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Potentiometric detector for histamine in biological samples: a tool for forensic application.

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The determination of the post-mortem interval is a big challenge in forensic practices. It is defined as the time between death event and the moment that the body is found. Over the years, several factors and signs have been analyzed for its determination, namely body temperature (*Algor Mortis*), cadaveric livors (*Livor Mortis*), corpse stiffness (*Rigor Mortis*) and cadaverous fauna (*Entomology*). However, these dating methods may not always be performed because they are too dependent on several factors. In this way, one of the main priorities of legal medicine is to have an easy, low cost and accurate method to evaluate the post-mortem interval. Using several analytical techniques, the study of the behavior of numerous biomarkers in the body was the most recent advance felt by pathologists. Although some controversies in the correlation between the concentration of analytical species (proteins, enzymes, electrolytes, amino acids, etc.) and the postmortem interval, these metabolites remain a study target.

Considering the enhanced production of biogenic amines after death, the aim of this work was to develop a reliable histamine sensor to be applied and validated in forensic field.

The histamine sensor with potentiometric transduction was developed based on two different macrocycle molecules as ionophores (cucurbit[6]uril and α -cyclodextrin), different solvent mediators and the presence or not of an anionic additive. The polymeric support and the use of multi-walled carbon nanotubes in different percentages in the membrane composition were also evaluated. The optimal formulation for the membrane comprises 1.0% (w/w) of cucurbit[6]uril, 66.8% (w/w) of 2-nitrophenyl octyl ether as plasticizer, 29.8% (w/w) of polyvinyl chloride, 0.3% (w/w) of potassium tetrakis(4-chlorophenyl) borate and 2.0% (w/w) of multi-walled carbon nanotubes. A slope of 30.9 ± 1.2 mV/dec, a detection limit of $(3.00 \pm 0.61) \times 10^{-7}$ mol/L and the lower limit of linear range of $(3.00 \pm 0.00) \times 10^{-7}$ mol/L are the main response characteristics of the developed histamine sensor. Afterwards, the histamine electrode was coupled to a sequential injection analysis system. The hydrodynamic parameters were optimized. Similar response characteristics were obtained by using samples volumes of 197 μ L propelled to the detection cell with a flow-rate of 30 μ L/s. The developed method was applied to real samples (serum blood samples) that were previously cleaned by using solid phase extraction. The recovery was determined for three different levels of histamine concentration (1×10^{-4} ; 1×10^{-5} and 1×10^{-6} mol/L). Recovery values of $(97 \pm 6)\%$; $(103 \pm 1)\%$ and $(118 \pm 9)\%$ were obtained, respectively.

Keywords: Potentiometry, Ion-selective electrodes (ISE), Cucurbit[6]uril, Histamine, Biological samples

A determinação do intervalo pós-morte é um dos grandes desafios nas práticas forenses. Este intervalo é definido como o tempo que decorre entre o evento mortal e o momento em que o cadáver é encontrado. Ao longo dos anos, vários fatores e sinais têm sido analisados para a sua determinação, nomeadamente a temperatura corporal (*Algor Mortis*), os livores cadavéricos (*Livor Mortis*), a rigidez corporal (*Rigor Mortis*) e a fauna cadavérica (*Entomologia*). Contudo, estes métodos de datação nem sempre podem ser aplicáveis pois são fortemente dependentes de diversos fatores. O estudo do comportamento de diversos biomarcadores no organismo, por recurso a diversas técnicas analíticas, foi o mais recente avanço sentido pelos patologistas. Apesar de algumas controvérsias na correlação entre a concentração da espécie analítica (proteínas, enzimas, eletrólitos, aminoácidos, etc.) e o intervalo pós-morte, estes metabolitos permanecem como um alvo de estudo.

Considerando o aumento da produção de aminas biogénicas após a morte, o objetivo deste trabalho foi desenvolver um sensor confiável para a histamina a fim de ser aplicado e validado no campo forense.

Para o desenvolvimento do sensor de histamina compararam-se duas moléculas macrocíclicas, (cucurbit[6]uril e α -ciclodextrina), que foram testadas em diferentes solventes mediadores na presença ou ausência de um aditivo iónico. O tipo de polímero e a introdução de nanotubos de carbono de paredes múltiplas na membrana foram também avaliados. O sensor com as melhores características analíticas apresentava 1,0%(m/m) de cucurbit[6]uril; 66,8%(m/m) de 2-nitrofenil octil éter; 29,8%(m/m) de cloreto de polivinilo; 0,3%(m/m) de tetrakis (4-clorofenil)borato de potássio e 2,0%(m/m) de nanotubos de carbono de paredes múltiplas. Um declive de $30,9 \pm 1,2$ mV/dec, um limite de deteção de $(3,00 \pm 0,61) \times 10^{-7}$ mol/L e um limite inferior de resposta linear de $(3,00 \pm 0,00) \times 10^{-7}$ mol/L foram as principais características de resposta do sensor desenvolvido para a determinação de histamina. O seu acoplamento num sistema de análise por injeção sequencial originou um comportamento semelhante, sempre que se usou um volume de amostra de 197 μ L, com um caudal de bombagem até ao detetor de 30 μ L/s. A percentagem de recuperação foi determinada para três níveis de concentração de histamina, nomeadamente 1×10^{-4} , 1×10^{-5} e 1×10^{-6} mol/L, cujos valores obtidos foram $(97 \pm 6)\%$; $(103 \pm 1)\%$ e $(118 \pm 9)\%$, respetivamente.

Palavras-chave: Potenciometria, Eléktrodos seletivos de iões (ESI), Cucurbit[6]uril, Histamina, Amostras biológicas

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Equation 1 - Nikolsky-Eisenman equation: E – Potential value (mV); E_0 – Constant that includes standard potential of the electrode, the reference electrode potential and the junction potential (mV); Z_A and Z_B – Primary ion charge and interfering ion charge; a_A and a_B – Primary ion activity and interfering ion activity (mol/L or mol/Kg); **$K_{A,B}^{pot}$** – potentiometric selectivity coefficient; R – Universal constant of ideal gas 8.3144 J/K/mol; T – Absolute temperature (K); F – Faraday constant 9.6486×10^4 C/mol. 14

CB[6] – Cucurbit[6]uril
CE – Capillary electrophoresis
DAO – Diamine oxidase
DBS – Dibutyl sebacate
FI – Flow injection
FNDPE – 2-fluorophenyl 2-nitrophenyl ether
GC – Gas Chromatography
HNMT – N-methyltransferase
HPLC – High performance liquid chromatography
ISE – Ion-selective electrode
 $K_{A,B}^{pot}$ – Potentiometric selectivity coefficient
KTPCIPB – Potassium tetrakis (4-chlorophenyl) borate
LLLR – Lower limit of the linear range
MWCNTs – Multi-Walled Carbon Nanotubes
o-NPOE – 2-nitrophenyl octyl ether
OPA – o-phthalaldehyde
OPT – o-phthaldialdehyde
PDL – Practical detection limit
PMI – Post-mortem interval
PVC – Polyvinyl chloride
PVC-COOH – Polyvinyl chloride carboxylated
SIA – Sequential injection analysis
SI-LOV – Sequential injection – Lab-on-Valve
SPE – Solid phase extraction
THF – Tetrahydrofuran
UV - Ultra-violet
 α -CD – alfa-cyclodextrin

General Introduction

The post mortem interval (PMI) is a common indicator in forensic practices, which evaluation is permanently challenging. It is defined as the time between death event and the moment that the body is found. The evaluation of PMI is crucial as it can contribute to the reconstruction of the crime scene, including the form and circumstances of death, to distinguish a suicide from murder, to establish a suspect's connection to the crime(s) scene(s) and to validate information provided by witnesses. From the point of view of the criminal law a precise estimation of PMI also helps to limit the number of suspects validates and establish the time slots for telephone or CCTV (Closed-circuit television) footage records associated with the murder investigation (1).

Classical methods based on temperature measurements (*Algor Mortis*) (2-4), corpse stiffness (*Rigor Mortis*) (5-7), formation of hypostasis or lividity (*Livor Mortis*) (8, 9), states of decomposition (*Putrefaction*) (10), formation of wax (*Adipocere*) (11-14) and the insect life cycle (*Entomologic methods*) (15) can be used to estimate the PMI. The precise assessment of these changes at a specific time can be used to estimate the time elapsed after death. However, these methods are based on subjective evaluation and inapplicable whenever there is total degradation of soft tissues and there is only skeleton. Besides the possibility of changes in the crime scene, these methods become time consuming and cannot be performed in situ, resulting in a delayed and in a less efficient investigation (16). Furthermore, the interpretation of the results depends on external factors such as temperature, humidity and pH (7, 9, 13, 17). In entomologic methods, several issues compromising reliable estimates must be accounted, namely associated with myiasis (18), drugs consumption (19), larval-mass effect (20), fluctuating temperatures (21), under size larvae (22) and effect of the photoperiod (23). To overcome all these problems, chemical and biochemical techniques that provides fast response, sensitivity and specificity, can improve the forensic evidences.

Luminescence (24, 25), radiocarbon dating (radiochemistry) (26, 27) and the determination of biochemical markers are the main alternatives used by forensic pathologists. However, luminescence and radiochemistry are both dating techniques choose to evaluate longer post-mortem interval. Biochemical markers, some of them are unchanged after death while others are extremely important because suffer variations in its concentrations with the time after death (table 1).

Table 1 - Behavior of the biochemical markers after death

Biochemical markers	Behavior after death
Electrolytes	
Sodium	Decrease of the serum and vitreous humor concentration (28-32)
Chloride	Decrease of the serum and vitreous humor concentration (28, 29, 32)
Potassium	Increase of the vitreous humor concentration (33) with a linear relationship (34-37)
Magnesium	No significant variations in serum samples (38, 39) or a slowly increase in vitreous humor (40-42)
Calcium	No significant changes in serum and synovial fluid after death (43-46)
Phosphate	Increase of the concentration in pericardial and ocular fluid (47-49)
Carbohydrates	
Lactate	Elevated levels in blood and cerebrospinal fluid after death (50-52)
Glucose	Decrease of the blood concentration (51, 53, 54)
Proteins	
Cardiac Troponin-I	Linear increase in heart tissue (55)
Cardiac Troponin-T	Increase of the concentration in serum, pericardial fluid and cardiac tissues (56-59)
Enzymes	
Aminotransferase	Increase of the levels in serum (60, 61)
Lactate dehydrogenase	Increase of the concentration with a linear relationship in serum and human liver (62, 63)
Nucleic acids	
Deoxyribonucleic acid	Increase of DNA fragmentation in blood and different tissues (64-66)
Ribonucleic acid	Stable values in brain after death (67-69) or a rapidly increase of the blood concentration (70)
Nitrogenous compounds	
Uric acid	Stable concentration in serum, vitreous humor and pericardial fluid (71, 72) or a decrease of the blood levels (73)
Creatinine	Stable levels in serum, vitreous humor and pericardial fluid (71-75) or a markedly increase in blood (76)
Urea nitrogen	Stable concentration in pericardial fluid and serum (72-75) or a slight rise in urea (71, 77)
Ammonia	Increase of the vitreous humor concentration (78)
Xanthine and hypoxanthine	Increase of the levels in vitreous humor and cerebrospinal fluid (79-81) with a linear correlation in vitreous humor (82, 83)
Amino acids	Significant increase in vitreous humor and cerebrospinal fluid concentrations (84)

Along the post-mortem period, specific amino acids undergo decarboxylation. Therefore, the quantification of resulting products can be useful to determine the time elapsed since death. Biogenic amines are an example. They are a group of basic nitrogenous compounds of low molecular weight (85) formed by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (86). Putrescine, cadaverine and histamine are biogenic amines obtained by the decarboxylation of amino acids and are typical examples associated with the state of putrefaction of the organic tissues of decomposing bodies. The profile of these biogenic amines is still somewhat unknown; therefore, it is important to evaluate and identify a possible correlation between the concentration and post-mortem interval. In this way, a new biomarker can be proposed.

1. Histamine

The chemical compound (2-[4-imidazolyl] ethylamine) commonly named as histamine was discovered by Dale and Laidlaw in 1910 (87). It is a polar and/or protonated molecule belonging to a group of metabolites called by biogenic amines (88). In that sense, it is the result of the enzymatic decarboxylation of the amino acid histidine catalyzed by the enzyme L-histidine decarboxylase (figure 1). Histamine is synthesized and released from diverse cells, namely mast cells, basophils, platelets, histaminergic neurons, and enterochromaffin cells. Depending on its location histamine can be metabolized according two different pathways: by diamine oxidase (DAO) also called histaminase (89), or by ring methylation by histamine N-methyltransferase (HNMT) (90). While DAO may be responsible for scavenging extracellular histamine after mediator release (e.g. after ingestion of histamine-rich food), the second most important enzyme inactivating histamine, HNMT, is a cytosolic protein which can convert histamine only in the intracellular compartment (91, 92). This way, the catabolism of histamine can originate different compounds in organism according to figure 1.

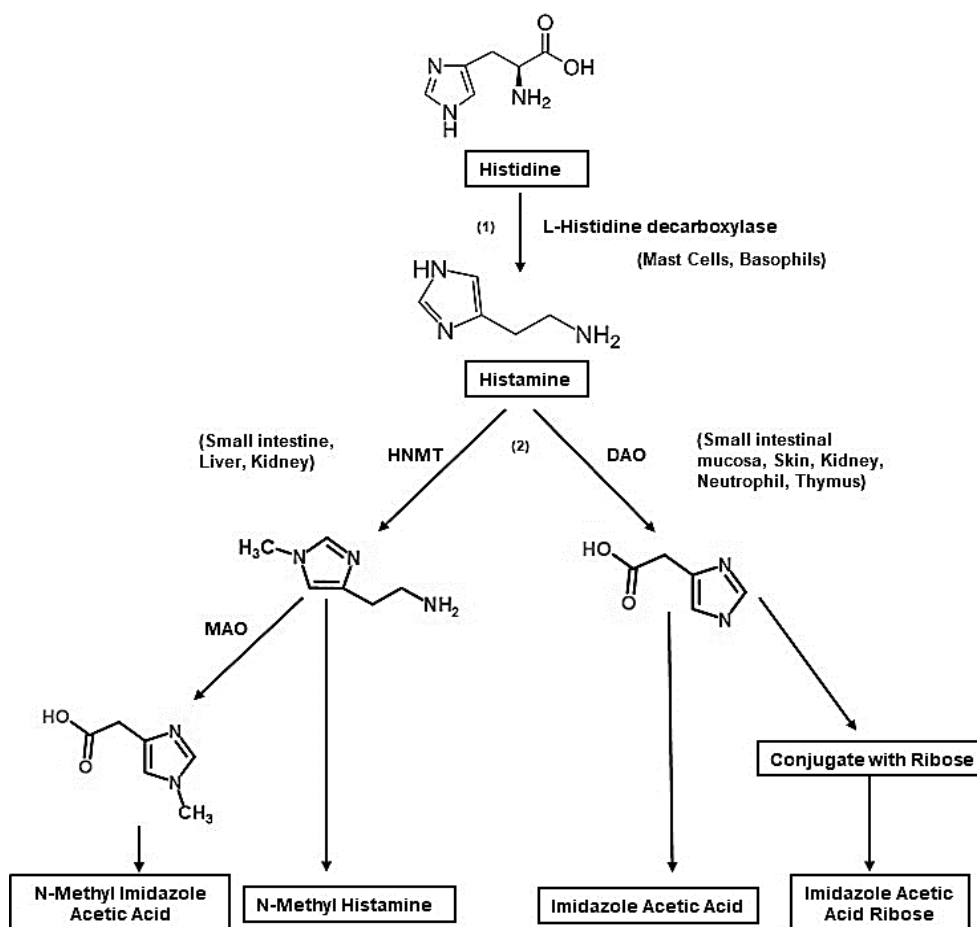


Figure 1- Synthesis and catabolism of histamine. Metabolic pathways and different metabolites of histamine. Image adapted from (93).

