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**Detection of biogenic amines in urine and plasma
by liquid chromatography coupled to
electrochemical detection (HPLC-ED) using
microextraction in packed syringe (MEPS)**

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Dissertação para obtenção do Grau de Mestre em
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Resumo alargado

As aminas biogénicas são neurotransmissores com importantes funções ao nível do sistema endócrino e ao nível do sistema nervoso central. Alterações e distúrbios na sua função normal estão frequentemente associadas a várias doenças e desordens psicológicas, nestas situações ocorre uma alteração, por excesso ou défice, dos níveis normais circulantes destes compostos. Estes níveis podem também estar alterados em pacientes sujeitos a terapias com fármacos psicoterapêuticos (por exemplo antidepressivos e antipsicóticos) e em casos de consumo de algumas drogas de abuso. Ainda, elevados níveis de aminas biogénicas são importantes marcadores para certo tipo de tumores que secretam estes compostos pelas suas propriedades hormonais. Deste modo, é de interesse clínico e neuroquímico a existência de métodos de deteção e quantificação destes compostos em amostras biológicas.

O objectivo deste trabalho foi o desenvolvimento e validação de um método analítico para a deteção e quantificação das aminas biogénicas, serotonina (5-HT), dopamina (DA) e norepinefrina (NE) em amostras de urina e plasma, usando a microextração em seringa empacotada (MEPS) e cromatografia líquida acoplada à deteção eletroquímica de coulometria (HPLC-ED). A 3,4-dihydroxybenzilamina (DHBA) foi utilizada como padrão interno. A optimização do protocolo de MEPS foi efectuada com recurso ao desenho experimental (DOE) e os compostos foram detectados num sistema de cromatografia líquida de *ultra performance* acoplada a um detector de fluorescência (UPLC-FLD). O uso de um desenho factorial fraccionado (2^{k-1}) demonstrou ser uma ferramenta útil para a optimização do método de extração, reduzindo o número de experiências, minimizando o tempo de processamento da amostra, reagentes e trabalho laboratorial. O protocolo de MEPS optimizado inclui 8 aspirações da amostra, sem passo de lavagem e duas eluições de 100 μ L de metanol.

O método foi completamente validado de acordo com critérios internacionalmente aceites, da *Food and Drug Administration* (FDA). O método demonstrou ser linear na gama de 50 a 1000 ng/mL para a 5-HT, e de 5 a 1000 ng/mL para a DA e NE, com coeficientes de determinação (R^2) superiores a 0.99 para todos os compostos. Os limites de deteção e quantificação foram, respectivamente, de 20 e 50 ng/mL para a 5-HT, e de 2 e 5 ng/mL para a DA e NE. Para todos os compostos, a precisão intra- e interdia variou entre 1 a 10 % e a exactidão esteve no intervalo de $\pm 15\%$.

A eficácia do método foi avaliada, por aplicação da metodologia validada a amostras reais de urina e plasma. Foi possível detetar e quantificar os 3 compostos em amostras de urina e a 5-HT em plasma. Por tudo anteriormente citado, podemos afirmar que o método desenvolvido é apropriado para a determinação destes compostos em análises laboratoriais de rotina nestas matrizes biológicas.

Este é o primeiro estudo que utiliza uma coluna de MEPS comercializada para a determinação simultânea de 5-HT, DA e NE em amostras de urina, sendo também o primeiro a aplicar MEPS para a determinação de 5-HT em amostras de plasma.

Como método de extração, MEPS demonstrou ser uma técnica de preparação da amostra simples e rápida, com baixo consumo de solventes, poupando tempo, dinheiro e trabalho aos laboratórios.

Palavras chave

Aminas biogénicas, 5-HT, DA, NE, MEPS, HPLC-ED, fluidos biológicos.

Abstract

Biogenic amines are neurotransmitters involved in several physiological processes. Changes and disturbs in their function are associated to several neurological disorders or diseases, as well as consumption of psychotherapeutic or psychotropic drugs. The detection of these compounds in biological fluids is therefore of clinical and neurochemical interest.

The goal of this work was to develop and validate an analytical method for the detection and quantification of the biogenic amines serotonin (5-HT), dopamine (DA) and norepinephrine (NE), using microextraction in packed syringe (MEPS) and liquid chromatography coupled to electrochemical detection (HPLC-ED) in both urine and plasma samples. The internal standard used was 3,4-dihydroxybenzylamine (DHBA). The MEPS extraction procedure was optimized using the design of experiments (DOE) tool, and the final conditions of 8 strokes, no washing, and two elutions of 100 μ L methanol were chosen.

The method was fully validated according to internationally accepted guidelines from the Food and Drug Administration. Linearity was established between 50-1000 ng/mL for 5-HT and between 5-1000 ng/mL for DA and NE, with determination coefficients (R^2) higher than 0.99 for all compounds. The limits of detection and quantification were respectively 20 and 50 ng/mL for 5-HT, and 2 and 5 ng/mL for DA and NE. Intra- and interday precision ranged from 1 to 10 %, while accuracy was within a $\pm 15\%$ interval for all compounds.

Authentic urine and plasma samples were analysed by the validated method, and the three compounds were detectable and quantifiable in urine, while only 5-HT was detected and quantified in plasma.

This is the first time that a commercially available MEPS column was used for the simultaneous detection of biogenic amines in urine, and also the first time that 5-HT was detected and quantified in plasma using MEPS. MEPS proved to be fast to perform with the use of less solvent volumes, saving time and money and being less laborious. The validated method is useful for the determination of biogenic amines in laboratorial routine.

Keywords

Biogenic amines, 5-HT, DA, NE, MEPS, HPLC-ED, biological specimens.

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List of abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
AD	Alzheimer's disease
BIN	Barrel insert and Needle assembly
C2	Ethylsilane
C8	Octylsilane
C18	Octadecylsilane
CAT	Catecholamines
CAS	Chemical Abstracts Service
CB1	Cannabinoid receptor type 1
CE	Capillary electrophoresis
COMT	Catechol- <i>O</i> -methyl transferase
CV	Coefficient of variation
DA	Dopamine
DAT	Dopamine transporter
DC	Direct current
DHBA	3,4-dihydroxybenzylamine
DOE	Design of experiments
DPBA	Diphenyl boronic acid
DS	Down's syndrome
E	Epinephrine
ED	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and drug administration
FLD	Fluorescence detection
GC	Gas chromatography
HClO ₄	Perchloric acid

HPLC	High Performance Liquid Chromatography
HVA	Homovanillic acid
ICH	International conference on harmonization
IS	Internal standard
KH ₂ PO ₄	Potassium dihydrogen phosphate
LC	Liquid chromatography
LLE	Liquid-Liquid extraction
LOD	Limit of detection
(L)LOQ	(Lowest) limit of quantification
M1	Mixed mode with C ₈ and cation exchanger
MAO	Monoamine oxidase
MCX	Mixed cation exchanger
ME	Metanephrine
MEPS	Microextraction in packed syringe
MDMA	3,4-methylenedioxy-N-methylamphetamine
MIP	Molecular imprinted polymer
MS	Mass spectrometry
NE	Norepinephrine
NET	Norepinephrine transporter
NME	Normetanephrine
OSA	Sodium octyl sulphate
PD	Parkinson's disease
R ²	Determination coefficient
RAM	Restricted-access media
RSM	Response surface methodology
SCX	Strong cation exchanger
SERT	Serotonin transporter
SPE	Solid phase extraction
SPME	Solid phase microextraction

THC	Δ^9 -tetrahydrocannabinol
TOF	Time-of-flight
UV	Ultraviolet
VMA	Vanillylmandelic acid
VMAT	Vesicular monoamine transporter

Justifications and Objectives

Neurotransmitters, including biogenic amines are since long known to play several important roles in the endocrine and nervous systems of the human organism.

Several psychiatric disorders and diseases (among others, depression, schizophrenia, Parkinson's disease and tumours), are related to some extent with disturbs or changes in biogenic amine transmission. Biogenic amines are also the main target of several psychiatric medications and psychotropic drugs. All this situations affect the neurotransmitters either at their production, storage, release, re-uptake and metabolism or their receptors; as a result the normal concentrations of circulating biogenic amines are altered.

For these reasons, the development of a method for detection of biogenic amines in biological fluids is thus a very useful tool for the diagnosis of psychiatric disorders and diseases, and for the monitoring of patients undergoing psychiatric medication or cases of psychotropic drug consumption.

Therefore, the objective of this work was to develop an analytical method for the clinical determination of three biogenic amines, serotonin (5-HT), dopamine (DA) and norepinephrine (NE) in urine and plasma, using microextraction in packed syringe (MEPS) and high performance liquid chromatography coupled to electrochemical detection (HPLC-ED). The three transmitters were chosen based on their importance in the situations mentioned above and compatibility with the electrochemical detection.

I. Revision of literature

1. Biogenic amines and amine transmission

1.1. Physiological properties of Biogenic amines

Biogenic amines are neurotransmitters and neuromodulator molecules with various important functions at the central and peripheral nervous system. They are involved in several psychological and hormonal signalling processes, ranging from homeostatic functions to emotional and motor behaviours. Defects in their function are often associated with psychiatric disorders and most drugs of abuse interfere with amine transmission [1-7]. This has made the pharmacology of biogenic amine synapses the target of several medications in modern pharmacology [1].

Biogenic amines are of basic nature, with pKa values ranging from 8 to 11 (table 1), and are therefore protonated at physiological conditions. Structurally, they are composed of an amino group connected to an aromatic ring and so they are also called as **monoamines** (figure 1). The catecholamines (CATs), dopamine (DA), norepinephrine or noradrenalin (NE), epinephrine or adrenalin (E) and the indolamines serotonin (5-hydroxytryptamine, 5-HT) and histamine are the major endogenous monoamines in most animal species including humans [1-4,6].

1.1.1. Serotonin

Serotonin was first identified in 1948 as a serum tonic factor, released by platelets during the clotting of blood, only latter was its function associated to a neurotransmitter molecule. The chemical structure of serotonin and related compounds contains a substituted indole moiety, consisting of a benzene ring and a nitrogen containing five-member ring, bonded to an amino group. The synthesis occurs through enzymatic hydroxylation and decarboxylation of the dietary amino acid L-tryptophan (figure 1a) [1-4,8]. Serotonergic neurons in the brain and enterochromaffin cells in mucosa of the gastrointestinal tract have the necessary enzymes for the biosynthesis process. Synthesis can also occur at the pineal gland, but as a precursor of melatonin. Most of the serotonin in the organism is stored at the serotonergic neurons, enterochromaffin cells and blood platelets (by uptake of circulating serotonin rather than secretion) [2,3].

In the nervous system transmission serotonin's action is mediated when it binds to one of its receptors on the postsynaptic neuron. A large number of receptors have been identified, and the synaptic transmission response is determined by the receptor to which serotonin binds and activates. Several behaviours have been associated to serotonin, as emotions, mood, sexuality, circadian rhythms, motor behaviours and mental state arousal. Other functions are also associated, such as the smooth muscle contraction, blood pressure regulation, gastrointestinal physiology, mediation of satiety and decreased food consumption [1-4,8,9].

1.1.2. Catecholamines

Catecholamines are structurally different from indolamines, mainly due to the fact that instead of a central indole ring, as in serotonin, they have a central catechol ring with an amine side chain. The major endogenous catecholamines are DA, NE and E, and their synthesis occurs at the adrenal medulla, sympathetic nervous system and brain. Synthesis of the three CATs occurs by a biochemical pathway starting from the amino acid tyrosine. In mammals both phenylalanine and tyrosine are precursors of CATs, since an enzyme, phenylalanine hydroxylase, located in the liver allows the conversion of dietary phenylalanine into tyrosine. The process occurs by a series of hydroxylation and decarboxylation reactions, originating sequentially dopamine, norepinephrine and epinephrine (figure 1b) [1-4]. Neurons only contain the necessary enzymes for their own production of neurotransmitters, thus the biosynthesis process ends either on DA, NE, or E in DA-, NE-, E-releasing neurons, respectively [2,4].

The catecholamines play important roles as hormones and as excitatory neurotransmitters, reflected by their synthesis and action at hormonal and nervous tissues. The CATs influence and control several physiological processes of the nervous system and systemic circulation as the stress system, heartbeat rate and blood pressure regulation. Dopamine has an essential role in the coordination of body movements, and is also involved in motivation, the reward system and reinforcement. NE and E functions have been implicated in control of arousal, attention, learning, memory and stress response influencing sleep and wakefulness, and also feeding behaviours [1,2,4,7].

