Development and validation of a novel method for serotonin and 5-hydroxyindole-acetic acid determination in plasma using liquid chromatography tandem mass spectrometry

By

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Thesis for the degree of
European Master in Quality in Analytical Laboratories

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''All models are wrong, but some are useful''

-George Box, 1978 (Statistician)

I therefore opine that a good model allows you to manipulate, play and make mistakes at low cost. However, models maybe quite different from the real thing.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AADC: Aromatic-L-amino acid decarboxylase

ACN: Acetonitrile

ADH: Aldehyde dehydrogenase

Alc DH: Alcohol dehydrogenase

Ald DH: Aldehyde dehydrogenase

CAD: Coronary artery disease

CNS: Central Nervous system

CT: Column temperature

CV: Coefficient of variance

DOE: Design of experiments

EC: European commission

ED: Electrochemical detection

EDTA: Ethylenediaminetetraacetic acid

ESI: Electrospray ionisation

FPD: Flame photometry detection

FR: Flow rate

FWHM: Full width at half maximum

GC-MS: Gas chromatography mass spectrometry

GIT: Gastrointestinal tract

HIOMT: Hydroxyindole-O-methyl transferase

HPLC: High performance liquid chromatography

5-CH$_3$O-HT: 5-methoxytryptamine

d_2-5-HIAA: 5-hydroxyindole-3-acetic-2,2-d_2 acid

5-HIAA: 5-indole-3-Acetic Acid

5-HTOL: 5-hydroxytyptophol

5-HT: 5-hydroxytryptamine

5-HTTP: 5-hydroxytryptophan

ICH: International Conference on Harmonisation

IS: Internal standard

ISO: International Standard Organisation

IUPAC: International Union of Pure and Applied chemistry

LC: Liquid chromatography

LC-MS/MS: Liquid chromatography tandem mass spectrometry

MAO-A: Mono amine oxygenase A

MDD: Major depressive disorder

MS: Mass spectrometry

NADP: Niacin adenine dinucleotide

N-Ac-5HT: N-acetyl-5-hydroxytryptamine

Na_2EDTA: Disodium ethylenediaminetetraacetic acid

RF: Response factor

RSD: Relative standard deviation

SD: Standard deviation

SERT: Serotonin transporters

SIL: Stable labelled internal standard

SRI: Serotonin reuptake inhibitor

TRP: Tryptophan

TPN: Triphosphate Nucleotide

TRP Hydr: Tryptophan hydroxylase

ABSTRACT

5-Hydroxytryptamine (5-HT) also known as serotonin is a biomarker in gastrointestinal disorder and several other pathological diseases where 5-HT and its metabolite 5-hydroxyindole-acetic acid (5-HIAA) are implicated. A sensitive and precise method has been developed and validated for the determination of serotonin and 5-hydroxyindole-acetic acid in human plasma. The method involves a simple protein precipitation step requiring no further downstream sample preparation. The method was developed using a Zorbax Eclipse-C8 RP (150mm × 4.6mm, 5μm) column (Agilent Technologies, Palo Alto, CA, USA). The column temperature was kept at 20°C and the solvent in gradient mode consisted of water with 0.1% formic acid (v/v) (B), acetonitrile with 0.1% formic acid (v/v) (C) and ethanol (D) and a UV detector at 254nm and the flow rate was maintained at 0.2mL/min. Linearity of the method was studied over the range of 0.5-50μg/mL. The correlation coefficient was $r^2 = 0.9823$ for serotonin and $r^2 = 0.9892$ for 5-hydroxyindole-acetic acid which indicates strong correlation between the studied concentration of the analytes and the signal. The precision of the method for 5-HT and 5-HIAA were achieved based on repeatability with RSD of 3.07-7.73% and 3.93-9.99% respectively. The percentage recoveries were 83-119% and 84-116% for 5-HT and 5-HIAA respectively, which shows good accuracy of the developed method. The limit of detection and limit of quantification were 0.5μg/mL and 1μg/mL respectively suggesting good sensitivity of the method. The developed method was applied in the analysis of human plasma samples from a project related to the determination of serotonin and its metabolite in plasma from pathological patients subjected to a diet rich in vegetables.

1. INTRODUCTION

1.1 Biology of serotonin

Neurotransmitters are chemicals that allow signal transmission, and thus communication among the nerve cells (neurons). One of the neurotransmitters used by neurons throughout the brain is 5-hydroxytryptamine also known as serotonin (5-HT). Serotonin is produced in and released from neurons that originate within discrete regions in the brain. Serotonin was originally discovered by Italian Vittorio Erspamer in Rome in 1935 [1] and American scientists, Maurice M. Rapport, Arda Green, and Irvine Page of the Cleveland Clinic isolated and named in 1948 [2]. The name "serotonin" is often referred to as a misnomer and reflects the circumstances of the compound's discovery [3]. It was initially identified as a vasoconstrictor substance in blood serum – hence "serotonin", a serum agent affecting vascular tone. This agent was later chemically identified as 5-hydroxytryptamine [2] and, as the broad range of physiological roles were elucidated, 5-HT became the most widely used and preferred name in the pharmacological field. Serotonin is a central and a peripheral neurotransmitter. It is biochemically synthesized from the amino acid tryptophan and it plays a great role in regulating various physiological functions such as sleep, hemostasis, and behavior regulation; in pathological conditions such as carcinoid syndrome, hypertension, thrombosis, and in cardiovascular diseases as well as psychiatric and neurological disorders such as schizophrenia, Huntington's disease, including many others [4, 5]. Serotonin is widespread in nature and can be found in foods, nuts, and animals.

It is synthesized in the serotonergic neurons in the central nervous system and in the enterochromaffin cells of the gut constitutes 80% of total production and storage and it is subsequently released when triggered by different stimuli [6] such as chemical and electrical synapses between taste cells and synapses and from taste cells to sensory afferent fibers.

Serotonin is generally interconnected to norepinephrine and dopamine (Fig.1). The three compounds are monoamine neurotransmitters. While serotonin is involved in cognitive impulses, relaxation as shown in the blue ring (Fig. 1), norepinephrine is involved in socialization, concentration etc, whereas dopamine is involved in behaviour, cognitive and voluntary movements. The three compounds are necessary for mind and emotional stability.
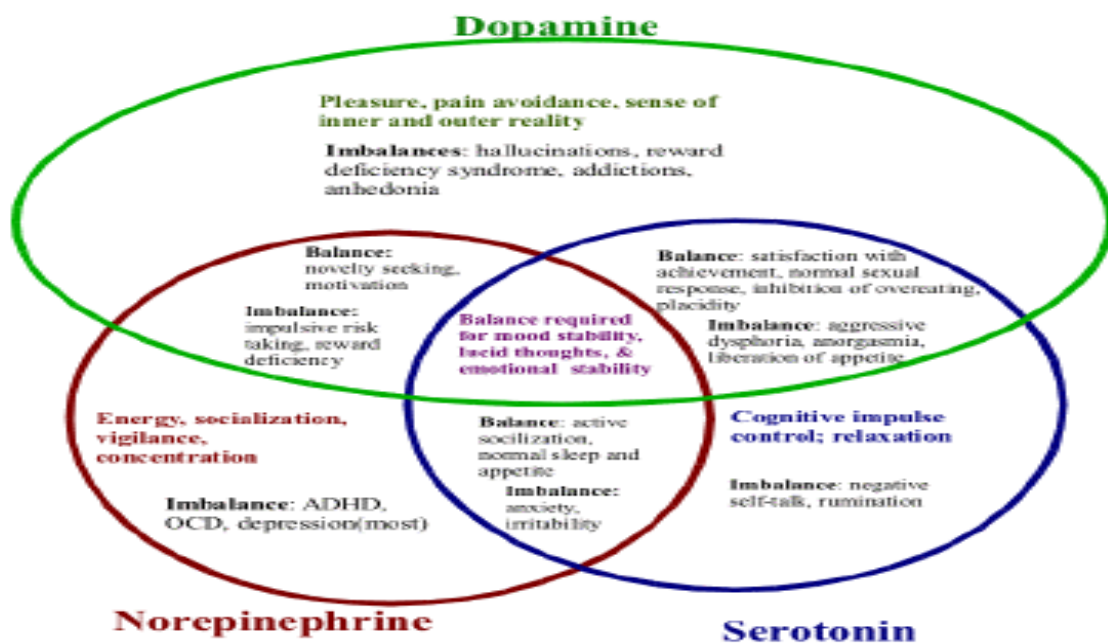
Figure 1. Interconnection between norepinephrine, dopamine and serotonin (monoamine neurotrasmitters).

(Source: http://www.horses-helping-troubled-teens.com/teen-depression.html)

Early detection of carcinoid tumour in the small intestine is diagnosed by measuring blood serotonin and 5-hydroxyindole-acetic acid (5-HIAA) secreted by the enterochromaffin cells [7]. Following the release of 5-HT, it is rapidly sequestered by platelets or otherwise metabolised by the liver or kidney to 5-HIAA by the catalytic action of the mitochondrial flavoprotein monoamine oxygenase (EC 1.4.3.4) and aldehyde dehydrogenase (EC 1.2.1.3). The 5-HIAA is a major metabolite of serotonin and it is often eliminated in urine; this will be highlighted in the course of the present research thesis. Serotonin is associated with coronary artery disease (CAD), [8]. Age has a defining trend in risk factor on most endogenous biological molecules such as cholesterol and homocysteine, these risk factors are often in increased levels in advancing age [9, 10], but serotonin seems to be inversely related to age, having higher levels in younger age groups in which it has a significant relationship with acute cardiac invents [8]. Serotonin is often tightly bound to protein and thus very small free serotonin concentrations can be found in the plasma [11, 12].

1.2 Neurophysiology of serotonin

Serotonin is a monoamine neurotransmitter that is primarily found in the gastrointestinal (GI) tract and central nervous system (CNS). The raphe nuclei (Fig. 2) is a cluster of nuclei found in the brain, they are distributed near the midline of the brainstem; hence, it functions to release serotonin to the rest of the brain. Furthermore, most serotonin inhibitors, like the selective serotonin reuptake inhibitors (SSRI) and antidepressants are generally believed to act on the raphe nuclei as their target site. However, there are other physiological functions of the raphe nuclei, but such detail is beyond the scope of the present thesis.
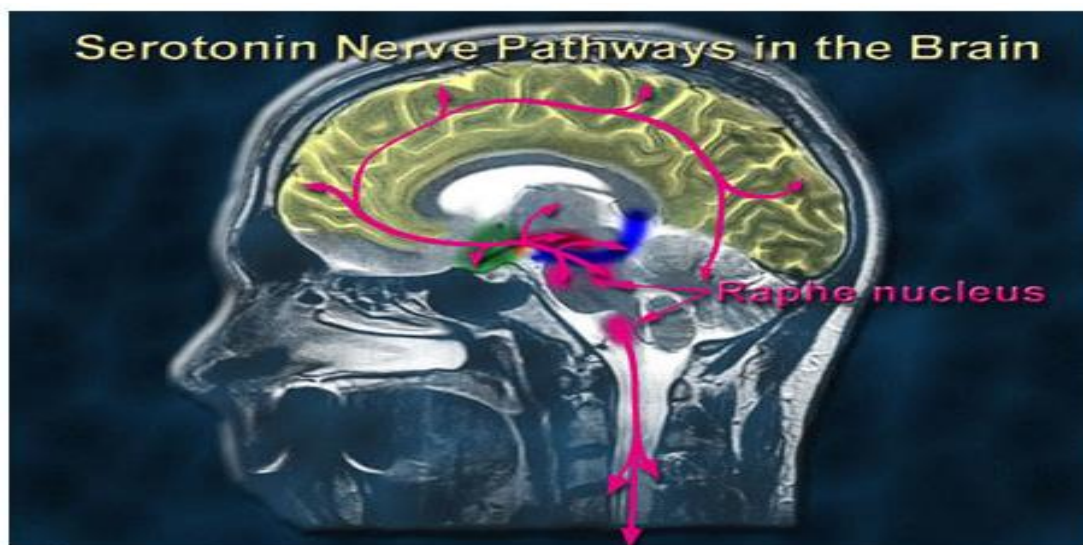
Figure 2. The raphe nuclei (where serotonin is active) represents the major nuclei with both ascending and descending serotonergic fibers projecting to the forebrain and the descending fibers that extend to the medulla and spinal cord.

(Source: http://www.bio.davidson.edu/courses/genomics/2003/mccord/5-htt.html)

As previously highlighted, about 80% or more of the human body's total serotonin is located in the enterochromaffin cells in the gut, where it is used to regulate intestinal movements. While the rest of the remnants are synthesized in serotonergic neurons in the CNS where it exerts pool of physiological roles, including but not limited to the regulation of mood,

appetite, sleep, muscle contraction, and some cognitive functions as well as memory and learning; and in blood platelets where it helps to control hemostasis and blood clotting.

The connection between serotonin receptors and the serotonin neuron (Fig. 3) defines its storage site in nerve terminal. Serotonin is stored in small vesicles within the nerve terminal of a neuron, the pink colored image in Fig. 3. Electrical impulses arising in the raphe nucleus traveling down the axon toward the terminal causes the release of serotonin from small vesicles into the synaptic space. Once in the synaptic space, the serotonin binds to special proteins, called serotonin receptors, on the membrane of a neighboring neuron. When serotonin binds to serotonin receptors

Figure 3. Connection between serotonin receptors and the serotonin neuron.
(Source:http://psychminded.wordpress.com/2011/10/10/and-i-guess-thats-why-they-call-it-the-blues/)

it causes a change in the electrical properties of the receiving neuron that generally results in a decrease in its activity rate.

The serotonin present in the synaptic space for a limited time (Fig. 4) is immediately removed if it is not bound to a receptor through special proteins called transporters (in green). The serotonin transporters are proteins located on the serotonin neuron terminals and they function to transport serotonin from the synaptic space back into the neuron where it can be metabolized by enzymes.
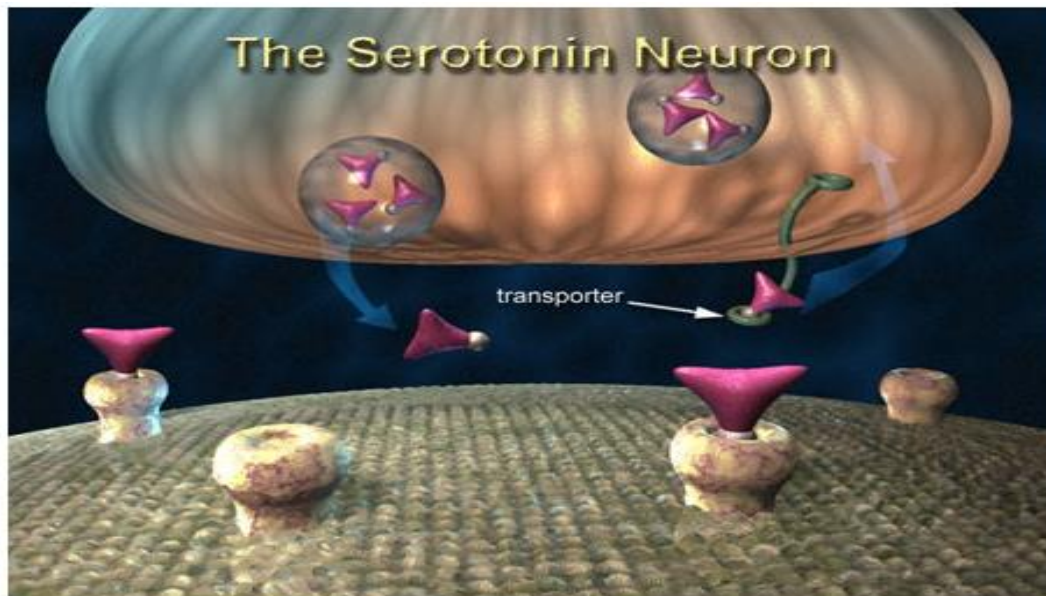
Figure 4. Connection between serotonin transporter and serotonin neuron.

(Source: http://www.drugabuse.gov/publications/teaching-packets/neurobiology-ecstasy/section-ii/3-serotonin-transporters)

1.3 Project scope

Development and validation of a novel method for the determination of serotonin and 5-hydroxyindole-acetic acid (5-HIAA) in human plasma using liquid chromatography tandem mass spectrometry (LC-MS/MS).

1.3.1 Project significance

The National Institute of Nutrition and Seafood Research (NIFES) has been involved in different human trials where the potential modulation of serotonin through diets and its determination in plasma has been proposed as key components to understand the functional role of serotonin in mental and physical health.

Therefore, NIFES is in urgent need of a reliable method for the analysis of serotonin (5-HT) and its 5-hydroxyindole-acetic acid metabolite (5-HIAA) in plasma samples. Unfortunately, there is a scarcity of published methods for the determination of 5-HT and 5-HIAA in plasma using LC-MS/MS. The main focus of the related literature has been on the analysis of 5-HT and 5-HIAA in urine. Whereas serotonin is more evident to emotional related routine measurements; 5-HIAA is more often measured in the assessment of carcinoid tumours. Consequently, it is important to develop a novel and robust method for the determination of 5-HT and 5-HIAA that can assist NIFES in the analysis of a high volume of plasma samples from various nutritional research projects. The new method will be optimised and validated and could become part of NIFES battery of nationally accredited analytical methods.

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1.3.2 Project brief at the onset

Serotonin is synthesized from the amino acid tryptophan. Tryptophan undergoes a hydroxylation reaction to form 5-hydroxy tryptophan (this reaction is catalysed by the enzyme tryptophan hydroxylase). 5-hydroxy tryptophan undergoes a decarboxylation reaction (catalysed by amino acid decarboxylase) to form serotonin in one of the fate of tryptophan's metabolic pathways. Serotonin subsequently undergoes oxidative deamination followed by concomitant oxidation to form 5-hydroxyindole-acetic acid (5-HIAA), which is the predominant metabolite in urine. Hence, this metabolite is mainly analysed and studied in urine. Therefore, the need for a robust and rapid method using liquid chromatography coupled to tandem mass spectrometry in the determination of the parent compound, 5-hydroxytryptamine and its acid metabolite in human plasma.

1.3.3 Objectives of the study

- To develop a novel and rapid method for extracting serotonin and 5-hydroxyindole-acetic acid from human plasma and further quantification by using liquid chromatography tandem mass spectrometry (LC-MS/MS).

