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SALIVA BIOFLUID SCREENING OF POTENTIAL STRESS BIOMARKERS FOR HEALTH DIAGNOSIS

DISSERTATION PROJECT- 2ND CYCLE

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MESTRADO EM BIOQUÍMICA PARA A SAÚDE

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To my beloved family who has always supported me and who is the reason I am here today to complete this master's degree.

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"Our Knowledge can only be finite, while our Ignorance must necessarily be infinite." (Karl Pooper).

ABSTRACT

Nowadays, biomarkers are widely used for diagnosis, allowing information about human health and well-being. In addition to blood, other fluids have been the subject of interest, such as sweat, tears and saliva, as they are less invasive and more practical methods. For biochemical evaluations, saliva represents an attractive medium, but still relatively little explored, and therefore time should be devoted to its study. The main objective of the project is to identify potential non-protein salivary biomarkers useful for the assessment of human health, more specifically with regard to stress monitoring.

A set of 11 small molecules (acetylcholine, dopamine, epinephrine, norepinephrine, glutamic acid, serotonin, tyrosine, tryptophan, ascorbic acid, lactic acid, and cortisol) was selected for their potential as relevant biomarkers for identifying stress in humans. These molecules were analysed using analytical chromatographic tools, specifically the liquid chromatography coupled with TANDEM mass spectrometry. To determine which of these molecules could be identified in saliva, samples were collected from 24 healthy individuals, comprising 12 men and 12 women under the age of 30. The samples were analysed using LC-MS/MS, successfully identifying and quantifying acetylcholine, dopamine, epinephrine, norepinephrine, glutamic acid, tyrosine, tryptophan, ascorbic acid, and cortisol in saliva. Lactic acid could not be quantified due to sampling limitations.

Electrochemical analysis of the saliva samples was also performed using screen-printed electrodes (SPEs) and the techniques of cyclic voltammetry (CV) and differential pulse voltammetry (DPV), allowing the identification of three regions where the biomarkers under study could be detected. The pH of the samples was also measured.

Keywords: Biomarkers, saliva, stress, LC-MS/MS, electrochemical techniques, biosensor

RESUMO

Atualmente, os biomarcadores são amplamente utilizados para diagnóstico, permitindo obter informações sobre a saúde e o bem-estar humano. Além do sangue, outros fluidos têm sido alvo de interesse, como o suor, as lágrimas e a saliva, por serem métodos menos invasivos e mais práticos. Para avaliações bioquímicas, a saliva representa um meio atrativo, mas ainda relativamente pouco explorado, devendo, portanto, ser dedicado tempo ao seu estudo. O principal objetivo do projeto é a identificação de potenciais biomarcadores salivares não proteicos úteis para a avaliação da saúde humana, mais especificamente no que se refere à monotorização do *stress*.

Foi escolhido um conjunto de 11 moléculas pequenas (acetilcolina, dopamina, epinefrina, norepinefrina, ácido glutâmico, serotonina, tirosina, triptofano, ácido ascórbico, ácido láctico e cortisol) com o potencial de serem biomarcadores relevantes da identificação do *stress* nos humanos. As mesmas foram analisadas através de ferramentas cromatográficas analíticas, mais especificamente recorrendo à cromatografia líquida acopolada à espetrometria de massa TANDEM (LC-MS/MS). De forma a se perceber quais destas moléculas se podiam identificar na saliva, foram recolhidas amostras de saliva de 24 indivíduos saudáveis, 12 homens e 12 mulheres com menos de 30 anos. As mesmas foram analisadas com recurso ao LC-MS/MS, conseguindo-se identificar e quantificar na saliva a acetilcolina, dopamina, epinefrina, norepinefrina, ácido glutâmico, tirosina, triptofano, ácido ascórbico e cortisol. O ácido lácteo não foi possível de quantificar devido a limitações de amostragem.

Foi também realizada uma análise eletroquímica das amostras de saliva, com recurso a SPEs (*screen-printed electrodes*) utilizando as técnicas de CV (voltametria cíclica) e DPV (voltametria de pulso diferencial), sendo possível a identificação de 3 zonas onde existe a possibilidade de se encontrarem os biomarcadores em estudo. A leitura do pH das amostras foi também efetuada.

Palavras chave: Biomarcadores, saliva, *stress*, LC-MS/MS, técnicas eletroquímicas, biossensor

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ACRONYMS

5-Ht	Serotonin
A	Area
Ach	Acetylcholine
AsA	Ascorbic Acid
BLK	Blank
Cal 01	Preliminary tests to see if the molecules under study were really solubilized
Cal 02	Calibration curve of 0.1 – 1000 $ng.mL^{-1}$, performed on the Accurore™ C 18 column, utilizing MeOH as dilution solvent for standards preparation
Cal 03	Calibration curve of 0.1 – 1000 $ng.mL^{-1}$, performed on the Accurore™ HILIC column, utilizing MeOH as dilution solvent for standards preparation
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Cal 06	Calibration curve performed from 0.1-50 $ng.mL^{-1}$, performed on the Accurore™ C 18 column, utilizing MeOH as dilution solvent for standards preparation
CE	Counter Electrode
Cort	Cortisol
Cv	Coefficient of Variation
CV	Cyclic Voltammetry
DA	Dopamine

DPV	Differential Pulse Voltammetry
E	Epinephrine
GC-MS	Gas Chromatography-mass Spectrometry
Glu	Glutamic Acid
HPLC	High-performance Liquid Chromatograph
Lac	Lactic Acid
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
NE	Norepinephrine
p*Ka*	- log *Ka*, where *Ka* is the acid dissociation constant
QC	Quality control standards
R^2	Squared correlation coefficient of calibration curves
RE	Reference Electrode
Rt	Retention Time
SPE	Screen Printed Electrodes
SWV	Square-Wave Voltammetry
Trp	Tryptophan
Tyr	Tyrosine
UHPLC	Ultra-performance Liquid Chromatography
WE	Working Electrode

1

INTRODUCTION

Stress is a complex concept, is defined as physiological and psychological reactions that prepare an organism's defence against external or internal threats stressors [1], causing an imbalance in body functioning, impair in the nervous system and tension.

Stress in general can be defined by the period of exposure, among other factors. Certain stressors, including extended mental and physical effort, different pathologies and specific behaviours, can induce an increase of these stress biomarkers, leading to pathological consequences on health [1].

Acute stress is defined here as a brief, short-term exposure to stressors. These can include situations like taking an examination, engaging in physical exercise, or completing a challenging laboratory task. In contrast, chronic stress refers to ongoing, repeated exposure to stress, whether real or perceived. This type of stress arises from more persistent pressures, such as relationship difficulties, long-term physical training, or managing chronic medical conditions like diabetes, depression, or high blood pressure [2].

1.1 Saliva for Clinical Diagnosis

Currently, blood is the principal fluid used for stress diagnostics. However, there is a clear lack of non-invasive, real-time sensors capable of measuring stress. Moreover, traditional methods like venipuncture for blood sampling can induce stress themselves, potentially leading to false-positive cortisol responses, particularly in individuals with needle phobias [1].

In addition to blood, other fluids have been targeted, such as sweat, tears and saliva, as they are non-invasive and more practical methods.

Sweat offers a good alternative for sampling, although factors such as humidity, temperature and pH can lead to inconsistent signals. Similarly, tears have shown some potential for detect-

ing stress hormones, yet they face analytical challenges due to lower concentrations of biomarkers such as cortisol and glucose, making it challenging to derive clinical outcomes from tear samples [1].

On the other hand, saliva has been revealing itself an attractive medium for biochemical evaluations, offering potential for the development of biosensor devices capable of continuous, real-time monitoring of human health, since saliva contains a wide range of biomarkers, including proteins, hormones, and enzymes that can be indicative of various health conditions such as stress, disease and poor health. [1].

In addition, saliva collection is relatively stress-free, easy to obtain in sufficient amounts, and can be self-collected with minimal training. This accessibility makes it ideal for a wide range of patients, including children and the elderly, and it can be collected in various settings such as at home or work. Biosensors could in the future be integrated with smartphone applications and other digital health technologies, enabling patients to track their health data more easily and share it with healthcare providers without the need for specialized laboratory facilities.

Whole saliva is a complex fluid produced by three major pairs of salivary glands—the parotid, submandibular, and sublingual glands—along with numerous minor salivary glands in the oral cavity. It contains not only secretions from these glands but also oral bacteria and food debris. The major glands account for 90% of total saliva production, while the minor glands contribute the remaining 10%.The normal daily production of saliva varies between 0.5 and 1.5 L [3].

There are two types of saliva: unstimulated and stimulated. Unstimulated saliva (produced by resting salivary glands) makes up the saliva typically present in the mouth without external triggers. In this state, production is distributed as 25% from the parotid gland, 60% from the submandibular gland, and 7-8% from minor glands [4].

Stimulated saliva is produced in response to external stimuli, such as chemical triggers (taste, smell, medications) or mechanical actions (chewing, vomiting), and saliva production increases fivefold, with the parotid gland contributing the most. During this phase, 50% of the saliva comes from the parotid gland, 25% from the submandibular gland, 7-8% from minor glands, and 7-8% from the sublingual gland [4].

The constituents of each type of saliva are shown in the following Table 1.

Table 1 - Values of sodium, potassium, phosphate, chloride and bicarbonate ($mol.m^{-3}$) in unstimulated and stimulated saliva

Compounds	Unstimulated ($mol.m^{-3}$)	Stimulated ($mol.m^{-3}$)
Sodium	6.53 [4]	26.09 [4]
Potassium	20.46 [4]	20.46 [4]
Phosphate	5.42 [5]	5.23 [5]
Chloride	14.1 [4]	28.21 [4]
Bicarbonate	1 [6]	60 [6]

This way saliva is a very dilute fluid, composed of 98.5% water, and 1% organic and 0.5% inorganic components [7]. Saliva contains three buffer systems: bicarbonate, phosphate and protein, which helps in maintaining the oral pH in a range between of 6.0-7.5 [3].

Saliva is also rich in electrolytes that include sodium, potassium, calcium and magnesium. It also contains immunoglobulins, proteins, enzymes, mucins, and nitrogenous compounds, which work together to fulfil several key functions.

Bicarbonates, phosphates, and urea help regulate pH and maintain saliva's buffering capacity, while macromolecule proteins and mucins assist in cleansing by aggregating, and/or attaching to oral microorganisms, contributing to plaque metabolism. Additionally, calcium, phosphate, and certain proteins act as anti-solubility agents, playing a crucial role in the processes of demineralization and remineralization. Immunoglobulins, proteins, and enzymes also provide antibacterial protection, enhancing oral health [8].

This way, despite the advantages presented for the use of saliva as a biofluid for clinical diagnosis several challenges must be considered. First, saliva collection can be influenced by factors such as the time of day, diet, hydration status, and even physical activity, resulting in inconsistent results.

However, since saliva is a complex mixture, the presence of various proteins, along with food debris, poses a significant challenge for the practical use of wearable biosensors. This complexity increases the risk of nonspecific adsorption, which can result in biofouling of the devices, ultimately compromising their reliability and accuracy [2].

Additionally, saliva can easily become contaminated with food particles, oral bacteria, and other contaminants, which can impact the accuracy of tests. So, the composition of saliva can

vary between individuals and under different physiological conditions, complicating the interpretation of results.

It's also important to note that despite being less invasive than other methods, some patients may still feel discomfort or aversion to saliva collection, particularly if specialized collection techniques are required.

1.2 Biomarkers

Biomarkers are measurable indicators of biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. For clinical diagnosis, they are measurable substances or indicators that can provide valuable information about a person's health status and they can help in the diagnosis, monitoring, and prognosis of various diseases and conditions [9].

Since the main goal of the project was to identify potential non-protein salivary biomarkers useful for the assessment of human health, more specifically with regard to stress monitoring, eleven molecules were chosen to evaluate their response. All of them were known to have some kind of connection with human's health, more specifically with stress-related conditions.

1.2.1 Neurotransmitters

Neurotransmitters (NTs) play a crucial role in responding to various environmental stimuli and alleviating stress. Nowadays, it's known that some of them are associated with stress levels.

Acetylcholine

Acetylcholine is a choline molecule that undergoes acetylated at the oxygen atom. It serves as a crucial neurochemical with a variety of functions across the brain and other organ systems of the body. As a neurotransmitter, acetylcholine acts as a chemical message that is released by neurons and that facilitates communication with another specialized cells, such as muscle cells (myocytes) and cells within glandular tissues [10].

Nowadays, recent studies indicate that stress increases the spontaneous release of Ach.

When a person experiences stress, the body's autonomic nervous system responds by activating the sympathetic nervous system (the fight-or-flight response). The fight-or-flight response refers to your body's response to a perceived or real stressful situation, such as needing to

escape danger [11]. After the stressor is removed, acetylcholine helps mediate the parasympathetic response, promoting relaxation and recovery.

This way some studies have shown that prolonged or chronic stress can lead to dysregulation acetylcholine levels and receptor sensitivity. This dysregulation can contribute to various stress-related disorders, including anxiety and depression, and that's why elevated levels of Ach were found in subjects experiencing active depression [3], as well as in those with a history of depression [12]. This excess can result in severe problems such as slurred speech, convulsions, coma, and respiratory depression.

On the other hand, high acetylcholine levels in the brain patients may cause headache, insomnia, giddiness, confusion, and drowsiness. In extreme cases, it may even lead to death due to effects on the heart, respiration, and brain function [13].

In this regard, acetylcholine has a complex role in the body's response to stress, affecting both physiological and psychological processes. By mediating the balance of the activities of the sympathetic and parasympathetic systems, acetylcholine aids in managing the body's stress response and promoting recovery.

1.2.1.1 Biological Amines

Biological amines, are organic compounds derived from amino acids that play crucial roles in various physiological processes, including the regulation of mood, stress responses, and neurotransmission.

Dopamine

Currently, dopamine is known to play a crucial role in almost all cognitive functions, including motor control, motivation, and learning. Its significance for health is widely discussed in myriad articles and social media posts, being often referred as the 'pleasure hormone' or 'reward molecule' [14].

Known as the "feel-good" hormone dopamine triggers feelings of pleasure and satisfaction by acting on specific brain areas. It also provides you motivation to do something when you're feeling pleasure. When you feel good, when you're achieve goals or experiencing something enjoyable, it's because you have an increase of dopamine levels in the brain.

On the other hand, under stress dopamine levels can fluctuate. Stressful situations can lead to an increased dopamine release, enabling the brain to focus and quickly prepare itself to

respond in an appropriate behaviours. As a hormone, dopamine is after released into your bloodstream, playing a small role in the "fight-or-flight" response [15].

Imbalanced dopamine levels, whether too high or too low, in some parts of the brain have been linked to mental health conditions such as depression, schizophrenia and psychosis [16]. Prolonged stress lead to downregulation of dopamine receptors, making the brain less responsive to dopamine lowering dopamine levels, leading to decreased motivation, concentration, excitement, and an overall sense of well-being.

In this context, dopamine is essential to the body's stress response, impacting motivation, reward processing, and mood regulation. Although acute stress can temporarily boost dopamine activity, chronic stress can disrupt this balance, potentially leading to mood disorders and negatively affecting overall well-being.

Serotonin

Serotonin (5-HT) is a key neurotransmitter in the central nervous system that plays an essential role in various physiological and pathological processes within the brain [17].

While dopamine is often linked to reward and motivation, serotonin is associated with enhancing mood, fostering happiness, and reducing anxiety. This way, 5-HT has two key functions: regulating anxiety and stress while promoting patience and ability to cope [17].

Researchers had established a clear link between low serotonin levels and increased risk of mental health conditions, including depression, anxiety, posttraumatic stress disorder, and schizophrenia [18]. Acute stress can initially increase serotonin release in certain brain regions, aiding in the regulation of the stress response. However, chronic stress often leads to a reduction of serotonin levels, potentially leading to much severe symptoms such as anxiety, irritability, and depression [19].

Epinephrine and Norepinephrine

Epinephrine and norepinephrine share many similarities, since they are the hormones responsible for triggering the "fight-or-flight", which prepares the body to face stressful or dangerous situations. When stress occurs, both hormones are activated and released in the bloodstream, to help the body respond quickly and in an effective way.

Epinephrine (commonly referred to as adrenaline), produced by the adrenal glands, triggers specific reactions in the body designed to ensure people's survival. It causes blood vessels to constrict, redirecting blood flow to vital muscles and organs, such as the heart and lungs, leading to increased heart rate, elevated blood pressure and enhanced blood flow to muscles [20].

This results in reduced pain perception, increased strength and physical performance, as well as heightened awareness and mental focus [21].

On the other hand collaborates with other hormones, including cortisol, to orchestrate the body's stress response. While epinephrine delivers quick energy and heightened alertness, cortisol plays a crucial role in maintaining this response over a longer duration and regulating a variety of bodily functions [22].

While it is essential for responding to stressors, chronic activation of the epinephrine response can lead to negative health consequences which can have detrimental effects on the body contributing to an increased risk of cardiovascular diseases, and weakened immune function. Elevated levels of epinephrine are associated with heightened emotions, leading to excessive arousal and anxiety symptoms.

Norepinephrine (sometimes referred to as noradrenaline) is a neurotransmitter and hormone that responds to stress and low blood pressure.

Same way as epinephrine, it enhances alertness, focus, and attention, making it easier for individuals to respond quickly in stressful situations, the difference is that, norepinephrine acts in bronchodilation (widening of the air passages in the lungs) and increase glucose availability, being this changes crucial for preparing the body to handle stress.

It also plays a role in regulating your ability to concentrate and maintaining your sleep-wake cycles, helping in the wake up in the morning, improving attention, and maintaining focus throughout the day [21].

An imbalance of norepinephrine, whether excessive or insufficient, can affect mental and emotional health, contributing to conditions such as depression, anxiety, addiction, substance abuse, and post-traumatic stress disorder [21].

1.2.2 Amino Acid Precursors of Biological Amines

Same as biological amines their precursors also play a significant role in the body's stress response.

Tryptophan

Tryptophan is an essential amino acid in humans and as a precursor of serotonin and melatonin, tryptophan intake is closely related to the reduction of depressive and anxious moods.

Since tryptophan cannot be synthesized in the body and must be obtained through dietary sources, [23] a research examining the impact of dietary tryptophan on affective disorders has found that tryptophan-rich diet can lead to fewer depressive symptoms and improved mood. As seen before during stressful situations, serotonin levels can be affected, since stress tends to inhibit the expression of this enzyme, reducing the serotonin levels, [24] leading to mood disturbances, anxiety, and depression. Therefore, sufficient tryptophan intake increases the availability of serotonin brain synthesis and help buffer the effects of stress. This may lead to favorable neuropsychological changes, boosting resilience to negative emotions [24].

On the other hand, as mentioned tryptophan is also a precursor to melatonin, a hormone that regulates sleep. Sleep disturbances are common during periods of stress, and adequate tryptophan levels can help promote restful sleep [25].

This way, sufficient levels of tryptophan can help alleviate the adverse effects of stress by facilitating serotonin synthesis, enhancing mood, and promoting improved sleep quality. In contrast, chronic stress may reduce tryptophan levels and impair serotonin production, which can contribute to anxiety and depression. Including foods rich in tryptophan in the diet may provide benefits for those dealing with stress and its associated symptoms [25].

Tyrosine

Tyrosine is a non-essential amino acid that play a crucial role in the synthesis of the catecholamine neurotransmitters dopamine, norepinephrine, and epinephrine.

Studies have been conducted to explore tyrosine's potential in mitigating the effects of stress [26], revealing that its release rate increases in response to stress, which in turn affects the production of dopamine, norepinephrine, and epinephrine.

As we seen before, under stressful conditions, the demand for these catecholamine's increases. So a tyrosine supplementation has been shown to improve cognitive flexibility and working memory during acute stress, helping individuals maintain performance in challenging situations [27].

The main effects of tyrosine that have been reported have been observed in its ability to prevent a decline in cognitive function during physical stress. The physical stressors include conditions of interest to the military, such as cold stress, the combination of cold stress and high-altitude stress, prolonged wakefulness and lower body negative pressure stress [28].

During this stressors the levels of the referred catecholamine's starts to decrease, potentially leading to mood disorders and fatigue, this way, adequate levels of tyrosine replenish these catecholamine levels, supporting mental health during prolonged stress.

This way, consuming a diet rich in tyrosine may be beneficial for individuals dealing with stress.

1.2.3 Amino Acids

Amino acids play a critical role in the body's response to stress by serving as precursors for neurotransmitters and hormones that regulate mood, cognition, and overall physiological functions.

Glutamic acid

Glutamic acid, also known as glutamate when referring to its ionic form, is a non-essential amino acid that plays several critical roles in the body, particularly in the brain.

Glutamic acid serves as an excitatory neurotransmitter in the central nervous system, is involved in various neurological functions, including learning and memory [29].
Recent studies, however, has linked central system glutamate dysregulation with various forms of physical distress, including pain intensity, sensitivity, and pain tolerance. Additionally, growing evidence suggests that glutamic acid plays a role in psychiatric distress, as it has been implicated in the pathophysiology of severe and chronic psychiatric disorders, such as psychotic, anxiety, and depressive disorders [30].
This way, maintaining proper glutamate balance is essential for resilience to stress and overall mental health, highlighting the importance of a balanced diet rich in glutamic acid-containing foods.

1.2.4 Carboxylic Acids

While carboxylic acids themselves may not be directly linked to stress, some of their derivatives and related compounds are associated with physiological and psychological stress mechanisms.

Ascorbic acid

L-ascorbic acid is commonly known as vitamin C, a water-soluble antioxidant vitamin, essential to human and several other mammals.
Vitamin C deficiency is often associated to stress-related diseases. While its effectiveness in treating anxiety spectrum disorders is less established, numerous studies have indicated that

ascorbic acid supplementation can produce antidepressant effects and enhance mood, [31] alleviating some of the harmful effects associated with chronic stress.

Ascorbic acid also plays an indirect role in the regulation of catecholamine's (dopamine and norepinephrine) by several mechanisms. First, ascorbic acid helps maintain the activity of tyrosine hydroxylase, so a diet deficient in vitamin C can lead to decreased serotonin levels. Second, ascorbic acid acts as a direct electron donor in the conversion of dopamine to norepinephrine, so ascorbic acid is important for regulating these catecholamine concentrations in the body [31].

This way, maintaining adequate levels of vitamin C through diet or supplementation may be beneficial for individuals experiencing stress and its related symptoms.

Lactic acid

Lactic acid is a substance produced by the body when cells metabolize glucose or other carbs to obtain energy. Increased blood lactate concentration is commonly observed during both physiological stress, such as exercise, and pathophysiological stress, referred to as stress hyperlactatemia [32].

Lactic acid normally accumulates in the muscles during intense physical activity. It's the way of the body to fuels the muscles providing energy when oxygen is limited. When people exert themselves too much, excessive exertion can lead to lactic acidosis, which could result in symptoms such as nausea, exhaustion and vomiting [33].

Moreover, the overproduction of lactate can lead to excessive lactic acid accumulation, that could cause another diseases including pulmonary disorders that reduce the blood oxygen levels, circulatory disorders that deprive oxygen delivery to tissues due to slow blood flow and haemoglobin disorders that impair the ability of red blood cells to carry oxygen effectively [23].

1.2.5 Steroid Hormone

Steroid hormones play a critical role in the body's response to stress, particularly in the regulation of various physiological processes.

Cortisol

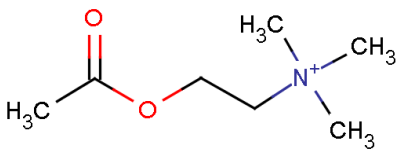
Cortisol, a glucocorticoid hormone produced by the adrenal glands [35], is a key mechanism that regulates how environmental factors influence the stress response, that's why is often referred as the "stress hormone".

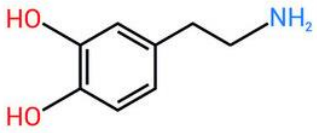
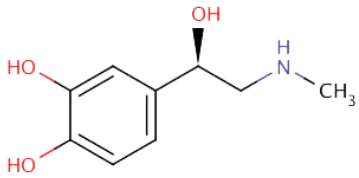
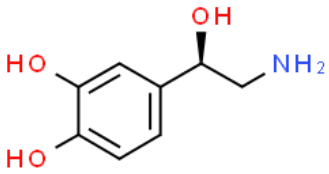
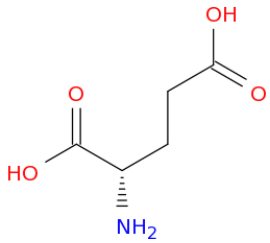
Cortisol plays a vital role in various physiological processes in the human body [36]. In acute stress situations, cortisol helps initiate the "fight or flight" response by mobilizing energy by increasing glucose availability and facilitating metabolic processes, to provide immediate energy and suppressing non-essential functions (like digestion and reproduction) to prioritize energy for dealing with the stressor. However in chronic stress, prolonged elevated cortisol levels can lead to various negative health outcomes, including immune suppression, increased blood pressure, weight gain, anxiety, and depression, which can escalate to suicide attempts [37].

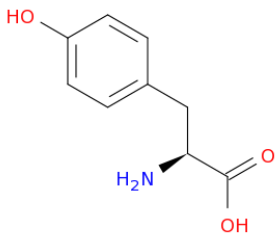
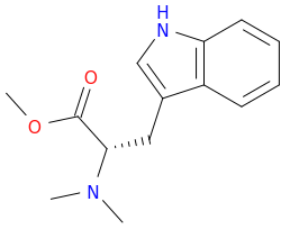
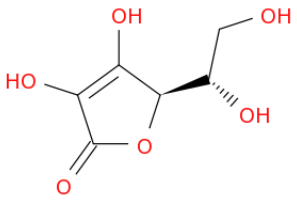
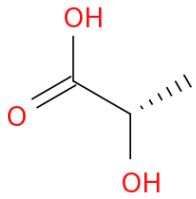
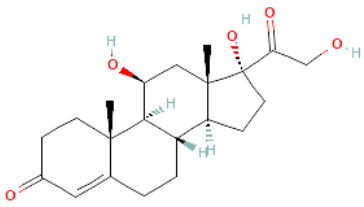
It is also important to take into account the natural fluctuations in cortisol throughout the day. In some studies, diurnal cortisol serves as a comprehensive assessment by capturing cortisol levels from morning to night and examining the diurnal cortisol slope, which describes how cortisol levels change over the course of the day. Typically, cortisol levels rise first hour after awakening before gradually declining throughout the day, followed by an increase at night [36].