The biological role in organism is related to the affinity for diverse types of histamine receptors (H1R, H2R, H3R, and H4R) on target cells in various tissues. Both, H1R and H2R receptors have a wide distribution and are present in most cells and tissues (94). The H3R is mainly expressed in the central nervous system and is associated with several neural diseases such as schizophrenia, sleep/wake disorders, epilepsy and neuropathic pain. The H4R is the newest identified member of the histamine receptor family and is expressed in hematopoietic cells.

1.1. Physiological role of histamine

Several roles have been assigned to histamine and consequently, its determination has been proposed in different matrices and in different contexts. For instance, it is commonly reported as an indicator of food deterioration and it is regarded as a biomarker for quality control during the food production and transportation. Many studies were done to determine histamine in different matrices such as fish and fisher products (95, 96), meat (97, 98), wine and beers (99, 100). Maximum admissible levels were establishing for histamine content in the food products by US Food and Drug Administration (FDA). Good quality fish should contain less than 10 mg/kg histamine. In turn, the level of 30 mg/kg indicates significant deterioration and a level of about 50 mg/kg is a definitive decomposition (101). A risk assessment study proposed a possible threshold of 500 mg/kg for cheeses and 400 mg/kg for fermented sausages (102).

The interaction of histamine with different cellular compartments (mast cells, basophils, glial cells, endothelial cells and neurons) is fundamental for the regulation of diverse physiological functions such as neuroendocrine system, circadian rhythms, psychomotor activity, learning, cognition and eating behavior (103). Alterations in histaminergic system have been reported in numerous pathological situations such as epilepsy, stroke, anxiety, depression, neurodegeneration and neuroinflammatory processes (Schizophrenia, Alzheimer and Parkinson diseases) (104-107).

During an allergenic reaction, histamine is the most important inflammatory mediator and it is released in high concentrations when mast cells and basophils are active after contact with a specific allergenic antigen (108, 109). Histamine plays a significant role in anaphylaxis. Anaphylaxis is the most severe form of an acute IgE-mediated allergenic reaction (type I hypersensitivity reaction) (110). It can cause different clinical manifestations such as nausea, vomits, asthma, diarrhea, urticaria, bronchospasm, edema of larynx and pharynx (111) or cause death due to cardiovascular collapse (anaphylactic shock) (110). Sudden anaphylactic death occurring outside the hospital are usually subject to forensic autopsies, but the diagnosis of anaphylactic shock is often difficult in autopsy because morphologic changes are nonspecific. Prognosis is based on the natural history of some

allergies, the amount of allergen required to trigger reactions (112) and on circumstantial evidence such as ingestion of certain foods, administration of antibiotics, anti-inflammatory drugs, anesthetics, contrast reagents and extracts of allergen (113).

In vivo, the histamine is a biomarker used for indicating mast cell activation. The normal levels for different biological matrices are indicated such as <1.0 ng/mL for plasma (114), < 0.5 ng/mL for serum (115), $(6-131) \times 10^3$ ng/24 hours for urine 24 hours (116) and 20.0-200.1 ng/mL for whole blood (117). The number of visible mast cell might substantially reduce in the post mortem tissues in anaphylactic situations. Measurement of mast cell degranulation involve histamine levels determination in body fluids, however the latter is readily metabolized in postmortem situations (half-life is minutes). It would be possible to measure its degradation product methylhistamine, which is more stable in urine, but again in sudden deaths significant amounts of methylhistamine might not have had time to appear in urine (118). It is necessary to be careful in histamine determination because it may originate from other cells during anaphylaxis or during sample processing (119).

After death, with increasing decarboxylation of amino acids, an increase in histamine levels occurs and these can be correlated with post-mortem interval. There are no established values for this parameter in the literature and as far as the author know no studies correlates them with time after death. So, it could be an opportunity to evaluate the histamine concentration in different organs and biological fluids as a possible biomarker for the forensic pathologists.

1.2. Analytical methods for histamine determination

Histamine is a relatively small, freely diffusible water-soluble compound of considerable biologic significance, generally at low concentrations in complex matrices (120). There are diverse methods for the determination of the histamine concentration, namely colorimetric (121, 122) and fluorometric methods (122, 123), enzyme-linked immunosorbent assay (ELISA) (124) and chromatography using kinds of detection (95, 125-127). These methods are characterized by analytical parameters when developed and optimized before to the analysis of the real samples containing histamine (table 2).

Table 2- Analytical parameters of the different methodologies described in literature for the determination of histamine

Analytical method	Sample	Linear range of the concentration (mol/L)	Detection limit (mol/L)	Interferences
Colorimetric	Fish samples (121)	8.90×10^{-5} - 5.40×10^{-3}	8.90×10^{-5}	----
Fluorometric	Seafood (128)	5.00×10^{-7} - 1.50×10^{-5}	5.00×10^{-8}	Histidine
	Brain tissues (129)	9.00×10^{-8} - 9.00×10^{-5}	9.00×10^{-8}	Spermidine
	Cheese (124)	9.00×10^{-5} - 9.00×10^{-3}	2.00×10^{-5}	----
Enzymatic assays	Korean pastes (130)	0.0 - 4.50×10^{-4}	2.00×10^{-6}	----
	Fish food (131)	0.0 - 9.60×10^{-5}	4.80×10^{-7}	----
Capillary electrophoreses	Pharmaceutical formulations (132)	1.80×10^{-5} - 7.20×10^{-4}	6.74×10^{-6}	No significant interferences
	Wine samples (133)	0.0 - 0.1	2.00×10^{-6}	----
High performance liquid chromatography	Brain tissues (126)	2.00×10^{-9} - 5.00×10^{-8}	4.00×10^{-10}	Amino acids
Gas-chromatography	Fish products (95)	3.60×10^{-5} - 3.60×10^{-2}	4.50×10^{-5}	----
	Fish sample (125)	9.00×10^{-4} - 9.00×10^{-3}	6.30×10^{-5}	----
Biosensors	Food products (134)	1.00×10^{-6} - 5.00×10^{-5}	5.00×10^{-7}	Lysine
	Fish products (135)	3.00×10^{-7} - 2.00×10^{-5}	1.70×10^{-7}	Amino acids
	Freshwater fish samples (136)	1.00×10^{-7} - 3.00×10^{-4}	2.54×10^{-8}	Biogenic amines and amino acids
Ion-selective electrodes	Human serum (137)	2.50×10^{-6} - 1.00×10^{-1}	1.00×10^{-6}	----
	Fish samples (138)	1.00×10^{-5} - 1.00×10^{-2}	4.70×10^{-6}	Biogenic amines
	Serum samples (139)	1.00×10^{-6} - 1.00×10^{-1}	5.00×10^{-7}	

A short description of those methods is introduced below to enhance true potentialities. The methods described will not be restricted to the forensic field.

1.2.1. Biochemical assays

Different biochemical assays of histamine in tissues and fluids have been developed over time such as colorimetric, fluorometric and enzymatic assays. Colorimetric assay involves a reaction between the purified histamine and chromophore. The color intensity of the product is proportional to the histamine concentration (121). The colorimetric assay has some advantages since it is easy to use, low cost, less technical skill requirement and has reasonable sensitivity (140). However, this method is more appropriate when elevated levels are naturally expected.

In turn, the chemical method leading to generation of fluorescent compound shows improved selectivity (141). Fluorometric assay has been modified for the measurement of low concentrations of histamine in different matrices such as urine, blood and small samples of tissues and body fluids. It is based on the reaction of histamine with o-phthaldialdehyde (OPT) at a highly alkaline medium to form a strong and stable fluorescent condensation product (123). The combination of the two aldehyde groups of OPT in the ortho position with the structure of the analyte increases the selectivity of the reaction because the commonly co-existing amines and amino acids do not interfere. Particularly if the reaction conditions are strictly controlled, even structurally similar compounds (e.g. histidine) do not interfere significantly (128). The fluorescence intensity of the histamine-o-phthaldialdehyde complexes is greater by one or two orders of magnitude than the known o-phthaldialdehyde complexes. Moreover, interferents can be separated from histamine by means of a DOWEX 50 ion exchange resin (142) or phosphoric acid cellulose (Cellex-F) (143). Solid phase extraction (SPE) as sample pretreatment step was also proposed to minimize spectral interference from histidine or other compounds (128). Hurahashy and Fujiwasa (129) concluded that the fluorometric method is simple, precise and sensitive.

Enzymatic methods can be an alternative method due to the well-known selectivity of the complex enzymes-substrates (115, 130). According to Lee (130), the obtained results display a good correlation with those determined by HPLC in the same samples. Histamine dehydrogenase (HDH) from *Rhizobium*, is a more selective alternative than histamine oxidase, but with identical affinity to putrescine and tyramine (131). Enzymatic methods allow a rapid histamine determination, but its kits are still expensive and sometimes more expensive than other methods (144). Immunoassay kits became popular due to their portability and they have been used for screening purposes (145). These methods are

described as sensitive, specific and accurate but require considerable number of samples at each run to be viable (146, 147). They are normally applied for the detection of histamine in food, human serum, and biological fluids (124). Most immunochemical methods requires a specific antibody against the compound to be measure (148). The major difficulty relies on the preparation of an antibody specific for histamine (147). Furthermore, it is necessary a previous chemical derivatization with *p*-benzoquinone, which is a toxic reagent (149, 150) or propionic acid esters, which either is time-consuming (151). Schneider et al.(152) describe a method to produce polyclonal antibodies against the parent compound, free histamine and its use in enzymatic immunoassay. Since the kits are expensive and it is necessary to carry out a calibration for each measurement (at least five determinations) new alternatives are proposed.

1.2.2. Capillary electrophoresis

In the last years, capillary electrophoresis appeared as a powerful analytical technique for the separation and quantification of many substances, especially when analyses do not require derivatization procedures (153). This technique has various advantages, such as the use of small sample volumes, low consumption of reagents, high separation efficiency, short analysis time and easy conditioning of columns (132). In 1998, Arce et al. (133) developed a flow-injection (FI) manifold to couple automatic derivatization to capillary electrophoresis with indirect ultra-violet (UV), aiming detection of biogenic amines in wine. The FI system afforded ion-exchange cartridges to allow clean-up of the wine samples and performed a preconcentration step prior analysis by CE. Therefore, the determination of a wide range of biogenic amines in less than 10 minutes was feasible. In 2002, Lange et al. (154) compared capillary electrophoresis and high-performance liquid chromatography methods to analyze biogenic amines in food. No laborious sample pretreatment was necessary to determine histamine by CE, while at least an automated precolumn derivatization with *o*-Phthalaldehyde (OPA) was required for the determination by HPLC. Furthermore, biogenic amines were separated by EC and HPLC in less than 9 and 20 minutes, respectively. Sun, Yang and Wang (155) proposed a method to separated and quantify different biogenic amines in milk by capillary electrophoresis with pulsed amperometric detection (CE-PAD). The procedure without the derivatization process, exhibited higher sensitivity than the one obtained by CE-UV. The method required small volumes of sample and was simpler to operate regarding GC or HPLC. However, alkalization of the eluent phase prior to detection was necessary and the detection cell required special maintenance (156). In 2004, Zhang and Sun (157) developed a rapid and sensitive method for simultaneous determination of histamine and histidine by capillary

zone electrophoresis with lamp-induced fluorescence detection, permitting its determination in P815 mastocytoma cells and beer samples. In 2006, a sensitive and quick (less than 15 minutes) capillary zone electrophoresis (CZE) method with conductometric detection was described for food samples analysis (158). The developed method was successfully applied, and some advantages were reported such as: the less laborious (is not require derivatization step or sample cleaning, but only a dilution or acidic extraction and filtration), low running cost, enough sensitivity and environmentally friendly. In 2008, it also developed and validated a capillary electrophoresis method with diode array detection for quantitative analysis of histamine in cheese (153).

1.2.3. Chromatography methods

1.2.3.1. High Performance Liquid Chromatography

The high-performance liquid chromatography is the usual choice for the simultaneous detection and quantification of biogenic amines, including histamine (126, 159). Almost all the procedures for HPLC analysis propose indirect detection, either UV or fluorescence, following by pre- or post-column chemical derivatization (134). This step is essential because the most of the biogenic amines don't have chromophore or fluorophore (154). Most frequently used derivatizing agent is the OPA, which leads to fluorescent reaction products providing sensitive detection. The reaction is strongly dependent of pH and requires a purification step of the sample for good resolution and high sensitivity (160). Nevertheless, these procedure steps add complexity to the determination, requiring additional skilled technicians, are time-consuming and can produce by-product interferences (120), resulting in low reproducibility (161).

1.2.3.2. Gas Chromatography

Gas chromatography is not often applied for histamine determination due to the incomplete histamine elution from the columns and peak tailing. In addition, the procedures require a derivatization step (125). However, there is reference to its determination by GC without this step (95). The method involves the histamine extraction with alkaline methanol before injection into GC. The reported detection limit is comparable with HPLC based methods with less procedure errors.

1.2.4. Sensors and biosensors

A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. The chemical information may originate from a chemical reaction of the analyte or from a physical property of the system investigated. Chemical sensors contain two basic functional units: a receptor part (convert chemical information into a form of energy) and a transducer part (transform the energy carrying the chemical information about the sample into a useful analytical signal). The receptor part of chemical sensors may be based upon various principles: physical, chemical or biochemical. In this latter case, the sensor is called a biosensor and a biochemical process is the source of the analytical signal (162). Some advantages are described for these devices like as quickness, reproducibility, accuracy, brief time analysis and low-cost detection (163).

In the case of histamine biosensors, the analytical response results from the reaction between the biogenic amine and amino oxidase, which convert the analyte in the corresponding aldehyde and generates ammonia (NH_3) and hydrogen peroxide (H_2O_2), both detected electrochemically. To circumvent the need to apply high potentials during the detection process a redox mediator to detect H_2O_2 at lower potentials (135, 164) or a combination of amine oxidases with peroxidases enzymes are used. Some authors refer the use of common amine oxidases such as DAO in construction of biosensors for the determination of histamine (134, 136, 165). However, there are different substrates for these enzymes, making the sensor selectivity low. It is described in literature that the use of highly specific enzyme (e.g. histamine dehydrogenase) produces a response targeted directly to the study molecule, and this way it is possible to increase the selectivity and the sensibility of the method (166).

The incorporation of molecularly imprinted polymers (MIPs) in the preparation of this type of sensing devices was also reported for histamine determination (167-169). These enzymatic polymers contain imprinted nanocavities that bear the exact shape, size and functional groups that are complementary to its target molecule. Some of the advantages of these compounds are related to the low cost of synthesis, its chemical robustness and to be inert over a wide range of different environments. They are suitable for detection of small molecules, show selectivity and specific covalent reversible binding characteristics and they are stable over a wide temperature and pH range (167, 168). Similarly, the Multi-Walled Carbon Nanotubes (MWCNTs) have been reported as suitable to be used as modifiers for electrodes due their exceptional properties including high conductivity, high chemical and thermal stability, and high elasticity (170).

Sensors based on electrochemical techniques, namely potentiometric sensors are an excellent alternative to the spectroscopic techniques due to be simple and faster, justifying its use.

1.2.4.1. Potentiometric sensors

Potentiometry based in ion-selective electrodes (ISEs) is one of the oldest and probably the most commonly used instrumental method of chemical analysis. This electroanalytical technique has been widely used in clinical, environment, food and pharmaceutical analysis (162, 171, 172). Potentiometry is based on the measurement of the electromotive force (emf) developed by an electrochemical cell (indicator and reference electrodes) immersed in the sample under zero-current flow conditions in an overall electrochemical cell arrangement (figure 2).

