Hinc patriam sustinet

Instituto Superior de Agronomia
Universidade Técnica de Lisboa

ENZYME-LINKED IMMONOSORBENT ASSAY (ELISA)

The development of a Direct Competitive ELISA for the determination of histamine in wine

Haline Garcia Victório

Dissertação para obtenção do Grau de Mestre em **Engenharia Alimentar**

Orientador: Doutora Maria Adélia Ferreira

Co-orientadores: Doutor Abel Oliva e Dra. Vitoria San Romão

Júri:

Presidente: Doutor Ricardo Manuel de Seixas Boavida Ferreira, Professor Catedrático do Instituto Superior de Agronomia da Universidade Técnica de Lisboa.

Vogais: Doutora Maria Adélia da Silva Santos Ferreira, Professora Auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;

Doutor Abel Martin González Oliva, Investigador Auxiliar do Laboratório de Diagnóstico Biomolecular – Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa;

Doutora Ana Isabel Amaro Gonçalves Domingos, Investigadora Auxiliar da Unidade de Tecnologia de Proteínas e Anticorpos Monoclonais do Instituto de Higiene e Medicina Tropical.

Lisboa, 2009

Acknowledgements

I am grateful to Dr. Maria Adélia Ferreira, Dr. Abel Oliva and Dr. Vitoria San Romão for the technical support and guidance over the development of the research and evaluation of this work.

I also thank Dr. Claudia Sánchez Lara and Elisa Campos from ITQB for excellent technical assistance.

Finally, I thank Dr. Ana Domingos, Dr. Fernando Cardoso and Dr. Carlos Novo for permitting the execution of the assays in the facilities of UTPAM/IHMT.

Abstract

The presence of biogenic amines in wines, specifically the histamine (0,1 mg/L to 20 mg/L) has been a constant source of concern for wine producers, since this substance presents toxic effects for human beings.

The goal of this work was the development of a fast, efficient and low cost technique for the determination and quantification of histamine in wine. For that end, the strategy was the use of a competitive ELISA method.

The first step for the development of this method was the acquisition of the antibody for histamine. Among the available options, it was decided to purchase the commercial polyclonal antibody for histamine.

The second step was the conjugation of the histamine with the peroxidase. Three methods of conjugation were performed, followed by their subsequent analysis.

Initially, it was necessary to perform assays for the optimization of the antibody and of the conjugate, as well as to test the blocking buffers (1,0% (w/v) gelatin and 0,5% (w/v) sodium caseinate).

Before the application of the ELISA competitive method, auxiliary tests such as indirect ELISA, ELISA inhibition test and ELISA binding test were performed. However, continuously unsatisfactory results were obtained, leading to the perception that there could be a low efficiency of the conjugation processes and/or a lack of specificity between the histamine used and the polyclonal antibody acquired for the assays.

Keywords: histamine, ELISA, conjugate, HRP, antibody

Resumo em Português

A presença de aminas biogénicas em vinhos, especificamente a histamina (0,1 mg/L a 20 mg/L) tem sido uma constante preocupação para o mercado vinícola, já que esta substância apresenta efeitos tóxicos para seres humanos.

O objectivo do presente trabalho foi desenvolver uma técnica rápida, eficaz e de baixo custo para a determinação e quantificação de histamina em vinhos. Para esse fim, foi proposto o método ELISA competitivo.

O primeiro passo para o desenvolvimento do método foi a obtenção do anticorpo anti-histamina. De entre as alternativas existentes, optou-se pela compra de um anticorpo policlonal.

O segundo passo foi a conjugação da histamina com a peroxidase. Foram feitos três processos de conjugação, seguidos de suas respectivas análises.

Inicialmente, foram realizados ensaios para a optimização do anticorpo e do conjugado, assim como foram testados dois tampões de bloqueio (1,0% (p/v) gelatina e 0,5% (p/v) caseinato de sódio).

Anteriormente à aplicação do método ELISA competitivo, foram realizados ensaios como ELISA indirecto, *ELISA inhibition test* e o *ELISA binding test*. Porém, os resultados foram pouco satisfatórios indicando uma possível falha no processo de conjugação e/ou baixa especificidade da histamina com o anticorpo utilizado.

Palavras chave: histamina, ELISA, conjugado, peroxidase (*HRP*), anticorpo

Resumo alargado em Português

A presença de aminas biogénicas em vinhos, especificamente a histamina (0,1 mg/L a 20 mg/L) tem sido uma constante preocupação para seus produtores e consequentemente para o mercado vinícola português, já que esta substância apresenta efeitos tóxicos para seres humanos. Uma das técnicas mais utilizadas para detecção dessa substância em vinhos é o HPLC (*High-Performance Liquid Chromatography*) porém, este método apresenta um custo elevado de equipamentos e manutenção, além de exigir técnicos treinados para o seu desenvolvimento.

O objectivo deste presente trabalho foi desenvolver uma técnica rápida, eficaz e de baixo custo. Para esse fim, foi desenvolvido um método imunoenzimático que se baseia na utilização de antigénios ou anticorpos marcados com enzimas que permitem a detecção e quantificação de substâncias de interesse biológico. Foi proposto portanto, o método ELISA competitivo, cujo princípio se baseia nas seguintes etapas: (i) o revestimento dos poços de uma placa ELISA com anticorpo específico contra a histamina; (ii) adição simultânea da histamina livre e da histamina marcada com a enzima peroxidase, com o objectivo de provocar uma competição pelo local de ligação do anticorpo; (iii) adição de um substrato cromogénico específico (TMB- tetrametilbenzidine); (iv) adição de ácido sulfúrico 1M com o objectivo de parar a reacção e medir fotometricamente a absorvância a 450 nm. Os resultados dos valores de absorvância esperados deveriam ser inversamente proporcionais à concentração de histamina da amostra.

Embora haja no mercado kits ELISA comercias para determinação de histamina em vinhos, todos esses métodos fazem um pré-tratamento (acilação ou derivatização) da histamina. O propósito do presente trabalho, foi avaliar o comportamento da histamina sem a etapa de pré-tratamento a fim de tornar o método mais fácil e rápido.

O primeiro passo para o desenvolvimento do método foi a obtenção do anticorpo anti-histamina. De entre as opções existentes, optou-se pela compra de um anticorpo policlonal anti-histamina.

O segundo passo foi a conjugação da histamina com a peroxidase. Este processo de conjugação é necessário para que seja possível quantificar através de um

espectrofotómetro a histamina marcada (neste caso com a enzima peroxidase), e diferenciá-la da histamina livre presente na amostra. Três métodos de conjugação foram realizados, um via periodato e os outros dois utilizando glutaraldeído. Foram realizadas análises para determinação da eficiência de conjugação e concluiu-se que o terceiro método de conjugação, usando glutaraldeído, era o que apresentava melhores condições para utilização nos ensaios subsequentes.

Inicialmente, foram realizados ensaios para a optimização do anticorpo anti-histamina e do conjugado (histamina + peroxidase), assim como foram testados dois tampões de bloqueio (1,0% (p/v) gelatina e 0,5% (p/v) caseinato de sódio); concluiu-se, portanto, que diluições na ordem de 1/1000 no caso do anticorpo e de 1/400 no caso do conjugado seriam apropriadas.

Após optimização da reacção entre anticorpo e conjugado, optou-se por iniciar os ensaios pelo método ELISA indirecto, utilizando ainda dois tampões de bloqueio, 1,0% (p/v) gelatina e 0,5% (p/v) caseinato de sódio. Porém, os resultados foram pouco satisfatórios indicando uma possível falha no processo de conjugação.

Outros ensaios foram realizados, como o *ELISA inhibition test* e o *ELISA binding test*. No primeiro ensaio, os resultados indicaram que este pode ser um possível método para determinação de histamina em alimentos que possuam maiores concentrações deste composto, já que somente a partir de 100 ug/µL de histamina foi possível diferenciar os valores de densidade óptica; no segundo ensaio, embora o conjugado não tenha sido utilizado e sim a histamina livre fixada na placa através da utilização da *poly-l-lysine*, resultados obtidos continuavam a ser muito aquém do esperado, o que poderia reforçar a ideia da falta de especificidade da histamina com o anticorpo em questão.

A fim de verificar a diferença entre trabalhar com a histamina na forma livre como foi proposto neste trabalho e a histamina derivatizada como é proposto pela maioria dos kits ELISA comerciais, considerou-se adequado realizar um ensaio com o kit comercial *Histamine ELISA kit (Ref: IB 89128, Immuno Biological Laboratories, Inc. Minneapolis, USA)*.

De entre as perspectivas apresentadas, outras hipóteses para o não funcionamento do método poderiam ser sugeridas. Uma delas seria a pouca especificidade entre a histamina utilizada nos ensaios e o anticorpo. Outra hipótese seria a de que o processo

de derivatização da histamina, como ocorre na maioria dos kits comerciais, seja fundamental para o sucesso dos ensaios – porém, para que isso ocorra, é fundamental que o processo de derivatização da histamina seja realizado, seguido da conjugação desta com moléculas como BSA (*Bovine Serum Albumin*), KLH (*Keyhole Limpet Hemocyanin*), HSA (*Human Serum Albumin*) e a imunização dos animais para que o anticorpo produzido seja específico para a histamina a ser utilizada.

Contents

Contents of figures

Contents of tables

Abbreviations

BA: Biogenic Amines

HA: Histamine

GA: Glutaraldehyde

HRP: Horseradish Peroxidase

BSA: Bovine Serum Albumin

TMB: 3,3', 5'5 Tetramethylbenzidine

Ab: Antibody

Ag: Antigen

Conjugate I (HA-HRP): conjugation of histamine with the enzyme via periodate method according to the Schneider protocol (Schneider E. *et al*, 1997).

Conjugate II (HA-GA-HRP): conjugation of histamine with the enzyme according to the Rice protocol (Rice T.K. *et al*, 1982).

Conjugate III (HA-GA-HRP): conjugation of histamine with the enzyme according to the Fujiwara protocol (Fujiwara *et al.*, 1997)

HA-GA-BSA: conjugation of histamine with BSA according to the Fujiwara protocol (Fujiwara *et al.*, 1997)

KLH: Keyhole Limpet Hemocyanin

HSA: Human Serum Albumin

PBS: Phosphate Buffered Saline

TBS: Tris Buffered Saline

EIA: Enzyme Immunoassay

1. Introduction

The presence of Biogenic Amines ("BA") in fermented foods, such as beer and wine, cheese and other dairy products, fish, sausages and meat has been receiving increasing attention due to its potential undesirable physiological effects in sensitive humans.

BA have been referred as being involved in relevant pathologies like immunological, neurological, and gastrointestinal diseases like gastric acid secretion (Marcobal *et al*., 2005, Aygun *et al*., 1999).

In wines, BA may be formed by lactic acid bacteria mostly after malolactic fermentation and/or during wine aging (Lonvaud-Funel, 2001). The main BA in wine is histamine, tyramine, putrescine, cadaverine and phenylethylamine. The presence of BA in wine is well documented and a wide range of biogenic amine concentrations are reported among different types of wine (from various origins and different winemaking processes), as reported by Lonvaud-Funel and Joyeux in a review issued in 1994.

There are two main reasons for the increasing interest in the study of amines in wines: (a) the toxicological risks associated with the ingestion of elevated amounts of these substances; and (b) the possible relationship between (i) high amine content and (ii) the quality of the grapes used in the production of the wine as well as the hygienic-sanitary conditions prevalent during processing (Souza *et al*., 2005).

The rising consumer demand for higher quality and safe food has led to a renewed interest in studies of biogenic amines. Nowadays, the analytical methods used for separation and quantification of BA in foods and beverage in general are mainly based on chromatography methods: gas chromatography (GC), thin-layer chromatography (TLC), and high-performance liquid chromatography ("HPLC") with precolumn or postcolumn derivatization techniques (Karovicova *et al*., 2003). HPLC methods are reported to be the most efficient, sensitive and reproducible ones. However, HPLC techniques require costly equipment, careful maintenance, expensive solvents and accessories, and specially trained personnel (Marcobal *et al*., 2006; Vidal-Carou *et al*., 2003). In order to enhance food control measurements, and possibly clarify the role of dietary histamine for human

health, it would be important the availability of easier, cheaper and faster methods for quantification of BA in food and beverage (Ayun *et al*., 1999).

Most immunochemical methods (enzyme immunoassays) for the detection of histamine in human serum, biological fluids, and food are based on antibodies against *N*-amino derivatives of histamine synthesized by reaction with, for example, *p*-benzoquinone or propionic acid esters. More recently, Claeys-Bruno *et al*., 2006, suggested methodological approaches for histamine quantification using derivatization by choroethylnitrosourea. However, since the antibodies used in these tests are not reactive with the natural histamine compound but only with the modified histamine, derivatization of histamine is required before analysis, which either implicates in a time-consuming process (propionic acid esters) or requires toxic reagents (*p*-benzoquinone, choroethylnitrosourea) (Ayun *et al*., 1999).

Among the several options in food and beverage products, it seemed appropriated to focus on wine, due to its relevance to the Portuguese economy as well as the rising interest on the subject worldwide.

Therefore, the goal of the study conducted was the pursuit of a simpler, more practical, faster and less costly method to quantify free histamine without previous derivatization by immunological methods (e.g. a direct competitive ELISA) in wine.

2. Definition – Biogenic amines

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxilation of amino acids or by amination and transamination of aldehydes and ketones. They are organic bases with low molecular weight and are synthesized by microbial, vegetable and animal metabolisms. Biogenic amines in food and beverage are formed by the enzymes of raw material or are generated by microbial decarboxylation of amino acids, although it has been found that some of the aliphatic amines can be formed "*in vivo*" by amination from corresponding aldehydes (Silla Santos, 1996).

The chemical structure of biogenic amines can either be: aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine, tryptamine). Several authors have classified cadaverine, putrescine, spermine, and spermidine among polyamines (Karovicova *et al*., 2003). The precursors of the main biogenic amines involved in food poisoning are the describe below:

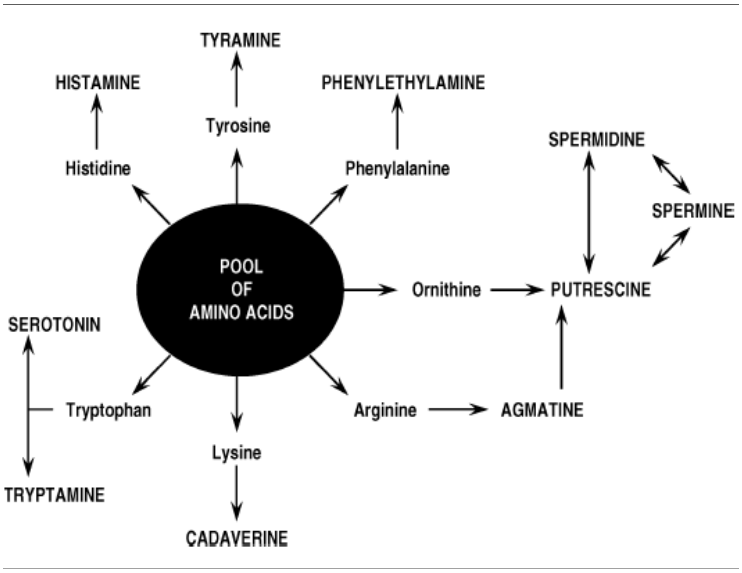

Figure 1. Precursor amino acids of biogenic amines. Source: Ancín-Azpilicueta, *et al*, 2008

The prerequisites for biogenic amine formation by microorganisms are: (i) availability of free amino acids, even if not always leading to amine production; (ii) presence of decarboxylase-positive microorganisms; and, (iii) conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity.

2.1. Biogenic amines in food.

Biogenic amines are usually found in virtually all food that contain proteins or free amino acids and are subject to conditions enabling microbial or biochemical activity. The total amount of the different amines formed strongly depends on the nature of the food and the microorganisms present. Biogenic amines are present in a wide range of food products including fish products, meat products, dairy products, wine, beer, vegetables, fruits, nuts and chocolate (Silla Santos, 1996).

In non-fermented food, the presence of biogenic amines above a certain level is considered as indicative of undesired microbial activity. However, the presence of biogenic amines in food does not necessarily correlate with the growth of spoilage organisms, because they are not all decarboxylase-positive.

Scombroid fish have most commonly been associated with incidents of histamine intoxication (scombrotoxicosis). The formation of histamine in scombroid and other marine fish containing abundant endogenous histidine has been attributed to microbial action rather than endogenous histidine decarboxilase activity (Karovicova *et al*., 2003; Silla Santos, 1996).

In fermented foods, it is expected that many kinds of microorganisms are present, some of them being capable of producing biogenic amines. Table 1 shows the lactic acid bacteria species capable of producing biogenic amines like histamine, tyramine, phenylethyllamine and putrescine.

Table 1. Species of lactic acid bacteria able to produce biogenic amines

	Species of lactic acid bacteria	References
Histamine	*Oenococcus oeni*	Lonvaud-Funel and Joyeux (1994)
		Coton et al. (1998b)
		Guerrini et al. (2002)
		Landete et al. (2005a)
	Lactobacillus hilgardii	Farías et al. (1993)
		Landete et al. (2005b)
		Constantini et al. (2006)
	Lactobacillus 30a	Moreno-Arribas et al., 2003
		Constantini et al., 2006
	Pediococcus damnosus	Aerny (1985)
		Delfini (1989)
	Pediococcus parvulus	Landete et al. (2005b)
Tyramine	*Lactobacillus brevis*	Moreno-Arribas et al. (2000)
		Lucas and Lonvaud-Funel (2002)
		Lucas et al. (2003)
		Landete et al. (2005a)
		Constantini et al. (2006)
	Lactobacillus hilgardii	Moreno-Arribas et al. (2000)
	Leuconostoc mesenteroides	Moreno-Arribas et al. (2003)
Phenylethylamine	*Lactobacillus brevis*	Moreno-Arribas et al. (2000)
		Landete et al. (2005a)
	Lactobacillus hilgardii	Moreno-Arribas et al. (2000)
Putrescine	*Oenococcus oeni*	Coton et al. (1999)
		Guerrini et al. (2002)
		Marcobal et al. (2004)
		Mangani et al. (2005)
	Lactobacillus 30a	Gale (1946)
		Tabor and Tabor (1985)
	Lactobacillus hilgardii	Arena and Manca de Nadra (2001)
	Lactobacillus plantarum	Arena and Manca de Nadra (2001)
	Lactobacillus buchneri	Moreno-Arribas et al. (2003)

Source: Ancín-Azpilicueta *et al*, 2008

2.2. Toxic action

Histamine is the most studied biogenic amine and its quantity in food products is usually taken as an indicator of freshness and quality. The effects produced by histamine are the well-known as they are associated with most cases of food poisoning. The intake of 5 mg/L of histamine in wine is considered a tolerable level; the amount of 10 mg/L is considered the maximum level admitted and a proportion higher than 20 mg/L induces a high toxicity (Rauscher-Gabernig *et al.,* 2009).The toxicological levels of biogenic amines have still not been established and depend on individual characteristics and the presence

of other amines. Legal upper limits of 100 mg/kg of histamine in food and 2 mg/L of alcoholic beverage have been suggested (Silla Santos, 1996).

The definition of wine quality should include a threshold quantity for biogenic amines related to the toxicological effects directly caused by their ingestion; however this limit is still under discussion, with a myriad of opinions being expressed by different countries. Thus, upper limits for histamine in wine have been recommended in Germany (2 mg/L), Belgium (5-6 mg/L), France (8 mg/L) and Switzerland (10 mg/L) (Landete *et al.*, 2005).

Among the amines found in wine, not all of them present toxic effects for consumers. Table 2 shows the main effects of amines that provoke toxic effects produce in human beings.