Table 2 present the properties of the biomolecules in study.

Table 2- Biomolecules in study. Chemical structures, molar mass (g.mol^{-1}) and p*K*a values; Solubility (mg.dm^{-3})

Compound	Chemical Structure, molecular formula, molar mass (*M* in g.mol^{-1}) and pKa	Solubility
Ach	 $C_7H_{16}NO_2$[38] ; *M*= 146.21[38]; pKa =12.31; p*K*a =13.65[39]	Soluble in water (6×10^{05} mg.dm^{-3} at 20°C), alcohol, DMSO (36 mg.dm^{-3} at 25°C), and ethanol (36 mg.dm^{-3} at 25°C). Insoluble in ether [39]

DA	 $C_8H_{11}NO_2$ [40]; *M*= 153.1 [40]; p*K*a =8.93 [40]	Soluble in water (1×10^{03} mg.dm^{-3}) [41], methanol (20 mg.dm^{-3}) [40]
E	 $C_9H_{13}NO_3$ [42]; *M*= 183.20 [42]; p*K*a =8.59 (at 25 °C) [43]	Soluble in water (180 mg.dm^{-3}); [44]
NE	 $C_8H_{11}NO_3$ [45]; *M*= 169.18 [45]; p*K*a =8.64; p*K*a[46]	Soluble in water (12.5 mg.dm^{-3}); slightly soluble in ethanol and di-ethyl ether [45]
Glu	 $C_5H_9NO_4$ [47]; *M*= 147.13 [47]; p*K*a =2.19; p*K*a =4.25; p*K*a =9.67 [47]	Soluble in water (8.57 mg.dm^{-3} at 25 °C); insoluble in methanol, ethanol, ether and acetone [47]
5-HT	 $C_{10}H_{12}N_2O$ [48]; *M*= 176.21 [48]; p*K*a =9.31 [49]	Soluble in water (25.5 mg.dm^{-3} at 25 °C); methanol and ethanol (5.3 mg.dm^{-3}) [50]

Tyr	 $C_9H_{11}NO_3$ [51]; *M*= 181.19 [51] p*K*a =2.06; p*K*a = 9.15 [51]	Soluble in water (0.479 mg.dm^{-3}) [52]; slightly soluble in ethanol; soluble in formic acid [51]
Trp	 $C_{11}H_{12}N_2O_2$ [53]; *M*= 204.22 [53]; p*K*a =7.38 (at 25 °C) [53]	Soluble in water (13.4 mg.dm^{-3} at 25 °C); Slightly soluble in acetic acid, ethanol; insoluble in ethyl ether [53]
AsA	 $C_6H_8O_6$ [54]; *M*= 176.12 [54]; p*K*a =4.17; p*K*a = 11.57 [54]	Soluble in water (400 mg.dm^{-3} at 40 °C) [54];
Lac	 $C_3H_6O_3$ [55]; *M*= 90.08 [55]; p*K*a =3.86 (at 25 °C) [55]	Soluble in water (562.0 mg.dm^{-3}); Completely soluble in ethanol, di-ethyl ether, and other organic solvents which are miscible with water [55]
Cort	 $C_{21}H_{30}O_6$ [56]; *M*= 362.5 [56]; p*K*a =12.31; p*K*a = 13.65 [57]	Soluble in water (0.32 mg.dm^{-3} at 25 °C); methanol (6.2 mg.dm^{-3}) [56]

1.3 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS)

To understand the response of the molecules of interest for study, the Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) technique was used.
Liquid Chromatography coupled with tandem mass spectrometry is an analytical technique that integrates the separation capabilities of liquid chromatography with the highly sensitive and selective mass analysis capability of triple quadrupole mass spectrometry [58]. This combination allows the effective separation, identification, and quantification of complex mixtures in various samples, making it a powerful tool in fields such as pharmaceuticals, environmental analysis, and clinical research.

In this technique, a sample solution containing the analytes of interest is pumped through a stationary phase with a mobile phase flowing at high pressure. The chemical interactions between the sample components, the stationary phase, and the mobile phase influence their migration rates through the column, resulting in effective separation. The wide variety of stationary and mobile phase combinations allows for tailored separations to suit different complex solutions, enhancing the technique's versatility and efficiency.

After elution from the column, the effluent is directed to the mass spectrometer. In an LC-MS/MS system, the mass spectrometer contains an ionization source where the column effluent is nebulized, desolvated, and ionized, resulting in the formation of charged particles. These ions are then analyzed to provide information about the mass-to-charge ratios, allowing for the identification and quantification of the analytes present in the sample.

These charged particles then migrate under high vacuum through a series of mass analysers (quadrupoles) by applying electromagnetic fields. A specific mass-to-charge precursor ion is selected to pass through the first quadrupole, while all other mass-to-charge ratio particles are excluded. In the collision cell, the selected mass/charge ions are then fragmented into product ions through collisions with an inert gas [59].

The third quadrupole is utilized to target specific product ion fragments. The resulting isolated product ions are quantified with an electron multiplier. This transition from precursor ions to

product ions is highly specific to the structure of the compound of interest, providing a high degree of selectivity. As a result after the ionization for MS, some of the ions are further fragmented, enabling detection of structurally related isomers [59].

LC-MS/MS offers several advantages over other analytical methods. It provides greater sensitivity and specificity compared to other methods, making it particularly effective for low molecular weight analytes. Its analytical specificity surpasses that of immunoassays and high-performance liquid chromatography (HPLC). Additionally, LC-MS/MS has higher throughput compared to gas chromatography-mass spectrometry (GC-MS), enabling more efficient analysis of samples.

LC-MS/MS challenges despite the numerous benefits of LC-MS/MS, it is not without challenges. Besides of being an expensive method, as a high complexity system, LC-MS/MS requires a high level of technical expertise to develop and validate laboratory-developed tests, as well as troubleshoot the instruments. In addition, the sensitivity of LC-MS/MS may allow lower limits of detection for some analytes, such as steroids, compared to immunoassays and other methods.

1.3.1 Column for UHPLC

Two different HPLC columns were used to evaluate the method's performance in terms of separation, peak area, and detection: a C 18 reversed-phase column and a HILIC column.

Hydrophilic interaction liquid chromatography (HILIC) and reversed-phase liquid chromatography (RPLC) are complementary techniques. RPLC employs non-polar stationary phases, such as the C 18 column, while HILIC uses polar stationary phases. Both methods commonly utilize mobile phases consisting of acetonitrile and water, but the elution strength of the solvents differs. In RPLC, acetonitrile is the strong eluting solvent, whereas in HILIC, water is the strong eluting solvent [60].
In RPLC, non-polar stationary phases like C18 (octyldecylsilane, with 18 carbon atoms bonded to silica) preferentially retain hydrophobic compounds, causing hydrophilic compounds to elute [61]. Conversely, HILIC employs highly polar stationary phases, such as silica, which adsorb water on their surfaces. This layer of water enhances the retention of hydrophilic compounds, while hydrophobic compounds are eluted earlier.
The resume of the differences between the two presented columns is in Table 3.

Table 3 - Comparison between C 18 and HILIC column in terms of their stationary phases, mobile phases, retention behaviour, and elution order.

C 18	HILIC
Nonpolar stationary phase	Polar stationary phase (for example, silica)
Polar mobile phase	Polar mobile phase
- Water/acetonitrile, water/methanol	- Water/acetonitrile
-Decrease retention by decreasing polarity of mobile phase	-Decrease retention by increasing polarity of mobile phase
- Increase acetonitrile in mobile phase to decrease retention	- Increase water in mobile phase to decrease retention
Elution order: polar to nonpolar	Elution order: nonpolar to polar

1.4 Electrochemistry

1.4.1 Electrochemistry Techniques

Electrochemistry involves chemical phenomena associated with charge separation. Often this charge separation leads to charge transfer, which can occur homogeneously in solution, or heterogeneously on electrode surfaces [62].

Among the various methods available for studying electrode processes, potential sweep methods are probably the most widely utilized.

Pulse voltammetric techniques, a subset of these methods, are particularly effective to detect species in very small concentrations, making them valuable tools in electroanalytical applications.

1.4.1.1 Cyclic Voltammetry (CV)

Cyclic voltammetry (CV) is a widely and powerful popular electrochemical technique that is commonly employed to investigate the reduction and oxidation processes of molecular species.
To perform cyclic voltammetric analysis, a solution containing an electrolyte and reference is placed in an electrochemical cell along three electrodes: a working electrode, a reference electrode, and a counter electrode. A potentiostat is then used to linearly sweep the voltage between the working and reference electrodes until it reaches a predetermined limit, at which point the sweep back in the reverse route [63].
Cyclic voltammetry offers several advantages over linear sweep voltammetry. The reverse sweep provides additional information that aids in the identification of materials. Furthermore, the peaks observed in the voltammogram offer valuable insights into the chemical reactions occurring at the electrode surface. For instance, if a peak indicating ion reduction appears during the forward sweep, a corresponding peak should appear during the reverse sweep, representing the re-oxidation of the ion back to its original state [64].

1.4.1.2 Differential pulse voltammetry (DPV)

In normal pulse voltammetry a base potential is selected, typically at a point where no faradaic reaction occurs, and this is applied to the electrode. From this value short pulses of increasing amplitude are applied, with the current being measured at the end of each pulse.
Differential pulse voltammetry on the other hand is a technique that is similar to normal pulse voltammetry but with two important differences: the base potential is incremented equally between pulses and the current is measured immediately before pulse application as well as and at the end of the pulse. The difference between the two currents is then recorded [65].

The primary advantage of differential pulse voltammetry (DPV) is its low capacitive current, which enhances sensitivity. Additionally, the small step sizes in DPV result in narrower voltammetric peaks, making it especially useful in applications where precise identification of closely related species is required. This allows for better discrimination between analytes with similar oxidation potentials.

1.4.2 Comparison between Screen-Printed Electrodes and Classic Electrochemical Cells

Screen-printed electrodes (SPEs) and classic electrochemical cells are both widely used in electrochemical analysis, but they differ significantly in terms of design, application, and performance.

SPEs are compact, all-in-one devices where the working, reference, and counter electrodes are printed onto a single substrate using conductive inks, such as carbon, silver, or gold. Carbon-based inks based in graphite, graphene or carbon nanotubes are commonly used to produce the WE area. This makes them highly portable and convenient for on-site testing [66]. In contrast, classic electrochemical cells consist of separate, larger components for each electrode (working, reference, and counter), typically made from materials like platinum or glassy carbon. These electrodes are individually immersed in solution within a dedicated electrochemical cell.

While SPEs provide sufficient sensitivity for many applications, they generally offer lower sensitivity and accuracy compared to classic electrochemical cells. This is due to their smaller surface area and simpler fabrication process. Classic cells, with their larger electrode surfaces and more precise construction, provide higher sensitivity and precision, making them better suited for detecting low concentrations of analytes.

SPEs are user-friendly and require minimal preparation, making them ideal for point-of-care diagnostics and field applications. Their quick setup and portability enable fast, on-site testing. In contrast, classic electrochemical cells require more expertise to assemble and maintain. They are typically used in laboratory environments for more complex electrochemical analyses. Additionally, the working electrode in classic cells often needs to be polished between experiments to remove any redox reaction products that accumulate during some experiments, which can slows down the process of analysis.

2
MATERIALS AND METHODS

2.1 Analytical Standards

All chemicals and reagents were of commercial origin. Analytical standard biomarkers tested: (-)-Epinephrine (E, ≥ 99%, Sigma®), (-)-Norepinephrine (NE, ≥ 98%, Sigma-Aldrich®), L-Glutamic Acid (Glu, ≥ 99%, Sigma®), L-Tryptophan (Trp, ≥ 98% HPLC, Sigma-Aldrich®), L-Tyrosine (Tyr, ≥ 98% HPLC, Sigma-Aldrich®), Sodium DL-lactate (Lac, ≥ 98% HPLC, Sigma®), Serotonin hydrochloride (5-HT, ≥ 98%, Sigma®), Ascorbic acid (AsA, Sigma-Aldrich®), Dopamine Hydrochloride (DA, E, ≥ 99%, Sigma®), Acetylcholine Chloride (Ach, Sigma-Aldrich®) and Hydrocortisone (Cort, ≥ 98%, Sigma®), Sodium Phosphate Dibasic Dihydrate Substance tampon (≥ 99%, Riedel-de Haën), Sodium Phosphate Monobasique Dihydrate (≥ 98%, Riedel-de Haën).
Analytical solvents, methanol and acetonitrile solvents for UHPLC-MS grade and Formic acid for LC-MS grade, were supplied from Carlo Erba® Reagents S.A.S.
Ultrapure water was supplied from a Milli-Q® ultrapure water system equipped at the end of assembly line with a Milli-Q® Reference and a Q-POD® element.

2.2 LC-MS/MS

2.2.1 Current Laboratory Material

The vortex used was a RSLAB®-6PRO. Volume measurements were carried out using micropipettes (Research Plus Eppendorf®; 2–20 mL, 20–200 mL, 100–1000 mL). Weighting measurements were performed using analytical balances Mettler Toledo® (AB204-S)

2.2.2 Material Washing

Since the LC-MS/MS device has high sensitivity for detecting low concentrations of the compounds present, it's necessary to carefully wash the material to be used, as any contamination can affect the analysis of the samples and consequently provide false positives.
This way, the material was washed and prepared according the laboratory protocol using a free phosphate and neutral detergent and glass bowls.
The common glass material to be used in the experimental performance was placed in a bowl, and hot water was poured into it in order to submerge all the material. The material was left to soak for a few minutes in a diluted detergent suitable for LC-MS/MS (2% to 5% in water)
Meanwhile, a second glass bowl was washed (which was used to place the already clean material), using diluted detergent (without phosphates) and hot water in the same way. The water was then poured out and the bowl was washed five times in distilled water and five times in Milli-Q water. After letting the detergent act for a few minutes, water was then poured out and the glassware began to be washed individually.
To do this, each material was washed ten times with distilled water and subsequently thirty times with Milli-Q water and placed in the previously washed cup. After the material was completely clean, it was taken to the oven for 6 hours at 120 ºC.
Afterwards, the material was removed from the oven and placed under ultraviolet light for 15 minutes, making it ready for use.

2.2.3 Standards Preparation

Standards for the 11 molecules under study were prepared. The objective was to prepare solutions of 1000 ppm in 5 mL volumetric flasks, 10 ppm in 10 mL volumetric flasks and 1 ppm in 1 mL vials for each compound. All standards were stored in the freezer.
Taking into account the solubility of each molecule, different solvents were used to solubilize them. As some molecules did not have a well-defined solubility, various ways of solubilizing them were tested.
For Epinephrine that was a problematic one, first we tried to solubilize it a mixture of 50% MeOH/50% H_2O, since it didn't solubilize we added DMSO, and still it didn't solubilize completely. Then we testes just water and same thing happened. After this we tested a 0.1% formic acid solution followed by methanol and E solubilized completely. The same thing happened for Tyr, NE and Trp when we tried just a mixture of 50% MeOH/50% H_2O. So the same thing done for E was done for these three compounds. They were first solubilized in formic acid and then methanol was added.

DA, 5-HT and Cort didn't have any problem so they solubilized quickly in methanol; Ach solubilized in a mixture of 50% MeOH/50% H_2O;
Other problematic compounds were AsA, Glu and Lac that didn't solubilize in methanol. After some tests they only solubilize completely in H_2O.
This way since some tests were performed before the compounds solubilize some of them ended up with concentrations below and above 1000 pm. So we needed to take that in consideration for the preparation of the remaining concentrations.

2.2.4 Mix Preparation

In order to verify whether the prepared standards were ready for use, a mixture containing all the prepared standards was prepared. For this purpose, 150 µL of the prepared 10 ppm solutions were removed. They were then analysed using LC-MS/MS. This preliminary tests were called Cal 01.

2.2.5 Instrumental

The LC-MS/MS analysis was performed using a Dionex® Ultimate 3000 System UHPLC+ focused and a TSQ Quantis™ triple-stage quadrupole mass spectrometer (Thermo Scientific).
A liquid chromatograph, ultra-performance liquid chromatograph (UHPLC), was equipped with four modules, namely, a SR-3000 Solvent Rack, a LPG-3400RS pump, an WPS-3000TRS auto sampler with temperature control, and a TCC-3000RS column compartment from Thermo Scientific Dionex Ultimate 3000 series UHLPC+ focused.
The triple-stage quadrupole mass spectrometer was equipped with an electrospray ionisation (ESI) source. The TSQ Quantis mass spectrometer was controlled using the TSQ Quantis Tune software and the LC-MS/MS operation and acquisition data system are controlled using the XCalibur™.

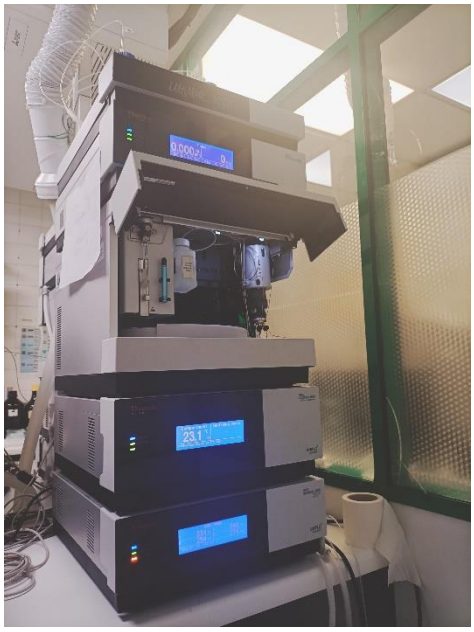


Figure 1 - UHPLC system

2.2.6 Chromatographic and mass spectrometry conditions

The sample injection volume was 10 µl. The separation of the 11 molecules was achieved using an Accurore™ C 18 Column Dim. (mm) 100 mm, 2.1 mm, Thermo Fisher Scientific and a an Accurore™ HILIC Column Dim. (mm) 150 mm, 2.1 mm, Thermo Fisher Scientific. The gradient mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B). The flow rate was 0.2 mL min-1. The equilibration time was 5 min with 10% of mobile phase B. The elution gradient for mobile phase B is as follows: 10% from 0 to 1 min; 25% from 1 to 3 min; 50% from 4 to 6 min; 25% from 7 to 7.5 min and 10% from 8 to 10 min.
Tandem Mass spectrometry (MS/MS) analysis was carried out using a triple-stage quadrupole mass spectrometer. Samples were injected in the select reaction monitoring (SRM) mode, using Multiple Reaction Monitoring (MRM) in positive or negative ionization spray voltage acquisition modes (ESI in positive or negative modes). These are presented in Table 4.

Table 4- Identified biomarkers. Biomarker, retention time (Rt), ESI operation mode, precursor ion (m/Z) and product fragment ions (m/z) and collision energy (eV).

Compound	Rt (min)	ESI mode	Percursor ion (m/z)	Fragment ion (m/z)	Collision Energy (eV)
Ach	0.91	+	146.138	42.917/60/87	28/12/15
DA	0.91	+	154.000	90.917/118.917/136.917	25/19/11
E	0.90	+	184.138	106.917/134.914/166.000	22/14/11
NE	0.90	+	170.060	106.917/134.917/151.917	21/16/9
Glu	0.97	+	148.000	83.917/101.917/129.917	18/12/10
5-HT	0.95	+	177.000	114.917/116.917/159.917	29/28/11
Tyr	1.15	+	182.100	90.917/118.917/122.917/135.917/164.917	17/14/10
Trp	1.39	+	205.112	114.917/118.27/145.917/187.917	41/27/20/18/11
AsA	0.82	-	174.962	71.083/86.917/115.083	12/18/13
Lac	1.16	-	88.950	43.083/71.167/86.917	12/9/9
Cort	8.21	+	363.100	121.000/327.000/344.917	26/17/16

2.2.7 Calibration Curves Preparation

Two calibration curves were prepared using two different solvents. The first was prepared using MeOH and the second using a mixture of 500 mL of H_2O and 500 mL of 0.1% AcFo.
In this way, a mixture of 10 ppm was prepared using the standards previously prepared in 5 mL flasks and the same was made with the solvents to be tested.

Solutions of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 200, 250, 300, 400, 500 and 1000 ng.mL^{-1} were then prepared from the mixes prepared previously.
Two different columns were also tested to separate the components, the Accurore™ C 18 and the Accurore™ HILIC columns, in order to understand which would provide better performance results for the method in terms of area, separation and detection.

2.2.8 New Calibration Curves Preparation

Based on the results obtained, a new calibration curve was prepared using methanol as solvent, presenting concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25 and 50 ng.mL^{-1} utilizing the Accurore™ C 18 column.

2.2.9 Extraction Optimization

In the first stage, blanks were performed on the salivettes®. To do this, a solvent mixture of 50% MeOH/50% H_2O and 50% ACN/50% H_2O was used for the extraction method.
To test the amount of solution that should be placed on each cotton to obtain 1 mL of extracted solution after centrifugation, two salivettes® were first separated and 2.5 mL of a 50% MeOH/50% H_2O mixture was placed on each cotton and the salivettes® were centrifuged for 2 minutes at 1000 ×g in a centrifuge, according to the values present in the instruction manual for collecting saliva samples from the salivettes®.
It was eventually realized that the extracted volume exceeded 1 mL, and therefore it was decided to reduce the volume to be initially placed on the cotton. Two more salivettes were then separated, and 1.5 mL of the 50% MeOH/50% H_2O mixture was placed in each cotton ball, repeating the centrifugation process. This time, an extracted volume of approximately 1 mL was obtained, as desired.
Therefore, the blanks were performed. Since it was intended to produce 6 blanks, six salivettes® were first separated. To see if it was possible to use a cotton ball that was not originally contained in the salivette®, in two of them the cotton ball was replaced by another packaged one.
Therefore, 1.5 mL of the 50% MeOH/50% H_2O mixture was then placed in each cotton pad, and the salivettes® were centrifuged as previously mentioned. The extracted volume in each salivette® was transferred to the vials. The process described above was repeated for the 50%

ACN/50% H_2O mixture, except for the replacement of the cotton, using the salivettes® as they originally came. The volume extracted at the end of the centrifugation was also transferred to the vials.
At the end, 2 extra vials of 2 mL were prepared, one containing only the 50% MeOH/50% H_2O and 50% ACN/50% H_2O mixture.
The vials were taken for analysis in the LC-MS/MS, with each sample being injected in triplicate.

2.2.10 Blanks Preparation

According to the data obtained, 10 blanks were then prepared. Ten salivettes® were separated and 1.5 mL of a mixture of 50% MeOH/50% H_2O were added and centrifuged for 2 minutes at 1000 g, using a CENTROMIX-BLT 230V 50/60Hz centrifuge.
The liquid-liquid extraction was performed with 250 µL of the pooled sample into a 2 mL vial (Alwsci) with screw cap and red PTFE/white silicone septa, adding 250 µL of the mixture of 50% MeOH/50% H_2O.
The sample was centrifuged for 10 min at 3000 rpm. The supernatant liquid extract as transferred using a 500 µL syringe (Gastight 1750 Hamilton) and filtered with a 13 mm, 0.22 µm nylon syringe filter (Filter Lab) into a conical insert (5.8 × 31.5 mm for 2 mL –N2004) into a sterile 2 mL vial with screw cap and red PTFE/white silicone septa.
The blanks were then analysed in triplicate on LC-MS/MS.

2.2.11 Calibration Curve Preparation for each compound

Based on the data obtained, calibration curves were constructed taking into account the particularities of each compound using the mixture of 50% MeOH/50% H_2O as solvent.
For Ach, a curve was constructed with concentrations of 1, 2.5, 5, 10, 15, 20, 30, 40 and 50 $ng.mL^{-1}$; for DA, Cort and E of 0.25, 0.5, 1, 2.5, 5, 10, 20, 30 and 40 $ng.mL^{-1}$; for NE of 50, 100, 150, 200, 300, 400, 500 and 900 $ng.mL^{-1}$; for Tyr of 5, 10, 20, 30, 50, 100, 150, 200 and 250 $ng.mL^{-1}$; for Trp of 5, 10, 25, 50, 100, 150, 200, 250 and 300 $ng.mL^{-1}$; for AsA and Lac of 25, 50, 100, 200, 300, 400, 500, 750 and 1000 $ng.mL^{-1}$; for Glu of 1, 2.5, 5, 10, 50, 250, 500, 750 and 1000 $ng.mL^{-1}$; for 5-HT of 0.25, 0.5, 1, 2.5, 5, 10, 20, 40 and 60 $ng.mL^{-1}$.

2.2.12 Method Validation

The method was validated according to Commission Decision 2002/657/EC [67] concerning the determination of analytes in products of animal origin guidelines, accordingly to SANCO/825/00 revision 8 (ref. 43). The validation was carried by the evaluation of measurement parameters of specificity, linearity, sensitivity, precision, accuracy, recovery, limits of detection (LOD), and limits of quantification (LOQ).

In order to assess the accuracy, through recovery and validate the method, 4 concentration levels were chosen from the calibration curves previously prepared for each compound. Since de dilution factor was 2, the contamination of salivettes® was performed with de double concentration of that 4 concentrations. Therefore, the following concentrations were prepared in 50 mL volumetric flasks for each compound:

For Ach concentrations of 2, 10, 40 and 100 $ng.mL^{-1}$; for DA, Cort and E of 0.5, 2, 20 and 80 $ng.mL^{-1}$; for NE of 100, 200, 800 and 1800 $ng.mL^{-1}$; for Tyr of 10, 40, 300 and 500 $ng.mL^{-1}$; for Trp of 10, 50, 300 and 600 $ng.mL^{-1}$; for AsA and Lac of 50, 200, 800 and 2000 $ng.mL^{-1}$; for Glu of 2, 20, 500 and 2000 $ng.mL^{-1}$; for 5-HT of 0.5, 2, 20 and 120 $ng.mL^{-1}$.

Six salivettes® for each concentration were contaminated with 1.5 mL of that same concentration. Then were centrifuged for 2 minutes at 1000 g, using a CENTROMIX-BLT 230V 50/60Hz centrifuge.

