lower molecular mass and lower boiling point, thus is the first compound to be eluted due to its volatility. Right after that, a new peak appears. Since acetonitrile has a higher molecular mass as well as a higher boiling point than methanol but lower than 1-propanol, is eluted afterward. The last peak in the chromatogram belongs to 1-propanol, which is coherent with its physicochemical properties.

Despite these results, when comparing retention times of the ones found from the starting study [113] different times were acquired. Some factors that might have conditioned the different times of retention, are the different temperatures used (final T= 60ºC vs final T=240ºC), and the material and size of both columns (DB-5MS vs DB-624). Nonetheless, from a quick search in the literature retention times are comparable and similar for the three compounds and coherent with their volatilities.

Mass spectrum data is crucial for identification purposes. Using the NIST library, the qualitative software was able to successfully identify all the target analytes and solvents. For the methanol standard, the most abundant and identifying ions were m/z 29, 31 and 32, whereas for the 1-propanol the most abundant ions were m/z 29,31, 39 and 42. As expected, m/z 31 is characteristic of primary alcohols, the reason why it appears in both target analytes. This is a representation of

a fragmentation of the hole alcohol molecule that loses a H+ proton. Contrarily, acetonitrile has a very distinctive ion (m/z 41), and it is readily identified.

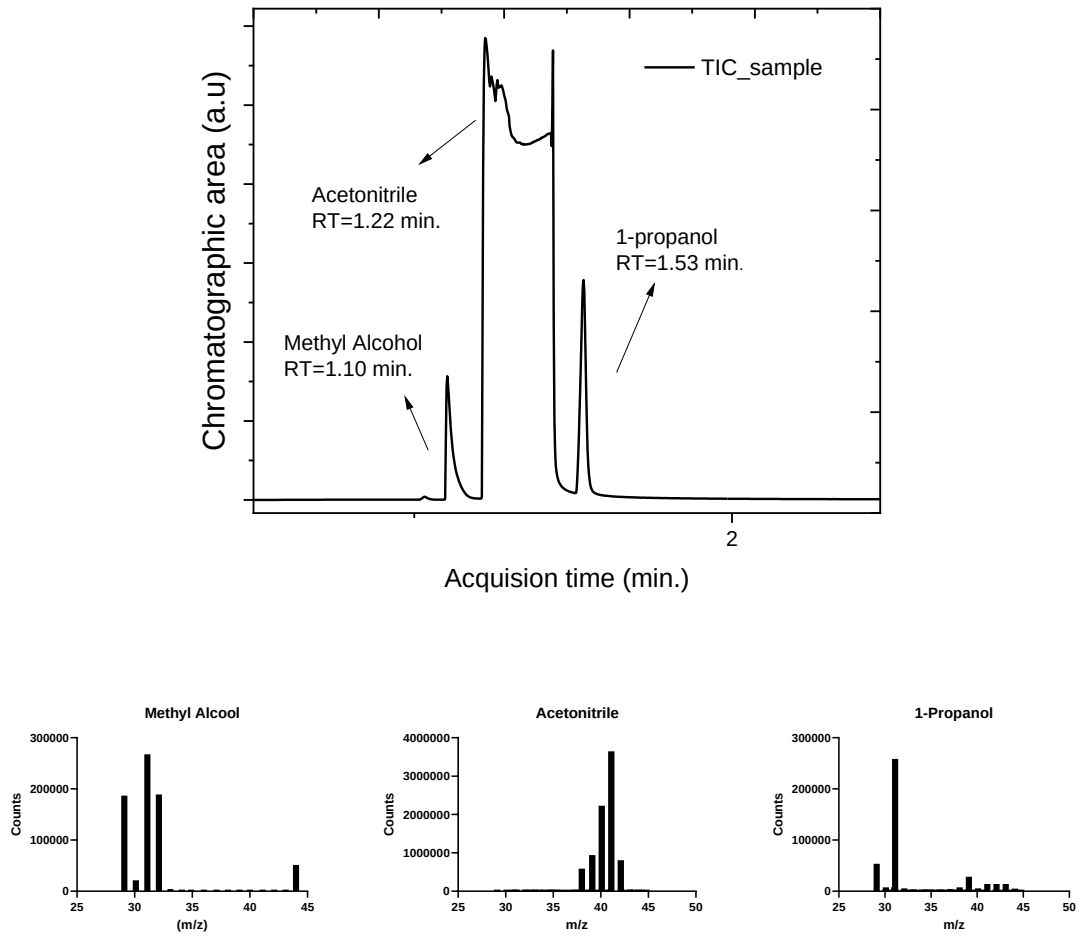


Figure 4.1.: GC-MS extracted chromatogram plot including the analytes respective mass spectrum. Each mass spectrum is unique to the molecular structure of the target analyte. As observed, volatile compounds are eluted first, while the least volatile compounds in a mixture are eluted later.

For the quantitative analysis, 1-propanol was chosen to perform the calibration curve. A set of standards were automatically injected into the GC injection port by setting up a sequence into the *software*. From the sequence, a quantitative analysis of each vial containing each standard was performed by the *MassHunter* software. Two identifying parameters were set manually into the quantitative program, such as retention time (± 1.57 minutes), determined by qualitative analysis software, and the most abundant ion of 1-propanol's mass spectrum (m/z = 31). Table 4.2 presents the chromatographic data given by the software after analysis of the batch.

Table 4.2.: Chromatographic data given by the MassHunter software for 1-propanol calibration, after "analyze batch" command.

Name	Level	Exp.Conc (µg/L)[a]	RT[b]	Response/ Area	Final Conc. (µg/L) [c]	Accuracy [d]	Ratio [e]
Inj.1	1	0.4020	1.540	33972	0.2206	54	12830
Inj.2	2	0.8040	1.576	73296	0.9086	113	10009
Inj.3	3	1.6080	1.567	145699	2.1753	135	4011
Inj.4	4	3.2160	1.529	153911	2.3190	72	4739
Inj.5	5	4.8240	1.540	320335	5.2306	108	1646

[a] Expected concentration of the target analyte, i.e., the added concentration at the preparation of the standard solutions.
[b] RI: Retention times obtained in minutes.
[c] Final concentration of the analyte based on the calibration curve.
[d] Accuracy: closeness of the measurements to a specific value.
[e] Ratio: signal-to-noise (S/N) ratio.

The linear equation was obtained as follow:

$$y = 5.7x10^5\ [1 - Propanol] + 2.1x10^4 \quad \textbf{Equation 3}$$

with a R^2 value of 0.95, as shown in Figure 4.2. The method for alcohols determination was validated and the obtained results illustrated that this workflow strategy can be well applied to BA's determination.

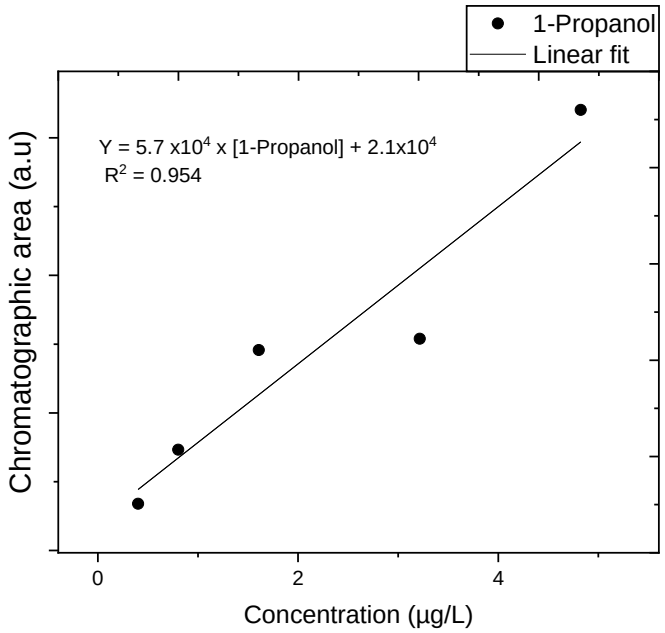


Figure 4.2.: Standard's calibration curve for five concentrations (0.4 to 4.8 µg/L) of 1-propanol as the target analyte.