Table 1: Chemical properties of the monoamines serotonin, dopamine and norepinephrine.

	Serotonin	Dopamine	Norepinephrine
CAS ^(a) number	50-67-9	51-61-6	51-41-2
Molecular formula	C ₁₀ H ₁₂ N ₂ O	C ₈ H ₁₁ NO ₂	C ₈ H ₁₁ NO ₃
Molar Mass (g/mol)	176.215	153.18	169.18
pKa	10.4 ^[10]	8.89 ^[11]	8.4 ^[11]

^aCAS - Chemical Abstract Service.

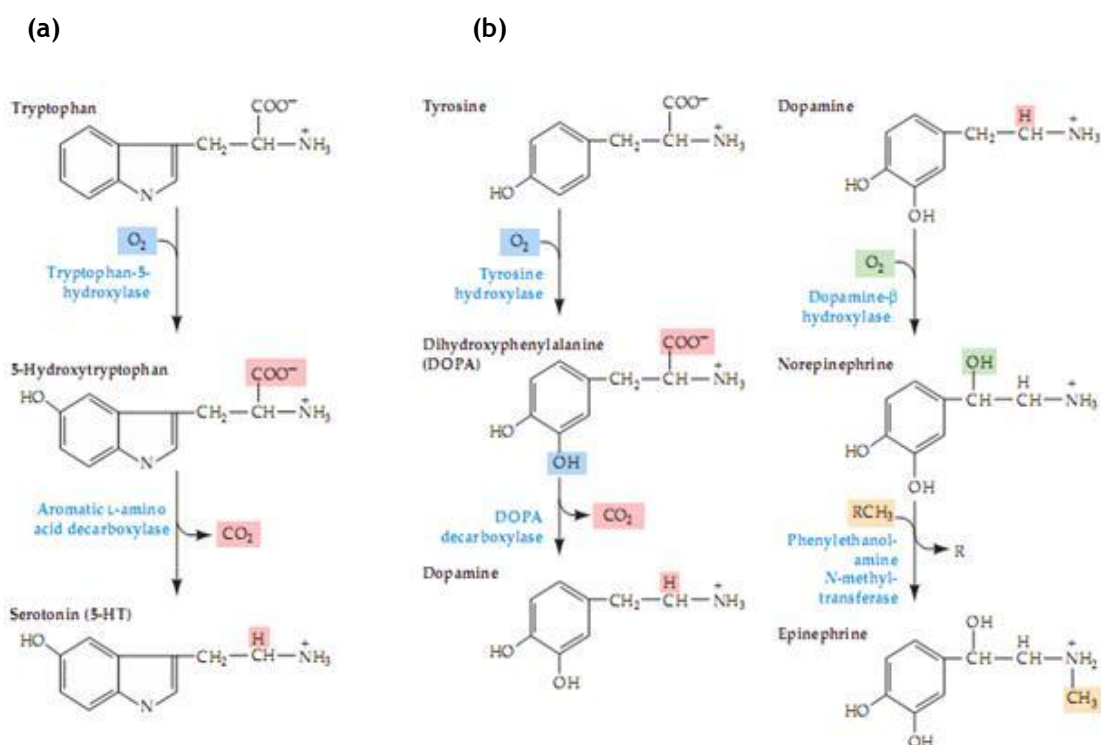


Figure 1: Biosynthesis process and structures of serotonin (a) and the catecholamines (dopamine, norepinephrine, epinephrine) (b). The enzymes involved in the several steps are shown as well. (Adapted from Purves D., *et al.*, 2004) [1]

1.2. Biogenic amine secretion, metabolism and re-uptake

After synthesis, serotonin and CATs are stored in vesicles, by the vesicular monoamine transporter (VMAT) through active transport from the cytoplasm. The neurotransmission occurs through the release of the monoamines from the vesicles into the synaptic cleft and binding to different several different receptors (5-HT, dopamine and adrenergic receptors) on the postsynaptic neuron [1-5]. The interaction with the relevant receptors initiates a biochemical cascade, resulting in several physiological responses [12].

The excess of neurotransmitter that remains in the synaptic cleft is removed mainly by a mechanism of re-uptake into the nerve terminal, through binding to specific membrane-associated monoamine transporter proteins, namely the serotonin (SERT), dopamine (DAT) and norepinephrine transporters (NET) [1-4].

The breakdown of monoamines is mediated mostly by the enzymes monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) [1-4,8,13-17], which are located in the cytoplasm in all mammalian tissues [2,13-16]. This is the main reason why monoamines have a short half life in the free form, and have to be stored into synaptic vesicles after synthesis [1-4]. Serotonin is metabolized by MAO to 5-hydroxyindoleacetic acid (5-HIAA) while CATs are metabolized both by MAO and COMT. The main metabolite of DA is homovanillic acid (HVA), NE and E originate normetanephrine (NME) and metanephrine (ME) respectively, and vanillylmandelic acid (VMA) as a final metabolite [2,4,14-17].

1.3. Disturbs in normal biogenic amine transmission

The normal action of biogenic amines in the organism may be compromised in a variety of situations that affect their synthesis, storage, re-uptake or catabolism (figure 2). Since biogenic amines are involved in such a variety of endocrine and neuronal functions, defects in their normal function often occurs in several neuropsychiatric and pathological situations [1-5]. This has made the pharmacology of amine synapses a crucial target for psychiatric therapy, with the development of drugs affecting the synthesis, binding to receptor and catabolism of these neurotransmitters [3]. On the other hand, amine synapse transmission is a target for most drugs of abuse, mainly psychoactive drugs (figure 2) [1,2,4,7].

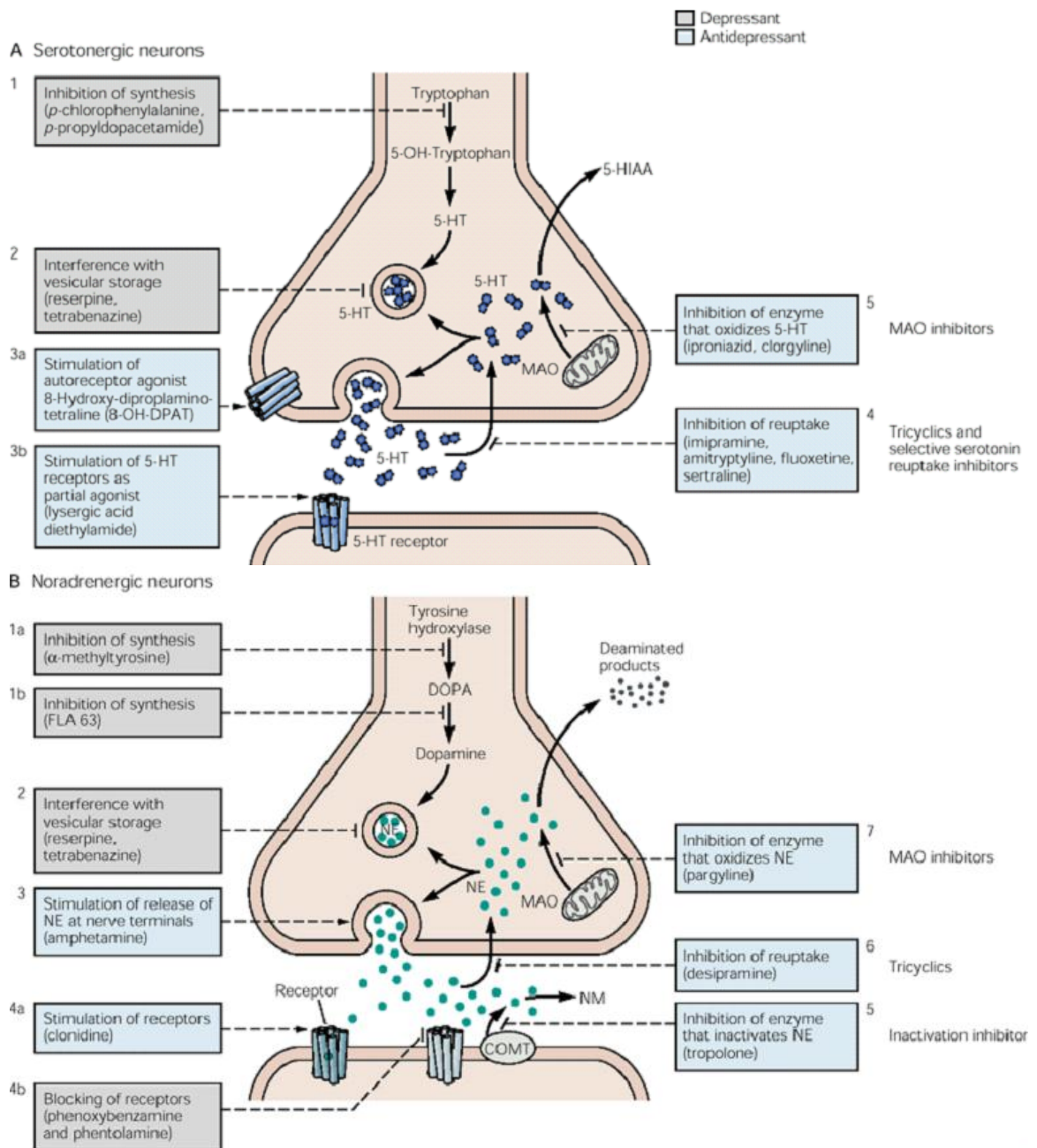


Figure 2: The serotonergic (A) and noradrenergic (B) synapses. Sites where most neuropsychiatric disorders, psychotherapeutic medicines and drugs of abuse have effect are shown as well. (Adapted from Kandel R.E. *et. al.*, 2000) [5]

1.3.1. Diseases and psychiatric disorders and biogenic amine transmission

Biogenic amines are implicated in several affective behaviours as euphoria, depression and anxiety. These responses provide important biological function and can become disordered in such a manner to constitute an illness, generally termed as mood disorders [5]. A decrease in the serotonergic and noradrenergic tone in the brain is commonly associated with depression, resulting in lack of the behavioural effects euphoria and pleasure. In patients with this disorder, low circulating levels of 5-HT and CATs are identified [3-5,8,9].

High levels of monoamine transmission, specially 5-HT and DA, are characteristic to psychosis, which can also occur as a long term side effect of several stimulatory psychotropic drugs [1,4]. Anxiety disorders and schizophrenia are strongly associated with altered levels of biogenic amines, often reflected by dysfunction of the catabolic enzymes MAO and COMT [1,3,14].

The underlying biochemistry of the major neuropsychiatric disorders is often not completely understood, the therapeutic effects of antidepressants and antipsychotics have mostly provided valuable information of the neurochemical functions of monoamines in these disorders. In fact, serotonin alone is administered as an antidepressant in some cases of depression [4].

Neurodegenerative diseases also result in either an excess or deficit in monoamine availability, most notable are Parkinson's disease (PD) and Alzheimer's disease (AD). In PD a destruction of the dopaminergic neurons of the substantia nigra occurs, with subsequent loss of the capacity to coordinate movements. Locus coeruleus, the principle site of the brain of NE synthesis is lost in AD, but also in PD and Down's syndrome (DS); compromising NE availability [2,3,14].

Some diseases of tumourigenic nature also affect normal biogenic amines function, and/or secretion. The hormonal effects of these neurotransmitters are also used as advantage for several tumours and blastomas. Notably serotonin is secreted in large amounts by carcinoid tumours, facilitating blood vessel irrigation of the tumour, resulting in a condition called carcinoid syndrome [9,15]. Neuroblastomas and other tumours affecting the brain will result in elevated monoamine levels. In fact, catecholamines are currently considered very important markers for neuroblastomas and pheochromocytomas diagnosis. 5-HT also plays a relevant role in several blood disorders, and both 5-HT and the CATs are involved and altered in cardiovascular diseases [9,14-19].

1.3.2 Medications and drugs of abuse interfering with biogenic amines pathways

As shown on figure 2, drugs can interfere in several steps of either serotonin or catecholamines pathway. Generally, drugs interfering with these pathways can be classified as depressant drugs and antidepressant drugs, either stimulating or inhibiting the monoamines transmission path [1,4,5,7,17].