The liquid-liquid extraction was performed with 250 µL of the pooled sample into a 2 mL vial (Alwsci) with screw cap and red PTFE/white silicone septa, adding 250 µL of the mixture of 50% MeOH/50% H_2O.

The sample was centrifuged for 10 min at 3000 rpm. The supernatant liquid extract as transferred using a 500 µL syringe (Gastight 1750 Hamilton) and filters with a 13 mm, 0.22 µm nylon syringe filter (Filter Lab) into a conical insert (5.8 × 31.5 mm for 2 mL –N2004) into a sterile 2 mL vial with screw cap and red PTFE/white silicone septa.

The solutions were then analysed in LC-MS/MS.

2.2.12.1 Specificity

The specificity was confirmed based on the presence of the two transition ions at the correct retention times corresponding to each compound, according to the EU Commission Decision [67].

2.2.12.2 Linearity

Linearity was evaluated by nine-point calibration curves (three replicates), using external calibration, according to the SANCO guidelines [68]. The precision and accuracy were evaluated for all the compounds. To evaluate the linearity, the equation model obtained by weighted least squares linear regression analysis with a weighting factor of 1/X was used. Each back-calculated concentration standard should be within ± 10% deviation.

The squared coefficient of determination for every substance was established by the quadratic regression of the calibration curves ($R^2 > 0.99$).

2.2.12.3 Limit of detection (LOD) and limit of quantification (LOQ).

The limit of detection (LOD) and the limit of quantification (LOQ) were estimated following the FDA approach guidelines [69]. LOQs were confirmed and determined as the lowest concentration of each of compounds in study, which could be quantified with the calibration curves.

2.2.12.4 Precision and accuracy.

Precision and accuracy of the method were determined using QC with 3 replicates at different concentrations defined for each compound. The accuracy was evaluated by comparing the found values with standard additions in spikes and was calculated as the ratio between the mean analytical result and the nominal concentration.

2.2.12.5 Recovery

The recovery was determined by comparing the MS/MS response level of spiked samples without and with extraction, according to the SANCO guide [69] using the average result of triplicates. The recovery percentage was calculated by dividing the value for the MS/MS response of the spiked sample without extraction by that of the extracted sample.

2.2.13 Saliva Sampling

In the next phase, saliva samples were collected from 24 healthy individuals, 12 men and 12 women under 30 years of age. The collection method uses Salivettes® and it was made in in accordance with the ethics committee of the NOVA School of Science and Technology.

Each individual rolled the cotton pad of salivitte® for 1 minute in mouth and put the cotton directly in salivitte® without touching the cotton with hand to avoid possible contaminations.

After saliva collection using the salivettes®, these were centrifuged for 2 minutes at 1000 × g, using a Sigma 3 K30 centrifuge. Liquid-liquid extraction was performed with 250 µL of the sample in a 2 mL vial (Alwsci), adding 250 µL of the 50% MeOH/50% H_2O mixture.

The sample was centrifuged for 10 min at 3000 rpm. The supernatant liquid extract is transferred using a 500 µL syringe (Gastight 1750 Hamilton) and filtered with 13 mm, 0.22 µm nylon syringe filter (Filter Lab) into a conical insert (5.8 × 31.5 mm for 2 mL –N2004) into a sterile 2 mL vial with screw cap and red PTFE/white silicone septa.

The samples were then taken to be analysed in the LC-MS/MS.

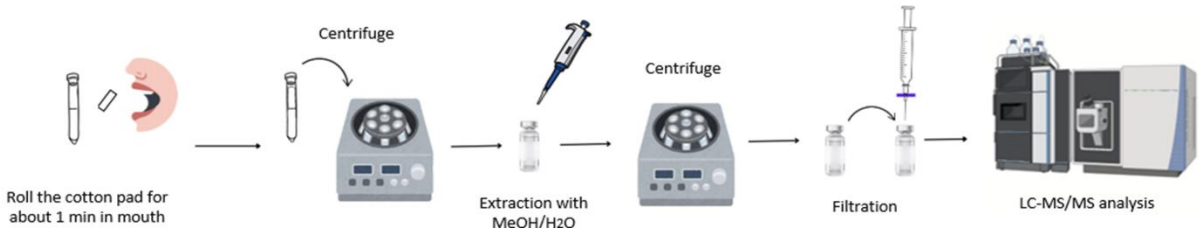


Figure 2 - Steps for Saliva Samples analysis

2.3 Electrochemistry

2.3.1 Buffer solution Preparation

Based on the data obtained for saliva, a 50 mM phosphate buffer solution with pH=7 was prepared in a 100 mL volumetric flask. After preparing the solution, its pH was measured using a METTLER TOLEDO FiveEasy F20-Basic Table pH meter, obtaining a pH of 6.7, so a few drops of a 1 mol.dm^{-3} NaOH solution were added to make the solution more alkaline. The pH was measured again and a solution with a pH of 7 was obtained.

2.3.2 Dopamine solution preparation for optimization of the experimental conditions of electrochemical techniques

Initially, a 5.5 mol.m^{-3} phosphate buffer solution/0.1 mol.dm^{-3} NaCl was prepared in a 50 mL volumetric flask. Then, a 1×10^{-3} mol.dm^{-3} DA solution was prepared in a 25 mL volumetric flask, making up to volume with the aforementioned buffer solution. The solutions were always stored in the refrigerator when not in use. Additionally, the DA solution was protected with greaseproof paper to prevent its rapid degradation by the refrigerator lights.

The three electrochemical techniques (CV and DPV) were then performed using a classical cell presented in Figure 3. The reference electrode (RE) was Ag/AgCl, the working electrode (WE) was pyrolytic graphite and the counter electrode (CE) was a platinum coil. It was used an Autolab ECO Chemie BV type 2 potentiostat/galvanostat electrochemical system for the study controlled by the Electrochemical System software 4.9 (GPES).

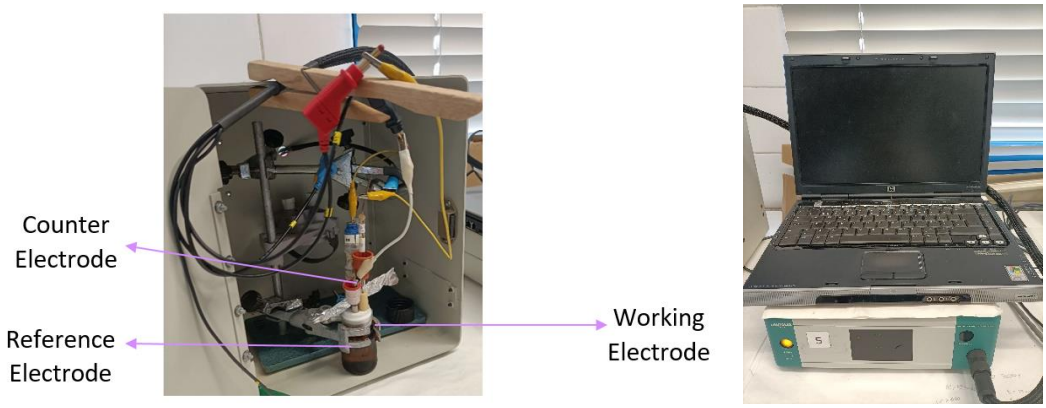


Figure 3 - Classic cell system (left) composed with RE of Ag/AgCl; WE of pyrolytic graphite and CE of platinum coil connected to Autolab ECO Chemie BV type 2 potentiostat/galvanostat (right)

2.3.3 Polishing of the Electrodes

In order to remove the redox reaction products that accumulate during some experiments, between each analysis it was performed the polishing of the working electrode. Since the WE utilized was pyrolytic graphite, the polishing was first performed utilizing a polishing powder (Alumina) of 1.0 µm n for 10 min, followed by a 10 min polishing in a polishing powder of 0.3 µm. During the 10 min, with the WE in a 90º angle, "number 8" is drawn on the polishing cloth as seen in Figure 4.

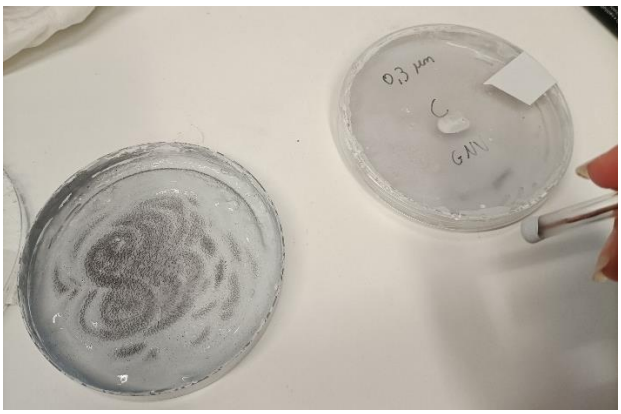


Figure 4 - Polishing of the WE

2.3.4 Saliva Sampling Electrochemical Performance

The saliva samples provided were transferred from the Salivettes®, to a sterile 2 mL vial with screw cap and red PTFE/white silicone septa and kept in ice whenever they were not being tested. The experimental conditions were defined in article [76]

The electrochemical data were acquired using an AUTOLAB PGSTAT 30 Potentiostat/Galvanostat and treated using GPES software. Instead of the classic cell system, were used SPEs (screen-printed electrodes).

SPEs were used with a three-electrode cell configuration, in which the working electrode material (4-mm diameter) were composed by single-walled carbon nanotubes (SWCNT), commercially available (Metrohm/Dropsens); the counter-eletrode were carbon ink and the reference eletrode silver ink.

First to make the SPE activation (pre-treatment of the electrodes), it was used 70 µL of a phosphate buffer solution (86×10^{-3} mol.dm^{-3}/NaCl 18×10^{-3} mol.dm^{-3}, pH 7) performing two voltammetric cycles at 50 mV of CV from 0 to 1.2 V.

Then 70 µL of saliva were analyzed using DPV form -0.35 to 1.50 V at a scan rate of 50 mV. The assays were performed at room temperature.

It was also made a pool from women and men saliva, where were add to a sterile 2 mL vial with screw cap and red PTFE/white silicone septa, 20 µL of each individual saliva. The analysis was performed the same way as mentioned before.

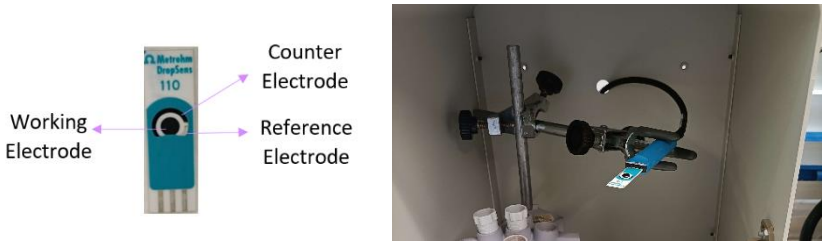


Figure 5 - Screen printed eletrctodes (Metrohm/Dropsens) composed by WE of single-walled carbon nanotubes, CE of carbon ink and RE of silver

2.3.5 Saliva pH Measurement

It was also measured in triplicate the pH of each individual saliva as well the pH from women and men pool using a METTLER TOLEDO FiveEasy F20-Basic Table pH meter, as seen in Figure 5.

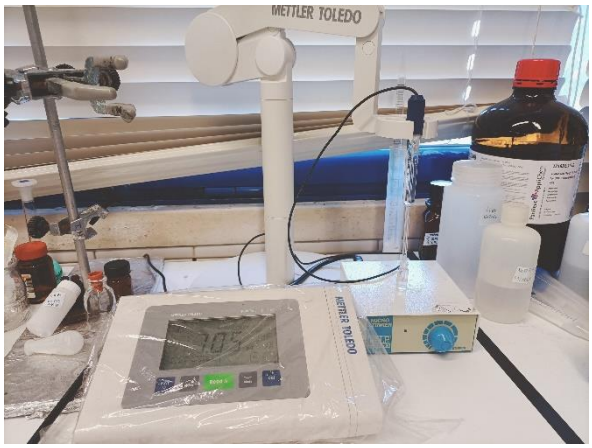


Figure 6 - Saliva pH measurement in METTLER TOLEDO FiveEasy F20-Basic Table pH meter

3
RESULTS AND DISCUSSION

3.1 LC-MS/MS

3.1.1 Method Optimization and Development

3.1.1.1 Selection of the Column to operate and the dilution solvent for standards preparation

In order to optimize the method, as mentioned before two columns were tested, an Accurore™ C 18 and an Accurore™ HILIC, to determine which of the two allows for better separation of the compounds under study.
In addition, the standards were tested using as dilution solvent for standards preparation methanol and a mixture of 50% H_2O/50% formic acid, in order to see in each of them the compounds would be more stable.

The data obtained were analysed and were calculated calibrations curves for each compound. Calibrations curves with higher correlation coefficient and better linearity will be factors to be considered when choosing the best and solvent for standards preparation for the analytical methodology.

For example, for Ach, the calibration curves obtained for each column/solvent standards preparation are presented in Figure 7.

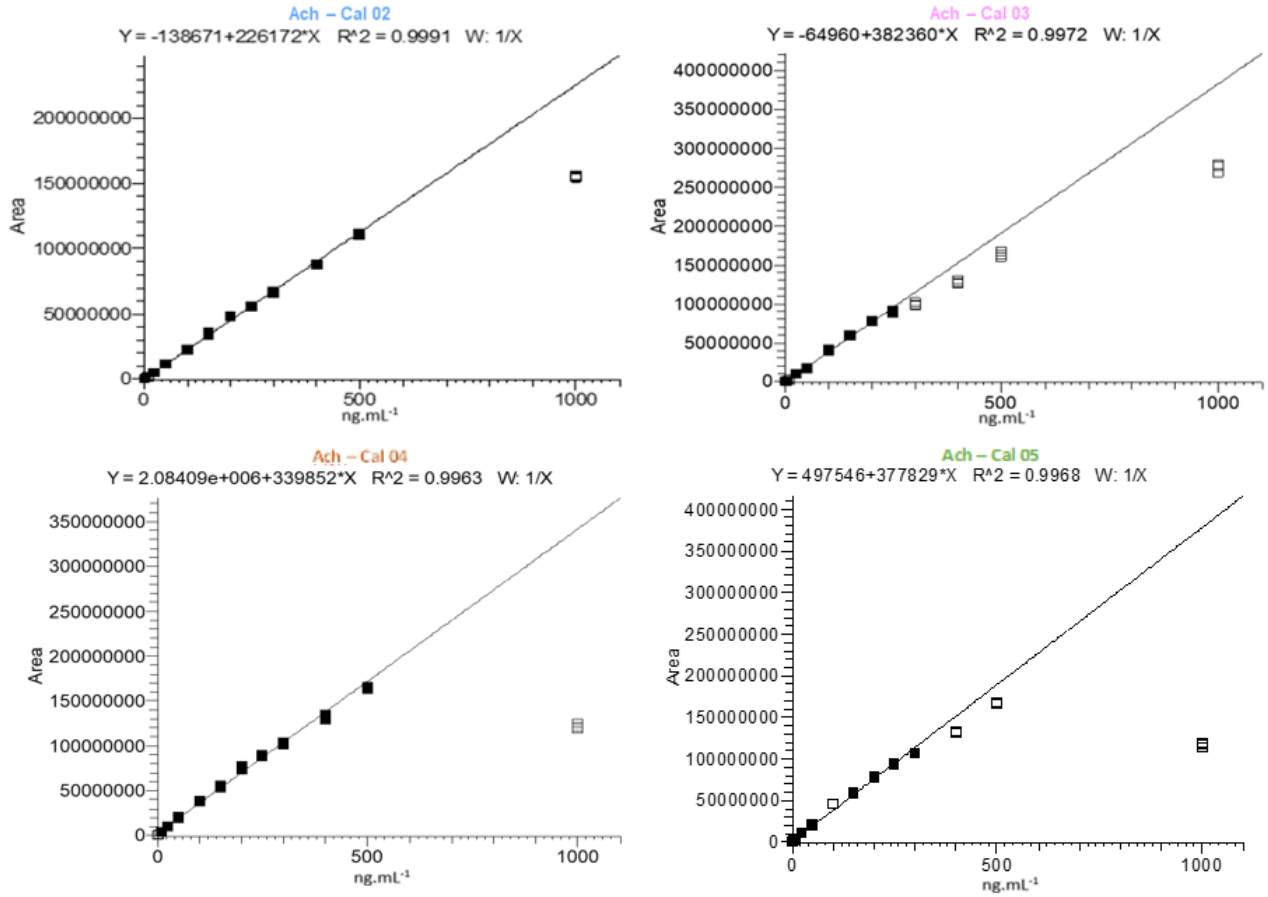


Figure 7 - Calibrations Curves obtained for Ach (Ach): Cal 02 – calibration curve of 0.1 – 1000 ng.mL^{-1}, performed on the Accurore™ C 18 column, utilizing MeOH as dilution solvent for standards preparation; Cal 03 – calibration curve of 0.1 – 1000 ng.mL^{-1}, performed on the Accurore™ HILIC column, utilizing MeOH as dilution solvent for standards preparation; Cal 04 – calibration curve performed from 0.1 – 1000 ng.mL^{-1}, performed on the Accurore™ HILIC column, utilizing a mixture of H_2O/50% AcFo as dilution solvent for standards preparation; Cal 05 – calibration curve performed from 0.1 – 1000 ng.mL^{-1}, performed on the Accurore™ C 18 column, utilizing a mixture of H_2O/50% AcFo as dilution solvent for standards preparation

The equations obtained for the the remaining compounds are present in Table 5 (Cal 02), and in appendix in Table A 1 (Cal 03), Table A 2(Cal 04) and Table A 3(Cal 05).

Table 5 - Linearity parameters of calibration curves from Cal 02 (R^2 and least squares fit) using external standard calibration for each compound.

Compound	Equation Curve	R^2	Linear range (ng.mL^{-1})
Ach	-137176+226164*x	0.9991	1-500
DA	-54029.8+41171.4*x	0.9985	2.5-500
E	-21954.1+20623.1*x	0.9975	2.5-500
NE	3426.13+302.205*x	0.9984	50-500
Glu	-146316+12235.8*x	0.9993	25-500
5-HT	11575.7+10118.1*x	0.9982	2.5-500
Tyr	-26182+30390.6*x	0.9986	2.5-500
Trp	-53724.9+60569*x	0.9992	2.5-1000
AsA	-4224.74+1835.51*x	0.9989	25-1000
Lac	14023.1+125.152*x	0.9992	10-400
Cort	-4210.31+13393.1*x	0.9988	1-500

Through the analysis of the results of the chromatograms, as well as evaluating the linearity of the calibration curves obtained for each compound, it was possible to see that in a generalized way the C 18 column is the one that presented the best results. Both the calibration curves obtained using MeOH and the mixture of 50% H_2O/50% AcFo presented a higher correlation coefficient and better linearity in most of the compounds using the C 18 column.

It was possible to see that Glu presented a higher correlation coefficient using the HILIC column, obtaining a R^2 of 0.9997 in a bigger linear range from 25-1000 ng-mL^{-1} using MeOH for standards preparation, in comparison to C 18 column, that obtained a R^2 of 0.9993 in a linear range of 25-500 ngmL^{-1}. Since the difference is not that significate and that with need to take into account all the compounds and not each one individually, the C 18 column is the one that performed better so is the most suitable for our system separation.

On the other hand, with regard to the dilution solvent for standards preparation, as we can see in Table A 1 and Table A 2, it was not possible to obtain calibration curves for Glu, 5-HT and AsA, utilizing the mixture of 50% H_2O/50% AcFo, so that's not the ideal solvent to prepare the present compounds.

This way it was possible to understand that utilizing MeOH gives more stability to all the compounds, and so higher correlation coefficients (always > 0.99) and better linearity as seen in Table 5.

Based on the optimized conditions for our system discussed previously, another calibration curve was constructed, Cal 06, in a lower range from 0.1-50 ng.mL^{-1}, to understand if the behavior of compounds at lowest concentrations would be different.

The equations obtain from each calibration curve for each compound in Cal 06 are present in Table 6.

Table 6 - Linearity parameters of calibration curves from Cal 06 – calibration curve performed from 0.1-50 ng.mL-1, performed on the Accurore™ C 18 column, utilizing MeOH as dilution solvent for standards preparation (R^2 and least squares fit) using external standard calibration for each compound.

Compound	Equation Curve	R^2	Linear range (ng.mL^{-1})
Ach	-878159+411918*x	0.9981	2.5-50
DA	-73867.2+81534.4*x	0.9971	2.5-50
E	-13793.7+46616*x	0.9997	0.5-50
NE	1500.38+1072.04*x	0.9937	5-50
Glu	-	-	-
5-HT	-2961.48+21790.2*x	0.9991	0.25-50
Tyr	7551.65+48160.8*x	0.9998	0.25-50
Trp	-9326.2+86241.9*x	0.9996	0.5-50
AsA	18986+2155.14*x	0.9991	2.5-50
Lac	-	-	-
Cort	3604.53+22027.7*x	0.9994	0.25-50

As we can see both of the compounds in study present a good correlation coefficient and good linearity. Although it was not possible to construct any calibration curve for Glu and Lac at this concentrations range.

3.1.1.2 Salivettes Blanks

Since the method selected for collecting saliva is salivettes®, it is necessary to understand whether the cottons present in them present any interference for the compounds under study that does not allow their quantification, so the blanks of salivettes® were performed.
The blanks were analysed based on the data obtained for the Cal 02 and Cal 06.
Taking into account the lowest concentration at which it was possible to determine each of the compounds in the two calibration curves, it was possible to calculate the average of the peak area corresponding to that concentration, for possible comparison with the peak areas determined in the blanks of the salivettes®.
When the peak area of the blank is greater than the peak area determined in the calibration curve, the concentration whose peak area value is greater than the peak area of the blank must be found in the calibration curve, so that the latter does not interfere when identifying the compounds in our real sample.

3.1.1.2.1 Extraction Solution - mixture of 50% MeOH/50% H_2O

From the solubility tests performed and from the data obtained in the calibration curves, it was possible to conclude that some compounds were better separated in water and other better in methanol, so we decided to use a "middle ground" utilizing an extraction solution of 50% MeOH/50% H_2O as well as selected for preparing the standard solutions.

To better visualize the comparison of the peak areas obtained in the blanks for each compound, with the peak areas obtained for the lowest quantifiable concentration determined by the calibration curves, the following diagram is presented in Figure 8.

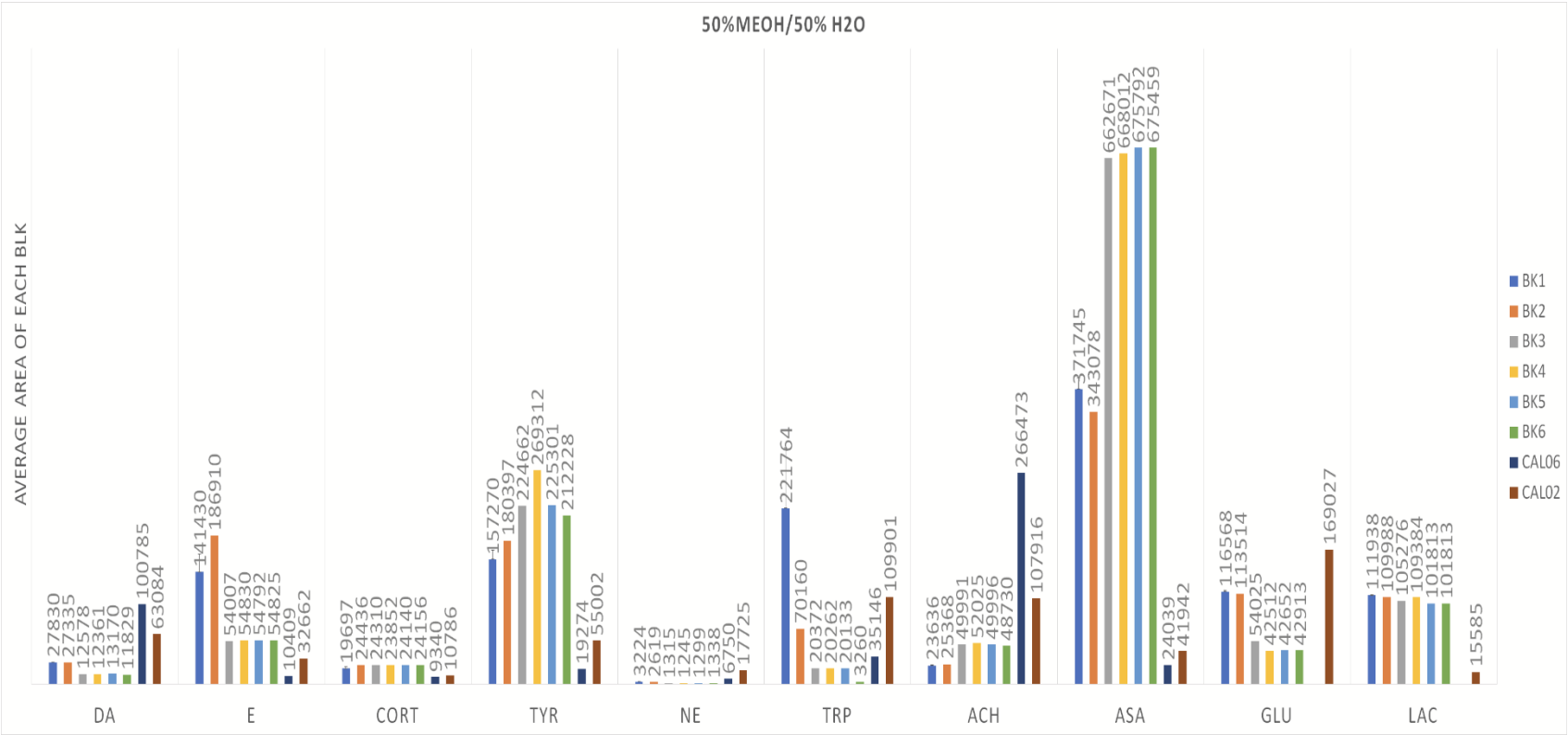


Figure 8- Average area of the triplicate blanks of DA, E, Cort, Tyr, NE, Trp, Ach, AsA, Glu, LAC, using a mixture of 50% MeOH/50%H_2O as extraction solution; Area corresponding to the lowest quantifiable concentration for each compound referred in Cal 02 and Cal 06

As a test to see whether the results obtained would be similar or not, blanks 1 and 2 were made in salivettes® whose cotton had been replaced by another that was sold separately, and so was no longer inside the salivette® like the cotton of the other blanks.
Therefore, the average areas and respective standard deviations of blanks 1 and 2 (called "Substitute Cotton") and of blanks 3 to 6 (called "New Cotton") were determined separately for possible comparison. The following data was obtained in Figure 9.