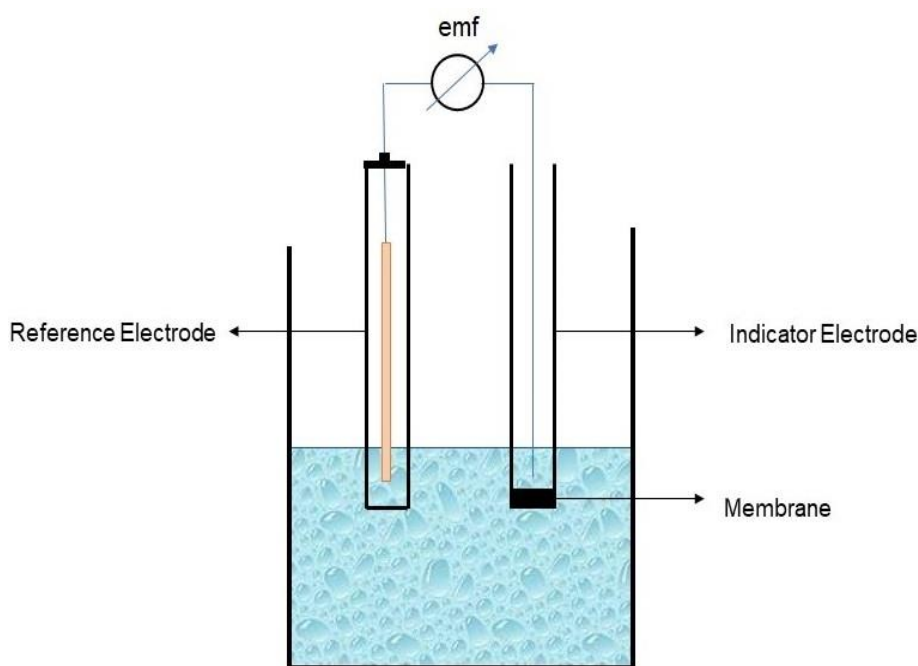


Figure 2- Scheme of a potentiometric cell.

The indicator electrode contains a selective membrane optimized according to the target ion to be recognized and measured. The variation of the concentration of the target ion in the solution is determined according to the electrode potential change. For dilute solutions or with ionic strength adjusted, the concentration match with the ionic activity and a numerical relationship can be established (according equation 1). However, real samples

contain other chemical species, and therefore, the potentiometric response can be defined by the following equation:

$$E = E_0 + \frac{RT}{Z_A F} \ln \left(a_A + \sum K_{A,B}^{pot} (a_B)^{Z_A/Z_B} \right)$$

Equation 1 - Nikolsky-Eisenman equation: E – Potential value (mV); E_0 – Constant that includes standard potential of the electrode, the reference electrode potential and the junction potential (mV); Z_A and Z_B – Primary ion charge and interfering ion charge; a_A and a_B – Primary ion activity and interfering ion activity (mol/L or mol/Kg); $K_{A,B}^{pot}$ – potentiometric selectivity coefficient; R – Universal constant of ideal gas 8.3144 J/K/mol; T – Absolute temperature (K); F – Faraday constant 9.6486×10^4 C/mol.

The main advantages of ISEs are its portability, are relatively inexpensive, simple to use, selectivity, low detection limit and have an extremely wide range of applications and wide concentration range (137, 173). Even more, the optimization of the membrane constituents can improve the detection limits at the picomolar level (174, 175) as also its potentiometric characteristics (176). Different selective membranes are described in literature for histamine determination (137-139, 177). Polymeric membranes consist of four components: (a) polymer matrix; (b) solvent mediator; (c) ionophore and (d) lipophilic additive.

The polymer provides mechanical stability to the membrane and increase its elasticity. Polyvinyl chloride (PVC) is the most commonly used but ethylene-vinyl acetate (178) and polystyrene (179, 180) can also be used .

The introduction of the solvent mediator contributes to the decrease of membrane viscosity and allow greater spacing of the polymer chain helping the mobility of membrane constituents. Certain characteristics such as polarity, lipophilicity, molecular weight, dissolution capacity and vapor pressure must be taken into account when preparing the sensing membrane (181). These variables, mainly lipophilicity, affect the ion extraction of organic phase and the complexation process with ionophore, so having influence in the selectivity and potentiometric response. There are many solvents that can be used in membrane preparation but the most common are phthalates (e.g. dibutyl phthalate) and phenolic ether derivatives (e.g. 2-nitrophenyl octyl ether) (182, 183).

The membrane selectivity is also related to the type of ionophore and the presence of ionic additives. The latter are ion-exchangers that promote the diffusion of some ions through the membrane interface and excluding others. The most common additive includes lipophilic molecules with high molecular weight such as quaternary ammonium or phosphonium salts and tetra-alkyl borates (184).

The ionophore or “ion carrier” is the key component in polymeric membranes in terms of selectivity. It is an organic compound with ability to selectively bind anions or

cations. There are many aspects that justify the interaction between analyte and ionophore namely if the size of analyte ion fits in a cavity in the ionophore structure, if several functional groups of the ionophore may specifically bind the ion target (185) and the binding strengths between ionophore and ions (186). Ionophore can be an ion exchanger or a macrocyclic compound, and they are classified according to the chemical nature and charge of the chemical specie to quantify. There are many ionophores that can be used in ion-selective membrane constitution (187) such as crown ethers (188), calixarenes (189, 190), metalloporphyrins (191, 192), and complexing agents like as cyclodextrin (193-195) and cucurbit[n]uril family.

Cucurbituril as ionophore

The first work published about the macrocycle cucurbit[n]uril, CB[n], was reported in 1905 and describes the results of the acid-catalyzed condensation reaction between glycoluril and formaldehyde (196). The chemical nature and structure of CB[n] had been unknown until 1981. In 1981, Freeman et al. (197) identified the molecular structure of CB[6], and more later, other macrocycles were isolated by changing the experimental conditions, such as CB[5], CB[7], CB [8] and CB[10] (198). The name of cucurbituril is due to the similar structure to a pumpkin, which belongs to the botanical family *cucurbitaceae*. These compounds family are made from n glycolurils bridged by $2n$ methylene groups and they are named according to the number of glycoluril units (figure 3) (199).

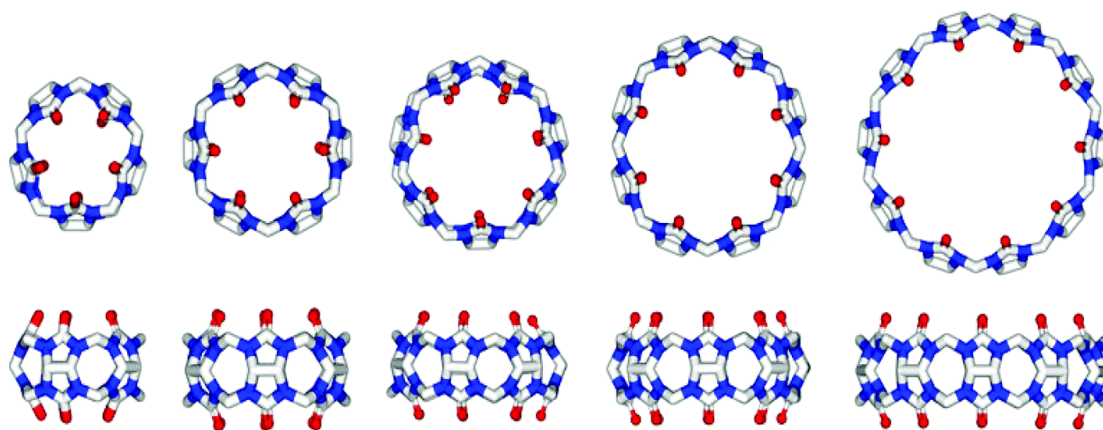


Figure 3- Chemical structures for cucurbit[n]uril family: cucurbit[5]uril, cucurbit[6]uril, cucurbit[7]uril, cucurbit[8]uril and cucurbit[10]uril containing 5,6,8 and 10 units, respectively. Image from (200).

These macrocycles have highly symmetric structure, with negatively charged carbonyl rims (201). They have a hydrophobic cavity, and the cavity sizes of CB[6], CB[7] and CB[8] are equivalent of α -, β - and γ - cyclodextrin, respectively. Although the cavity size of the cucurbiturils with cyclodextrins is similar, they have different chemical properties. While cyclodextrins are natural products formed of chiral glucopyranose units (chiral molecular receptors), cucurbiturils are achiral compounds that exhibit an equatorial symmetric plane allowing an identical opening of the cavity (202). In general, the cucurbiturils have higher binding constants, much higher binding ratios, and a high affinity for guests carrying a cation or guests with some degree of hydrophobicity. As these compounds have a higher guest binding rate than cyclodextrins, they are more attractive for biological applications (203). Cucurbituril are soluble in acidic solution or aqueous solution containing alkali metal ion, and it has low solubility in common solvent, except CB[5] and CB[7] which have a moderate solubility in water. The cucurbituril family has high thermal stability, so the decomposition process is only observed up to 420°C for CB[n] (n=5,6, and 8) and 370°C for CB[7] (204). The rigid structure and capability of forming stable complexes with molecules and ions (such as drug molecules, amino acids and peptides, saccharides, dyes, hydrocarbons, and proteins) make them good candidates in supramolecular chemistry including recognition, catalysis, separation, and transport (205). It is described in literature other applications as well as artificial ion channels, vesicles, stationary phases in chromatography, ISEs, polymers, and nanomaterials (206).

In the ion-selective electrodes, these macrocyclic receptors allow fabrication of membrane electrodes for ion sensing. They form complexes with the target ion. In the case of the amino acids, the substrates lysine, arginine, histidine and ornithine have low affinity for the cucurbit[n]uril family because they are weak competitors and did not interfere with the formation of the complex. Decarboxylation produces the corresponding amines (cadaverine, agmatine, histamine, putrescine) which exist in their ammonium ions form. The high affinity of these products is a directly consequence of the cation receptor propensity of cucurbiturils because the decarboxylation increases the net positive charge and thereby the affinity of the competitor by removal of the carboxylate group (207).

As discussed so far, there is an opportunity to evaluate histamine as a forensic biomarker to estimate the post-mortem interval.

The advantages of ion selective electrodes seem to be an interesting alternative to the other analytical methods found in the literature, since is simples, accessible, fast, inexpensive, accurate and reliable.

In this way, the aim of this work was to develop a potentiometric sensor for histamine determination based on cucurbit[6]uril as ionophore. The influence of the different constituents (ionophore, additive, polymer and mediator solvent) and its proportions in the membrane will be optimized. The sensor will be evaluated in blood serum samples.

Experimental Procedure

1. Reagents and solutions

Deionized water (conductivity $<0.1 \mu\text{S/cm}$) and analytical grade chemicals were used without further purification, unless otherwise stated. All weighing's were performed on an analytical balance (KERN, ACS 80-4).

Polyvinyl chloride carboxylated (PVC-COOH, ref. 18-331-95) was from Janssen Chimica; polyvinyl chloride (PVC, ref.81392), potassium tetrakis(4-chlorophenyl) borate (KTPCIPB, ref.60591), α -cyclodextrin (α -CD, ref.28705-256), 2-fluorophenyl 2-nitrophenyl ether (FNDPE, ref.47390-5ML-F), ammonium chloride (ref. 09702) were from Fluka Analytical; tetrahydrofuran (THF, ref.186562), cucurbit[6]uril (CB[6], ref.94544-1G-F), 2-nitrophenyl octyl ether (o-NPOE, ref.73732-25ML), dibutyl sebacate (DBS, ref.84838-5ML), magnesium chloride (ref.M8266-100G), calcium chloride dihydrate (ref.C8106), lithium chloride (ref. 310468-500G), histamine hydrochloride (HA, ref. H7250-5G), putrescine dihydrochloride (ref.P7505), cadaverine dihydrochloride (ref.33220) and multi-walled carbon nanotubes (MWCNTs, ref.308068-56-6) were from Sigma; potassium chloride (ref.26764.298) and sodium chloride (ref.27810.295) were from Analab NORMAPUR; sodium hydroxide (ref.28244.295) and formic acid (HCOOH, ref.131030.1611) were from Panreac; lithium hydroxide (LiOH, ref.1127980) was from Merck; sulfuric acid (ref.524541) was from Carlo Erba Reagents; ethanol abs. 100% a.r. (EtOH, CL00.0505.2500) was from Chem-Lab NV and methanol (MeOH, ref.20864.320) was from VWR chemicals.

The serum samples were obtained from healthy and volunteer patients. The samples to be analyzed by SI-LOV system were cleaned-up using a solid-phase extraction cartridge Water Oasis MCX 3cc (60 mg, ref.186000253).

2. Construction and evaluation of potentiometric histamine sensor

2.1. Preparation of conventional electrodes

For the preparation of conventional units hollow acrylic cylinders with dimensions of approximately 100 mm of length, 10 mm of external diameter and 7 mm of internal diameter were used (Figure 4a). The body of the electrode was filled with a conductive paste properly homogenized and formed by 33% (w/w) of Araldite M, 13% (w/w) of hardener REN HY5162 and 54% (w/w) of graphite powder (see Figure 4b). At the opposite end was placed a shielded coaxial cable with about 20 mm of length and the internal copper wire directly contacted the graphite conductive support (Figure 4c). The assembly was maintained for 48 hours at room temperature and after that, the cable was attached to the top with a rubber. Finally, the graphite surface was polished with sandpaper P100 to obtain a smooth surface

for deposition of sensor membrane and then cleaned with tetrahydrofuran to eliminate possible residues deposited in surface.

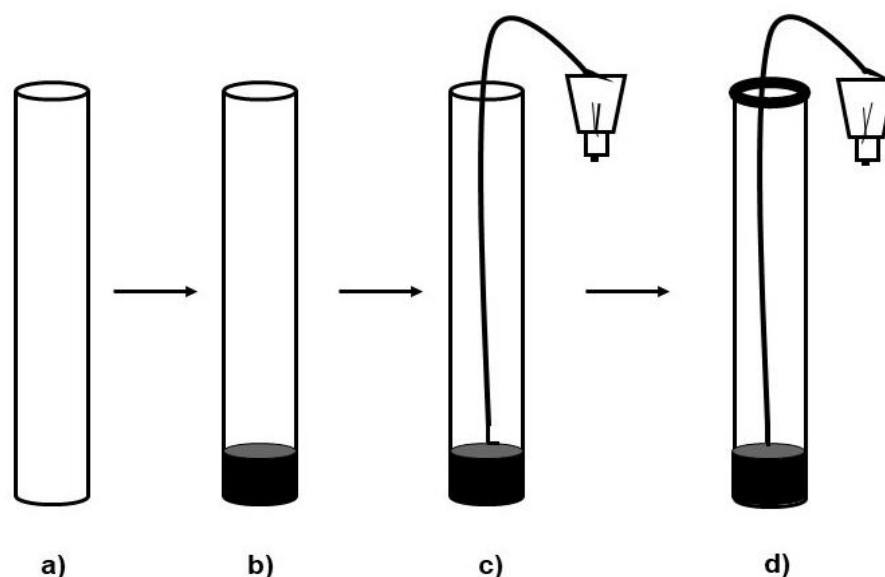


Figure 4- Preparation of conventional electrodes units with hollow acrylic tube (a) filled with conductive paste of graphite (b). A coaxial cable was introduced, and the internal copper wire directly contacted the graphite conductive support (c). Finally, the coaxial cable was attached to the top with a rubber. Image adapted from (184).

2.2. Polymeric membrane preparation

For the construction of the histamine electrodes, different sensor membranes were prepared to select the one with the best characteristics. All membranes were prepared by initial dissolution of ionophore in a plasticizer solvent in the presence or absence of lipophilic ion-exchanger. This solution was then mixed with 180 mg of PVC or PVC-COOH previously dissolved in tetrahydrofuran. Potassium tetrakis(4-chlorophenyl) borate was used as ion-exchanger and α -CD or CB[6] were used as ionophores. Three solvent mediators: 2-fluorophenyl 2-nitrophenyl ether, 2-nitrophenyl octyl ether and dibutyl sebacate were used in membranes preparation. Then, the membrane was directly dropped onto the graphite conductive of the electrode and dried at room temperature until the next day. Before each calibration, the electrodes were hydrated in water for 1-2 hours.