- To optimise and validate the developed method.

- To apply the novel method in the analysis of plasma samples from nutritional intervention studies.

1.4 Biosynthesis of serotonin

Serotonin is derived from the amino acid tryptophan. The metabolic pathway through which tryptophan is converted to serotonin is outlined in Fig. 5. The first step involves hydroxylation of tryptophan to form 5-hydroxytryptophan, 5-HTP. The reaction requires oxygen and it is catalysed by a hydroxylase, which is dependent upon the presence of the reduced form of coenzyme, niacin adenine dinucleotide phosphate (NADP) also known as triphospyridine nucleotide (TPN). In the next step of the biosynthetic pathway, the hydroxylated tryptophan (5-HTP) undergoes a decarboxylation reaction to yield 5-hydroxytryptamine (serotonin), this decarboxylation reaction is catalysed by a decarboxylase enzyme (aromatic amino acid decarboxylase) also known to be involved in the decarboxylation of other amino acids such as tyrosine to form tryramine or of 3-4-dihydrophenylalanine to form dihydrophenylalanine, an intermediate precursor of norepinephrine and subsequently epinephrine, another member of monoamine neurotransmitter and neuromodulator. The decarboxylase enzyme requires a pyridoxal phospahate (vitamin B6) as a cofactor. This route of production of serotonin from tryptophan is thus one of the minor fates of the ubiquitous routes of tryptophan metabolism; consequently about one percent of tryptophan takes this pathway.

On the contrary, the major route of tryptophan metabolism proceeds through the kynurenine, hydroxyanthanilic acid, and quinolinic acid and to nicotinic acid, a very important end product of tryptophan metabolism proved by isotopic studies which revealed that the nitrogen of the indole ring of pyridine ring of the tryptophan is retained as the nitrogen of the pyridine ring of nicotinic acids [13].
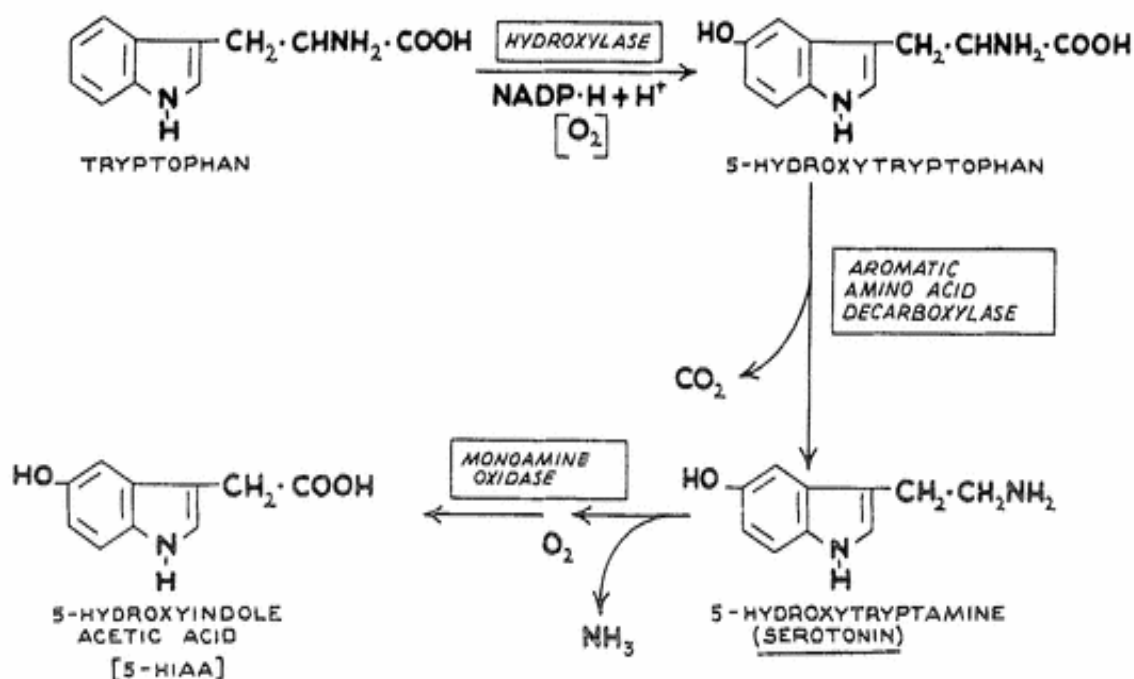
Figure 5. The metabolic pathway of tryptophan metabolism to serotonin (5-HT) and subsequently to 5-hydroxyindole-acetic acid (5-HIAA), [13].

In many animals, the conversion of tryptophan to nicotinic acid renders the supply of vitamin in the diet unnecessary as long as protein containing tryptophan is ingested. In other mammals such as rat, rabbit, dog and pig for instance tryptophan can replace completely the vitamin in the diet. In humans, about 60 mg of tryptophan produces 1 mg of nicotinic acid, thus this implies that the nutritional deficiency of nicotinic acid that often occurs in pellagra must be considered a protein and vitamin (tryptophan and nicotinic acid) deficiency respectively [13].

Biochemically, it may be necessary to observe that carcinoid produces abnormality of tryptophan metabolism in which as much as 60 %, other than the normal 1 %, of tryptophan may follow the serotonergic pathway. In addition to the production of the excess amines, the switch in the metabolism shuts adequate production of nicotinic acid which would lead to nitrogen imbalance and subsequently nicotinic acid deficiency.

Furthermore, the majority of serotonin produced in the tissues is further metabolized via oxidative deamination to form 5-hydroxyindole-acetic acid (5-HIAA). The series of this event involves the serotonin produced from L-tryptophan subsequently undergoing oxidative deamination followed by concomitant oxidation and conjugation in the liver, lungs and thrombocytes catalysed by monoamine oxygenase A (MAO-A) and aldehyde dehydrogenase (ADH) to form 5-HIAA, which is the predominant metabolite in urine. Hence, this metabolite is mainly analysed and studied in urine. Consequently the pertinent of a robust and rapid method using the tandem mass spectrometric technique that can simultaneously determine the parent compound, serotonin and its metabolite, 5-HIAA in human plasma. A comprehensive biosynthetic and degradative pathway of serotonin metabolism is shown in Fig. 6.
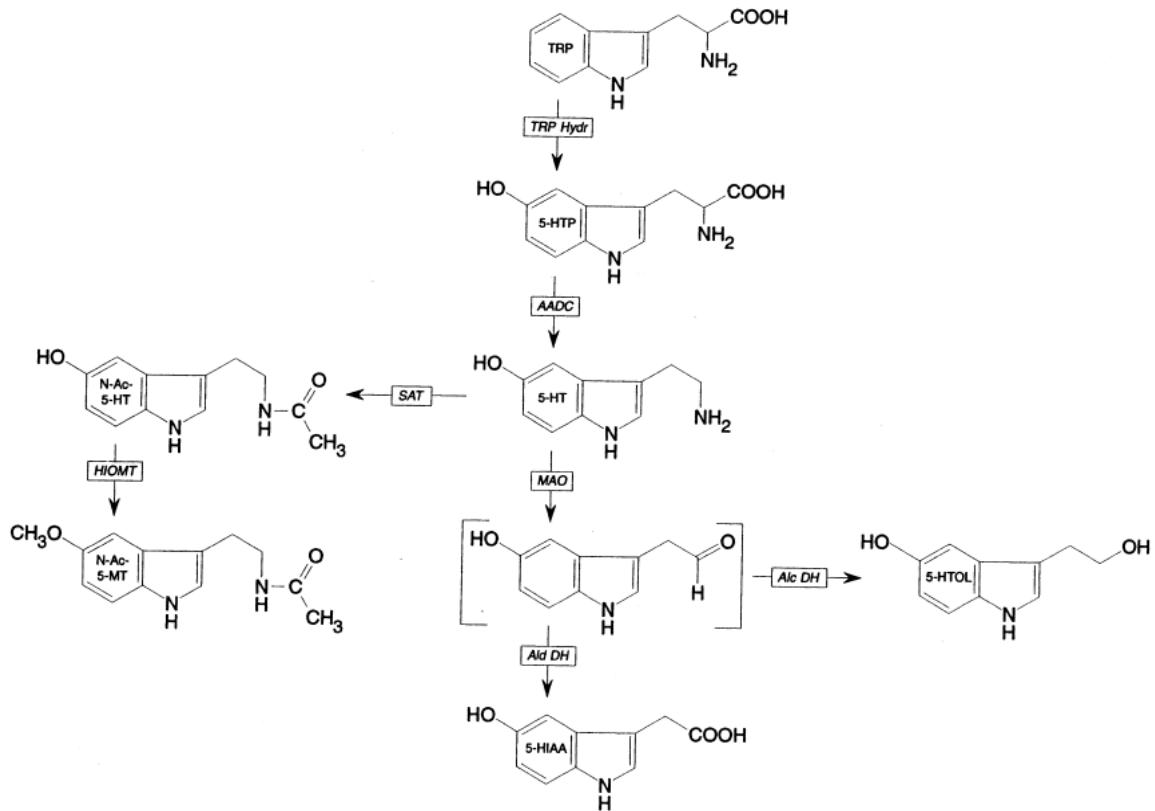
Figure 6. Biosynthetic and degradative routes in the metabolism of serotonin.

Abbreviations: AADC, aromatic-L-amino acid decarboxylase; Alc DH, alcoholdehydrogenase; Ald DH, aldehyde dehydrogenase; HIOMT, hydroxyindole-O-methyl transferase; MAO, monoamine oxidase; N-Ac-5HT, N-acetyl-5-hydroxytryptamine; N-Ac-5Mt, N-acetyl-5-methoxytryptamine, melatonin; N-Ac-5-HT, N-acetyl-5-hydroxytryptamine; TRP, Tryptophan; TRP Hydr, tryptophan hydroxylase; 5-HT, Serotonin, 5-hydroxytryptamin; 5-HTOL, 5-hydroxytryptophol; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindole acetic acid.
(Adapted from, I. P. Kema et al, 2000: Review of Clinical chemistry of serotonin and metabolites).

1.5 5-HIAA/5-HT ratio in depression

Depression is often characterised by feelings of guilt and hopelessness, appetite loss, insomnia, and suicidal thoughts, consistent sadness, extreme fatigue, etc and serotonin has been undoubtedly implicated in this effects. Serotonin is thought to play the critical role in depression because of its effects on mood state listed above as well as other cognitive processes. Serotonin influences the initiation and then gradual "relaxation" of thoughts. Imbalance in serotonin results in ruminating negative self-talk (worry), that is, negative self-

talk thoughts that keep reoccurring and will not go away, this constitutes a major problem in depression. Most of the antidepressants exact their effects by inhibiting serotonin reuptake (SRI's). During serotonergic neurotransmission, serotonin is released into the synaptic cleft (junction between two neurons), Fig. 4. After exerting its action in the postsynaptic neuron, part of the serotonin is transported again into the presynaptic neuron by specific transporters (SERT). Once again in the presynaptic neuron, part of this serotonin is incorporated again into vesicles (Fig. 3) and part is metabolized by the monoamine oxidase (MAO) and other enzymes to form 5-HIAA. In summary, when the serotonergic activity is high, more serotonin is released in the synapses, and consequently, metabolized to 5-HIAA. Therefore, the 5-HIAA/5-HT ratio would be higher. In addition, metabolism of certain body molecules means that there is both anabolism (syntheses, in this case from L-tryptophan to 5-HT) and catabolism (from 5-HT to 5-HIAA) of that molecule. It is possible to assume on the basis of 5-HIAA levels that only the impairments in catabolism step, from 5-HT to 5-HIAA (impairments in MAO or aldehyde dehydrogenase activity) and not in the syntheses step.

Serotonin turnover has been reported to be indeed related to stress, and it is known that chronic stress may lead to depression. In different animal models it is known that stress induce an increase in serotonergic turnover, but often also in dopaminergic and noradrenergic turnover [14]. These alterations are transient if the stress is acute (for example a persecution by a predator). If the stress becomes chronic (for example, cohabitation with a dominant specimen), important and long-lasting changes in the monoaminergic systems could occur.

Also, chronically stressed animals may present symptoms similar to those observed in Major Depressive Disorder, (MDD) patients [14]. The 5-HIAA/5-HT ratio could be useful for different purposes. One of them is its use as an estimator of the serotonergic activity (for example, a group of acutely stressed rats is expected to have a higher 5-HIAA/5-HT ratio than control rats, due to their higher serotonergic turnover).

Furthermore, as pointed earlier the biosynthesis of serotonin in humans represents only a minor route for tryptophan and in normal conditions this accounts for less than 2% of ingested tryptophan, the major part of tryptophan is utilized in protein synthesis and catabolism proceeds to give kynurenine and 3-hdroxyanthranilic acid [15,16]. Serotonin synthesis in brain is controlled by mechanisms that activate or inhibit tryptophan hydroxylase (the rate limiting enzyme in the 5-HT biosynthetic route). Calcium-induced phosphorylation renders the enzyme inactive, while an intra-neuronal serotonin pool inhibits it through negative feedback mechanism. Irregular variation in tetrahydrobiopterin concentrations could also be involved in the regulation of tryptophan hydroxylation roles and actions [17].

Serotonin and 5-HIAA are mainly excreted in free forms whereas the minor catabolic product of serotonin; 5-hydroxytryptophol is predominantly excreted as a conjugate. As a potent vasoactive amine, serotonin in circulation is almost completely confined to platelets [12] and thus functionally rendered inactive.

Elevated plasma serotonin are hazardous, many rapid elimination mechanisms have evolved to clear excess 5-HT. Platelets possess an active serotonin reuptake system [18], the liver catabolises serotonin, pulmonary endothelial cells take up serotonin while some macromolecules binds free serotonin. Liver is also involved in the clearance of excess serotonin in the plasma with subsequent formation of 5-HIAA.

1.6 Stability of serotonin

Serotonin is well known to be an unstable compound, it degrades almost completely if not properly and carefully treated. For example 5-HT decomposes at high temperature and at acidic pH, but at pH 6 it is stable up to 35°C [19]. Serotonin can be preserved in EDTA because the compound chelates metal ion thus prevents decomposition. In a broader sense, the instability of serotonin can be circumvented by employing several pre-analytical

considerations such as immediate refrigeration after sample collection, avoidance of repeated thawing and freezing, acidification (pH>2), and addition of antioxidants such as ascorbic acid, perchloric acid, sodium metabisulfite, L-cysteine, EDTA etc.

It has been reported that the stability of serotonin and 5-HIAA is poor in acidic medium that only contain Na_2EDTA [19]. Markedly improvement can be noticed with the addition of L-cysteine and ascorbic acid. It had been observed that the ability of Na_2EDTA to complex metal ions diminishes at pH below 5 and the strength of ascorbic acid to act as antioxidant diminishes at lower pH, since the protonation reduces its ability to become oxidized.

2. THEORETICAL BACKGROUND

2.1 Analysis of serotonin by chromatography

There has been an increased interest in serotonin chemistry. This interest could be attributed to the increased availability of HPLC methods for research and routine studies. A considerable number of articles published in the mid-80s employed this technique with different detections in the study of serotonin in biological matrices. Analyses of serotonin and its major metabolite, 5-hydroxyindole-acetic acid (5-HIAA) are indispensable for the study of their pathophysiological roles. While serotonin is more evident to emotional related routine measurements, its metabolite, 5-HIAA is more often measured in the assessment of carcinoid tumours. Compared to other biogenic amines such as catecholamines and histamine, serotonin can be said to be recently discovered. Studies have elucidated its main function significance in recent years.

The presence of serotonin in tissues is still a subject of investigation [20] and such investigation will not have been possible without the development of accurate, reliable, precise, and sensitive methods for the analyses of serotonin and its metabolites in biological fluids. Several analytical methods have been applied in quantitative and qualitative determination of serotonin in various derivatives of blood. An overview (Table 1) of the different methods for the analysis of serotonin in human plasma revealed that 33.3% of the reported articles used HPLC with fluorometric detection, 40.4% electrochemical detection, 10.1% employed radioimmunoassay techniques, while only 1.01% used gas chromatography and 6.06% used other types of assays such as electrochemical sensors and biosensor based techniques. It is evident from (Table 1) that electrochemical detection is the most popular method for serotonin analysis in plasma [21, 22].

Table 1. Overview summary of the analysis of plasma serotonin using different analytical techniques and their respective detected concentrations of serotonin in (nmol/L).