- *Biogenic Amines*

As previously mentioned, even small amounts of water can interfere with the silylation reagent which can interfere with the amine's derivatization process. Since the existing methodology for type of reagents includes having a dried sample, a control of the humidity was performed. The traditional and most convenient way of testing the water content in amines recipient is to incubate it in its liquid or dried form, and subject it to heat. The accompanying graphs Figure 4.3 show the variation of relative humidity with temperature (60ºC) over liquid (150 µL) and dried (0.025g) PUT.

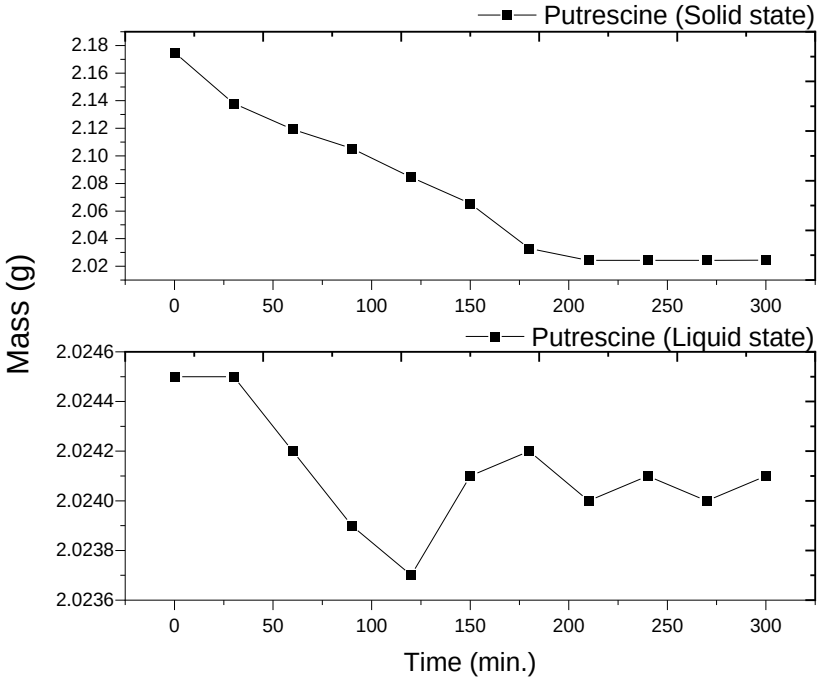
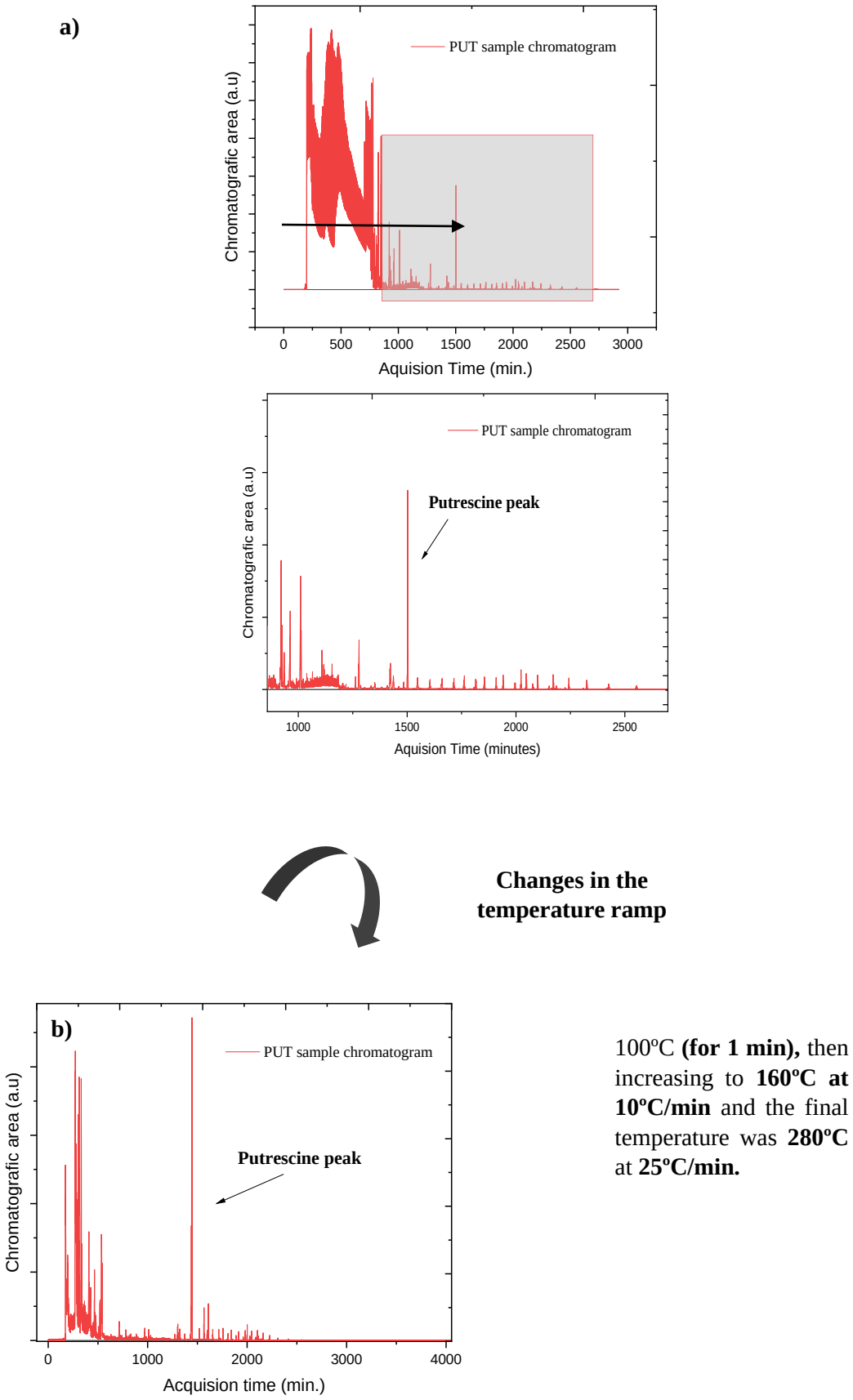


Figure 4.3.: Variation of relative humidity with temperature (60ºC) over PUT sample: (a) solid and (b) liquid state.

From the observation of the previous figure it was considered that solid PUT sample have minimal amount of water, thus it will not interfere with the derivatizing agent. In its liquid state, and as required to protocol, liquid samples (standard) need more than 3 hours at 60ºC to be completely dry. Taken this results in consideration, temperature of incubation was set to 70ºC for a faster and optimized evaporation step.