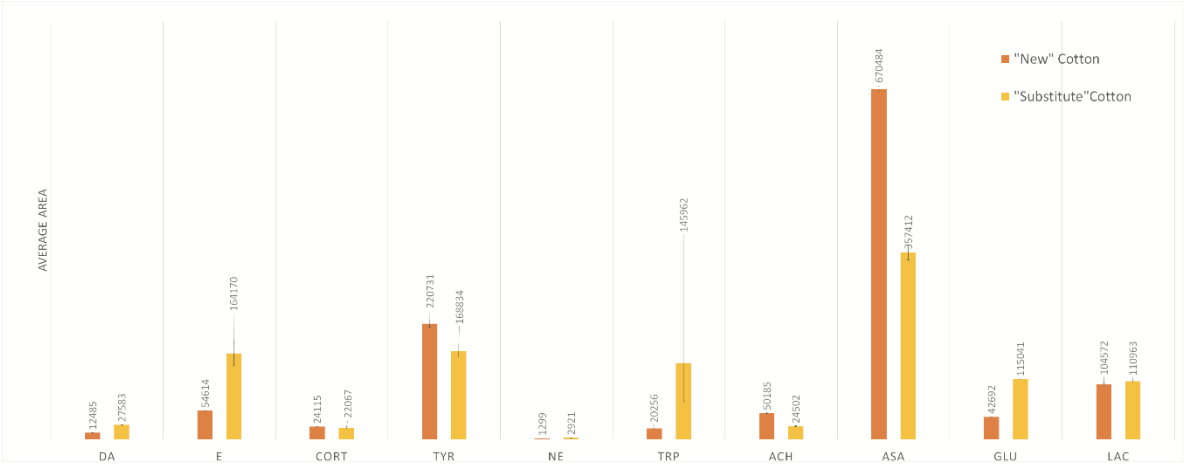


Figure 9– Average peak areas of BLKs 1 and 2 ("Substitute" Cotton) and whites 3 to 6 ("New" Cotton), and their respective standard deviations

As seen in Figure 9, the peak areas obtained for blanks 1 and 2 vary considerably when compared to the other blanks, and with a few exceptions, they are usually larger. Therefore, it is possible to see that analyses of real saliva samples should only be performed using cottons that already come with the salivettes® in order to obtain more accurate results.
Therefore, to determine the lowest concentration at which the compound can be determined without interference from the blank, only blanks 4 to 6 will be considered, in other words, those whose cotton was not replaced in the salivette®.
Therefore, the minimum quantifiable concentrations for each compound where there is no interference of blanks, are presented in Table 7 (Cal 02) and in Table 8 (Cal 06).

Table 7- Lowest quantifiable concentration ([C]) for each compound without interference from the blank for Cal 02 using a mixture of 50% MeOH/50% H_2O as extraction solvent.

Molecule	Lowest [C] no Interference of BLK ($ng.mL^{-1}$)
DA	2.5
5-HT	2.5
E	5
Cort	2.5
Tyr	10
NE	50
Trp	5
Ach	1
AsA	25
Glu	25
Lac	

Table 8 - Lowest quantifiable concentration ([C]) for each compound without interference from the blank for Cal 06 - using a mixture of 50% MeOH/50% H_2O as extraction solvent.

Molecule	Lowest [C] no Interference of BLK ($ng.mL^{-1}$)
DA	2.5
5-HT	0.25
E	2.5
Cort	1
Tyr	5
NE	5
Trp	2.5
Ach	2.5
AsA	10
Glu	*
Lac	

* It was not possible to construct a calibration curve for Glu

In both Cal 02 and Cal 06, a lowest concentration of 2.5 $ng.mL^{-1}$ was obtained for DA. In turn, 5-HT was able to obtain lower concentrations in Cal 06, being these 0.25 $ng.mL^{-1}$. E and Trp were obtained a lower concentration of 2.5 $ng.mL^{-1}$ in Cal 06.

Tyr and NE were able to obtain a lowest concentration without blank interference at 5 ng.mL^{-1} in Cal06. Same way, Cort and AsA obtained a lowest concentration of 1 and 10 ng.mL^{-1}, respectively in also Cal 06.

As it was not possible to construct the calibration curve for glutamic acid in Cal 06, the data for the minimum concentration where there is no interference from the blank will be the data from Cal 02, being this 25 ng.mL^{-1}.

On the other hand Ach has a lowest concentration without blank interference in Cal 02.

Lac was not possible to determine because it had always blank interference in our concentration range, so the sampling method utilized is not appropriate for the study of this compound.

It is also important to take into account that 5-HT was not found in blanks chromatograms during analysis, so sampling method used do not affect this compound.

3.1.1.2.2 Extraction Solution - mixture of 50% ACN/ 50% H_2O

It was also tested another extraction solution, a mixture of 50% ACN/50% H_2O, in order so see if the results obtained would be better, in other words, if it was possible to obtain lowest blanks interference for the compounds in study.

The analysis of the blanks was carried out in the same way as for the extraction method described previously with methanol, the only difference being that only cottons that were already inside the salivettes® were used. The average peak area of the blanks for each compound was then calculated and the results obtained are presented in Figure 10.

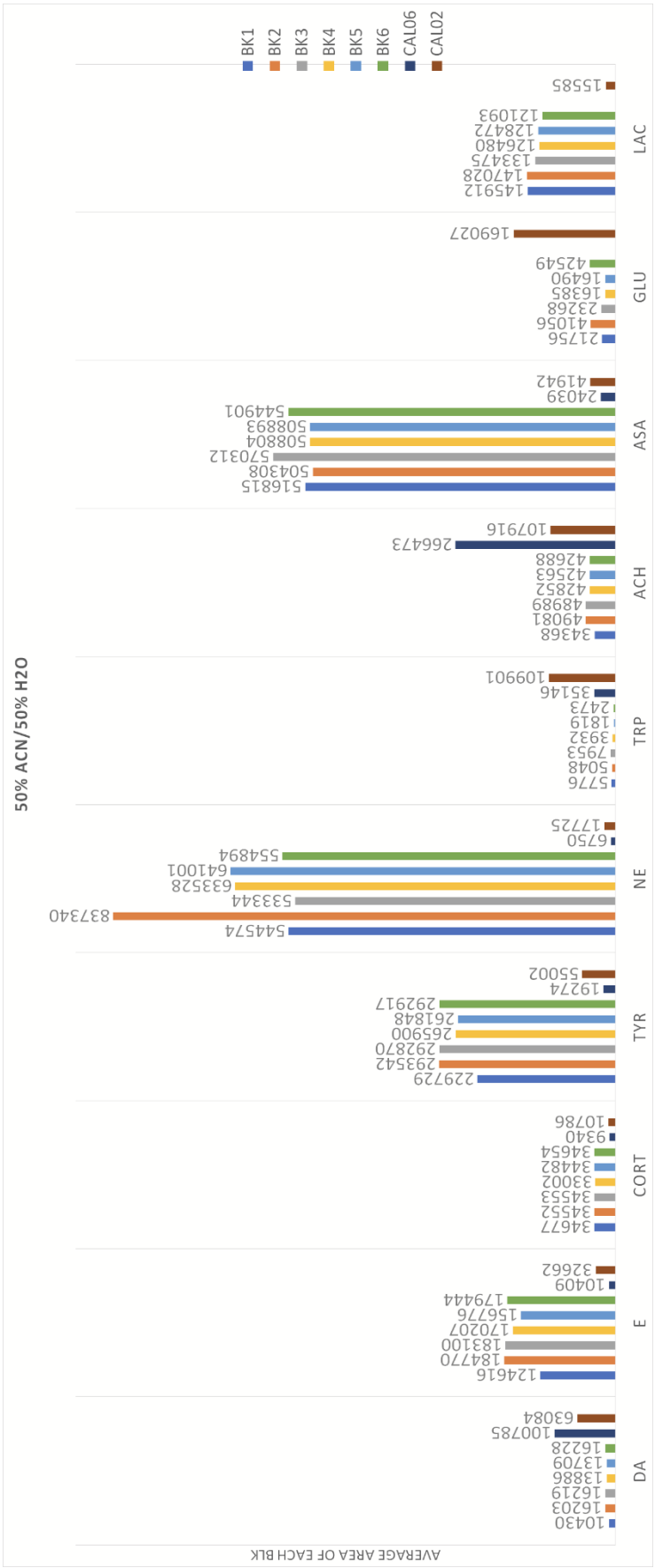


Figure 10 - Average peak area of triplicate blanks of DA, E, Cort, Tyr, NE, Trp, Ach, AsA, Glu, LAC, using a mixture of 50% ACN/50% H_2O as extraction solution; Area corresponding to the lowest quantifiable concentration for each compound referred in Cal 02 and Cal 06

The Figure 11 illustrates the averages of the blanks obtained for each compound. Trp blanks were not concordant between them so it was not possible to calculate its interference.

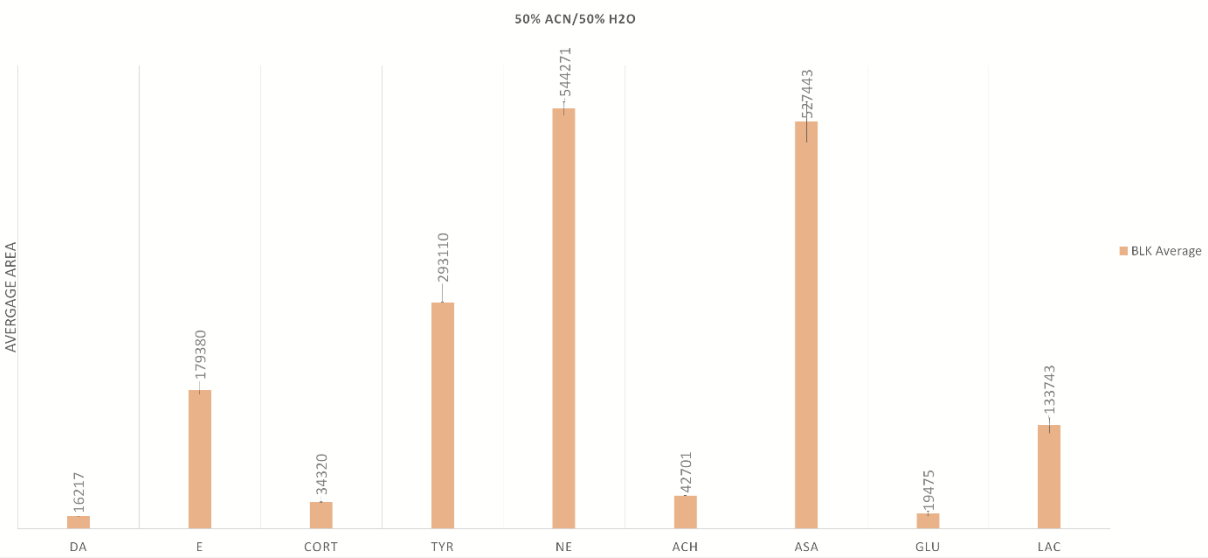


Figure 11 - Average of the peak areas of the blanks obtained from the extraction with 50% ACN/50% H_2O for each compound and respective standard deviation

Same way as for the extraction solution of 50% MeOH/50% H_2O, 5-HT was not found in blanks chromatograms during analysis, so blanks do not affect this compound.

Therefore, the minimum quantifiable concentrations for which there is no interference the minimum quantifiable concentrations for each compound where there is no interference of blanks, are presented in Table 9 (Cal 02) and in Table 10 (Cal 06).

Table 9 - Lowest quantifiable concentration ([C]) for each compound without interference from the blank and correspondent average area for Cal02 using a solvent mixture 50% ACN/50% H_2O as extraction solvent.

Molecule	Lowest [C] no Interference of BLK ($ng.mL^{-1}$)
DA	2.5
5-HT	2.5
E	25
Cort	5
Tyr	25
NE	-
Trp	-
Ach	1
AsA	100
Glu	25
LAC	-

Table 10 - Lowest quantifiable concentration ([C]) for each compound without interference from the blank and correspondent average area for Cal06 using a solvent mixture 50% ACN/50% H_2O as extraction solvent.

Molecule	Lowest [C] no Interference of BLK ($ng.mL^{-1}$)
DA	2.5
5-HT	0.5
E	5
Cort	2.5
Tyr	10
NE	-
Trp	-
Ach	2.5
AsA	50
Glu	*
LAC	-

* It was not possible to construct a calibration curve for Glu

In both Cal 02 and Cal 06, a lowest concentration of 2.5 $ng.mL^{-1}$ was obtained for DA. In turn, 5-HT, was able to obtain lower concentrations in Cal 06, being these 0.5 $ng.mL^{-1}$. E was obtained a lower concentration of 5 $ng.mL^{-1}$ in Cal 06.

Same way, Cort, Tyr and AsA obtained a lowest concentration of 2.5, 10 and 50 ng.mL^{-1}, respectively in also Cal06.

In the other hand, for Ach the lowest concentration that it is possible to quantify without blank interference is 1 ng.mL^{-1} in Cal 02.

As it was not possible to construct the calibration curve for Glu in Cal 06, the data for the minimum concentration where there is no interference from the blank will be the data from Cal 02, being this 25 ng.mL^{-1}.

NE, Trp and Lac were not possible to determine because blank had always interference in our concentration range.

To better compare the results obtained for each extraction solution, Table 11 was constructed.

Table 11 - Comparison between the values obtained for the lowest quantifiable concentration without interference from blanks in calibration curves 02 and 06, in the extraction methods using the solvent mixture 50% MeOH/50% H_2O and 50% ACN/50% H_2O

	50% MeOH/50% H_2O		**50% ACN/50% H_2O**	
Molecule	**Lowest [C] no Interference of BLK Cal 02 ng.mL^{-1})**	**Lowest [C] no Interference of BLK Cal 06 (ng.mL^{-1})**	**Lowest [C] no Interference of BLK Cal 02 (ng.mL^{-1})**	**Lowest [C] no Interference of BLK Cal 06 (ng.mL^{-1})**
DA	2.5	2.5	2.5	2.5
5-HT	2.5	0.25	2.5	0.5
E	5	2.5	25	5
Cort	2.5	1	5	2.5
Tyr	10	5	25	10
NE	50	5	-	-
Trp	5	2.5	-	-
Ach	1	2.5	1	2.5
AsA	25	10	100	50
Glu	25	*	25	*
LAC	-	-	-	-

* It was not possible to construct a calibration curve for Glu

As we can see by comparison of the values obtained in the Table 11, it's by using the mixture of 50% MeOH/50% H_2O that we obtained lower concentrations where it's possible to avoid interference from the blank for the majority of the compounds, as is the case of 5-HT, E, Cort, Tyr and AsA, which is the most desirable situation.

On the other hand, in the extraction solution using 50% ACN/50% H_2O, the values obtained for the various blanks were not very precise among themselves, which also does not provide us with great reliability for choosing this mixture. This fact also affects tryptophan (Trp). The values between triplicates of the blanks were not precise among themselves, not allowing the determination of the interference of the blank.
As for norepinephrine (NE), it couldn't be identified and quantified without the interference of the blank when using the extraction solution mentioned above, something that could be done using the MeOH/H_2O.
Therefore, the extraction solution chosen for carrying out the analyses will be the mixture of 50% MeOH/50% H_2O.

3.2 Method Validation

3.2.1 Linearity, LOD and LOQ

After analyzing how the sampling method affected the identification and quantification of the compounds under study, new calibration curves were constructed for each compound according to the results obtained.

The linearity of the method was evaluated using external standard calibrations. Table 12 reports the linearity parameters of calibration curves, R^2 and least squares fit 1/X weighted, using external standard calibration for each compound. Good correlation coefficients (R^2), higher or equal to 0.99, were obtained.

Table 12-Linearity parameters of calibration curves (R^2 and least squares fit) using external standard calibration for each compound with LODs ($ng.mL^{-1}$) and LOQs ($ng.mL^{-1}$)

Compound	Equation Curve	R^2	Linear range ($ng.mL^{-1}$)	LOD ($ng.mL^{-1}$)	LOQ ($ng.mL^{-1}$)
Ach	-133215+832946*x	0.9997	1-50	1.02	3.09
DA	7719.43+54176.7*x	0.9998	0.25-40	0.61	1.85
E	-2669.98+24960.5*x	0.9997	0.25-40	0.66	1.99
NE	6226.06+250.735*x	0.9996	50-900	11.02	33.39
Glu	57012.7+46514*x	0.9999	2.5-1000	15.20	46.06
5-HT	934.438+12200.8*x	0.9998	0.25-60	0.71	2.15
Tyr	126.782+30060.5*x	0.9999	5-250	2.01	6.09
Trp	-108789+121104*x	0.9997	5-300	6.54	19.83
AsA	55438.4+157.674*x	0.9930	25-1000	2.8	28.50
Lac	29463.9+153.756*x	0.9998	25-1000	15.24	46.19
Cort	1800.77+28683.9*x	0.9999	0.25-40	0.55	1.66

The chromatograms obtained for each compound are shown in the appendix in section A.2 of the Appendix A.

3.2.2 Analysis of 10 blanks in triplicate

Ten blanks were then performed on the salivettes®, using a mixture of 50% MeOH/50% H_2O as extraction solution. Each blank was injected in triplicate.
Blanks values that didn't agree with each other were discarded, so the intermediate results obtained for the Area of each compound are presented in section A.3 of appendix.

The average of the blanks obtained for each compound was then calculated to be later subtracted in the real saliva samples. The values obtained are presented in Table 13.

Table 13 - Retention time obtained for Ach, DA, E, NE Glu, Tyr, Trp, AsA, Lac and Cort in Blanks; average area of the BLKs with respective standard deviation (% Stdev) and precision (% Cv);

Compound	Rt (min)	Average A_{BLK}	Stdev (%)	Cv (%)
Ach	0.82-0.83	46723.88	36457.29	7.8
DA	0.97-0.98	3969,99	220.15	5.5
E	1.17-1.18	97803.25	6947.63	7.1
NE	0.82	395159,20	36703.28	9.3
Glu	0.96-0.97	342986.78	37048.12	10.8
5-HT	-	-	-	-
Tyr	1.16-1.17	107216.98	9415.81	8.8
Trp	1.41	21758.55	222.69	10
AsA	0.82	736876.96	40821.90	5.5
Lac	1.16	250488.56	18768.70	7.5
Cort	8.05-8.07	16270.00	1560.71	9.6

When evaluating the peaks present in the blanks chromatograms, it was possible to understand that 5-HT was not present, so the sampling method as no interference in this compound. As seen in Table 13, all the Cv (%) are above or equal to 10 %, as the parameters demand to validate the method. In the case of Glu is 10.8 % but is still acceptable since is on the threshold.

3.2.3 Recovery, Accuracy and Precision

Then for contamination, four concentrations were selected in the range chosen to create the calibration curve for each compound, in order to be possible to calculate the recovery of the extraction method.

The following results present in Table 14 were obtained for recovery with respective standard deviation and precision.

Table 14 - Recovery of each compound with the respective standard deviation (% Stdev) and precision (% Cv) for each chosen concentration ($ng.mL^{-1}$)

Compound	$C_{Theorethical}$ ($ng.mL^{-1}$)	$C_{Experimental}$ ($ng.mL^{-1}$)	Recovery (%)	Stdev (%)	Cv (%)
Ach	1	0.9	87.4	0.0387	7.03
	5	4.3	85.5	0.0593	2.50
	20	17.0	85.1	0.2576	2.65
	50	42.5	85.1	0.7980	3.19
DA	0.25	0.2	76.5	0.0120	2.10
	1	0.85	82.9	0.0045	4.13
	10	8.5	84.8	0.0531	2.32
	40	34.0	85.0	0.1324	3.10
E	0.25	0.2	91.4	0.0198	1.27
	1	0.8	85.0	0.0028	1.23
	10	8.5	85.0	0.3529	6.80
	40	34.0	85.0	0.9747	7.75
NE	50	38.8	77.6	28.54	7.79
	150	123.8	82.5	133.46	7.5
	400	336.3	84.1	346.70	5.4
	900	761.3	84.6	559.27	3.2
Glu	1	0.7	66.6	0.1234	3.19
	10	8.3	83.2	0.1284	1.15
	250	212.3	84.9	2.6407	4.17
	1000	849.8	84.9	21.2728	8.68
5-HT	0.25	0.2	80.4	0.0076	2.76
	1	0.8	83.9	0.0082	1.49
	10	8.5	84.9	0.0759	5.09
	60	51.0	84.9	0.3048	3.99
Tyr	5	4.2	85.0	0.2381	3.43
	20	17.0	85.0	0.6769	6.52
	100	85.0	85.0	2.9469	4.29
	150	127.5	85.0	4.1699	4.76
Trp	5	4.4	87.7	0.15356	4.71

	25	21.4	85.5	0.5956	4.15
	150	127.6	85.1	3.2619	3.64
	300	255.1	85.0	9.2587	4.96
AsA	25	-31.5	-125.9	4.1699	7.49
	100	32.2	32.3	21.2728	2.28
	400	287.2	71.8	78.5489	0.51
	1000	797.1	79.7	61.3287	0.61
Cort	0.25	0.2	81.2	0.0319	5.04
	1	0.8	84.1	0.04124	2.06
	10	8.5	84.9	0.1299	1.52
	40	34.0	85.0	0.4608	1.27

It was possible to obtain for the majority of the compounds recoveries between 70-110% [70], and precision inferior to 10%, which are the values accepted the recovery of the method. These compounds are Ach, DA, E, NE, 5-HT, Tyr, Trp and Cort.

In the case of Glu, from the data obtained it's only possible to quantify this compound from 1 $ng.mL^{-1}$ since it's recovery in first landing is 66.6%. Same way for AsA, but this compound could only be quantified from the third landing, meaning that we can only quantify AsA from 400 $ng.mL^{-1}$. Since the compounds decomposes relatively fast and can establish molecular interactions with each other, it's normal to obtain lower recoveries in lower concentrations.

With regard to Lac, the values obtained for the concentrations were always negative. Was already seen that the blanks for Lac had always interference, so is normal that Lac can't be quantified at this concentrations because this is not the appropriate sampling method to study this compound.

3.3 Sampling Saliva Performance

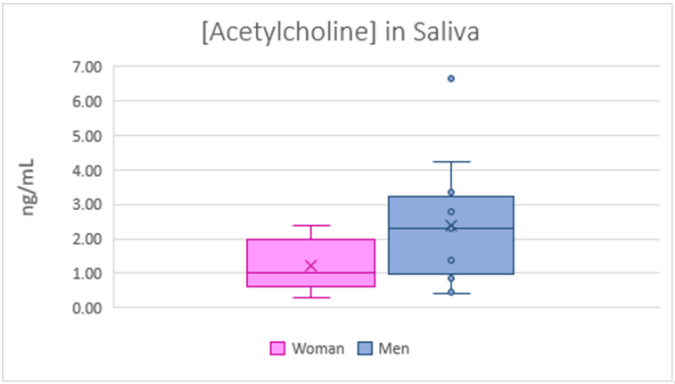
In the next phase, saliva samples were collected from 24 healthy individuals, 12 men and 12 women under 30 years of age. The collection method is presented in the appendix.

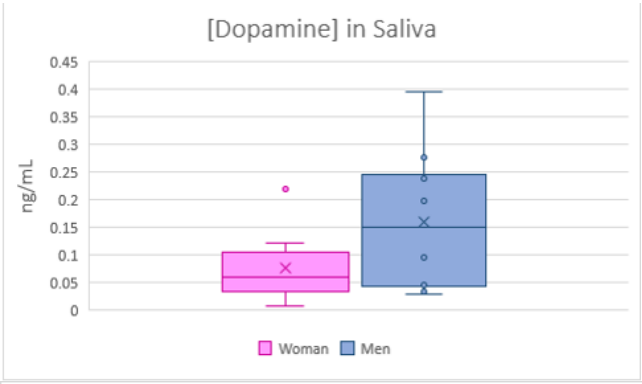
By analysing the chromatograms obtained for each compound from each of the individuals, the peak areas obtained were obtained (triplicates were performed as mentioned). Using the average of the peak areas obtained, it was possible to calculate the concentration of each compound taking into account the following equation:

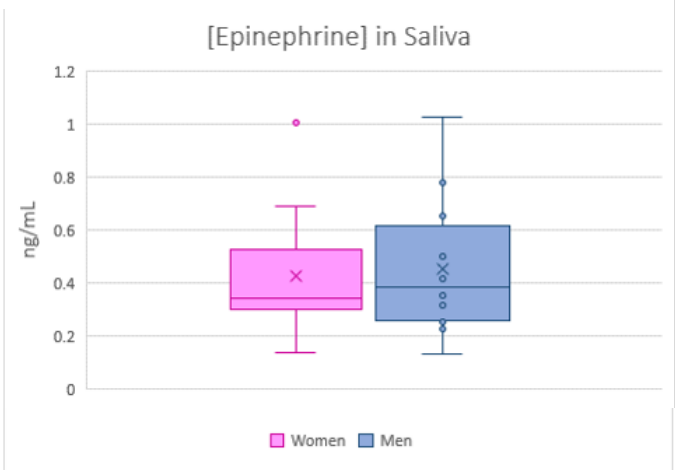
$$c_{saliva} = \frac{(c_{calibration\ curve} - c_{BLK}) \times D \times V_{extraction}}{m_{sample}}$$

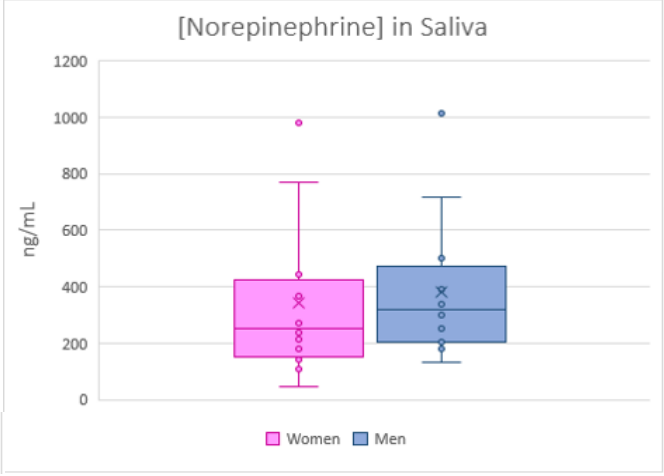
Where $c_{calibration\ curve}$, is he concentration obtained by replacing the average of the area obtained in the calibration curve; D, the dilution factor (in case 2); $V_{extraction}$, the volume used to perform the extraction (0.250 mL) and m_{sample} the mass of saliva obtained from each individual discounting the weight of the salivette® (~7.00 g).