Most drugs of abuse interact at some level with amine neurotransmission, including opiates, tranquilizers, hypnotics, designer drugs, stimulant drugs, hallucinogens, tryptamines and piperazines. Stimulant psychotropic drugs like cocaine and amphetamines stimulate monoamine release from the nerve terminal and block the monoamine transporters DAT and NET. The 3,4-methylenedioxy-N-methylamphetamine (MDMA) or “ecstasy” blocks both membrane SERT and vesicular VMAT transporters, preventing the re-uptake process, resulting longer period of the monoamines 5-HT, DA and NE availability in the synaptic cleft [1,2,4,7]. The Δ^9 -tetrahydrocannabinol (THC), a hallucinogenic drug, interacts with a presynaptic receptor, CB1, where it reduces the release of neurotransmitters, exhibiting a neuromodulator function [2,4,5].

Psychotherapeutic drugs also have their major effect on aminergic transmission pathways. Based on their effects, they can be divided into antipsychotics, antidepressants, stimulants and antianxiety drugs [1].

Antipsychotics reduce the amount of monoamines available for nervous transmission. Reserpine, a traditional folk medicine used for hypertension and psychoses, inhibits the VMAT transporter, thereby keeping NE and DA in the cytoplasm where they undergo catabolism by MAO. Other commonly used antipsychotic drugs reduce monoamine levels by blocking specific receptors on the postsynaptic nerve [3,8].

Antidepressant drugs, as the tricyclic compounds, raise the circulating monoamine levels by inhibiting the re-uptake of both serotonin and norepinephrine. Another class of antidepressants selectively inhibit the re-uptake of serotonin or dopamine, by blocking their re-uptake receptors, SERT and DAT. These are called as selective serotonin re-uptake inhibitors [1,5]. Monoamine oxidase inhibitors block the activity of the enzyme MAO, thus preventing breakdown of biogenic amines. The consequence of this inhibition is longer persistence (longer half life) of monoamines in the organism, and is one of the mechanisms used for the treatment of anxiety disorders and depression [1,5].

Another class of therapeutic agents, not directly focused on the treatment of neuropsychiatry disorders, can significantly affect in a definite manner brain structures specific for biogenic amine transmission. Two methods used for the treatment of brain tumors and leukemias, radiotherapy and chemotherapy, are the most notable. Survivors of this type of treatment may develop neurocognitive deficits, primarily in the area of attention, resulting in loss of biogenic amine availability, especially DA [20,21].

2. Biogenic amine detection

The quantification of monoamines in biological samples is of great interest for clinical areas and neurochemistry sciences. The evaluation of circulating concentrations of neurotransmitters in the organism is a very useful tool for the diagnosis of neuropsychiatric diseases and malignant tumours. Even if not to directly diagnose the disease, the detection of circulating monoamines can give an important idea of the neuronal signalling pathway affected and evaluate the physiological extent of the disease. With the same principle, biogenic amine detection can also be a very helpful tool to accompany patients undergoing neuropsychiatric and cardiovascular medications [16-18,22-24]. At last, knowledge of the circulating biogenic amine concentration could also be used as a secondary tool to monitor cases related to the consumption of drugs of abuse.

2.1 Biological fluids

After secretion monoamines are distributed through various tissues in the organism. For a successful quantification of the circulating levels in the body, it's important the use of a sample or biological fluid of easy acquisition and usable for routine clinical tests. On the other hand, the sample matrix should have a good correlation between its monoamine concentration levels and the concentration in the brain and other functional organs.

Clinical analysis of biogenic amines in humans is mostly done in urine and plasma. Urine is obtained non-invasively and high volumes are often obtained, oppositely to plasma. Urinary monoamine concentrations reflect the activity of the sympatho-adrenal system and are useful markers for the diagnosis of several types of tumors. Plasma monoamines also provide very useful information about normal and pathological sympatho-adrenal activity. This sample is especially used to detect biogenic amines for the prognosis of several cardiovascular diseases and neuroendocrine tumors [9,12,14-19,22-25]. It has been found that levels of biogenic amines and metabolites in urine and plasma are increased in patients with cancer diseases, and decreasing levels directly reflect the level of applied therapy [14]. Both samples are not easy to adulterate, but several factors can influence the monoamine concentration, like food consumption and states of anxiety [15]. In plasma, sample collection has to take into account the ease of oxidation of the amines by MAO, and it is necessary to add antioxidants [17].

2.2 Extraction techniques

When handling with complex samples like urine and plasma, a preparation step is crucial for a successful analysis [27,28]. The purpose of this step can be resumed to isolation of the compounds of interest from the matrix and concentration, since they may be present only in vestigial quantities. Removal of interfering compounds is an important step since they can have similar properties to the analytes of interest thus interfering with detection. Urine and plasma samples also contain macromolecules like proteins, which are incompatible with some chromatographic systems. A good extraction procedure must therefore be reproducible, selective, easy to automate and have a high recovery rate [26,29].

Samples can be submitted to a previous step before extraction, depending on the compound of interest, hydrolysis of conjugates and protein precipitation are often used before extraction protocols. In urine 5-HT, DA, E and NE are present in the free form, as sulphoconjugates and as glucuronides; in some studies determining total monoamine concentration, dissociation of this complexes is required [30,31].

In humans, urine and plasma monoamines are classically extracted by absorption on alumina or on boric acid gel and liquid-liquid extraction (LLE) [12,19,22,23]. But some limitations may be assisted with these methods, like lack of selectivity, low extraction yields, labor intensive and time consuming procedures, large volume of samples, and the use of toxic organic solvents in LLE. Recent works have focused mostly on solid phase extraction (SPE) for its easier automatization, high recovery rates, relatively fast procedures and use of lower organic solvent volumes [12,19,24,25,32-34].

SPE is presently the most commonly used sample preparation technique for several compounds, including biogenic amines. In SPE, the extraction, cleanup and concentration of compounds from the sample matrix occurs on a solid support [35]. Several papers have been published employing SPE in urinary and plasmatic biogenic amine sample preparation (Table 3). The recovery rate is often above 90 %, varying with the kind of cartridge used. Most used columns employed for monoamine SPE sample preparation are silica-based sorbents, containing octadecylsilane (C_{18}) or octylsilane (C_8) groups, and strong cation exchanger (SCX) groups mixed with C_8 (mixed cation exchanger, MCX) [12,19,24,25,32-34,36]. A specific one step LLE procedure employing diphenyl boronic acid (DPBA) ethanolamine ester followed by SPE with a C_{18} sorbent is also reported [33,37].

For clinical monoamine detection, LLE and SPE are the two most frequently used sample preparation technique [22]. Table 3 resumes the most recent published techniques used for the determination of these compounds.

2.2.1 Microextraction in packed syringe

Microextraction in packed syringe (MEPS) is a recent microextraction technique developed in 2004 for AstraZeneca, Sweden. MEPS is considered as a miniaturized version of SPE, employing the basic principles of SPE but in form of small packed columns inserted into a syringe. This method is relatively fast to perform, simple and robust, and compared to SPE, MEPS has some considerable advantages. The use of organic solvents is reduced, the sample amount can be as low as 10 μL and the column can be re-used up to 100 extractions for plasma and 400 for water samples, oppositely to SPE columns which are discarded after usage. Another great achievement of MEPS is the possibility of directly injecting the eluates (typically 20-50 μL) into liquid chromatography (LC), gas chromatography (GC) and capillary electrophoresis (CE) systems without any modifications to the device, facilitating on-line coupling to any of these systems [27,28,35,38,39].

Physically MEPS consists of a syringe similar to the ones used for LC injection (100-250 μL), and a barrel insert and needle assembly (BIN) that contains the SPE column. In the BIN approximately 1 mg of solid sorbent is packed as a plug, protected by two inert frits on both sides to prevent its motion (figure 3). The sorbent chemistry of both SPE and MEPS are similar, commercially available BINs for MEPS include silica-based absorption materials [C_{18} , C_2 , C_8 , and M1 (mixed mode with C_8 and cation exchanger)], restricted-access media (RAM) and molecular imprinted polymers (MIPs) [27,28,38,39].

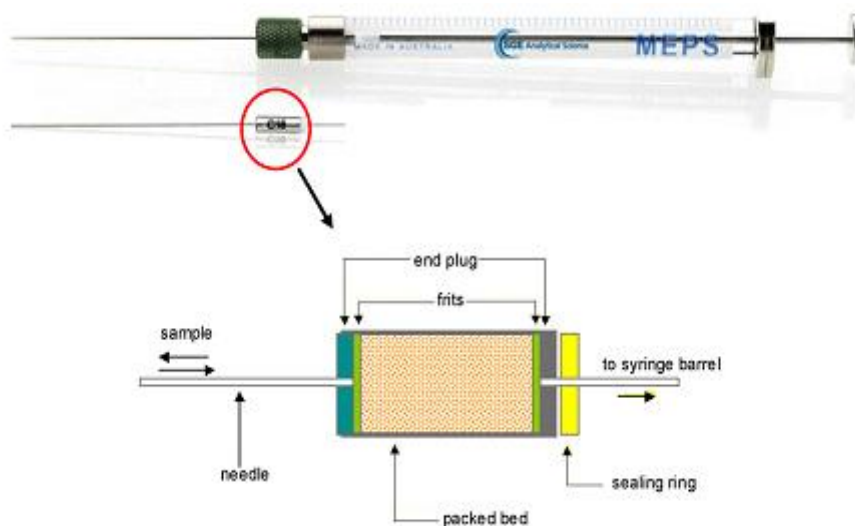


Figure 3: A MEPS syringe (250 μL) and BIN from SGE, and a scheme showing the composition of the extraction column. (Source: Abdel-Rehim M., 2010) [38]

The main objective of MEPS is to simultaneously remove interfering compounds present in the matrix and to selectively isolate and concentrate the analytes of interest. The

sample flows through the solid sorbent bed (extractant) by withdrawing the sample with the syringe. The analytes present in the liquid sample are isolated through retention on the solid phase. The column is then washed to remove interferences and the retained compounds are eluted with an organic solvent (figure 4) [38,39].

MEPS performance is affected by many factors, such as sample dilution, pH, composition and volumes of the washing and elution solutions, sorbent amounts and most importantly the sorbent material. The main step in the development of a MEPS extraction procedure requires optimization of these variables [35,38,39].

Initially the sorbent material is activated with an organic solvent like methanol, increasing the exposure of the functional groups for maximal interaction with the analytes in the sample. This is the conditioning step. For a successful passing of the sample through the sorbent, samples require dilution to reduce viscosity (1:5 for urine and plasma, up to 1:25 for blood) and removal of any unnecessary macro particles by centrifugation and eventually deproteinization. The adjustment of the pH of the sample is a crucial step for reverse-phase sampling. The aspiration speed of the sample should not be too high (normally ranging from 10 to 20 $\mu\text{L/s}$) to allow a strong interaction between analytes and sorbent, as well as not to physically force and destroy the column sorbent. Repeated circles of draw-ejection from the sample can be used to increase analyte retention, thus obtaining higher recoveries [38,39].

To remove any interfering species and proteins that may have interacted with the sorbent, a washing step is usually performed with water, buffers or aqueous solutions containing a small proportion of organic solvents. The volume and constitution of this solution is very important, it should be able to remove any unwanted compounds from the stationary phase and at the same time interact to the least extent possible with the analytes of interest [38,39].

Finally an elution step is done using an organic solvent for which the analyte has affinity to disrupt the interaction between analyte and stationary phase. This solvent should be able to elute all analytes in small volumes and be miscible with the mobile phase of the chromatographic system, normally methanol, isopropanol or acetonitrile are used [38,39]. In some cases the volatile eluent can be dried under a gentle nitrogen stream and re-dissolved in the mobile phase or another solution before injection. This allows concentration of the eluted analyte.