For the first tests, PUT alone was studied. For identification purpose, selected ions (specific for derivatized PUT; m/z 73, 174 and 175) were used in MS parameters and a SCAN mode of those ions were performed during each run. When PUT analyte was eluted, one peak was shown for each window ion at that retention time. After PUT identification, a strategy to change the temperature ramp was carried out. Since temperature can largely influence the final chromatogram, more ramps were added, increasing the run time. Thus, based on the literature for the column in use (DB-5MS for BAs determination), a change in the temperature ramp was performed and a much clearer chromatogram was achieved (Figure 4.4). For that reason, the GC-MS conditions were set as definitive and used in the following samples.



100ºC **(for 1 min),** then increasing to **160ºC at 10ºC/min** and the final temperature was **280ºC** at **25ºC/min.**

Figure 4.4.: Effects of temperature on the final chromatogram: (a) PUT chromatogram with the initial temperature parameters; (b) PUT chromatogram after a change in the temperature parameters.

Another strategy used for identification purposes, was to add sample in solid form directly into the 300 µL reagent solution. Here, a thick peak was achieved, which helped reinforce the identification previously made.

For the calibration four amines were used: PUT, CAD, TYR and HIS. The BAs were chosen based on the concentrations found in literature, which are known to be the most relevant ones. GC-MS conditions used were the same as for the PUT alone. For each amine, samples containing analyte in the solid form were prepared for a rapid identification, confirmed by the PubChem mass-spectrum section. Additionally, a mix standard solution was prepared by adding 250 µL (500 ppm) of each amine to a 1 mL final volume solution. After that, the previously mentioned methodology was followed (Figure 4.5).

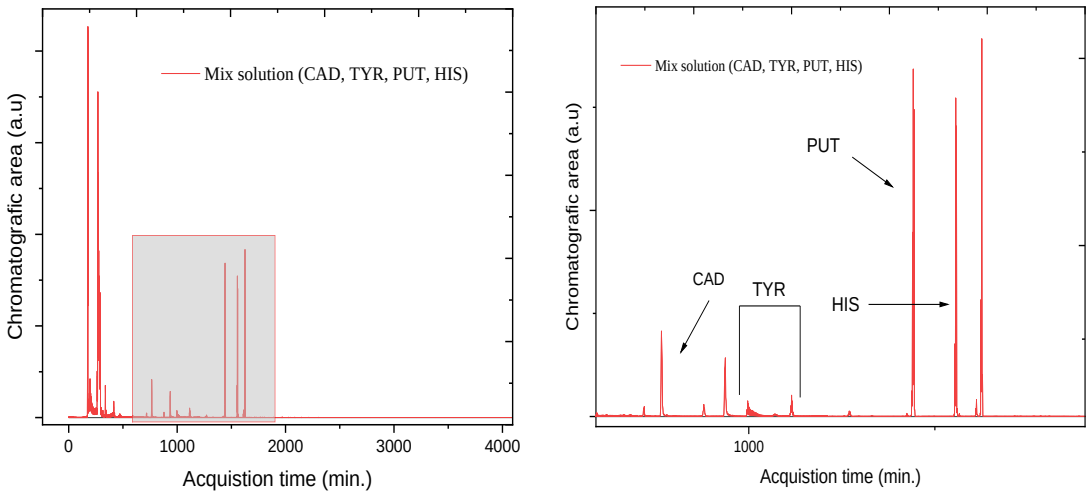
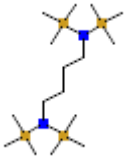
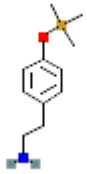
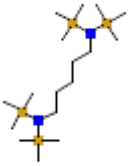
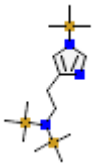


Figure 4.5.: GC-MS chromatogram containing the peaks of all four Bas in the study (approximated close-up is shown on the right). Apolar compounds are the last to be eluted due to the stationary properties of the column.

CAD was the first compound to be eluted at 3.8 minutes, consistent with the mix standard solution. Since the structure of CAD is similar to PUT it was expected that CAD would be eluted at similar retention times. However, that was not the case. The column used is the 5% phenyl equivalent phase that provides a boiling point elution order with a slight increase in selectivity, especially for aromatic compounds. Since the column DB-5MS is non-polar, the volatility of the analytes should be taken into consideration as well. With that in mind, and considering the basis of chromatography, those differences can be explained by the amine interaction with the stationary-phase, i.e., polar compounds will be diluted first and have less interaction with the non-polar column.

Table 4.3 shows the BAs determined by GC-MS. Since CAD has an extra chain of carbon and hydrogen, it is more polar than PUT, therefore is eluted first. Clearly, CAD was the only amine that could not be detected in the lower concentration range. The peak would only appear for 300 ppm of CAD, which is a confirmation of its detection limit. The second compound to be eluted is TYR. This last, fragments into two peaks, however, only the one that had the m/z 180 as the highest peak was considered. TYR is a primary amine, thus, is more polar than PUT that has an extra amine group but less polar than CAD which can be explained by the aromatic group in its structure. Thirdly, PUT was eluted at 7.4 minutes and sooner than the literature (~9 minutes) [111]. The last compound identified was HIS, with the highest retention time 8.2 minutes. Taking into consideration the column specifications, it is expected that HIS, being the most structurally complex amine (one aromatic compound and two amine groups), is the last amine to be eluted.

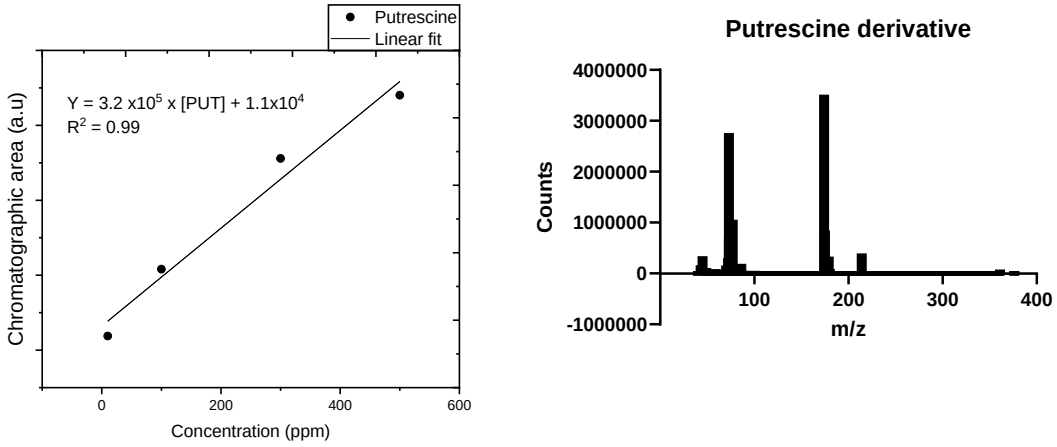
Table 4.3.: BAs determined by GC-MS, including respective CAS number, molecular structure, retention times and linearity range.

Compound name/ TMCS derivative	CAS	Structure	M/Z ions[a]	Retention time (min)	Linearity (ppm)
Putrescine tetra-TMS	39772-63-9		174, 73, 175	7.4	10-500
Tyramine, TMS derivative	--		180, 73, 165	4.7	25-500
Cadaverine tetra-TMS	65898-76-2		174, 73, 175	3.8	300-500
Histamine, 3-TMS	--		174, 73, 312	8.2	-

[a] based-on PubChem data.

- *BAs calibration curve*

PUT calibration curve, derived from the peak (RT=7.4 min.) shown in Figure 4.5, is presented in Figure 4.6. The linearity is good, however peak area integration by software is sometimes hard to perform. Correlation coefficient of 0.99 was obtained.



(a) (b)

Figure 4.6.: (a) Putrescine calibration curve and (b) mass spectrum of putrescine derivative obtained from MassHunter Qualitative Analysis.