2.3. Calibration curves

The working characteristics of the electrodes were evaluated through the calibration curves, determining the following parameters: the slope, practical detection limit (PDL) and lower limit of linear range (LLLR). To a strict volume of buffer solution (0.01 mol/L HCOOH/LiOH, pH=4) increasing volumes of histamine solutions prepared in the same ionic strength adjustor were added. For each addition, the corresponding potential was recorded. Figure 5 is an example of the calibration curve. The slope corresponding to the linear part translates the sensibility of the measurement.

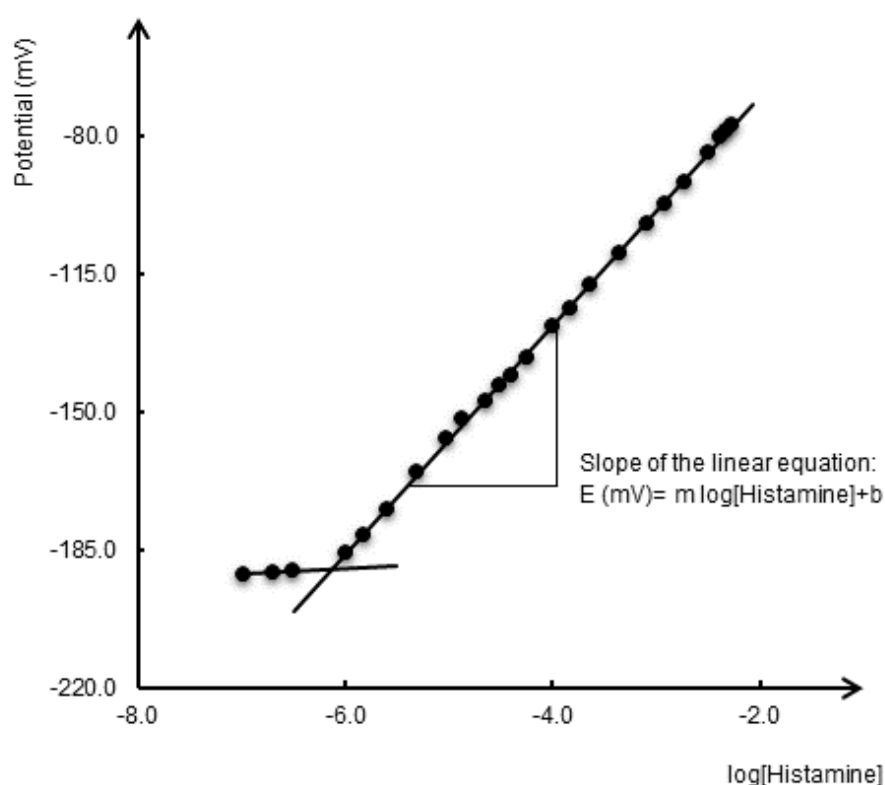


Figure 5 - Typical potentiometric response of a sensor based on a polymeric membrane (PVC) with potassium tetrakis(4-chlorophenyl) borate as additive and CB[6] as ionophore dissolved in o-NPOE.

According to Buck and Lindner (208), the practical detection limit is define as the activity (or concentration) of chemical specie which determines the potential corresponding to the point of interception of the two lines tangent to the calibration curve. The point corresponding to the intersection of the two lines represented in figure 6 the practical detection limit being the lower limit of linear range the first value of concentration where the electrode began to present linear analytical response in function of histamine concentration.

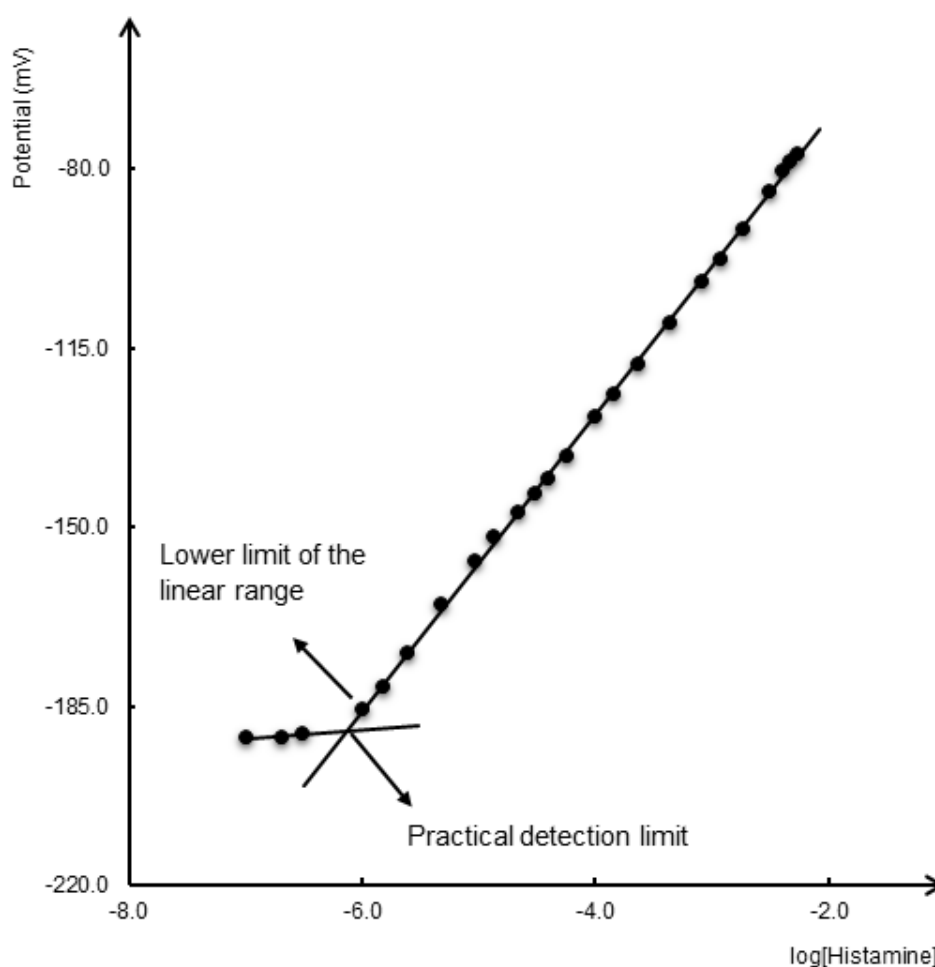


Figure 6- Determination of Practical Detection Limit (PDL) and Lower Limit of the Linear Range (LLLR) for the polymeric membrane with additive and CB[6] as ionophore dissolved in *o*-NPOE.

To perform the calibration curves, the histamine solutions were daily prepared. The stock solution of 0.01mol/L concentration was prepared by weighing about 184 mg of histamine reagent and afterwards transferred into a 100mL volumetric flask with subsequent dilution until the mark with a LiCl solution ($I=0.01\text{mol/L}$) or with a 0.01mol/L buffer solution (HCOOH/LiOH) at a pH 4. The standard solution with lower concentration was prepared by rigorous dilution with the same solvent.

An Orion 90-02-00 double junction AgCl/Ag reference electrode (with a 0.01 mol/L LiCl as an external reference solution) and the indicator (histamine electrode) were immerse in 20.00mL of buffer solution (HCOOH/LiOH) under constant stirring. The values of potential for each addition of standard histamine solution was measured with a decimillivoltmeter (CRISON, microPH 2002) (figure 7).

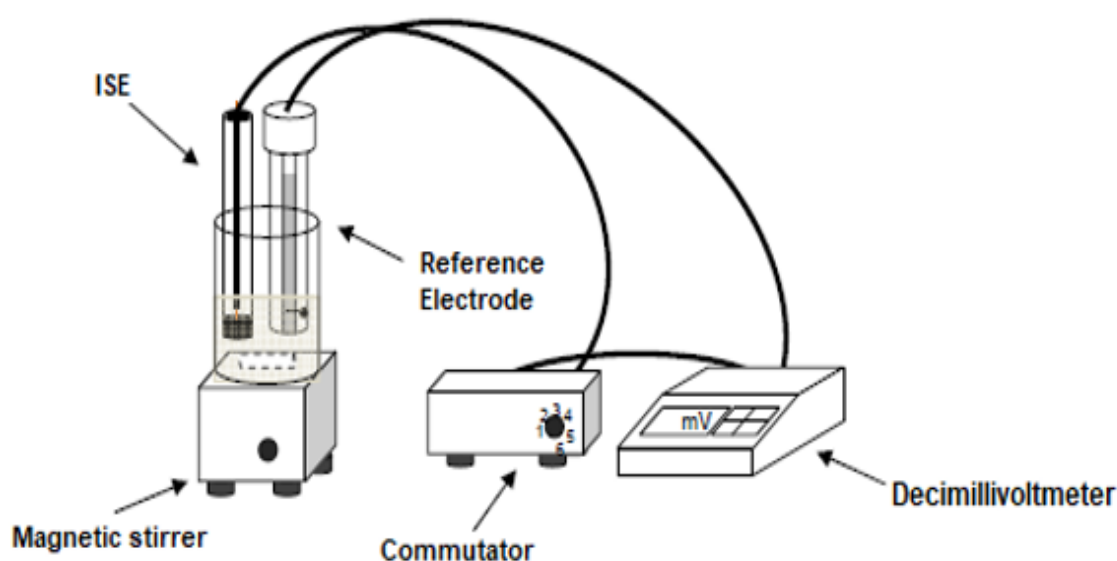


Figure 7- Assembly scheme used for the evaluation of conventional electrodes. Image adapted from (184).

2.4. Determination of response time

Some time-dependent properties such as response time, signal stability and lifetime, are notable features for sensor characterization. Response time has impact not only on batch measurements but above all on non-stationary conditions (flow conditions). According its definition (208), the evaluation of this parameter was carried out by measuring the time elapsed from each addition of histamine solution to the potential stabilization (potential variations less than 1mV) along the calibration curve. This parameter can be affected by many experimental conditions such as stirring and temperature of the solutions, the region of the calibration plot and memory effects to interfering (209).

2.5. Study of pH influence on the potentiometric response

The pH can condition the dissociation of the analyte in the sample as well as in the membrane and, consequently, influence the response of the electrode. Thus, the evaluation of the pH influence on the potentiometric response was evaluated through the Reilley Diagrams. The analytical assays were performed with histamine solutions of 1.00×10^{-4} and 1.00×10^{-5} mol/L without ionic strength adjusted. A volume corresponding to about 200mL of histamine solution was used for performing this assessment. The indicator electrode, reference electrode and pH electrode (Hach, sensION™+ pH31) previously calibrated with three solutions at pH=4, 7 and 10 where immersed in this solution. The pH of the solutions was changed by addition of concentrated sulfuric acid or concentrated sodium hydroxide solutions. For each pH variation, the correspondent potential was register.

2.6. Evaluation of the drift of electrical potential

The determination of the potential drift allows us to evaluate how the baseline potential for an ISE can vary over time. This determination was carried out by recording the electrode potential over time. This procedure was performed when the electrode is immersed in histamine solutions of 1.00×10^{-3} mol/L, 1.00×10^{-4} mol/L and 1.00×10^{-5} mol/L. A diagram of electrical potential vs. time was drawn for each concentration.

2.7. Determination of selectivity coefficient – study of some interfering substances

Potentiometric selectivity coefficient, $K_{A,B}^{pot}$, is defined as the ability of an ion-selective electrode to differentiate the main ion in the presence of interfering ions. The activities of the primary ion, A, and the interfering ion, B, at which $K_{A,B}^{pot}$ is determined should always be specified, as the value of $K_{A,B}^{pot}$ is defined by the modified Nikolsky- Eisenman equation (Equation 1). Highly selective electrode to a primary ion, $K_{A,B}^{pot}$ values are less than one unit. These values can be determined by different methods such as mixed solution methods (that includes fixed interference method; fixed primary ion method; two solution method and matched potential method) and separate solution method (210).

In this work, the selectivity coefficient was determined using the fixed interference method. To choose the best ionic strength adjuster, different interfering cations were studied. Therefore, potassium chloride, lithium chloride, sodium chloride, ammonium chloride, calcium chloride dihydrate and magnesium chloride were used. Calibrations curves with these salts were performed at fixed concentration of 1.00×10^{-2} mol/L. As shown in figure 8, the potential values obtained were plotted as a function of the logarithm of the primary ion activity (or concentration). The intersection resulting from the extrapolation of the linear portions of the curve correspond to the value of a_A which is to be used to calculate $K_{A,B}^{pot}$ from the equation: $K_{A,B}^{pot} = \frac{a_A}{(a_B)^{Z_A/Z_B}}$, where both Z_A and Z_B have the same signs, positive or negative (211).

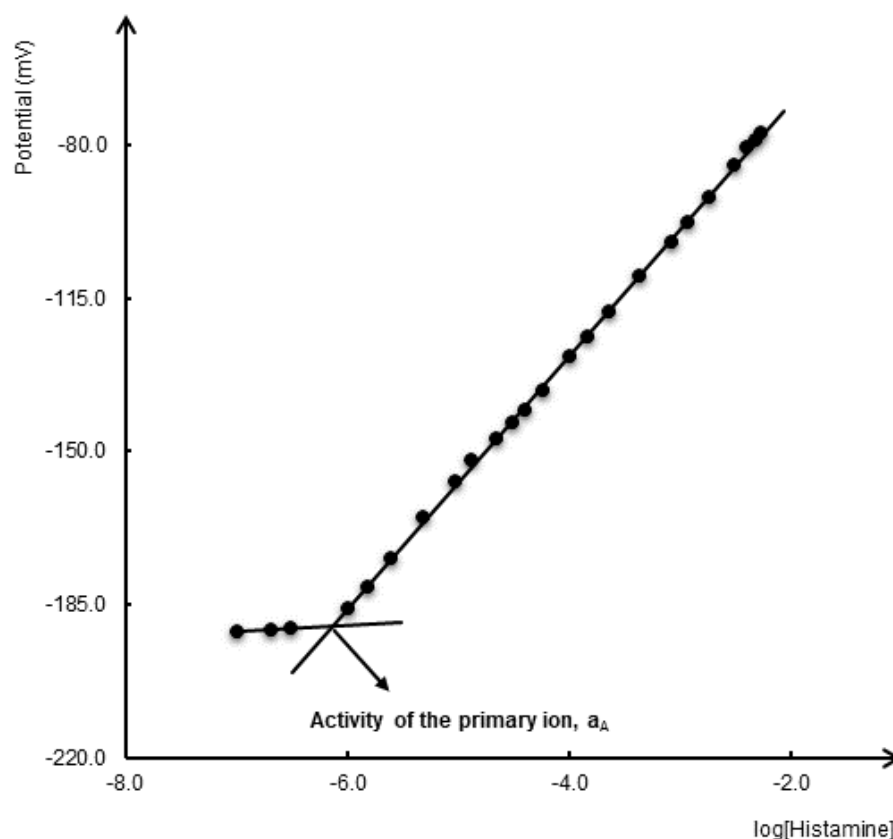


Figure 8 - Example of the determination of the activity of the primary ion

Putrescine ($C_4H_{12}N_2$) and cadaverine ($C_5H_{14}N_2$) are biogenic amines, with similar structure, that can be associated with the interval post-mortem because they are produced during of the process of putrefaction in decaying corpses. Consequently, it is important to determine the degree of interference of these chemical species in the analytical measurement with the histamine electrode. Therefore, calibration curves were carried out for putrescine and cadaverine in solutions with adjusted ionic strength. Each biogenic amine standard solution of 0.01 mol/L was prepared by weighing an amount of about 0.1611g of putrescine dihydrochloride and 0.1751g of cadaverine dihydrochloride, and afterwards transferred into a 100mL volumetric flasks with subsequent dilution with a 0.01mol/L buffer solution ($HCOOH/LiOH$) at a pH 4. The standard solution with lower concentration was prepared by rigorous dilution with the same solvent.