In one recent work, El-Beqqali A. *et. al.* (2007) developed a protocol, for the determination of 5-HT and DA in urine, using MEPS online with LC-MS-MS detection. In this method a polystyrene polymer sorbent (silica-C₈) was manually injected inside a MEPS

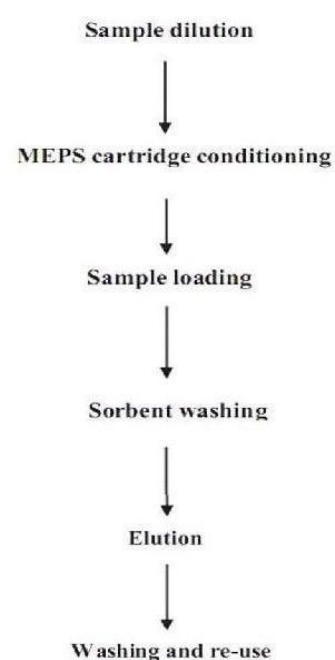


Figure 4: Schematic representation of the different steps in a MEPS extraction procedure.

syringe, validation was done in urine and a low sample volume (30 μL) was used. The limits of quantification (LOQ) and detection (LOD) for both compounds were 50 ng/mL and 1 ng/mL respectively, and the extraction recovery was about 50 % [40]. This is the only published work that uses this extraction technique for the determination of biogenic amine neurotransmitters.

2.3 Separation and detection techniques

The range levels of neurotransmitters in plasma and urine vary mostly at very low concentrations, very often in the order of ng/mL and pg/mL (table 2). At this level it is required highly sensitive and selective detection and separation techniques, especially considering the chemical instability of these compounds, the complex matrix from which they are extracted and the metabolites present [12].

Historically, analysis of monoamines has been done by immunoassays [41], voltammetry [42], and gas chromatography coupled to mass spectrometry (GC-MS) [43]. But these methods have proven to be either time consuming, expensive, may require derivatization reactions or in some cases are not sensitive enough to detect the physiological concentration ranges (table 2). Due to these limitations, monoamine analysis in biological samples has focused to more rapid and sensible methodologies. High performance liquid chromatography (HPLC) is one of the most popular separation techniques used [44]. Several studies have reported methodologies using HPLC coupled to electrochemical (ED) [12,14,15,21-23,25,30,31,33,34,45,46], ultraviolet (UV) [47,48], fluorescence (FLD) [46,49] and mass spectrometry (MS) [50,51,52] detectors. Several studies also employ capillary electrophoresis (CE) coupled with UV [19,24,34], MS [34] and time of flight/mass spectrometry (TOF/MS) [24,32]. Table 3 shows an overview of recent published detection techniques for biogenic amines.

Table 2: Normal range levels of NE, DA and 5-HT in urine and plasma.

	Urine	Plasma
Norepinephrine	$\leq 80 \mu\text{g}/24\text{H}^{[53]}$	70-750 pg/mL ^[55]
Dopamine	$\leq 400 \mu\text{g}/24\text{H}^{[53]}$	< 30 pg/mL ^[55]
Serotonin	$\leq 210 \mu\text{g}/24\text{H}^{[54]}$	101-283 ng/mL (serum) ^[56]

2.3.1 HPLC coupled to electrochemical detection

Electrochemical detectors (ECD or ED) are amongst the most sensible methods used nowadays for monoamine detection. These detectors rely on an electrochemical reaction, wherein a certain constant potential (in DC mode) is applied by an electrochemical cell (electrode) to the flowing mobile phase. If the potential is high enough it will oxidize or reduce the elements in the mobile phase, which produces a current signal measured in function of time, originating a result given in a current vs. time chromatogram [57].

Compounds must be of electroattractive nature to be suitable for this type of detection. By selecting a specific potential under which the compound of interest undergoes reduction or oxidation, a signal is produced proportional to the quantities present in the sample. The applied potential is characteristic to each compound, and is dependent on various factors as the nature of the electrochemical cell surface, composition of the mobile phase and pH. A cyclic voltammogram consists of a peak area/potential curve, and can be obtained by injecting separately the compound of interest at a range of voltages. The resulting curve gives the information of the maximum oxidation potential of the compound, and can be used to choose a potential at which no interferences are oxidized optimizing the signal to noise ratio [57].

There are two types of electrochemical detectors, amperometric and coulometric. In an **amperometric** detector, the electrochemical cell has a design as which allows the mobile phase to flow over its surface, being only partly exposed to the electrode's surface hence only part of the electroattractive species present in the flow undergo an electrochemical reaction. The corresponding signal of this fraction (typically 5-15 % of the total amount) is then converted into an estimation of the total of species present in the sample [57].

Although it's relative high sensitivity, amperometric detectors usually do not allow reaching very low concentrations of electroattractive compounds. Enhanced sensitivity can be obtained with the use of a **coulometric** detector cell; in this design the flow does not pass over the surface but rather through a porous graphite electrode. Resulting in a higher exposure area of the solvent to the electrode, consequently most of the electroattractive species are oxidized or reduced, giving a higher signal without a corresponding increase of background noise, thus allowing detection of lower quantities of compound of interest. In this detection the signal typically corresponds directly to almost 100 % of the analytes present in the sample [57].

Several studies reported detection methods for serotonin and catecholamines, using HPLC coupled to amperometric or coulometric detection (table 3). Coulometric detection has shown to obtain lower detection limits, almost twice as low when compared to amperometric detection [25].

Table 3: An overview of recent published techniques for biogenic amine determination in urine and plasma.

Compound (s)	Sample	Sample Preparation	Detection mode	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%)	Reference
DA, NE	Plasma	SPE	HPLC-ED (Coulometric)	12	40	DA - 98 NE - 95	[12]
DA	Urine	SPE	CE-UV	107	127	103.5	[19]
DA, NE	Urine	SPE	HPLC-ED (Amperometric)	DA - 7 NE - 8.8	N/A	DA - 93 NE - 82.5	[22]
DA, NE	Plasma	Alumina Adsorption	HPLC-ED (Coulometric)	N/A	N/A	DA - 60 NE - 87.5	[23]
DA, NE	Urine	SPE	CE-UV	DA - 17 NE - 20.3	N/A	DA - 85.95 NE - 83.7	[24]
			CE-TOFMS	DA - 46 NE - 50.75	N/A	DA - 85.95 NE - 83.7	
DA, NE	Plasma	SPE	HPLC-ED (Amperometric)	DA - 0.15 NE - 0.10	DA - 0.20 NE - 0.20	DA - 75.3 NE - 91.7	[25]
			HPLC-ED (Coulometric)	DA - 0.06 NE - 0.05	DA - 0.12 NE - 0.10	DA - 75.7 NE - 92	
5-HT	Plasma	SPE	CE-TOFMS	26.5	N/A	71.6	[32]
DA, NE	Urine	LLE and SPE	HPLC-ED (Coulometric)	DA - 4 NE - 2.4	DA - 9.8 NE - 5.4	DA - 92.25 NE - 87.5	[33]

Compound (s)	Sample	Sample Preparation	Detection mode	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%)	Reference
DA	Urine	SPE	HPLC-ED	N/A	N/A	44.5	[34]
			CE-UV	107	N/A		
			CE-MS	183.8	N/A		
DA, NE	Plasma	SPE	HPLC-ED (Coulometric)	DA - 0.01 NE - 0.02	N/A	DA - 64.6 NE - 74.6	[37]
DA, NE	Urine	MEPS	LC-MS/MS	1	50	50	[40]
5-HT, DA	...	SPME	HPLC-ED (Amperometric)	0.1	N/A	N/A	[45]
5-HT, DA, NE	Urine	SPE	HPLC-FLD	8	N/A	DA - 90.2	[46]
			HPLC-ED	DA - 0.1 NE - 0.2	N/A	NE - 91.3	
DA, 5-HT	Urine	Deproteinization with HClO ₄	HPLC-FLD	DA - 10 5-HT - 8	DA - 30 5-HT - 24	DA - 96.36 5-HT - 87.5	[49]
DA, NE	Urine	LLE	LC-MS/MS	N/A	DA - 2.5 NE - 10	71	[50]
5-HT	Urine and plasma	On-line SPE	LC-MS/MS	N/A	Urine - 5.3 Plasma - 158.6	Urine - 84 Plasma - 57.5	[51]
DA, NE	Urine	On-line SPE	LC-MS/MS	N/A	DA - 1.8 NE - 2.7	N/A	[52]

LOD: limit of detection, LOQ: limit of quantification, N/A: not available on original paper, SPME: solid phase microextraction, HClO₄: perchloric acid.

II. Experimental part

1. Materials and methods

1. 1. Reagents and standards

Serotonin hydrochloride, dopamine hydrochloride, (-) norepinephrine (≥ 98 %, crystalline) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) were acquired from Sigma-Aldrich (Sintra, Portugal).

Methanol and acetonitrile (HPLC-grade) were acquired from VWR Internacional (Carnaxide, Portugal). EDTA, citric acid, OSA, dibutylamine and phosphoric acid were acquired from Sigma-Aldrich (Sintra, Portugal). Sodium acetate, triethylamine and acetic acid (HPLC-grade) were acquired from Sigma-Aldrich (Sintra, Portugal). Potassium dihydrogen phosphate and phosphoric acid were acquired from Panreac Química (Cascais, Portugal)

1.2 Instrumentation

- Nylon membrane filter from Pall Corporation (VWR Internacional, Carnaxide, Portugal);
- Mili-Q Advantage A10 water purifying system from Interface (Amadora, Portugal);
- Analytical balance, model CP225, from Sartorius S.A. (Lisboa, Portugal);
- Vortex Mixer 230V model from Labnet International (VWR Internacional, Carnaxide, Portugal);
- Magnetic agitation plate, model ASINCRO, from J.P. Selecta (ILC, Oporto, Portugal);
- Refrigerating chamber (at 4 °C) from Dagard Ibérica (Odivelas, Portugal);
- pH meter 744 model from Metrohm (Soquímica, Lisboa, Portugal) ;
- Automatic micropipettes from Gilson (VWR Internacional, Carnaxide, Portugal), maximum volumes of 20, 200 and 1000 μL ;
- Ultrasound system, model Transonic 460/H from Elma (VWR Internacional, Carnaxide, Portugal);
- Heraeus Multifuge IS-R- Thermo Electron Corporation centrifuge (Osterode, Germany);
- Waters XTerra MS C₁₈ ODS reverse phase analytical column (5 μm , 250 x 4.6 mm i.d.) acquired from Via Athena (Sacavém, Portugal);

- Agilent Zorbax 300SB C₁₈ reverse phase analytical columns, (5 µm, 150 x 4.6 mm i.d. and 5 µm, 250 x 4.6 mm i.d.) were obtained from Soquímica (Lisbon, Portugal);
- MEPS syringe (100-250 µL) from SGE - Analytical Science (Australia), acquired from ILC (Oporto, Portugal);
- MEPS columns (M₁ and C₁₈) from SGE - Analytical Science were acquired from ILC (Oporto, Portugal);

1.3. Internal standard

When analysing biological samples it's useful to add a compound of known concentration (internal standard) to the sample before extraction. This is done to correct the effect of analyte loss during sample extraction. To be effective, the internal standard (IS) should have similar physical and chemical properties to the compounds of interest. Yet the internal standard should not normally be present in the biological sample and should not have overlaying signals with any of the target analytes [61].

For biogenic amines detection in biological fluids, 3,4-dihydroxybenzylamine (DHBA) is the most common used IS. Identification and separation of this compound was successful in both electrochemical and fluorescence systems under the already optimized conditions.

1.4. Stock and working solutions

Individual stock solutions of the neurotransmitters and internal standard were prepared in methanol at a final concentration of 1 mg/mL. For this, 5 mg of each compound were weighted and dissolved in methanol, then added to a 5 mL volumetric flask, and filled up with methanol. For NE, the weighted solid was first dissolved in 200 µL of acetic acid and then filled up with methanol to 5 mL. Working solutions of NE, DA and 5-HT were obtained by direct dilution of the standards to final concentrations of 0.1, 1 and 10 µg/mL. The internal standard was diluted to a 10 µg/mL working solution. All standards and working solutions were kept in amber glass vials and stored at 4 °C in the absence of light.

1. 5. Chromatographic and detection systems

For the compound detection and extraction protocol optimization, an electrochemical amperometric and a fluorescence detection system were used. Validation was done in an electrochemical coulometric system.

For amperometric detection, a Waters HPLC system including a quaternary pump with controller (600), an in-line degasser (AF), a manual injector (Rheodyne 7725) and an electrochemical amperometric detector (ECD-2465) was used. All instrument parts were controlled by Empower software supplied by Waters (Milford, MA, USA). The separation was achieved using a XTerra MS C₁₈ ODS reverse phase analytical column (5 µm, 250 x 4.6 mm i.d.) and a precolumn (5 µm, 10 x 4.6 mm i.d.) from Waters. Electrochemical oxidation of the compounds was achieved with a flow-single cell equipped with a 3 mm diameter glassy carbon working electrode over and ISAAC reference electrode.