Table 2. Biogenic amines and their pharmacological effects

Amine	Precursor	Pharmacological effects
Histamine	Histidine	Liberates adrenaline and noradrenaline
		Excites the smooth muscles of uterus, the intestine and the respiratory tract
		Stimulates both sensory and motor neurons
		Controls gastric acid secretion
Tyramine	Tyrosine	Peripheral vasoconstriction
		Increases the cardiac output
		Causes lacrimation and salivation
		Increases respiration
		Increases blood sugar level
		Release noradrenaline from the sympathetic nervous system
		Causes migraine
Putrescine and cadaverine	Ornithine and lysine	Hypotension
		Bradycardia
		Lockjaw Paresis of the extremities
		Potentiate the toxicity of other amines
Phenylethylamine	Phenylalanine	Releases noradrenaline from the sympathetic nervous system
		Increases the blood pressure
		Causes migraine
Tryptamine	Tryptophane	Increase the blood pressure

Source: Ancín-Azpilicueta *et al*, 2008

It must be said, however, that Kanny *et al*. (2001), using histamine-poor or histamine-rich wines claims that there is no correlation between the histamine content of wine and human wine tolerance, based on the argument that the presence of others compounds in wine, such as acetaldehyde, could explain the fact that some people are intolerant to this beverage as these compounds could provoke the liberation of endogenous histamine thus provoking such intolerance (Ancín-Azplilicueta *et al*, 2008).

2.2.1. Histamine and histamine metabolism

Histamine is synthesized by mast cells, basophils, platelets, histaminergic neurons, and enterochromaffine cells and exerts its effects by binding to its 4 receptors [histamine 1 receptor (H1R), H2R, H3R, and H4R] on target cells in various tissues (figure 2). It causes smooth muscle cell contraction, vasodilatation, increased vascular permeability and mucus secretion, tachycardia, alterations of blood pressure, and arrhythmias, and it stimulates gastric acid secretion and nociceptive nerve fibers. In addition, histamine has been known to play various roles in neurotransmission, immunomodulation, hematopoieses, wound healing, day-night rhythm, and the regulation of histamine and polyamine induced cell proliferation and angiogenesis in tumor models and intestinal ischemia (Maintz and Novak, 2007).

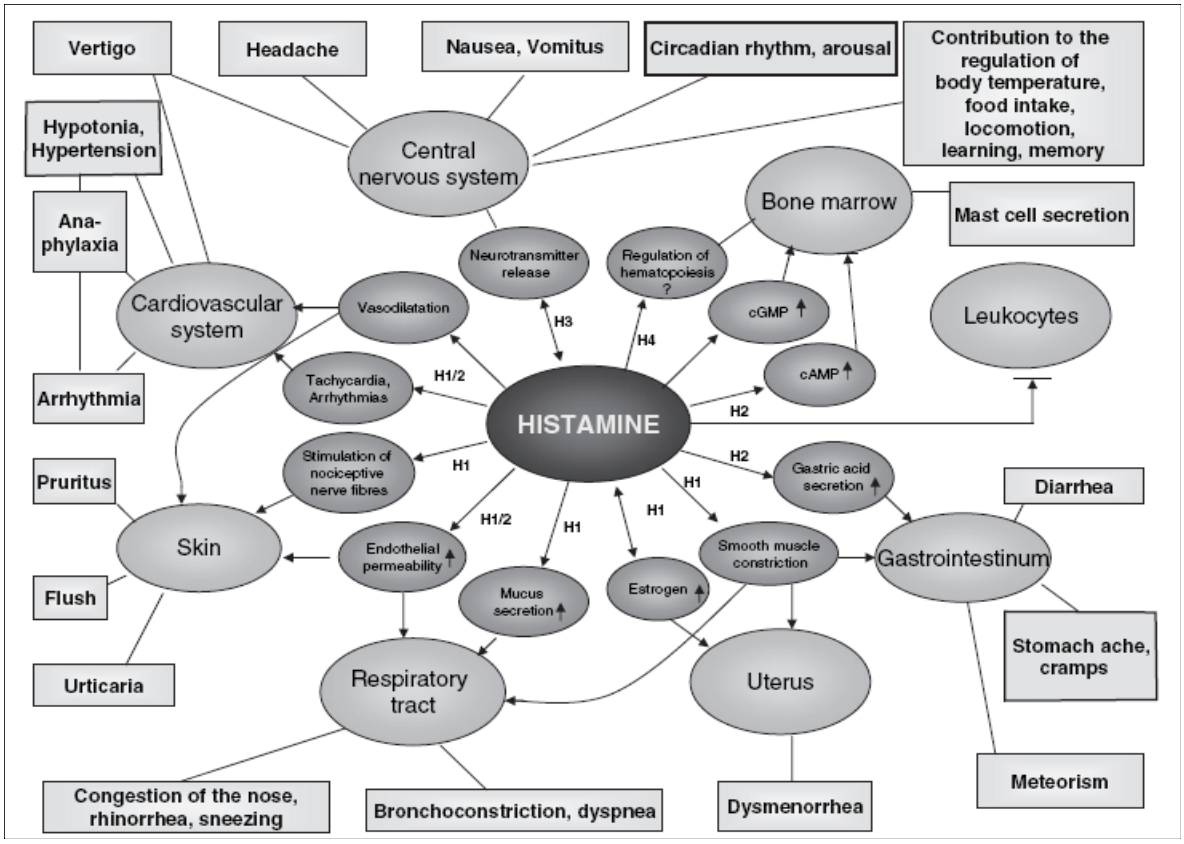

Figure 2. Summary of histamine-mediated symptoms. Adapted with permission from Maintz L et al. Dtsch Artzebl 2006;103:A3477-83. Source: Maintz and Novak, 2007

Histamine can be metabolized in 2 ways: by oxidative deamination by DAO - Diamine Oxidase - (former name: histaminases) or by ring methylation by histamine-N-methyltransferase (HNMT) (fig 3). Whether histamine is catabolized by DAO or HNMT is supposed to depend on the localization of histamine. The DAO protein is stored in plasma membrane-associated vesicular structures in epithelial cells and is secreted into the circulation on stimulation. Therefore, it has been proposed that DAO may be responsible for scavenging extracellular histamine (e.g. after ingestion of histamine-rich food) (Maintz and Novak, 2007).

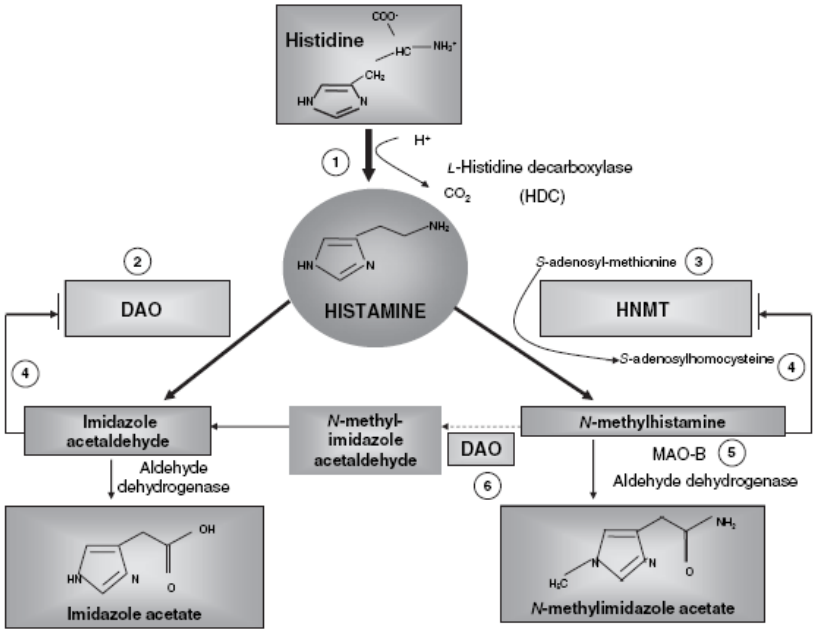

Figure 3. Summary of the histamine metabolism. The biogenic amine histamine is synthesized by decarboxylation of the amino acid histidine catalyzed by l-histidine decarboxylase (HDC) (1). Histamine can be metabolized by extracellular oxidative deamination of the primary amino group by diamine oxidase (DAO) (2) or intracellular methylation of the imidazole ring by histamine-N-methyltransferase (HNMT) (3). Therefore, insufficient enzyme activity caused by enzyme deficiency or inhibition may lead to accumulation of histamine. Both enzymes can be inhibited by their respective reaction products in a negative feedbackloop (4), N-Methylhistamine is oxidatively deaminated to N-methyl-imidazole acetaldehyde by monoamine oxidase B (MAO B) (5) or by DAO (6). Because the methylation pathway takes place in the cytosolic compartment of cells, MAO B (5) has been suggested to catalyze this reaction in vivo (35). Source: Maintz and Novak, 2007

2.2.2. Histamine intolerance

Histamine intolerance results from a disequilibrium of accumulated histamine and capacity for histamine degradation. The main enzyme for metabolism of ingested histamine is diamine oxidase (DAO). An impaired histamine degradation based on a reduced DAO activity and the resulting excess of histamine may cause numerous symptoms mimicking an allergic reaction. Ingestion of histamine-rich food, alcohol, or drugs that release histamine or block DAO may provoke diarrhea, headache, congestion of the nose, asthmatoid wheezing, hypotension, arrhythmia, urticaria, pruritus, flushing, and other conditions in these patients (Maintz and Novak, 2007).

2.2.3. Histamine and headache

Headache can be induced dose-dependently by histamine in healthy persons as well as in patients with migraine. Histamine-induced headache is a vascular headache caused mainly by nitrate monoxide. Histamine releases endothelial nitrate monoxide upon stimulation of H1R, which is also expressed in the large intracranial arteries. In migraine patients, plasma histamine concentrations have been shown to be elevated both during headache attacks and during symptom-free periods. An increase in the number of brain mast cells is associated with pathologic conditions such as migraine, cluster headache, and multiple sclerosis. Many migraine patients have histamine intolerance evidenced by reduced DAO activity, triggering of headache by food rich in histamine (e.g. wine), and the alleviation of headache under a histamine-free diet and therapy with antihistamines (Maintz and Novak, 2007).

2.2.4. Histamine and gastrointestinal

Besides headache, gastrointestinal ailments including diffuse stomachache, colic, flatulence, and diarrhea are leading symptoms of histamine intolerance. Elevated histamine concentrations and diminished DAO activities have been shown for various inflammatory and neoplastic diseases such as Crohn disease, ulcerative colitis, allergic enteropathy, food allergy, and colorectal neoplasmas. In the colonic mucosa of patients with food allergy, a concomitant reduced HNMT and an impaired total histamine

degradation capacity (THDC) have been found, so that the enzymes cannot compensate each other (Maintz and Novak, 2007).

2.3. Origin of biogenic amines in wine

The first report on the levels of histamine in wines was published in 1965, due to histamine poisoning associated with wine samples. In the 1980s interest was extended to other amines, among which tyramine, putrescine and cadaverine, due to technological and toxicological aspects. From a technological point of view, high levels of these amines are associated with low quality products or with defective winemaking practices, indicating poor hygienic conditions during processing. Furthermore, putrescine and cadaverine were observed to potentiate the toxic effect of histamine and tyramine. Some amines are also significant to wines in terms of aroma and flavor. In general, a weakening of the flavor impression is attributed to amines, whereby an unpleasant bitter aftertaste has been described in wines with high amine levels. Furthermore, putrescine and cadaverine can negatively affect the sensory quality of wines (Manfroi *et al*., 2009).

Beside the amines already present in grapes, several amines can be formed and accumulated during winemaking. The formation of amines depends on the pH of the wine, addition of sulphur dioxide, use of clarification agents, ageing with or without lees and, length of barrel aging (Manfroi *et al*., 2009).

2.4. Factors that influence the concentration of amines in wine

The total content of amines in wine is variable. This variability is due to the fact that numerous factors could influence the formation of biogenic amines. Some of these factors could have an indirect influence due to a modification in the concentration of precursor amino acids of the biogenic amines in the medium. Other factors could modify the development of microorganisms with amino-genic capacity. The factors which can influence the concentration of amines in wine, are described below (Ancín-Azpilicueta *et al*., 2008).

2.4.1. Raw materials: grapes and must

In general, low concentrations of amines are present in both the grape and the must. However, different concentrations of these compounds have been found in raw materials because amines, especially polyamines, are indispensable components of all living cells (Ancín-Azpilicueta *et al*., 2008).

Some amines are normal constituents of grapes, being in variable amounts in different types of grapes. Among biogenic amines detected in grapes, putrescine and spermidine are usually abundant (20 and 45 mg/kg of fresh fruit, respectively), whereas ethanolamine, agmatine, cadaverine, spermidine, histamine and tyramine have been found in lower amounts (Landete *et al*., 2005).

2.4.2. Influence of pH.

The wines that have higher pH generally have higher amine concentrations. Wines that exhibited pH values higher than 3.6 set a threshold for high histamine content (Fig.4). This is consistent with the fact that white wines (which by rule have lower pH values, i.e. around 3.11) show lower histamine concentration. The relationship between pH and amines is explained by the fact that the higher the pH, the greater the amount of bacteria that can develop, thus increasing the probability of having strains able to form amines (Landete *et al*., 2005).

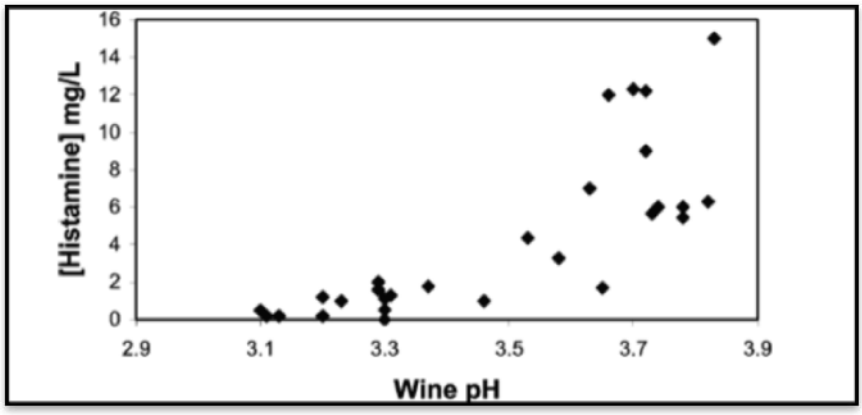

Figure 4. pH values versus histamine concentration from 28 different red, rosé, and white wines analyzed with malolactic fermentation accomplished. Source: Landete *et al*, 2005

2.4.3. Amino acid concentration

The content of free amino acids in the must could be related to the quantity of amines in wine. The amino acids present in grapes constitute a major source of yeast assimilable nitrogen and they are used by yeast as sources of nitrogen during alcoholic fermentation. Furthermore, amino acids also act as nutrients for the bacteria during the secondary fermentations. The concentration of amino acids in must depends on many factors such as grape variety, geographical origin, nitrogenous fertilization, degree of grape maturity, vintage and climatic conditions. In addition, different winemaking technologies like prefermentation, clarification, crushing and duration of maceration process have an effect on the amino acid fraction in must.

Therefore, there is a correlation between higher amounts of amino acids in must and higher amounts of biogenic amines after malolactic fermentation. However, in real conditions, it is difficult to establish a correlation between the concentration of biogenic amines and the consumption of their precursors amino acids during alcoholic fermentation. This could be due to the fact that during the alcoholic fermentation, yeasts mainly use amino acids directly in biosynthesis or as a nitrogen source and to a lesser extent for decarboxylation reaction where biogenic amines are formed (Ancín-Azpicueta *et al*., 2008).

2.4.4. Influence of winemaking

Some technological practices can influence on biogenic amines content in red wines. Studies showed that the longer time period of skin maceration increased the formation of histamine, tyramine, and putrescine and that the wine aging on lees mainly increased the concentration of putrescine and methylamine. It would seem that the fungi that attack the grape in certain vintages could also influence the amino acid concentration in wine (Ancín-Azpicueta *et al*., 2008).

Some techniques used in winemaking can reduce the biogenic amine content in wine, such as the use of bentonite (clarififying agent). Another technique proposed for elimination of histamine in wine is a thermal treatment of the grape after pressing. In

addition, there is the possibility of making refermentations with histaminolytics, but it does not seem to have been any positive results (Ancín-Azpicueta *et al*., 2008).

2.4.5. Influence of vinification conditions

The type of vinification conditions can influence the amine contents in wine; thus, white and rosé wines present lower amine concentrations than red wines. This may be attributed to various causes: (i) lower amino acids content in berries because they are harvested earlier than red grapes; (ii) short or no maceration with skins; (iii) short contact time with lees; and (iv) no malolactic fermentation (Landete *et al*., 2005).

Landete *et al*. (2005) observed that malolactic fermentation increases histamine concentration, which is probably due to the combination of two factors: the increase of amino acids in wine as a consequence of yeast lysis after alcoholic fermentation and the proliferation of lactic acid bacteria with decarboxylase activity. In a medium poor in nutrients such as the wine, lactic acid bacteria can obtain energy to grow from decarboxylation of amino acid precursors.

Accordingly, Aerny (1990) found that high pH values favored the proliferation of bacteria strains, which can be responsible for the formation of biogenic amines in wines. In such study, it was also argued that the addition of SO_2 after alcoholic fermentation, even in small quantities, slowed down the malolactic fermentation and favored the development of Pediococcus, which are bacteria with a high aminogenic capacity (Ancín-Azpilicueta *et al*., 2008).

Vidal-Carou *et al* (1990) studied the relationship between (i) the concentration of histamine and tyramine in wine and (ii) the level of SO_2 and volatile acidity. These authors found that there existed a correlation (99.9%) between total sulfur dioxide level and biogenic amines in red wines. The highest amine contents were found in wines with low total sulfur dioxide level.

The influence of other wine compounds such as malic acid, citric acid, ethanol and sugar has also been studied. Rollan *et al*. (1995) found that high ethanol (12%v/v), l-lactic acid,

and citric concentrations reduced the histidine decarboxylase activity of cell suspensions of a strain of *Oenococcus oeni (Leuconostos oenos* 9204) (Ancín-Azpilicueta, *et al*., 2008).

In conclusion, the factors which favor the formation of biogenic amines during red wine vinification are high fermentation temperatures, maceration of the solid parts of the grape, excessive non-acid pHs, high yeasts biomass, development malolactic fermentation, and low levels of free SO_2 (Ancín-Azpilicueta, *et al*., 2008).

2.4.6. Influence of malolactic fermentation and storage on biogenic amines concentration.

According to figure 5, it is possible to observe the increase of histamine after malolactic fermentation; the others amines also increased, but to a lesser extent. During the first six months of storage in bottles, the histamine showed another increase, although smaller than that observed after malolactic fermentation.

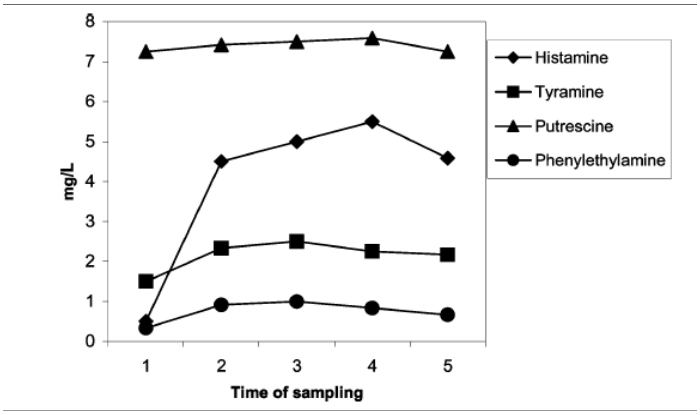

Figure 5. Evolution of histamine, tyramine, phenylethylamine, and putrescine concentration of wines from Utiel-Requena, before (1) and after (2) malolactic and 3 (3), 6 (4), and 12 (5) months of storage. Source: Landete *et al*, 2005

2.4.7. Formation of amines during alcoholic and malolactic fermentation

Among other possibilities, BA are formed during alcoholic fermentation, even from the very beginning, since it has been observed that the apiculate yeasts posses the capacity for the formation of these substances. During alcoholic fermentation, the formation of amines will depend, among others factors which have been previously outlined, on the strain of

Saccharomyces cerevisiae that predominates during fermentation (Ancín-Azpilicueta *et al*., 2008).