3. Application of potentiometric sensor in a sequential injection analysis system

In the last years, the development and application of automatic analysis facilitated the preparation of the samples, making the measurements quicker and with less operator interventions. In some cases, the reduction of sample volume is advantageous, especially when it comes to biological material. Based on it, the histamine ion selective electrode was coupled in a Sequential Injection Analysis (SIA) system. In this system, a multiposition selection valve (Valco Instruments, Houston, TX) operate in synchronization with a Minipuls 3 Gilson (Villiers-le-Bell France) peristaltic pump with a PVC pumping tube ($\varnothing_{int}=0.90$ mm) of the same brand, a VICI C25-3118E (figure 9). Seven channels ($\varnothing_{int}=0.5$ mm) were drilled in a single acrylic block with 20 mm thick to, respectively, access the central and six lateral ports of the selecting valve. In one of these channels a transverse hole with 0.5 mm diameter was drilled to screw the reference electrode in a perpendicular position. The histamine electrode is in a wall-jet position at the end of the flow channel and it is in perpendicular position to the reference electrode. When the pump is operated in reverse, aliquots are sequentially drawn into a holding coil (with 30 cm and flow lines with $\varnothing_{int}=0.5$ mm PTFE (polytetrafluoroethylene) tubing), and upon forward propulsion the resultant stack of sample and reagents are sent to the detector. This system was controlled by means of a PCL-711 Advanced interface card coupled to a microcomputer running a home-made software written in Quick Basic 4.5.

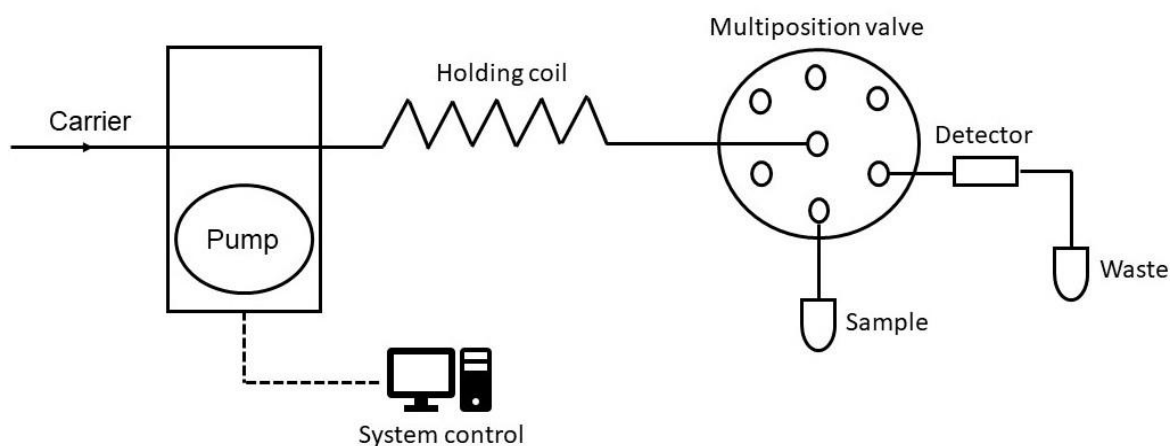


Figure 9 - Schematic diagram of Sequential Injection Analysis (SIA) system.

The possibility of reducing the size of SIA systems led to the development of a new analytical concept: SI-LOV (Sequential Injection – Laboratory on Valve). This new instrument has been designed (figure 10) to operate with sample volumes from nanoliters to microliters, while maintaining relatively large bore conduits that minimize surface contamination and clogging. Furthermore, the main advantage is its robustness because it is possible to work with common instruments used in conventional flow systems (valves, flow tubes, detectors) without need a more sophisticated equipment (212). The center channel of the selector valve does the connection between different ports. When peristaltic pump operates in reverse direction, the sample and reagents are aspirated by the different ports of selector valve to the holding coil. By changing the direction, the final solution is conducted to the detector port.

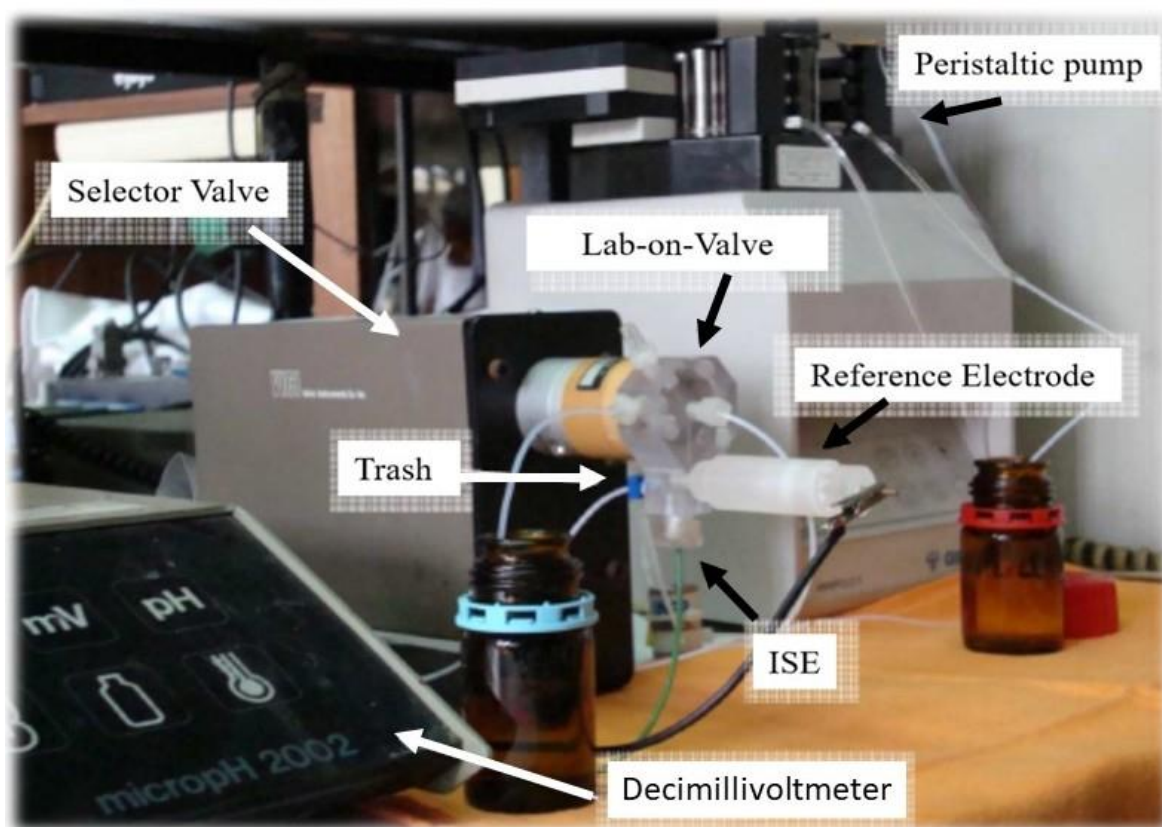


Figure 10 - Schematic representation of Sequential Injection valve- Lab-on-valve

The possibility of controlling any hydrodynamic condition in the system enables to control different steps of the analytical process. Before to optimization of sample volume and flow rate, the peristaltic pump was calibrated.

3.1. Optimization of the flow conditions

Sample volume was optimized after performing calibration curves for the histamine selective electrode coupled to SI-LOV system, using variable aspiration times (9, 12 and 14 seconds). For that, a dilution process prepared a set of histamine standard solutions with concentrations between 1.00×10^{-2} mol/L and 1.00×10^{-7} mol/L. All standard solutions were daily prepared.

After optimization of the aspiration time, it was evaluated the influence of the flow rate on analytical signal. Studies were performed in the same range of concentrations at different flow rates of 22, 24 and 30 $\mu\text{L/s}$. For each standard solution, three aspirations were made, and the peaks obtained were converted in potential values.

4. Sample treatment

The initial objective of this work was to develop an analytical methodology to detect histamine in forensic material. According the time constraints and bureaucratic difficulties in obtaining such materials, it was decided to test the analytical usefulness of the sensor in human blood serum samples. Due to the presence of several interferences, namely protein fouling in the membrane, different sample preparation methods were evaluated – protein precipitation procedure and solid phase extraction (SPE).

4.1. Protein Precipitation procedure

The protein precipitation is a procedure frequently used in analysis of serum and plasma. The protein procedure described by Polson (213), but with some modifications was used in this work. First, a pooled serum sample was mixed with ethanol absolute (3:1) ($T=4^{\circ}\text{C}$) and vortexed for 20 seconds, left to stand for 20 minutes at $\approx 4^{\circ}\text{C}$ and centrifuged (Thermo Electron Corporation) for 10 minutes at 3000 rpm. The supernatant was evaporated to dryness and reconstituted in 1mL of 0.01 mol/L buffer solution (HCOOH/LiOH , $\text{pH}=4$). Two different portions of the final solution were evaluated. One of them was analyzed directly in the flow system without changes and another portion was doped with 200 μL of standard histamine solution before to be studied. To improve the protein precipitation, the contact time of the serum with ethanol and the centrifugation time was increased to 40 minutes and to 12 minutes, respectively. To guaranty total protein precipitation, part of the obtained sample was subjected to another cycle of protein precipitation.

4.2. Solid-phase extraction procedure

Solid phase extraction (SPE) is an increasingly useful sample preparation technique. According to Zhang (214), the SPE cartridge was conditioned with 6 mL of methanol followed by 6 mL of water. One milliliter of the serum sample passed through the cartridge and then it was washed twice with 1 mL of deionized water. The elution was carried out with 1 mL of MeOH-5% NH₄OH. This procedure was repeated three times. The eluate was evaporated to dryness with nitrogenous flow and reconstituted in 1mL of 0.01 mol/L buffer solution (HCOOH/LiOH, pH=4). An aliquot of the final product was analyzed directly in SI-LOV and another one was doped with a known standard solution of histamine before to be analyzed. To evaluate the success of the SPE method was determined the recovery considering the procedure (figure 11) and the mathematical formula describe by Waters (215):

$$\% \text{Recovery} = 100 \times \frac{\text{Response extracted sample (with standard)}}{\text{Response pos-extracted spiked sample}}$$

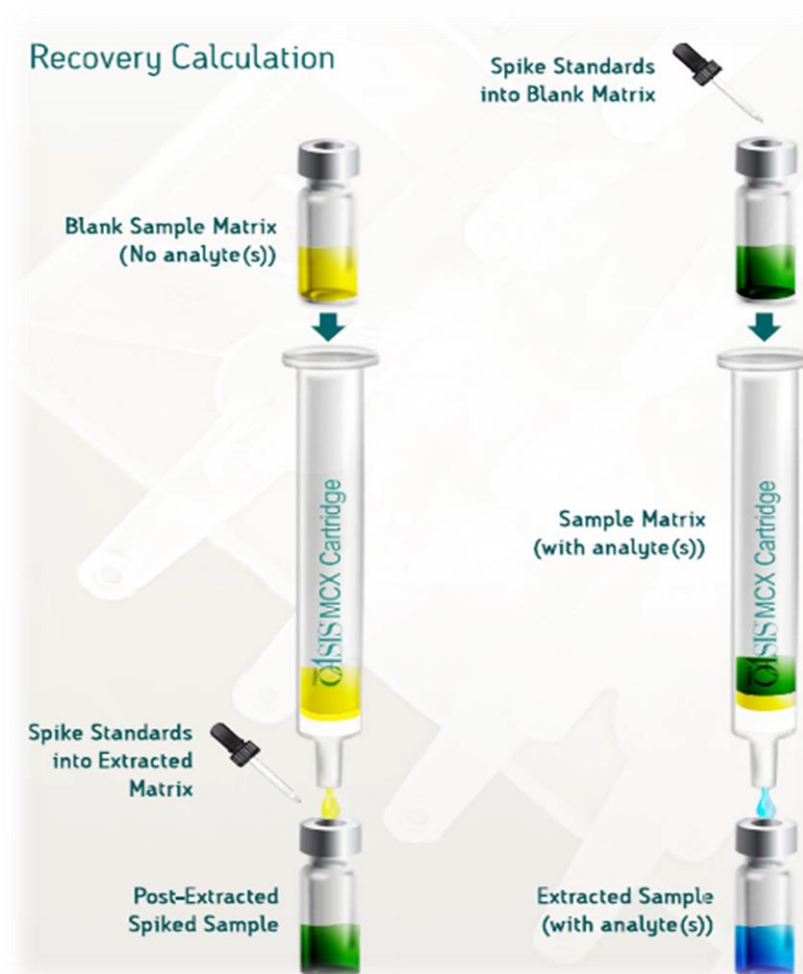


Figure 11 - Analytical procedure for the sample cleaning and subsequent calculation of recovery percentage. Image from (215).

Results and Discussion

***I. Results for the evaluation of
potentiometric sensor for
histamine determination***

One of the objectives of this work was the development of a potentiometric sensor for histamine. After the construction of solid contact electrodes based on graphite, different membranes were tested to select the best composition table 3. The working characteristics were evaluated by the determination of the slope, practical detection limit and lower limit of the linear range values.

Table 3 - Membrane composition (%w/w) of histamine electrodes. α -CD, α -cyclodextrin; CB[6], cucurbit[6]uril; FNDPE, 2-fluorophenyl 2-nitrophenyl ether; o-NPOE, 2-nitrophenyl octyl ether; DBS, dibutyl sebacate; KTpCIPB, potassium tetrakis (4-chlorophenyl) borate; PVC, polyvinyl chloride; PVC-COOH, polyvinyl chloride carboxylated; MWCNT, multi-walled carbon nanotubes; PDL, practical detection limit; LLLR, lower limit of the linear range.

Membrane type	I	II	III	IV	V	VI	VII	VIII	IX	X
α -CD (%w/w)	1.0	----	----	----	----	----	----	----	----	----
CB[6] (%w/w)	----	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
FNDPE (%w/w)	68.4	68.2	68.6	68.4	68.3	----	----	----	----	----
o-NPOE (%w/w)	----	----	----	----	----	68.9	----	67.7	66.8	65.0
DBS (%w/w)	----	----	----	----	----	----	68.3	----	----	----
KTpCIPB (%w/w)	0.3	0.3	----	----	0.3	0.3	0.3	0.3	0.3	0.3
PVC (%w/w)	30.3	30.4	30.4	----	----	30.0	30.4	29.9	29.8	29.0
PVC-COOH (%w/w)	----	----	----	30.6	30.4	----	----	----	----	----
MWCNT (%w/w)	----	----	----	----	----	----	----	1.0	2.0	5.0
Response parameters										
Slope (mV/dec)	(25.3±1.4)	(25.9±1.9)	(15.2±1.9)	(21.3±1.8)	(34.8±3.0)	(30.5±1.3)	(22.3±1.6)	(30.2±2.4)	(30.9±1.2)	(31.3±1.4)
PDL (mol/L)	$(3.73 \pm 2.42) \times 10^{-5}$	$(2.57 \pm 1.19) \times 10^{-7}$	$(2.55 \pm 1.14) \times 10^{-5}$	$(1.75 \pm 1.09) \times 10^{-4}$	$(8.89 \pm 2.67) \times 10^{-7}$	$(5.70 \pm 1.63) \times 10^{-7}$	$(2.04 \pm 0.60) \times 10^{-4}$	$(3.83 \pm 0.83) \times 10^{-7}$	$(3.00 \pm 0.61) \times 10^{-7}$	$(3.83 \pm 0.89) \times 10^{-7}$
LLLRL (mol/L)	$(8.43 \pm 5.24) \times 10^{-5}$	$(3.63 \pm 0.79) \times 10^{-7}$	$(3.59 \pm 1.91) \times 10^{-5}$	$(5.19 \pm 3.29) \times 10^{-4}$	$(9.92 \pm 0.36) \times 10^{-7}$	$(9.18 \pm 2.09) \times 10^{-7}$	$(8.72 \pm 1.39) \times 10^{-4}$	$(4.99 \pm 0.00) \times 10^{-7}$	$(3.00 \pm 0.00) \times 10^{-7}$	$(4.97 \pm 0.00) \times 10^{-7}$
pH range	[3.1 - 5.9]	[2.3 - 4.2]	----	----	[2.5 - 3.6]	[4.7 - 10.4]	[4.0 - 10.0]	----	[2.7 - 5.4]	----

1. Study of different components in the membrane

1.1. Ionophore study

The study started by reproducing a previously described electrode (242) which used α -CD as ionophore (membrane I). Then, it was evaluated the main potentiometric characteristics of the replacement of α -CD by CB[6] in the membrane, maintaining all other constituents (table 3).