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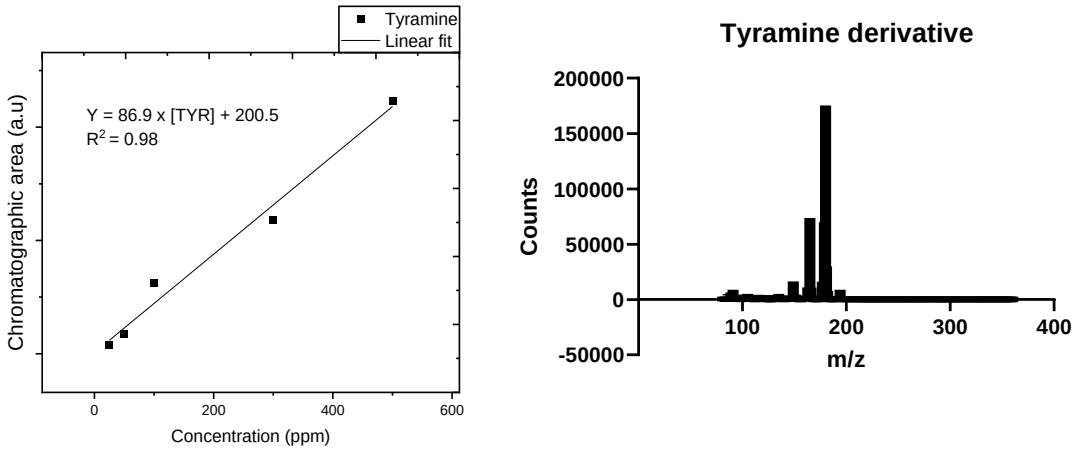
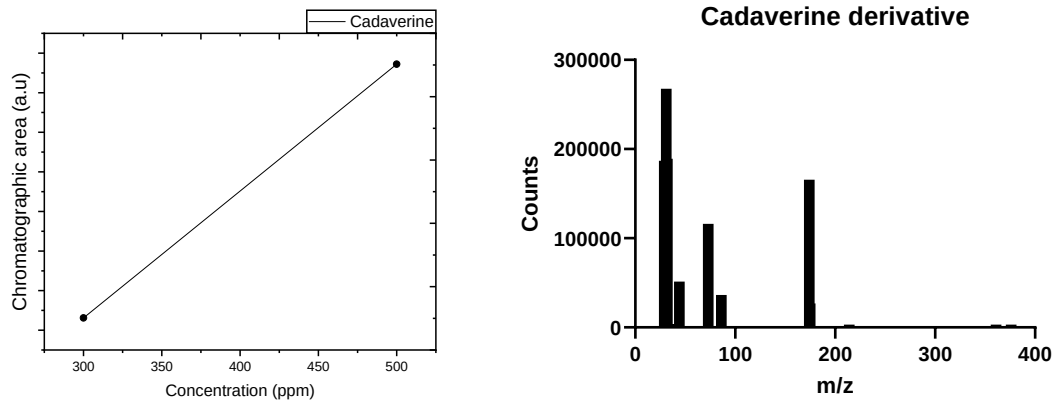
TYR calibration curve, derived from the peak (RT=4.7 min.) shown in Figure 4.5, is presented in Figure 4.7. The linearity is great and correlation coefficient of 0.98 may suggest higher affinity of GC column towards this amine.



(a) (b)

Figure 4.7.: (a) Tyramine calibration curve and (b) mass spectrum of tyramine derivative obtained from MassHunter Qualitative Analysis.

CAD calibration curve, derived from the peak (RT=3.8 min.) shown in Figure 4.5, is presented in Figure 4.8. For lower concentration range CAD peak was absent. It was possible to detect CAD concentrations up to 300 ppm.



(a) (b)

Figure 4.8.: (a) Cadaverine calibration curve and (b) mass spectrum of cadaverine derivative obtained from MassHunter Qualitative Analysis..

4.3. Conclusions

An identification and quantification approach was successfully applied to BAs determination. Derivatization of BAs was accomplished using the silanization method that produced trimethylsilyl fragments to be easily identified by the GC-MS. After the derivatization step, BAs temperature ramp was optimized based on literature findings, which helped achieve a much more clear chromatogram. A qualitative analysis was performed by two strategies that included mixing all the BAs in the study and using one single BA on a solid form. The practical utility of this strategy was to easily identify (e.g., retention times, mass spectrum, and volatility) all the BAs in each sample to be further used in calibration curves. For the quantitative analysis, a calibration curve based on area peaks was performed for each BAs. PUT linearity ranged from 10 to 500 ppm with a R^2 of 0.97 , thus exhibiting a very high affinity towards the column and a lower detection limit. TYR linearity ranged from 25 to 500 ppm, with a R^2 of 0.98. This BA was very easy to identify since the peak area was very well defined. CAD had a linear range of 200 to 500 ppm. For this particular amine, the limit of detection was higher and CAD peak only appears at higher concentrations which suggests that the affinity of CAD fragment towards the stationary phase is weaker. The last amine to be analyised was HIS. Although HIS peaks were predominant in the qualitative chromatogram, these last did not show on a quantitative chromatogram for any of the concentrations. Thus, HIS full characterization was not completed.

Overall, it was concluded that further application of these calibration curves into real meat samples could be used to either validate the biosensor after optimization and compare the results with the microbial analysis in chicken breast samples, as it would be the goal for this chapter.

Chapter 5. Product control: microbiological analysis of chicken meat and biogenic amine index

BAs are mainly formed by the decarboxylation of specific amino acids present in food through the action of enzymes. For that reason, BAs content can be related to bacterial quality and used as a quality indicator. Additionally, to the microbiological analysis, physicochemical parameters, such as pH and color, were measured over the days of this study. Some of the bacterial species that can be found in meat include Enterobacteriaceae and certain lactobacilli, pediococci and enterococci. Considering the growth curve of bacteria, they follow a trendy and similar behavior divided into four stages: lag phase, exponential phase, stationary phase and decline or death phase. This suggests that after storage, bacteria start to proliferate and can spoil food right after a few days stored in domestic fridges. Moreover, this process strongly depends on multifactorial conditions during rearing, slaughtering, and processing of animals, thus microbiological content can vary from product to product. This study aimed to determine the relationship between BAs production via decarboxylase activity and microorganisms [total aerobic mesophilic bacteria (TVC), Enterobacteriaceae and lactic acid bacteria (BAL)] counts in the breasts of poultry meats during storage at 8 ºC for 12 days. Aerobic storage of chicken meat allowed high final population levels, highlighting higher values for TVC, whereas BAL remained at lower values during experiment days. Physical properties (pH and color) have demonstrated some chicken meat degradation, more specifically changes from acidic to alkaline and lighter to darker tones, respectively.

The parameters gathered during this period were a relevant contribution to the construction of Food quality chart, an useful tool that may be used as a complement to food security department, by indicating meat spoilage and predict meat's expiration date. Moreover, considering the literature's biogenic amine index and using it as a base for white meat quality assessment, it is possibly to discard a deteriorated product and account for their toxicological effects on human health. In summary, the presented study relates some parameters that can give in-depth knowledge about the presence of BAs in meat.

5.1. Materials and methods

5.1.1. Samples

Chicken breasts were bought from a local supermarket and cut in fillets of 3×4×1 cm, weighing approximately 10 g. Portions of chicken meat were packed in air overwrapped with polyethylene (PE) film. Following packaging, samples were stored at 8ºC and examined at 2-day interval. While 4ºC is chosen as the ideal storage temperature of chicken meat, 8ºC was maintained as the fixed storage temperature in order to obtain a higher BA content after 12 days.