The control of malolactic fermentation is one of the most important measures to take into account in order to avoid accumulation of biogenic amines in wine.

2.5. Immunochemical methods

2.5.1. Definition of immunochemical methods

All immunochemical methods are based on a biospecific linkage between an antigen (hapten) and an antibody, or occasionally, on the non-specific interactions of free or bound antibodies with certain compounds (complement, protein A) or cell receptors (Fc-receptors). Immunochemical methods are used to demonstrate the presence of an antigen or to determine its quantity (when specific antibody is available) or the quantity of an antibody (when a specific antigen or hapten is available). The course of the antigen-antibody reaction is measured by a decrease of one of the pair of reacting components or, more frequently, by the formation of a reaction product, the antigen-antibody complex.

In the formation of the antigen- antibody complex, four basic factors should be considered – type of antigen, type of antibody, environment in which the reaction takes place and method of identifying complexes.

The immune complex produced can be detected by the naked eye (precipitate, agglutinate), after staining with common histochemical stains (usually reacting with proteins), by different optical methods (turbidimetry, conventional and laser nephelometry, spectrofluorimetry) or after labeling one of reacting components (antigen, hapten or antibody) with a radioactive isotope, or, occasionally, with another compound functioning as a label. The antigen or antibody must be labeled before their mutual interaction. Radioactive isotopes such as ^{14}C, ^{3}H, ^{131}I and ^{75}Se (radioimmunoassay – RIA), enzymes (enzyme-immunoassay – EIA), *etc*. may be used as labels.

Some authors distinguish several generations of immunochemical methods. In this work we will concentrate in the third generation that includes highly sensitive methods to

quantify antigens, antibodies and haptens. This group of methods includes EIA, method used in this work.

2.5.2. Immunoenzyme methods

Immunoenzyme methods (EIA) can be classified according to the type of compound to be determined (antigen, antibody), according to (i) which component is labeled; (ii) reaction type (competitive or non-competitive); and (iii) whether the free labeled component must be separated from the bound component. For any type of EIA, the most advantageous enzyme and most suitable procedure for conjugating it to the antigen or antibody must be chosen. All immunospecifically reacting sites, i.e. antigenic determinants or binding sites on the antibody, in the resultant conjugate must be preserved. Damage to immunospecifically reacting sites would reduce reaction specificity. Depending on the catalytic activity of the enzyme, the enzyme activity of the conjugate may change or remain unchanged. According to these criteria, EIA methods can be divided into two main groups: (i) Homogeneous EIA methods – the catalytic activity of the enzyme in the conjugate decreases or increases; (ii) heterogeneous EIA methods – the catalytic activity of the enzyme in the conjugate does not change.

In this work we will concentrate in heterogeneous EIA methods.

2.5.2.1. Heterogeneous EIA – ELISA techniques

Compared to homogeneous EIA, heterogeneous EIA methods require that the free labeled reacting component is separated from the labeled reacting component bound in complex with the antibody. The aim of this method is the immobilization of one of the pair of reactants by binding to a solid carrier (usually the wall of the test tube or well of the microplate). Separation of the free and bound components is readily achieved by washing. As the procedure is based on immunoadsorption the technique is known as the enzyme-linked immonosorbent assay (ELISA).

Enzymes used in these techniques should have a low relative molecular weight, high stability and enzyme activity; also, they should bind covalently to antibodies and to various functional groups of antigens; finally, the product of the enzyme reaction should be colored

or otherwise readily detectable and the enzymes must be readily available and inexpensive. Among the enzymes fulfilling these conditions, horseradish peroxidase (EC 1.11.1.7) is one of the most widely used, although alkaline phosphatase (EC 3.1.3.1) is also used.

There are several types of ELISA methods which differ in the analytical procedure (competitive or non-competitive) and the type of labeled reactant (antigen or antibody).

i. Competitive ELISA for antigens and haptens

The antibody specific for the test antigen is bound (immobilized) to the solid phase (Fig.6). A known amount of the enzyme-labeled antigen and either a known (standard) or unknown (test) amount of the unlabeled antigen are then added. During the incubation, the labeled antigen competes with the standard or test unlabeled antigen. After incubation, the reaction vessel is washed with an appropriate buffer to remove free reactants. A substrate solution is then added, which, following degradation by the enzyme present in the immune complexes, yields a colored product. The color intensity (absorbance) of the reaction mixture is assayed spectrophotometrically.

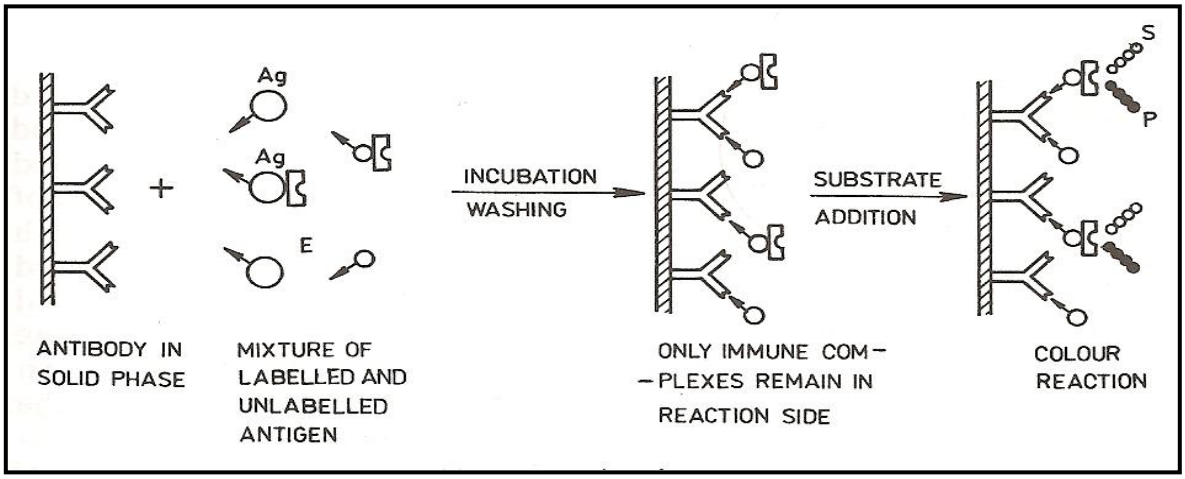

Figure 6. Competitive ELISA of haptens and antigens (source: Frenencík Miroslav (1993). *Handbook of Immunochemistry*)

ii. Sandwich ELISA for antigens

In this type of assay, the antibody is also first bound to the solid phase (fig.7). Known (standard) or unknown (test) amounts of the antigen are the bound to it. After washing, a second, enzyme-labeled antibody, which reacts with the remaining free determinants of the antigen bound to the immobilized antibody, is then added. The reaction vessel is washed again and the substrate to detect the enzyme activity is added.

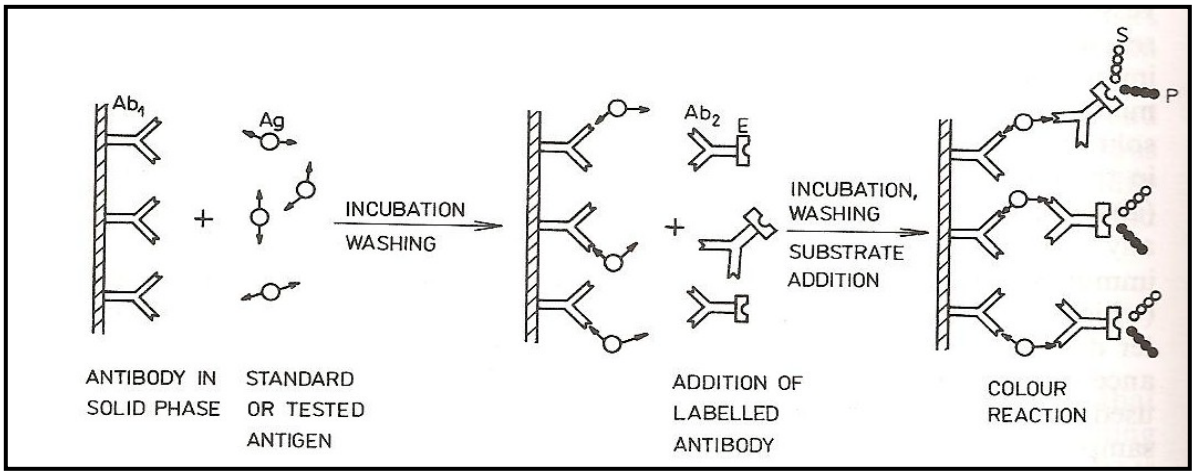

Figure 7. Sandwich ELISA for antigens (source: Frenencík Miroslav (1993). *Handbook of Immunochemistry*)

iii. Indirect ELISA for antigens

The antigen is bound in the solid phase (after its saturation) and the reaction vessel is washed (Fig.8) When a small antigen is used, it must first be bound to a larger carrier, e.g. bovine serum albumin. The test sample containing the antigen to be measured is then mixed with a specific labeled antibody and the mixture incubated with the immobilized antigen. After incubation, the reaction wells are washed, the substrate is added and the color reaction develops. Indirect ELISA for antigens can also be modified so that an unlabeled antibody (e.g. rabbit IgG) is used instead of the labeled antibody. The immunocomplexes are detected by a labeled anti-immunoglobulin as the second antibody (e.g. pig anti-rabbit IgG).

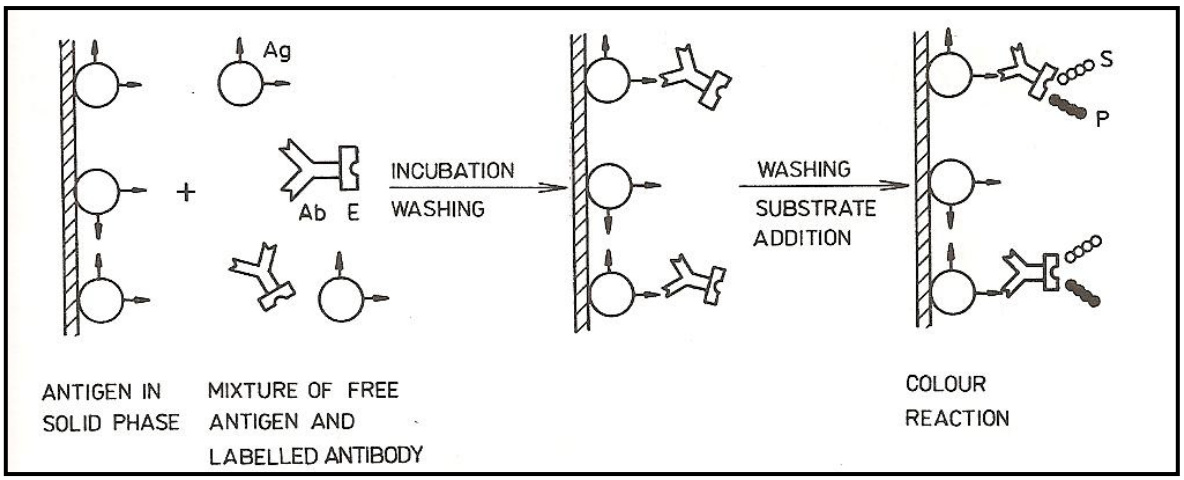

Figure 8. Indirect ELISA for antigens (source: Frenencík Miroslav (1993). *Handbook of Immunochemistry*)

iv. Sandwich ELISA for antibodies

This modification is also known as indirect ELISA for antibody detection and may be used to demonstrate or titrate antibodies specific for a certain antigen. Its principle is shown in fig.9. An antigen is bound to the solid phase and washed; the diluted antiserum, in which the presence of antibodies is to be determined, is then added. The mixture is incubated to allow the antibodies present to react with the immobilized antigen. The wells are washed and the enzyme-labeled second antibody is added. After another washing, the enzyme substrate is added and the intensity of the color reaction is measured.

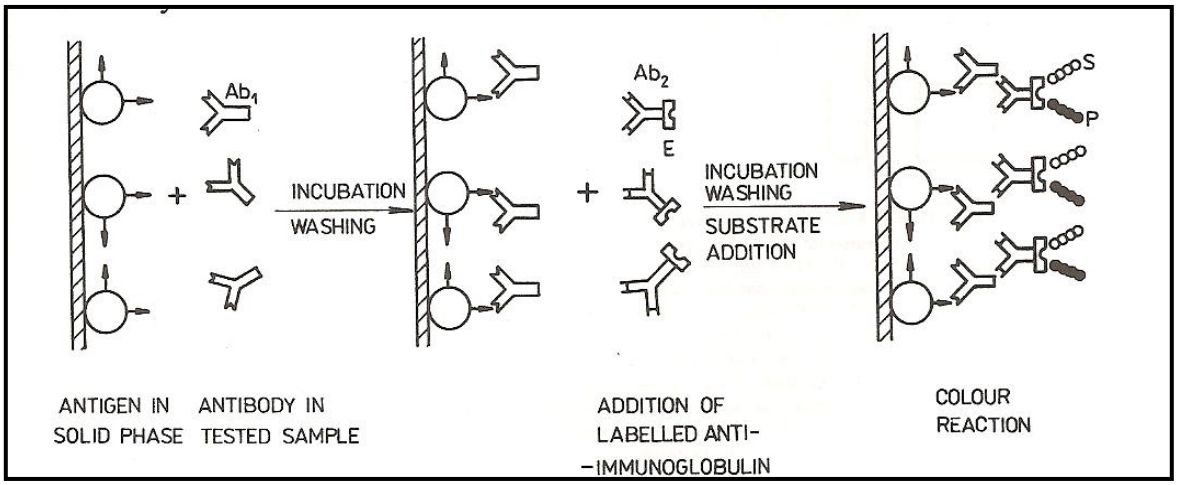

Figure 9. Sandwich ELISA for antibodies (source: Frenencík Miroslav (1993). *Handbook of Immunochemistry*)

2.5.3. ELISA (Enzyme Linked Immunosorbent Assay)

ELISAs by definition exploit the use of an enzyme attached to one of the reagents utilized in the test. Subsequent addition of relevant enzyme substrates/chromogens causes a color change; the results can be read both by eye and quantified using specially designed spectrophotometers. The fact that proteins (including antibodies) can be passively attached to plastics has been exploited in most applications of ELISAs. Since one of the components is attached to a solid phase by passive adsorption, subsequent reagents can be added, and after a period of incubation, unreacted material can be simply washed way.

ELISAs provide highly sensitive and precise methods for the estimation of biological parameters, with the added advantage that they can handle large numbers of samples that may then be analyzed rapidly.

These assays are versatile, having been applied to a wide range of biological fields, e.g., viruses, bacteria, fungi, and protozoan and metazoan parasites.

2.5.3.1. Principles of ELISA

The performance of ELISA and related solid phase assays depend on four major principles:

i. A variety of enzymes, including horseradish peroxidase (HRP) and alkaline phosphatase (AP) – the most popular – can be chemically coupled to either antibody or antigen under conditions which retain the biological properties (i.e. substrate interaction, antigen binding, antigenicity) of both components of the conjugate;
ii. Most antigens, for example proteins, peptides, polissaccharides and bacterial lipopolysaccharides, bind spontaneously to plastic surfaces such as the wells of polystyrene microtitre plates. Antibodies, as proteins, also attach whilst retaining their antigen-binding activity. Thus antigen or antibody-coated plates can be prepared as the initial step. Once antigens or antibodies applied to coat or “sensitize” the solid phase are bound, they become resistant to vigorous washing in detergent buffer whilst excess unbound reagent is simply removed by this process;

iii. In subsequent steps one or more layers of a solid phase captured immune complex are formed, with unbound entities again efficiently washed away. This affordsthe basis for high specific to non-specific signal ratio when captured enzyme reacts with the substrate; and,
iv. An enzyme conjugated antibody or antigen when bound in the immune complex leaves the enzyme component available for substrate interaction. Addition of substrate, in the usual form of the assay, results in a progressive substrate solution color change. The reaction can be stopped at an appropriate stage and the color signal determined by visual comparison with standards or by optical density measurement.

Examples of specific areas to which ELISAs have been applied in research and diagnosis are:

i. Detection and identification of whole or parts of disease agents, e.g., typing;
ii. Discrimination of disease agents, e.g., subtyping;
iii. Quantification of whole or part of the agent, e.g., estimation of parasite load;
iv. Identification of specific antibodies, e.g., serodiagnostics for epidemiological studies; and,
v. Quantification of specific antibody isotypes, e.g., diagnostic usefulness of IgM/IgG ratio, usefulness of IgE in parasitic disease diagnostics.

2.5.3.2. Steps in ELISA

There are some important steps to be considered in an ELISA assay:

i. Coating step: ELISA plates are prepared by coating either with antigen or antibody and are then exposed to a potentially complementary antibody or antigen;
ii. Blocking step: blocking non-specific sites of the solid phase;
iii. Washing steps: remove excess unbound antigens/antibodies, but antigens/antibodies coated to the solid phase become “resistant” to vigorous washing;

iv. Detection step: consists of the addition of a substrate resulting in a progressive substrate solution color exchange which absorbance values can be measured, for instance, through an ELISA reader; and,
v. Controls: in an ELISA assay the positive and negative controls, as well as, the controls of the blocking steps and cross-reactions must always be present.

2.5.3.2.1. Solid-Phase

The most widely used solid-phase process, by far, is the 96-well microtiter plate manufactured from polyvinyl chloride (PVC, flexible plates) or polystyrene (inflexible "rigid" plates).

i. Immobilization of antigen/antibody on solid-phase-coating.

A major feature of the solid-phase ELISA is that antigens or antibodies can be attached to surfaces easily by passive adsorption. This process is commonly called coating. Most proteins adsorb to plastic surfaces, probably as a result of hydrophobic interactions between non polar protein substructures and the plastic matrix. The interactions are independent of the net charge of the protein, and thus, each protein has a different binding constant. The rate and extent of the coating depend on:

- The diffusion coefficient of the attaching molecule;
- The ratio of the surface area being coated to the volume of the coating solution;
- The concentration of the substance being adsorbed;
- The temperature; and,
- The time of adsorption.

These factors are linked and it is very important to determine the optimal antigen/antibody concentration for coating in each system by suitable titrations.

ii. Coating time and temperature

The rate of the hydrophobic interactions depends on the temperature. The higher the temperature, the greater the rate. There are many variations on incubation conditions. The most usual regimens involve incubation at 37°C for 1-3h, overnight at 4^{o}C, or a combination of the two.

iii. Covalent Antigen Attachment

Precoating of plates with polymers, such as polyglutaraldehyde and polylysine, has been used to prevent desorption, the antigen being covalently bound. These polymers bind to plates with a high efficiency and act as nonspecific adhesive molecules. This method is particularly useful for antigens with high carbohydrate content, since these normally bind poorly to plastic.

2.5.3.2.2. Blocking conditions and nonspecific reactions

In general, measures have to be taken to prevent nonspecific adsorption of proteins to wells from samples. Nonspecific adsorption of protein can take place with any available plastic sites not occupied by the solid-phase reagent.

There are two methods used to eliminate such binding. One is the addition of high concentrations of immunologically inert substances to the dilution buffer of the added reagent. Commonly used blocking agents are: Human serum albumin (HAS); Bovine serum albumin (BSA); Casein; Gelatin, etc, and they act by competing with nonspecific factors in the test sample for available plastic sites. Such blocking agents can also be added as a separate step before the addition of the sample. This increases the competing ability of the blocker. Nonionic detergents (i.e., Tween 20, Tween 80, Triton X-100, SDS) have also been used to prevent nonspecific adsorption. These are used at low concentration, so as to allow interaction of antigen and antibody. Occasionally, both detergents and blocking substances are added together.

2.5.3.2.3. Washing

The purpose of washing is to separate bound and unbound (free) reagents. The liquid used to wash wells is usually buffered, typically PBS (0,1M, pH 7.4), in order to maintain isotonicity, since most antigen-antibody reactions are optimal under such conditions. Sometimes detergents, notably Tween 20 (0,05%), are added to washing buffers. This can cause problems where excessive frothing takes place producing poor washing condition, since air is trapped and prevents the washing solution from contacting the well surface.