As verified by the above results presented, the replacing of α -CD (membrane I) by CB[6] (membrane II) improved the PDL and the LLLR in two decades, making possible the determination of histamine concentration of 0.1 μ mol/L instead of 10 μ mol/L. Therefore, the results obtained for membrane II led to the selection of CB[6] as ionophore in the construction of future electrodes.

1.2. Effects of the anionic additive

The effect of the presence of negative charges in membrane was evaluated by using CB[6] as ionophore. In this way, a new membrane was introduced in the study, with similar composition of membrane II, but without the anionic additive (membrane III). After a set of calibrations, the results obtained for these membranes are in table 3.

The potentiometric characteristics of membrane III is worse than membrane II, namely in the slope, PDL and LLLR. These results can be explained by the influence of the additives in the membrane's perm-selectivity. The results are supported by some authors which defend the idea that the presence of the additive contributes to exclude interferences of the opposite charge of the primary ion and consequently to improve the response to histamine, and to increase the membrane conductivity, favoring the histamine binding (216, 217).

1.3. Effect of the polymer composition

In literature is described that the presence of COOH groups opens the possibility for the chemical modification of the matrix membrane. Thank to good electrochemical characteristics and properties due to the presence of ionizable groups, PVC-COOH became popular alternative to PVC (218). Moreover, according to the previous discussed results, the presence of negative charges improves the main electrode characteristics, and for that, membranes containing PVC-COOH were prepared (table 3). This approach permits a better understanding about the influence of the type and quantity of ionic charges in the characteristics of histamine electrode.

The results showed that the amount and the type of negative charges present in PVC-COOH were not enough to improve the general electrode performance (II, IV, V). Maintaining the anionic additive amount in the membrane and changing the type of polymer (II and V), PVC-COOH membranes presents an improvement in the slope value but impaired by the PDL and LLLR. These results showed that membranes based on PVC-COOH does not contribute for better membrane characteristics.

1.4. Effect of the solvent mediator

After concluding that the anionic additive is very important in the membrane composition and the presence of carboxylate groups is not a favorable modification, the following study was to determine the effect of the solvent mediator in the electrode characteristics. The choice of the mediator is important, because the characteristics of the electrode such as sensitivity, selectivity, PDL and LLLR are strongly influenced by its dielectric constant (219, 220). The presence of this component on the membrane and its polarity determine the degree of the ions diffusion and ensures membrane viscoelasticity. Therefore, three membranes (II, VI and VII) were prepared by using solvent mediators with different dielectric constants (table 3).

According to other authors (221, 222), the results obtained confirm the effect of the dielectric constant in the slope, PDL and LLLR values. DBS mediator (membrane VII) provided the worse response for histamine in comparison with o-NPOE and FNDPE. In this case, a very low slope was obtained, compared to the membranes II and VI, and the PDL and LLLR were significantly higher. These results likely arises from the difference in the dielectric constant of the solvents (o-NPOE, $\epsilon=23.1$ (223); DBS, $\epsilon=4.54$ (224)). A higher dielectric constant for o-NPOE membrane increases the polarity and decreases the electrical resistance of the membrane. In this situation, the extraction of histamine into the membrane phase is facilitated.

Membrane II based on FNDPE, a more polar solvent ($\epsilon=50$ (225)), present better results concerning the main electrode characteristics than the ones prepared with other solvent mediator. However, the slope didn't have the same behavior. The o-NPOE membrane had more Nernstian slope, although being less polar. According to Moreira et al. (222) polarity changes the membrane characteristics, but no simple correlation with its polarity alone is to be expected.

Therefore, the membrane VI was chosen for subsequent studies due to the Nernstian slope (30.5 mV/dec) and lower PDL and LLLR values (10^{-7} mol/L).

1.5. Effect of the presence of Multi-Walled Carbon Nanotubes

Multi-walled carbon nanotubes have been used as novel nanomaterial in electrochemical sensor applications because its conductivity properties make them suitable to be used in ion-selective membranes (170, 226).

In this sense, the effect of MWCNTs on the optimized membrane (VI) was also evaluated. This strategy was followed to improve the histamine selective electrode characteristics mainly decrease both PDL and LLLR.

Different amounts of MWCNTs were dispersed in membrane during its preparation. As shown in table 3, the presence of these particles in membrane composition improve the PDL and LLLR, namely for membrane IX, when compared with the same membrane but without MWCNTs. Furthermore, it also verified higher reproducibility of the results (less standard deviation). The high conductivity associated of these particles favors ionophore-histamine binding allowing better results. Therefore, the membrane composition selected to continue this work was membrane IX.

2. Determination of response time

The variation of the response time of electrode with the histamine concentration for membrane IX is shown in figure 12.

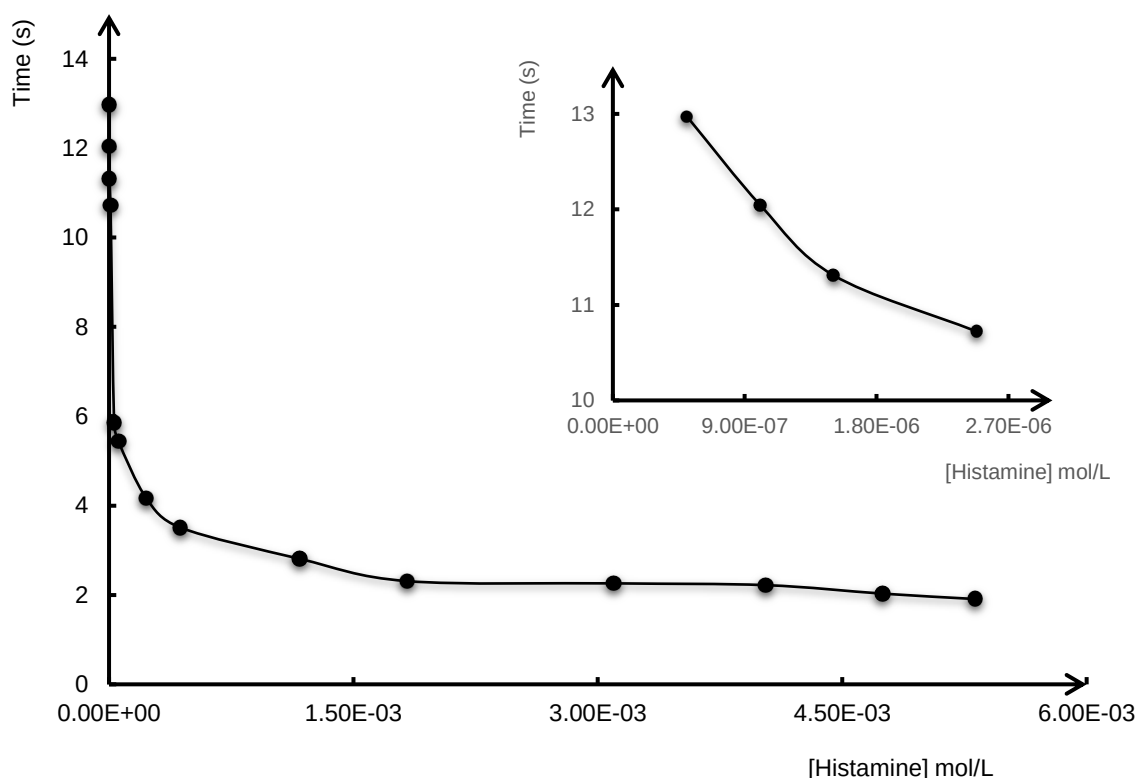


Figure 12 - Response time (membrane IX) determined from the calibration curve.

As expected and according to the results obtained, lower histamine concentrations (until range concentrations of 10^{-4} mol/L) has response time longer than higher concentrations (<1 s). However, in this selective electrode the response time for histamine concentration of 10^{-5} mol/L is around 13 seconds, a very fast response when compared with others (138).

3. Study of pH influence on the potentiometric response

The influence of pH on the potentiometric response was evaluated for the different membranes prepared. To define the operational pH range, a potential variation of ± 5 mV was established as a criterion. Figure 13 shows the different Reilley diagrams obtained for the various membranes when immersed in a histamine solution of 1.00×10^{-4} mol/L, and the results for membrane IX for the two levels of concentration.

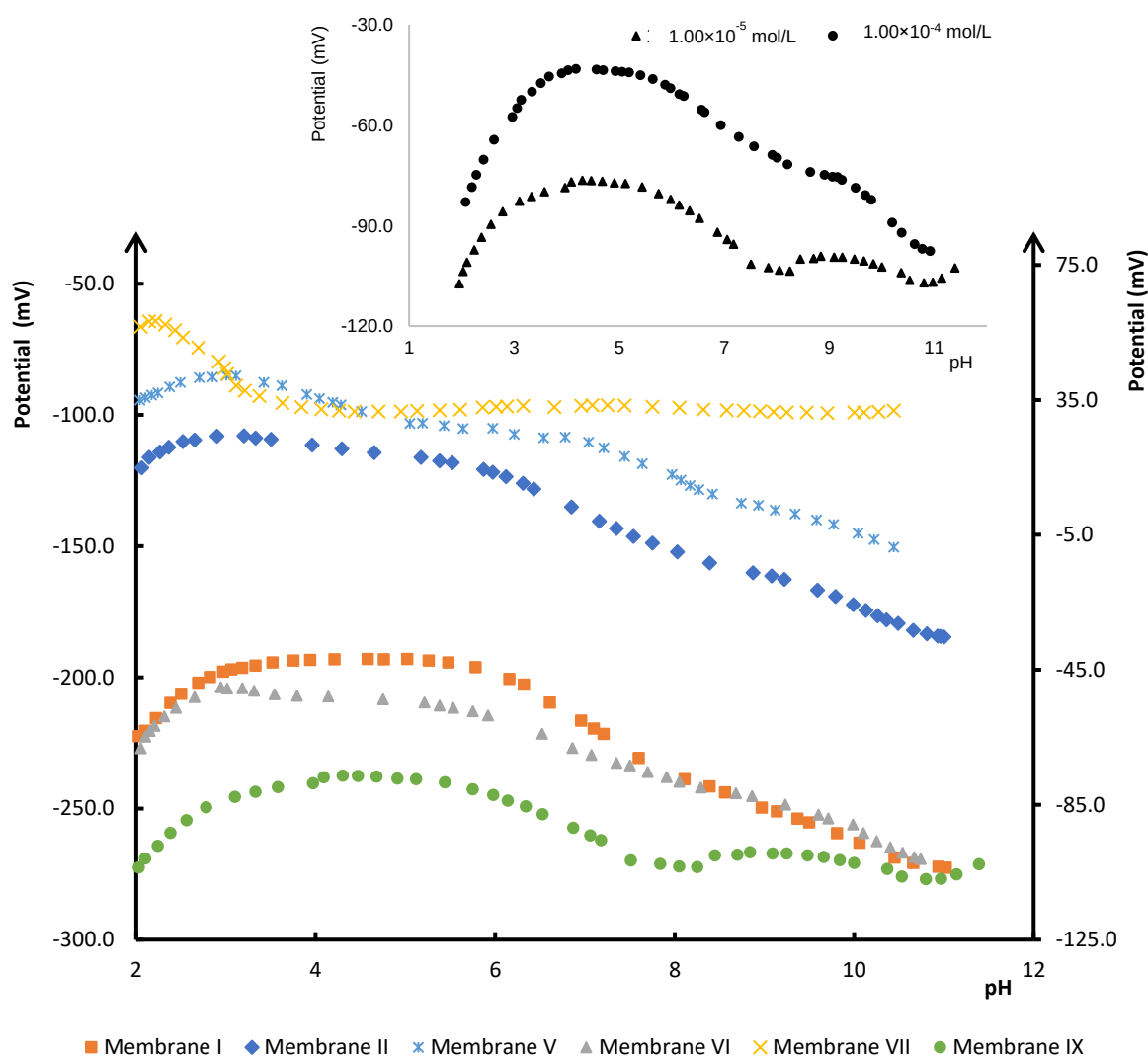


Figure 13 - Reilley diagrams for different polymeric membranes. The potential values of membranes I, II, and V are plotted on the left axis while the potential values for membranes VI, VII and IX are read on the right axis. Graphic representation of the results obtained for membrane IX for the two levels of concentration.

A similar profile was obtained for all the units except for the membrane prepared with DBS as plasticizer, where the potential decrease until pH 4 and remains stable in the pH range between 4 and 10. In the other membranes, for lower pH, the potential increased, indicating that the membrane sensor responds to hydrogen ions. Then, the results show that the potential is independent of the pH in the range 2 – 5 (approximately). In this pH range, histamine is ionized (di-cationic form (227)) allowing higher number of interactions. For higher pH values it was observed a decrease of potential may be due to the deprotonation of histamine with pK_{a1} : 6.9 and pK_{a2} : 9.2 (228).

In table 3 are describe the specific pH ranges for each membrane. Considering these results, all subsequent assays were performed in solutions with the pH adjusted to 4 to ensure that the histamine was in di-cationic form.

4. Baseline electrode deviation (Drift)

According to the IUPAC (208), drift is the slow non-random change with time of the baseline potential of an ion-selective electrode cell assembly maintained in a solution of constant composition and temperature. The deviation determination is achieved through a set of potential measurements over a given period in a constant position and temperature solution. The slope of baseline potential Vs. time line is called drift. Figure 14 shows the drift obtained for histamine electrode in different concentrations.

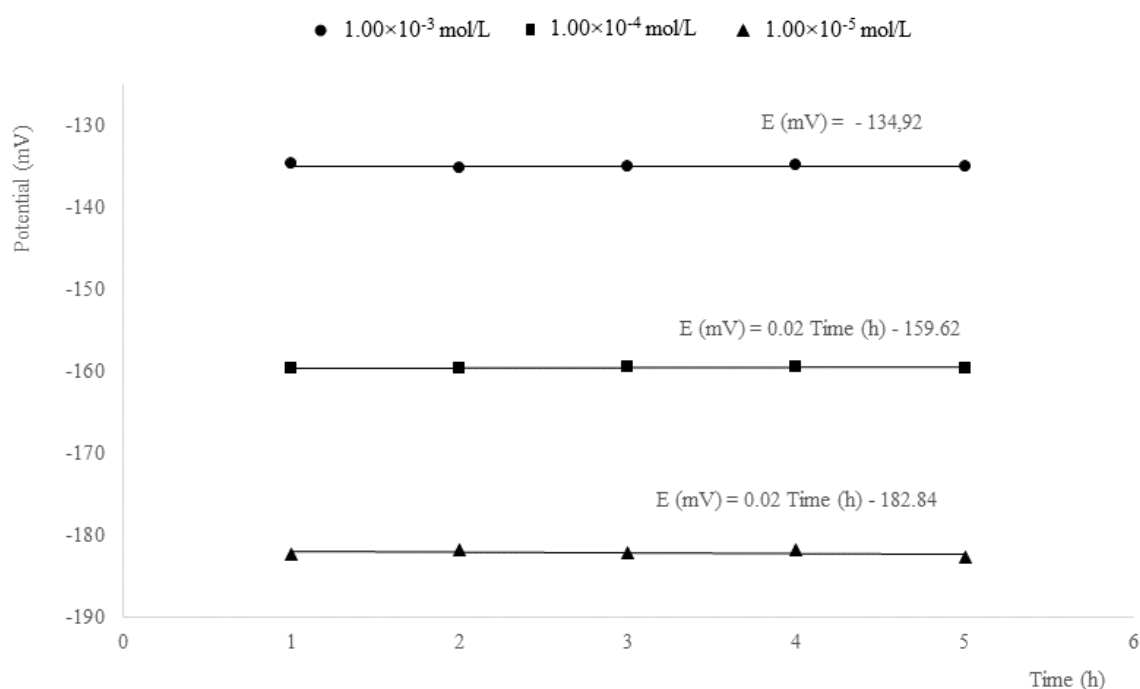


Figure 14- Baseline electrode deviation for different concentrations

For this electrode, the results obtained show that for histamine concentration of 1.00×10^{-3} mol/L the drift is null. For concentrations of 1.00×10^{-4} mol/L and 1.00×10^{-5} mol/L the values obtained were of about 0.02 mV/h, but it can be considered negligible. It is possible to conclude that this electrode does not exhibit significant drift in electrical potential and therefore, does not requires frequent standardization and hence makes its daily use very simple.