For fluorescence detection, an ultra high-performance liquid chromatography (UPLC) system from Agilent Technologies (1290) with auto sampler and binary pump, coupled to a fluorescence detector (1260) was used. The separation was achieved using a Zorbax 300SB C₁₈ reverse phase analytical column (5 µm, 150 x 4.6 mm i.d.).

For coulometric detection, a HPLC system (1260) from Agilent, with auto sampler and quaternary pump was used. The system was coupled to a Coulochem III from ESA (Dias de Sousa S.A., Lisbon, Portugal) Compounds separation was done using a Zorbax 300SB C₁₈ reverse phase analytical column (5 µm, 250 x 4.6 mm i.d.). Electrochemical oxidation of the compounds was achieved using a high sensitivity dual electrode analytical cell (5011A) from ESA.

Both fluorescence and coulometric chromatography systems were controlled by Chemstation software supplied by Agilent Technologies (Waldbronn, Germany).

1.6. Chromatographic and detection conditions

For the amperometric system the mobile phase was constituted by 0.145 mM EDTA, 0.1 M sodium acetate, 0.1 mM citric acid, 0.5 mM sodium octyl sulphate (OSA), 1 mM dibutylamine and 10 % methanol (v/v), adjusted to pH 3.5 with perchloric acid 70 % (v/v). An isocratic flow of 1 mL/min was used with a running time of 30 min. The applied potential of the working electrode was set at +750 mV.

For the fluorescence system a mobile phase containing 50 mM sodium dihydrogen phosphate (KH₂PO₄) and 1 % acetonitrile (v/v) was used. The temperature of the column oven was 20°C. An isocratic flow of 0.5 mL/min was used with a running time of 25 min. For detection a timetable was used, with constant excitation wavelength (280 nm) but varying emission wavelength (0-10 min: 310 nm, 10-20 min: 340 nm).

For the coulometric system, separation of the compounds was achieved with a mobile phase consisting of 75 mM KH_2PO_4 , 1.7 mM OSA, 0.01 % Triethylamine (v/v), 25 μM EDTA and 10 % acetonitrile (v/v) adjusted to pH 3.5 with phosphoric acid. The temperature of the column oven was 23 °C. An isocratic flow of 1 mL/min was used with a running time of 25 min. The potential of the analytical cell was set at +300 mV in the channel 1 (oxidation channel) and -150 mV in the channel 2 (reduction channel).

The auto sampler temperatures of both fluorescence and coulometric system were kept at 4 °C.

All mobile phases were prepared with MilliQ water to a final volume of 1 or 2 L, filtered through a 0.2 μm pore nylon membrane and degassed for a minimum of 15 min before use. Storage of the mobile phases was done under 4 °C, and before use, they were allowed to reach room temperature.

1.7. Biological samples

Urine samples were obtained from laboratory staff and kept at 4 °C until use. Plasma samples were obtained from the excess supplies of the Instituto Português do Sangue (outdated transfusions). These samples were stored at -20 °C until analysis.

1.8. Optimized extraction procedure

Final conditions for the extractions were as following: 500 μL of sample were diluted in 500 μL of 0.1M KH_2PO_4 (1:2 dilution), each sample was spiked with 25 μL of IS (at 10 $\mu\text{g}/\text{mL}$). The mixture was slightly vortex-mixed for 30 s. The MEPS sorbent was activated 3 times with 250 μL methanol and 3 times with 250 μL of water. The sample was manually drawn through the sorbent and ejected in the same vial 8 times (strokes). The analytes were eluted from the stationary phase twice with 100 μL methanol. After each extraction, the sorbent was washed sequentially with 3×250 μL methanol and 3×250 μL water. This step aimed at decreasing memory effects (carry-over), conditioning simultaneously the sorbent for the next extraction.

2. Results and discussion

2.1. Optimization of the mobile phase

In order to obtain a good chromatographic separation of the compounds, the composition of the mobile phase was optimized. Since different chromatographic systems were used, it was necessary to repeat the optimization procedure for each chromatographic system. Electrochemical methods require certain components (e.g. counter ions) which are not necessary for the fluorescence method. Also, the dimension of the columns in the three methods is not the same. Therefore different compositions for each system were tested in isocratic flow, based in relevant information found on literature.

2.1.1. Amperometric system

Initially a mobile phase composed of 0.145 mM EDTA, 0.1 M sodium acetate, 0.1 mM citric acid, 0.5 mM sodium octyl sulphate (OSA), 1 mM dibutylamine and 5 % methanol (v/v), at pH 3.5, was tested in isocratic mode with a flow of 1 mL/min and a potential of +750 mV as reported [58]. These conditions allowed a good separation of the compounds, but serotonin's retention time was 42.45 minutes which would require a total running time of almost 45 minutes. In order to decrease the retention time, the content of organic solvent (methanol) was raised, as shown in table 4. Methanol percentages ranging from 5-12.5 % were tested.

Table 4: Retention time variation of 5-HT, DA and NE with different methanol contents in the mobile phase.

	Methanol (5 %)	Methanol (7.5 %)	Methanol (10 %)	Methanol (12.5 %)
Serotonin	42.45 min	30.48 min	27.12 min	26.18 min
Dopamine	15.38 min	13.46 min	10.95 min	10.20 min
Norepinephrine	6.77 min	6.44 min	5.53 min	5.40 min

Initially epinephrine was used as well in the method, and with methanol percentages higher than 10 %, overlay of the peaks from NE and E was observed (Figure 5).

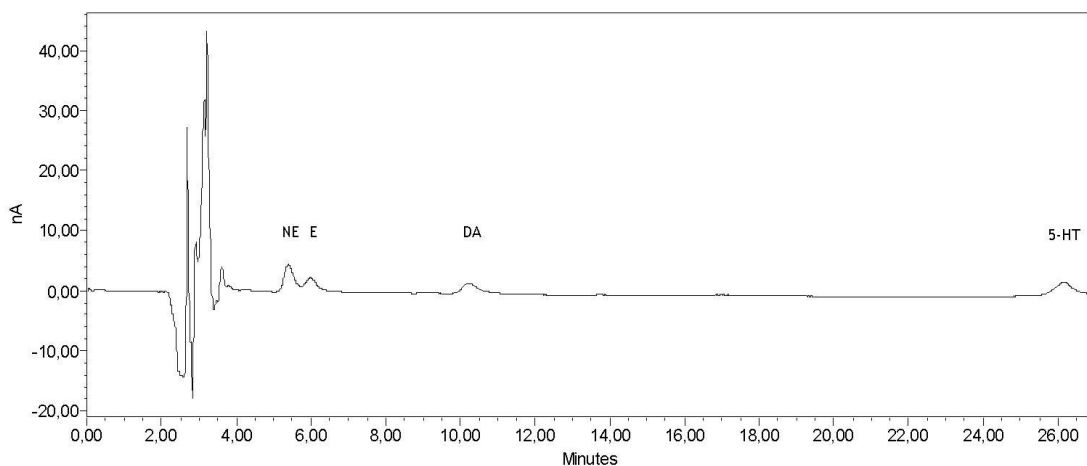


Figure 5: Chromatogram of 5-HT, DA, NE and E at 1 µg/mL using a mobile phase with 12.5 % methanol; an overlay of the peaks from NE and E is observable.

Since serotonin's retention time didn't lower significantly over 10 % of methanol and in order to keep a shorter running time, this percentage was chosen by compromise for the mobile phase. Figure 6 shows a typical chromatogram of the mobile phase with 10 % methanol.

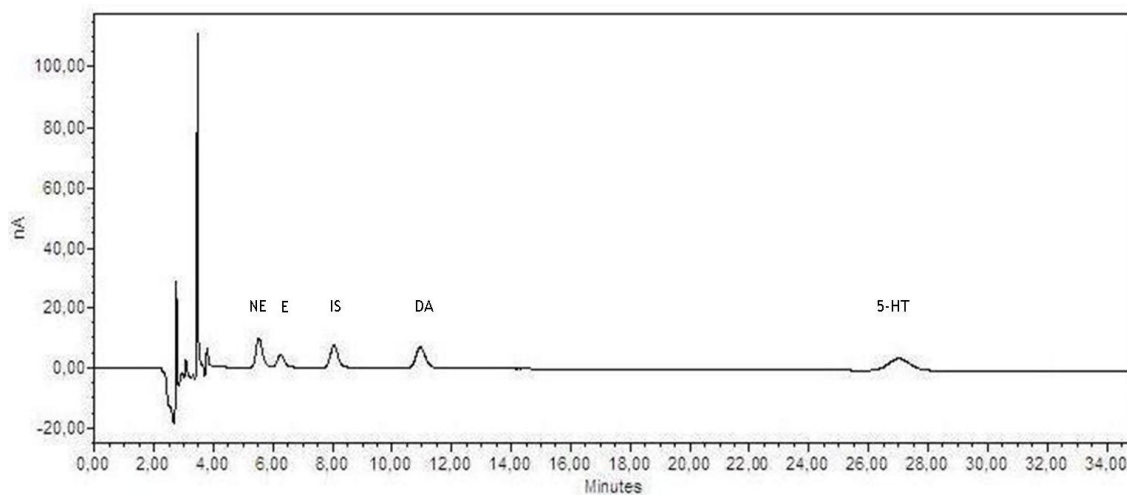


Figure 6: Chromatogram of 5-HT, DA, NE, E and internal standard (DHBA) at 1 µg/mL using a mobile phase with 10 % methanol.

2.1.2. Fluorescence system

A mobile phase composed of 50 mM KH_2PO_4 was used as reported [59] with 1 % of acetonitrile added, and the temperature of the column oven set to 20°C. The compounds were detected with an excitation wavelength of 280 nm, and emission of 310 nm (catecholamines) and 340 nm (serotonin) with a flow of 0.5 mL/min. The conditions showed a good separation of 5-HT, DA, NE and the internal standard (DHBA) (Figure 7). For better intensity of the peaks, a timetable was created where the emission wavelength changed from 310 nm to 340 nm at 10 min of run.

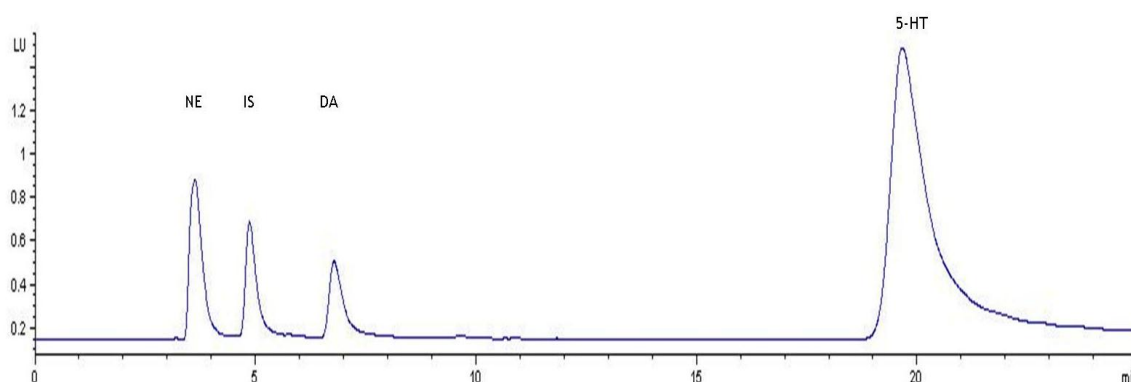


Figure 7: Chromatogram obtained by fluorescent detection of 5-HT, DA, NE and internal standard (DHBA) at 50 ng/mL using the final optimized conditions. The retention times are 19.66 min for 5-HT, 6.77 min for DA, 3.63 min for NE and 4.85 min for DHBA.

2.1.3. Coulometric system

Initially, a mobile phase reported by Hubbard *et al.* [21] was used with a flow of 1 mL/min, which allowed a good separation of the compounds. But the peak of NE showed a slight overlay with an unidentified peak from the mobile phase or from the system (Figure 8). Changes in flow or column temperature did not separate those peaks, but better resolution appeared at 23°C.