Method	Conc.	Year	Ref no.
Other	11	1954	70
Spectrophotoflurometric	79.5	1962	71
Spectrophotoflurometric	114	1963	72
Spectrophotoflurometric	73.8	1965	73
HPLC (fluorometric)	34.1	1968	74
Spectrophotoflurometric	114	1975	75
Immunoassay	36	1976	76
HPLC (fluorometric)	40.3	1979	77
Radioenzyme	38	1979	78
Other	100	1979	79
HPLC (fluorometric)	179	1979	80
HPLC (fluorometric)	99.6	1981	81
Radioenzymatic assay	22.6	1981	82
Immunoassay	9	1982	24
Radionenzymatic assay	12	1984	25
HPLC (EC)	44	1984	83
HPLC (EC)	15.9	1985	84
HPLC (fluorometric)	160	1985	85
HPLC (fluorometric)	10.8	1985	86
HPLC (fluorometric)	13.6	1985	87
HPLC (EC)	5	1986	88
HPLC (fluorometric)	3.3	1987	89
HPLC (EC)	14.6	1987	90
HPLC (EC)	9.1	1988	91
HPLC (fluorometric)	4.86	1988	92
HPLC (EC)	13.9	1988	93
HPLC (EC)	14	1988	94
HPLC (EC)	5.28	1988	95
HPLC (fluorometric)	10.2	1989	96
HPLC (EC)	30.1	1989	97
HPLC (EC)	8.51	1989	98
HPLC (EC)	15	1989	99
Radioenzymatic assay	2	1990	100
Radioenzymatic assay	2.5	1990	101
HPLC (EC)	26.1	1990	102
Other	66	1990	103
HPLC (EC)	2.2	1990	104
HPLC (fluorometric)	4.31	1990	105
Immunoassay	19	1991	106
HPLC (EC)	31	1991	107

HPLC (EC)	141	1992	108
HPLC (fluorometric)	28	1992	109
Radioenzymatic assay	2.8	1992	110
HPLC (fluorometric)	17	1992	111
HPLC (EC)	119	1992	21
Radioenzymatic assay	2.6	1993	112
HPLC (EC)	6.7	1993	113
HPLC (fluorometric)	18.5	1993	114
GC-MS	0.77	1993	115
HPLC (EC)	94.3	1993	116
HPLC (EC)	2.8	1993	117
Radioenzymatic assay	3.4	1994	118
HPLC (fluorometric)	39.7	1994	119
HPLC (fluorometric)	61	1995	120
Radioenzymatic assay	1.05	1995	121
Radioenzymatic assay	0.6	1995	122
Other	9.08	1995	123
Other	14.2	1995	124
HPLC (EC)	14	1996	125
HPLC (fluorometric)	15.5	1997	126
HPLC (fluorometric)	3.5	1998	127
HPLC (EC)	16.85	1998	128
HPLC (EC)	14.6	1998	129
HPLC (EC)	39.6	1998	130
HPLC (EC)	10.8	1999	131
Spectrophometry	54.2	1999	132
HPLC (EC)	161	1999	133
Radioenzymatic assay	9.6	2000	134
HPLC (EC)	11	2000	135
HPLC (fluorometric)	16.9	2000	136
HPLC (EC)	3.52	2000	137
HPLC (fluorometric)	32.9	2000	138
HPLC (EC)	32.3	2003	139
HPLC (EC)	3.73	2003	140
HPLC (fluorometric)	25.5	2003	141
HPLC (fluorometric)	5.7	2004	142
HPLC (fluorometric)	6	2004	143
HPLC (EC)	42.6	2004	144
HPLC (EC)	26	2004	145
HPLC (EC)	9.8	2004	146
Immunoassay	2.6	2004	147
HPLC (fluorometric)	1.8	2005	148
HPLC (EC)	33	2005	149
HPLC (EC)	15	2005	150

HPLC (fluorometric)	13.1	2006	151
HPLC (fluorometric)	21.3	2006	152
HPLC (EC)	33.5	2006	153
HPLC (EC)	90.8	2006	154
Immunoassay	50	2006	155
Immunoassay	10	2007	156
Other	10.1	2007	157
HPLC (EC)	90	2007	158
HPLC (EC)	63.1	2007	159
HPLC (fluorometric)	10.2	2007	160
HPLC (fluorometric)	12.7	2007	161
Immunoassay	40	2008	162
Immunoassay	0.63	2008	163
HPLC (EC)	44	2009	164
Immunoassay	12.3	2010	165
LC-MS/MS	4.6	2010	53

However, fluorometric methods for serotonin determination are not specific and are preceded by poor separations from interfering compounds (poor selectivity), while radio-isotopic method are outrageously expensive to be employed in routine analysis. Thin layer chromatography [23], radio immunoassay [24, 25], enzyme immunoassay [26], gas chromatography mass spectrometry [27-31], HPLC with UV [32], electrochemical detection (EC), [33], and mass spectrometry [34] all have been employed in the determination of serotonin and consequently other related indoles and biogenic amines in biological fluids. Although different kinds of detectors have been applied in conjunction with the HPLC, EC detectors are the most popular [35-38]. Notwithstanding the popularity of HPLC with EC detectors, liquid chromatography mass spectrometry either single or tandem have been successfully employed in the determination of 5-HT in a wide range of biological fluids or tissues [39-48].

In addition, the use of Liquid chromatography tandem mass spectrometry (LC-MS/MS) for determining serotonin in other body fluids are widely reported but to the best of our

knowledge there are few reported validated method using the LC-MS/MS technique in determination of serotonin in plasma. Liquid chromatography tandem mass spectrometry has a wide application in the analyses and quantification of biological fluids; its robust nature cannot be over emphasized [49]. LC-MS has evolved into a technique characterized by sensitivity, selectivity, and specificity, allowing for the analysis of trace amounts of target analytes in complex mixtures such as biological fluids. LC-MS either tandem or single has been applied in determination of serotonin in wide variety of biological samples such as gut lavage fluid [50], tissues [39], plasma, whole blood [41], serum [49] urine [51, 52], brain and cell cultures including many others. Furthermore, it has been reported that the use of LC-MS/MS in the analysis of serotonin offers merits in relation to specificity and linear range, as well as permitting for simultaneous determination of its metabolites [41]. There are a number of clinical applications of LC-MS, the technique is more generally applicable than gas chromatography mass spectrometry (GC-MS); this is due to the broader range of biological molecules that can be analysed and more powerful use of LC separations in clinical laboratories. This trend of preferring LC-MS over LC with conventional detectors could be traced to the high specificity and its ability to handle complex mixtures.

Moreover, it is generally supposed that the highly specific nature of LC-MS/MS allows the use of short chromatographic run time, minimal sample clean-up and devoid of derivatisation which is often the case with GC. However, as the demand continues to grow for the analyses of drugs in body fluids, the most common preferred chromatographic technique is gas chromatography; it permits efficient separation with sensitive detection of the analytes in complex matrices.

2.2 Mass spectrometry and chromatography

Mass spectrometry (MS) is an analytical technique that produces spectra of the masses of the atoms or molecules of a sample. The spectra are employed to determine the elemental or isotopic identity and composition of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules, such as serotonin [53] and wide variety of chemical compounds. An MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. A diagram of tandem mass spectrometry is show in Figure 7.
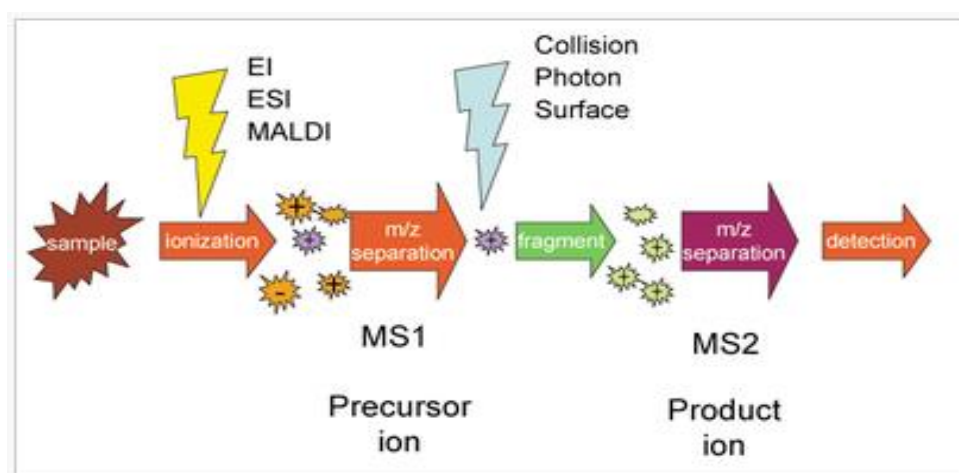
Figure 7. Schematic of tandem mass spectrometry

(Source: http://en.wikipedia.org/wiki/Tandem_mass_spectrometry)
A sample is injected into the mass spectrometer, ionized and accelerated and then analyzed by mass spectrometry (MS1). Ions from the MS1 spectra are then selectively fragmented and analyzed by mass spectrometry (MS2) to give the spectra for the ion fragments.While the diagram indicates separate mass analyzers (MS1 and MS2), some instruments can utilize a single mass analyzer for both rounds of MS.

MS are usually classified on the basis of how the mass separation is accomplished, but they all can be described as ion optical devices which separate ions according to their mass-to-charge (m/z) ratios by utilizing electric and/or magnetic force fields.

The concept of MS is to form ions from a sample, to separate the ions based on their m/z ratio (this can be considered to be the same as the mass because the ion has only a single charge in

most cases), and to measure the abundance of the ions. In modern MS instrumentation, all of the functions (ionization, separation of the ions, rate of data acquisition, detection of the ions, and storage of the data) are under computer control. Gaseous molecules are ionized in the ion source to form molecular ions in which some will fragment. By various processes, ions of differing m/z values pass through the mass analyzer once at a time to reach the detector. When the ions strike the detector, they are converted into an electrical signal, which in turn is converted into a digital response that can be stored by the computer. Furthermore, powerful new technologies of ion-analyses (tandem MS, time-of- flight MS, ion-trap MS) substantially increased the capabilities of MS analyzers with respect to specificity and to the extent of data read out. These developments suggest a more widespread use of MS techniques superior to other analytical methods in routine laboratory medicine. The knowledge of the m/z of the ions enables one to determine what is present, while the measured ion intensities answer the question of how much is present. Only ions are detected in mass spectrometer and any nonionic particles that have no charge are removed from the mass spectrometer by the continuous pumping that maintains the vacuum. The effluents from the chromatographic column are introduced into the mass spectrometer and it then enters the ionisation chamber through the capillary to which a charge is applied. The charge droplets emerge from the tip of the capillary and the charge to volume ratio increases as the droplets evaporate. The positive charges are repelled and free proton-adducts of the molecules emerge. The adducts are selected in an electromagnetic field in the first quadrupole according to their mass to charge ratio (m/z). From there they enter the second quadrupole, where they are collided with gas molecules, usually nitrogen, and thus fragment to form product ions, also called daughter ions. Consequently, the abundant product ion is selected in the third quadrupole according to its m/z ratio and allowed to reach the detector.

Furthermore, since tandem mass spectrometry is a selective method, several ions may be measured even if they are not distinctly chromatographically separated; this is achieved by monitoring several molecular transitions intermittently, also called multiple reactions monitoring (MRM). The amount of analyte in a sample may not correlate directly with the ion-current intensity of its mass spectrometric signal.

2.2.1 Low pressure chromatography

Chromatography systems are often defined by their pressure characteristics, acronyms such as LPLC and HPLC are used to refer to low-pressure and high performance liquid chromatography respectively. Low pressure chromatography operates at a pressure <50psi (~3bar), thus they are often used for sample purification and simple protein separations that do not require high resolutions. Low pressure chromatography techniques are time consuming, their retention times are excessively long (over 30 minutes for 5-HT/5-HIAA), they exhibit poor limit of detection and thus may not be suitable for a routine analysis because of the poor sample through-put, also such techniques are often encompassed by poor sensitivity and thus unreliable [54-56]. Low pressure chromatography systems can only force liquid along flow path provided the pressure capabilities of the systems exceeds the resistance or back pressure after the pump. However, LPLC has some advantages over HPLC in that the tubing and pumps used in low pressure chromatography are easy to clean or replace and thus clogging the pump and cross contamination are often not a source of worry. They are inherently easier to use and maintain and are less prone to licking etc.

2.2.2 High performance liquid chromatography

HPLC is an improved form of column chromatography that is used to separate, purify, identify and quantify chemical compounds. Chromatography uses the principle of the differences in surface interaction between analytes and eluent molecules to separate compounds within one sample. In HPLC system, the analytical instrument composes of two

phases. The stationary phase (absorbent) is the solid support situated within the column often packed with silica bonded hydrocarbons. The mobile phase (eluent) refers to the solvent which is continually added to the column. When injecting a sample into the instrument it will migrate through the column according to its affinity to the mobile and stationary phases, this means compounds that have less strong affinity to the mobile and stationary phases will elute faster than compounds that have stronger affinity to the phases. In HPLC system the continuously applied pressure ensures that the analytes and the mobile phase are forced through the densely packed column. A HPLC block diagram is shown in Figure 8.
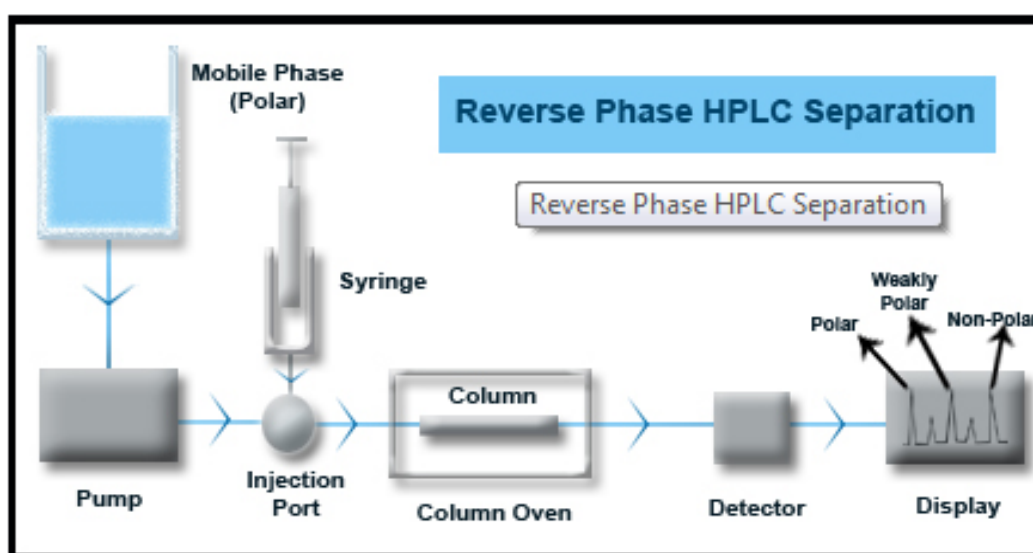
Figure 8. Set up of a reverse phase HPLC system showing the pump, injection port, column, detector and a read out (display) system.

(Source: http://www.lab-training.com)

The majority of HPLC applications are covered under reversed phase chromatography. Stationary phases mostly comprise of non-polar alkyl hydrocarbons such as C-8 or C-18 chains bound to silica or other inert supports. Mobile phases maybe polar or weakly polar and the elution order of the compounds is polar followed by less polar or weakly polar and non-polar compounds in the end.

2.3 Sample preparation/extraction methods for serotonin in plasma

There are several clean-up procedures during the analysis of biological fluids. This includes the selective clean-up of sample by selective removal of all endo- and exogenous compounds in the matrix that could clog the chromatographic column or interfere with the separation of the analyte of interest. Several sample preparation techniques have been used for clean-up of complex matrices in plasma and other bio-fluids. Such techniques include solid phase extraction (SPE) [57-59], protein precipitation [60], liquid-liquid extraction (LLE) as well as online solid phase extraction (SPE) [49]. In most bioanalytical methods, the sample preparation is usually done by liquid-liquid extraction or solid phase extraction.

LLE is a method used to separate compounds based on their solubility in two immiscible liquids, usually water and organic solvents; thus, it is an extraction of a substance from one liquid into another liquid. LLE is highly selective, depending on the choice of the solvent and pH, the analytes can be extracted from most of the endogenous compounds and recoveries can be improved by successive extractions. On the other hand, SPE is a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties; with SPE technique many of the drawbacks associated with LLE can be avoided, such as incomplete phase separation, poor recoveries, and use of expensive fragile glassware as well as use of large quantities of organic solvents. SPE is more efficient than the LLE; it is more rapid and can be automated. However, both of these methods are laborious and tedious and often imprecise. Thus, since the present work also aims at developing simple and reliable sample preparation techniques for the analysis of serotonin and its metabolite, 5-hydroxyindole-acetic acid, it is therefore, particularly important to consider sample preparations that is less laborious, but still efficient. Protein precipitation with miscible organic solvents (such as acetonitrile) has been successfully employed in preparation of plasma samples because of its

low cost and minimal method development requirements. This method involves the addition of organic solvent, vortex mixing and subsequent centrifugation to separate the resultant protein precipitates from the analytes and it provides sufficient clean-up for LC-MS/MS analyses.

2.4 Theory on internal standard

Internal standard (IS) is a chemical substance with very similar but not necessarily identical physicochemical properties to the analyte and it provides several advantages in the analysis of biomolecules by liquid chromatography tandem mass spectrometric (LC-MS/MS). It is often added in a constant amount to the samples in a chemical analysis to aid in quantification, this is also known as spiking. It is very advantageous in that: it improves intra injection reproducibility, it corrects the loss of analyte during sample preparation and it markedly reduces matrix interferences and ionization effects. An IS can be a structural analogue or stable labelled internal standard but the latter is often preferred in bioanalysis.

During chromatographic quantitative analysis using the IS method, the analyte concentration can be determined using the response factor (the ratio of the sensitivities of the analyte and the internal standard). The IS needs to provide a signal that is similar to the analyte in most ways but markedly distinguishable by the instrument. It is believed that stable labelled internal standard yield better assay performance in bioanalytical liquid chromatography mass spectrometry assays. Internal standard with similar chemical properties as the analyte may cover up assay problems with stability, recovery, and ion suppression. However, stable labelled internal standards are not always available and are very expensive; thus structural analogues can also be used as IS.

Using IS in quantitative bioanalysis by liquid chromatography tandem mass spectrometry is mainly aimed to compensate for matrix/measurement effects [61].

Mass detection unarguably represents unequalled sensitivity and selectivity and it is thus a clear candidate for LC-based quantitative assays. Quantitative detection is often complicated by effect of matrix interferences, for example in plasma or urine constituents. When an analyte is introduced into the ion source it will compete with other compounds introduced into the ion source simultaneously. The matrix components decreases the analyte signal, this is known as ion suppression, most notably in electrospray ionisation (ESI)-based MS detection as used in this thesis. The extent of ion suppression will largely depend on the chemical structure of the analyte of interest; which entails that if an analyte and IS are not structurally similar, the ratio of analyte and IS detectors responses may vary due to different degrees of ion suppression, thereby compromising the quantitation.

In HPLC analysis a solution of a known analyte ([A]) and internal standard ([IS]) concentration is often first run under certain column conditions. The ratio of the detector signal of the analyte (S_A) and the internal standard (S_{IS}) is calculated to measure the response factor (RF) using Eq.1. Subsequently a known concentration of the internal standard and the calculated RF are used to determine the concentration of the unknown analyte by solving [A] in (Eq.1).

$$RF = \frac{[IS]}{[A]} \times \frac{S_A}{S_{IS}} \qquad\qquad Eq.\ (1)$$

2.5 Experimental design and optimisation

Experimental design and optimization are tools that are used to systematically examine different types of problems that arise within research, development and production. Experimental design such as response surface and factorial design are multivariate approach suitable for development of analytical methods. Experimental design is a powerful tool to reduce large number of data to manageable size to examine the effects of experimental

variables and their interactions and to optimise simultaneously methodological and/or instrumental conditions.