When using detergents, it is necessary to carefully avoid adversely affecting the reagents (denature antigen), and greater care is needed to prevent frothing in the wells.

2.5.3.2.4. Addition of sample

Immunoassays involve the accurate dispensing of samples in relatively small volumes. The sample, before addition would be diluted in appropriated buffer generally PBST, TBS and TBS with 0,1,0% (w/v) BSA.

2.5.3.2.5. Incubation

The reaction between antigens and antibodies depends on their distribution, time, temperature, and pH (buffering conditions) at which the incubation step takes place. Two types of incubation conditions are common: (i) incubation of stationary plates and (ii) incubation of rotating plates. These conditions affect the times and temperatures required for successful ELISAs.

2.5.3.2.6. Enzyme conjugates

Intrinsic to the ELISA is the addition of reagents conjugated to enzymes. Enzyme is the substance that can react at low concentration as a catalyst to promote a specific reaction. Assays are then quantified by the build-up of colored product after the addition of substrate. In this study, the antigen (histamine) was conjugated with HRP and the substrate used was TMB.

2.5.3.2.7. Development of color

The substrate is a chemical compound with which an enzyme reacts specifically, usually chosen to yield a colored product. The rate of color development will be proportional, over a certain range, to the amount of enzyme conjugate present. On a kinetic level, reactions are distinguished by their kinetic order, which specifies the dependence of reaction rate on the concentrations of reactants. Under the conditions generally employed in ELISA, the reaction exhibits zero order with respect to the substrate. It can be seen that too little substrate will limit the rate of product production. Thus, sufficient substrate must be

present to prevent the substrate and/or cofactors from being rate-limiting. There are some physicochemical parameters that affect the development of color including (i) buffer composition and pH, (ii) reaction temperature, (iii) substrate and/or cofactor concentration and stability, (iv) product stability, (v) enzyme stability, and (vi) substrate and product stability. For the present work, the chosen enzyme was horseradish peroxidase (HRP).

2.5.3.2.8. Stopping reactions

Reagents are added to prevent further enzymatic reaction in ELISA. This is performed at a time as determined in the specific assay. This process is usually called “stopping”, and the reagent used, the “stopping reagent”. The stopping is usually made at a time when the relationship among the enzyme-substrate-product is in the linear phase. Molar concentrations of strong acids or strong bases stop enzymatic activity by quickly denaturing enzymes. For this work, the chosen stopping reagent was H_2SO_4 1M.

2.5.3.2.9. Spectrophotometric reading

The product of the substrate catalysis by enzyme is measured by transmitting light of a specific wavelength through the product and measuring the amount of adsorption of that light, if any, by an equipment (spectrophotometer, e.g. SPECTRAmax ^{tm} 340 Microplate Reader, Sunnyvale, California).

2.5.3.3. Advantages in ELISA

There are some advantages to use ELISA as compared to the use of others analytical methods such as HPLC. The advantages are listed below:

i. Simplicity, because (a) reagents are added in small volumes; (b) separation of bound and free reactants is made by simple washing procedures; (c) passive adsorption of proteins to plastic is easy; (d) specialized equipment is usually readily available;
ii. Facilitated reading, because (a) colored end-product can be read by eye to assess whether tests have worked (avoiding waiting for results with machine reading as in

RIA); (b) multichannel spectrophotometers quantify results that can be examined statistically;

iii. Rapidity, because (a) tests can be performed in a few hours (depending on incubation time); (b) Spectrophotometric reading of results is fast (96 wells read in 5 s);

iv. Sensitivity detection levels of 0.01 to 1 µg/mL are easily and consistently achievable. These levels are ideal for most diagnostic purposes;

v. Commercially available reagents offer great flexibility in ELISA design and achievement of specific assays;

vi. Adaptability, as different configurations allow different methods to be examined to solve problems. This is useful in developing tests and research science;

vii. Cost as startup costs are low and reagent costs are low;

viii. Acceptability, as fully standardized ELISAs in many fields are now accepted as "gold-standard" assays;

ix. Safety, because safe non-mutagenic reagents are available. Disposal of waste poses no problem (unlike radioactivity);

x. Availability, because ELISAs can be performed anywhere, even in laboratories where facilities are less than state of the art;

xi. ELISA kits are widespread and often successful; and,

xii. Standardization, as quantification of data allows easier standardization.

3. Materials and Methods

3.1. Chemicals

For this work, histamine (2-[4-Imidazolyl]ethylamine), Sodium periodate, Sodium borohydrate, 3,3', 5'5 Tetramethylbenzidine (TMB), Peroxidase type VI-A from Horseradish, Alkaline phosphatase Yellow (pNPP) liquid substrate system for ELISA and Anti-Rabbit IgG (whole molecule)–Alkaline Phosphatase antibody produced in goat were purchased from Sigma-Aldrich, Sintra, Portugal. Rabbit Anti - histamine unconjugated was purchased from AbD serotec, Oxford, UK. Histamine ELISA Kit was purchased from Immuno Biological Laboratories,Inc. Minneapolis, USA.

3.2. Preparation of a histamine-HRP conjugate I via periodate method

With regard to the enzyme-labeled antigen in direct competitive EIA, histamine was coupled to horseradish peroxidase (HRP) via the periodate method (Schneider *et al*, 1996). HRP (27 mg), dissolved in 2 mL distilled water, was stirred with 0,4 mL of an aqueous sodium periodate solution (21,4 mg/mL) for 20 min. The active HRP was dialyzed (dialysis cassettes - Slide-A-lyser 3,5K - 3,500MWCO 0,5-3,0 mL sample volume from Pierce Protein Research Products, Rockford, USA) against 1 mM acetate buffer (pH 4,4); then, 2mg histamine (dissolved in 1 mL distilled water) was added. The pH of this mixture was adjusted to 9,5 with 0,1M NaOH and incubated for 2h at room temperature. Then 0,1 mL sodium borohydride solution (4mg/mL distilled water) was added and incubated at 4°C for one hour. The conjugate was dialyzed against PBS, and stored lyophilized at -18°C.

3.3. Determination of the percentage of peroxidase in the conjugate I

For the determination of the percentage of peroxidase in the conjugate, Laurenti protocol (Laurenti *et al*., 2000) was followed. Spectrophometric measurements were performed by using a SPECTRAmax ^{tm} 340 Microplate Reader, Sunnyvale, California. Also, HRP (6mg/mL) was dissolved in bidestilled water at pH 6,3. The optical density of HRP was characterized at 403 nm.

3.4. Determination of the effect of HRP enzyme - TMB substrate reaction time on absorbance

The HRP enzyme induced TMB substrate oxidation reaction is limited by either unreacted substrate or hydrogen peroxidase. For this study, the Theegala and Suleiman protocol (2000) was adapted.

For the calibration curves, 3µL of different dilutions of HRP and labeled antigen were added to 100µL TMB. After specific reaction time, the reaction was stopped with 100µL H_2SO_4 1M and the absorbance was measured at 450nm.

3.5. Preparation of a histamine-HRP conjugate II

A solution of 5mg histamine and 12mg horseradish peroxidase in 1mL of 0,1M aqueous phosphate buffer (pH 6,8) was prepared. A dropwise 0,05mL of 1,0% (v/v) aqueous glutaraldehyde solution was added to this stirred solution, and then stirred for two hours. The resulting solution was dialyzed (dialysis cassettes - Slide-A-Lyser 3,5K - 3,500MWCO 0,5-3,0 mL sample volume from Pierce Protein Research Products, Rockford, USA) for 16 hours at 4°C with 2L of phosphate-buffered saline solution. The dialysate was centrifuged for 30 min at 4°C at 20000 rpm to remove particulate matter and to produce the horseradish peroxidase-histamine conjugate.

3.6. Synthesis of histamine-enzyme conjugate III

Histamine (12mg) in 1 mL of a solution containing 1 M sodium acetate was incubated with 1 mL of 30mM glutaraldehyde for 30 s with stirring. After that, 10 mg of a carrier protein (BSA) in 1 mL of sodium acetate was added to this mixture, and incubated for 10 min with slow stirring at room temperature. Then, 5mg of $NaBH_4$ was added so that the double bonds were saturated. The coupling mixture was dialyzed (dialysis cassettes - Slide-A-Lyser 3,5K - 3,500MWCO 0,5-3,0 mL sample volume from Pierce Protein Research Products, Rockford, USA) against several changes of 10 mM sodium acetate overnight at 4°C.

3.7. Chessboard titration (titration of antigen and antibody)

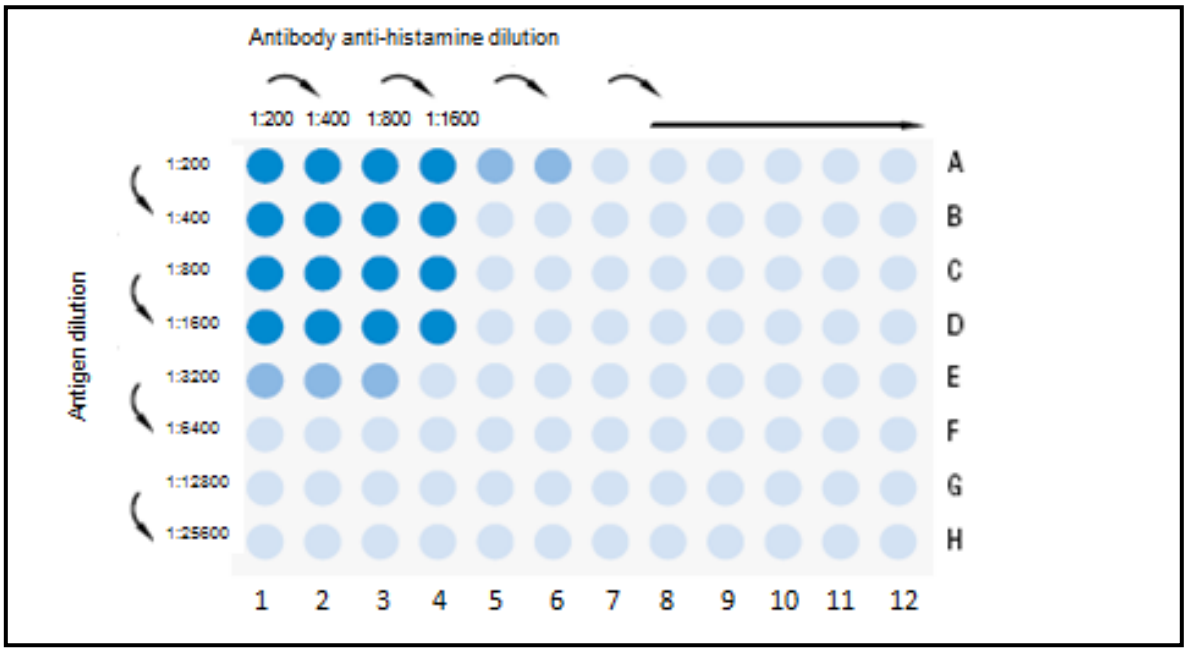

Figure 10. Chessboard titration of antigen and antibody. Source: adapted from www.piercenet.com

The strategy of this assay is to dilute the Ab across the plate one way (in a buffer that allows passive adsorption), incubate the plate at 37°C for 1 hour or 4°C overnight, wash the plate, and then dilute the conjugate (HA-GA-HRP) across the plate, the opposite way to the Ab, obtaining a chessboard titration of Ab against conjugate. The conjugate is diluted in a buffer to prevent nonspecific adsorption of the conjugate to any free protein binding sites on the wells. After washing, all the wells receive a solution containing the substrate. Thus, the color developing in each well depends on: (i) the amount of antibody and (ii) the amount of conjugate that has remained bound to that antibody.

3.7.1. Detailed procedures of the chessboard titration

The following steps were performed in this assay:

i. Add 50µL of carbonate buffer to each well of plate;
ii. Dilute of the antibody (5 mg/mL) in carbonate/bicarbonate buffer (1:100);
iii. Add 50µL of the diluted antibody to all the wells of column 1;
iv. Mix contents by pipeting up and down;

v. Transfer 50 µL to column 2, and so on, until column 11;
vi. Incubate at 4°C overnight;
vii. Wash the plate four times with PBS;
viii. Addition of dilutions of conjugate: make up 1 mL of a 1/200 dilution of the conjugate in a 5-mL bottle; add 5 µL of conjugate to 1 mL of blocking buffer;
ix. Add 50µL of blocking buffer to every well of the microplate;
x. Add 50µL of the conjugation dilution into the first row (A 1-12) of the plate. Thus, we have 100 µL of a 1/400 dilution of conjugate in this row.
xi. Mix and transfer 50µL of the conjugate from row A to row B. Repeat the transfer of dilutions to the end of the plate. There should now be 50µL of conjugate dilutions in each wells, in a dilution range from 1/400 in row A to 1/51200 in row H;
xii. Leave at room temperature for 1 h;
xiii. Wash the plate;
xiv. Add 50µL/well of TMB;
xv. Examine the color changes at 1, 3, 5, 8 e 10 min;
xvi. Add 50 µL of a 1M H_2SO_4 to each well after 10 min to stop color development;
xvii. Read the plate using a spectrophotometer at 450nm;

3.8. Indirect ELISA

Wells in a microtiter plate were coated with 100µL of a labeled-histamine diluted in 0,5M of bicarbonate buffer pH 9,5 and incubated overnight at 4°C. The wells were further blocked with the blocking buffer (1,0% (w/v) gelatin or 0,5% (w/v) sodium caseinate) specific for each of the assays. The wells were incubated with 100µL of a fixed concentration of anti-histamine antibody (dilution 1/1000 in 0,1% (w/v) specific blocking buffer) and incubated at 37°C. The wells were then incubated with 100 µL goat anti-rabbit IgG labeled with alkaline phosphatase (dilution 1/10000 in TBS/T plus 1% (w/v) BSA) for 1 h at room temperature. The bound enzyme activity was measured using 100 µL of alkaline phosphatase Yellow (pNPP) liquid substrate system for ELISA, incubated for 30 minutes at room temperature and the absorbance was measured at 405 nm.

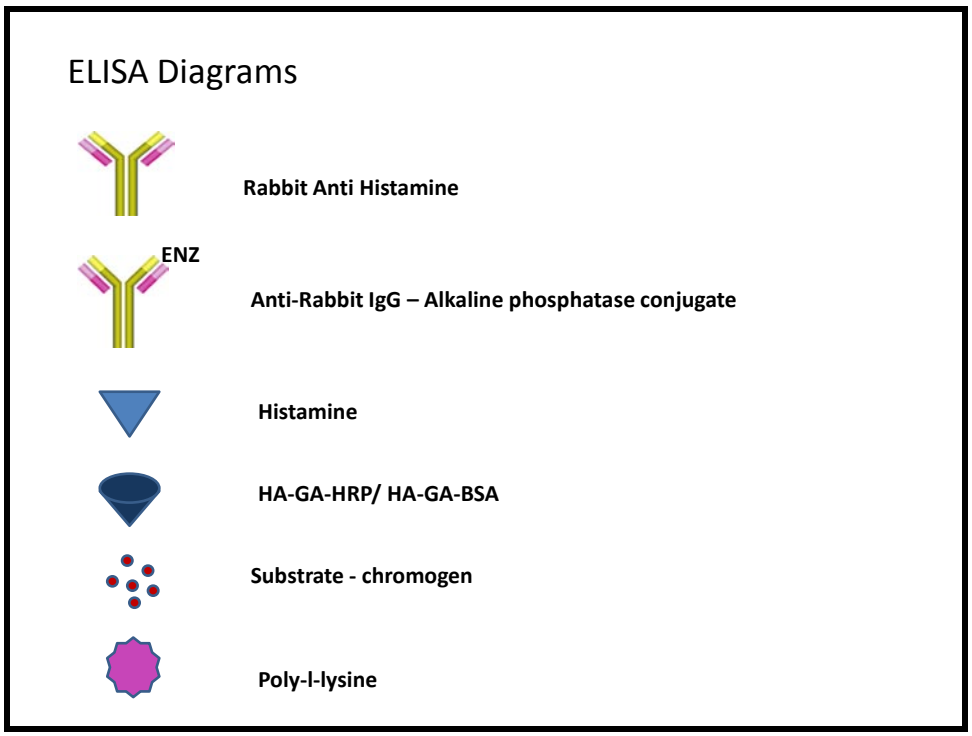

Figure 11. Diagrams of ELISA

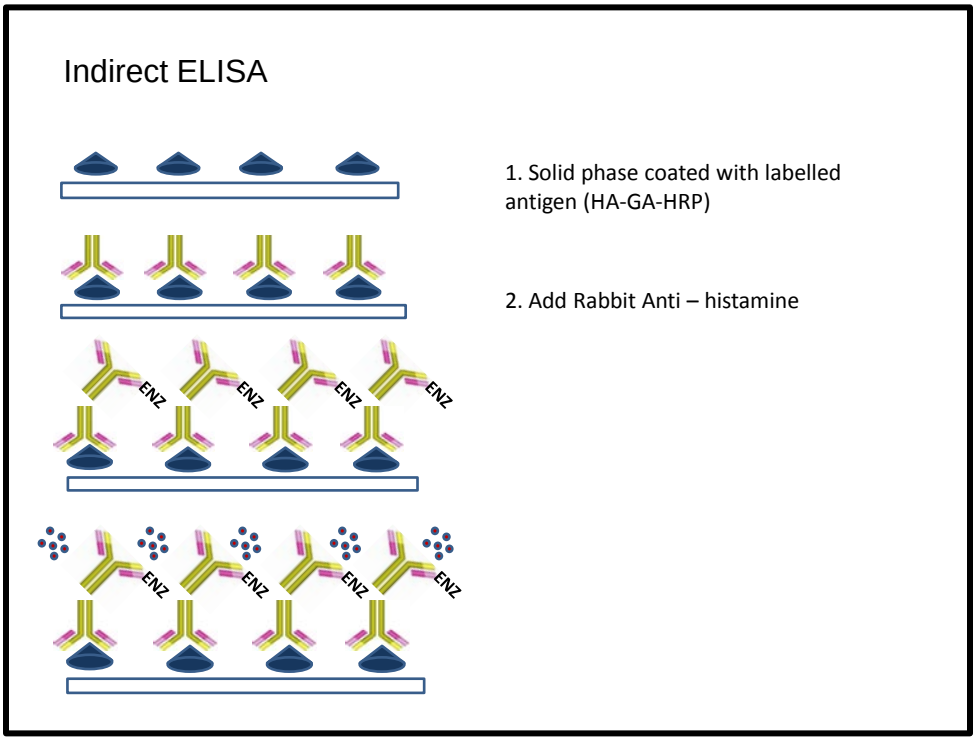

Figure 12. Indirect ELISA

3.9. ELISA – Inhibition test

Wells in a microtiter plate were coated with 100µL of a fixed concentration of conjugate (20 µg/mL) and incubated overnight at 4°C. The wells were further blocked with specific blocking buffer (1% (w/v) gelatin). The wells were then incubated with 50µL of a fixed concentration of anti-histamine antibody (dilution 1/1000 in 0,1% (w/v) gelatin) and 50µL of free histamine at various concentrations overnight at 4°C, followed by incubation with goat anti-rabbit IgG labeled with alkaline phosphatase (dilution 1/10000 in TBS/T 0,05%(v/v)) for 1 h at room temperature. The bound enzyme activity was then measured as described in item 3.8 above.