5.1.2. Microbial analysis

The number of microorganisms potentially associated with chicken meat spoilage (LAB, Enterobacteriaceae and TVC) were analyzed by homogenized meat cut samples with tryptone salt (tryptone 0.1 % and NaCl 0.85 %) in a Stomacher for 90 s. Serial decimal dilutions were prepared in the same solution for microbiological determinations. TVC were obtained on Plate Count Agar (CM325, OXOID, England) (30ºC, 72 h); LAB on double layer on Man Rogosa Sharpe agar (CM361, OXOID, England) (30ºC, 72 h); Enterobacteriaceae on double layer Violet Red Bile Glucose agar (CM485, OXOID, England) (37ºC, 24 h), according to ISO 4833:2003 [115], NF V 04-503 (1988) [116], ISO 21528-2:2004 [117].

5.1.3. Physical-Chemical Measurements: pH and color

The pH was measured directly in the muscle using a penetration electrode with a pH meter (Crison Instruments, Spain) and was evaluated in duplicate immediately after opening the packages and consequently for 12 days (2-day interval). Onecolor app was used to performed color measurements of chicken samples.

5.2. Results and discussion

5.2.1. Bacteriological analysis

Chicken meat has become a popular food product easily accessed and used as a raw material for processed and frozen products, sold either raw or pre-cooked, which have been cut, shredded, ground, or minced into small pieces [39]. Due to the evolution of meat processing, meat is today far more susceptible to chemical, biological and physical changes. This includes microorganisms and microbial enzymes that can accelerate meat spoilage making it inappropriate for consumption. The organoleptic test is the fastest, well-established method to detect the quality of fresh meat, however, it can be subjective.

The present study's main goal was to monitor the changes in microbial flora (LAB, TVC and Enterobacteriaceae) of breast chicken meat stored at 8ºC for 12 days (Table 5.1).

Table 5.1.: Changes in microbial flora of fresh breast chicken breast stored at 8°C in aerobic conditions.

	Storage time (days)						
	0	2	4	6	8	10	12
Total viable counts	5.2[a]	7.0	9.0	9.8	9.9	10.1	10.9
Lactic acid bacteria	3.1	4.9	5.4	5.6	5.9	6.0	4.2
Enterobacteriaceae	4.5	5.4	6.9	7.0	7.0	7.5	6.9

[a] Values are expressed as mean of three determinations (Log CFU g-1).

These are known to be linked with the production of free amino acids involved in BAs metabolic formation. Since TVC englobes all of the microorganisms such as bacteria, yeast or mold spores in a sample, its higher concentration in meat samples is expected. On the first day, TVC concentration was 5.1 log CFU g^{-1}, thus suggesting an overall good quality of the chicken breast meat at the moment of buying. After being stored at 8ºC for 12 days, TVC concentrations increased substantially reached values of 10.8 CFU g^{-1} which is attributed to spoilage meat. Initial (day 0) Enterobacteriaceae counts were 4.5 log CFU g^{1}, thus suggesting good hygiene practices from the meat supplier. These last reached, at a fast rate, 7.4 log CFU g^{-1} after 10 days. Interestingly, BAL counts were always slightly above compared to the previous, where the initial (day 1) and final (day 12) counts of the chicken samples were 3.1 and 6.0 log CFU g^{-1},

respectively. These results suggest that both BAL and Enterobacteriaceae respond similarly to the storage conditions in terms of meat spoilage. Since meat quality is dependent on many exterior factors namely animal breed type, feeding source (grains, pasture, and grass), genetics of animal and postmortem techniques, which will translate into different types and concentrations of substrates for microorganisms to proliferate, meat samples can differ in counts at the initial moment. Although count values were low at the initial days, from the literature it is possible to conclude that the day 0 chicken breast samples can differ [41], [118], [119], however, results are comparable and the microbial behavior is similar.

Bacteria growth is well-established through kinetics models. Farber et. al., [120] has reported that packing such as vacuum ones extend the lag phase of microorganisms, therefore decreasing growth rate during the exponential phase. Figure 5.1 presents the microbial growth kinetics obtained for this study. Lag phase is considered to be missing since from day 0 to day 2 it is already visible an increase of biomass. The duration of each stage (exponential, stationary, and death phase) for BAL, TVC, or Enterobacteriaceae is similar, however, for BAL and Enterobacteriaceae it is possible to observe a decline that is correspondent to the death phase.

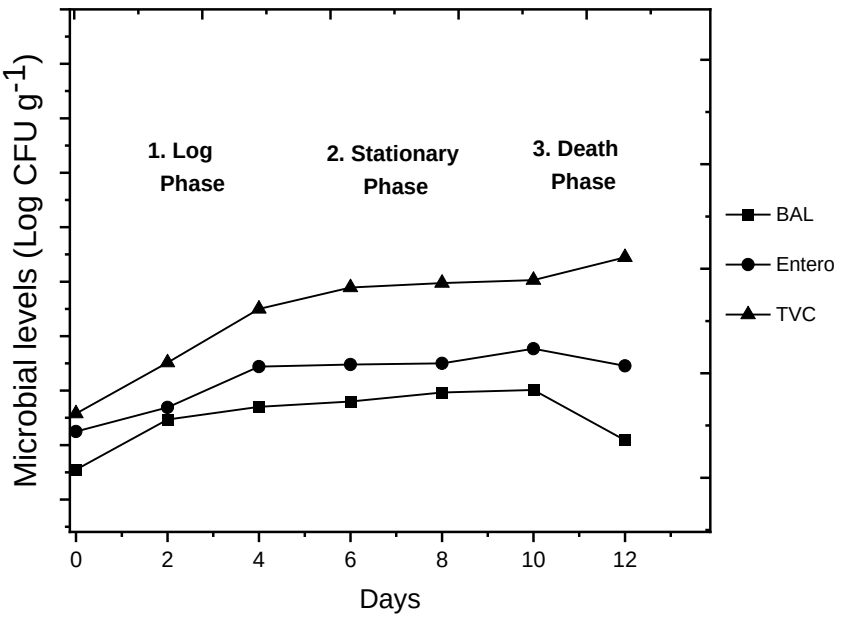


Figure 5.1.: Time evolution of the total viable counts (TVC), lactic acid bacteria (LAB), Enterobacteriaceae (Entero) for chicken breast fillets stored aerobically at 8ºC.

5.3.2. Chemical and physical proprieties: pH and color

The presence of microorganisms in meat is the major contributor to the reduced shelf-life. Since bacteria typically grow in neutral environments (pH=7), an increase in pH is linked with microbial contamination. This is explained by the rate of the glycolytic pathway during post-slaughtering [121]. Figure 5.2 shows the influence of meat spoilage in pH values during storage at 8ºC for 12 days. Significant changes in pH values, from acidic to alkaline environment, were observed from days 0 to 12, thus suggesting a rapid proliferation of microorganisms. The pH values of breast chicken meat ranged from 6.0 to 8.3 from days 0 to 12, respectively. Storage temperature of 8ºC

is considered to be extreme, which means that for lower temperatures pH is generally above 7 [122], [123].