2.5.1 Doehlert design

Doehlert design is a type of multivariate experimental design where different factors are studied at different number of levels. It is often referred to as a uniform shell design in that there is a regular distribution of experimental points on the surface of the spherical shells. A diagram of Doehlert uniform shell design for two variables is shown in Fig. 9.
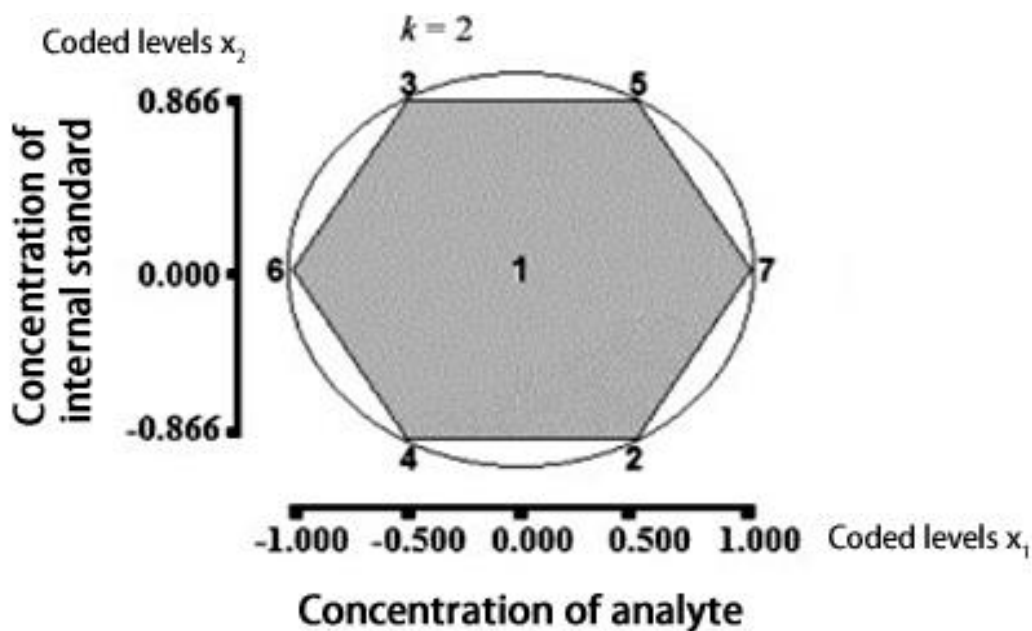
Figure 9. Spatial distribution of experimental points in a two-factor Doehlert uniform shell design, [62]

The design displays an equally spaced distribution of experimental points circumscribed to a sphere of radius 1, in this way the variance of the estimated response is the same at all points on the sphere centered at the origin [62]. For a number of K factors, the total number of experiments to be measured is given by $k^2 + k + 1$; hence the total number of experiments for two variables is 7.

The two-factor Doehlert design (Fig. 9), is a hexagon with vertices 2, 3, 4, 5, 6 and 7 and a central point 1, The hexagon in question define five levels (-1.00, -0.50, 0.00, +0.50, +1.00) for factor 1 (X_1) and three levels (-0.866, 0.00, +0.866) for factor 2 (X_2).

Doehlert uniform shell design is an appropriate tool to model the response factor (RF) behaviour as a function of the concurrent variation of the concentrations of internal standard and analyte [63]. Such model can be a first (Eq. 2) or second (Eq. 3) order polynomial function.

$$RF = b_0 + b_1[A] + b_2[IS] + b_3[A][IS] \qquad \text{Eq. (2)}$$

$$RF = b_0 + b_1[A] + b_2[IS] + b_3[A][IS] + b_4[A]^2 + b_5[IS]^2 \qquad \text{Eq. (3)}$$

where b_0 is the intercept

b_1 and b_2 - the linear term coefficients

b_3 - the interaction coefficients of the analyte [A] and [IS].

b_4 and b_5 represents the effect of the second order coefficients.

2.6 Matrix effects

Matrix effect is the combined effect of all components of the sample other than the analyte to be quantified. This often affects the selectivity of a bioanalytical techniques and it is frequently the case when using liquid chromatography tandem mass spectrometry in bioanalysis. In order to minimize matrix interferences in LC-MS/MS a number of approaches can be applied. Such approaches can be instrumental modifications or method improvements, such as extraction modification, ionisation switching as well as monitoring the elution solvents.

Matrix effect is not solely unique to LC-MS/MS detectors but has also been observed in other detectors such as fluorescence [64] and electron capture techniques.

Other detectors used in gas chromatography can cause matrix irreproducibility, for instance flame dampening caused by lipids and some plasma constituents has been observed in flame photometric (FPD) or mass spectrometry (MS) detectors in GC [65]. Thus matrix effects may not be solely related to the interferences during ionisation process of LC-MS/MS or sample preparation and extraction but should be well-thought-out when developing new analytical methods in which reproducibility, robustness, limit of detection and quantification as well as other analytical parameters are of paramount importance.

2.7 Theory on method validation

Method validation encompasses the procedures of establishing the performance characteristics and limitations of a method and the identification of the influences which may change these characteristics and to what extent. Specifically, according to the International Standard Organisation (ISO/IEC 17025), method validation is the process of proving that an analytical method conforms and fulfills the requirements for the intended use. It is aimed at demonstrating that the procedure when correctly applied, produces results that are fit for purpose [66].

The analytical parameters that are often considered in method validation include: accuracy, precision, repeatability, intermediate precision, specificity, detection limit, quantitation limit, linearity and range.

2.7.1 Accuracy

The accuracy of an analytical procedure is defined as the closeness of agreement between an accepted conventional true value or an accepted reference value and the experimental value. This is often referred to as trueness.

There are different approaches used to assess accuracy of an analytical method, these technique includes but not limited to (i) comparing the result of the analytical measurement with a certified reference value, this approach assumes that the uncertainty of the reference method is known; (ii) analysing a sample of known concentration and comparing the results of the analytical method in question with an established reference method; (iii) when the certified control samples are not available, a blank matrix of interest can be spiked with a known concentration of the analyte and the percentage of recovery is calculated; (iv) use of the standard addition method [67-69].

Furthermore, following the extraction of the analyte from the matrix and subsequent submission to the analytical instrument, its recovery can be determined by comparing the calculated ($C_{calculated}$) and nominal ($C_{nominal}$) concentrations by using the following expression [68].

$$Recovery = \frac{c_{calculated}}{c_{nominal}} \times 100 \qquad\qquad \text{Eq. (4)}$$

2.7.2 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the same analytical conditions. The International Conference on Harmonization (ICH) has defined precision to contain three components: repeatability, intermediate precision and reproducibility.

Precision of an analytical method can be determined based on standard deviation (SD), relative standard deviation (RSD) also known as coefficient of variation (CV), or Variance (SD^2) of the analytical measurement results.

The equations used for determining the SD and CV parameters are:

$$\boldsymbol{SD} \; = \sqrt{\frac{\sum_{i=0}^{n}(xi-\bar{x})^2}{n-1}} \qquad\qquad \text{Eq. (5)}$$

$$\boldsymbol{\bar{x}} \; = \frac{\sum_{i=0}^{n} xi}{n} \qquad\qquad\qquad \text{Eq. (6)}$$

$$\boldsymbol{CV} \; = \frac{SD}{\bar{x}} \times 100 \qquad\qquad\qquad \text{Eq. (7)}$$

where $\bar{x}$ is the mean value, n is the number of measurements.

2.7.3 Specificity/selectivity

Specificity/selectivity refers to the ability of an analytical method to differentiate the analytes or the internal standard from closely related endogenous matrices. According to recommendation of the International Union of Pure and Applied Chemistry (IUPAC), the term selectivity is defined as the extent to which an analytical method can determine particular analytes under given conditions in mixtures or matrices, simple or complex, without interferences from other components. Specificity is the "ultimate" of selectivity [68, 69]. Notwithstanding, the term specificity entails that there is no interference or that there is 100% selectivity.

There are several approaches to assessing the selectivity of a method: (i) comparison of chromatogram of a blank sample with and without spiking known analytes; (ii) analysis of standard or certified reference material; (iii) comparison of the chromatographic response of the sample with or without matrice interference [68].

Specificity in liquid chromatography is obtained by selecting optimal columns and setting chromatographic conditions, such as eluent composition, column temperature and detector wavelength.

2.7.4 Linearity

The linearity of an analytical method is its ability to obtain a test result which is directly proportional to the concentration of analyte in the same sample within a given range. Linearity is generally demonstrated by using a series of standards (dissolved in pure solvent or spiked in a blank sample) at a minimum of five levels of concentrations, determining the intensity of the signals generated by the standards, constructing the signal versus concentration curves and performing regression and statistical analysis to judge whether or not the linearity over the studied range is fulfilled. Validation of linearity is often regarded as a verification of the assumed response function without or with an acceptable deviation. It is primarily performed by means of graphical evaluation of the experimental data from the assumed response model (residual analysis), also called residual plots. For linear ranges, the deviations should be equally distributed between positive and negative values when the residual are plotted. Linearity is expressed mathematically as:

$$y = mx + b \hspace{4cm} \text{Eq. (8)}$$

where y is the analytical response, x is the amount (or concentration) of analyte, m is the sensitivity (or slope of the calibration curve), b is the intercept of the calibration curve (error of measurement of the dependent variable - y).

There are statistical established means of determining each of the defined parameters above based on least square method of linear regression analysis:

$$m = \frac{n\sum xiyi - \sum xi \sum yi}{n\sum(xi^2) - (\sum xi)^2}$$ Eq. (9)

$$b = \frac{\sum(xi^2)\sum yi - \sum xi \sum xiyi}{n\sum x^2 - (\sum xi)^2}$$ Eq. (10)

$$r = \frac{n\sum xiyi - \sum xi \sum yi}{\sqrt{[n\sum xi^2 - (\sum xi)^2][n\sum yi^2 - (\sum yi)^2]}}$$ Eq. (11)

The correlation coefficient (r) is often used to explain linearity, and an analytical method is most times reported as being linear if r^2 is close to unity. The term is used as a measure of the strength of the relation between the dependent and independent variables. In other words it measures the relationship between a concentration and the signal. However, low r does not necessarily mean that there is no relationship between the variables, just that it is not a linear relationship.

2.7.5 Range

The range of an analytical method is the interval between the upper and lower values that is proven to have a suitable level of precision, accuracy, and as well demonstrate to be linear using a particular analytical method. The range must be expressed in the same unit as the test results.

2.7.6 Limit of detection and Limit of quantification

Limit of detection (LOD) is the lowest concentration that can be measured (detected) but not necessarily quantified by a given analytical procedure with statistical significance. It is the point at which a measured point is larger than the uncertainty associated with it.

In chromatography, the detection limit is the amount injected that produces a peak with a height at least two or three times higher than the baseline or noise level. Limit of quantification (LOQ) is the smallest amount or the lowest concentration of a substance that can possibly be determined by means of a given analytical procedure with established accuracy, precision and uncertainty.

There are several approaches to determine the detection and quantification limits of an analytical method [68], such approaches include:

- ❖ Visual assessment: in this approach the detection limit is determined by analysing samples of known concentration and establishing the minimum level at which the analyte can be conveniently detected.
- ❖ Based on the standard deviation of the blank: this involves the measurement of the analytical background performed by analysing an appropriate number of blank samples and calculating the standard deviation of the responses.

By using a minimum of six blank analyses results, the limit of detection is expressed as the analyte concentration in relation to the sample blank value added to three times the standard deviation.

$$LOD \ = \ \bar{X} + 3(SD_{blank}) \qquad\qquad \text{Eq. (12)}$$

$$LOQ \ = \ \bar{X} \ + 10(SD_{blank}) \qquad\qquad \text{Eq. (13)}$$

where $\bar{x}$ is the mean concentration of the analyte in a sample, SD_{blank} is the standard deviation of the blank sample. However, there are other procedures to determining the LOQ other than using Eq.13, such as using 6 or $7xSD_{blank}$ with acceptable accuracy and precision.

❖ Determination from the calibration curve at low concentration, whereby a minimum of three concentrations near to the estimated quantification limits are analysed and linear regression of the points computed to determine the LOD and LOQ.

$$LOD = 3.3 \times \frac{SD_{res}}{m} \qquad\qquad \text{Eq. (14)}$$

$$LOQ = 10 \times \frac{SD_{res}}{m} \qquad\qquad \text{Eq. (15)}$$

where SD_{res} residual standard deviation

A graphical illustration of the analytical paramaters is show in Fig. 10.
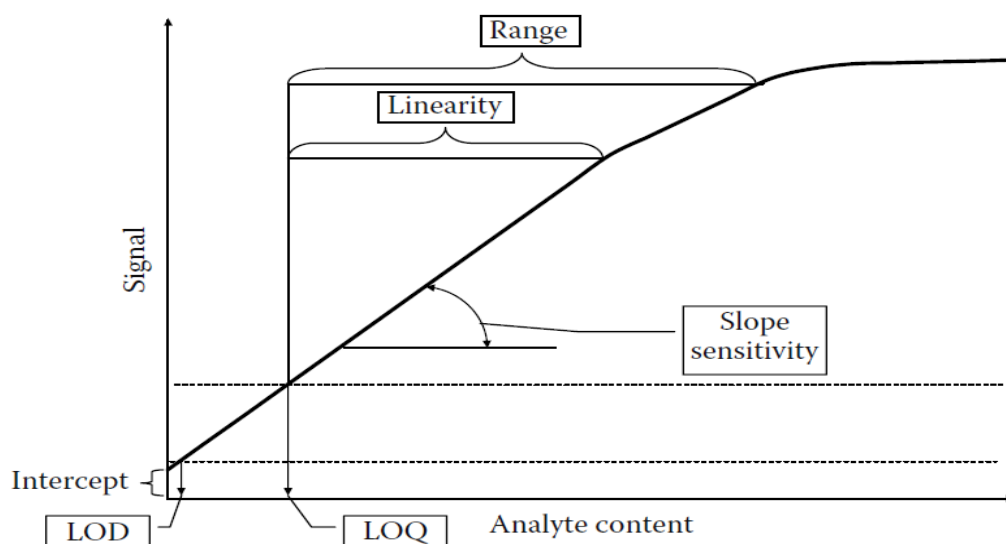
Figure 10. Graphical illustration of linearity, measuring range, limit of detection, limit of quantitation, and sensitivity [68].

3. METHOD DEVELOPMENT

3.1 Reagents

Serotonin hydrochloride (cat No. H9523) ≥ 98% purity and internal standard 5-methoxytryptamine (Cat No. 286583) ≥ 98% purity, 5-hydroxyindole-3-acetic acid, ≥ 98% purity (CAS No.54-16-0) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Deuterated internal standard, 5-hydroxyindole-3-acetic-2,2-d_2 acid (CAS No. 56209-31-5) ≥ 99% purity was purchased from CDN Isotopes Inc. (Pointe-Claire Quebec, Canada). HPLC-grade acetonitrile and ethanol were from Merck (Darmstadt, Germany) and formic acid (≥ 98% purity) was from Fluka Chemie (Basel, Switzerland). De-ionised water was purified in a Milli-Q system (Millipore, Milford, MA, USA).

3.2 Plasma samples

Blank plasma used for developing and validating the method was from an anonymous donor. A set of 10 plasma samples from an anonymous clinical biobank involving pathological patients subjected to a diet rich in vegetables (Cancer and Diet Project Code CV-2012-2013) were analysed by using the validated method. All plasma samples considered in this thesis were collected in EDTA tubes and stored in -80˚C prior to use. Patient written consent and Ethical approval from Research Ethics Committee were obtained prior to the study.

3.3 Analytes extraction and optimisation

Blank plasma sample (approx. 15mL) from an anonymous donor was used for the analysis carried out during the development of the extraction method. From the plasma samples 5mL was spiked with 500µg/mL of the analytes (5-HT and 5-HIAA) and kept at -80˚C until further experiments.

3.3.1 Selection of extraction solvents

Based on literature search, protein precipitation was proposed as the most suitable, simple and rapid method to be employed in the extraction of the analytes for a selective LC-MS/MS method analysis. During the development of the extraction protocols, the first step was the selection of suitable solvent (s) composition to extract 5-HT and 5-HIAA from the plasma samples. The choice of solvents to be evaluated was based on published articles using protein precipitation techniques. The commonly applied solvents are acetonitrile (ACN), methanol, water formic acid and their mixtures. A pseudo 3-dimensional chart showing several extraction trials is shown in Fig. 11.
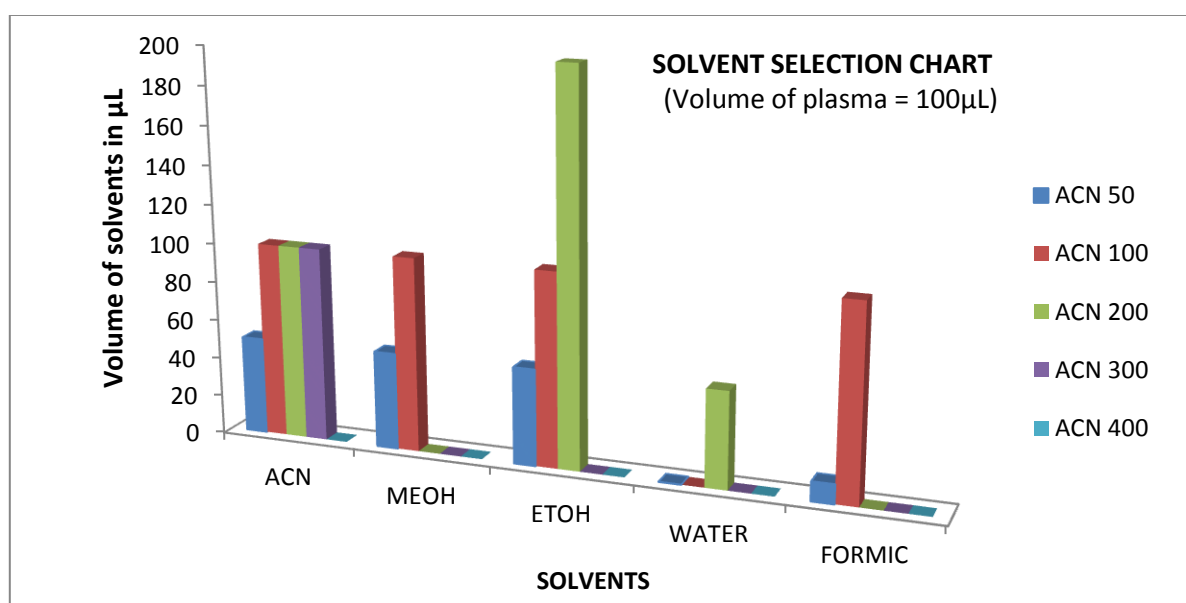

Figure11. A chart of the solvent composition trials.