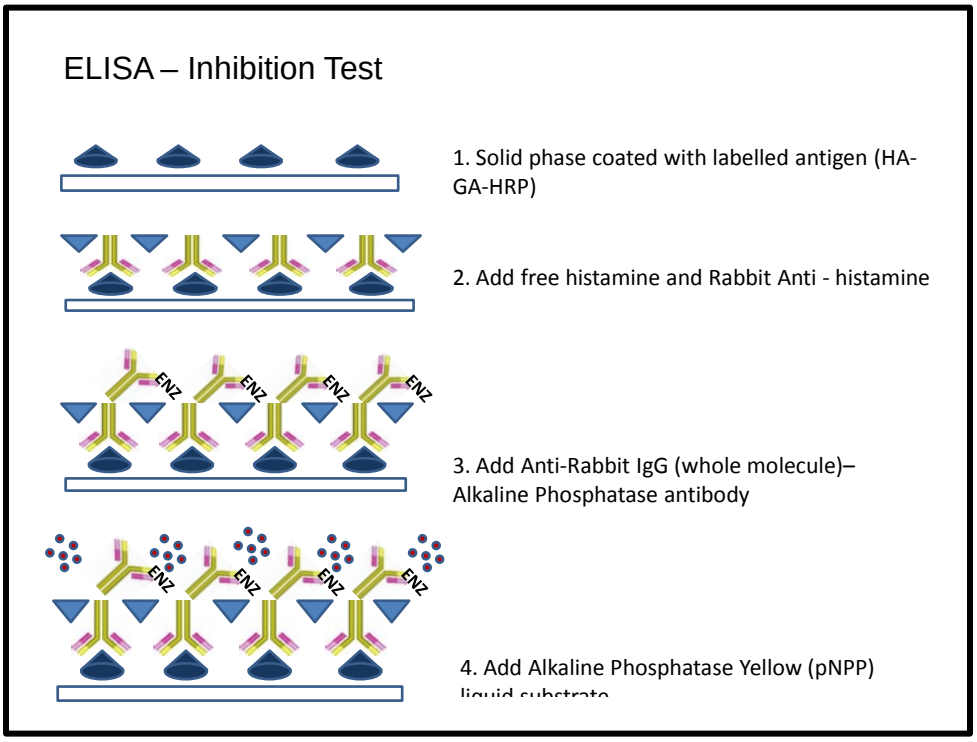

Figure 13. ELISA Inhibition test

3.10. ELISA binding test (poly-l-lysine)

Wells in a microtiter plate, coated with poly-l-lysine (30µg/µL), were activated with 2,5% (v/v) de glutaraldehyde (GA) for 1 h. Histamine at various concentrations were added to the wells and incubated for 1 h at room temperature. Excess aldehyde groups were blocked with 0,5% (w/v) NaHB4 for 10 min. The wells were further blocked with specific

blocking buffer (1,0% (w/v) gelatin) for 1 hour for protein binding sites, and then incubated overnight at 4°C with rabbit anti-histamine (dilution 1/1000 in 0,1% (w/v) gelatin). The wells were then incubated with goat anti-rabbit IgG labeled with alkaline phosphatase (dilution 1/10000 in TBS plus 1% (w/v) of BSA) for 1 h. The bound enzyme activity was then measured as described in item 3.8 above.

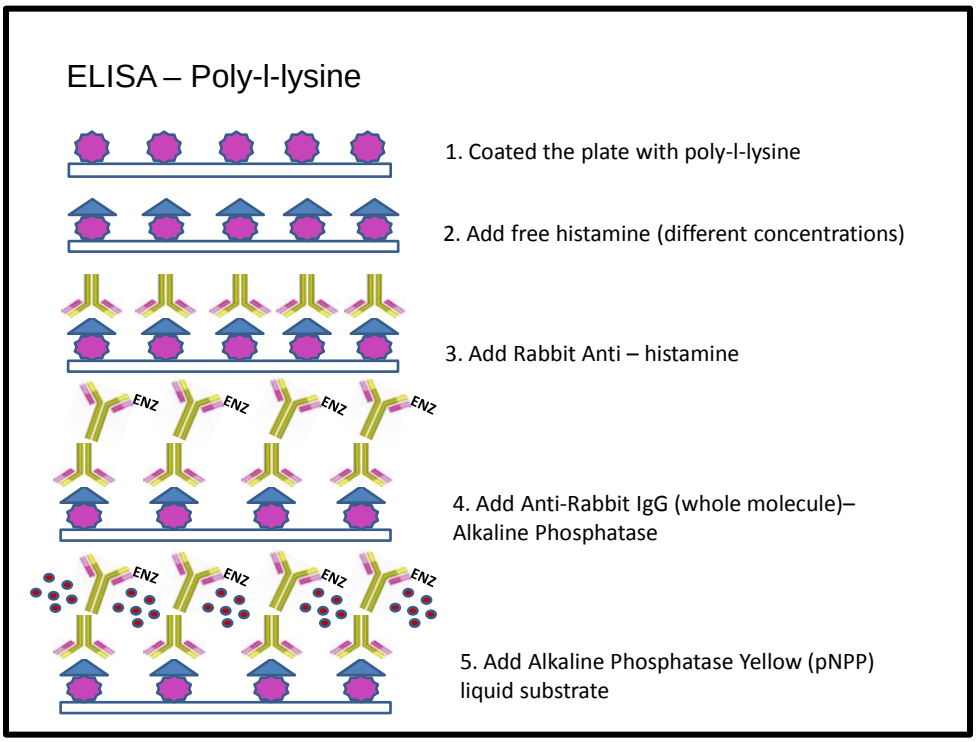

Figure 14. ELISA binding test

3.11. Direct competitive enzyme immunoassay

The wells of a microtiter plate were coated with 100 µL/well rabbit anti-histamine (diluted 1:1000 with bicarbonate buffer) overnight. Free protein binding sites were blocked with a specific blocking buffer (1% (w/v) of gelatin) for 1 h. The plate was washed three times with PBS and drained. Then 50 µL of histamine standard at different concentrations was added, followed by 50 µL of histamine-HRP (in 0,1% (w/v) gelatin/PBS) solution in a dilution 1/400. After incubation for 2 h at room temperature, the plate was washed again and further treated with 100 µL of enzyme-substrate solution (3,3', 5'5 Tetramethylbenzidine –TMB). After 15 min, the enzyme reaction was stopped with 1M H_2SO_4 (100µL per well) and the absorbance was measured at 450 nm.

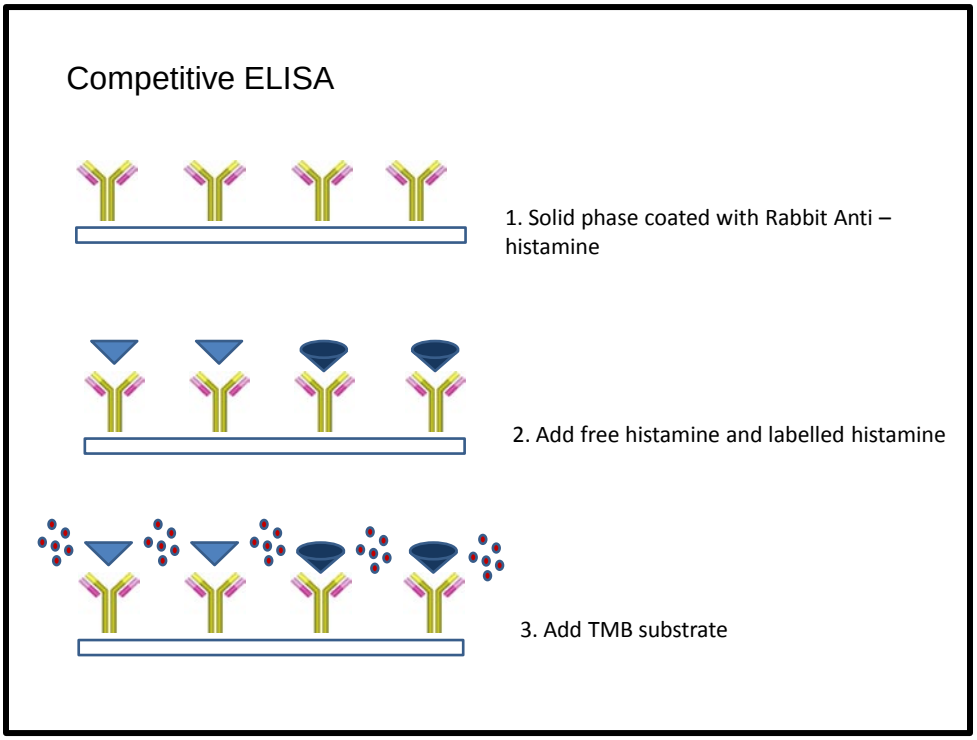

Figure 15. Competitive ELISA

3.12. Histamine ELISA Kit (Immuno Biological Laboratories,Inc. Minneapolis, USA)

The test is supposed to be conducted according to a pre-defined set of parameters.

First, histamine is quantitatively acylated. The subsequent competitive ELISA kit uses the microtiter plate format. The antigen is bound to the solid phase of the microtiter plate. The acylated standards, controls and samples and the solid phase bound analyte compete for a fixed number of antiserum binding sites. After the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgGperoxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm.

The determination of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standard concentrations.

4. Results and Discussion

For the performance of this work, it was suggested the use of an enzyme immunoassay, since this is the most suitable method for the purposes of achieving a fast, efficient and low cost determination and quantification of histamine in wine.

Notwithstanding the fact that there exist ELISA commercial kits for the same purposes available in the market, such kits require the previous acylation of the histamine before the performance of the tests. Accordingly, for this work, the purpose was the evaluation of the behavior of the histamine without the acylation step.

The chosen method was the direct competitive ELISA, as described by the Ayun protocol (Ayun *et al*, 1999) and the Schneider protocol (Schneider *et al*, 1996). Both authors worked with intact histamine and both obtained successful results.

The first step of the work was the acquisition of the antibody for histamine. Considering that the histamine is a hapten (i.e. one sole molecule is not immunogenic), it was necessary to conjugate it with a bigger molecule (e.g. a protein like BSA). As a result, there were three hypotheses: (i) to derivatize the histamine (according to Clayes-Bruno *et al*., 2006, the derivatizing reagents increase the hapten size and exclude the hapten-carrier linker from the immunodominant site), and the antibodies would be produced for derivatized histamine; (ii) to conjugate the free histamine with BSA, for example, and the antibodies would be produced for free histamine; (iii) to purchase commercial antibody anti-histamine. Due to the limited time available for the development of this project, it was decided to purchase commercial antibody anti-histamine.

The second step for the execution of this work was the conjugation of histamine with an enzyme peroxidase. The conjugation process is required in order to permit the quantification of the enzyme-labeled histamine and therefore differentiate it from the free histamine contained in the sample. In a competitive method as suggested for this work enzyme-labeled histamine and free histamine compete for specific antibody binding sites. Bound enzyme conjugate converts the colorless chromogen into a blue product. Because color development is directly related to the amount of captured conjugate while binding of

free and conjugated histamine directly compete for binding, color development is indirectly proportional to histamine concentration of the sample.

The first conjugation was made via periodate method (Schneider E., *et al*, 1996).

In order to quantify the amount of enzyme after the conjugation process, it was necessary to run a comparison between the best spectrum of absorbance of the enzyme (before conjugation) and the absorbance of the conjugate (HRP and histamine). Such comparison allowed for the determination of the efficiency of the conjugation process, which, in this case, is important since the higher the enzymatic activity in the conjugate, the lower the quantity of material would be required for the tests.

The result of the conjugation was not satisfactory and was decided to make another process of conjugation according Rice protocol (Rice *et al.*, 1982).

A study was performed to determine the ideal reaction time for the quantification of enzyme peroxidase and of the conjugates that react with the substrate (Theegala and Suleiman, 2000). Through the results obtained, it was possible to determine the efficiency of the conjugation processes.

The conjugate obtained through the Rice protocol (Rice *et al.*, 1982) presented the most satisfactory results with regard to the enzymatic activity and was then chosen to be used in the subsequent assays.

After the execution of the second conjugation process, and the determination of its efficiency, the next step was the execution of assays for the optimization of the concentration of the anti-histamine antibody and of the labeled histamine with the peroxidase, in order to find the optimal relation between these components.

The results obtained demonstrated that the enzymatic activity of the second conjugation process was indeed excellent, but the amount of histamine conjugated with the enzyme could be insufficient to react with the antibody.

Therefore, it was decided to perform a third conjugation method, this time according to Fujiwara (Fujiwara *et al.*, 1997).

Similarly to the previous conjugates, assays were also performed with the third conjugate, in order to determine the efficiency of the conjugation and of the optimization reaction between the antibody and the conjugate, as above described.

The results of the reaction efficiency for the third conjugate were not as good as the ones presented by the second conjugate but were nonetheless acceptable (as compared with the reference values for the reaction of the enzyme with the TMB substrate). However, the results of optimization reaction (chessboard titration) of the third conjugate were far better than the results of the second conjugate, even if not yet within the ideal range desired for the assays to be performed. In any case, considered all these aspects, the third conjugate was chosen to be used in the ELISA assays.

The ELISA assays were performed through indirect ELISA as this is the most simple and effective test, and because any possible flaws are easily detectable. However, as this method was significantly different from the initially proposed approach, it was necessary to obtain a second antibody (anti-rabbit IgG (whole molecule) – alkaline phosphatase antibody) that would react with the rabbit anti – histamine and with the substrate specific for the enzyme in question.

Due to the unsatisfactory results obtained, it was then decided to test yet another ELISA method, the ELISA inhibition test. This assay was initially performed with the conjugate histamine-BSA and later with the conjugate histamine-HRP. The results of ELISA inhibition test were again not satisfactory.

In order to rule out the possibility that the conjugate histamine + peroxidase could be interfering in the results, it was then performed an ELISA binding test. For that, the wells were coated with poly-l-lysine, a compound often used as an attachment factor which improves adherence. In this case, its purpose was to capture the free histamine. The results were not satisfactory.

Finally, it was decided to perform an assay with a Histamine ELISA Kit (Immuno Biological Laboratories, Inc. Minneapolis, USA), in order to compare the efficiency between the tests using the free histamine and the histamine that was subject to a derivatization process in an ELISA assay.

4.1. Determination of the percentage of peroxidase in the conjugate

For the determination of the HRP enzyme activity, after the first conjugation with the histamine via periodate method, or in other words, to test enzyme conjugation efficiency, in accordance with Laurenti protocol (Laurenti *et al*., 2000), the optical spectrum of HRP was determined through the spectrophotometer.

Adapting Laurenti protocol (Laurenti *et al*., 2000), HRP was dissolved in bidestilled water. The optical spectrum of HRP was measured at 403 nm.

4.1.1. HRP optical density

The determination of the optical density of HRP is shown in figure 16.

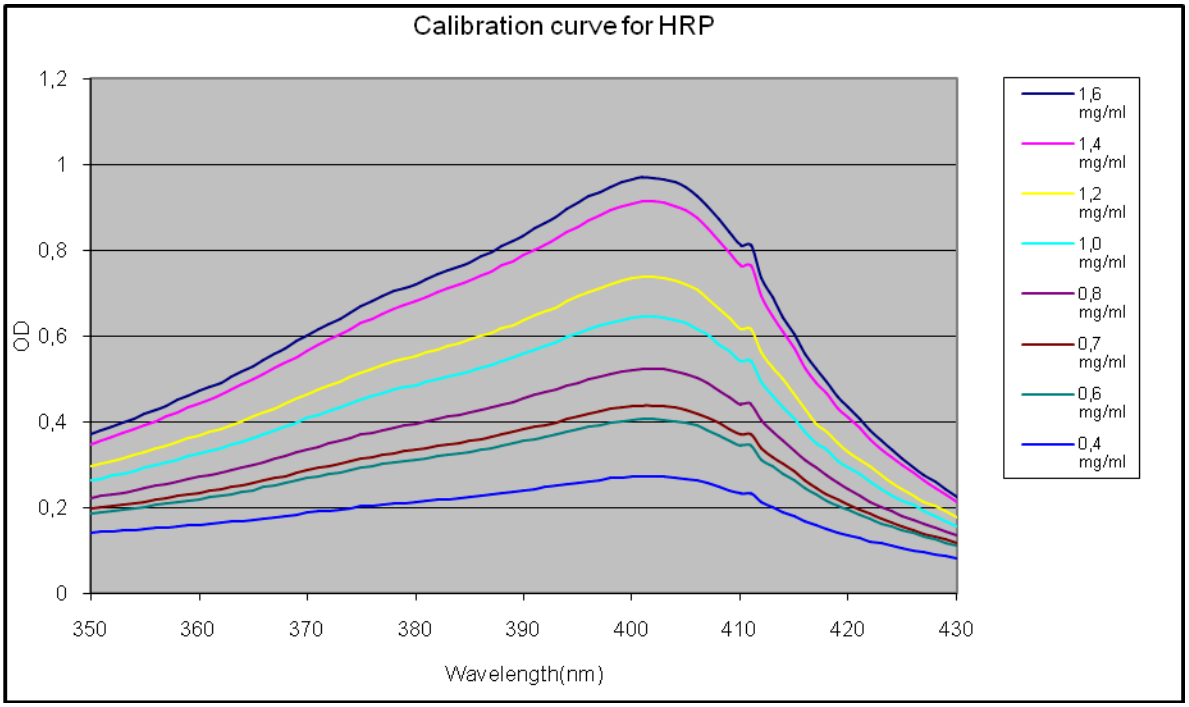

Figure 16. Determination of optical density of HRP

This determination is consistent with previous observations made by Laurent *et al* 2000 in similar experimental conditions.

From the determination of the optical density of HRP, the HRP calibration curve was measured at 403 nm and the respective straight-line equation is shown in (Fig. 17).

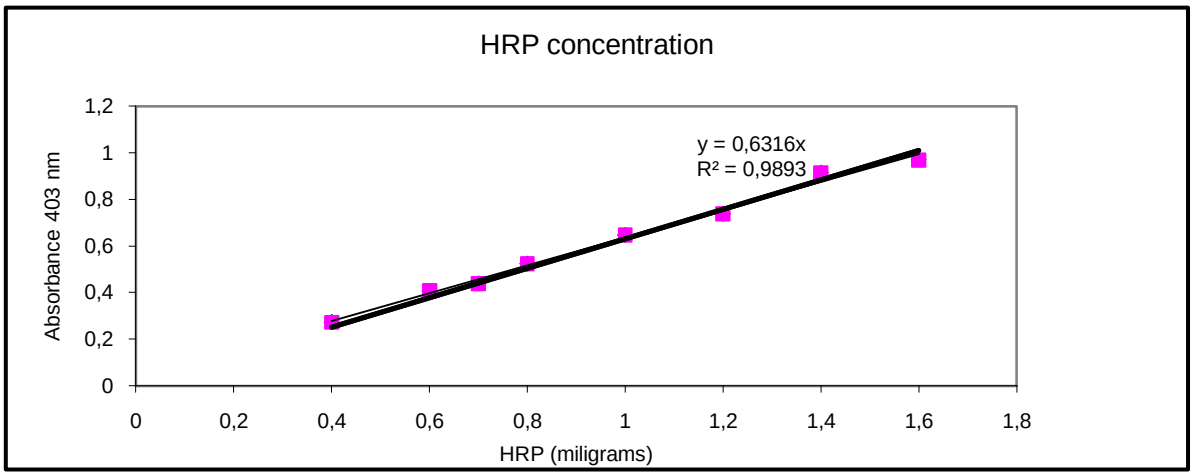

Figure 17. Calibration curve of HRP at 403 nm

According to this calibration curve and its straight-line equation, it was possible to measure the peroxidase concentration in the conjugate I.

4.1.2. Measurement of the peroxidase in the conjugate I through absorbance

Using the straight-line equation (y = 0,6316 x) and the values of absorbance of the conjugate at 403 nm in different concentrations, it was possible to determine the efficiency of the conjugation reaction.

Table 3. Determination of the percentage of peroxidase in the conjugate I

%E	concentration of the conjugate (mg/mL)	x	y (absorbance values of the conjugate I)
20	6	1,180	0,745
16	5	0,809	0,511
17	4	0,687	0,434
19	3	0,576	0,364

%E = x values*100 / concentration of the conjugate

According to the results, it could be concluded that the enzyme conjugation efficiency (i.e. the enzyme activity in the conjugation) was only 20% (table 3).