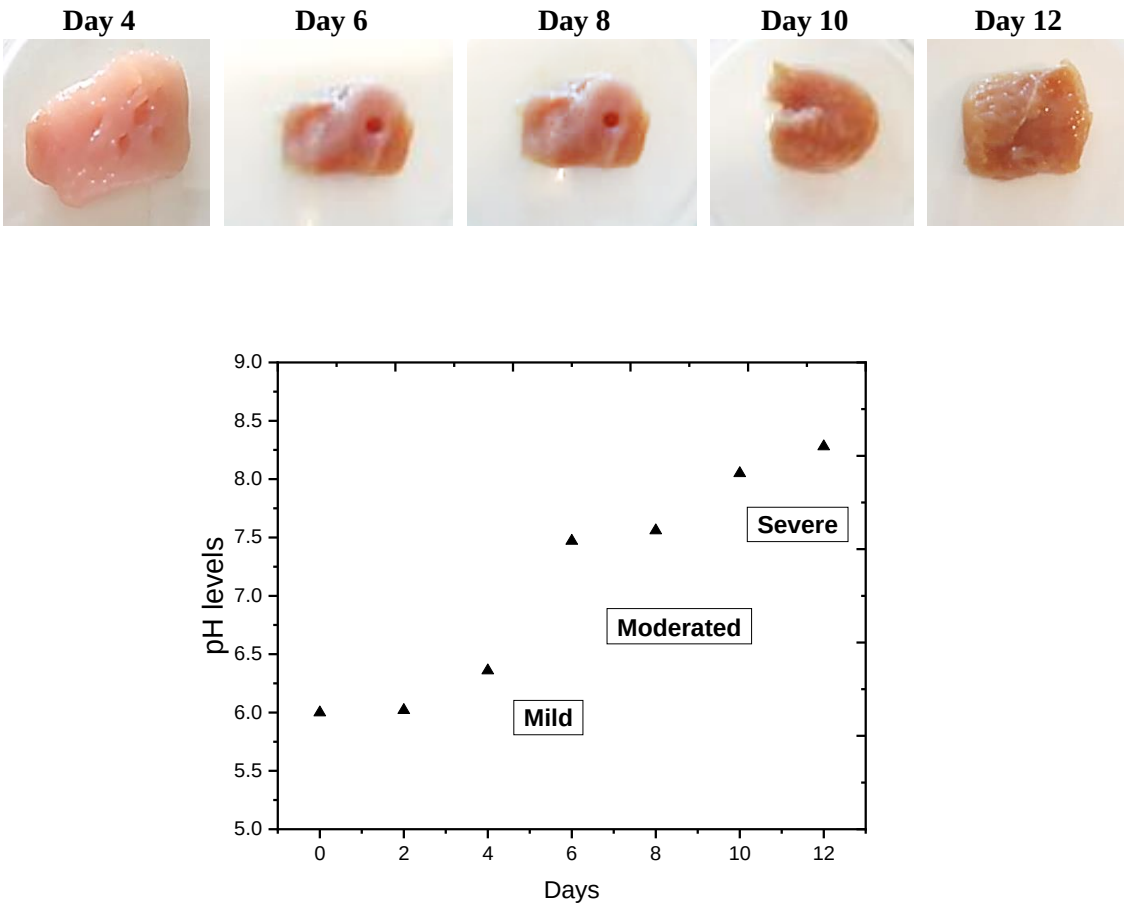


Figure 5.2.: Influence of meat spoilage in color and pH parameters during storage at 8ºC for 12 days.

Besides pH measurements, perception of meat color during storage time is also used as an indicator of the freshness of meat. Besides, from a consumer point of view meat color is an important quality attribute at the moment of buying. As a consequence of meat storage prior to slaughtering, the presence of microorganisms and its reproduction accelerates the change of fresh meat. Results of the color measurements during the storage time are presented in Table 5.2. During aging, Lightness (L*) decreased significantly from 48.8 to 33.5. Similarly, redness (a*) and yellowness (b*) also decreased with aging for all the samples, ranging from 23.2 to 18.9 and 20.9 to 18.3 respectively, indicating a decreased of the initial bright pink aspect to a much darker and opaque color. Jung et al. (2015) [124] has reported a relationship with ultimate pH and water holding capacity, where the change of pH in meat was negatively correlated with the L∗ value, and positively correlated with a∗ value of breast chicken meat. The discoloration of meat is also linked with an increased in oxidative processes from lipid oxidation, for the formation of metmyoglobin, which accumulation results in loss of light reflectivity [121]. The results obtained for L*, a* and b* parameters are according to the literature [121], [123], [125], where an accentuated decrease of L* parameter is presented. Chicken breast oxidation is lower for lower storage temperatures [126], thus limiting its deterioration over stored days.

Table 5.2.: Effects of storage color characteristics (L*, a*, b*) of chicken breast samples during storage time at 8ºC.

	Storage time (days)				
Color	4[1]	6[1]	8[1]	10[1]	12[1]
Lightness (L*)	48.8	37.5	37.7	34.9	33.5
Redness (a*)	23.2	14.0	17.0	18.1	18.9
Yellowness (b*)	20.9	10.3	19.1	17.0	18.3

[1] Values are expressed as mean of three determinations.

5.3.3. Biogenic amine's index: shelf-life assessment

The quality of chicken is more suitable to be measured in terms of the biogenic amine index (BAI) than red meat [40]. Currently, BAI is widely accepted and can be calculated by summing TYR, HIS, PUT and CAD levels in the different meat types or by using concentration proportions in a direct way: BAI < 5 $mg.g^{-1}$ means good meat quality; between 5–20 $mg.kg^{-1}$ means acceptable meat quality; between 20–50 $mg.kg^{-1}$ means poor meat quality; and BAI > 50 $mg.kg^{-1}$ means spoiled meat [40]. Thus, considering BAI and using the physicochemical and biological data obtained from the previous study, a new representation and quality assessment for breast chicken meat is proposed (Table 5.3).

Table 5.3.: Food quality chart: an easy tool for chicken breast meat quality and shelf-life assessment.

Chicken breast meat shelf-life	Biogenic amines concentration (ppm)	Microbial Counts (range)			Color (range)			pH (range) from 6 to 8,5
		BAL	Entero	TVC	L*	a*	b*	
	50	5.4 to 6	6.9 to 8	9+	35 to 33	19 to 18.9	19.1 to 18.27	6.4 to 8.3
	20 to 50	5 to 5.4	5.4 to 6.9	7.1 to 9	37.5 to 35	20 to 19	19.8 to 19.1	6.1 to 6.4
	5 to 20	3 to 5	4.4 to 5.4	5.1 to 7.1	48.8 to 37.5	23.2 to 20	20.9 to 19.8	6 to 6.1

This tool allows an easy and user-friendly visualization of breast chicken meat quality based on several parameters, namely BA's concentration, microbial analysis (that include the main responsible bacteria for BA's formation), color and pH. In the first column of the table, it is possible to observe a 3-color system that represent the deterioration state of the chicken meat

sample, based on green ("Product is in good condition for at least two more days"), yellow ("Product is in satisfactory condition and has an expiration date of approx. two more days") and red ("Product is expired and in bad condition") labels. Food quality chart and BAI have a common basis, once BA's content is the eliminatory factor determined by BAI index (good, acceptable, and poor quality). However, the new information added augmented the information of the quality of meat when meat is at least in an acceptable quality, where shelf-life information is given at the final stage of the Food quality chart. Users will be able to enter each value associated with certain parameter, aiming for a final and complete score in a form of color and shelf-life time (days). This tool would potentially help consumers with useful information of the industry regarding freshness or spoilage and sanitary quality of chicken breast meat.