(MEOH=methanol, ACN=acetonitrile, ETOH=ethanol, Formic=Formic acid)

The preliminary experiments for selection of extraction solvents were carried out using blank plasma samples spiked with equal amounts of 5-HT and 5-HIAA (10μg/mL). The general procedures applied involves: test tubes containing 100μL plasma samples were treated with extracting solvents as shown in Fig. 11. At each step of the solvent addition, the test tubes were vortex mixed for approximately 30s then centrifuged for 5 min at 3500 rpm. The resultant supernatants were visually evaluated for their clarity. From the test tubes that provide clear supernatants, extracts were taken and submitted to LC-MS/MS, the resulting signals and clear separation of the monitored peaks were visually assessed. The remaining solutions were kept over-night to check if there would be further precipitation.

Furthermore, based on the obtained results the extraction solvent system yielding clear supernatant, well resolved peaks was selected as the optimal system for extracting 5-HT and 5-HIAA from human plasma.

3.4 Mobile phase selection and optimisation of the gradient system

The selection of the mobile phase was based on previous articles that employed gradient mode in the study of biogenic and monoamines. Acetonitrile, formic acid, water and methanol are the frequently most applied solvents in the study of such compounds.

Varying compositions of these solvents and/or their mixtures were tried randomly during the selection of the mobile phase gradient condition. Some of the solvent programs tried during the optimisation process are shown in Fig 12 (A-J). The chromatograms of the different conditions will be shown in section 4.2.

A
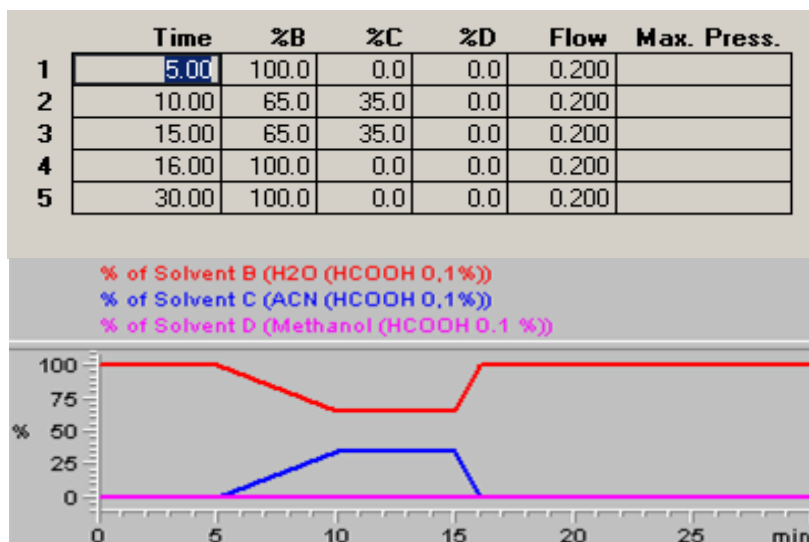
	Time	%B	%C	%D	Flow	Max. Press.
1	5.00	100.0	0.0	0.0	0.200	
2	10.00	65.0	35.0	0.0	0.200	
3	15.00	65.0	35.0	0.0	0.200	
4	16.00	100.0	0.0	0.0	0.200	
5	30.00	100.0	0.0	0.0	0.200	

% of Solvent B (H2O (HCOOH 0,1%))
% of Solvent C (ACN (HCOOH 0,1%))
% of Solvent D (Methanol (HCOOH 0.1 %))

(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=methanol, 0.1% formic acid) FR=0.2mL/min, CT=20˚C.(CT=column temperature, FR=flow rate).

B
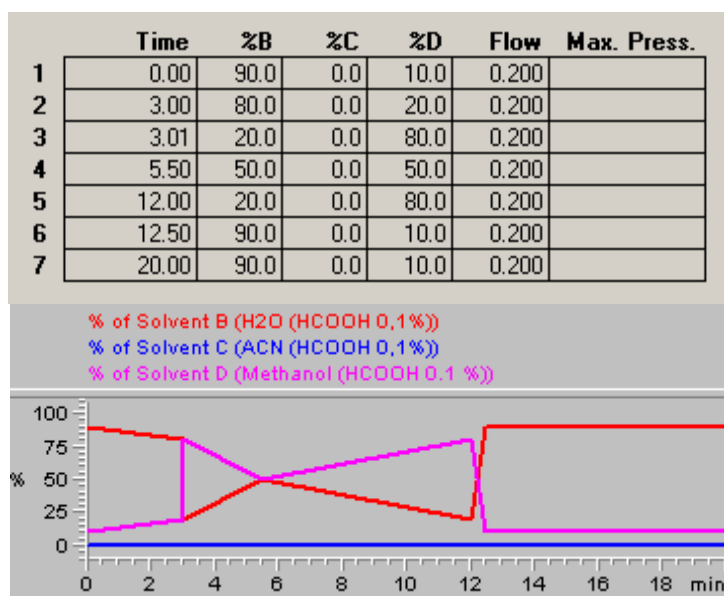
	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	90.0	0.0	10.0	0.200	
2	3.00	80.0	0.0	20.0	0.200	
3	3.01	20.0	0.0	80.0	0.200	
4	5.50	50.0	0.0	50.0	0.200	
5	12.00	20.0	0.0	80.0	0.200	
6	12.50	90.0	0.0	10.0	0.200	
7	20.00	90.0	0.0	10.0	0.200	

% of Solvent B (H2O (HCOOH 0,1%))
% of Solvent C (ACN (HCOOH 0,1%))
% of Solvent D (Methanol (HCOOH 0.1 %))

(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=methanol, 0.1% formic acid) FR=0.2mL/min, CT=20˚C.

C

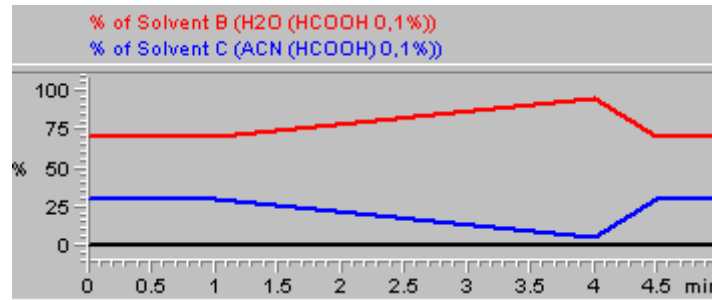|	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	70.0	30.0	0.0	0.200	
2	1.00	70.0	30.0	0.0	0.200	
3	4.00	95.0	5.0	0.0	0.200	
4	4.50	70.0	30.0	0.0	0.200	
5	5.00	70.0	30.0	0.0	0.200	

% of Solvent B (H2O (HCOOH 0,1%))
% of Solvent C (ACN (HCOOH) 0,1%))

(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid)
FR=0.2mL/min, CT=20˚C.

D

	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	80.0	0.0	20.0	0.500	
2	5.00	80.0	0.0	20.0	0.500	
3	15.00	17.0	65.0	18.0	0.500	
4	17.00	80.0	0.0	20.0	0.500	
5	40.00	80.0	0.0	20.0	0.500	

% of Solvent B (H2O (HCOOH 0,1%))
% of Solvent C (ACN (HCOOH 0,1%))
% of Solvent D (Ethanol)

(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=ethanol)
FR=0.5mL/min, CT=20˚C.

E
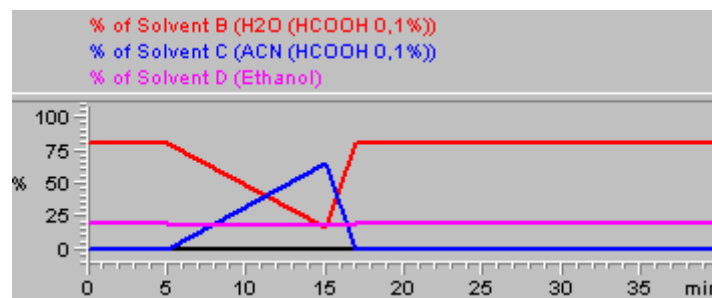
	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	80.0	0.0	20.0	0.200	
2	5.00	80.0	0.0	20.0	0.200	
3	12.00	17.0	65.0	18.0	0.200	
4	17.00	80.0	0.0	20.0	0.200	
5	20.00	80.0	0.0	20.0	0.200	

(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=ethanol)
FR=0.2mL/min, CT=20˚C.

F

	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	80.0	0.0	20.0	0.200	
2	5.00	80.0	0.0	20.0	0.200	
3	12.00	17.0	65.0	18.0	0.200	
4	17.00	80.0	0.0	20.0	0.200	
5	40.00	80.0	0.0	20.0	0.200	

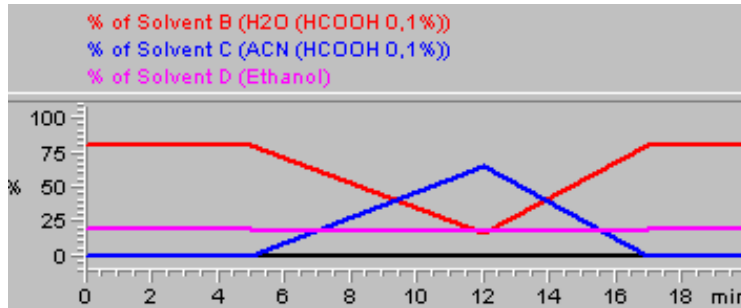(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=ethanol)
FR=0.2mL/min, CT=20˚C.

G

	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	80.0	0.0	20.0	0.200	
2	5.00	80.0	0.0	20.0	0.200	
3	10.00	17.0	65.0	18.0	0.200	
4	17.00	80.0	0.0	20.0	0.200	
5	40.00	80.0	0.0	20.0	0.200	

(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=ethanol)
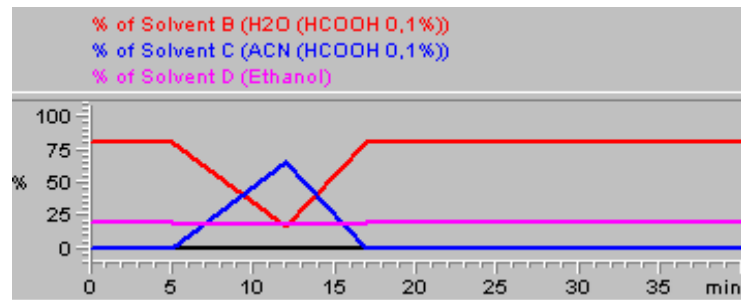FR=0.2mL/min, CT=20˚C.

H

	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	80.0	0.0	20.0	0.200	
2	5.00	80.0	0.0	20.0	0.200	
3	15.00	17.0	65.0	18.0	0.200	
4	17.00	80.0	0.0	20.0	0.200	
5	20.00	80.0	0.0	20.0	0.200	
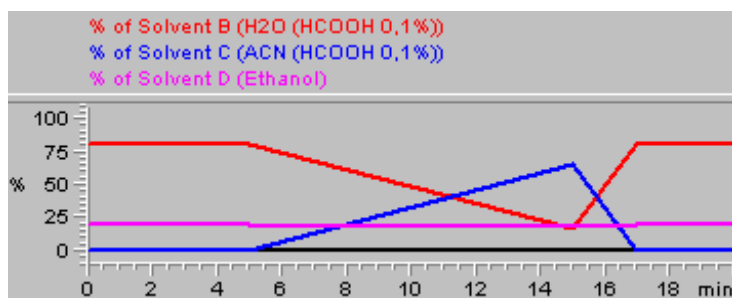
(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=ethanol)
FR=0.2mL/min, CT=20˚C.
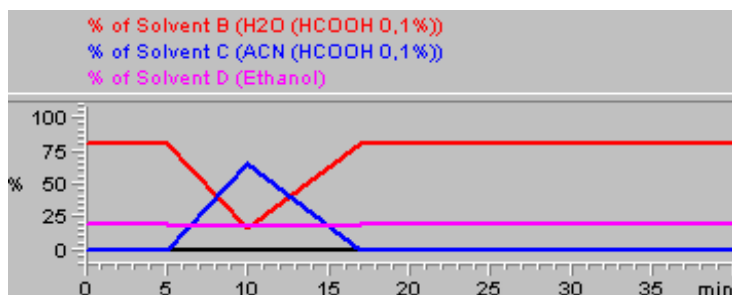
I

	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	80.0	0.0	20.0	0.500	
2	5.00	80.0	0.0	20.0	0.500	
3	15.00	17.0	65.0	18.0	0.500	
4	17.00	80.0	0.0	20.0	0.500	
5	40.00	80.0	0.0	20.0	0.500	

(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=ethanol)
FR=0.5mL/min, CT=20°C.

J

	Time	%B	%C	%D	Flow	Max. Press.
1	0.00	80.0	0.0	20.0	0.200	
2	5.00	80.0	0.0	20.0	0.200	
3	15.00	17.0	65.0	18.0	0.200	
4	17.00	80.0	0.0	20.0	0.200	
5	40.00	80.0	0.0	20.0	0.200	
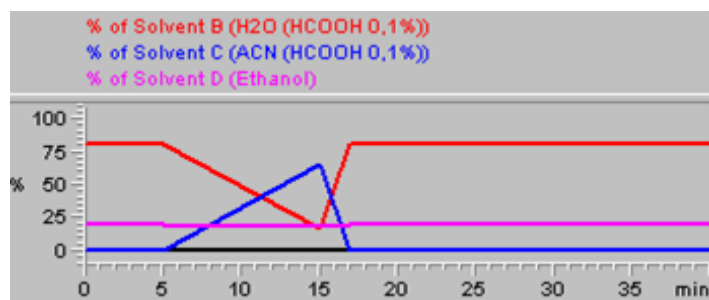
(B=water, 0.1%formic acid, C=acetonitrile, 0.1% formic acid, D=ethanol)
FR=0.2mL/min, CT=20°C.
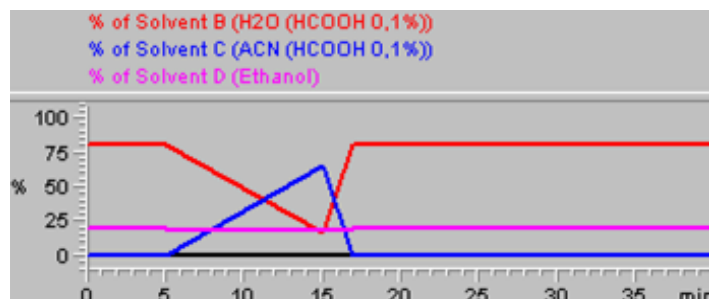
Figure 12 (A-J). Trials during the selection of gradient condition and other instrumental parameters.

During the course of developing the best gradient program and other instrumental optimisation parameters (flow rate and column temperature), injection volumes were studied from 30, 25, 20, 10, 5 and 3μL.

3.5 Optimising the addition of internal standard

During the course of developing the protocol for adding the internal standards to the blank plasma, two procedures of addition were studied. The first involves adding directly the solution of internal standard in ACN to the blank plasma samples and the second procedure involves drying completely the solvent (ACN) in the stream of Nitrogen before adding the blank plasma. The solutions of the two procedures were injected into the LC-MS/MS and the signals were obtained and compared. The chromatograms of the two prcedures will be shown in section 4.3.

3.5 Application of Doehlert design to select optimal amounts of internal standards.

In this work Doehlert uniform shell design was used to optimise the response factor in order to select an optimal amount of internal standard to be used in the method. An initial blank plasma sample containing 5-HT and 5-HIAA (100 µg/mL of each analyte) was diluted with blank plasma to 1.00, 13.25, 25.50, 37.70, and 50.00 µg/mL Furthermore, a stock of 100µg/mL of the internal standards (5-CH$_3$O-HT and d$_2$-5-HIAA) in acetonitrile was diluted with pure ACN to 4.30, 25.50, and 46.70 µg/mL for optimising the response factor applying Doehlert uniform design. The approriate extraction protocol was carried out and the resultant supernatants were submitted to LC-MS/MS analysis.

The response factor (RF) behaviour was studied and modelled using a Doehlert uniform shell design in which the concentrations of the analytes (5-HT and 5-HIAA) with their respective internal standards (5-CH$_3$O-HT and d$_2$-5-HIAA) were varied simultaneously (Table 2). In the design in question (Fig. 13), LC-MS/MS analysis data from the samples of seven experimental points in triplicates were used to develop models using regression analysis.