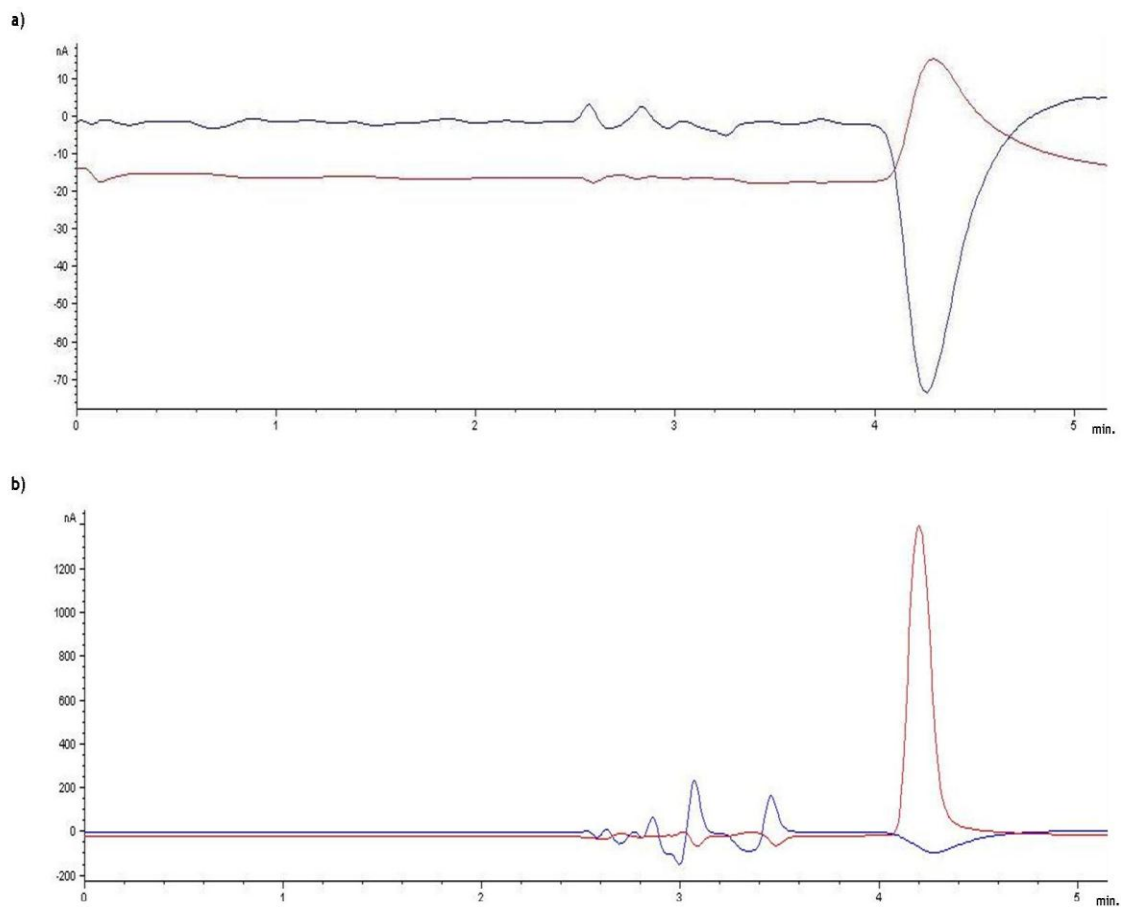


Figure 8: Chromatogram showing the baseline signal of an injection of mobile phase (a) and the peak of NE at 50 ng/mL (b). The retention time of NE (4.20 min) overlays with an unknown peak seen in the mobile phase injection. The upper line shows the signal of channel 1 (+300 mV), and the under line the signal of channel 2 (-150 mV).

Afterwards, a different mobile phase designed for determination of biogenic amines was used [60]. The established composition was 75 mM sodium dihydrogen phosphate monohydrate, 1.7 mM OSA, 0.01 % triethylamine (v/v), 25 μ M EDTA and 10 % acetonitrile (v/v) adjusted to pH 3.5 with phosphoric acid. The unidentified peak was still present; however, at 23 °C a good separation with NE was observed (Figure 9).

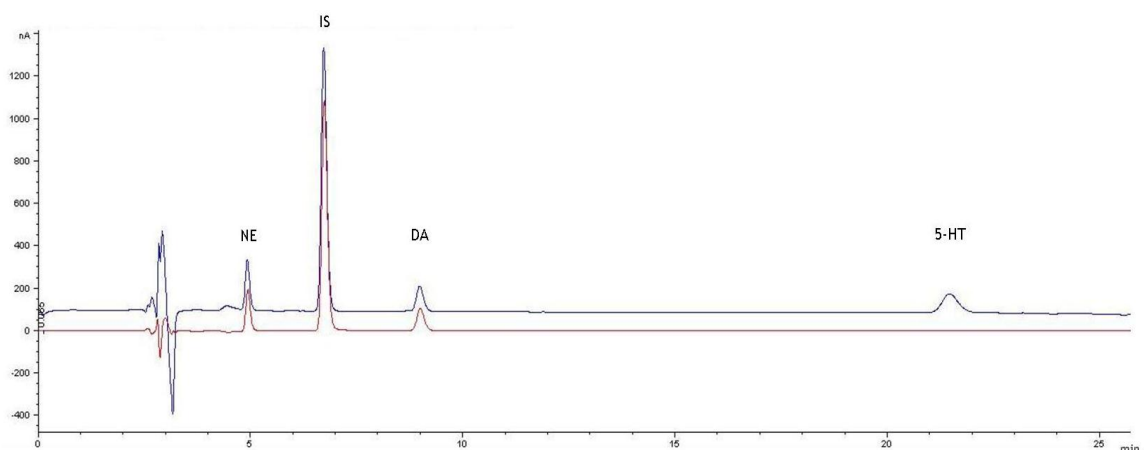


Figure 9: Chromatogram of 5-HT, DA, NE (50 ng/mL) and internal standard (DHBA, 250 ng/mL). The upper line shows the signal of channel 1 (+300 mV), and the under line the signal of channel 2 (-150 mV).

These conditions were used for subsequent analysis, and the final retention times of the compounds were: 21.46 min for 5-HT, 8.98 min for DA, 4.92 min for NE and 6.72 min for the internal standard. As reported by Hubbard *et al.* [21], electrochemical oxidation of the catecholamines and serotonin was most effective with an oxidation potential of +300 mV in channel 1, -150 mV were set for the second channel. For the intended concentrations used in the calibration curve (5-1000 ng/mL), a sensitivity under 100 nA resulted in the saturation of the signal with the higher concentrations. A sensitivity of 100 nA was considered sufficient for detection in these ranges.

2.2. Optimization of the extraction procedure

The efficiency and performance of a MEPS extraction protocol depends on several variable factors, most importantly, the employed column (type of sorbent), sample dilution, number of sample drawings, washing and elution.

Based on literature [39], the conditioning step of the extraction protocol was set to 3 drawings of 250 μ L of methanol and water. Washing was done only with water, since adding an amount of organic solvent to the washing solution resulted in a drastic loss of analytes. Different elution solutions were also tested (methanol with 0.1 % formic acid, isopropanol and acetonitrile), but solutions containing methanol showed better results. The presence of formic acid did not seem to affect the extraction yield, and therefore only pure methanol was used. Two types of columns were tested, a reverse phase C_{18} and a mixed mode (C_8 and SCX, M_1). During initial extraction experiments, the area of the compounds extracted with the two

types of sorbent were compared. The areas resulting from extraction with M_1 columns were much lower than those observed with the C_{18} columns, and therefore all subsequent experiments were done using C_{18} columns.

In order to obtain better yields of extraction, an experimental design or design of experiments (DOE) tool was used.

2.2.1. Design of experiments (DOE)

When developing a method, including extraction protocols, the several variable factors that have to be considered make this a very time-consuming task. Most variable factors are chosen based on existing literature, or otherwise an attempt to identify the factors is done through a trial-and-error approach or varying one factor at the time, while maintaining the other constant. Such is of course very time-consuming, exhaustive and expensive, while it can also provide misleading information since some interactions between factors may not have been taken into account [62].

DOE is a useful statistical tool for the planning of the entire procedure, assessing several intervening factors and minimizing the effects of uncontrolled factors. This spares a lot of time, work, resources and money to the laboratories, while better results are obtained [62].

With this in mind, a 2^k factorial design was applied using the statistical program MINITAB® version 15 for optimization of the extraction protocol.

The peak area of each analyte after extraction by MEPS was divided by the peak area of the IS (added after extraction) in order to compensate injection differences ($n=3$); this peak area ratio was used as the measured response (dependent variable). An initial screening design was done to identify significant factors influencing the extraction response. The following factors were assessed for statistical significance: number of sample drawings (2 or 8 strokes), number of washing steps (1 or 2), washing solution volume (10 or 50 μL), number of elutions (1 or 2) and volume of elution solvent (50 or 100 μL). Each factor was studied at two levels, low (-1) and high (+1) (Table 5).

Table 5: Factor levels in the design for MEPS optimization.

Factor	Low (-1)	High (+1)
Strokes	2	8
Washing volume (μL)	10	50
Number of washing steps	1	2
Methanol volume (μL)	50	100
Number of elutions	1	2

Considering these five factors, a two level full factorial design (2^5) would require 32 experiments and a high number of these experiments ($n=16$) would assess only fifth-order interactions. In order to reduce the number of experiments, the interactions of fifth order were considered negligible and a fractional factorial design (2^{5-1}) was used. This design reduces the experiments to half ($n=16$) by running only part of the full factorial design, while still evaluating the main interactions, sparing time, reagents and labour.

These experiments were carried out in a random order, to avoid the influence of noise factors, minimizing systematic errors.

The experimental design matrix as well as the response in the different extraction conditions is shown in table 6.

Table 6: Experimental design matrix showing the experimental factors, and response to each factor.

Run Order	Experimental factors					Response (peak area ratio)		
	Strokes	Washing volume (µL)	N° of Washing steps	Volume of methanol eluent (µL)	N° of elutions	5-HT	DA	NE
1	8	10	2	50	2	0.156	0.009	0.014
2	2	10	1	100	1	0.145	0.008	0.014
3	8	50	1	100	1	0.109	0.005	0.006
4	8	50	2	50	1	0.052	0.005	0.004
5	8	10	1	50	1	0.147	0.010	0.016
6	8	10	1	100	2	0.141	0.011	0.011
7	2	10	1	50	2	0.122	0.011	0.011
8	2	50	1	100	2	0.101	0.006	0.009
9	8	50	2	100	2	0.076	0.008	0.006
10	2	50	2	50	2	0.065	0.006	0.003
11	8	10	2	100	1	0.116	0.009	0.010
12	2	10	2	100	2	0.122	0.010	0.010
13	2	10	2	50	1	0.098	0.004	0.007
14	2	50	1	50	1	0.076	0.005	0.006
15	2	50	2	100	1	0.038	0.005	0.004
16	8	50	1	50	2	0.087	0.005	0.006

Based on these responses, the following Pareto charts were obtained showing the experimental factors in decreasing order of the effect magnitude. If a factor has a magnitude high enough to cross the red line, it's considered statistically significant and is assumed as to influence the response.