5.3. Conclusions

In summary, the different microbial groups that potentially contributed to the spoilage of chicken breast studied all increased after storage time. At 8ºC TVC was the dominant microorganism, while BAL remained at lower levels. Physicochemical properties of the chicken meat were determined, such as pH and color. The pH levels over the storage days have changed from acidic to alkalinity, as it would be expected and correlated with microbial content during storage. Color system LAB was performed, where L*, a* and b* parameters have decreased from days 4 to 12, indicating a decreased of the initial bright pink aspect to a much darker and opaque color. All the collected parameters were indicative of breast chicken deterioration over the 12 days of storage at 8ºC and contained useful information for the construction of the Food quality chart. This last can be used to renovate and upgrade the existing BAI index, once the impact of this tool may be emphasized through its potential contribute and utility for the current trend in food security.

Chapter 6. Final conclusions and future work

Food quality has gained special attention in the last decades. Increased awareness over compounds that could contaminate the food we eat should be taken as a signal towards new sustainable developments in the analytical chemistry field. The literature highlights some of the most commonly found BAs in food products, among those the most toxic to human health. Herein, the combination of biosensors to the existing sensors should be the key to ensure global food safety.

This master thesis accomplished one of the main objectives, i.e., the characterization of a sensor to H_2O_2 detection and its further application in milk samples. To achieve this goal a chemiluminescence sensor based on a sensing membrane was developed. In summary, the sensing membrane approach for the chemiluminescence reactions allowed the characterization of a biosensor for H_2O_2 detection, thus contributing either to a green method for its detection, but also as an alternative one. The developed methodology for the sensing membrane contributed to the final proposed method. Calibration curve obtained was linear from 0.0001% to 0.001% w/w H_2O_2 and 0.001% to 0.01% w/w H_2O_2 with a correlation coefficient of 0.996 and 0.998, respectively. The limit of detection and quantification based on signal to noise ratio of 3 and 10 ranges from $2.0x10^{-5}$ to $1.2x10^{-4}$ % w/w and $6.8x10^{-5}$ to $3.97x10^{-4}$ % w/w, respectively. From the concentration's range used in this study (0.0001% to 0.01% w/w H_2O_2), it was possibly to lower the LOD to values almost incomparable with the ones found in the literature. Moreover, % RSD was found to be less than 10% which demonstrate a very good repeatability. The developed method was eco-friendly and was found to be sensitive, rapid, and cost-effective. It has a great potential to be used as a method for H_2O_2 detection in the most diverse areas (e.g., environmental, food quality, and agriculture) due to the reported sensitivity.

One viable route for BAs elimination on food matrices is when enzyme decarboxylase is present in a favourable medium. This strategy is widely used in an investigation context, however, not very often applied to the food market. Thus, to develop a methodology that can detect BAs in meat matrices based on an enzymatic biosensor, the strategy was to use enzymes and let them contact with BAs so that an enzymatic reaction can follow. This study has allowed us to notice some patterns of absorbance of this DAO enzyme in a liquid medium, which was not expected.. DAO absorbance spectrum has confirmed the visual characteristics of the enzyme. With such an intense color, and since chemiluminescence is an optical reaction, it wasn't observed any signal in the presence of the enzyme. With a complementary colorimetric method (DR1900) it was intended to find out if H_2O_2 was being release during reaction. Results given by DR1900 equipment were negative to the presence of H_2O_2. The use of a white powder enzyme has confirmed that the darker tone characteristics of the last bought enzyme were interfering with the measured signal, since signals obtained with the optimized detector were positive for the presence of H_2O_2, altought at a lower concentration than the one expected.

Complementary to the biosensor, it was developed a methodology to detect BAs in real samples based on GC-MS. Silanization was the chosen method for the determination of four BAs (TYR, PUT, CAD and HIS). This method was used both to confirm the identities of the biogenic amine derivatives but also to quantified them. However, and considering the results obtained using GC-MS, it was possible to conclude that the equipment worked better for qualitative purposes when working with BAs. The limit of detection was above 10 ppm for PUT alone and 25 ppm for TYR and CAD. HIS was successfully detected when mixed with the other three amines and in the solid form, however, when the approach was mixing HIS in liquid form signal was not obtained. Temperature ramp [100ºC (1 min); 160ºC at @10ºC/min until 280ºC at @ 25ºC/min] was optimized for BAs and a clean chromatogram was obtained. Overall, the chromatograms were not

very reproducible, which could be explained by the use of a longer column (30 m) as opposed to a shorter one (15m), as well as maintenance of the GC inlet end by cutting the tip after 25 runs. The proposed methodology did not accomplish its main goal of detecting BAs in real meat matrices, however, allowed a better understanding of GC-MS components, as well as its main disadvantages.

For food security purposes, the freshness of a product can be defined by various indicators, including microbial analysis, that includes total viable counts (TVC), BAs index, estimate oxidation rate through colour and pH determinations. Thus, portions of breast chicken meat were evaluated during storage days at 8ºC. From that study, it was concluded that the evolution of microbial content in chicken meat was coherent with both pH and color determinations, that are dependent on microorganisms grow. As expected, all microorganisms have behaved accordingly to the microorganism's growth kinetic curve. After 10 days, BAL and Entero have reached a maximum and started to decrease its counts. Contrarily, TVC did not show death phase. As for pH levels and color parameters these have changed accordingly during storage time, from acidic to alkalinity, and from the initial bright pink aspect to a much darker and opaque color, respectively. This is correlated with microbial content since they are responsible for the pH changes as well as the color features. All the collected parameters were indicative of breast chicken deterioration over the 12 days of storage at 8ºC and have contributed to the construction of the Food quality chart. It can be used as new BAI and contribute to an easier way of determining meat quality in a new era of emerging control of food quality.

This master thesis allowed a significant improvement of the scientific knowledge of the BAs present in food products, therefore going further on the development of new and important tools for its determination. Specifically, it opens new perspectives regarding food contaminants and analytical techniques, which will endorse innovation in the food market. The results obtained can be improved and pushed forward, namely, to study the solution for the absorbance problem, which can be accomplished either by the process of enzyme immobilization or by changing the source of the enzyme; and to use chromatographic methods to validate the biosensor using real samples.

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ANNEX

Annex from Chapter 4.

Biogenic amines determination by GC-MS

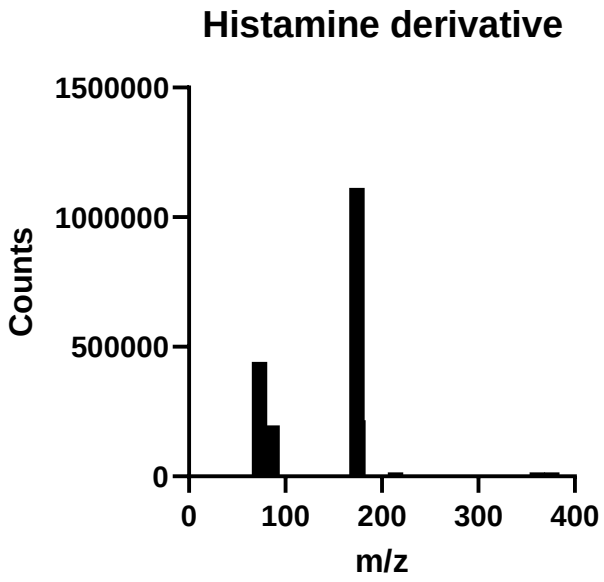

Figure 1.: Mass spectrum of histamine derivative obtained from MassHunter Qualitative Analysis.

Annex from Chapter 5.