Table 2. Concentration of serotonin and 5-hydroxyindole-acetic acid and their respective internal standards in the plasma at each experimental point in the two-factor Doehlert design

Exp. no.	Coded levels x_1	Coded levels x_2	Natural levels (5-HT)	Corresponding IS (5-CH$_3$O-HT)	Natural levels (5-HIAA)	Corresponding IS (d$_2$-5-HIAA)
1	-1.00	0.000	1.00	25.5	1.00	25.5
2	-0.50	0.866	13.25	46.70	13.25	46.70
3	1.00	0.866	37.75	46.70	37.75	46.70
4	0.00	0.000	25.50	25.50	25.50	25.50
5	-0.50	-0.866	13.25	4.30	13.25	4.30
6	1.00	-0.866	37.75	4.30	37.75	4.30
7	0.50	0.000	50.00	25.50	50.00	25.50

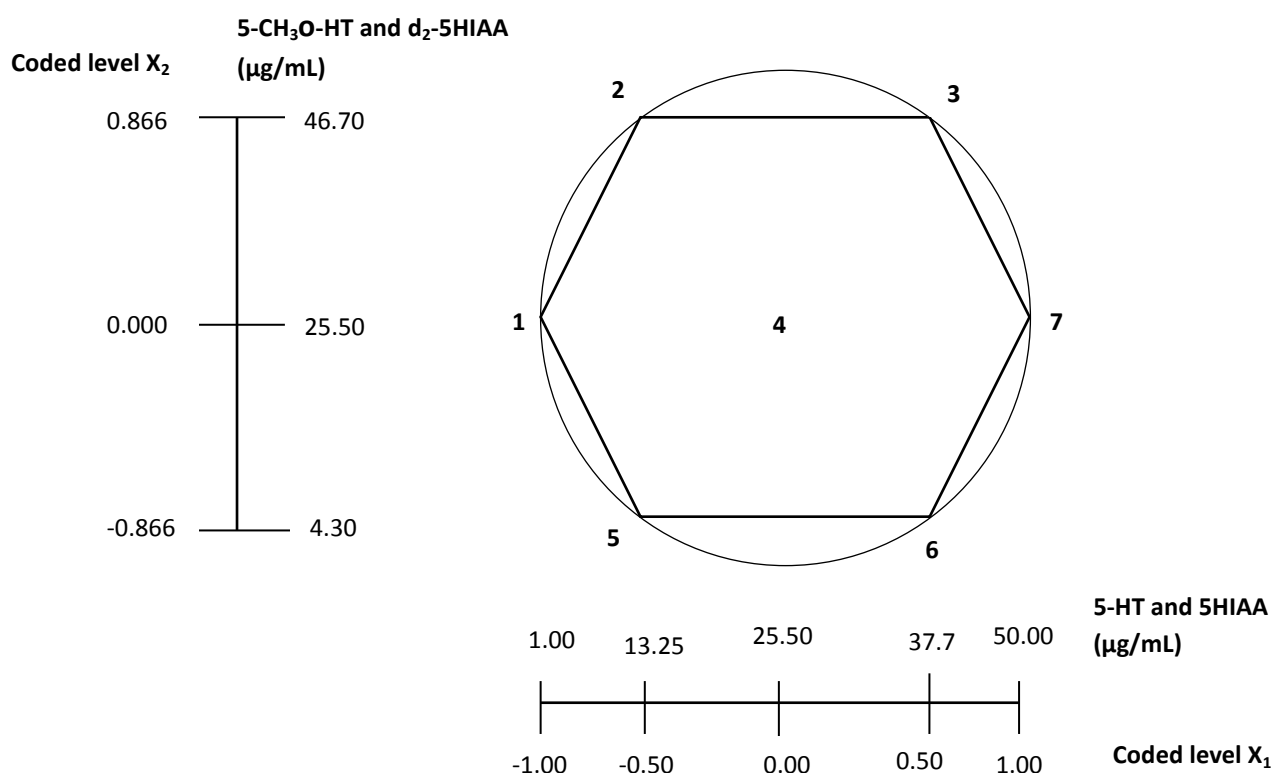Figure 13. Two-factor Doehlert design showing the coded and natural levels of the analytes and internal standard at each experimental point.

At each experimental point in the design the obtained peak area signal ratio of analyte and the internal standard was used to determine the response factor (RF) according to the expression in (Eq. 1). The RF was modelled based on the second order polynomial model (Eq. 3).

The adequacy of the developed models from the obtained data was evaluated by statistical approach that describes the variation of the obtained values to that of the predicted model; where by the variances of the lack-of-fit and pure error are estimated by dividing their summation with respective degrees of freedom. The variance ratio of the lack-of-fit error to that of pure error known as experimental F-value (F_{exp}) is used to conclude if the model fits the data by comparing with the theoretical F-value (F_{theo}). If the F_{exp} is lower than the F_{theo} (obtained from F-distribution table) it therefore means that the model is fitted. The procedure (Eq. 16-20) employed for calculating the F_{exp} involves: calculation of the experimental RF (RF_{exp}) according to (Eq. 1) and the average $\overline{(RF)}$ of each replicates were obtained. From the regression model, calculated RF (RF_{cal}) was obtained and the sum of residual error (v_{RE}), pure error (v_{PE}) and lack of fit (v_{LOF}) were calculated according to the following equations:

$$v_{RE} = (RF_{exp} - RF_{cal})^2 \qquad\qquad \text{Eq. (16)}$$

$$v_{PE} = (RF_{exp} - \overline{RF})^2 \qquad\qquad \text{Eq. (17)}$$

$$v_{LOF} = (RF_{cal} - \overline{RF})^2 \qquad\qquad \text{Eq. (18)}$$

$$\frac{\sum(RF_{cal} - \overline{RF})^2/(P-K)}{\sum(RF_{exp} - \overline{RF})^2/(W-P)} \qquad\qquad \text{Eq. (19)}$$

$$\frac{V_{LOF}/DF}{V_{PE}/DF} \qquad\qquad \text{Eq. (20)}$$

where V_{PE} is the variance of pure error, V_{RE} is variance of residual error, V_{LOF} is variance of lack-of-fit, K is the number of regression coefficients, N is the number of experiments, P is the number of the experimental points, and DF is the degree of freedom.

In the present work, the F-values were obtained at 97.5% confidence level.

3.6 Plasma sample protocol

The plasma was thawed at room temperature. 100 µL of plasma containing the analytes was added to eppendorf tubes, and 100 µL of acetonitrile (ACN) solution containing the internal standards (20 µg/mL) was added to the eppendorf tubes containing the plasma. The solution was vortex-mixed (Banderlin RK 100 ultra-mixer, Berlin Germany) for approximately 30s and centrifuged (Eppendorf AG centrifuge, Hamburg, Germany) at 3500 rpm for 5 min. The supernatants were collected and 100 µL of pure ACN was added to each collected supernatant. To completely precipitate the proteins and prepare the samples for LC-MS/MS analysis, the supernatants were again vortex-mixed and centrifuged at 3500 rpm for 5min.

A simplified and concise flow chart of the sample preparation protocol is shown in Fig. 14.
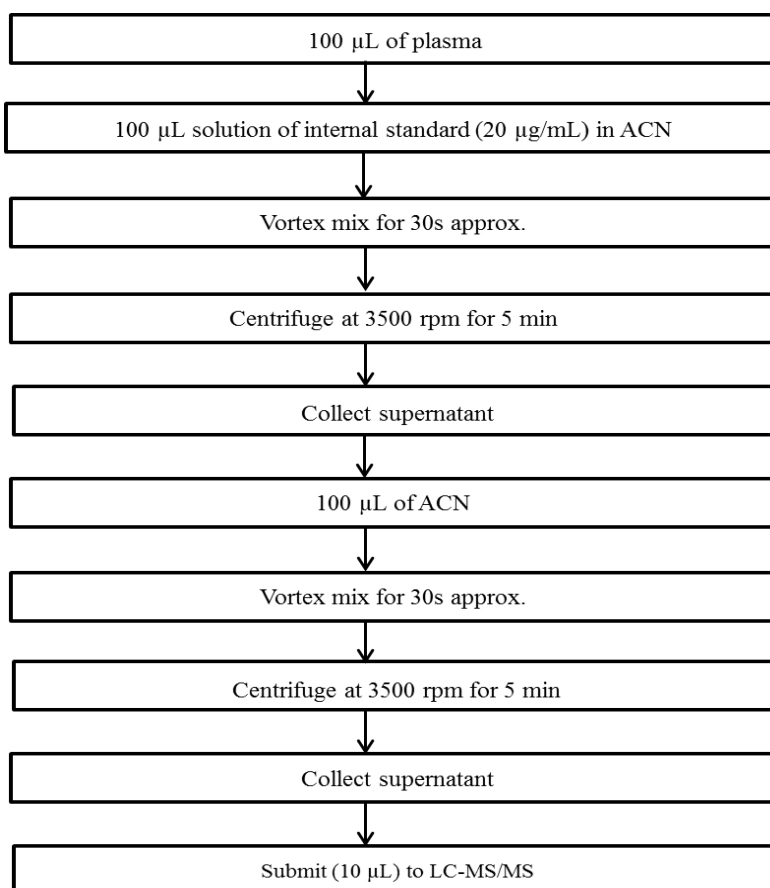

Figure 14. Flow chart of sample preparation protocol.

3.7 Method validation

The selectivity of the method was evaluated by spiking a blank sample with the analytes and the internal standards (5-HT, 5-HIAA, and 5-CH$_3$O-HT, d$_2$-5-HIAA). The extracted ion chromatograms (177→160,129 *m/z* for 5-HT, 192→157,146 *m/z* for 5-HIAA, and 191→174,143 *m/z* for 5-CH$_3$O-HT, 194→175 *m/z* for d_2-5HIAA) of the spiked samples were compared to the ones from the blank samples. The calibration experiment was performed using blank plasma samples spiked with 5-HT and 5-HIAA in the concentration range of 0.5-50 µg/mL. Six concentration levels (0.5, 1.0, 13.25, 25.5, 37.7 and 50 µg/mL) in triplicates were prepared according to the protocol previously described in section 3.6 using 20 µg/mL of 5-CH$_3$O-HT and d$_2$-5-HIAA internal standards. A linear regression analysis was performed for both 5-HT and 5-HIAA. The results will be presented in section 4.5. The LOD ($3\times SD_{blank}$) and LOQ ($6\times SD_{blank}$) were estimated visually by using sequential dilution of the spiked analytes in plasma. The recovery was determined by comparison of the nominal (spiked blank samples) and the calculated concentrations based on the constructed calibration curve models. The precision was determined based on repeatability and this was evaluated by determining the relative standard deviation (RSD) values of the signal ratio of the analyte to that of the internal standard data for the calibration curves.

3.8 Liquid chromatography ion trap mass spectrometry

An Agilent 110 series LC/MSD trap, SL model with an electrospray interface (ESI), a quaternary pump, degasser, autosampler, thermostatic column compartment, and a variable wavelength UV detector was used. The injection volume was 10µL. The column, a Zorbax Eclipse-C8 RP 150mm × 4.6mm, 5µm (Agilent Technologies, Palo Alto, CA, USA) was a double endcapped column 100% water compatible, which could be operated at a pH range of

2-9. It provided improved basic peaks of amines such as serotonin, because of the polar groups embedded in the stationary phase and fast conditioning column.

The column temperature was kept at 20°C and the solvent in gradient mode consisted of water with 0.1% formic acid (v/v) (B), acetonitrile with 0.1% formic acid (v/v) (C) and ethanol (D). The flow rate was maintained at 0.2mL/min and the UV detector at 254nm.

The initial condition 80% of B, 20% of D was maintained from 0-5 min and then changed to 17% of B, 65% of C and 18% of D in 10 min, returned to 80% of B, 20% of D in 2 min held for 23 min. The total analysis time was 40 min.

Using this gradient condition, reproducible retention times were obtained and peak areas were monitored from sample to sample. Nitrogen was used as nebulizing (50 psi) and drying gas (8L/min) at 350°C. The ESI source was operated in positive mode and ion optics responsible for getting the ions in the ion trap such as capillary exit, skimmer, lens and octapoles voltage were controlled by using the smart view option with a resolution of 13000 m/z/s (Full width at half maximum, FWHM/m/z = 0.6-0.7). A complete system control, data acquisition and processing were done using the Chem Station for LC/MSD version from Agilent. The transitions were monitored at, m/z 177→160,129 for serotonin, m/z 192→157,146 for 5-hydroxyindole-acetic acid, and m/z 191→174,143 for 5-CH$_3$O-HT, m/z 194→175 for d_2-5HIAA internal standards respectively. The magnitude of the signals was recorded in ion counts per seconds (icps).

3.9 Statistics

The response factors for the analytes (5-HT and 5-HIAA) are expressed as mean values and the standard deviation. The models were generated and simplified using linear regression analysis in Microsoft Excel 2010. The statistical adequacy of the models was established by an F-test at a 97.5% confidence level as previously mentioned.

4. RESULT AND DISCUSSIONS

4.1 Selection of optimal extraction solvent

Different extraction protocols were evaluated using several solvents, such as methanol, formic acid, acetonitrile (ACN) and water as previously discussed in section 3.3.1. Following several trials acetonitrile proved to be the most effective extraction solvent, since it leaves the plasma sample free of more protein precipitates unlike other solvents (methanol, formic acid and water) or their mixtures that leaves the extract cloudy and thus unsuitable to be injected into the LC-MS/MS. The volume of ACN to be used was as well optimised, 1:1, 1:2, 1:3 and 1:4 of plasma to ACN respectively were evaluated. It was found that; 1:1 leaves the supernatant cloudy after long standing hours. The supernatant were only clear for up to 8 hours post deproteinisation after which it started to form more precipitates, therefore making it unsuitable to be submitted to the LC-MS/MS after long hours. The plasma with high volume of ACN was concentrated in vacuum drier (stream of nitrogen was also tried) and re-dissolved in ACN during the development of the extraction protocols. During this process, 1:2 of plasma to ACN was selected as the most suitable ratio for the precipitation process. Using this ratio the supernatant were clear when kept overnight. This is good because the method can be successfully applied in routine analysis where numerous samples are often analysed and some samples are bound to be kept for long hours after preparation.

4.2 Selection of the optimal gradient system

During the course of optimising the gradient program several gradient system, flow rates and injection volumes were studied (section 3.4). The chromatograms of the conditions studied are shown in Fig. 15. Generally, as can be seen in Fig. 15, some of the gradient conditions presented no clear signal, poor separation and/or some background noise. The best gradient condition (Table 3) was used with a flow rate of 0.2mL/min, and reproducible retention times and well resolved peaks were obtained (Fig. 16).

Furthermore, it was found that larger volume of injection (30, 25, 20µL) resulted to poor peak separation and poor resolution (Fig. 17). 10µL was found to be the best injection volume, giving well separated and well resolved peaks.
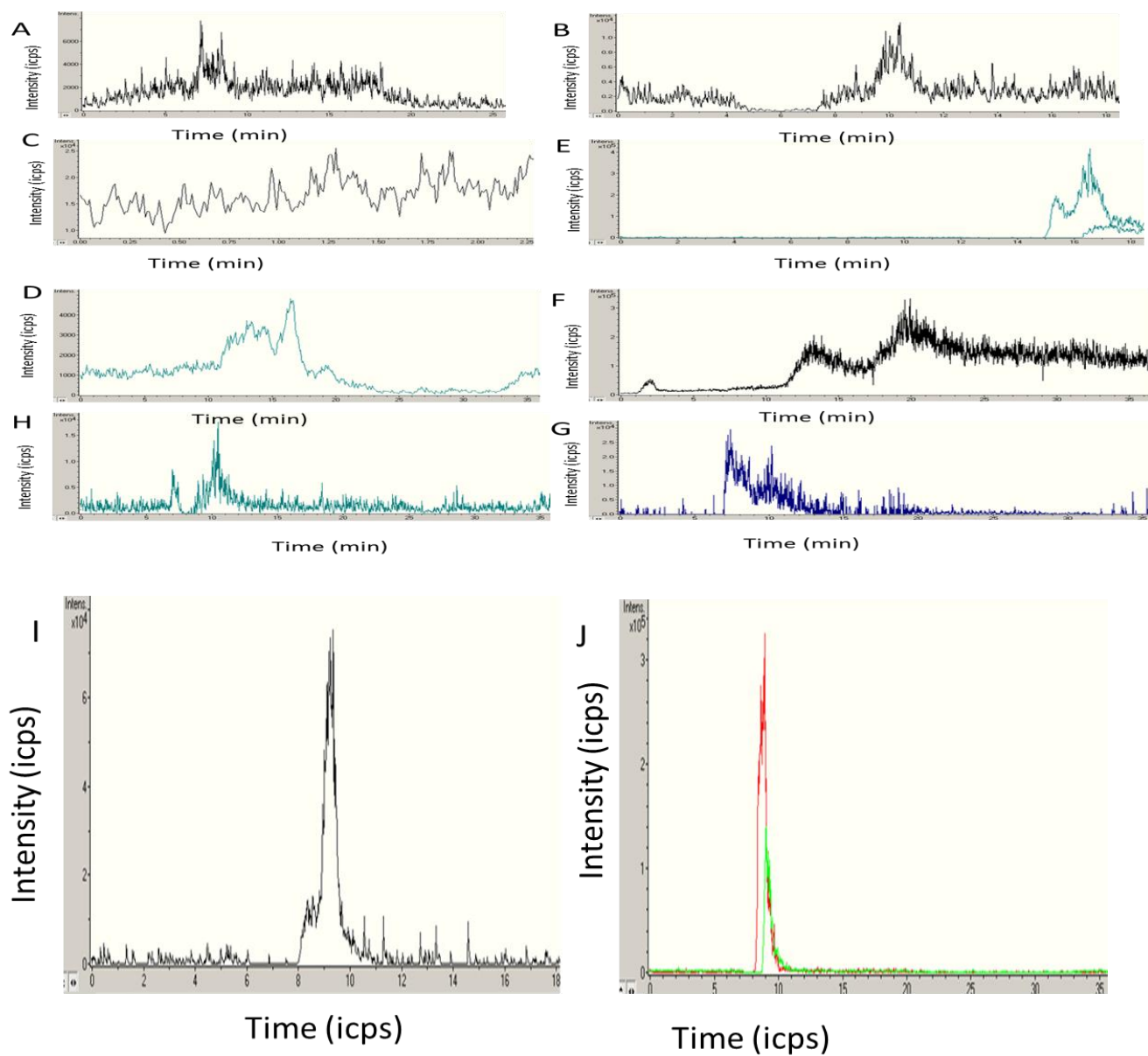

Figure 15. The chromatograms of the different gradient conditions

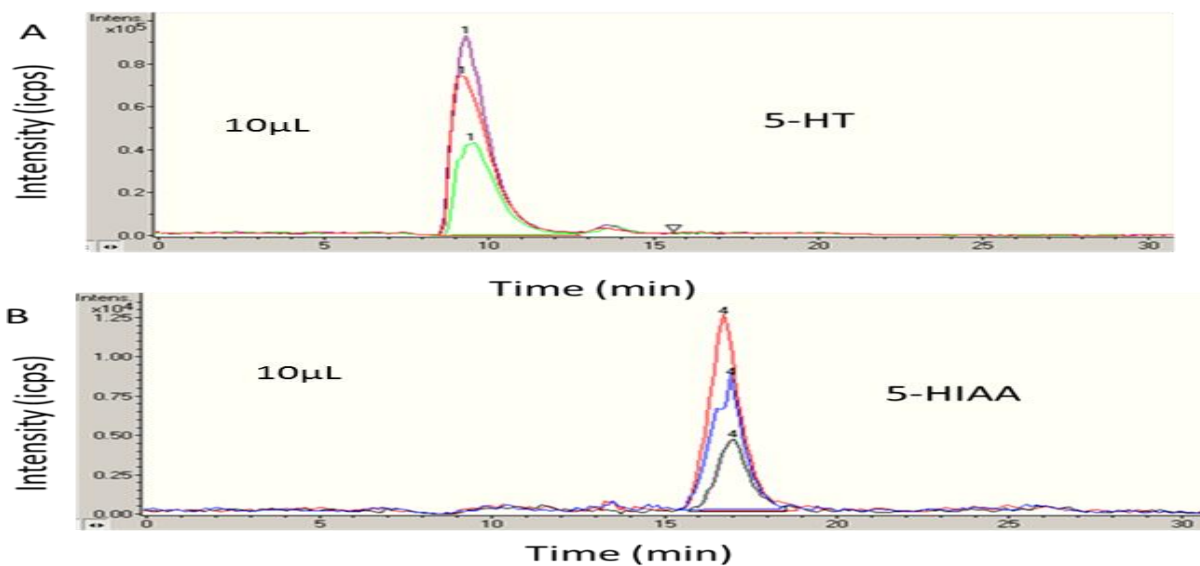Figure 16. The chromatogram of the best gradient condition showing reproducible retention times of the analytes.
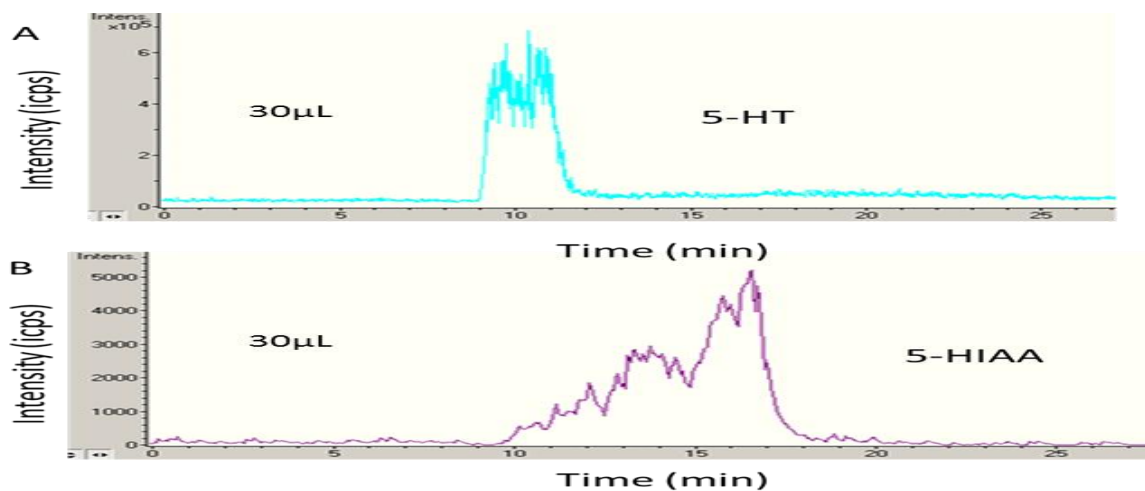

Figure 17. The chromatogram of large injection volume.