This process presented a very low efficiency (i.e. low enzymatic activity), therefore not satisfying the premise that the enzyme should be highly active. As a result, it was decided to perform additional conjugation processes.

4.2. Determination of the effect of HRP enzyme - TMB substrate reaction time on absorbance.

This study was conducted to determine the optimal reaction time needed for the quantification of HRP or labeled antigen that react with the substrate. The reaction time then determined was later used in the subsequent ELISA assays.

For this reaction, the protocol described (item 3.4) was followed. The dilutions of HRP used was 1,50E-06; 7,50E-07; 6,00E-07; 5,00E-07 ug/µL. The calibration curve for HRP enzyme – TMB substrate reaction is represented in figure 18.

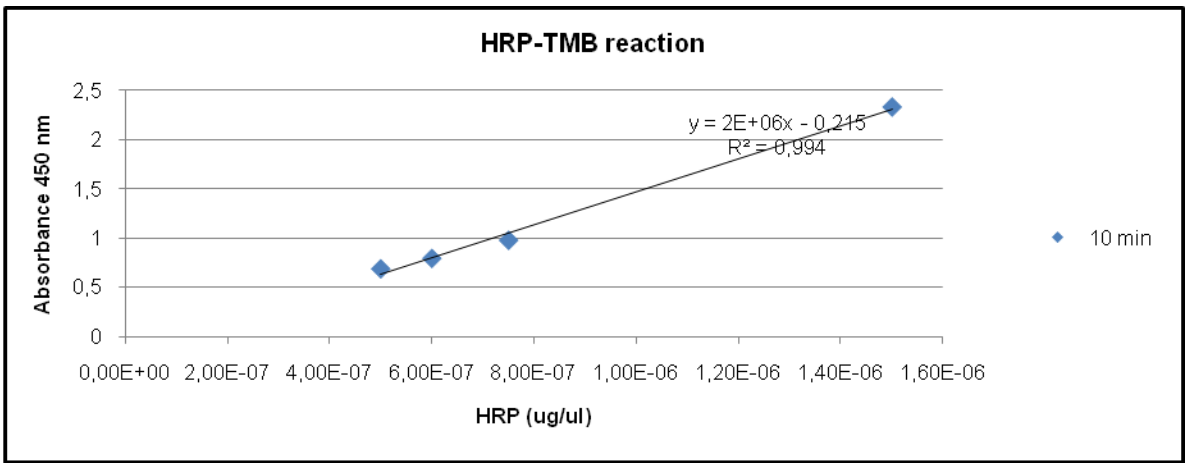

Figure 18. Calibration curve of HRP

The results from this experiment (Fig. 18) suggest that the absorbance of the reaction HRP-TMB substrate increased as a consequence of the increase of the HRP concentration. However, considering the Beer-Lambert law, it is possible to empirically establish the ideal range of absorbance between 0,5 and 1,0. Therefore, concentrations of HRP between 7,50E-07 ug/µL and 6,00E-07 ug/µL, and 10 min of reaction time seemed to be the good reference to use in the following assays.

This result is also consistent with previous observations indicated in the Theegala and Suleiman protocol (Theegala and Suleiman, 2000) for similar experimental conditions.

4.2.1. Determination of the effect of conjugate I - TMB substrate reaction time on absorbance

According to the results of the determination of the HRP-TMB substrate reaction time, the calibration curve of the conjugate was performed.

For this reaction, the protocol described (item 3.4) was followed. The dilutions of HRP used was 2,80E-04; 2,40E-04; 2,00E-04; 1,80E-04; 1,60E-04 ug/µL. The calibration curve for conjugate I – TMB substrate reaction is represented in figure 19.

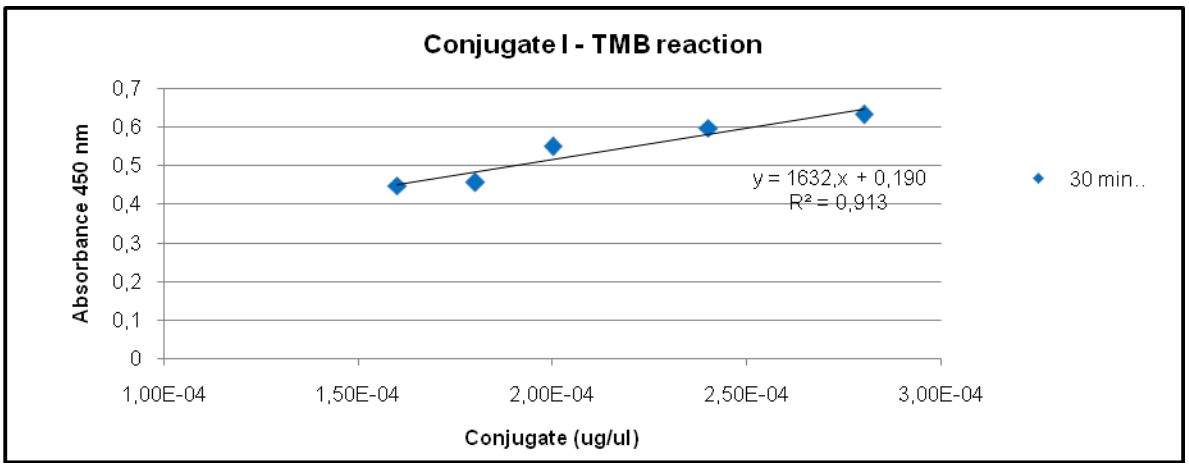

Figure 19. Calibration curve conjugate I HRP-HA via periodate method

After determination of the conjugate's reaction time with TMB (fig.19), results showed that higher concentrations of conjugate and additional time were necessary to achieve the approximate absorbance results identified in the reaction with the enzyme (fig.18). In this case, the results of the reaction with the conjugate confirm that this conjugation presents a very low enzymatic activity. Therefore, this conjugate was dispensed in subsequent assays.

4.2.2. Determination of the effect of conjugate II - TMB substrate reaction time on absorbance

According to the same protocol used in the tests described above, the calibration curve of the conjugate was determined.

For this reaction, the protocol described in item 3.4 was followed. The dilutions adopted for the HRP were 9,00E-06; 4,50E-06; 3,00E-06 µg/µL. The calibration curve for conjugate II – TMB substrate reaction is shown in figure 20.

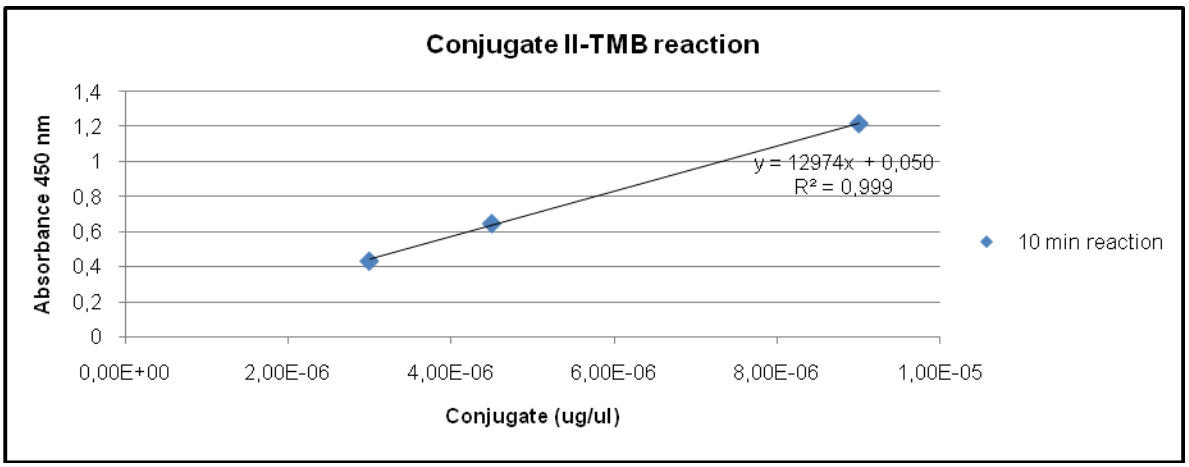

Figure 20. Calibration curve for the conjugate II HRP-GA-HA.

According to the results (Fig. 20), it is possible to suggest that the new protocol of conjugation seemed more efficient in relation to the enzyme activity than the conjugation via periodate method. These results are closer to the results obtained with the enzyme before the conjugation.

This conjugate was then used in the subsequent assays.

4.2.3. Determination of the effect of conjugate III - TMB substrate reaction time on absorbance

The same procedure applied to both the previous conjugates was also used for this new conjugate.

For this reaction, the protocol described in item 3.4 was followed. The dilutions adopted for the HRP were 1,20E-04; 2,40E-04; 6,00E-04; 1,20E-03ug/µL. The calibration curve for conjugate III – TMB substrate reaction is presented in figure 21.

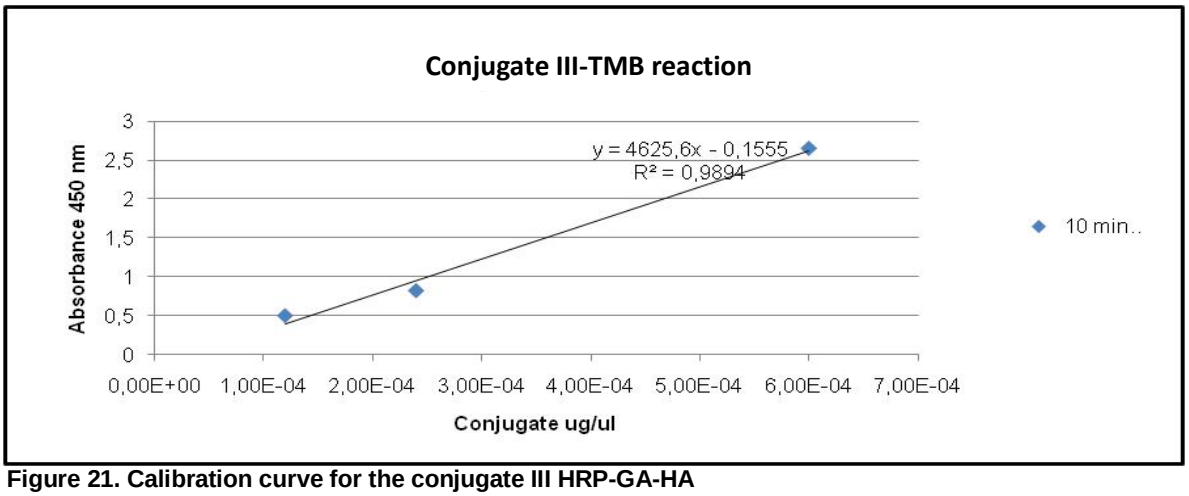

Figure 21. Calibration curve for the conjugate III HRP-GA-HA

After determination of the conjugate reaction with TMB (fig.21), the results showed that higher concentrations of conjugate were necessary to achieve the approximate absorbance results identified in the reaction with the enzyme (fig.18). Therefore, the reaction time was 10 min as indicated in the reference values obtained with the reaction with the enzyme and it was decided to use this conjugate in future assays.

Up until this point, from the three conjugation processes undertaken, the conjugation according to the Rice protocol (Rice *et al.*, 1982) was the one that demonstrated the best results regarding enzyme activity.

4.3. Chessboard titration

In order to optimize the reaction between the antibody and the conjugate, several assays with the two conjugates were performed, where the sole variation was the kind of blocking buffer in which the conjugates were diluted.

For the performance of this assay, the protocol described in item 3.7 was followed. Gelatin 1,0% (w/v) and sodium caseinate 1,0% (w/v) were chosen in order to avoid nonspecific adsorption of the conjugate to any free protein binding sites on the wells.

For the achievement of optimal absorbance effectiveness and considering the specifications of the equipment (SPECTRAmax^{tm} 340 Microplate Reader, Sunnyvale,

California) used in the assays, it was possible to establish the ideal range the absorbance at 450 nm values between 0 and 3,0.

The results of the chessboard titration (conjugate II) using 1% (w/v) of gelatin as blocking buffer are shown in figure 22.

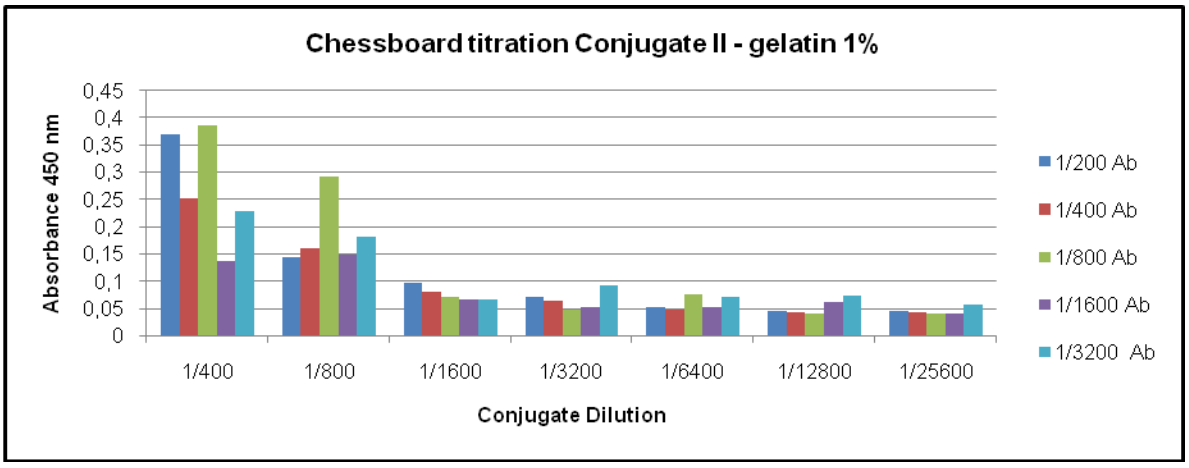

Figure 22. Chessboard titration conjugate II (HA-GA-HRP) – 1,0% (w/v) gelatin

The results shown in figure 22 were not satisfactory, as they lacked coherence. For example, at 1/400 of concentration of the conjugate, it should be expected that the levels of absorbance would diminish along with the reduction of the concentration of the antibody, but a certain oscillation was observed. This could indicate that notwithstanding the good results obtained with regard to enzymatic activity in this conjugate, the conjugation might have been inefficient with respect to the histamine conjugated with the enzyme.

The results of the chessboard titration (conjugate II) using 1% (w/v) of sodium caseinate as blocking buffer are shown in figure 23.

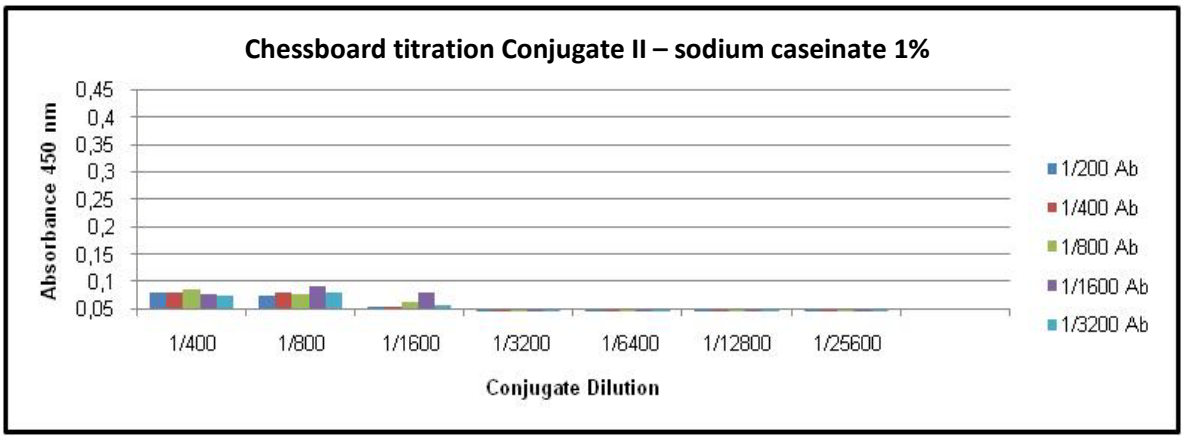

Figure 23. Chessboard titration Conjugate II (HA-GA-HRP) – 1,0% (w/v) sodium caseinate

In the assay where the sodium caseinate was used (fig.23), it seems that his compound might inhibit the enzymatic activity. Therefore, this conjugate was dispensed in subsequent assays.

Considering the poor results obtained in the previous assays, yet a new attempt was performed, this time in accordance with the Fujiwara protocol (Fujiwara *et al*., 1997), and new optimization assays (chessboard titration) were conducted.

The figure 24 displays the results of the chessboard titration (conjugate III) using 0,5% (w/v) of sodium caseinate as blocking buffer.

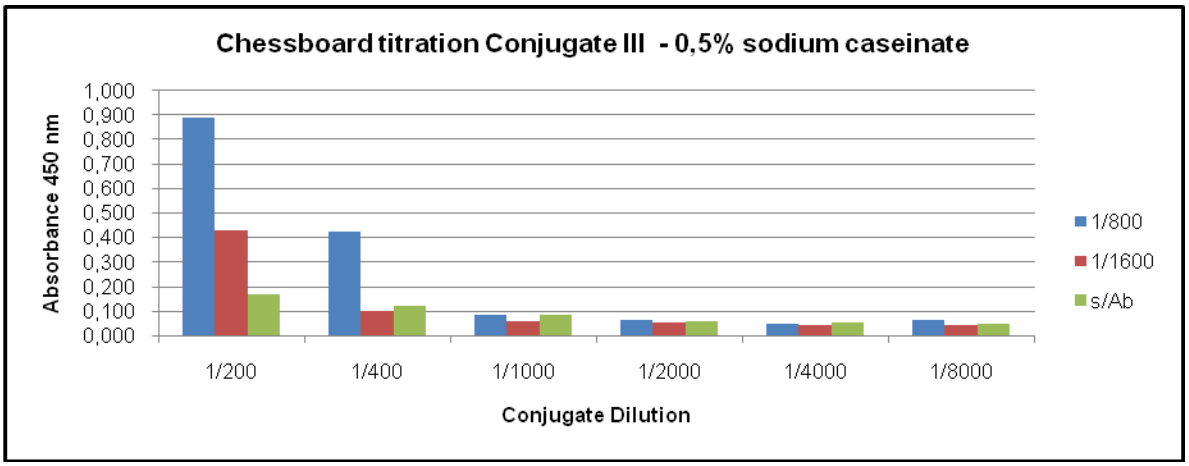

Figure 24. Chessboard titration Conjugate III (HA-GA-HRP) – 0,5% (w/v)sodium caseinate

The results obtained in this assay (fig. 24) were more coherent that the ones observed for the conjugate II, even if not yet as expected, since it would be desirable that dilutions of the conjugate would be measurable up to 1/20000, according to reports from Schneider (1996) – and with this assay the measurement of the dilutions was only possible until 1/400.

Therefore, the conjugate III was chosen to be used in the subsequent ELISA assays. The following reference values were adopted for the reagents: 1/400 for the conjugate with an average of 1/1000 of the antibody.

4.4. Indirect ELISA

After optimizing the reaction between the conjugate and the antibody, and proceeding with the ELISA competitive assay, it was decided to start the assays by ELISA indirect. Two blocking agents were tested, gelatin 1,0% (w/v) and sodium caseinate 0,5% (w/v). The tests were performed in triplicate.

For the performance of this assay, the protocol described in item 3.8 was followed. The conjugate dilutions were 1/200; 1/400; 1/1000; 1/2000; 1/4000; 1/8000 and the washing buffer used in these assays was TBS/T (TBS + Tween 20 0,05%).

The results indicated in figure 25 refer to the indirect ELISA using 1% (w/v) gelatin as blocking buffer.