The following values were obtained for the concentration of each biomarker in saliva, taking into account that the average density of saliva is equal to 1.007 g.mL^{-1} [71].









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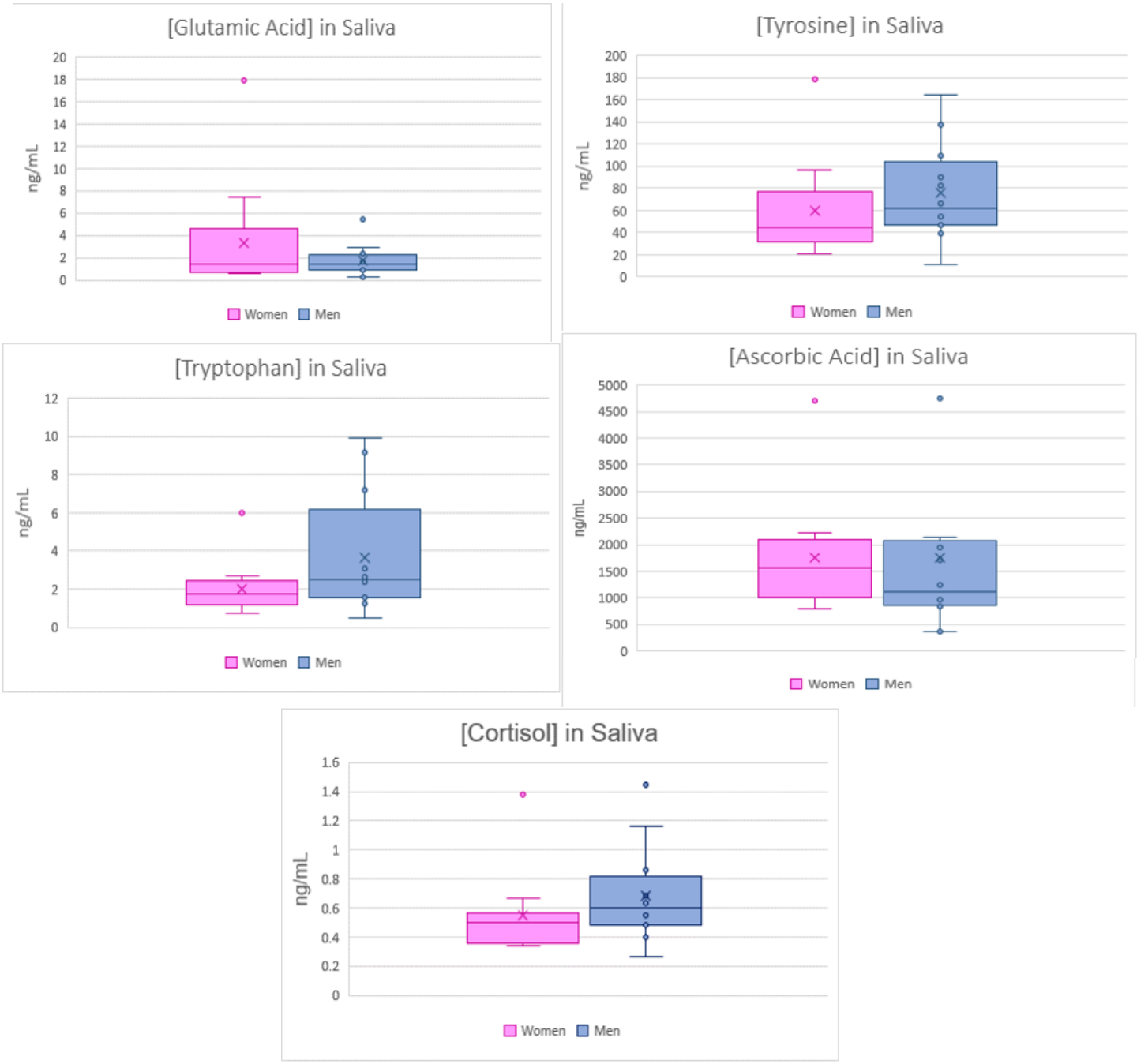

Figure 12 - Concentration (ng.mL^{-1}) obtained in saliva for Women1 to Women12 and Men1 to Men12 with respective Stdev (%) for Ach, DA, E, NE, Glu, Tyr, Trp, AsA and Cort.

As can be seen, in Figure 12 the Ach concentrations in saliva varied between 0.28 – 6.68 ng.mL^{-1}. It's know that in human blood the normal values of this *NT* are between 29.2-191.5 ng.mL^{-1} [72], but there is no data available for its normal values in saliva. This way, we only can see that Ach concentrations in saliva can be found in lower concentrations than in the blood.

In blood the normal values for DA are between 0 - 0.03 $ng.mL^{-1}$, from 0-0.14 $ng.mL^{-1}$ for E and from 0.07-1.7 $ng.mL^{-1}$ to NE [73]. The measurement of salivary catecholamine's in more than 300 samples taken from about 50 healthy volunteers indicated that the normal values of norepinephrine and dopamine were very low, about 0.02 $ng.mL^{-1}$, each [74]. There are limited research focused on the detection of the parent compounds themselves as they display limited chemical stability and are rapidly metabolized to yield, so it's more likely that their metabolites will be found in higher concentrations in saliva, such DOPA, HVA and 3-MT (DA metabolites), and NMN and MN (E and NE metabolites).

The DA concentrations in saliva obtained from the experimental results varied between 0.03 – 0.40 $ng.mL^{-1}$, from 0.13-1.03 $ng.mL^{-1}$ for E and from 48.05-1015.00 $ng.mL^{-1}$ for NE.
Since the literature values are not stress-related, it's not possible to identify if the subjects in this study whether or not present values within the limits, but in case, all the individuals presented higher values.
With regards to NE, the values are really high when compared to the literature values so another tests must be performed in this compound to confirm the data.

It's also important to note, that Women 1, Men 6 and Men 7 presented higher levels of E and NE when compared with the rest of the individuals and Women 9 and Women 10 the lowest. Also Men 6 and Men 7 presented higher levels of DA than the rest. On the other hand, Women 10 presented the lower values.
Usually this three catecholamines work together, playing role in the "fight-or-flight" response, preparing the body to deal with danger and stressful situations, so it's normal that if one of them is in high concentration, especially E and NE, the others also will be.

In regard to Tyr, the experimental concentrations in saliva range from 11.23-178.94 $ng.mL^{-1}$. There is no stress related data for Tyr concentration neither in blood or saliva, it's only known that Tyr is a precursor for the synthesis of DA, E and NE, that helps mitigating the effects of stress by increasing the availability of these catecholamines.
As seen in Figure 12, Women 1, Men 2 and Men 7 present higher concentration values compared to the other subjects. Also Women 1 presented high concentration of E and NE and Men 7 high concentration of DA, E and NE, something that is expected.
On the other hand, Woman 9 and Men 10 presented low levels of Tyr, and also low levels of DA, E and NE.
Usually depressed people often have low levels of tyrosine, cause it's depleted when people face stress situations [27], and consequently the concentrations of the referred catecholamines

are lower too, but since we don't have a normal range in saliva for Tyr is not possible to affirm that they are depressed, we could only suppose that they could be dealing with some stressful situation.
Same for Tyr, there is no stress related data for Trp concentration neither in blood or saliva, Trp concentrations in saliva then range from 0.49-9.90 $ng.mL^{-1}$. Tryptophan is related to improved resistance to negative emotions and as it is a precursor of tyrosine, low levels of tryptophan imply low levels of tyrosine and vice versa. [24]
Again as with tyrosine, Men2 and Men 7 have high tryptophan values. Women 9 and Men 11 again presents values that are lowest, something that is expected. Once again, we could only suppose that they could be dealing with some stressful situation since there is no data available.

The experimental concentrations of AsA in saliva range from 360.19-4754.87 $ng.mL^{-1}$.
Ascorbic acid is an important agent maintain the activity of tyrosine hydroxylase, so a diet deficient in vitamin C can lead to decreased serotonin levels. Second, ascorbic acid acts as a direct electron donor in the conversion of dopamine to norepinephrine, so ascorbic acid is important for regulating these catecholamine concentrations in the body. As can be seen Women1, Men6 and Men7 have high AsA values and Women11 low when compared to other subjects, something that's is in agreement with what as mentioned before fore DA, E, NE and Tyr. Men12 also has low AsA levels, and low DA and NE, something in agreement with AsA role.

Glu concentrations in saliva range from 0.28-17.93 $ng.mL^{-1}$. It is important to note that Woman1 and Women10 present high values and Men 10 and Men 11 low concentrations. There is no reference data stress related for AsA or Glu.

Cort concentrations in saliva range from 0.26-1.44 $ng.mL^{-1}$.
Cortisol has long been recognized as the "stress biomarker" in evaluating stress related disorders, since it's important for treating anxiety and depression, in order to provide better long-term recovery and it is know that the normal values of Cort in saliva are between 1.0-1.6 $ng.mL^{-1}$ [75], and so only Women1, Men6 and Men7 are in the normal range.
Over again Women 11 as real low concentrations of Cort, as for other biomarkers, maybe indicating some stress related disorder.

It was not possible to find 5-HT in saliva, so only its metabolites, such as 5-HIIA, and therefore a study of them should be carried out in the future to study their presence in saliva, in order to study 5-HT stress related influence.
It was possible to find Lac in saliva, but since it was not possible to validate the method the method of extraction because the salivette® interference, the Lac concentrations determined in saliva would not be reliable and another method of sampling should be performed.

In summary, the Table 15 shows the ranges values obtained for the concentration of each compound in study in human saliva.

Table 15 - Concentration obtained for each compound in human saliva ($ng.mL^{-1}$) for LC-MS/MS analysis

Compound	Concentration ($ng.mL^{-1}$)
Ach	0.28 – 6.68
DA	0.03 – 0.40
E	0.13-1.03
NE	48.05-1015.00
Glu	0.28-17.923
Tyr	11.23-178.94
Trp	0.49-9.90
AsA	360.19-4754.87
Cort	0.26-1.44

It's important to note that some of the concentrations obtained were below the LOQs determined for each compound, such as for Ach, DA, E, Glu, Trp and Cort, meaning that only qualitative concentrations will be presented. It will be necessary to carry out a study that includes lower concentrations in the calibration curves to obtain more accurate and quantitative results. Also will be necessary to test other sampling procedures.

3.4 Electrochemistry

3.4.1 Optimization of Cyclic voltammetry and Differential pulse voltammetry conditions for Identification and Quantification of Dopamine in order to learn the Basics of Electrochemistry

In order to learn the basic concepts of electrochemistry, cyclic voltammetry and differential pulse voltammetry were performed in a classic cell. The tests were performed utilizing a solution of Dopamine.

As referenced before there are two types of saliva: unstimulated saliva and stimulated saliva. Since the studies are intended to be carried out in unstimulated saliva, it is clear that phosphate is predominant. Therefore, in order to simulate the study environment and at the same time delay dopamine degradation, phosphate buffer was used for the electrochemical study.

- For Cyclic Voltammetry:

Based on the following experimental conditions:

- Solução DA 1.00×10^{-3} mol.dm^{-3}/phosphate buffer 5.5 mol.m^{-3}/0.1 mol.dm^{-3} NaCl
- WE – Pyrolytic graphite
- RE – Ag/AgCl
-CE – Platina Coil
- Initial Potencial: -0.1 V
-First Vertex Potencial: 0.6 V
-Second Vertex Potencial: -0.1 V

The scan rate was then varied: 5, 10, 20, 30, 50, 75 and 100 mV.s^{-1}, obtaining the following voltammograms:

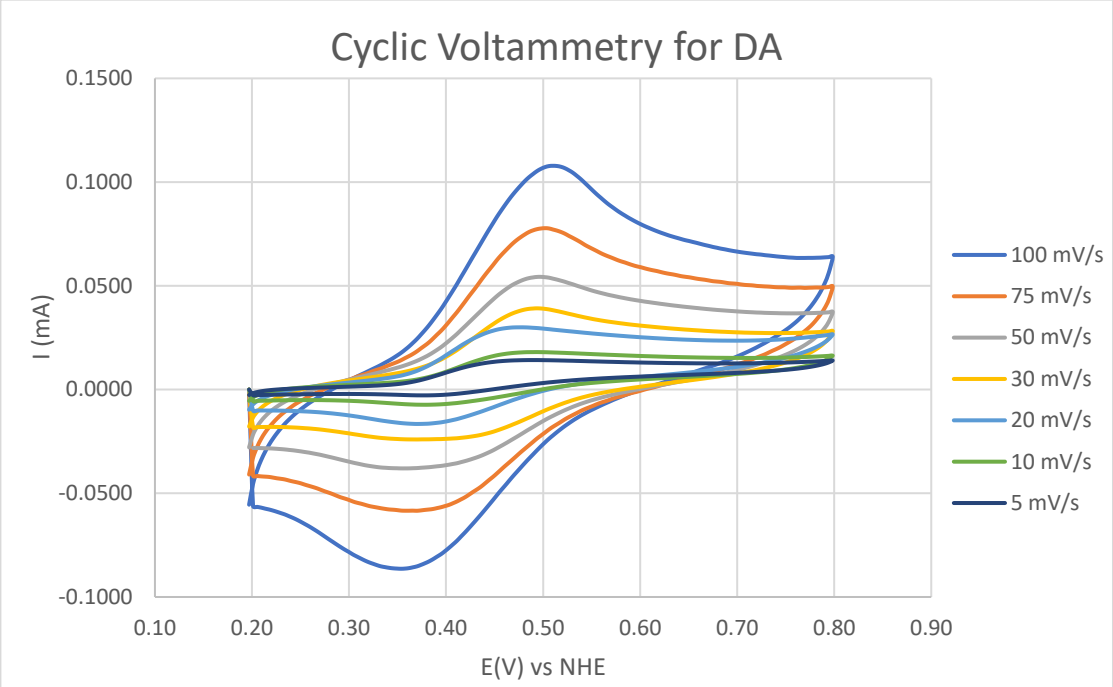

Figure 13- Cyclic Voltammetry for DA 1.00×10^{-3} mol.dm^{-3}/ phosphate buffer 5.5 mol.m^{-3}/0.1 mol. dm^{-3} NaCl at a scan rate of 5, 10, 20, 30, 50, 75 and 100 mV.s^{-1}; WE (Pyrolytic graphite), RE (Ag/AgCl), CE (Platina Coil)

As is possible to see in Figure 13, the scan rate which allowed a better identification of the DA peaks was the 100 mV.s^{-1}. Although since we want smaller steps for a better analysis, it was decided to use 20 mV.s^{-1} for the DA identification.

The values obtained for the oxidation and reduction peak obtained for DA with a scan rate of 20 mV.s^{-1} are present in Table 16.

Table 16 - Potential (V vs NHE) and intensity (mA) for Oxidation and reduction peaks obtained for DA with a scan rate of 20 mV.s^{-1} by cyclic voltammetry

Oxidation Peak		**Reduction Peak**	
E (V) vs NHE	I (mA)	E (V) vs NHE	I (mA)
0.440	2.27×10^{-2}	0.386	-2.39×10^{-2}

- For Differential Pulse Voltammetry

Tests were then performed to determine the best window for performing DPV in our Dopamine system.

The experimental conditions were as follows:

Modulation time = 0.01 s

Interval time = 0.2 s

Initial Potential = -0.1 V

End Potential = 0.7 V

Step potential = 0.00255 V

The variable that was varied was the modulation amplitude, varying by: 0.0075 V, 0.01245 V and 0.025 V. The voltammograms presented in Figure 14 were obtained after performing a smooth of 4 and a baseline correction of 0.01.

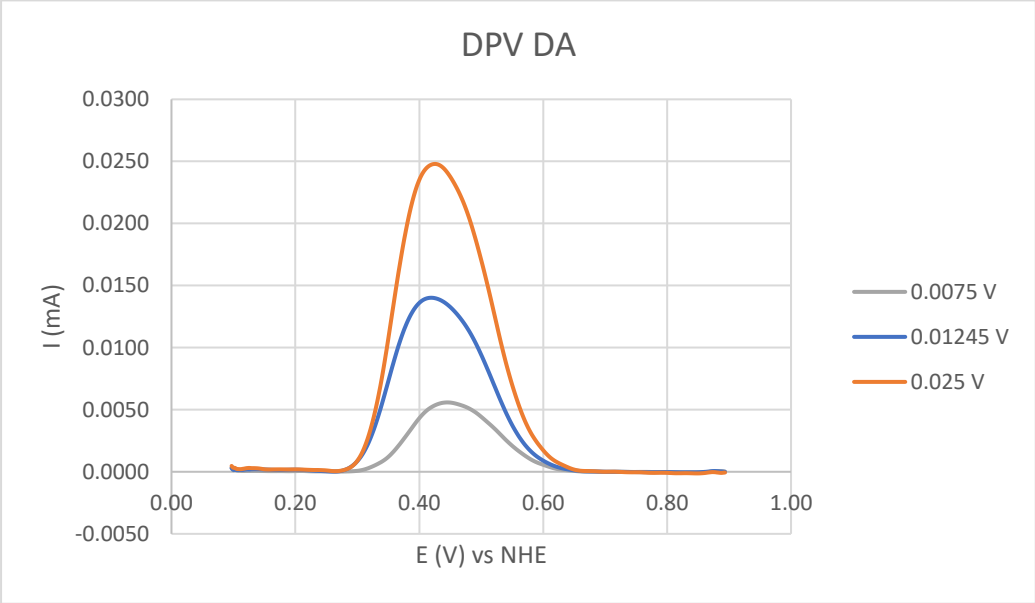

Figure 14 - Voltammograms obtained for differential pulse voltammetry for DA 1.00 × 10^{-3} mol.dm^{-3}/ phosphate buffer 5.5 mol.m^{-3}/0.1 mol.dm^{-3} NaCl at modulation amplitude of 0.075, 0.01245 and 0.025 V; WE (Pyrolytic graphite), RE (Ag/AgCl), CE (Platina Coil)

As can be seen, the test where the DA peak is best identified is the one corresponding to a modulation time of 0.025 V.

Then choosing the modulation time of 0.025 V, the interval time was changed: 0.1 s and 0.5 s. After performing a smoothing of 4 and a baseline correction of 0.01, the voltammograms obtained are presented in Figure 15.

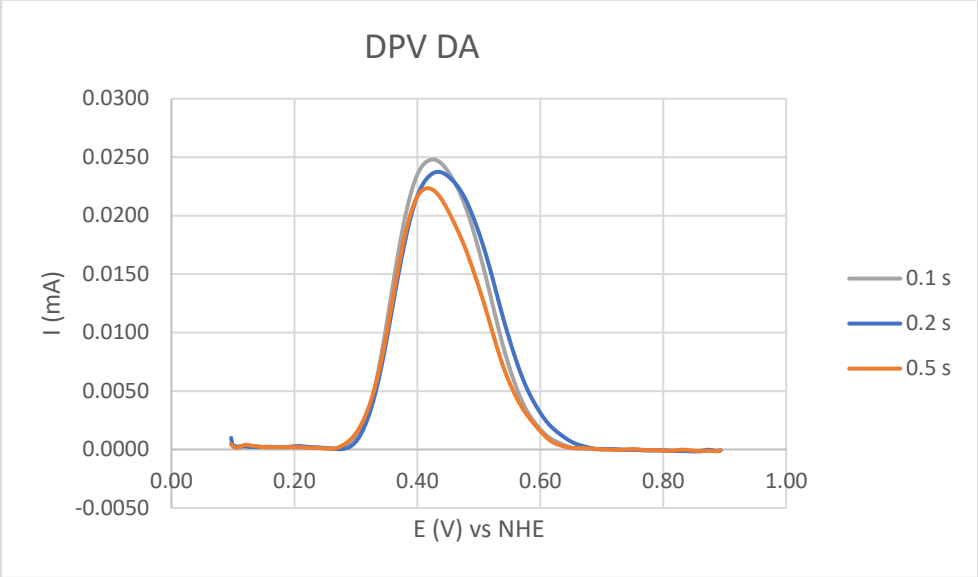

Figure 15 - Voltammograms obtained for differential pulse voltammetry for DA 1.00×10^{-3} mol.dm^{-3} phosphate buffer 5.5 mol.m^{-3}/0.1 mol.dm^{-3} NaCl at modulation amplitude of 0.025 V and Interval time of 0.1, 0.2 and 0.5 s ; WE (Pyrolytic graphite), RE (Ag/AgCl), CE (Platina Coil)

By comparison with the voltammogram performed with an interval time of 0.1 s, it can be seen that the best experimental conditions for our system, where the peak is more defined, are when using an interval time of 0.1 s with a modulation amplitude of 0.025 V.
The peak potential (V vs NHE) and intensity (mA) obtained for DA is present in Table 17.

Table 17 - Peak Potential (V vs NHE) and intensity (mA) obtained for DA with a modulation time of 0.025 V and an interval time of 0.1 s by DPV

DA	
E (V) vs NHE	I (mA)
0.421	2.48×10^{-2}

3.4.2 Sampling Saliva Performance

3.4.2.1 Electrochemical study using SPE

A previous study with sweat was performed to optimize the experimental conditions but instead of performing electrochemical techniques in the classic cell, were used Screen Printed

Electrodes (SPEs). Since what we wanted to know, is that if it is possible to identify the presence of some of the molecules in study in human saliva, the same experimental conditions were used and the data obtained will be used for comparison with saliva [76].

Using Screen Printed Electrodes (SPEs), the saliva samples collected from the 23 individuals mentioned above were analyzed. For the study only the saliva of the two women and two men was analyzed, and a pool of saliva was created for the women and men with 20 µL of saliva from each. It is also important to note that the saliva of some individuals was not analyzed due to the low amount of saliva provided (due to the low level of salivation) and that left over from the LC-MS/MS analysis.

Taking into account the following experimental conditions already defined [76].

Modulation time = 0.05 s

Interval time = 0.5 s

Initial Potential = -0.35 V

End Potential = 1.50 V

Modulation amplitude = 0.02505 V

Blank tests were performed on the SPEs, obtaining the following voltammograms in Figure 16.

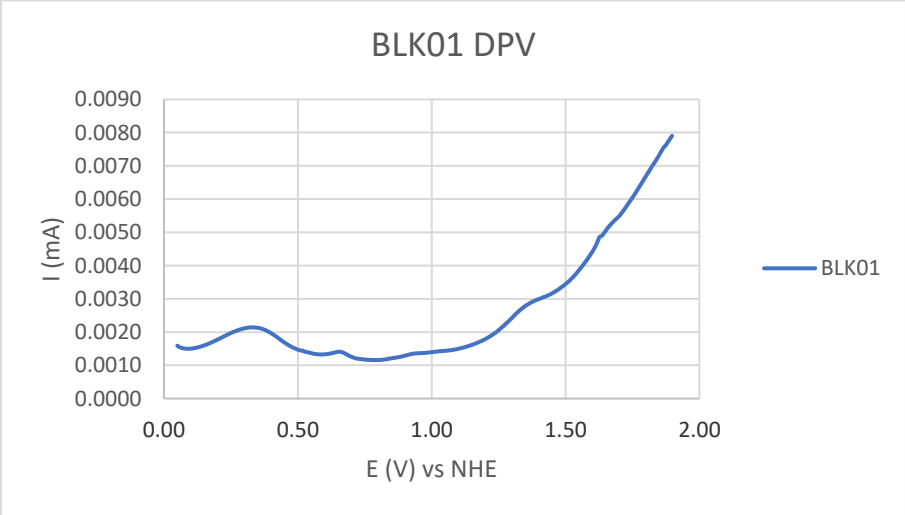

Figure 16 - DPV for phosphate buffer solution 86 × 10^{-3} mol.dm^{-3}/NaCl 18 × 10^{-3} mol.dm^{-3}, pH 7, using SPEs (Metrohm DropSens 110); Modulation time of 0.05 s, Interval time of 0.5 s, Initial Potential of -0.35 V, End Potential of 1.50 V and Modulation amplitude of 0.02505 V

Blank 1 was taken for discounting the voltammograms obtained for human saliva. By making a baseline correction of 0.01 and by discounting Blank 1. The voltammograms obtained for human saliva are present in appendix in Figure A 13 and Figure A 14.

From the data obtained it was possible to identify 3 zones that present defined peaks, which are presented in Figure 17.