Table 3. Gradients programme of the mobile phase (eluent).

TIME (min)	B% (H_2O, 0.1% HCOOH)	C% (ACN, 0.1% HCOOH)	D% (ETHANOL)
0	80	0	20
5	80	0	20
15	17	65	18
17	80	0	20
40	80	0	20

4.3 Procedure for addition of internal standard

Two procedures were studied during the course of developing the protocol for adding the internal standards to the blank samples. The results showed that there was no clear difference in the obtained signals from the two procedures (Fig. 18). Therefore, since the present method also aims at developing a simple, less time consuming protocol, the procedure that involves adding the internal standard in ACN solution into the plasma without drying was preferred as the procedure of choice for the developed method.
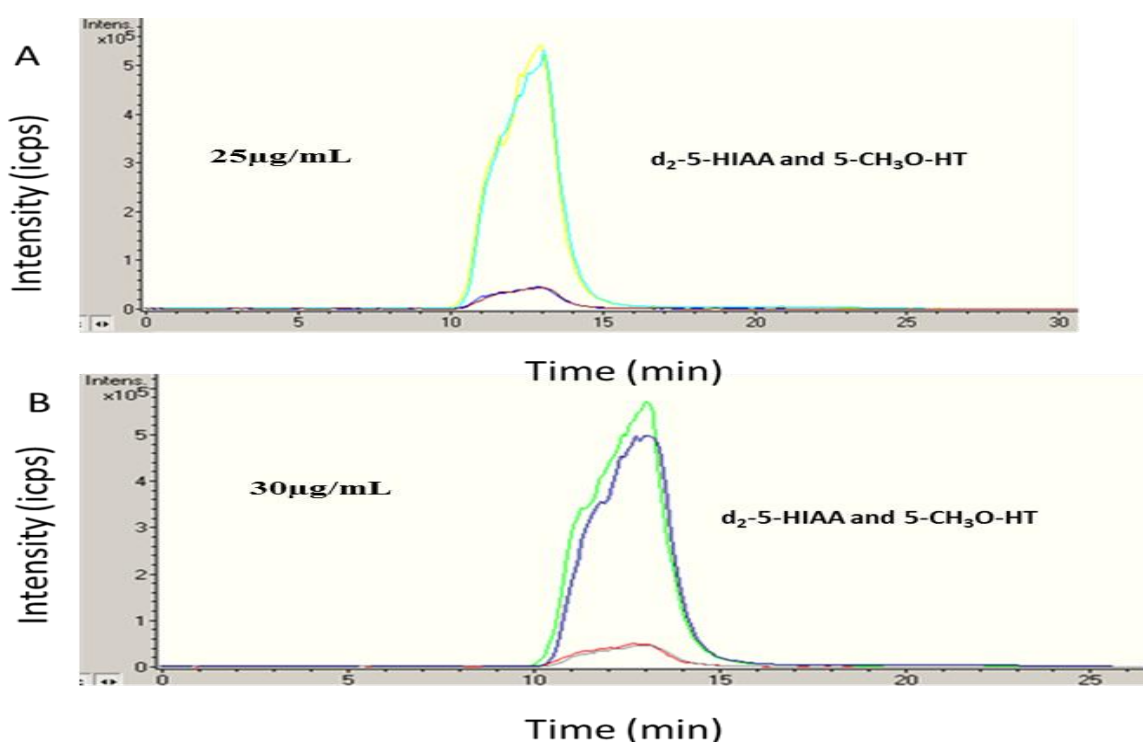
Figure 18. Overlap chromatogram of the two procedures for adding internal standard using two different concentrations (A=25µg/mL, B=30 µg/mL).

4.3.1 Optimisation of the internal standard

A protocol for simultaneous analyses of serotonin and 5-hydroxyindole acetic acid has been developed. The behaviour of the analyte and internal standard has been studied and modelled using Doehlert uniform shell design (section 3.5) to select the optimal amount of both internal standards. Furthermore, the selection of the optimal internal standard was based on

mathematical models using regression analysis and visualizing the concentration in the response surface plot of the obtained model to find a region where the response factor remain relatively constant over the studied analytical concentration range.

The seven experimental point solutions of plasma prepared in triplicates containing different concentration of 5-HT, 5-HIAA and their corresponding internal standards (Table 2) were injected randomly into the chromatographic system and detected by the ion trap tandem MS. The computed response factors at the seven experimental points are presented in Table 4.

Table 4. Summary of RFs for the analytes (5-HT and 5-HIAA) at each experimental points expressed as mean±standard deviation

Exp. No	Analytes conc. (µg/mL)	IS conc. (µg/mL)	RFs for 5-HT	RFs for 5-HIAA
1	1	25.5	1.8±0.13	2.63±0.26
2	13.25	46.70	2.81±0.02	11.90±1.05
3	37.75	46.70	4.07±0.11	15.22±1.66
4	25.50	25.50	4.31±0.13	18.56±0.72
5	13.75	4.30	5.52±0.22	39.71±16.65
6	37.75	4.30	7.63±0.26	34.91±7.28
7	50.00	25.50	5.87±0.16	21.56±5.48

The transitions were monitored at, $177 \rightarrow 160,129$ m/z for serotonin, $192 \rightarrow 157,146$ m/z for 5-hydroxyindole-3-acetic acid, and $177 \rightarrow 174,143$ m/z for 5-CH$_3$O-HT, $194 \rightarrow 175$ m/z for d_2-5HIAA internal standards respectively. The magnitude of the signals was recorded in ion counts per seconds (icps).

The analyses time for the solution was 40 min and the elution times were approximately 9.2, 13.4 for 5-HT, 5-CH$_3$O-HT and 16.0, 13.6 for 5-HIAA, d$_2$-5-HIAA respectively. The extracted ion chromatograms (EICs) were extracted, integrated and the RFs calculated using (Eq. 1).

4.4 Modelling the RF as a function of 5-HT and 5-HIAA and their internal standards (5-CH₃O-HT and d₂-5-HIAA respectively).

Various polynomial models were evaluated for the system 5-HT, 5-HIAA and their internal standards (5-CH₃O-HT, d₂-5-HIAA respectively) and it was found that full second order polynomial model with six coefficients (Eq. 3) was adequate to describe the behaviour of serotonin and 5-hydroxyindole-acetic acid and their corresponding internal standards. The adequacy of the model was established by comparing the experimental lack of fit to pure error variance ratio (F_{exp} = 3.57 for 5-HIAA, F_{exp} = 6.24 for 5-HT) with the theoretical (F_{theo} = 6.42 for 5-HIAA, 6.93 for 5-HT at 97.5% confidence levels) values at determined degrees of freedom (Eq. 20). The compared variances gives enough evidence to support the adequacy of Eq. 21 and 22 to predict the RF over the experimental domain in the study.

During the modelling, models with lesser number of coefficients were also considered (Eq. 2), but it was found that this type of model with lower number of terms was not statistically adequate for modelling the behaviour of the RF as a function of the analytical concentrations. The models did not fulfil the condition stated in section 3.5 (that if the F_{exp} is lower than the F_{theo} it means that the model is fitted).

The result of the RFs are presented as mean values in the surface plot (Fig. 19) generated from the obtained model. The models are shown below (Eq. 21 and 22).

$$RF = 36.88 + 6.43 \times 10^{-1}[5 - HIAA] - 1.75[d_2 - 5 - HIAA] + 7.82 \times 10^{-3}[5 - HIAA] \quad \text{Eq. (21)}$$
$$\times [d_2 - 5 - HIAA] - 1.21 \times 10^{-2}[5 - HIAA]^2 + 1.93 \times 10^{-2}[d_2 - 5 - HIAA]^2$$

$$RF = 4.34 + 1.45 \times 10^{-1}[5 - HT] + 1.48 \times 10^{-1}[5 - CH_3O - HT] - 8.16 \times 10^{-4}[5 - HT] \quad \text{Eq. (22)}$$
$$\times [5 - CH_3O - HT] + 9.30 \times 10^{-4}[5 - HT]^2 + 1.86 \times 10^{-3}[5 - CH_3O - HT]^2$$

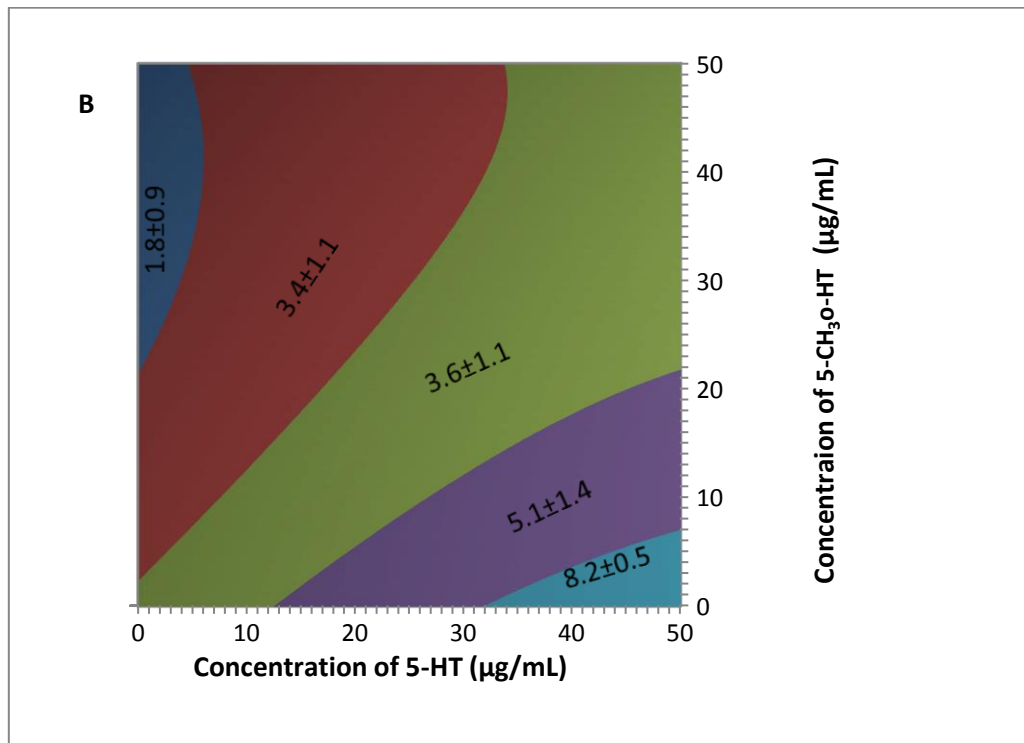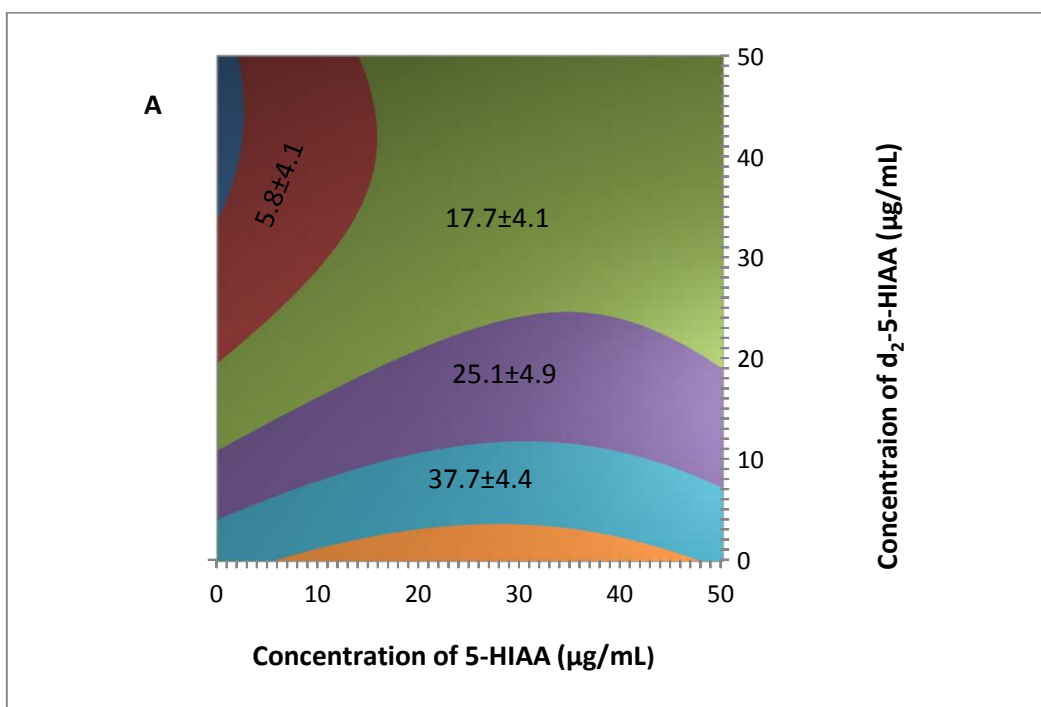The response surface plots are shown in (Fig. 19A and B).

Figure 19. A. Modelling of the response factor as a function 5-HIAA and d_2-5-HIAA, B. Modelling of the response factor as a function 5-HT and 5-CH$_3$o-HT

The RF values 5.8±4.1, 17.7±4.1, 25.1±4.9, 37.7±4.4 were estimated in the ranges of 25-50 µg/mL, 21-24 µg/mL, 8-25 µg/mL 0-7 µg/mL respectively for d_2-5-HIAA (Fig. 19A) and 1.8±0.9, 3.4±1.1, 3.6±1.1, 5.1±1.4, 8.2±0.5 were estimated in the ranges of 25-50 µg/mL, 8-50 µg/mL, 23-50 µg/mL, 9-22 µg/mL, 0-8 µg/mL respectively for 5-CH_3O-HT (Fig. 19B).

Every region in the surface plot encompasses concentration regions for both analytical species where it is expected to find constant RF. Furthermore, it is shown that the system described by the analytes (5-HT and 5-HIAA) and their internal standards (5-CH_3O-HT and d_2-5-HIAA) in Fig. 19A and B exhibits an inverse relationship. This means that as the concentration of the internal standards increases the mean value of the RF decreases proportionally and vice versa (Fig. 19A and B). This trend suggests a decreasing sensitivity of the analyses as the concentration of internal standard increases. Consequently, a critical look at the response surface plots in Fig. 19A and B for a region where the surface plot remains relatively constant to select optimal concentration of internal standards to be spiked in the plasma samples. The optimal concencentrations of 5-CH_3O-HT and d_2-5-HIAA to be used in analysis of 5-HT and

5-HIAA can be from 9-22 µg/mL for 5-CH_3O-HT and 8-25 µg/mL for d_2-5-HIAA according to (Fig. 19A and B). As a result 20 µg/mL was considered as the optimal amount of internal standard for the quantification of 5-HT and 5-HIAA in the plasma samples. At this concentration of d_2-5-HIAA the RF values remains relatively constant over the entire analytical range while for 5-CH_3O-HT the RF values was not however constant over the entire analytical range but this concentration was satisfactory to be considered as the most adequate concentration for the developed method. The average RF values at this region is 3.6±1.1 for 5-CH_3O-HT and 25.1±4.9 for d_2-5-HIAA.