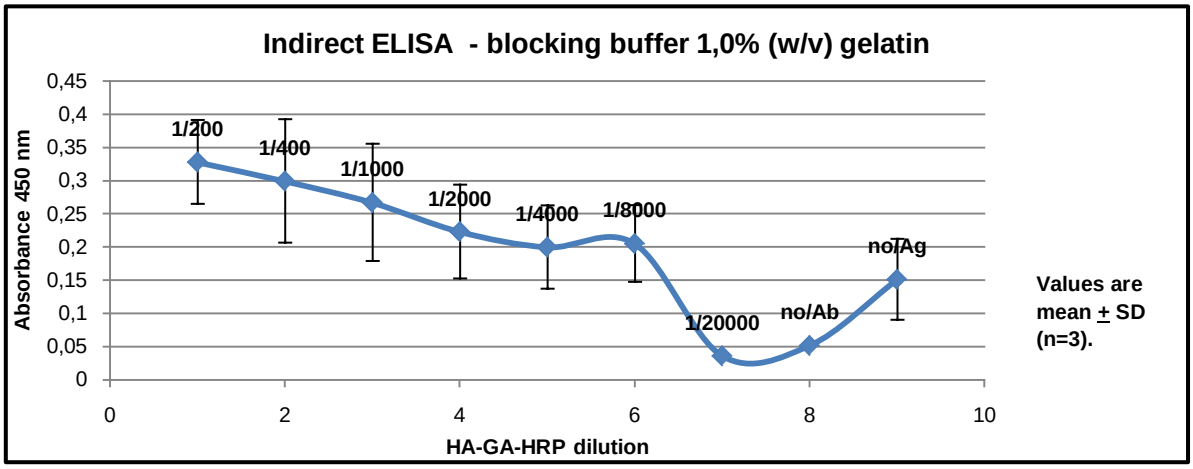

Figure 25. Indirect ELISA – blocking buffer 1,0% (w/v) gelatin

The results of the Indirect ELISA using 0,5% (w/v) sodium caseinate as blocking buffer are represented in figure 26.

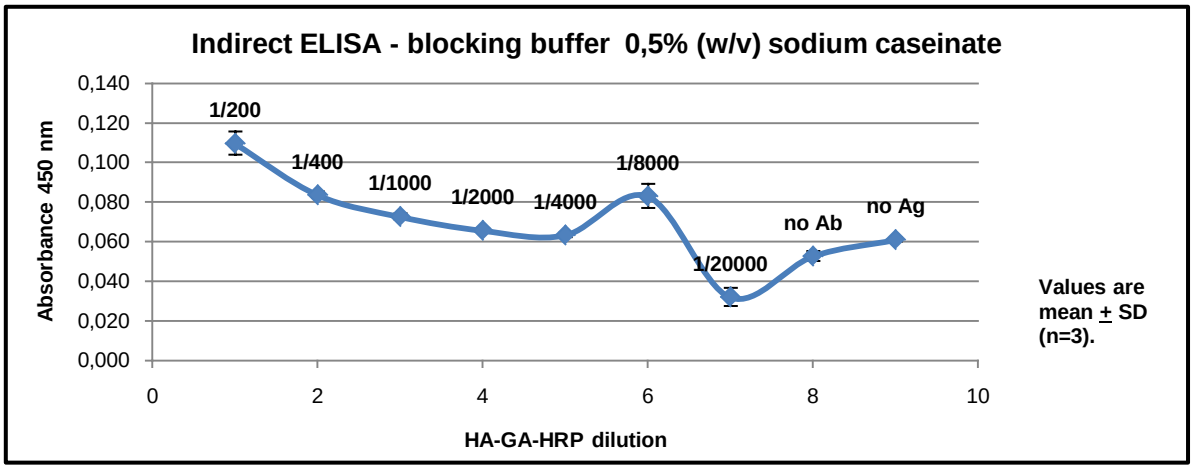

Figure 26. Indirect ELISA – blocking buffer 0,5% (w/v)sodium caseinate

In the first assay (fig. 25), although there were variations in the concentrations of the conjugate, the values of absorbance did not vary significantly. Another relevant observation is that in the among the wells with no conjugate, the absorbance values was very similar, being therefore possible to infer that the antibody (anti-histamine) ended up binding to the plate and/or to the blocking agent. These results could indicate that the conjugation process could have been inefficient in relation to the histamine conjugate to the enzyme.

In the second assay (fig. 26), the results were very similar to the ones from the previous assay, however, the absorbance values were too low, indicating a possible inhibition of the caseinate in relation to the enzyme.

4.5. ELISA inhibition test

After the non-satisfactory results obtained in the indirect ELISA assays, possibly caused by the conjugate, it was decided to perform other kinds of assays.

In this specific case, before executing the conjugation III (Fujiwara *et al*., 1997) with the peroxidase enzyme, it was decided to conjugate the histamine with BSA in order to test the effects of the conjugation in the ELISA assays.

The first ELISA inhibition test was made with the conjugate HA-GA-BSA (fig.27 and 28).

For the performance of this assay, the protocol described (item 3.9) was followed. The blocking buffer used was 1,0% (w/v) of gelatin.

Figure 27 displays the results of the ELISA inhibition test using 1,0% (w/v) of gelatin as blocking buffer.

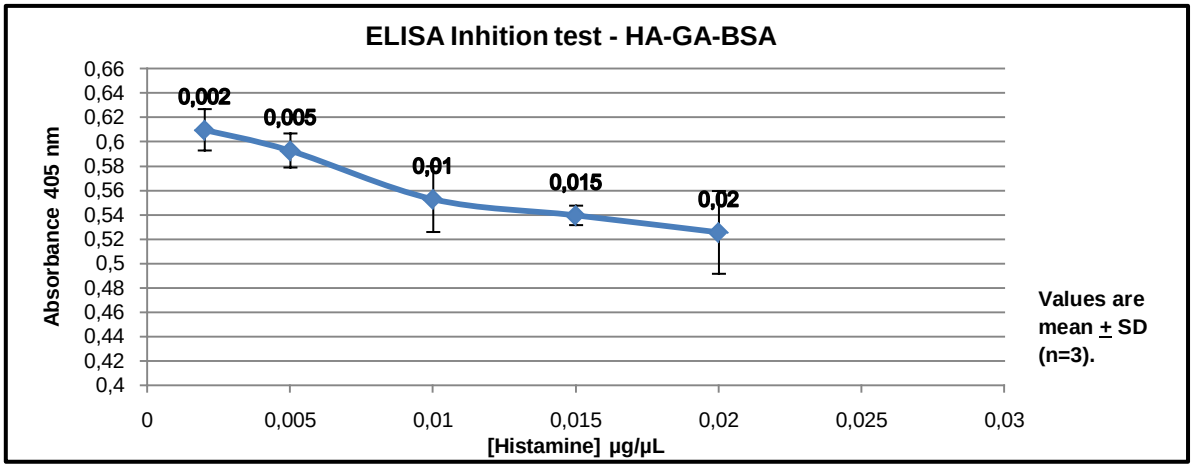

Figure 27. ELISA Inhibition test – HA-GA-BSA

Figure 28 displays the results of the ELISA inhibition test using higher concentrations of histamine.

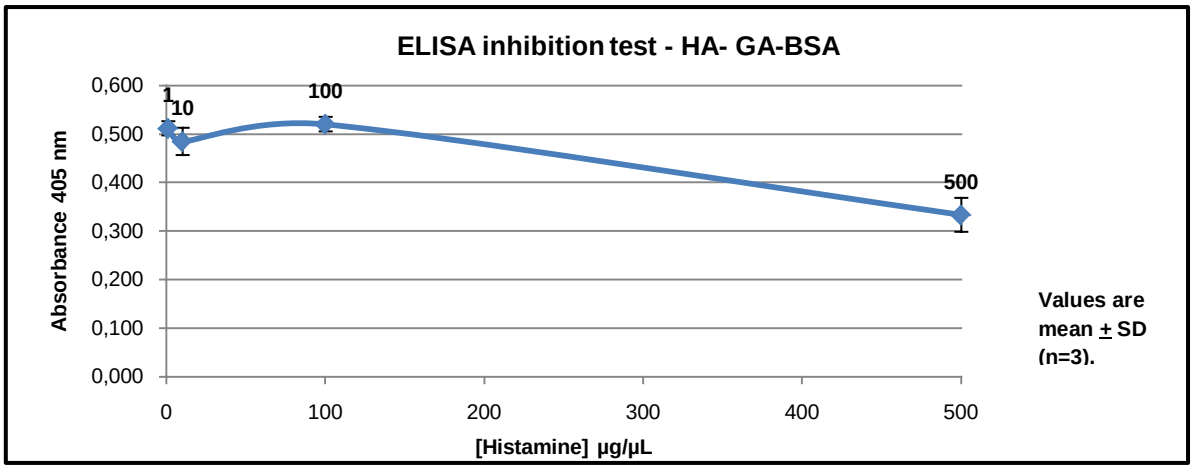

Figure 28. ELISA Inhibition test – HA-GA-BSA. Higher concentration of histamine

The results of the first assay (fig. 27) demonstrated that absorbance values did not change significantly as the concentration of histamine increased. One possible reason for this result could be the low concentrations of histamine used in this assay.

In a second assay (fig.28), higher concentrations of histamine were used, and only then it was possible to notice alterations in the absorbance levels. Based on these results, it was possible to observe that this kind of assay can be an efficient test to determine histamine in types of food that present a higher concentration of this substance.

Following the same protocol of earlier assays, a new test was performed with the conjugate HA-GA-HRP in order to verify the performance of the conjugation with the peroxidase for this method.

Figure 29 displays the results of the ELISA inhibition test using the conjugate HA-GA-HRP.

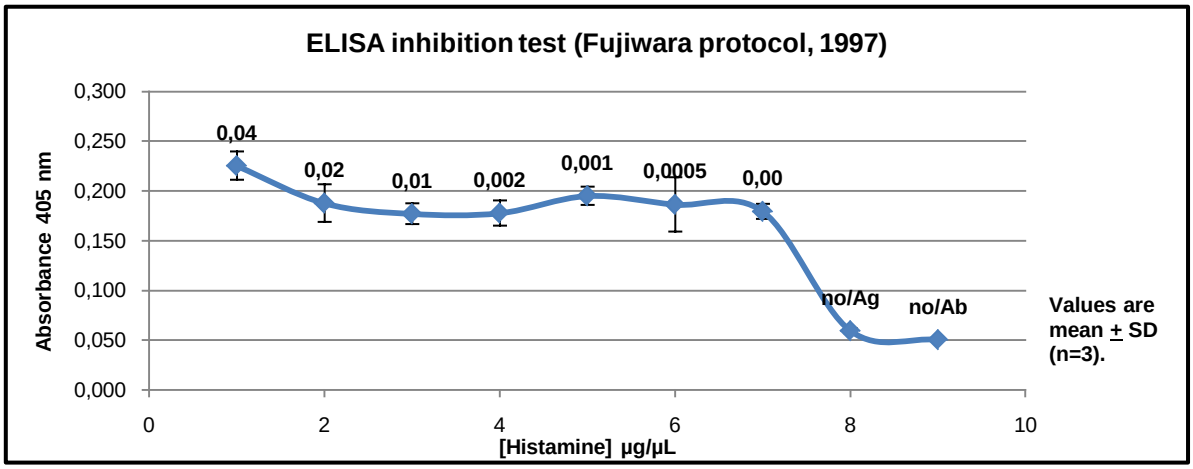

Figure 29. ELISA Inhibition test – HA-GA-HRP.

The results indicated that even when there is a variation in the concentration of histamine in the sample, the absorbance values do not change. At the point 7 of Fig.29, for example, the absorbance values should be higher, since there is no competition for the antibody, as there is no histamine and the conjugate should attach to all of the available antibodies. These results strengthened the hypothesis that the conjugate might have been the reason for the unsatisfactory results achieved so far.

4.6. ELISA binding test

The goal of this assay was to work with the histamine that was not conjugated with the enzyme, as an attempt to avoid the possibility of enzyme conjugation interference in the assays. Two assays were performed, one with low concentration of histamine and the other with higher levels of histamine.

For the performance of this assay, the protocol described (item 3.10) was followed. The blocking buffer used was (1,0% (w/v) of gelatin).

The results of the ELISA binding test using low concentration of histamine are presented in figure 30.

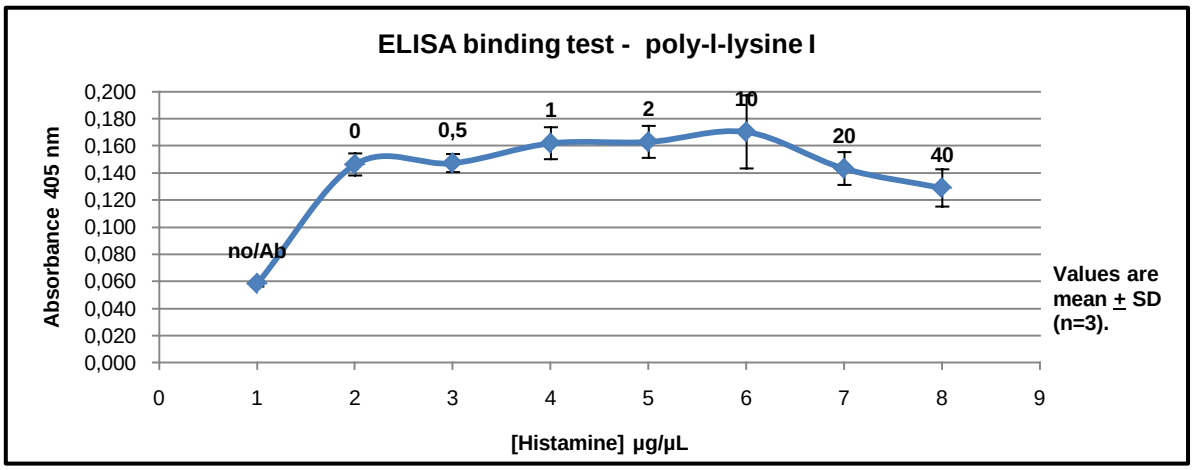

Figure 30. ELISA binding test – poly-l-lysine

The results of the ELISA binding test using higher concentrations of histamine are presented in figure 31.

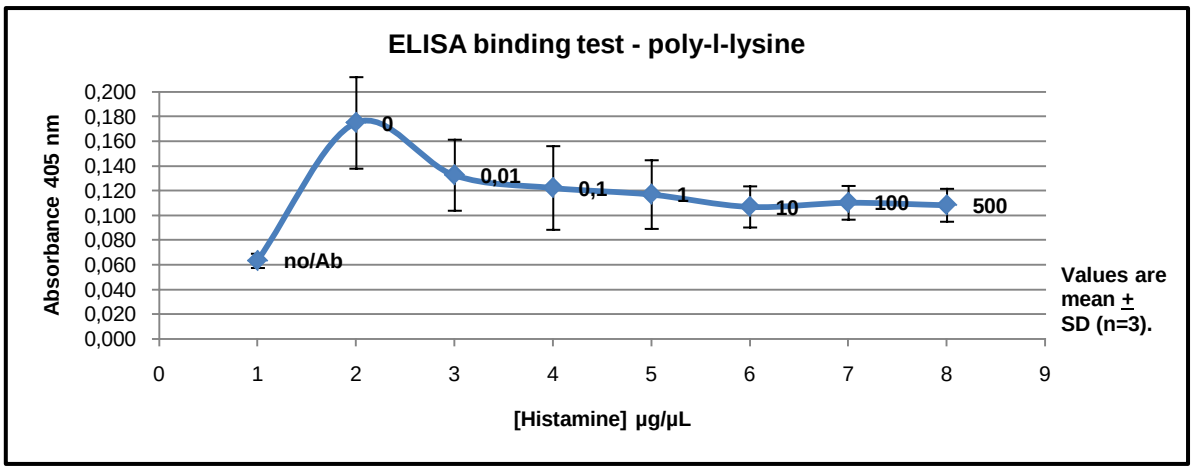

Figure 31. ELISA binding test – poly-l-lysine. Higher concentration of histamine

In the first assay (Fig.30), it was possible to observe that absorbance values did not represent significant differences when exposed to different concentrations of histamine. Therefore, it seemed that either the concentrations of histamine were too close to verify any differences among them, or this was not an efficient method to determine histamine levels. Therefore, a second assay was performed, with higher concentrations of histamine, in accordance with Fujiwara protocol (Fujiwara *et al*., 1997) whom reported the possibility to determine the presence of histamine with concentrations equal or higher than 10 ug/µL).

In this second assay (Fig.31), not only the absorbance values did not increase in wells with a concentration of histamine higher than 10 ug/µL (as reported by Fujiwara *et al*., 1997), but also the results showed highest absorbance values in wells where there was no presence of histamine. One possible reason for such results is that the antibody binds to the blocking agent or to the poly-l-lysine.

Another observation from this second assay was that in wells where there was no presence of antibody (anti-histamine), absorbance values were low in relation to other wells, which could indicate that binding between antibodies (anti-histamine and anti-rabbit IgG) would be efficient. Therefore, it seems that either histamine does not bind to poly-l-lysine, or the antibody used is not specific for the referred histamine. In any event, this could not be a suitable assay to determine histamine in wine as the Fujiwara protocol (Fujiwara *et al.,* 1997) indicates that it is only possible to detect its presence in concentrations which are equal or higher than 10 ug/µL, and the range that is supposed to be tested (histamine in wine) vary between 0,1 mg/L and 20 mg/L.

4.7. ELISA competitive

Notwithstanding the poor results observed in all the previous assays, it seemed important to conduct a trial of a competitive ELISA test, since this is the method chosen to be developed in this work.

This assay was conducted according to the protocol described in item 3.11. For this assay, histamine standard concentrations adopted were: 10; 1; 0,05; 0,01; 0,001; 0,0005 ug/µL

However, contrarily to what was expected, the bound enzyme conjugate did not convert the colorless chromogen into a blue product, and in the absence of such reaction, the following steps of the assay were not possible to be performed.

In this case, it is possible to suggest that there has been no connection between the conjugate and the antibodies. This might indicate that the conjugate did not contain sufficient histamine to react with the antibody or that there was a low specificity between the antibody and the histamine in question.

Although it was not possible to obtain sucessful results for this assay, other authors have reported successful experiences with this method.

In the protocol developed by Ayun *et al.* (1999), related to the presence of histamine in cheese, it was suggested that a direct competitive ELISA method could be considered as a reliable method for the quantitative determination of histamine in cheese at levels higher than 10 mg/kg.

In the protocol developed by Schneider *et al*. (1996), for the determination of histamine in fish and its by-products, a preliminary study to check the applicability the competitive ELISA method for the detection of histamine in salmon meat showed that levels as low as 10 µg of histamine per gram of tissue could be detected.

However, it is worth mentioning that both these authors have produced the specific antibodies for the particular histamines used in their assays, through immunogen synthesis, where the histamine is conjugated with KLH (Keyhole Limpet Hemocyanin) via

glutaraldehyde, and this conjugate (histamine plus KLH) is subsequently used for the antiserum production.

4.8. Histamine ELISA Kit (Immuno Biological Laboratories, Inc. Minneapolis, USA)

In order to verify the difference in performance and results between the free histamine (as it was originally the concept of this work) and the derivatized histamine (as it is proposed in the majority of the commercial ELISA kits), it was considered adequate to perform an assay with a commercial Histamine ELISA kit (Ref: IB 89128, Immuno Biological Laboratories, Inc. Minneapolis, USA).

The method was performed in accordance with the protocol indicated in the kit supplier's instructions.

A typical standard curve of the CD ELISA for histamine is illustrated in figure 32.