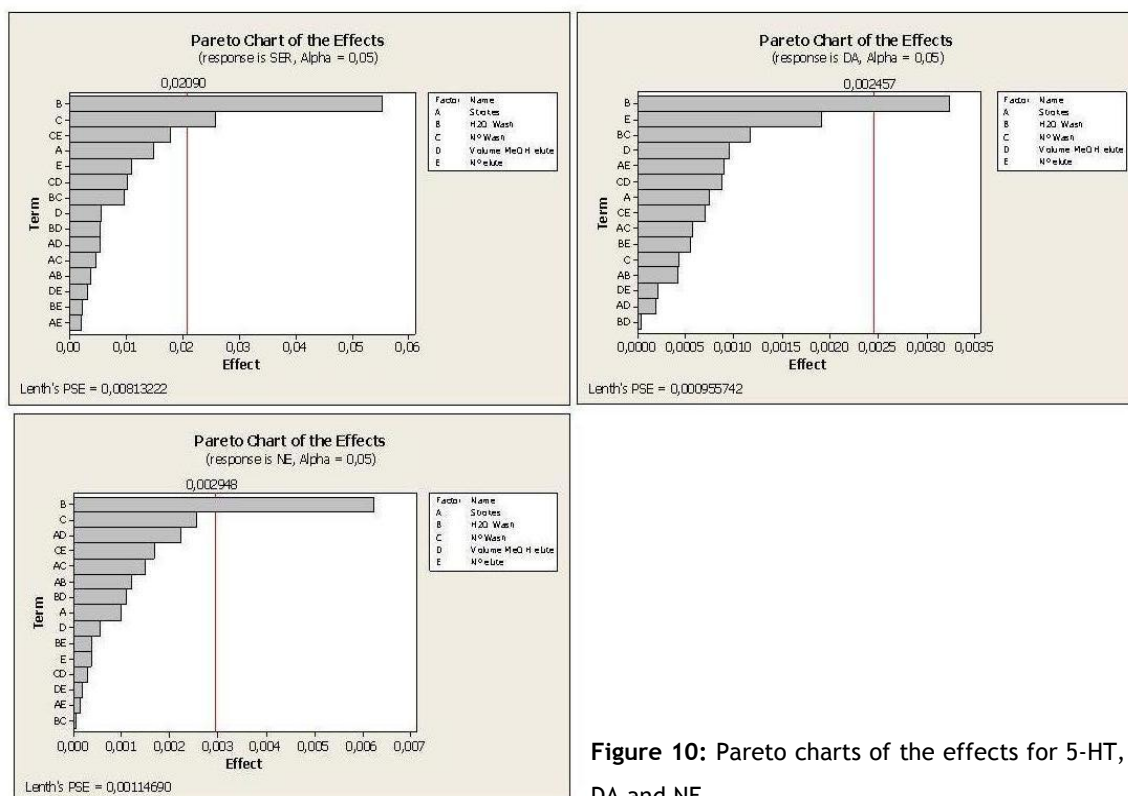


Figure 10: Pareto charts of the effects for 5-HT, DA and NE.

For all compounds the volume of water used in the washing step was found to be statistically significant, and in the case of serotonin the number of washing steps.

Regarding the main effects plot (figure 11), we observed that a washing volume of 10 μL originates significantly better responses for the three compounds. Generally, using one single washing step and two elutions (each with 100 μL methanol) showed better responses.

As shown in the interaction plots (figure 12), an interaction of the number of washing steps with the number of elutions is observable for 5-HT and NE. For DA, a slight interaction of volume of washing solution and number of washing steps is seen as well. However, none of those interactions had a significant effect as regards the response.

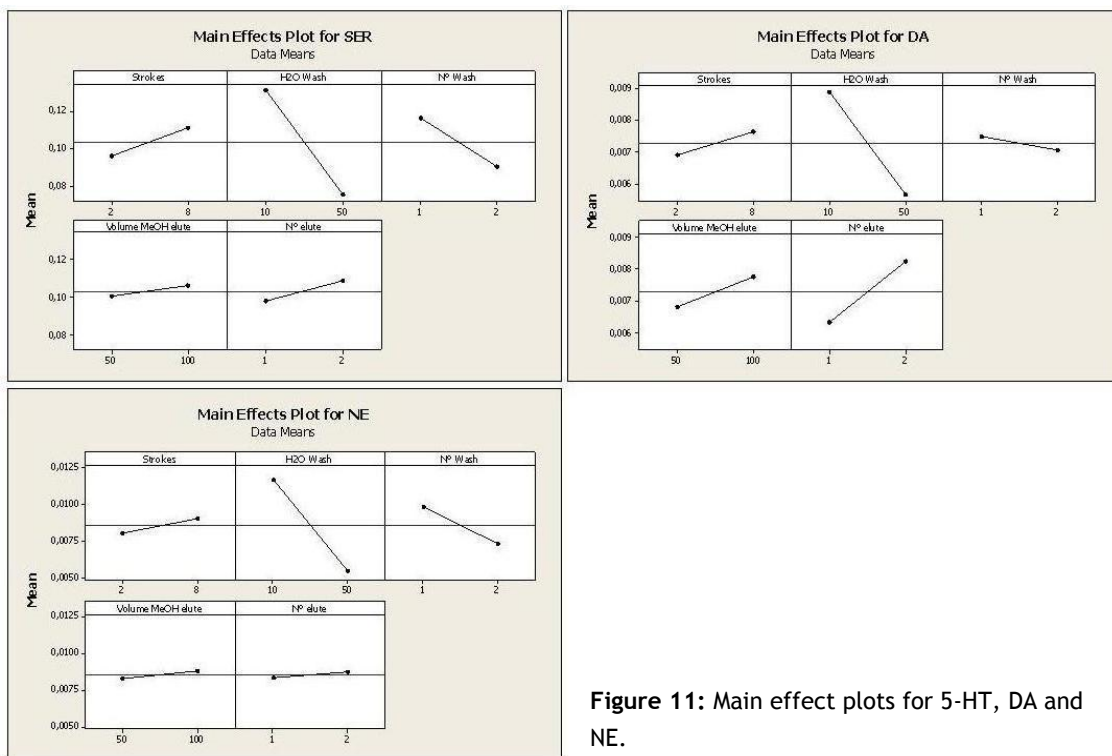


Figure 11: Main effect plots for 5-HT, DA and NE.

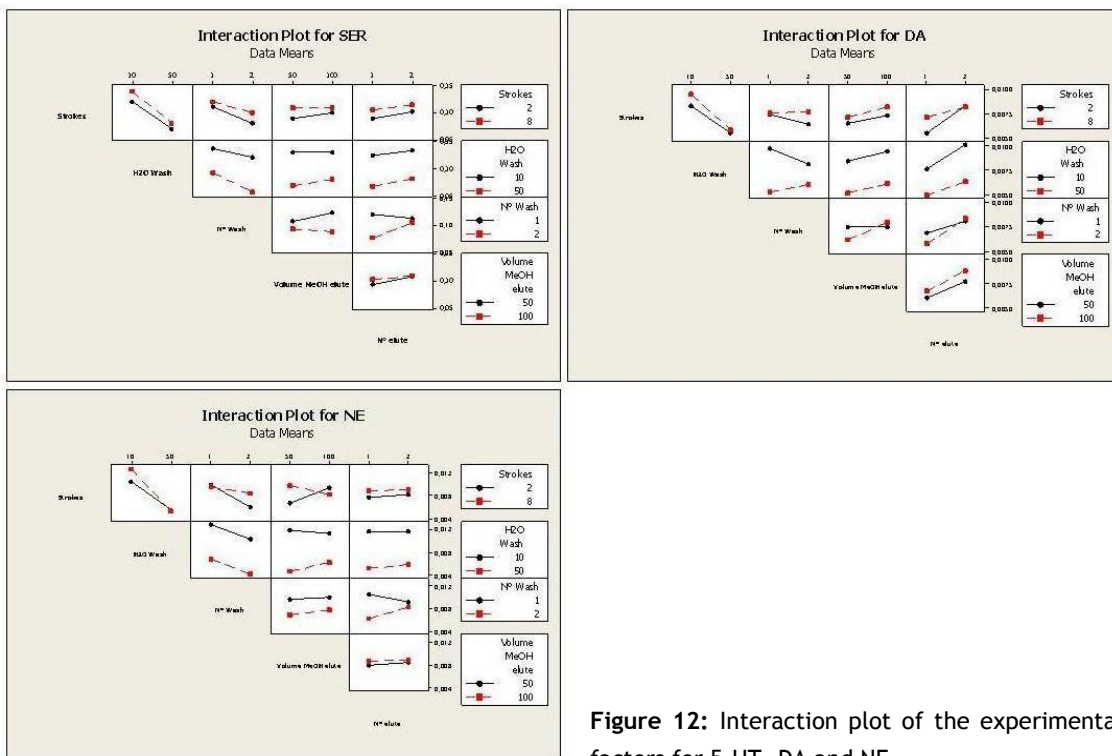


Figure 12: Interaction plot of the experimental factors for 5-HT, DA and NE.

Based on these results, the number of strokes, volume of eluent and number of elutions were kept constant, and 8 sample drawings and 2 elutions of 100 μ L of methanol were used in all subsequent extractions. In order to optimize the volume of water used for washing the column and the number of washes, a response surface methodology (RSM) was used (table 7).

Table 7: Response surface matrix of the experimental factors and the respective response.

Run Order	Experimental factors		Response (peak area ratio)		
	H ₂ O Wash (μ L)	N ^o Wash	5-HT	DA	NE
1	10	1	0.148	0.009	0.016
2	15	2	0.091	0.004	0.005
3	10	1	0.152	0.008	0.016
4	5	0	0.228	0.014	0.031
5	17	1	0.140	0.006	0.011
6	3	1	0.165	0.012	0.021
7	5	2	0.182	0.012	0.017
8	10	1	0.162	0.010	0.015
9	10	1	0.155	0.009	0.015
10	15	0	0.227	0.025	0.036
11	10	0	0.276	0.016	0.032
12	10	3	0.113	0.005	0.011
13	10	1	0.153	0.010	0.015

The surface response plot did not show statistical significance for the responses. However, the results show that the highest response is obtained when no washing step is done. As a compromise, the column washing step was skipped and the final extraction conditions were used as follows: 8 sample drawings, no washing and two elutions with methanol, 100 μ L each.

2.3. Method Validation

In order to evaluate the adequacy of the developed protocol, the method was validated according to parameters accepted by the Food and Drug Administration (FDA) [61] and the International Conference on Harmonization (ICH) [63]. The validation was done with a

five day validation protocol, evaluating the method's selectivity, linearity, precision and accuracy, limit of detection and quantification, and recovery.

Since neurotransmitters are endogenous molecules, they are normally present in urine and plasma, and thus obtaining a blank sample without monoamine neurotransmitters is not possible. Therefore, the validation was done using a solution (water) with similar properties to urine and plasma.

2.3.1. Selectivity

Selectivity is the capability of a method to differentiate and quantify the analyte in the presence of other compounds in a complex sample [61,63]. Since the extraction was performed using spiked water samples, the extraction of a blank water sample did not originate a signal besides the mobile phase baseline. Additionally, other neurotransmitters (NE, acetylcholine and histamine) present in urine and plasma were analysed, but no signal overlay was found at retention times of the analytes at the chosen potential.

2.3.2. Linearity

The calibration curves for each compound (n=5) were obtained using water (500 μ L) spiked with increasing concentrations of 5-HT, DA and NE and a constant concentration of internal standard with the extraction protocol mentioned above. The data for the calibration curves was obtained by the ratio between analyte and internal standard peak area versus the analyte concentration. Calibrators, ranging from 50 to 1000 ng/mL (5-HT) and 5 to 1000 ng/mL (DA and NE), were prepared, in the concentrations of 5, 10, 20, 100, 200, 800 and 1000 ng/mL. Using the linear regression equation of the curve, each calibrator was quantified in order to calculate the mean relative error or accuracy (bias) as shown in the following equation:

$$\text{bias} = \frac{\text{measured concentration} - \text{theoretical concentration}}{\text{theoretical concentration}} \times 100 \quad (1)$$

The bias values obtained from the calibration curves didn't allow correct quantification of the samples, since the error values were higher than the accepted values, especially at the lower concentrations. This fact is due to the wide calibration range adopted, and to compensate for heteroscedasticity weighted least squares regressions had to be

adopted. Therefore, six weighting factors were evaluated for each analyte: $1/X$, $1/X^2$, $1/Y$, $1/Y^2$, $1/\sqrt{X}$ and $1/\sqrt{Y}$. For each compound one factor was chosen based on the best bias value for the different concentrations. The lowest relative error was obtained using the weighting factor $1/Y$ for serotonin, and $1/X^2$ for dopamine and norepinephrine (table 8).

Table 8: Linearity data for 5-HT, DA and NE (n=5).

	Weighting factor	Calibration range (ng/mL)	Regression equation		R ²	LLOQ (ng/mL)
			m	b		
5-HT	1/Y	50 - 1000	0.0067 ± 8E-04	0.1389 ± 0.0738	0.9959 ± 0.0013	50
DA	1/X ²	5 - 1000	0.0018 ± 1.1E-04	-0.0012 ± 0.0023	0.9906 ± 0.0046	5
NE	1/X ²	5 - 1000	0.0013 ± 4.5E-05	0.0025 ± 0.0082	0.9942 ± 0.0039	5

m - Slope, b - intercept, LLOQ - lower limit of quantification

The bias is considered valid if the value is situated between ± 15 % of the nominal concentration, except for the lowest calibrator (LLOQ) which is accepted at ± 20 % [61,63]. With the application of regression equation analysis using weighting factors, the method showed linearity in the shown calibration range since the R² values were superior to 0.99 and the relative error was situated within the accepted intervals.