5. Determination of the potentiometric selectivity coefficient - Interferences study

The determination of selectivity coefficients of various ions was carried out for different membranes according to the fixed interference method. The results obtained are represented in table 4.

Table 4 - Potentiometric selectivity coefficient values for different ions in membranes I, II and IX

Interference (1.00×10^{-2} mol/L)	$K_{Hist^{2+}, Interf^{+}}^{pot}$ (mol/L)		
	Membrane I	Membrane II	Membrane IX
Ammonium	$(1.50 \pm 0.08) \times 10^{-1}$	$(4.83 \pm 0.52) \times 10^{-2}$	$(2.08 \pm 0.16) \times 10^{-1}$
Potassium	$(3.92 \pm 0.48) \times 10^{-1}$	$(1.80 \pm 0.21) \times 10^{-1}$	$(5.59 \pm 2.12) \times 10^{-1}$
Sodium	$(2.06 \pm 0.69) \times 10^{-2}$	$(1.47 \pm 0.42) \times 10^{-2}$	$(3.28 \pm 0.25) \times 10^{-2}$
Lithium	$(7.74 \pm 1.54) \times 10^{-3}$	$(1.57 \pm 0.11) \times 10^{-2}$	$(9.05 \pm 4.50) \times 10^{-3}$
Magnesium	$(8.30 \pm 2.02) \times 10^{-5}$	$(1.30 \pm 0.11) \times 10^{-4}$	$(1.66 \pm 0.68) \times 10^{-4}$
Calcium	$(1.77 \pm 0.71) \times 10^{-4}$	$(2.79 \pm 0.16) \times 10^{-4}$	$(4.29 \pm 0.32) \times 10^{-4}$

In literature is mentioned that an electrode has selectivity for a principal ion when the value of potentiometric selectivity coefficient is smaller than one unit. However, in IUPAC Recommendations for nomenclature of ion-selective electrodes (208) is referred that a lower value of $K_{Hist^{2+}, Interf+}^{pot}$ means a better the electrodes preference for the principal ion. The selectivity coefficients for the described membranes are low enough to conclude that membrane I, II and IX are selective to histamine. Calcium and magnesium present lower K_{pot} . However, to proceed with this work it was decided to use lithium ion.

5.1. Determination of putrescine and cadaverine

After death biogenic amines are produced in decaying cadavers and they can be detected affecting the results for histamine determination. Putrescine and cadaverine are biogenic amines that belongs to the family of nitrogenous organic bases. Therefore, it is important to evaluate the optimized potentiometric sensor (IX) against them as it is described in table 5.

Table 5 – Membrane IX characterization for cadaverine and putrescine. Comparison with the results obtained for histamine

Biogenic amine	Slope (mV/dec)	PDL (mol/L)	LLLR (mol/L)
Histamine (n=12)	30.9 ± 1.2	(3.00 ± 0.61)×10 ⁻⁷	(3.00± 0.00)×10 ⁻⁷
Cadaverine (n=12)	28.4 ± 0.4	(1.43 ± 0.70)×10 ⁻⁶	(2.17 ± 1.68)×10 ⁻⁶
Putrescine (n=12)	24.8 ± 0.9	(2.70 ± 1.07)×10 ⁻⁶	(3.86 ±2.14)×10 ⁻⁶

Although of this potentiometric sensor has satisfactory results in histamine determination, it is possible to verify that the sensor responds to cadaverine and putrescine. The slopes values obtained for these biogenic amines are very close to the Nernstian slope, having cadaverine a higher value than putrescine. Regarding PDL and LLLR, the values are about a decade lower than for histamine. So, this mean that this membrane can serve as a potentiometric detector for the determination of these biogenic amines. If the study sample contain putrescine and cadaverine, it is necessary choose a sample pre-treatment or an analytical procedure suitable that ensures only histamine determination, as the chromatographic separation.

6. Determination of the lifetime of membrane

The construction of ion-selective electrodes is easy and cheap, but the durability of the detector is a very important characteristic that should be evaluated. For that, the lifetime of membrane IX was studied by performing calibrations in different days, on the same conditions. The parameters as slope, PDL and LLLR were compared over time (table 6).

Table 6 - Evaluation of the lifetime for the optimized membrane.

Number of days	Slope (mV/dec)	PDL (mol/L)	LLLR (mol/L)
1	29.3	3.00E-07	3.00E-07
2	29.0	2.56E-07	3.00E-07
3	27.2	3.00E-07	3.00E-07
4	25.4	3.00E-07	3.00E-07
5	24.6	6.77E-07	9.89E-07
11	24.3	7.62E-07	9.94E-07
24	24.5	7.01E-07	9.92E-07
32	26.4	7.34E-07	9.96E-07
39	24.5	8.02E-07	9.96E-07
49	25.4	1.82E-06	2.44E-06
155	25.3	1.46E-06	1.49E-06

The values obtained show a slight decrease of the slope over time and then a stabilization to a value approximately of 25 mV/dec until the end of the study. The practical detection limit and lower limit of the linear range showed a similar behavior by losing the detection limits obtained for a new electrode. However, after five months the sensor can only be used for concentrations higher than 1.49×10^{-6} mol/L. Moreover, if lower histamine concentration must be determined, a new membrane can be dropped on the electrode once it is easy and economical to recycle the sensor.

II. Coupling of the potentiometric sensor to the Sequential Injection Analysis (SIA) system

1. Optimization of analytical conditions in SIA system

1.1. Optimization of the sample volume (aspiration time)

The sample volume was the first parameter evaluated using the optimized membrane (membrane IX). For that, the peristaltic pump was switch on in a reverse mode to aspirate the sample. Three aspiration times (9, 12 and 14 seconds) were studied by a set of calibrations with different concentrations of standard histamine solutions. By previously calibration of the pump, these aspiration times were converted in sample volumes as show in table 7. The main characteristics of the sensor were determined as represented in table 7 and figure 15. In the same figure, there is inserted a graphic record of the respective calibration.

Table 7 - Sample volume optimization. Slope, practical detection limit and lower limit of the linear range values for different sample volumes

Aspiration time (s)	Sample volumes (μL)	Slope (mV/dec)	PDL (mol/L)	LLLR (mol/L)
9	127	23.2	6.27×10^{-6}	1.00×10^{-5}
12	170	23.3	1.00×10^{-6}	1.00×10^{-6}
14	197	27.7	1.00×10^{-6}	1.00×10^{-6}

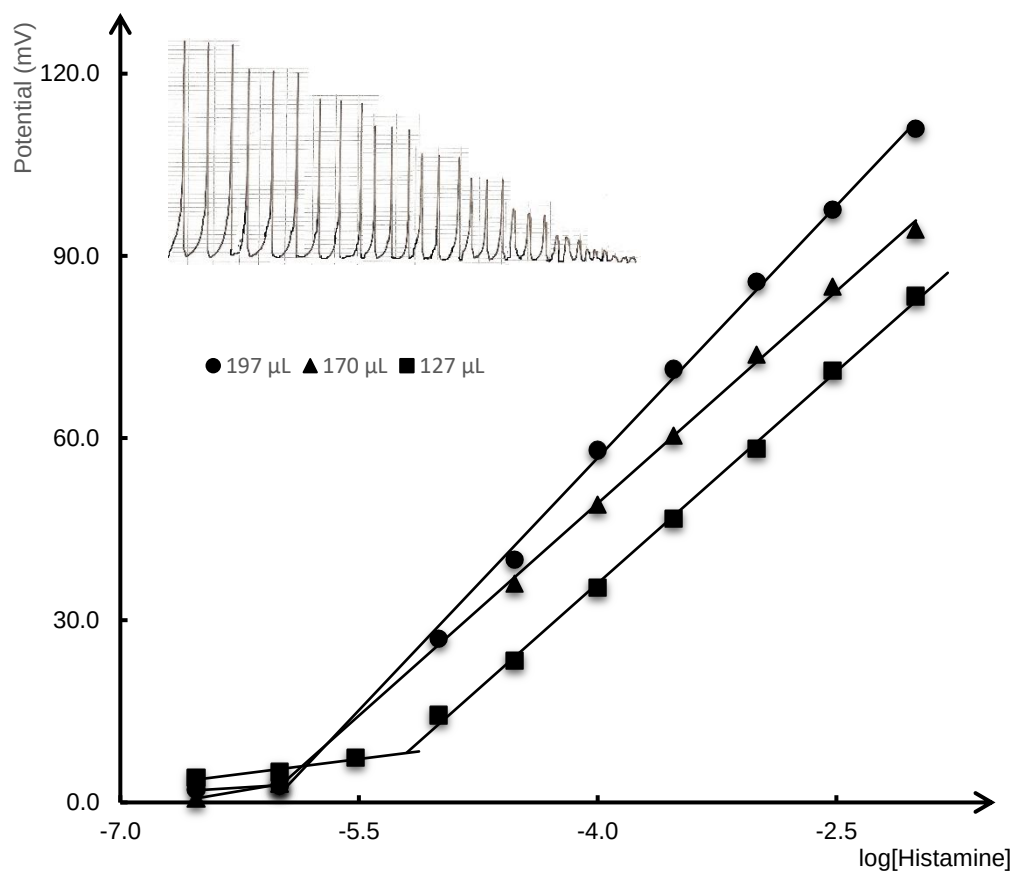


Figure 15- Graphic representation of calibration curves for ion-selective electrode in SI-LOV system. Influence of the sample volume.

The optimization of the sample volume allowed the choice of the best condition for histamine determination. The best analytical characteristics were obtained for 197μL. The Nernstian slope (≈ 29 mV/dec) was maintained concerning its evaluation in batch conditions but the PDL and LLLR both of 1.00×10^{-6} mol/L, were half decade concentration worse than in batch. When the sample volumes decrease to 170μL and 127μL, the slope values also decrease 23.3 mV/dec and 23.2 mV/dec, respectively. The practical detection limit also suffers a slight decrease when the sample volume decreases to 127μL.

1.2. Optimization of the flow rate in the detector

In the same way, the flow rate in the detector was optimized. For this, a set of calibrations were made for different flow rates, namely: 22.4 $\mu\text{L/s}$, 24 $\mu\text{L/s}$ and 30 $\mu\text{L/s}$ (table 8 and figure 16).

Table 8 - Flow rate optimization. Slope, practical detection limit and lower limit of the linear range values for different flows.

Speed	Flow ($\mu\text{L/s}$)	Slope (mV/dec)	PDL (mol/L)	LLLR (mol/L)
18.5	22.4	22.6	1.00×10^{-6}	1.00×10^{-6}
20	24	27.1	1.00×10^{-6}	1.00×10^{-6}
25	30	28.8	1.00×10^{-6}	1.00×10^{-6}

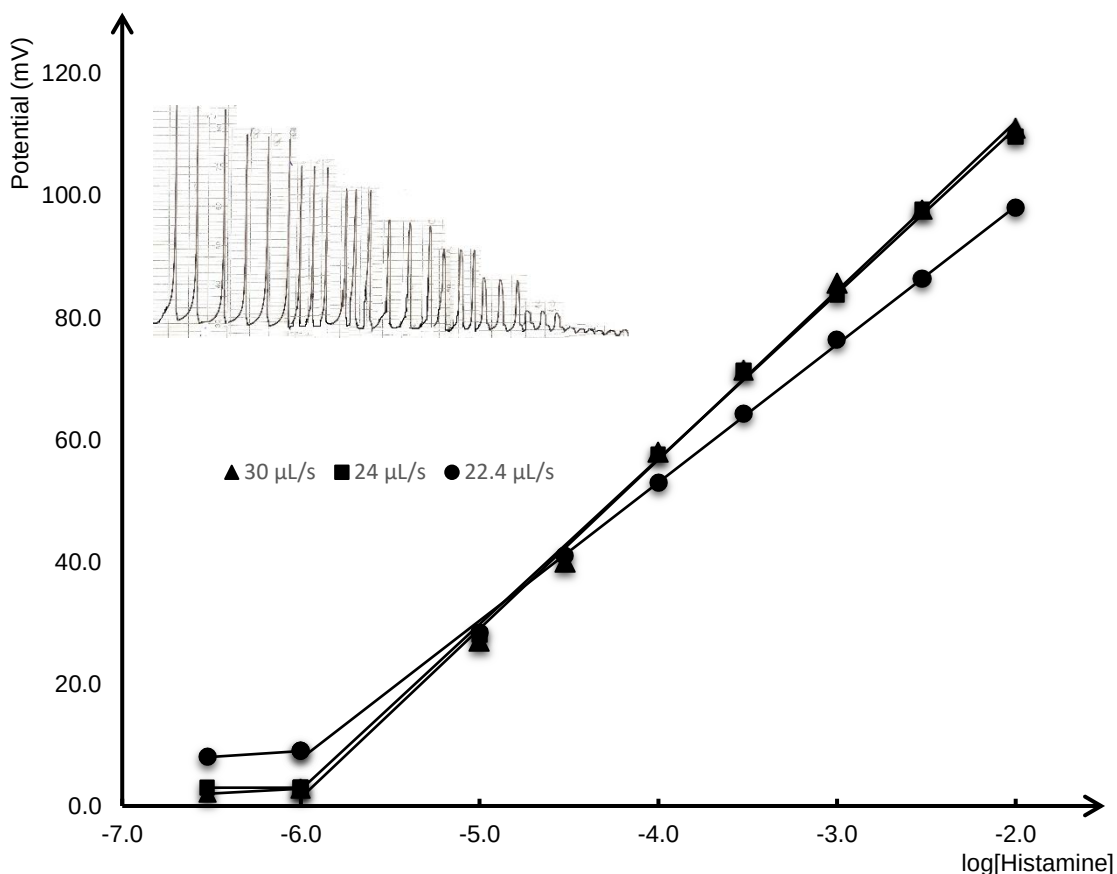


Figure 16- Typical recording of a calibration curve for 30 $\mu\text{L/s}$. Graphic representation of a calibration curves in flow rate optimization of histamine ion-selective electrode based in CB[6] with 2% of MWCNTs, coupled in SI-LOV system.

According to the figure 16, a flow rate of 24 $\mu\text{L/s}$ enables a calibration curve with a Nernstian slope and a PDL and LLLR of $1.00 \times 10^{-6} \text{ mol/L}$. Increasing the flow rate, there isn't any improvement in the electrode characteristics. Nevertheless, 30 $\mu\text{L/s}$ seems to clear the detection cell easier, and consequently the baseline reestablishment is faster.

The optimal hydrodynamic conditions were also used for histamine determination in real samples (table 9).