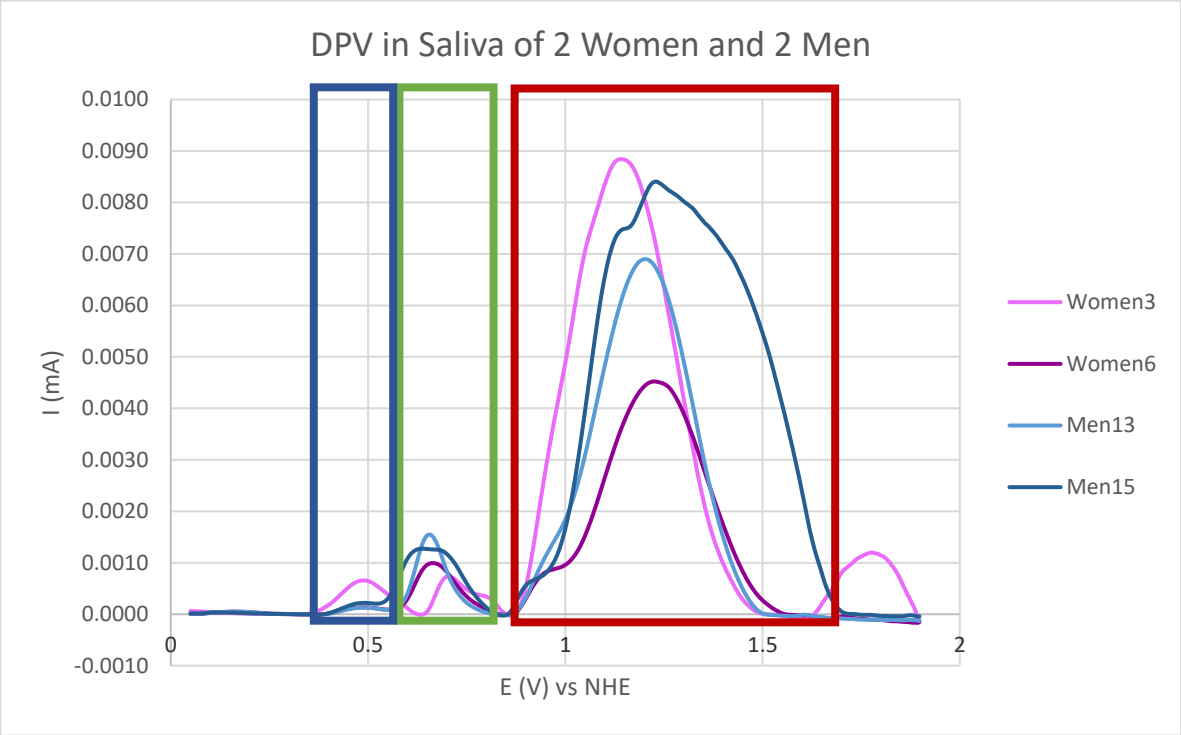

Figure 17 -DPV in Human saliva for women and men using SPE (Metrohm DropSens 110), Modulation time of 0.05 s, Interval time of 0.5 s, Initial Potential of -0.35 V, End Potential of 1.50 V and Modulation amplitude of 0.02505 V, with 3 defined zones: ZI (blue) – 0.4-0.6 V; ZII (green) – 0.6-0.8 V; ZIII (red) – 0.85-1.7 V.

The ZI, which is in blue, is between 0.4-0.6 V, the ZII, which is in green, is between 0.6-0.8 V and the ZIII is between 0.85-1.7 V.

In the same way, the same zones can be observed in the pool of female and male saliva, as can be seen in the Figure 18.

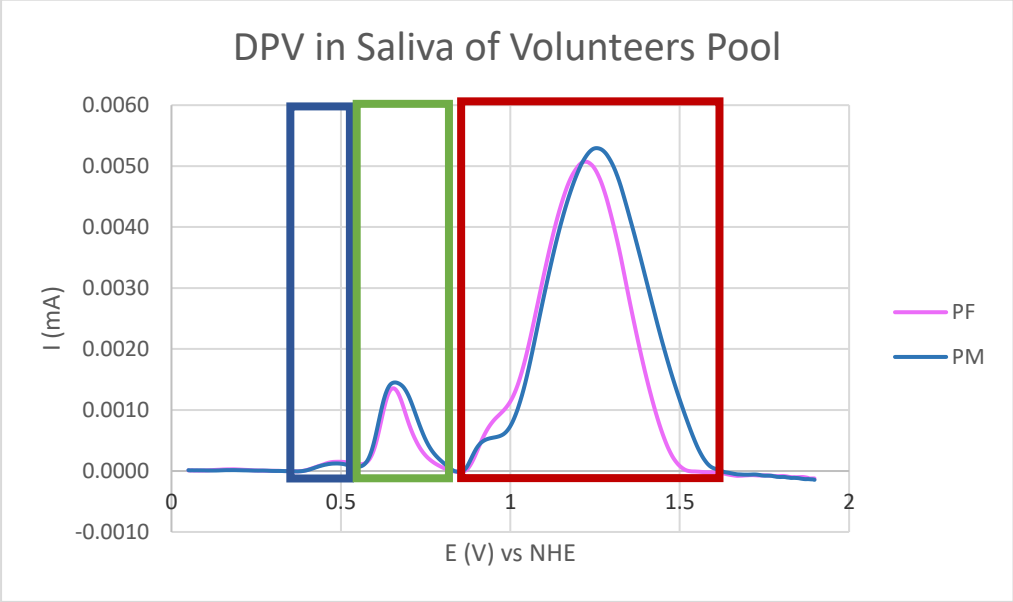

Figure 18 - DPV in Human saliva in feminine pool (PF) and masculine pool (PM) using SPE (Metrohm DropSens 110); Modulation time of 0.05 s, Interval time of 0.5 s, Initial Potential of -0.35 V, End Potential of 1.50 V and Modulation amplitude of 0.02505 V, with 3 defined zones: ZI (blue) – 0.4-0.6 V; ZII (green) – 0.6-0.8 V; ZIII (red) – 0.85-1.7 V.

The data obtained for the peaks present in the 3 zones identified for each individual of potential energy and current intensity are present in Table 18.

Table 18 - Potencial Energy (V) of the peaks selected in each zone and corresponding Intensity (mA) for each saliva individual using DPV

Individuals	E(V) vs NHE	I (mA)
Women3	0.488	6.63×10^{-04}
	0.705	7.84×10^{-04}
	1.138	8.88×10^{-03}
Women6	0.473	8.34×10^{-05}
	0.664	9.42×10^{-04}
	1.228	4.57×10^{-03}
Men3	0.473	7.28×10^{-05}
	0.654	1.50×10^{-03}
	1.203	6.92×10^{-03}
Men5	0.488	1.65×10^{-05}
	0.674	1.16×10^{-03}
	1.228	8.44×10^{-03}

In order to understand in which range(s) of potential energy each compound could be identified, data from a study previously carried out for the molecules under study were used.

The data for each molecule according to this study are presented in Table 19.

Table 19 - Potentials and Intensity of each biomarker at a concentration of 1×10^{-3} mol.dm^{-3} without and with PBS pH = 7 [76]

	Biomarker without PBS		**Biomarker in PBS**	
Biomarker	E (V) vs NHE	I (mA)	E (V) vs NHE	I (mA)
Acetylcholine - Ach	1.3894	5.72×10^{-04}	1.3894	5.72×10^{-04}
Dopamine - DA	0.5535	3.81×10^{-02}	0.5585	3.80×10^{-02}
Epinephrine - E	0.6240	3.03×10^{-02}	0.6189	3.03×10^{-02}
Norepinephrine - NE	0.5434	3.16×10^{-03}	0.5434	3.16×10^{-03}
Glutamic Acid - Glu	1.8577	1.49×10^{-02}	1.8577	1.49×10^{-02}
Serotonin - 5-HT	0.7096	9.16×10^{-05}	0.7096	8.97×10^{-05}
Tyrosine - Tyr	0.8758	2.23×10^{-04}	0.8758	7.95×10^{-05}
Tryptophan - Trp	0.5765	2.21×10^{-04}	0.5865	2.02×10^{-04}
Ascorbic Acid - AsA	0.6240	4.86×10^{-03}	0.6239	4.86×10^{-03}
Lactate - Lact	0.3219	5.32×10^{-04}	0.2413	5.22×10^{-03}
Cortisol - Cort	1.6160	1.24×10^{-02}	1.6159	1.24×10^{-02}

According to the data obtained, it is possible that Ach is present in ZII. In turn, DA may be present in ZI, however its intensity is very low, meaning that its metabolites are present. E may be present in ZII and NE in ZI. In the case of NE, the intensity is quite low, meaning that only metabolites may be observed.
Tyr, in turn, may be present in ZIII and Trp may be present in ZII, although both present a low current intensity In LC-MS/MS it was not possible to identify 5-HT in saliva, and since it detects lower concentrations than electrochemistry, probably 5-HT is not present. If so 5-HT may be contained in ZII, but probably what is seen are its metabolites may also be found
AsA may be present in ZI and Cort in ZII, both presenting good current intensities.

In the case of Glu and Lac, these are found outside any of the identified zones, so it is not possible to identify them in saliva in the chosen electrochemical window.

3.4.2.2 Measurement of Saliva pH

The pH of each sample was also analyzed and the results are presented Figure 19, as well as of the female and male pools presented in Figure 20.

Since LC-MS/MS analysis was performed first and that saliva samples needed to be analyzed quickly in order to the compounds don't start to degrade, it was not possible to measure same individuals pH because there was not enough amount of saliva left. So the data obtained are presented in the following graphs:

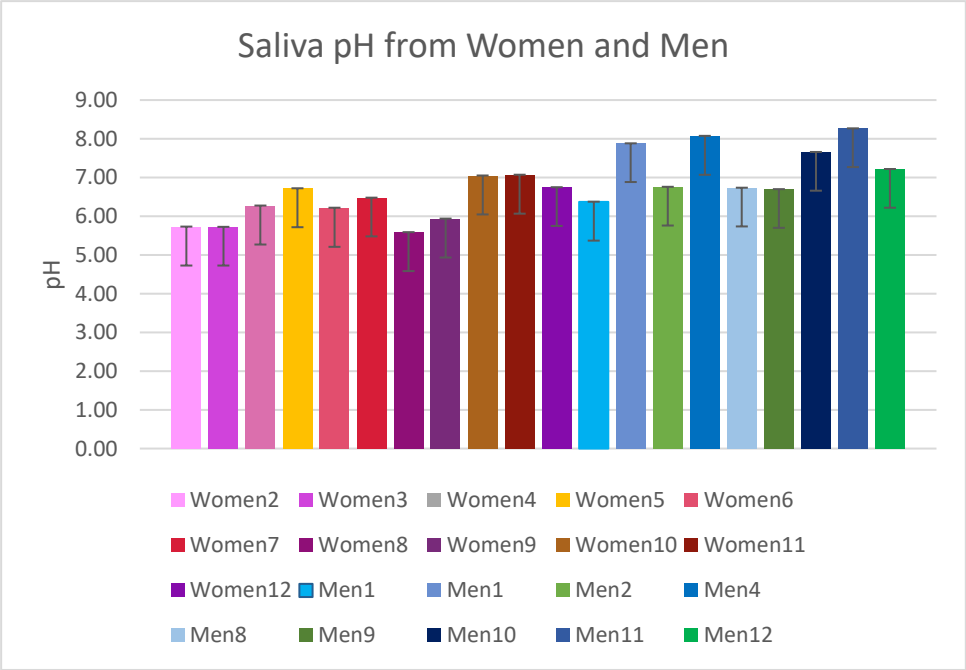

Figure 19 - Saliva pH from women and men with respective standard deviation (%)

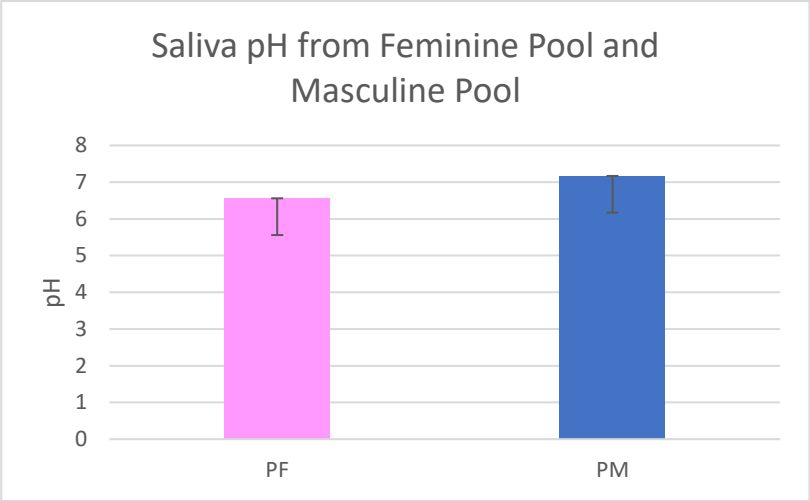

Figure 20- Saliva pH from feminine and masculine pool with respective standard deviation (%)

The pH of saliva typically varies between individuals and can differ based on factors such as diet, health, and time of day. For healthy individuals, the average saliva pH ranges between 6.2 and 7.6 [77].

In general, what can be seen is that the pH of men's saliva is higher than that of women's saliva. Men' saliva pH is often slightly higher on average compared to women, potentially due to differences in hormonal levels, salivary flow rate, and metabolism. The pH of saliva in women can fluctuate more significantly due to hormonal changes during menstrual cycles, pregnancy, or even menopause.

On the other hand the pH of saliva in individuals under stress can vary, generally becoming more acidic (lower pH) due to increased cortisol production and other physiological changes associated with stress. Men under stress saliva pH tends to range between 5.5 and 6.5, depending on the severity of stress and other individual factors and women under stress saliva pH can be slightly lower than that of men, often ranging between 5.0 and 6.0, due to greater hormonal susceptibility and stress responses.

4
CONCLUSIONS AND FUTURE WORK

The LC-MS/MS screening method for saliva, targeting the selected eleven compounds, was validated according to international guidelines and demonstrated high selectivity. It also exhibited good linearity ($R^2 > 0.99$) for all compounds analysed. Accuracy and precision were confirmed, with recoveries ranging from 70% to 110% and a coefficient of variation below 10%, both within acceptable literature references. The compounds whose all levels meet the aforementioned requirements were Ach, DA, E, NE, 5-HT, Tyr, Trp and Cort. The lowest recovery percentage obtained was 71.8% for AsA, and the highest was 91.4% for E.

In the case of Glu, from the data obtained it's only possible to quantify this compound from 1 $ng.mL^{-1}$ since it's recovery in first level is 66.6%. Same way for AsA, but this compound could only be quantified from the third level, meaning that we can only quantify AsA from 400 $ng.mL^{-1}$.

The application of the method developed by LC-MS/MS analysis, to the real samples, saliva samples, verified the presence of the molecules in study, being these Ach, DA, E, NE, Glu, Tyr, Trp, Asa, Lac and Cort, with exception of 5-HT.

In the case of 5-HT probably what we could found better in saliva, would be its metabolites, such us 5-HIIA, and for other compounds too, for example for DA, that also has metabolites such as DOPAC, 3-MT and HVA. There are limited research focused on the detection of the metabolites compounds themselves as they display limited chemical stability and are rapidly metabolized. A future study need to be performed with metabolites of the referred compounds in order to evaluate if it is possible to identify and quantify them utilizing the salivettes® as sampling method.

In regard to Lac, it was only possible to identify its presence in saliva, but since the blanks presented always interference in the experimental range utilized, it was not possible to quantify this compound, so another sampling method need to be tested in the future to quantify Lac in saliva. Instead of utilizing the cottons inside salivettes®, for example, test with compresses.

The concentration results obtained in human saliva concentrations were 0.28 – 6.68 $ng.mL^{-1}$ for Ach, 0.03 – 0.40 $ng.mL^{-1}$ for DA, 0.13-1.03 $ng.mL^{-1}$ for E, 48.05-1015.00 $ng.mL^{-1}$ for NE, 0.28-17.923 $ng.mL^{-1}$ for Glu, 11.23-178.94 $ng.mL^{-1}$ for Tyr, 0.49-9.90 $ng.mL^{-1}$ for Trp, 360.19-4754.87 $ng.mL^{-1}$ for AsA and 0.26-1.44 $ng.mL^{-1}$ for Cort.
It's important to note that some of the concentrations obtained were below the LOQs determined for each compound, such as for Ach, DA, E, Glu, Trp and Cort, meaning that only estimated concentrations will be presented as qualitative results. It will be necessary to carry out a study that includes lower concentrations in the calibration curves to obtain more accurate results.

With regard to electrochemistry, it was possible to understand that compounds under study present peaks with very similar energies, making it possible to only estimate the zone where these are likely to be. It was possible to identify three zones, The ZI varied between 0.4-0.6 V, the ZII between 0.6-0.8 V and the ZII between 0.85-1.7 V.
It's possible that Ach is present in ZII. In turn, DA may be present in ZI, however its intensity is very low, meaning that its metabolites are present. E may be present in ZII and NE in ZI. In the case of NE, the intensity is quite low, meaning that only metabolites may be observed.
Tyr, in turn, may be present in ZIII and Trp may be present in ZII, although both present a low current intensity In LC-MS/MS it was not possible to identify 5-HT in saliva, and since it detects lower concentrations than electrochemistry, probably 5-HT is not present. If so 5-HT may be contained in ZII, but probably what is seen are its metabolites may also be found
AsA may be present in ZI and Cort in ZII, both presenting good current intensities.
In the case of Glu and Lac, these are found outside any of the identified zones, so it's not possible to identify them in saliva through electrochemical techniques.

In the future, the development of the sensor requires the study of each of the compound individually, in order to understand the concentrations up to which it's possible to determine each compound in electrochemistry, but the present work was only to see if it was possible to identify any peaks for this compounds in saliva, something that it's indeed possible.
Regarding the pH of saliva, we found that men's pH (~7) is higher than women's (~6.5), and, which could be the subject of another topic, in order to understand the processes involved in this difference.

5
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A

APPENDIX

A.1 Linearity parameters of calibration curves

Table A 1 - Linearity parameters of calibration curves from Cal 03 (R^2 and least squares fit) using external standard calibration for each compound.

Compound	Equation Curve	R^2	Linear range ($ng.mL^{-1}$)
Ach	-64960+382360*x	0.9972	0.25-250
DA	-67547.2+77638.7*x	0.9986	1-500
E	-93738.7+40900*x	0.9974	5-500
NE	-	-	-
Glu	187339+16694.4*x	0.9997	25-1000
5-HT	-30680.2+21648.6*x	0.9991	5-500
Tyr	-28397.7+85821.2*x	0.9982	2.5 -500
Trp	-95797.1+110400*x	0.9992	2.5-500
AsA	-50439.2+3143.26*x	0.9987	25-400
Lac	23996.4+169.74*x	0.9991	100-1000
Cort	5218.23+10472.3*x	0.9986	0.5-500

Table A 2 - Linearity parameters of calibration curves from Cal 04 (R_2 and least squares fit) using external standard calibration for each compound

Compound	Equation Curve	R^2	Linear range (ng.mL^{-1})
Ach	2.08409e06+339852*x	0.9963	10-500
DA	-74792.8+31214.9*x	0.9809	2.5-500
E	4133.17+19730*x	0.9987	1-300
NE	45252+345.827*x	0.9787	50-500
Glu	-	-	-
5-HT	-	-	-
Tyr	-16709.3+43047.8*x	0.9977	2.5-500
Trp	-36752.3+84636.9*x	0.9971	1-400
AsA	-	-	-
Lac	3748.53+89.1982*x	0.9661	100-500
Cort	-24799.6+13277.8*x	0.9970	5-500

Table A 3 - Linearity parameters of calibration curves from Cal 05 (R_2 and least squares fit) using external standard calibration for each compound

Compound	Equation Curve	R^2	Linear range (ng.mL^{-1})
Ach	497546+377829*x	0.9968	2.5-300
DA	11621.5+33507.9*x	0.9923	5-300
E	309715+19396.9*x	0.9878	25-400
NE	28041.3+367.72*x	0.9725	10-500
Glu	-	-	-
5-HT	-	-	-
Tyr	2257.84+69925.7*x	0.9985	2.5-400
Trp	1820.99+100300*x	0.9980	1-400
AsA	-	-	-
Lac	8168.63+153.414*x	0.9260	2.5-500
Cort	3729.59+18061.2*x	0.9987	0.25-500

A.2 Chromatograms obtained for Calibration Curve of Each compound

- For Ach

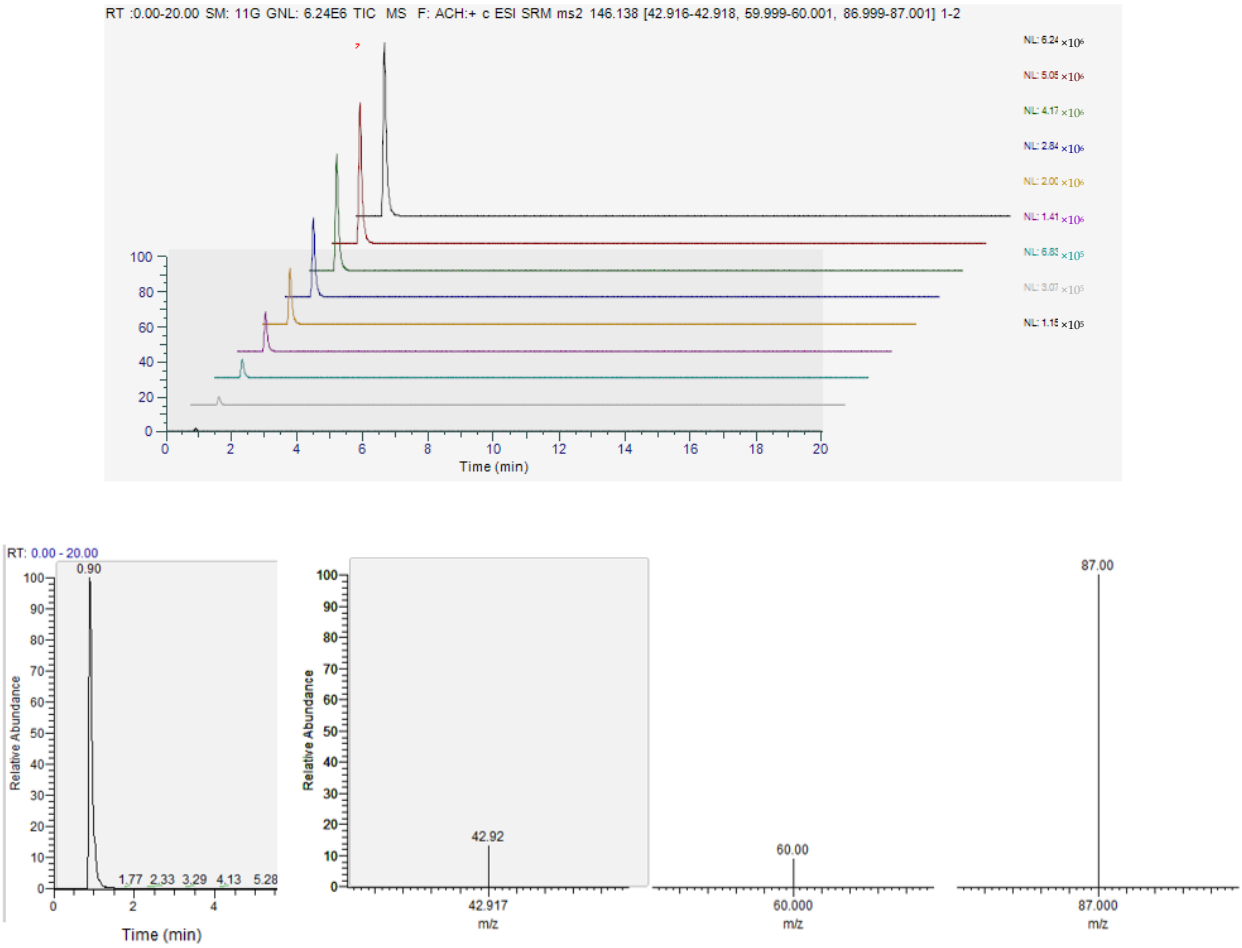


Figure A 1 - Chromatograms obtained for Ach for concentrations of 50, 40, 30, 20, 15, 10, 5, 2.5 and 1 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For DA

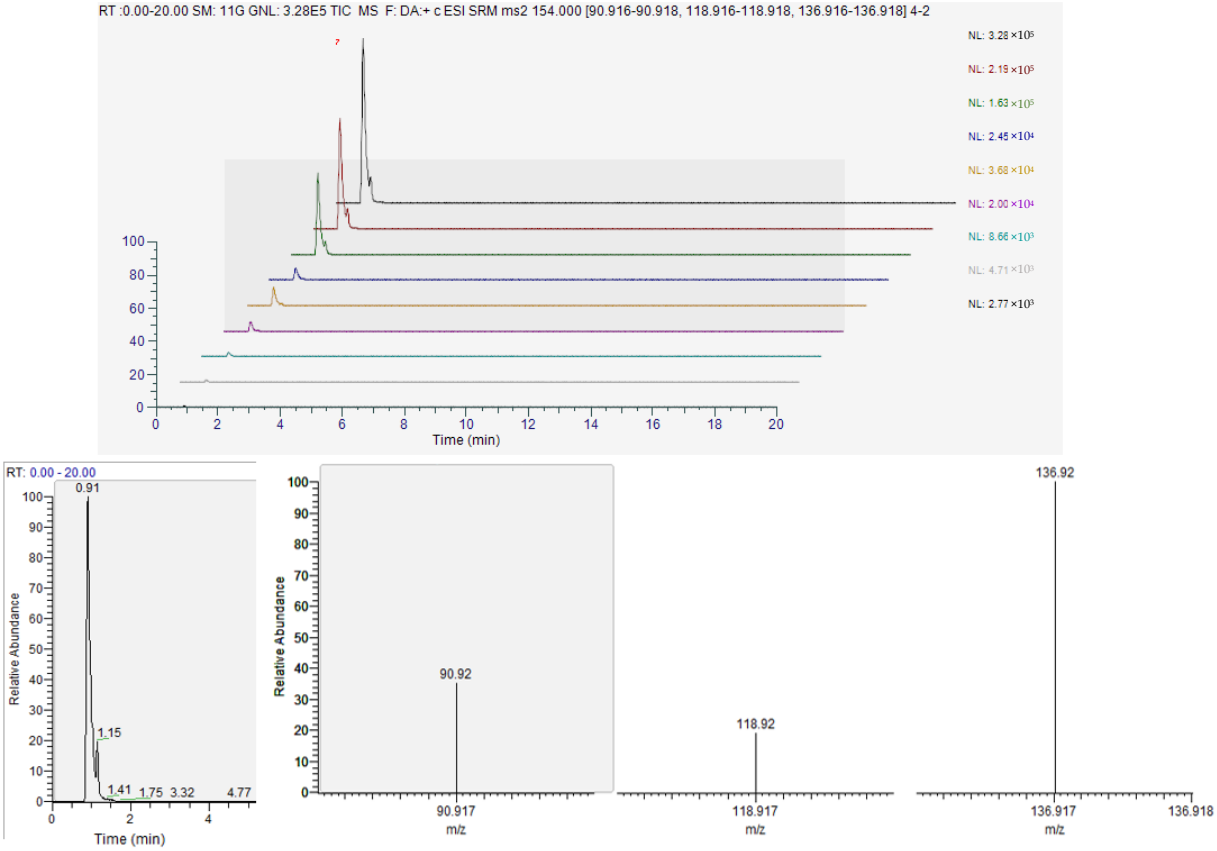


Figure A 2 - Chromatograms obtained for DA for concentrations of 40, 30, 20, 10, 5, 2.5, 1 and 0.25 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For E