Food Quality Chart (FQC)

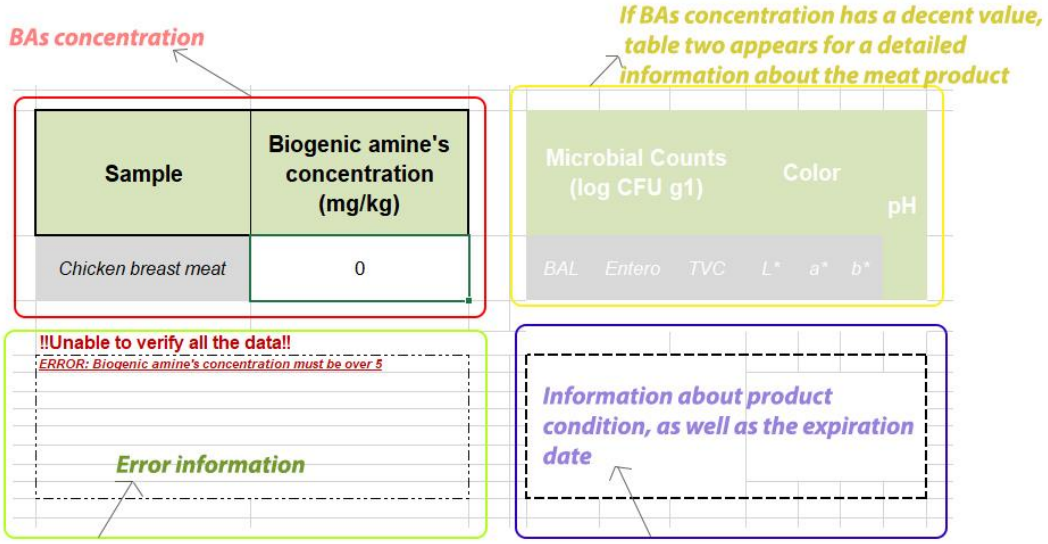

Figure 2.: Information about how the table works.

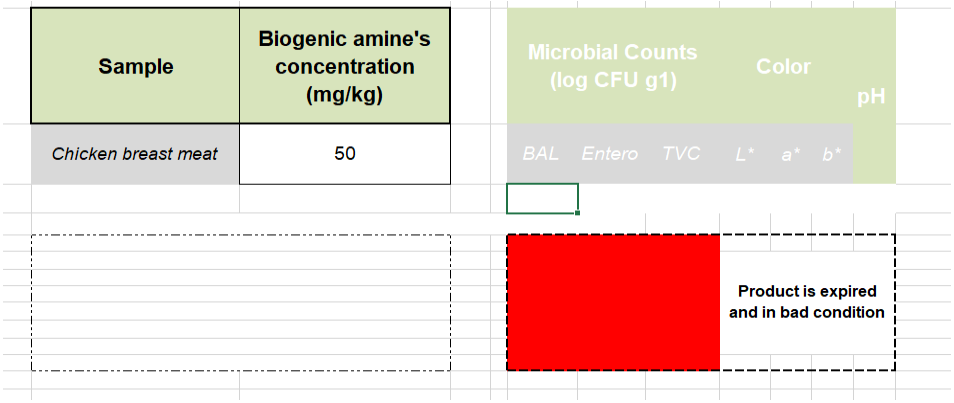

Figure 3.: Example of the FQC when BAs concentration is excedding 50 ppm.

BAs concentration of 15 means that the chicken meat is in “fresh” condition

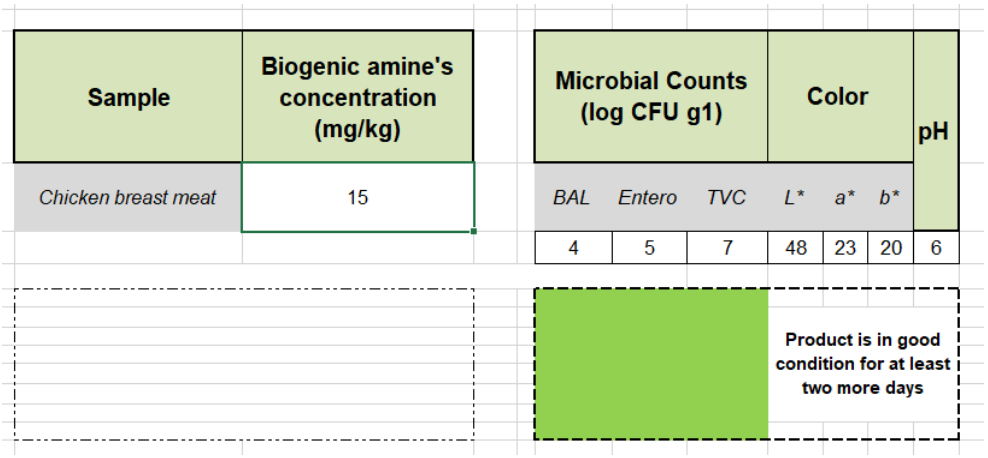

Figure 4.: Example of the FQC when BAs concentration is low and all the parameters are aceptable.

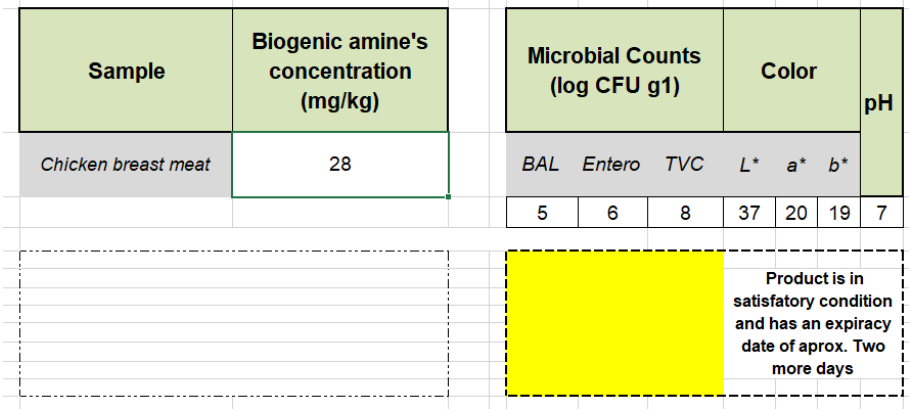

Figure 5.: Example of the FQC when BAs concentration is still above 50 ppm and all the parameters are changing.

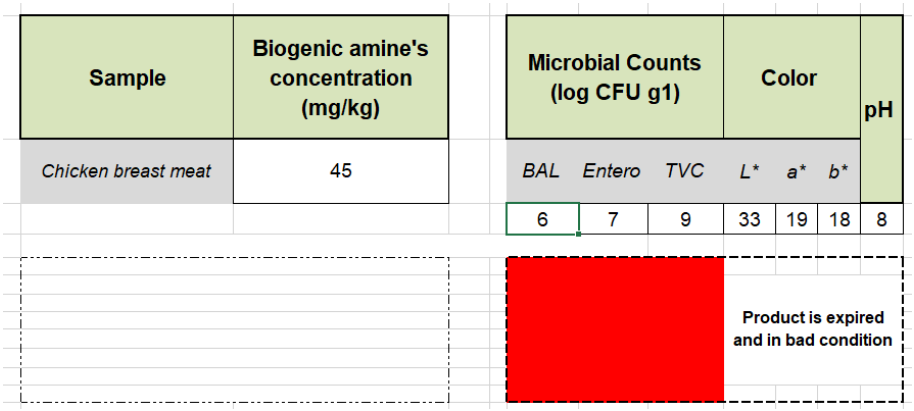

Figure 6.: Example of the FQC when BAs concentration is still above 50 ppm but all the parameters are indicative of the putrefication state.