Table 9 - *Optimized conditions for the sequential injection analysis in histamine determination*

Port	Time (s)	Flow ($\mu\text{L/s}$)	Direction
6	14	14.1	aspirate
1	200	30	pumping

III. Results of the analysis of real samples

The analytical usefulness of the prepared sensor was verified by actual analysis of samples of serum from volunteer donors. The first idea was to determine the content of histamine in serum sample without any sample pretreatment. The ion-selective electrode was previous calibrated, and the biological samples were analyzed in the optimized flow system previously described. Figure 17 shows a calibration curve for histamine solutions obtained in the flow system depicted in figure 10, and the analysis of serum before and after being doped with histamine. As it is observed, no potential variation occurred when samples were doped with 10^{-2} mol/L histamine solution. This result evidences a high matrix effect resulting from the presence of high protein contents that have high affinity for the cucurbituril molecule (229-232).

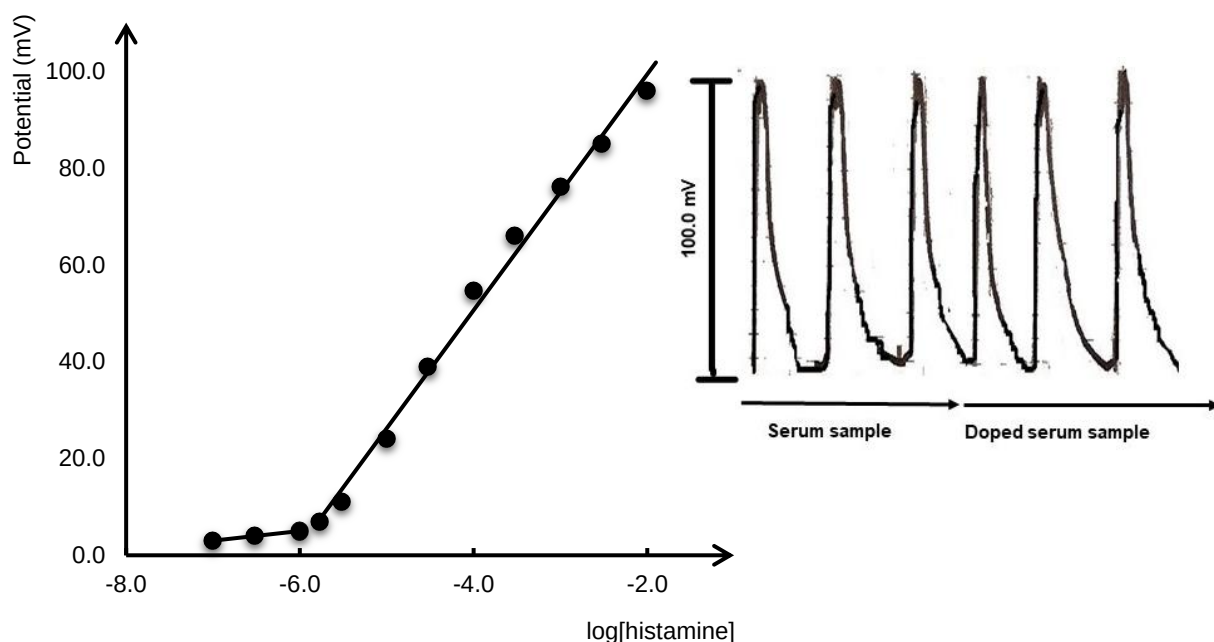


Figure 17- Typical calibration curve of histamine ion-selective electrode coupled in SI-LOV system. Recording of the serum sample and serum sample doped with a concentration of 1.00×10^{-2} mol/L.

Since the presence of protein-cucurbit[6]uril complex affect the results and overestimate the histamine value the sample pretreatment is advisable in this situation. Different protein elimination techniques were tested, namely protein precipitation and cleaning of the sample behind the use of solid-phase extraction.

1. Sample pretreatments

1.1. Protein precipitation

The first procedure applied in the pretreatment of the sample was the protein precipitation. After calibration of the electrode, an aliquot of human serum was treated according the procedure described in section 4.1. After precipitation and subsequent centrifugation, the supernatant was evaporated to dryness on a hot plate and reconstituted in 0.01 mol/L buffer solution (HCOOH/LiOH, pH=4). The resulting solution was analyzed (figure 18).

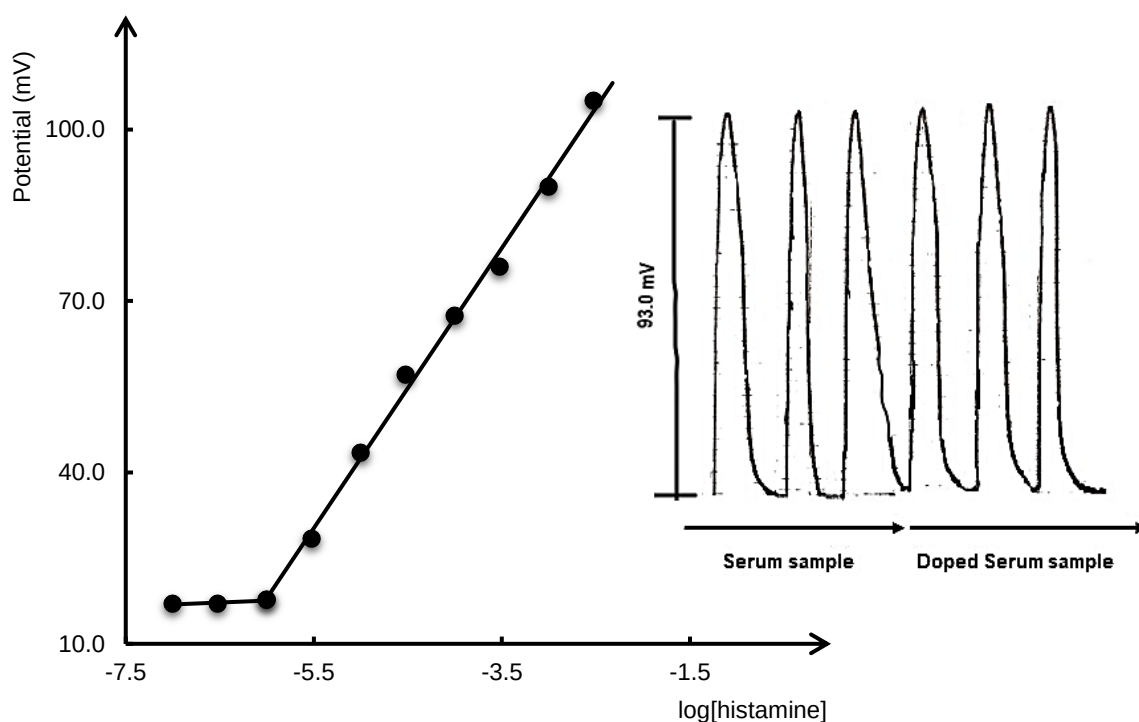


Figure 18 - Typical calibration curve of histamine ion-selective electrode coupled in SI-LOV system. Recording of serum sample and a serum sample doped with a concentration of 1.00×10^{-2} mol/L after the protein precipitation procedure.

The results showed that this procedure was not efficient and the same effect resulting from the presence of the proteins was observed. The peaks for the non-doped serum sample have the same height when compared with the peaks for doped sample with histamine standard solution. This indicates that the procedure of removal of the proteins was not sufficiently effective.

To obviate the situation, changes to the procedure previously used were introduced. The cooling time and the centrifugation time were increased as described in the experimental procedure. In the same way, the electrode was calibrated and then a non-doped serum sample and a doped serum sample were analyzed.

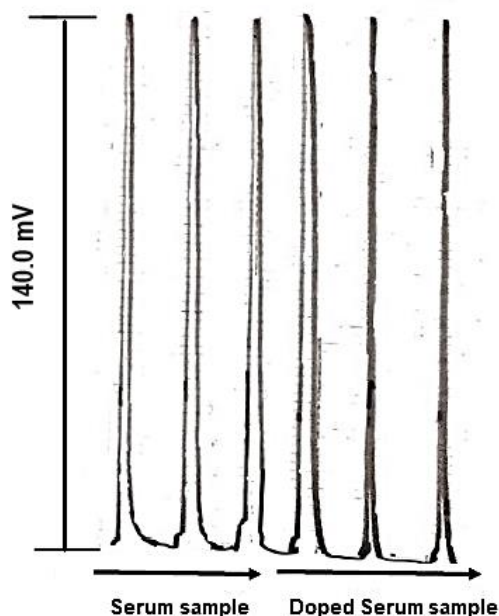


Figure 19 - Recording of serum sample and serum sample doped with a concentration of 1.00×10^{-2} mol/L after the first changes in a protein precipitation procedure.

However, the results showed that the modifications introduced did not improved the results (figure 19). The peaks for both serum samples remain high and with no difference between them. According to Peng et al. (233) the temperature is an important variable for extra histamine production in biological samples (above 60°C). As during protein precipitation procedure, the supernatant was evaporated to dryness on a hot plate at high temperature (>60°C), this strategy could have been responsible for the poor results obtained. Because of this, the same authors (233) proposed the use of MCX SPE cartridges, for cation exchange, due to the chemical properties of histamine (a polar basic compound). Recoveries of 95% were reported when compared with 50% of recovery for the most common used cartridges (C18, HBL).

1.2. Solid-phase extraction (SPE)

Since the previous procedures did not result, a sample treatment was attempted based on the use of SPE. According to Mahendradata et al. (234), the Lichrolut-SCX column was used for histamine determination. The main advantage of this SPE was the possibility to use a buffer solution for washing and eluting the sample. However, for the elution the buffer solution was mixed with isopropanol. For that, it was necessary to analyze the effect of this alcohol on the polymeric membrane. Standard histamine solutions were prepared in 0.01 mol/L buffer solution (HCOOH/LiOH, pH=4) with 8% (v/v) of isopropanol and were analyzed in flow system. In parallel, a calibration with standard histamine solutions in buffer solution was performed (figure 20).

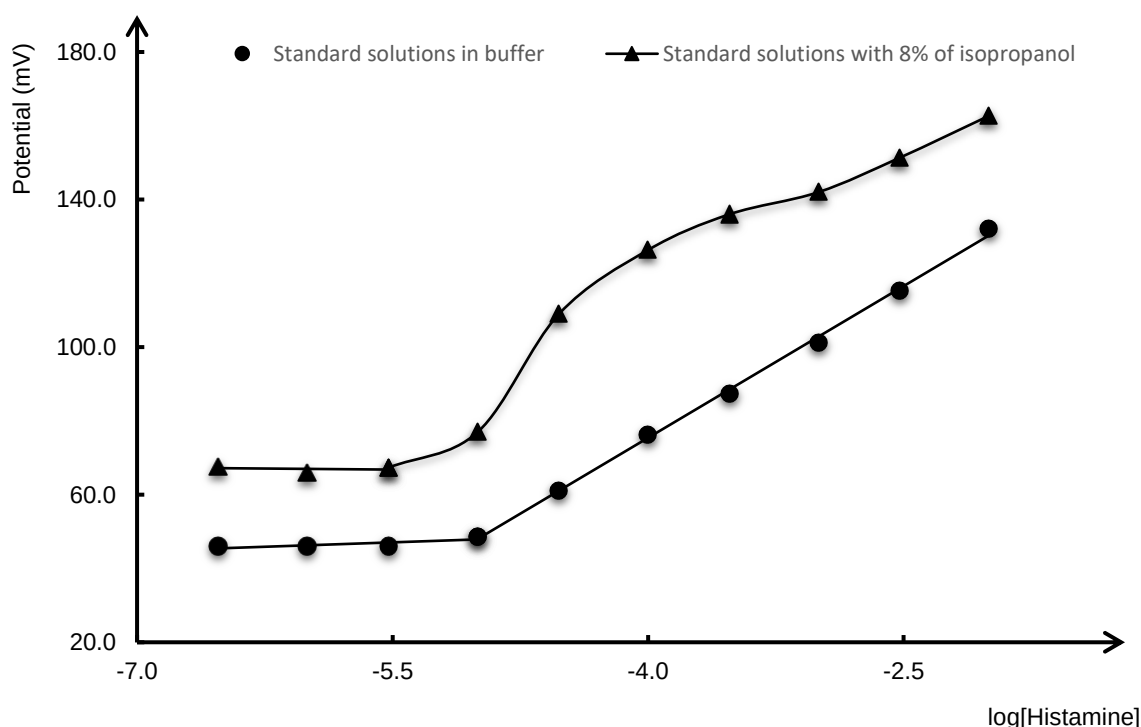


Figure 20- Graphic representation of calibration curves for normal standard solutions and standard solutions with 8% (v/v) of isopropanol.

As representing in figure 20 the results showed a different behavior for calibration curve if they are carried out in the presence or absence of isopropanol. This shows the incompatibility of using this extraction procedure with the use of the proposed histamine selective membrane. However, based on the work presented by Zhang et al. (214) another column (Oasis MCX column) was used. Following the protocol described by them, the conditioning with MeOH and water, the sample loading, the washing with water and the elution with MeOH-5%NH₄OH was the procedure used. The recovery was determined for

three levels of histamine concentration, namely 1×10^{-4} ; 1×10^{-5} and 1×10^{-6} mol/L. Recovery values of (97 $\pm$ 6)%; (103 $\pm$ 1)% and (118 $\pm$ 9)% were obtained, respectively. Relative standard deviations are acceptable (235) considering the concentrations levels measured (table 10).

Table 10 - Determination of the recovery and the respective standard deviation and relative standard deviation for different concentrations of histamine in biological samples.

Concentration	Recovery _(n=6)	Standard deviation _(n=6)	Relative standard deviation (RSD)
1.00×10^{-4} mol/L	97%	$\pm 6\%$	6%
1.00×10^{-5} mol/L	103%	$\pm 1\%$	1%
1.00×10^{-6} mol/L	118%	$\pm 9\%$	8%

Final considerations and prospects

The first objective of this work was the development of a potentiometric sensor for the determination of histamine. The study of different membrane components allowed the selection of the optimized composition that provided the best potentiometric response. Thus, it was found that CB[6] was better ionophore than α -CD improving 100x the detection limit. The use of KTpCIPB as additive affects positively the membrane response concerning to its absence. Replacing PVC by PVC-COOH no improvement was identified in the potentiometric response, even in the presence of additive. The solvent mediator, o-NPOE, was the best plasticizer and the use of 2% of MWCNTs in the membrane composition clearly affects the mechanical and electrical properties of the sensor. The optimal formulation for the membrane comprises 1.0% (w/w) of CB[6], 66.8% (w/w) of o-NPOE, 29.8% (w/w) of PVC, 0.3% (w/w) of KTpCIPB and 2.0% (w/w) of MWCNTs. The electrode responded with a slope of 30.9 ± 1.2 mV/dec, a practical detection limit of $(3.0 \pm 0.61) \times 10^{-7}$ mol/L and a lower limit of linear range of $(3.00 \pm 0.00) \times 10^{-7}$ mol/L. Coupling of the potentiometric sensor to a sequential injection analysis system enabled the automatization of the procedure, using around 0.2mL of blood serum after cleaning with SPE-MCX column. The analysis of real samples give rise to recoveries of about $(97 \pm 6)\%$; $(103 \pm 1)\%$ and $(118 \pm 9)\%$ for the histamine concentration of 1×10^{-4} ; 1×10^{-5} and 1×10^{-6} mol/L, respectively.

The big challenge of this work was the collection of forensic samples and its analysis by the developed ion-selective sensor. However, as forensic sample collection involves ethical constraints that could not be overcome in a timely manner, it was chosen to show the validation of the sensor through the analysis of a biological samples. The choice of this matrix is due to the clinical importance of histamine, since it plays an important role in the human/animal organism.

In the future, the analysis of different samples of forensic origin is predicted to correlate the levels of histamine with the time after death.

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