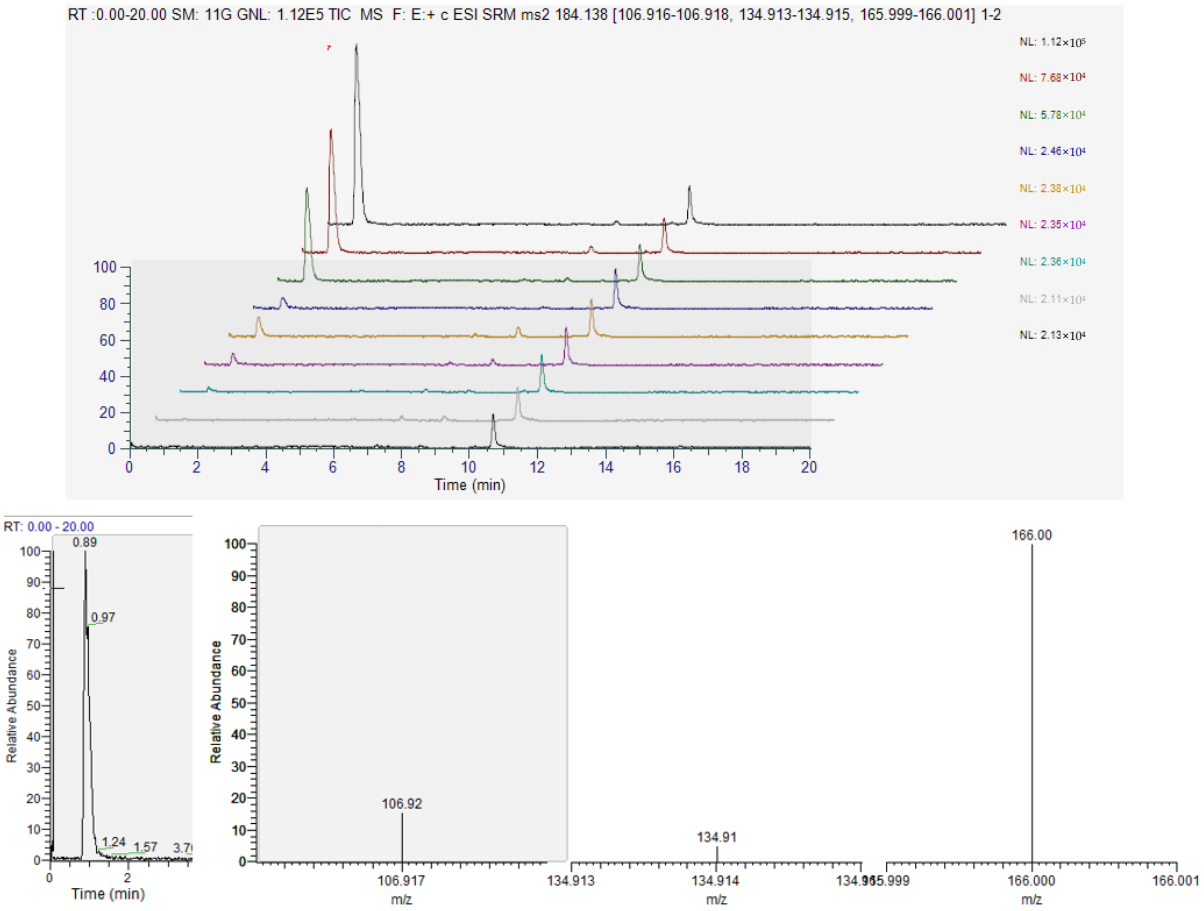


Figure A 3 - Chromatograms obtained for E for concentrations of 40, 30, 20, 10, 5, 2.5, 1 and 0.25 ppb using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For NE

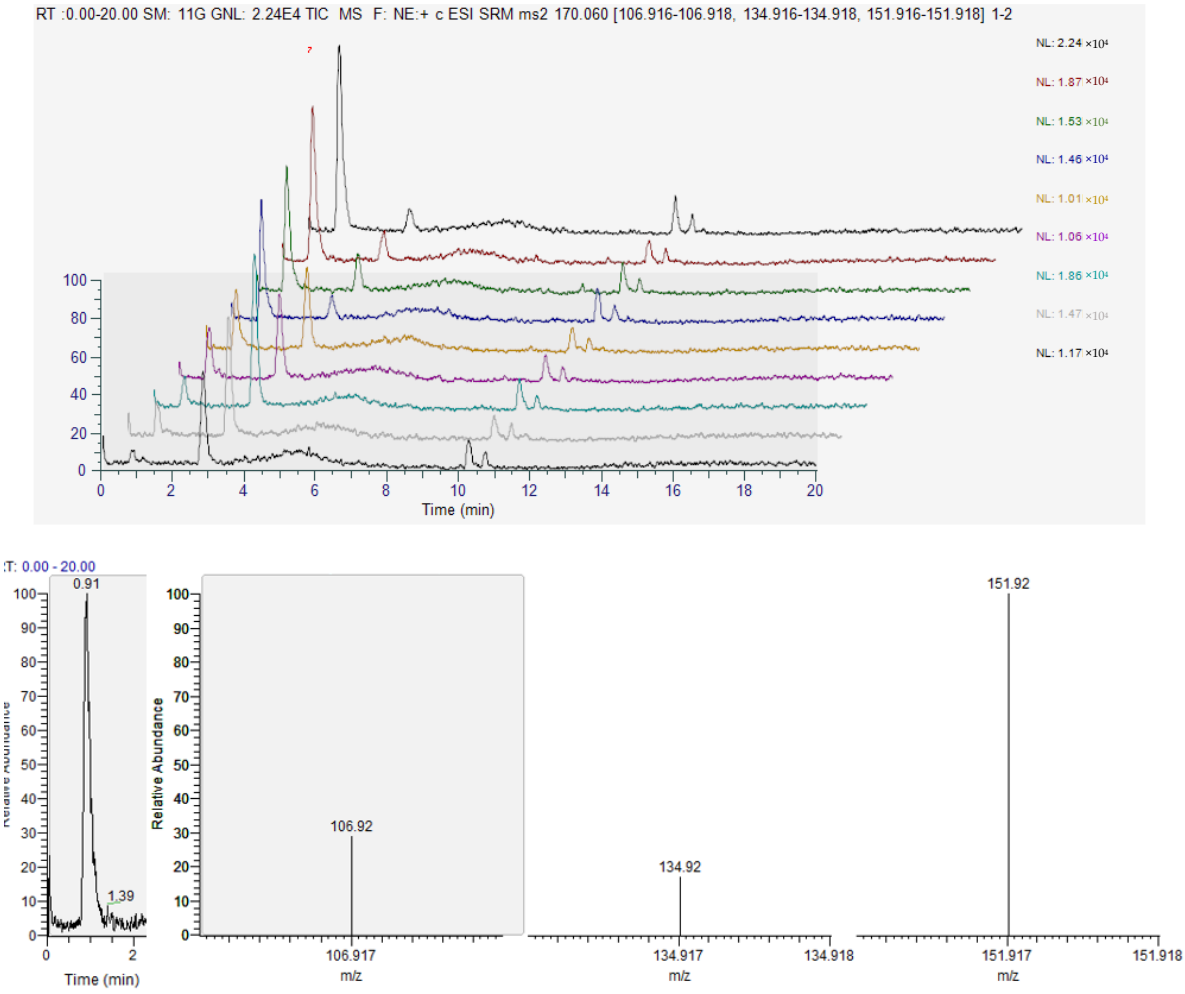


Figure A 4 - Chromatograms obtained for NE for concentrations of 900, 700, 500, 400, 300, 200, 150, 100 and 50 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For Glu

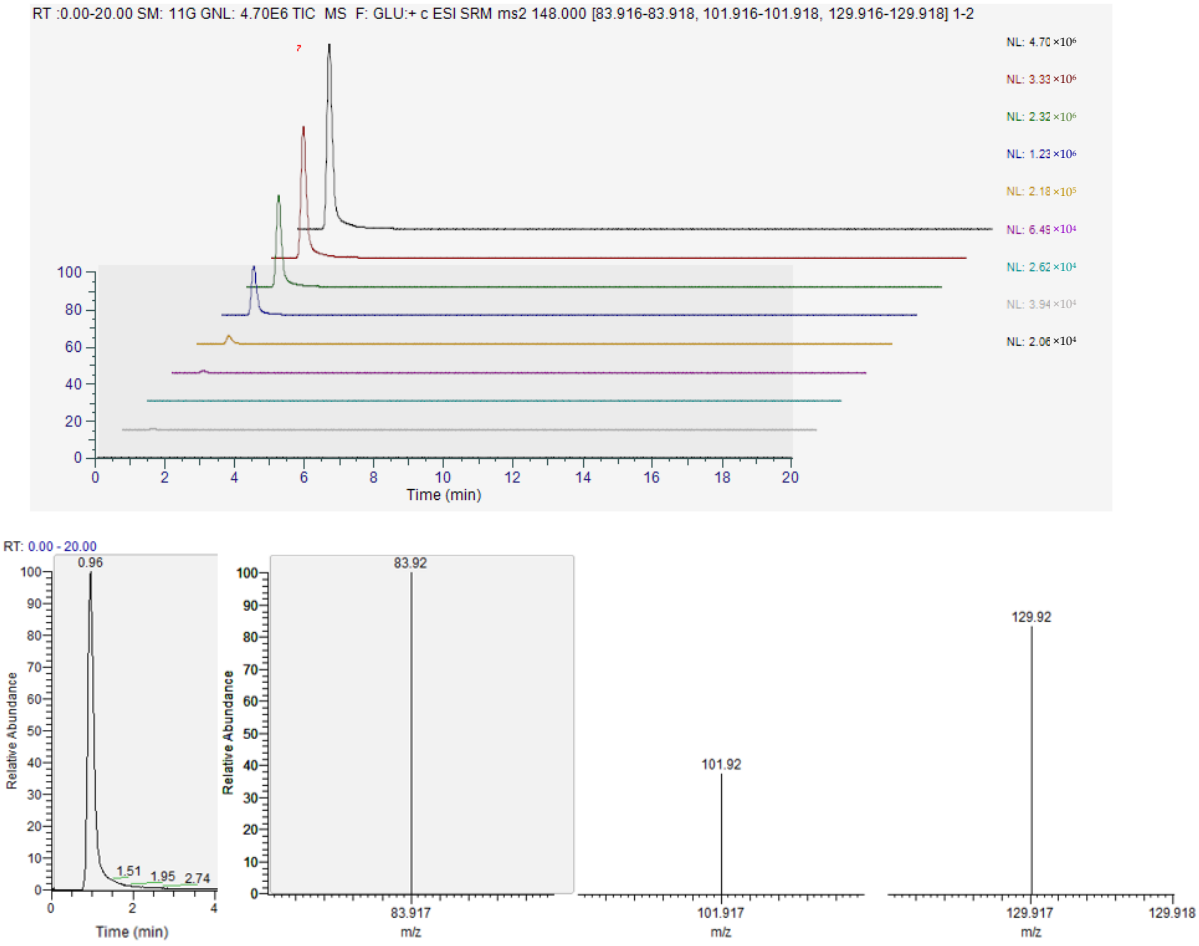


Figure A 5 - Chromatograms obtained for Glu for concentrations of 1000, 750, 500, 250, 50, 10, 5, 2.5 and 1 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For 5-HT

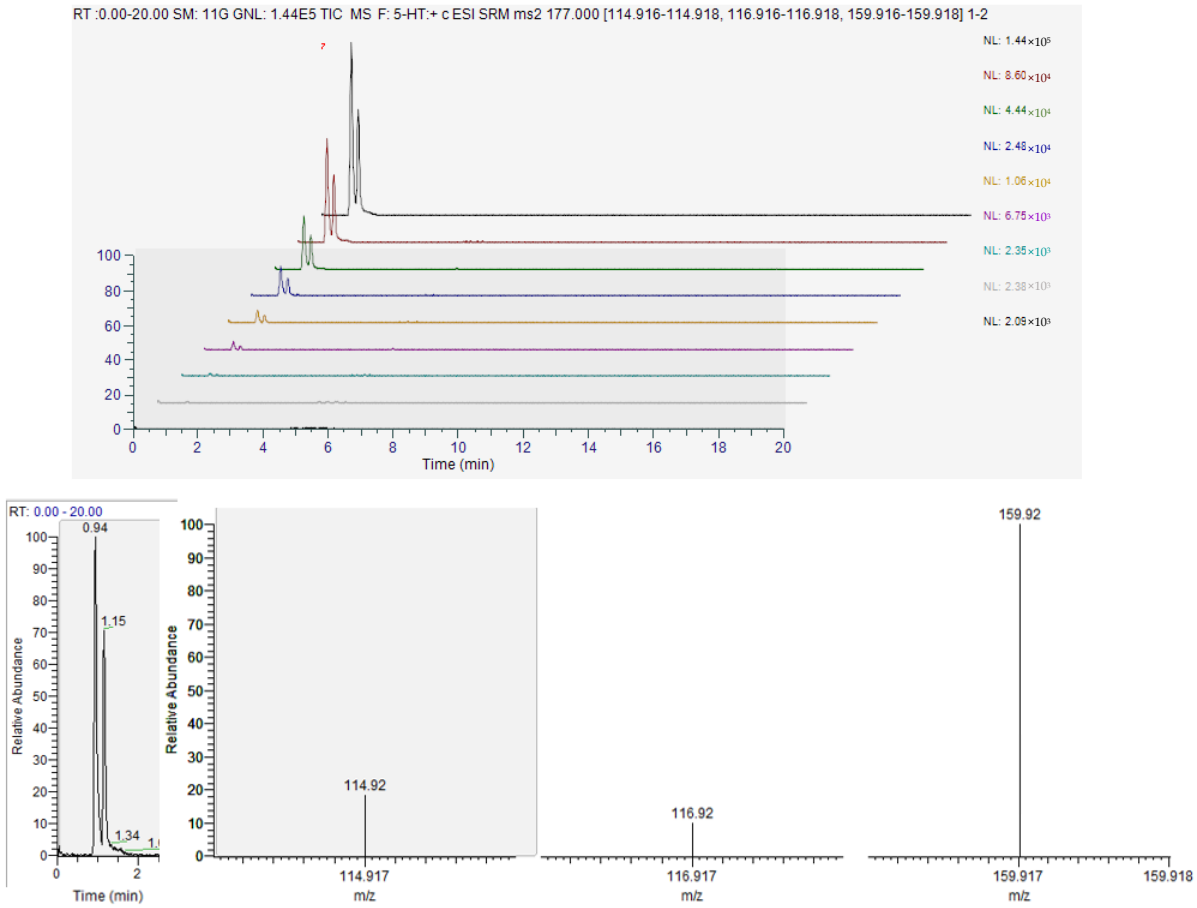


Figure A 6 - Chromatograms obtained for 5-HT for concentrations of 60, 40, 20, 10, 5, 2.5, 1 and 0.25 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent;; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For Tyr

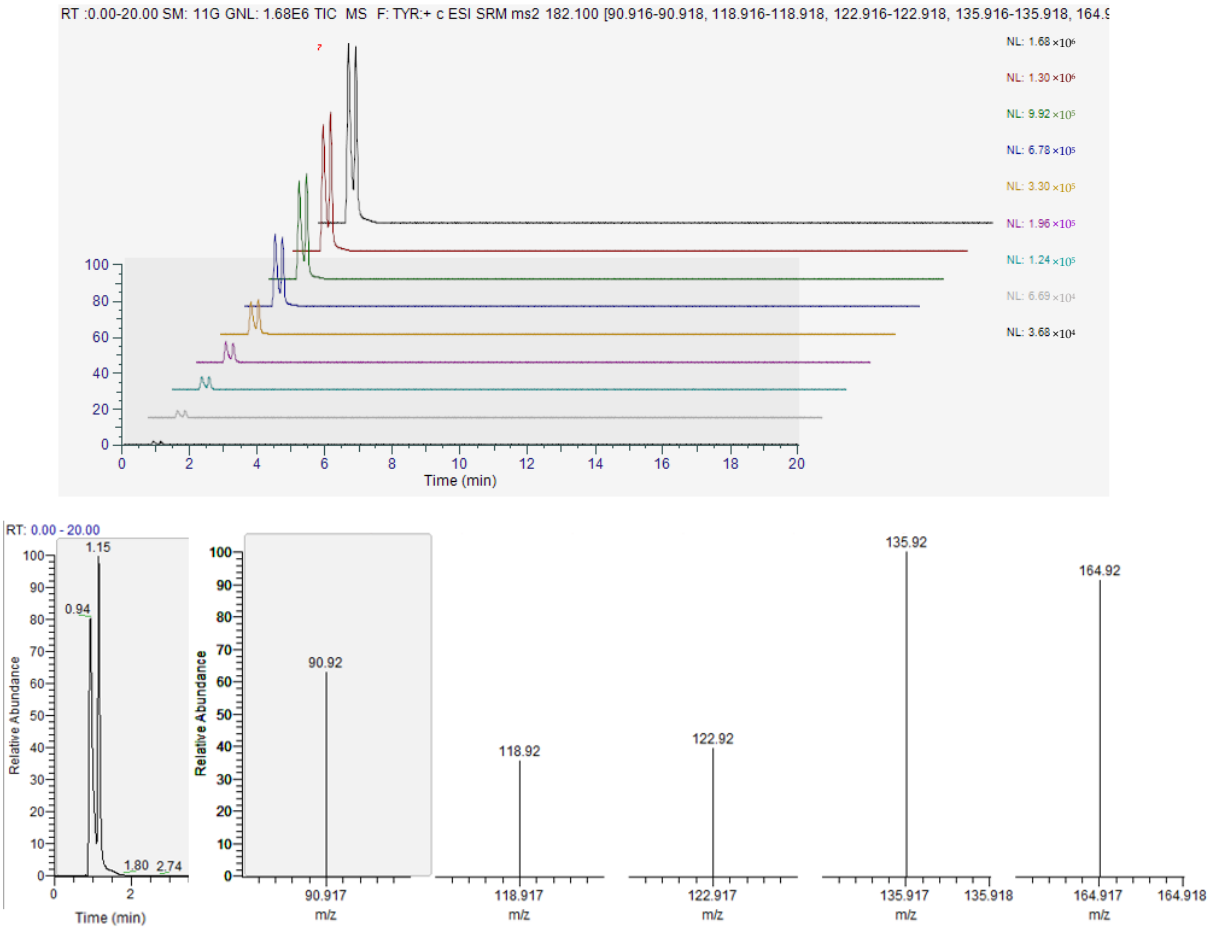


Figure A 7 - Chromatograms obtained for Tyr for concentrations of 250, 200, 150, 100, 50, 30, 20, 10 and 5 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For Trp

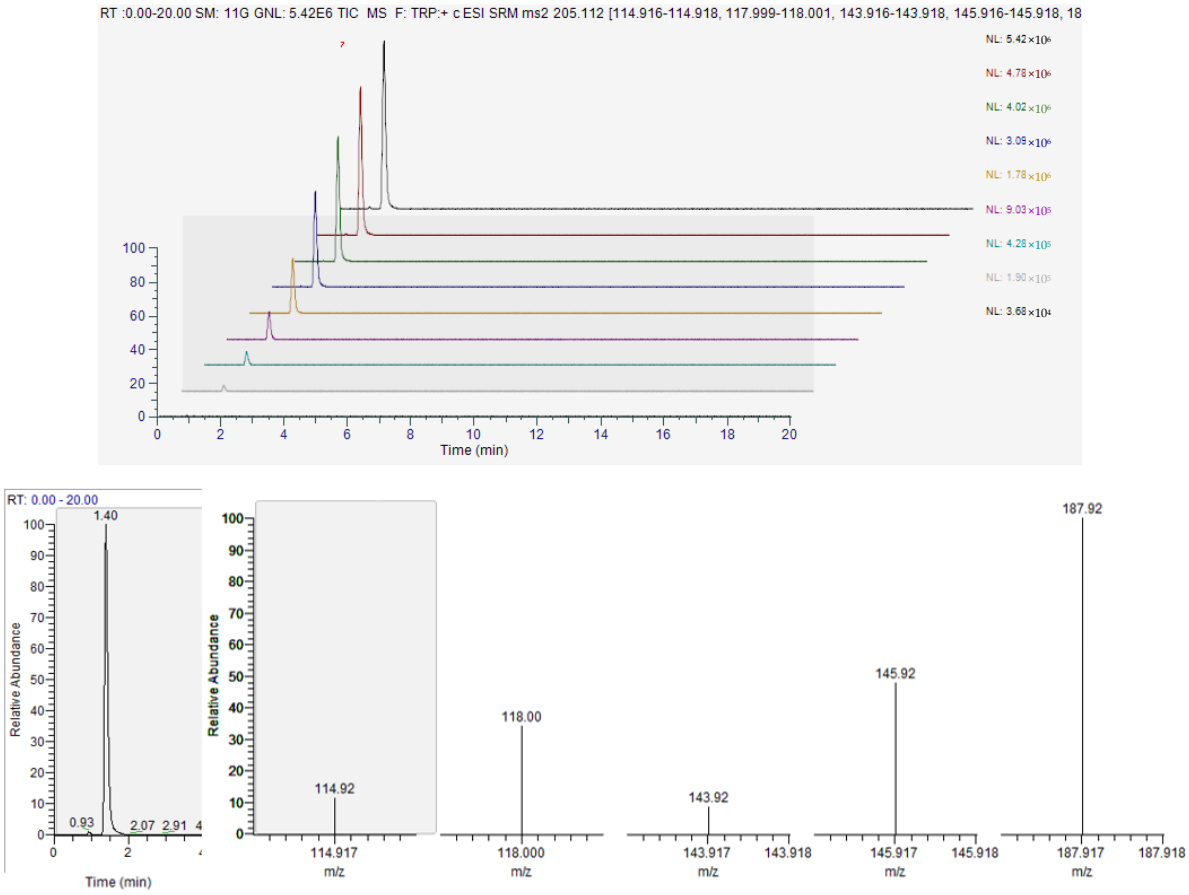


Figure A 8 - Chromatograms obtained for Trp for concentrations of 300, 250, 200, 150, 100, 50, 25, 10 and 5 ng.mL-1 using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For AsA

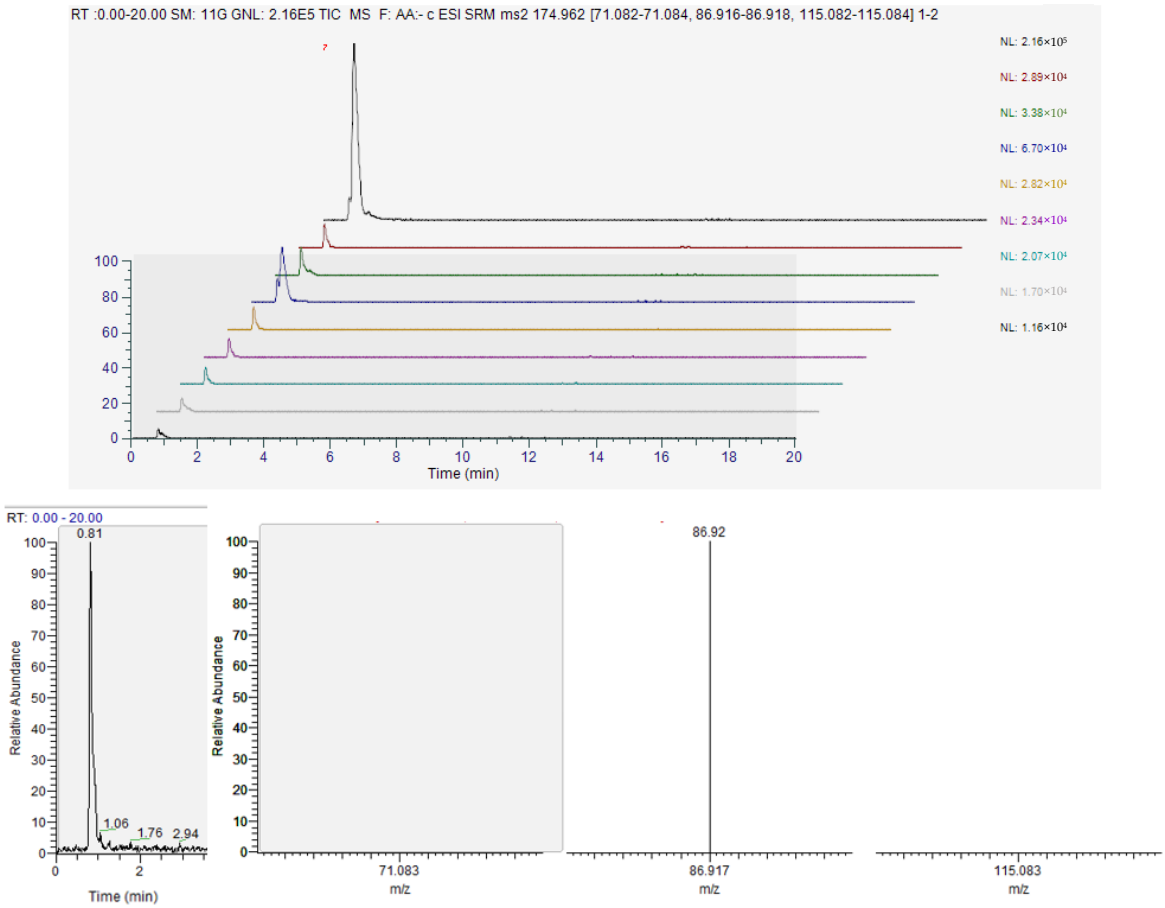


Figure A 9 - Chromatograms obtained for AsA for concentrations of 1000, 750, 500, 400, 300, 200, 100, 50 and 25 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For Lac