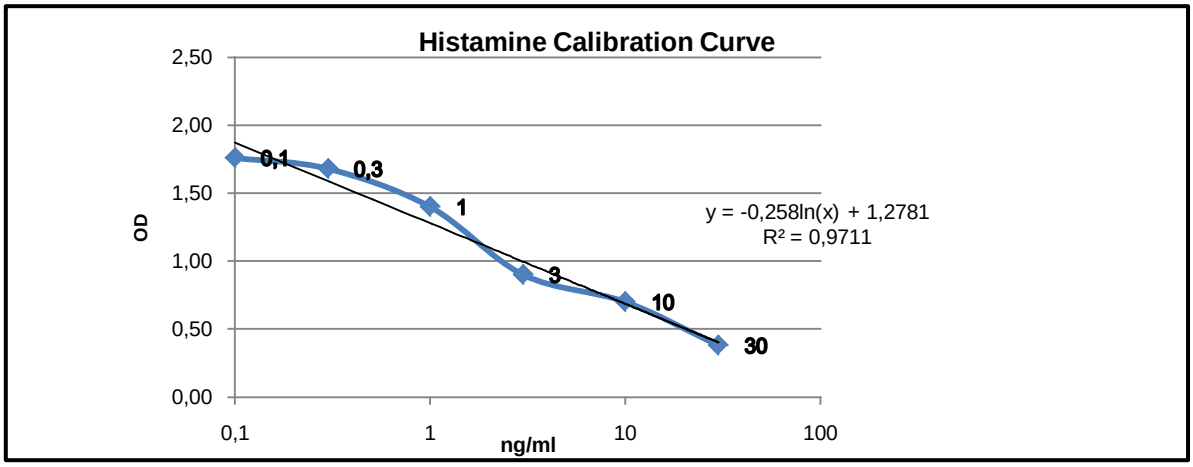

Figure 32. Kit ELISA histamine – standard calibration curve

Table 4. Determination of the concentration of histamine in the sample

Standard conc. ng/mL	OD standards	Straight- Line equation of standards	y (absorbance values of the sample)	x conc. of histamine in the sample ng/mL
0,1	1,76	y = -0,258Ln(x) + 1,2781	0,941	**3,69**
0,3	1,68	y = -0,258Ln(x) + 1,2781	0,904	**4,26**
1	1,40	y = -0,258Ln(x) + 1,2781	0,624	**12,62**
3	0,90	y = -0,258Ln(x) + 1,2781	0,617	**12,97**
10	0,70	y = -0,258Ln(x) + 1,2781	0,627	**12,47**
30	0,38	y = -0,258Ln(x) + 1,2781	0,598	**13,96**

With the purpose of certifying that the pH in the histamine sample would not interfere with the results of the assay (i.e. being sure that the pH of the histamine sample is not substantially different from the pH of the standard sample provided with the kit), before the performance of the assay with the commercial kit, the pH of the two samples was measured and presented the pH levels of 4,9 (histamine sample) and 0,88 (kit sample). This difference would be undesirable for the performance of the assay. However, when the adjustment buffer (provided with the kit) was added to the samples, the pH levels of both samples was re-measured and the result was a pH of 9,98 for both samples.

It was thus possible to verify that the pH levels do not interfere with the results of the kit, provided that the adjustment buffer is utilized. According to a study by Claeys-Bruno *et al*., 2006, the pH determination is considered an important matter for the performance of this type of assays, as the pH has a relevant role in the process of derivatization of the histamine.

Initially a calibration curve and its straight-line equation were determined (Fig.32) in accordance with the parameters that are indicated in the kit's instructions.

Through the straight-line equation and the absorbance values obtained in the analysed sample, it was possible to determine the concentration of histamine in the sample.

From the results obtained, it is possible to verify in both samples (standard sample and histamine sample) that the higher the quantity of histamine, the lower the absorbance level, which is consistent with the principle of the ELISA competitive method. In other words, the higher presence of histamine in the sample corresponds to a lower level of connection between the labeled histamine (histamine plus peroxidase) and the antibody, and therefore, the absorbance levels verified will be lower.

In this case, it is possible to suggest that the process of derivatization could be the important step for the determination of histamine in food and beverages samples.

5. Conclusion

For the preparation of a direct competitive ELISA, two main aspects were critical: (i) the availability of an antibody that was specific for the histamine being tested; and (ii) the conjugation of the histamine with the peroxidase.

The first step was the acquisition of antibody. The ideal scenario would have been the production of an antibody specific for the chosen histamine, through an immunization process with animals (as reported by Ayun *et al*, 1999 and Schneider *et al*, 1996). However, the period available for the performance of this work did not allow sufficient time for this process, and it was therefore decided to purchase a readily available polyclonal antibody from the market.

The second step was the conjugation of the histamine with the peroxidase. For that, three subsequent conjugation methods were performed, along with the reaction tests for each of them for (i) the determination of enzymatic activity; and (ii) the optimization of the conjugate with the antibody.

However, after the first few ELISA assays towards optimization of the antibody/antigen binding (chessboard titration), none of the conjugates seemed effective for the performance of the method, i.e. they did not reproduce consistent results between themselves or when compared to others tests. Nonetheless, even with the absence of an ideal conjugate, the last one (Fujiwara *et al.*, 1997) was chosen to perform the assays as the remaining conjugates presented less satisfactory results.

After performing the assays and taking into consideration the poor results observed, it seemed possible that the conjugates could be the reason for such difficulties. The reason for this possibility was the fact that all the conjugates were subjected to tests for the evaluation of the enzymatic activity, but no tests were performed in order to evaluate the amount (if any) of histamine in such conjugates.

In an attempt to rule out that possibility, an ELISA binding test was performed using poly-l-lysine. This kind of assay allows for the use of poly-l-lysine, a polypeptide with the

characteristic of attaching itself to the wells and of capturing the free histamine. The results, however, were again unsatisfactory.

At this point, there were two main possible hypotheses to explain the poor results so far: either (i) there was some sort of problem with the performance of the ELISA binding test (e.g. a non-functional poly-l-lysine) and/or the lack of satisfactory results were in fact due to a low efficiency of the conjugation processes; or (ii) there was a lack of specificity between the histamine used and the polyclonal antibody acquired for the assays.

Leaving on hold the work developed so far, it was decided to experiment with the differences between working with free histamine (as done up until that moment) and derivatized histamine. Therefore, another assay was performed, this time with a commercial kit – Histamine ELISA Kit (Immuno Biological Laboratories, Inc. Minneapolis, USA).

This assay was performed with the purpose of evaluating the efficiency, in an ELISA assay, of using the derivatized histamine. The positive results obtained with the commercial ELISA kit suggest that the derivatization process could be relevant for the efficiency of the assays.

With all the above considered, and having reached this stage, the only available option would be to dispose of the material in use and start the whole process all over again.

Taking into account that the main objective of this work is to experience the process of an academic research, and that the time available for this training experience is limited, it seems that this work has fulfilled the main goal, as herein reported. Further work to improve and test the proposed ELISA could be performed, but this would exceed the possibilities and the available resources of this research.

6. Possible steps for further studies on this subject

In relation to possible further studies on this subject, it might be suggested:

(a) the development of a method to quantify the amount of histamine in the conjugate; and,

(b) the optimization of the reaction between antibody and antigen through the use of a bio-sensor equipment (e.g. Biacore), that would make it possible to determine the interaction between the molecules and could be important for the success of the assays.

7. References

- Aerny, J.1985. Origène de l'histamine et dáutres amines biogènes dans les vins. Off. Int. Vigne Vin, 656-657:1017-1020.

- Ancin-Azpilicueta, C. González-Marco A., Jiménez-Moreno N. 2008. Current Knowledge about the Presence of Amines in Wine. Critical Reviews in Food Science and Nutrition, 48:257-275.

- Aygun. O., Schneider E., Scheuer R., Usleber E., Gareis M., Martlbauer E. 1999. Comparison of ELISA and HPLC for the determination of histamine in cheese. Journal Agriculture and Food Chemistry. 47: 1961-1964.

- Claeys-Bruno, M., Vandenabeele-Trambouze O., Sergent M., Geffard M., Bodet D., Dobrijevic M., Commeyras A., Phan Tan Luu R. 2006. Methodological approaches for histamine quantification using derivatization by chloroethylnitrosourea and ELISA measurement. Part I: Optimization of derivated histamine detection with coated-plates using optical design. Chemometrics and Intelligent Laboratory Systems 80: 176-185.

- Claeys-Bruno, M., Vandenabeele-Trambouze O., Sergent M., Geffard M., Bodet D., Dobrijevic M., Commeyras A., Phan Tan Luu R. 2006. Methodological approaches for histamine quantification using derivatization by chloroethylnitrosourea and ELISA measurement. Part II: Optimization of the derivatization step. Chemometrics and Intelligent Laboratory Systems 80:186-197.

- Crowther, J.R.(1995) ELISA Theory and Practice. Methods in Molecular Biology vol 42. Institute for Animal Health, Woking, UK. Humana Press Totowa, New Jersey.

- Frenencík Miroslav (1993). *Handbook of Immunochemistry.* Ed. Chapman & Hall. First Edition.

- Fujiwara K., Kitagawa T., Inoue Y., Alonso G. 1997. Monoclonal antibodies against glutaraldehyde-conjugated histamine: application to immunocytochemistry. Histochemistry and Cell Biology 107:39-45.

- Harlow E., Lane D. *(*1988). *Antibodies, A Laboratory Manual.* Cold Spring Harbor Laboratory.

- Herbert P; M. J. Cabrita ; N. Ratola ; O. Laureano; A. Alves. 2006. Relationship Between Biogenic Amines and Free Amino Acid Contents of Wines and Musts from Alentejo (Portugal). Journal of Environmental Science and Health Part B, 41:1171–1186.

- Hill, S.J., Ganellin C.R., Timmerman H., Schwartz J.C., Shankley N.P., Young J.M., Schunack W., Levi R., Haas H.L. 1997. International Union of Pharmacology. XIII. Classification of Histamine Receptors. Pharmacological Reviews. Vol.49, n.3.

- Kanny G., Gerbaux V., Olszewski A., Frémont S., Empereur F., Nabet F., Cabanis J.C., Moneret-Vautrin, D.A. 2001. No correlation between wine intolerance and histamine content in wine. Journal Allergy Clin. Immunol., 107:375-378.

- Karovicová J. and Kohajdová Z., 2003. Biogenic amines in Food. Department of Food Technology, Slovak University of Technology, SK – 812 37 Bratislava.

- Landete J.M., Ferrer S., Pardo I. 2004. Improved enzymatic method for the rapid determination of histamine en wine. Food Additives and Contaminants, vol.21, n°12 pp.1149-1154.

- Landete J.M., Ferrer S., Polo L., Pardo I. 2005. Biogenic Amines in Wines from Three Spanish Regions. Journal Agric. Food Chem.53: 1119-1124.

- Laurenti, E., Suriano G., Ghibaudi E.M., Ferrari R.P. 2000. Ionic strength and pH effect on the Fe(III)-imidazolate bond in the heme pocket of horseradish peroxidase: an EPR and UV–visible combined approach. Journal of Inorganic Biochemistry 81:259–266.

- Lehane, L. and Olley, J. 2000. Histamine fish poisoning revisited. International Journal of Food Microbiology 58: 1–37.

- Leitão M.C., Marques A.P., San Romão M.V. 2005. A survey of biogenic amines in commercial Portuguese wines. Food Control 16: 199-204.

- Liu, S.Q., Davis C.R., Brooks J.D. 1995. Growth and Metabolism of Selected Lactic Bacteria in Synthetic Wine. Am. J. Enol.Vitic. vol. 46, n°2.

- Lonvaud-Funel A and Joyeux A, 1994. Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of Leuconosfoc oenos. Journal of Applied Bacteriology, 77:401-407.

- Lonvaud-Funel, A. 2001. Biogenic amines in wine: role of lactic acid bacteria. FEMS Microbiol. Lett. 199: 9–13.

- Maintz L. and Novak N., 2007. Histamine and histamine intolerance. Am.J.Clin.Nutr. 85: 1185-96. American Society for Nutrition.

- Manfroi L., Silva P.H.A, Rizzon L.A., Sabaini P.S., Glória M.B.A. 2009. Influence of alcoholic and malolactic starter cultures on bioactive amines in Merlot wines. Food Chemistry. Vol. 116, Issue 1: 208-213.

- Marcobal A., Polo M.C., Martín-Álvarez, P.J., Moreno-arribas, M.V. 2005. Biogenic amine content of red Spanish wines: Comparison of a direct ELISA and HPLC method for the determination of histamine in wines. Food Research International 38: 387-394.

- Muscarella, M., Iammarino, M., Centonze, D., and Palermo, C. 2005. Measurement of histamine in seafood by HPLC, CE, and ELISA: Comparison of three techniques. Veterinary Research Communications 29: 343–346.

- Rauscher-Gabering, E. Grossgut R., Bauer F., Paulsen P. 2009. Assessment of alimentary histamine exposure of consumers in Austria and development of tolerable levels in typical foods. Food Control 20:423-429.

- Rice T.K., Therese A., 1985. Receptor-based histamine assay. United States Patent.4,544,629.

- Rollan G.C., Coton E., Lonvaud-Funel A. 1995. Histidine decarboxylase activity of *Leuconostos oenos* 9204. Food Microbiol., 12:455-461.

- Schneider E., Usleber E., Martlbauer E. 1996. Production and Characterization of Antibodies Against Histamine. ACS Symposium Series 621. *Immunoassays for Residue Analysis. Food Safety.* Ed. Beier & Stanker.

- Serrar, D., Breband, R., Bruneau, S., and Denoyel, G.A. 1995. The development of a monoclonal antibody-based ELISA for the determination of histamine in food: application to fishery products and comparison with the HPLC assay. Food Chemistry 54:85-91.

- Sessa, M., Desiderio A and Perin, A. 1984. Effect of acute ethanol administration on diamine oxidase activity on the upper gastrointestinal tract of rat. Alcoholism:Clinical and Experimental Research, 8,2:185–190. Published Online: 28 Jun 2008

- Shalaby A R.. 1996. Significance of biogenic amines to food safety and human health. Food Research International, 29, 7:675-690

- Silla Santos, M.H., 1996. Biogenic amines: their importance in foods. International Journal of Food Microbiology 29: 213-231

- Souza C. S., Theodoro K.H., Souza E.R., Motta S., Glória M.B.A. 2005. Bioactive amines in Brazilian wines: types, levels and correlation with physic-chemical parameters. Brazilian Archives of Biology and Technology. An International Journal. Vol.48, n.1: pp.53-62.

- Theegala, C.S. and Suleiman A.A. 2000. Quantification of antibody immobilization using peroxidase enzyme-substrate reaction. Microchemical Journal 65:105-111.

- Timmerman H. Histamine and histaminergic ligands; a playground for medicinal chemists. 2002. Il Farmaco 57: 549-550.

- Vidal-Carou M.C., Ambatle-Espunyes, A., Ulla-Ulla M.C., Mariné-Font A., 1990. Histamine and Tyramine in Spanish wines: their formation during the winemaking process . Am. J. Enol. Vitic., 41: 160-167.

- Vidal-Carou M.C., Lahoz-Portolés F., Bover-Cid S. and Marine-Font A., 2003.Ion-pair high-performance liquid chromatographic determination of biogenic amines and polyamines in wine and other alcoholic beverages, Journal of Chromatography A, 998 : 235–241.

Web pages:

http://www.inbio.com/protocols/solution.html 16-02-09

http://biotech.about.com/od/buffersandmedia/ht/phosphatebuffer.htm 24-07-09

http://www.rndsystems.com/tsg_detail_objectname_elisa_development.aspx 14-09-09

8. Annexes.

8.1. Preparation of solutions used in this work

Preparation of acetate buffer 1mM pH 4.4:

Table I *Preparation of acetate buffer*		
Acetate buffer 1 >, 1 L, pH 4.4, 1 mM		
Solute	**CH_3COOH**	**CH_3COONa**
Database nº	44	8
Supplier		Merck
Weight	0.0415 g/ 0,040 mL	0.0253 g
Density	1.05 g mL^{-1}	-
Volume	39.5 µL	-
Mw	60.05 g moL^{-1}	136 g moL^{-1}

http://www.egr.msu.edu/biofuelcell/tools/acetate/acetate.html

The solutes were added to 800 mL of bidistilled water, while stirring, at RT. To adjust the solution to 1 L, bidistilled water was added. After, the pH was measured and adjusted to 4.4.

Preparation of PBS buffer:

Table II *Preparation of 1 >PBS*				
PBS 1 >, 1 L				
Solute	**KCl**	**KH_2PO_4**	**Na_2HPO_4**	**NaCl**
Database nº	34A	57	3	23B
Supplier	Merck	Merck	Riedel-deHaën	Merck
Weight	0,200 g	0,240 g	1,440 g	8,000 g
Mw	75 g moL^{-1}	136 g moL^{-1}	142 g moL^{-1}	58 g moL^{-1}

The solutes were added to 800 mL of bidistilled water, one by one, while stirring, at RT. To adjust the solution to 1 L, bidistilled water was added. After, the pH was measured and adjusted to 7.4 (normally, with few drops HCl 0.1 M).

Preparation of Carbonate buffer:

Table III *Preparation of 1x Carbonate buffer*		
Carbonate Buffer 1X, 1L pH 9,6, 50 mM		
Solute	**Na2CO3**	**NaHCO3**
Database nº	7	37
Supplier	Merck	Merck
Weight	1.59g	2.93g
Mw	105.99 g/moL	84.01 g/moL

The solutes were added to 800 mL of bidistilled water, one by one, while stirring, at RT. To adjust the solution to 1 L, bidistilled water was added. After, the pH was measured and adjusted to 9,6.

Preparation of TBS buffer:

Table IV *Preparation of 1x TBS buffer*			
TBS 1X , 1L pH 7,4			
Solute	**0,05 M Tris**	**0,138 M NaCl**	**0,0027 M KCl**
Database nº	74	23	34
Supplier	Roth	Merck	Merck
Weight	6,05g	8,064g	0,2013g
Mw	121.14 g/moL	58.44 g/moL	74.55 g/moL

The solutes were added to 800 mL of bidistilled water, one by one, while stirring, at RT. To adjust the solution to 1 L, bidistilled water was added. After, the pH was measured and adjusted to 7,4.

Preparation of Blocking buffers:

Solution of gelatin in PBS: 1g of gelatin in 100mL of PBS;
Solution of 0,5% (w/v) sodium caseinate in PBS: 0,5 g of sodium caseinate in 100 mL of PBS.

8.2. Problems and Solutions in ELISA

Problem	Possible cause	Solution
High background	• Insufficient washing	• Increase number of washes • Add a 30 second soak step in between washes
	• Insufficient blocking	• Check blocking solution calculations • Increase blocking time
	• Incubation times too long	• Reduce incubation time
	• Interfering substances in samples or standards	• Run appropriate controls
	• Buffers contaminated	• Make fresh buffers
No Signal	• Reagents added in incorrect order, or incorrectly prepared	• Repeat assay • Check calculations and make new buffers, standards, etc. • Review protocol
	• Contamination of HRP with azide	• Use fresh reagents
	• Not enough antibody used	• Increase concentration
	• Standard has gone bad (if there is a signal in the sample wells)	• Check that standard was handled according to directions • Use new vial

Problem	Possible cause	Solution
	• Capture antibody did not bind to plate	• Dilute in PBS without additional protein
	• Buffers contaminated	• Make fresh buffers
Too much signal – whole plate turned uniformly blue	• Insufficient washing/washing step skipped – unbound peroxidase remaining	• Increase number of washes • Avoid detergents in wash solution; ensure no air bubbles are trapped in wells
	• Too much HRP	• Check dilutions
Standard curve achieved but poor discrimination between points (low or flat curve)	• Not enough HRP	• Check dilutions
	• Capture antibody did not bind well to plate	• Dilute in PBS without addition protein
	• Not enough detection antibody	• Check dilutions
	• Plate not developed long enough	• Increase substrate solution incubation time
	• Incorrect procedure	• Go back to general ELISA protocol
	• Improper calculation of standard curve dilutions	• Check calculations
Poor duplicates	• Poor washing	• Avoid detergents in wash solution; ensure no air bubbles are trapped in wells
	• Uneven plate coating due to procedural error or poor plate quality (can bind unevenly)	• Check coating and blocking volumes, times and method of reagent addition.

Problem	Possible cause	Solution
	• Variations in protocol	• Adhere to the same protocol from run to run
Very low reading across the plate	• Insufficient development time	• Increase development time
	• Capture antibody did not bind to the plate	• Dilute in PBS without additional protein

Source: http://www.rndsystems.com/tsg_detail_objectname_elisa_development.aspx