2.3.3. Limits of detection (LOD) and quantification (LOQ)

When handling a sample, the LOD is defined as the lowest analyte concentration that shows a clear visible signal, distinguishable from the background noise. This concentration doesn't necessarily have to be measurable as an exact value. The lower limit of quantification (LLOQ) is the concentration of analyte in a sample that can be measured with acceptable precision and accuracy, with a coefficient of variation (CV) not exceeding 20 % and the bias within ± 20 % [61,63].

The LODs were determined as the lowest concentration that showed a clear peak during the 5 days of validation, allowing identification of the compounds, and was found to be 20 ng/mL for 5-HT and 2 ng/mL for DA and NE. The determination of LLOQ was based on the lowest calibrator concentration that showed linearity in the regression equation, allowing quantification of the compound. The obtained limits of quantification were 50 ng/mL for 5-HT, and 5 ng/mL for both DA and NE. Compared to available literature using MEPS [40], the limit of quantification was lower for DA and equal for 5-HT, although the authors used a lower sample volume (30 µL) when compared to our work (500 µL). However a non-commercially

available sorbent was used. Comparing to other extraction methods (table 3) the LLOQ is situated within the average of obtained limits.

Considering the normal range levels of the monoamines in plasma and urine (table 2), the obtained LOQ is sufficient for daily routine analysis of 5-HT, DA and NE in urine and 5-HT in plasma.

2.3.4. Precision and accuracy

The precision of an analytical method is defined as the repeatability of individual measurements of an analyte, when these are done repeatedly with multiple aliquots of the same biological sample, normally expressed by means of the coefficient of variation (% CV). The accuracy describes the difference between the mean measured concentrations for a sample obtained by the linear equation, and the true concentration of the sample. Accuracy can be expressed as % bias.

The precision and accuracy of a method should be measured using at least five determinations for each concentration, and at least three concentrations of the calibration range should be measured (including the highest and lowest concentration) [61].

Precision is evaluated using two approaches, within-run and between-run repeatability. In the first approach, the precision during a single analytical run is assessed, while the latter assesses precision with time and may involve different analysts, equipment, materials and laboratories [61].

Within-run precision was determined using water spiked with three concentrations (50, 100 and 1000 ng/mL) of 5-HT and four concentrations (5, 50, 100 and 1000 ng/mL) of DA and NE. Each concentration was measured on a different day and in replicates of six measurements (table 9).

Table 9: Within-run precision and accuracy (n=6) for all compounds.

	Concentration (ng/mL)	Measured concentration (ng/mL)	CV (%)	Bias (%)
5-HT	50	54.91 ± 3.91	7.13	9.82
	100	91.97 ± 3.96	4.30	-8.03
	1000	931.9 ± 55.60	5.97	-6.80
DA	5	5.52 ± 0.19	3.50	10.34
	50	48.59 ± 2.95	6.09	-3.01
	100	92.53 ± 5.79	6.26	-7.47
	1000	1020.09 ± 30.17	3.74	2.01
NE	5	5.58 ± 0.18	3.30	11.53
	50	46.47 ± 3.40	7.32	-7.05
	100	95.13 ± 8.95	9.41	-4.87
	1000	1057.73 ± 46.65	4.41	5.77

The CV obtained from the within-run precision was lower than 10 % for all compounds. All bias values were situated within ± 15 % of the nominal concentration.

The between-run precision was measured using five concentrations for 5-HT, and seven concentrations for DA and NE, during a five day period (table 10).

Table 10: Between-run precision and accuracy (n=5) for all compounds.

	Concentration (ng/mL)	Measured concentration (ng/mL)	CV (%)	Bias (%)
5-HT	50	57.35 ± 1.58	2.76	12.76
	100	93.01 ± 2.99	3.22	-7.6
	200	178.55 ± 6.65	3.72	-12.13
	800	817.76 ± 6.87	0.84	2.22
	1000	1010.53 ± 9.15	0.91	1.04
DA	5	5.10 ± 0.17	3.24	1.85
	10	10.10 ± 0.55	5.44	0.79
	20	18.31 ± 0.61	3.33	-9.33
	100	93.77 ± 5.34	5.70	-6.92
	200	187.11 ± 5.97	3.19	-6.98
	800	874.78 ± 37.82	4.32	8.40
	1000	1087.86 ± 35.39	3.25	7.99
NE	5	5.07 ± 0.19	3.70	1.28
	10	9.68 ± 3.89	9.36	-5.13
	20	20.72 ± 11.51	3.24	1.17
	100	95.97 ± 6.27	6.54	-4.56
	200	190.22 ± 5.18	2.72	-5.21
	800	842.85 ± 27.19	3.23	5.01
	1000	1050.46 ± 41.05	3.91	4.69

The combined within- and between-run precision was also evaluated. This was done using two control samples prepared during five days at the concentrations of 50 and 500 ng/mL, each one in triplicate (n=15) (table 11).

Table 11: Combined within- and between-run precision and accuracy (n=15) for all compounds.

	Concentration (ng/mL)	Measured concentration (ng/mL)	CV (%)	Bias (%)
5-HT	50	55.52 ± 2.76	4.96	11.04
	500	535.40 ± 44.22	8.25	7.08
DA	50	50.27 ± 4.20	8.36	0.54
	500	567.56 ± 8.52	1.50	13.51
NE	50	48.15 ± 3.47	7.20	-3.69
	500	525.41 ± 33.21	6.32	5.08

2.3.5. Recovery

The recovery of the extraction was assessed at three sample concentrations (50, 100, 1000 ng/mL). For each concentration three samples were extracted (x % of concentration), and the equivalent volume of working solution added to the elution solvent (100 % of concentration), also in triplicate. The internal standard was added only after extraction to the elution solvent of both extracted and non-extracted samples. The recovery was calculated dividing the average of the extracted samples signal ratio with the average of the non extracted ratios, and was expressed in percentage. The recovery values are shown in table 12.

Table 12: Recovery at three different concentrations (n=3) for all compounds.

	Recovery (%)		
	50 ng/mL	100 ng/mL	1000 ng/mL
5-HT	3.76 ± 0.35	3.92 ± 0.80	2.72 ± 0.61
DA	2.82 ± 0.33	2.98 ± 0.71	1.62 ± 0.09
NE	1.41 ± 0.05	1.55 ± 0.52	1.51 ± 0.95

These recovery values are very low when compared to other extraction techniques (table 2). However with the obtained LOQ values of the developed method, these recoveries are sufficient in order to apply this methodology to authentic samples.

2.4. Method application to urine and plasma samples

In order to verify the applicability of the method, two urine samples (500 μ L) and one plasma sample were analysed by the described protocol; the plasma sample was diluted 1:4 in order to facilitate syringe drawing. The method allowed for detection and quantification of all analytes in urine, and serotonin in plasma (table 13).

Table 13: Obtained concentrations of monoamines in urine and plasma samples. ND - not detected.

Sample	Obtained concentration (ng/mL)		
	5-HT	DA	NE
Urine 1	103.17	143.46	589.62
Urine 2	237.55	455.56	858.90
Plasma	180.86	ND	ND

The obtained concentrations were within the normal ranges in urine and plasma (table 2), figure 13 and 14 show obtained chromatograms for urine and plasma.

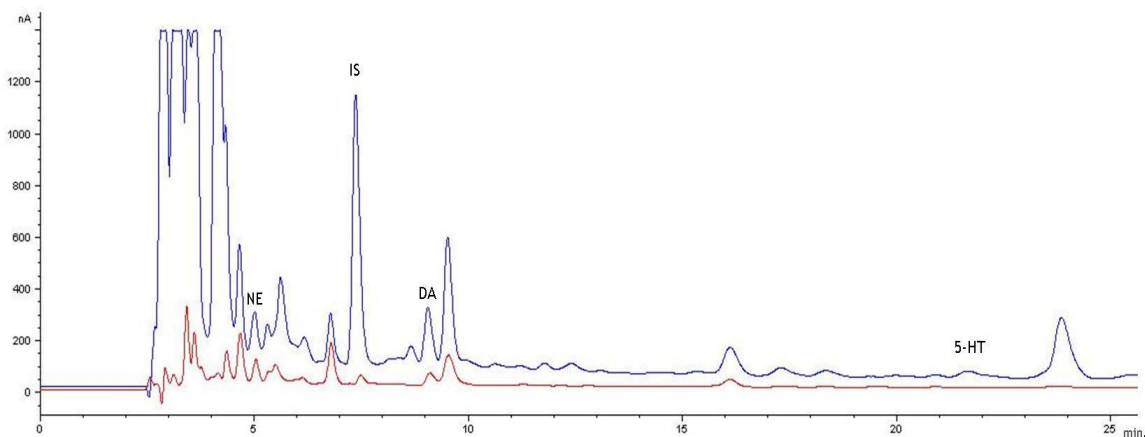


Figure 13: Chromatogram of an extracted urine sample (Urine 1).

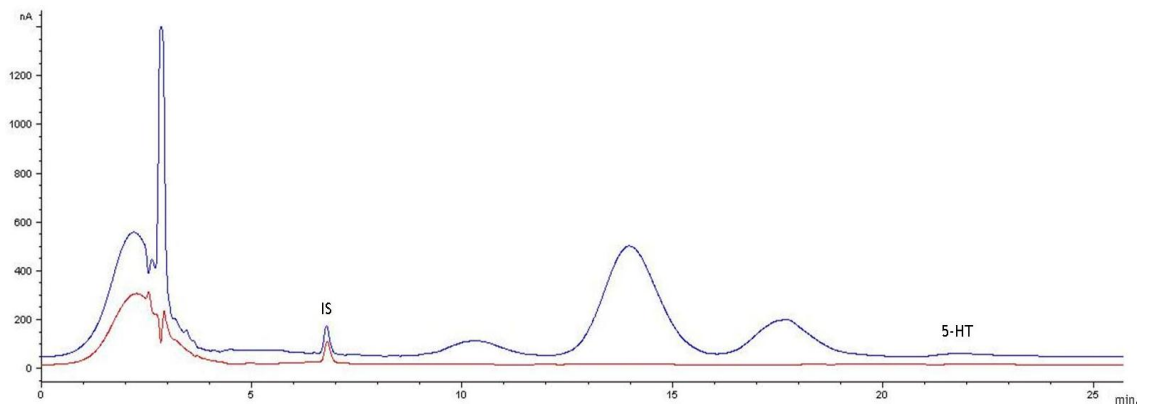


Figure 14: Chromatogram of the extracted plasma sample.

III. Conclusions

- A fast and simple method was developed for the detection of biogenic amines, 5-HT, DA and NE, in urine and 5-HT in plasma, using MEPS and HPLC coupled to coulometric electrochemical detection.
- The established chromatographic conditions, allowed the simultaneous detection and quantification of 5-HT, DA and NE, with good separation of the analytes in less than 25 minutes.
- As a sample preparation step, MEPS showed to be a relatively fast method (with an average of 5 minutes per extraction), using small solvent and sample volumes with the possibility to re-use the column for more than 100 extractions, sparing time, money and reagents.
- The design of experiments (DOE) was used for the optimization of the extraction process in order to obtain better extraction recoveries. A fractional two level factorial design showed to be useful for the optimization, reducing the number of necessary experiments and sparing money, time and labour.
- The method was validated according to internationally accepted guidelines, and showed to be linear within the studied concentration range, with adequate precision and accuracy. The limits of detection and quantification were low and allowed for 5-HT, DA and NE quantification in urine and 5-HT in plasma.
- Under optimized conditions, the extraction recoveries of the MEPS protocol were significantly low. However, within the quantification range, the method is suitable for routine detection and quantification of 5-HT, DA and NE in urine and 5-HT in plasma under physiologically normal and elevated levels.
- This is the first study for the simultaneous detection and quantification of 5-HT, DA and NE in urine using a commercially available MEPS column, and the first study for the detection and quantification of 5-HT in plasma using MEPS.

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