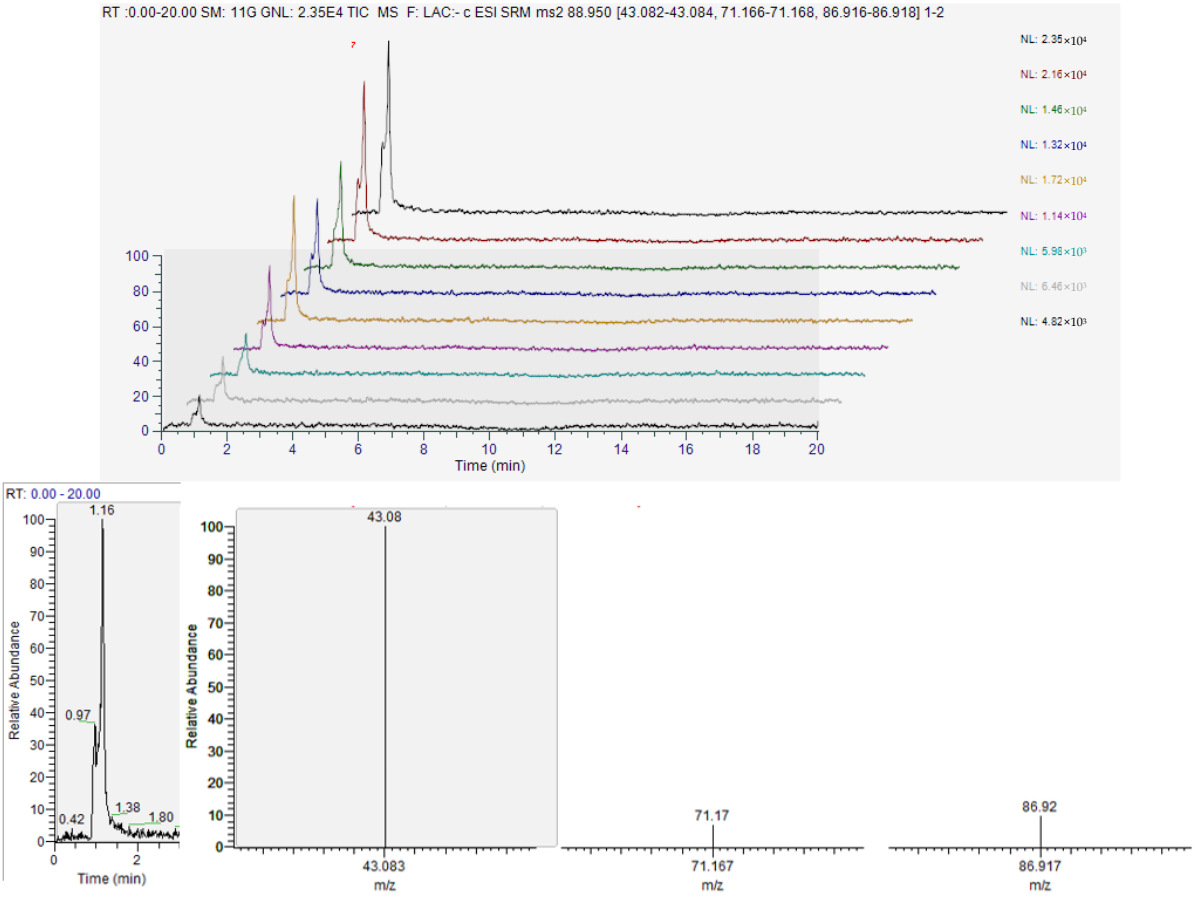


Figure A 10 - Chromatograms obtained for Lac for concentrations of 1000, 750, 500, 400, 300, 200, 100, 50 and 25 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

- For Cort

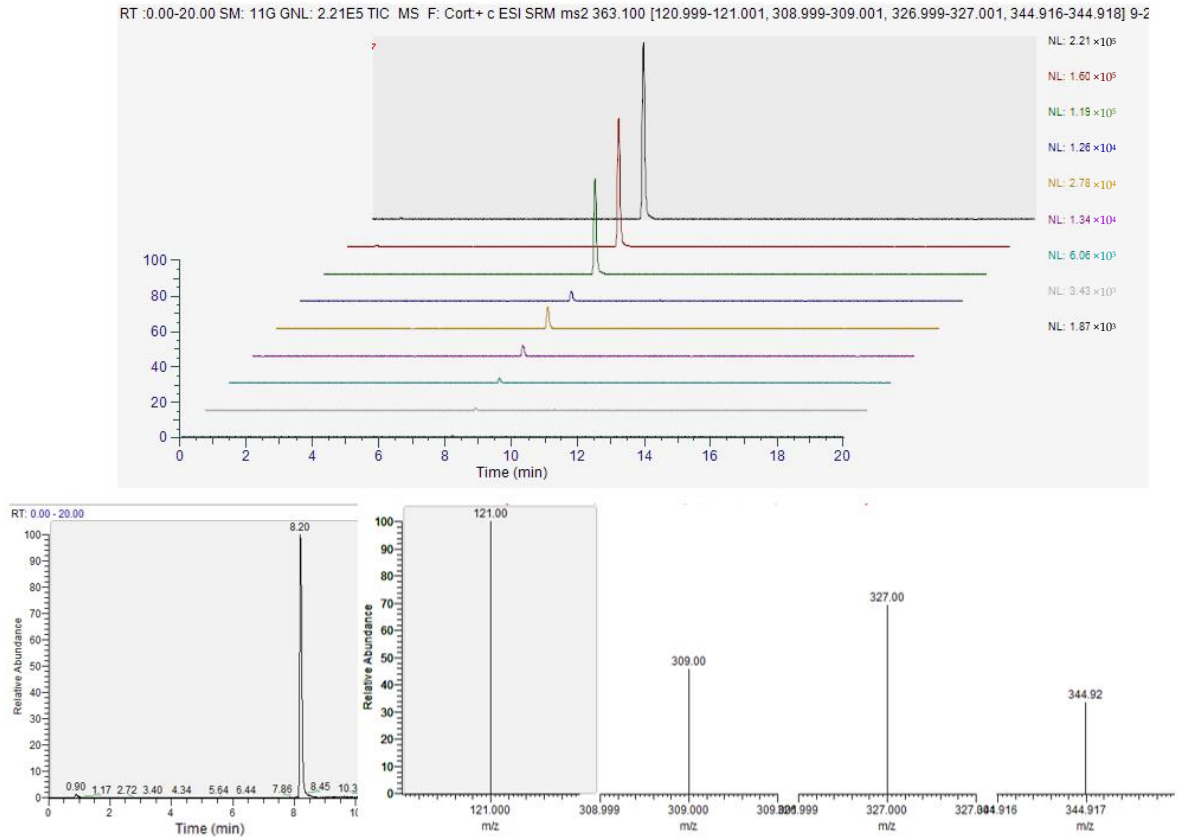
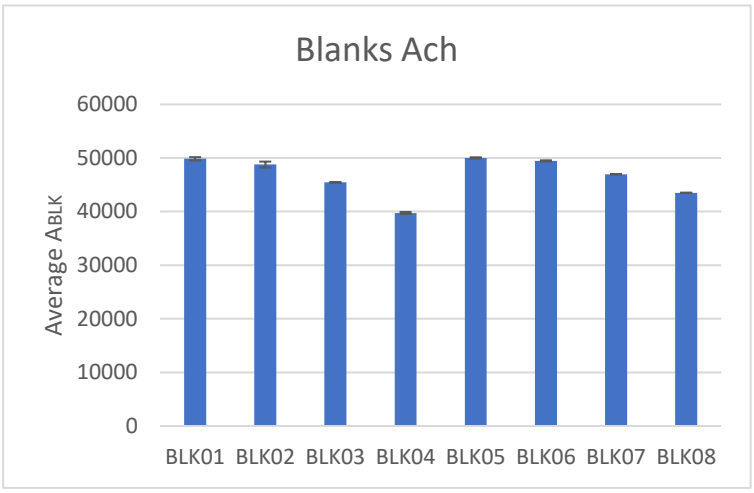
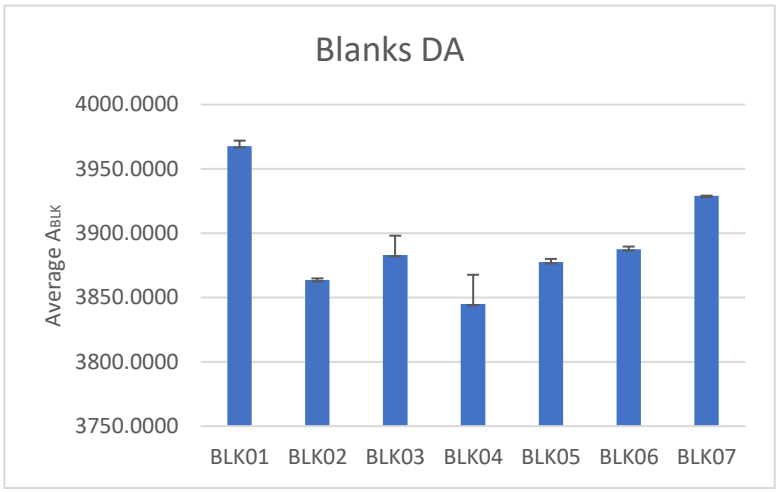
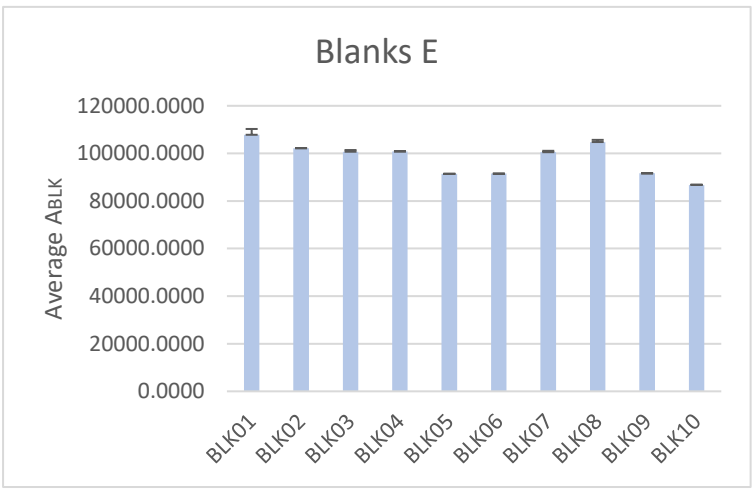


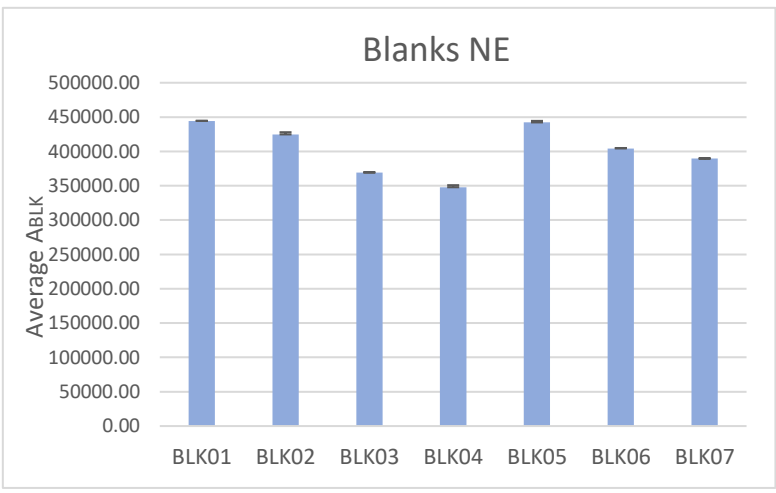
Figure A 11 - Chromatograms obtained for Cort for concentrations of 40, 30, 20, 10, 5, 2.5, 1 and 0.25 ng.mL^{-1} using a mixture of 50% MeOH/50% H_2O as extraction solvent; retention time (Rt) , precursor ion (m/Z) and product fragment ions

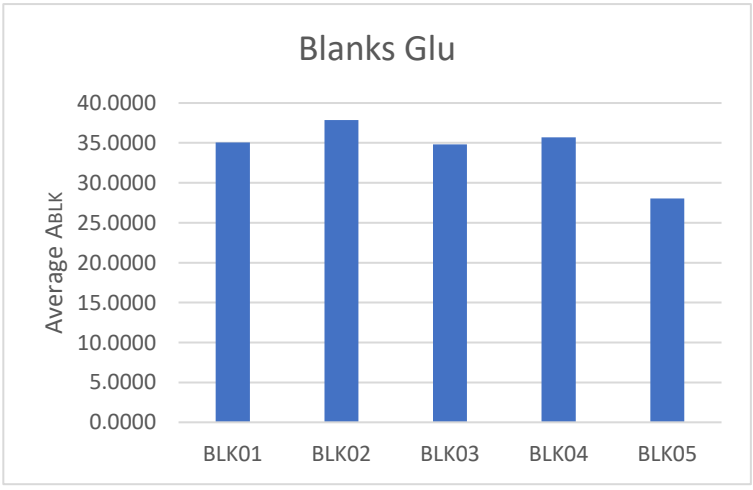
A.3 Intermediate Results of Blanks

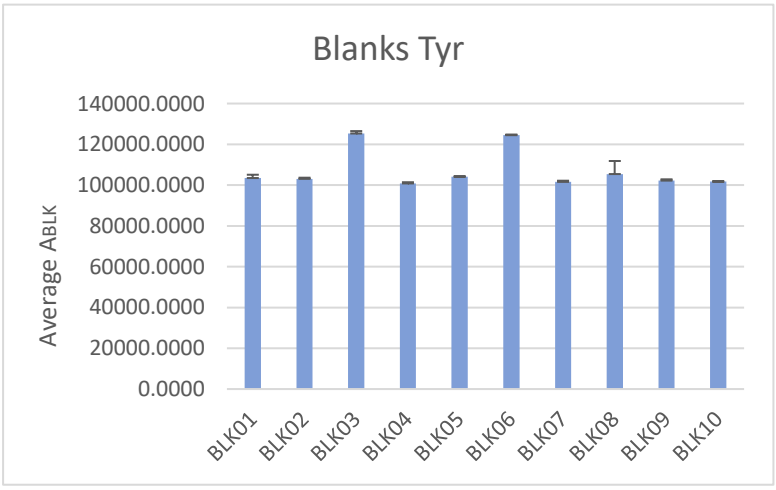












Cont.

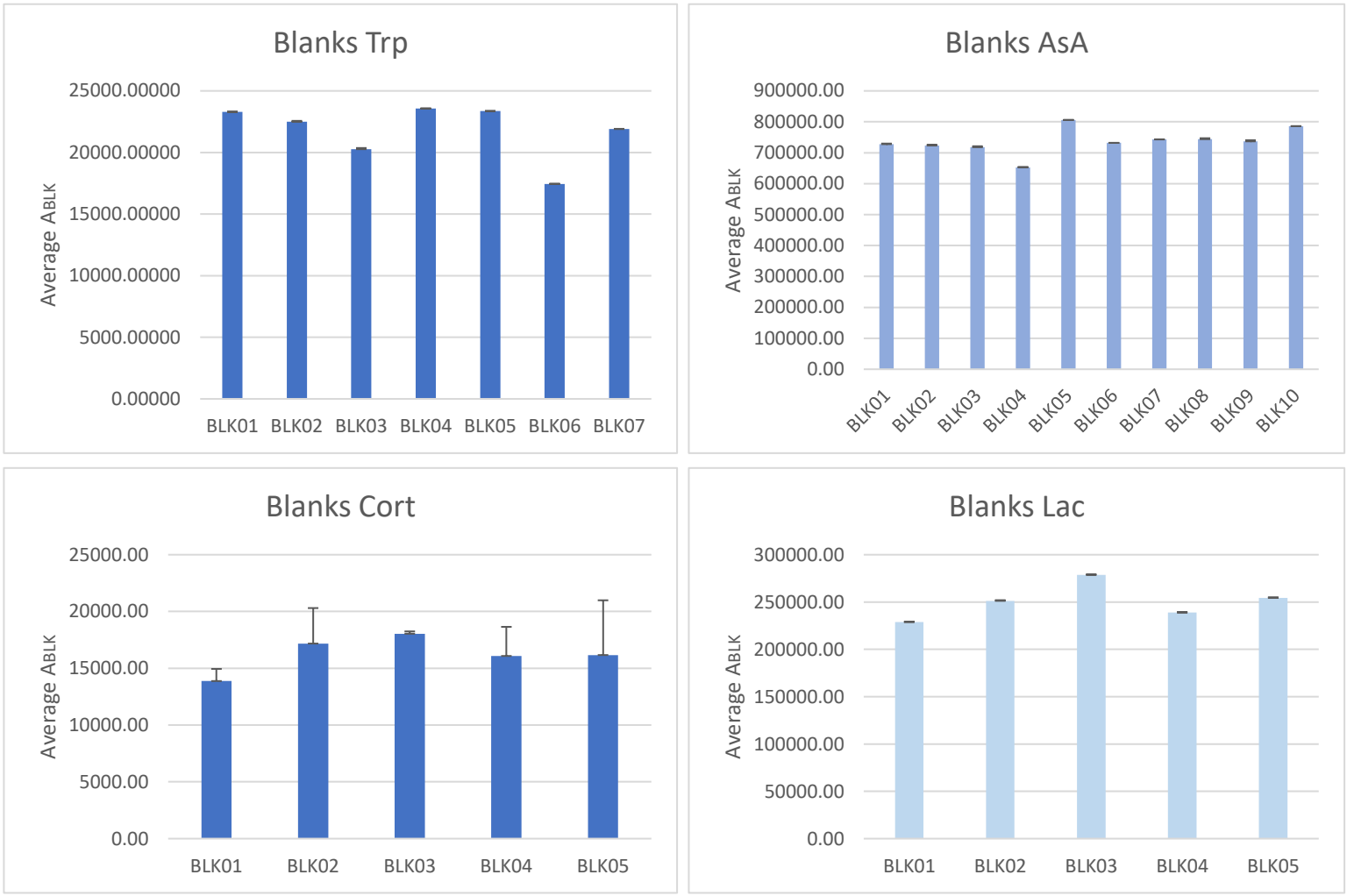


Figure A 12 - Average Area of the BLKs for Ach, DA, E, NE, Glu, Tyr, Trp, AsA, Lac and Cort with respective standard deviation (% Stdev)

A.4 Voltammograms obtained for Human Saliva

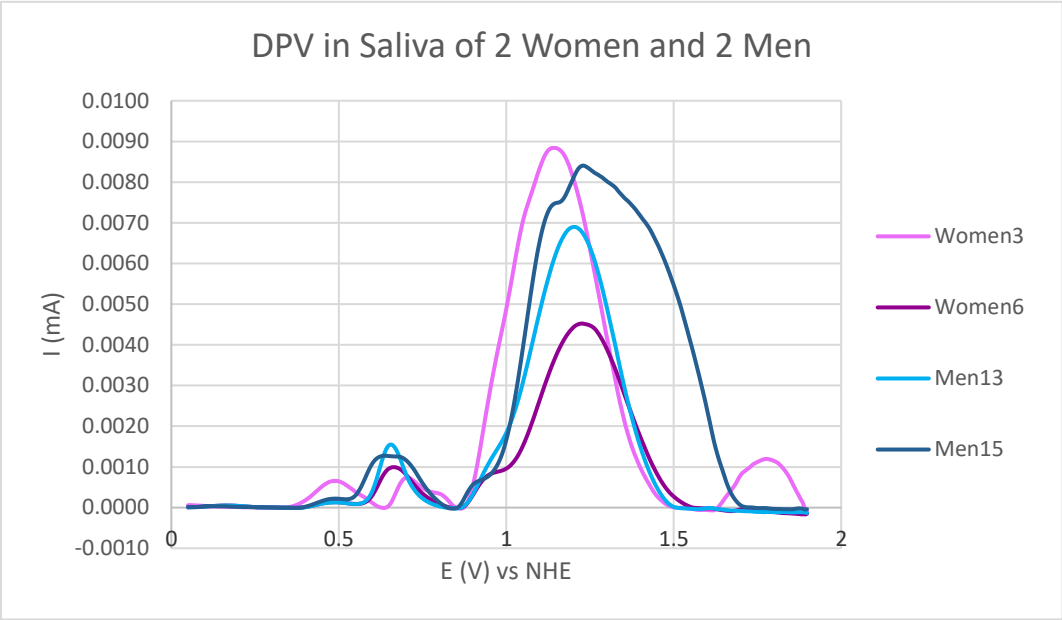


Figure A 13 - DPV in Human saliva for women and men using SPE (Metrohm DropSens 110); Modulation time of 0.05 s, Interval time of 0.5 s, Initial Potential of -0.35 V, End Potential of 1.50 V and Modulation amplitude of 0.02505 V.

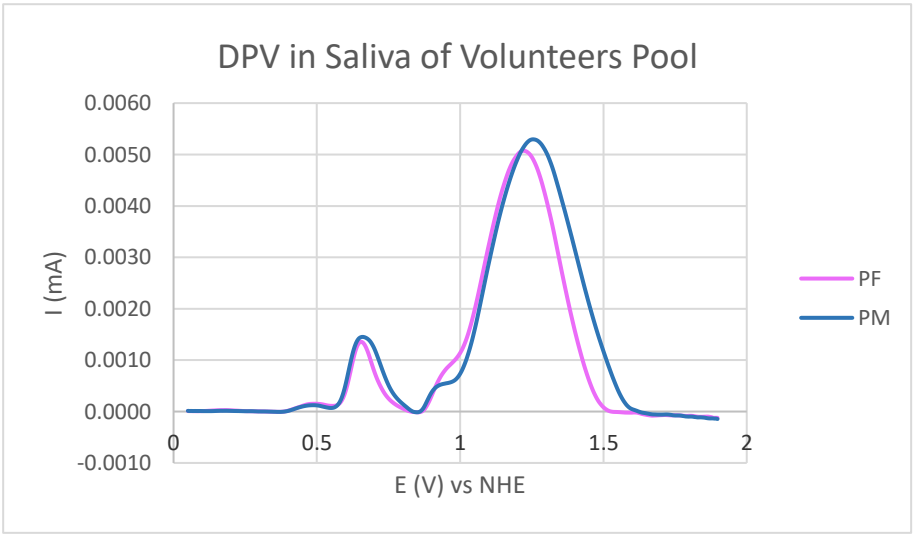


Figure A 14 - DPV in Human saliva in feminine pool (PF) and masculine pool (PM) using SPE (Metrohm DropSens 110); Modulation time of 0.05 s, Interval time of 0.5 s, Initial Potential of -0.35 V, End Potential of 1.50 V and Modulation amplitude of 0.02505 V.

A.5 Restarting the UHPLC Equipment

The UHPLC equipment in question had been out of operation for approximately 4 years, so it was necessary to restart the equipment in order to be able to clean it, since the intention was to couple this equipment to a triple quadrupole tandem mass spectrometer (MS/MS) detector. So I had a formation of how to perform this restart. The report made is present next in this section.

RESTARTING THE UHPLC EQUIPMENT (ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY)

Report by Rute Viera

Restarting the UHPLC Equipment (Ultra High Performance Liquid Chromatography)

The UHPLC equipment in question had been out of operation for approximately 4 years, so it was necessary to restart the equipment in order to be able to clean it, since the intention is to couple this equipment to a triple quadrupole tandem mass spectrometer (MS/MS) detector.

Washing the Eluent Bottles

First, it is necessary to wash the 4 eluent bottles to be used in the system, in order to decontaminate them. Since the UHPLC equipment will be connected to the MS/MS apparatus, it is important that the bottles are thoroughly washed to avoid possible contamination, given that the MS/MS detector is quite sensitive and can be contaminated.

The washing should be done by leaving the bottles immersed in hot distilled water containing a small amount of phosphate-free detergent. Afterwards, each bottle should be washed 10 times in distilled water and 30 times in milliQ water.

1. Place the bottles in the oven at 120 °C until they are dry.

Preparing the Eluents

1. Prepare the following eluents:
   (A) H_2O/AcFo 0.1%
   (B) Buffer 5 mM NH_4Fo/AcFo 0.1%
   (C) MeOH
   (D) ACN
2. Also prepare the Piston and Syringe solutions. The Piston solution consists of a mixture of 10% MeOH/90% H2O and the Syringe liquid of 50% MeOH/50% H2O.
3. In order to avoid air in the system, degas all the eluents and liquids prepared in the ultrasound device at room temperature for 5 min.

Connecting the Device

1. Set up the Piston and check if the Piston seal that is dry remains in solution, thus being operational.
2. Purge each eluent twice, always taking care to open the pump valve before starting the purge, so that the flow goes into the sewer and not into the system, so as not to damage the equipment. Finally, close the valve.
3. Wash the syringe – 100 cycles and check that it does not contain any air bubbles that could interfere with the analyses.
4. In order to remove the air from the syringe and avoid possible contamination, another 5 x 100 cycles should be performed.
5. Wash the loop with buffer (Wash buffer loop) – 35 times, in order to eliminate any contamination in the loop.
6. Wash the needle externally (Wash needle externally) – 100 times.
7. Wash the syringe again 4 x 100 cycles. Steps 4, 5 and 6 can be repeated in the same way as long as possible contamination is suspected.

Note: The pressure displayed must always be taken into account when carrying out the steps mentioned above, and it must always be at 0.

Initiating the Column

1. The percentage of eluents to be used should be a mixture of 50% H2O/0.1% AcFo and 50% ACN.
2. Start the flow pump, gradually increasing the flow until the flow indicated in the program is established.
3. Start the flow pump, gradually increasing the flow until the flow indicated in the program is established.
4. Wait approximately 30 minutes to confirm that the system is stable under the desired conditions.

Methanol Test

1. Prepare a vial with methanol for analysis, in order to verify that the system and the equipment column were already decontaminated and ready to inject the samples under study.
2. Perform the analysis according to the method established in the program.

Note: this initial installation was performed using a DAD detector at a wavelength of 340 nm. The equipment was then coupled to the MS/MS after being configured.