Extracted ion chromatograms of 5-HT, 5-HIAA, 5-CH$_3$O-HT and d$_2$-5-HIAA and their corresponding mass spectra are shown in Fig. 20-23.
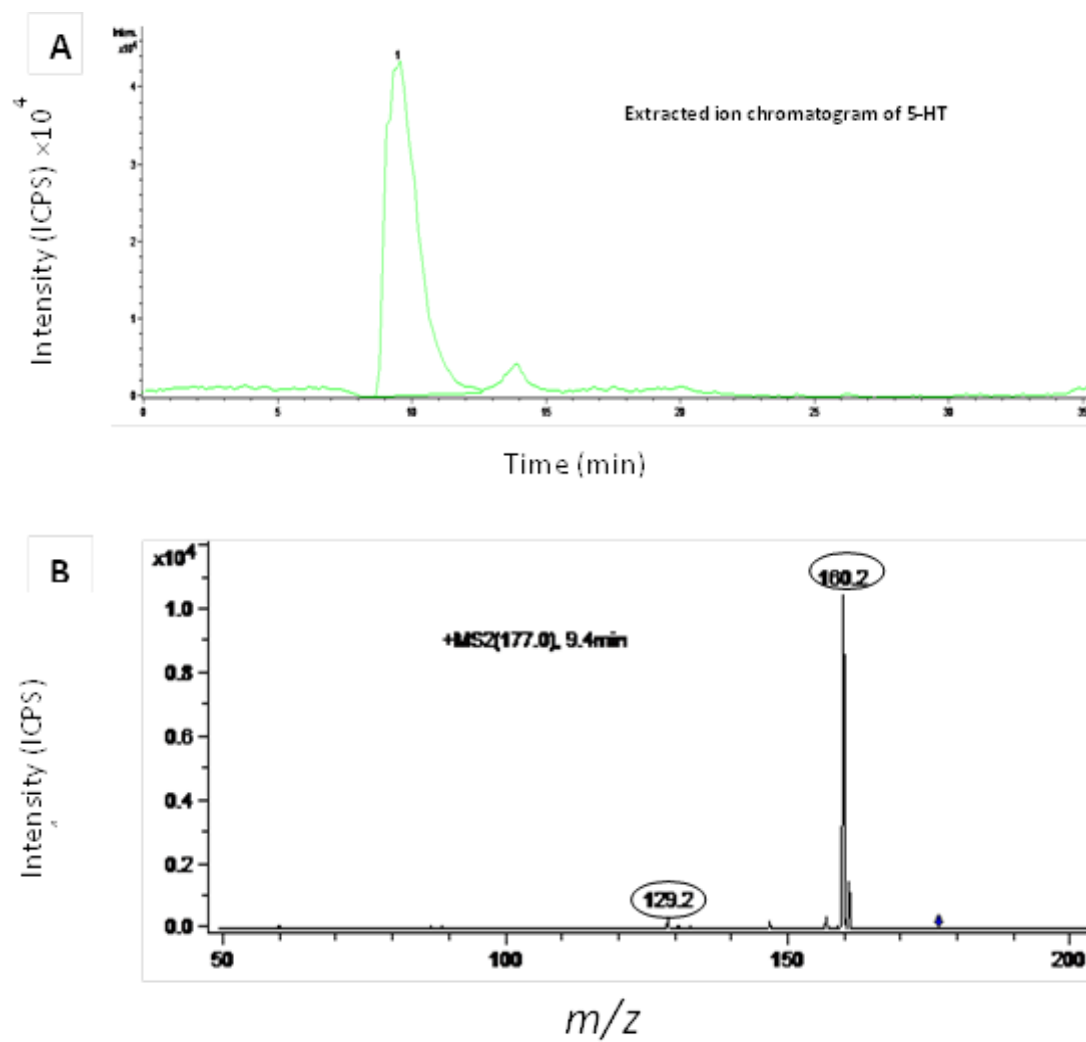
Figure 20. Chromatogram of 5-HT (A) and its mass spectrum (B).
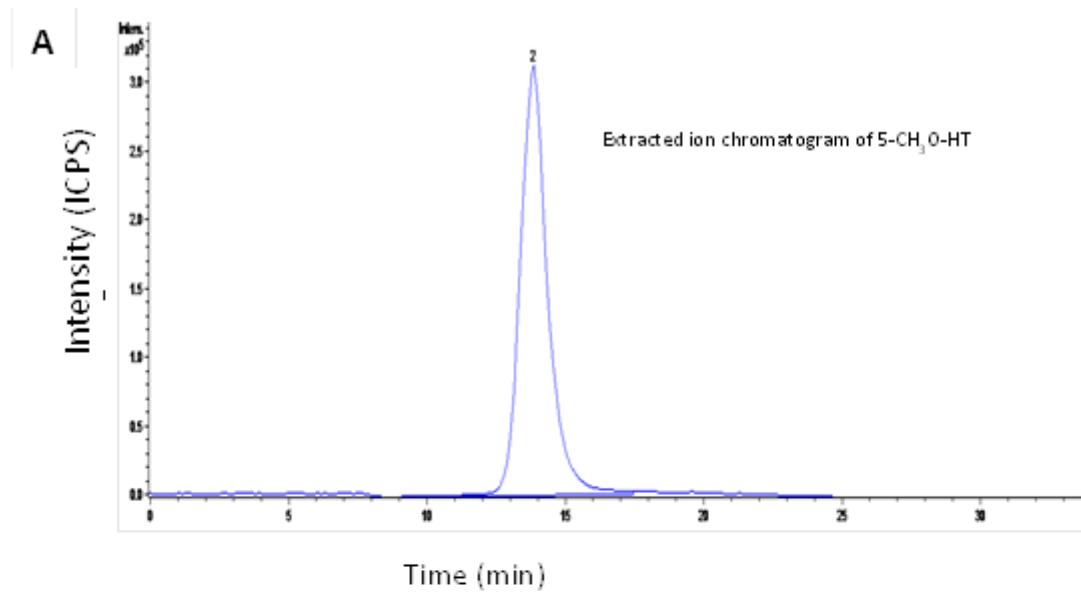
Figure 21. Chromatogram of 5-CH₃o-HT (A) and its mass spectrum (B).

Figure 22. Chromatogram of 5-HIAA (A) and its mass spectrum (B).

A

Extracted ion chromatogram of d$_2$5-HIAA
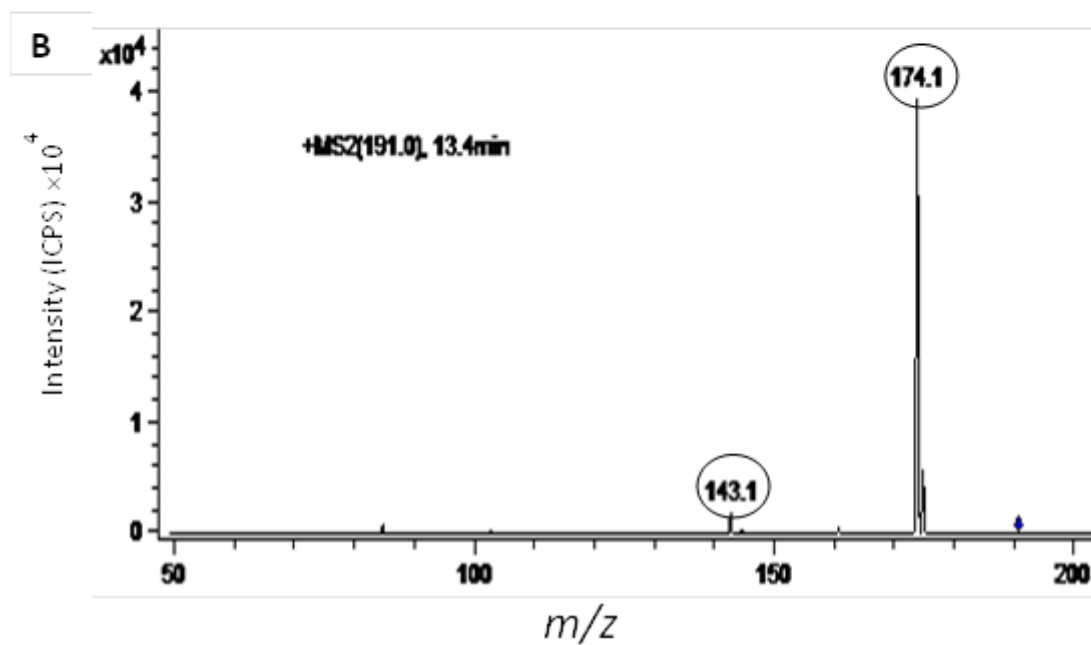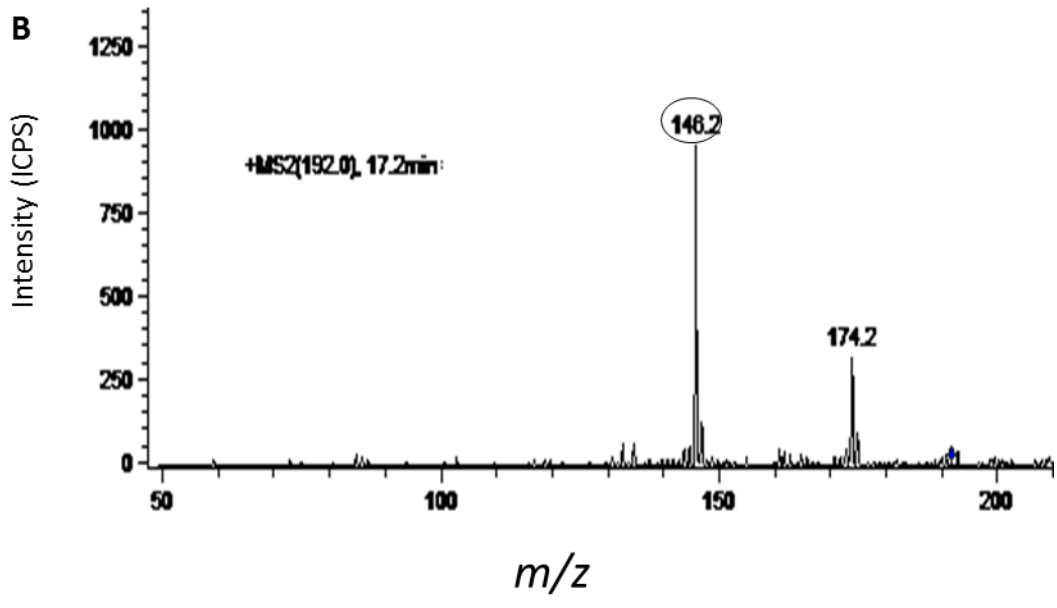
B

+MS2[194.0], 14.3min

175.2

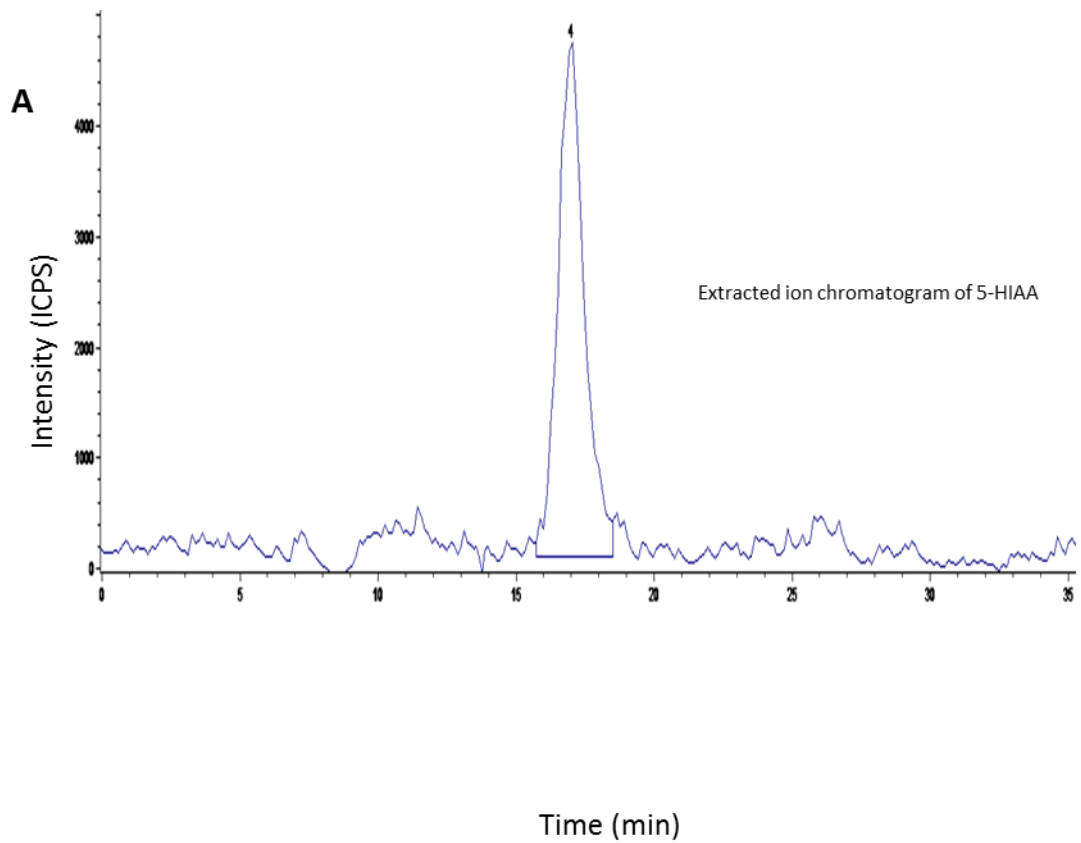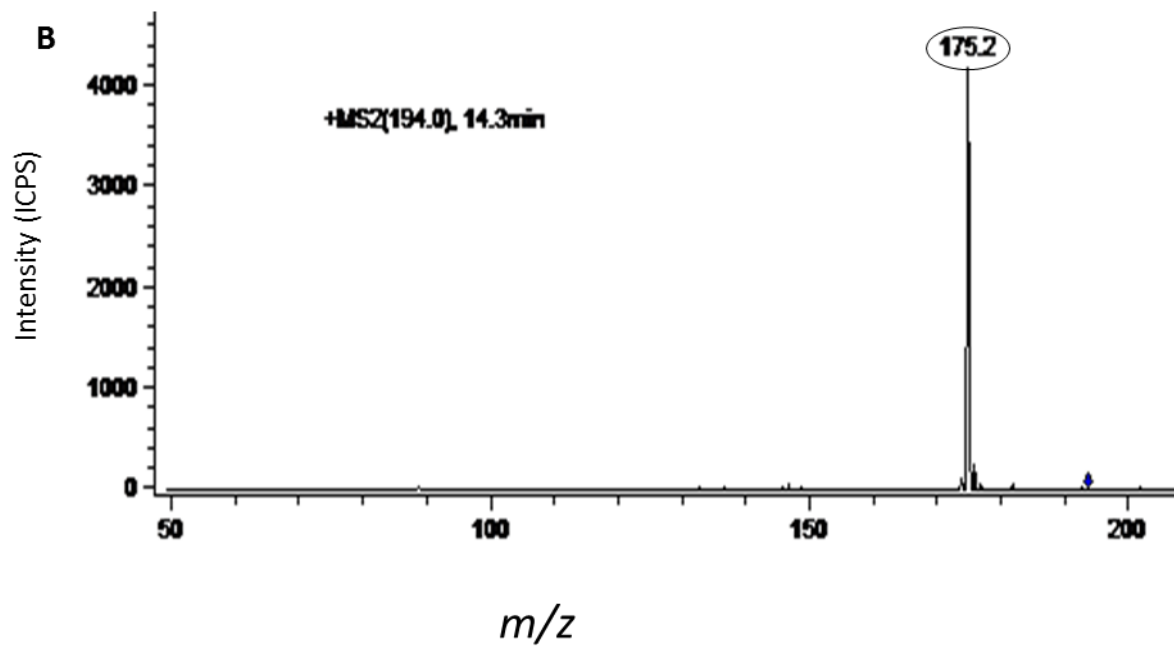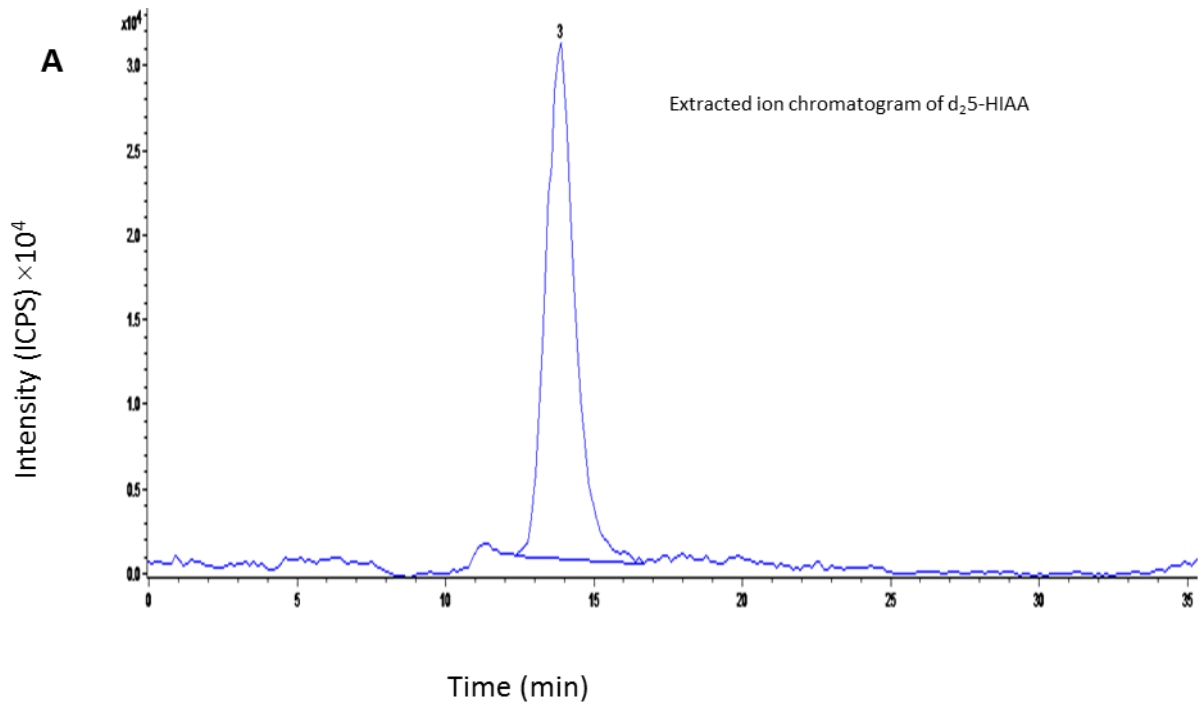Figure 23. Chromatogram of d$_2$5-HIAA (A) and its mass spectrum (B).
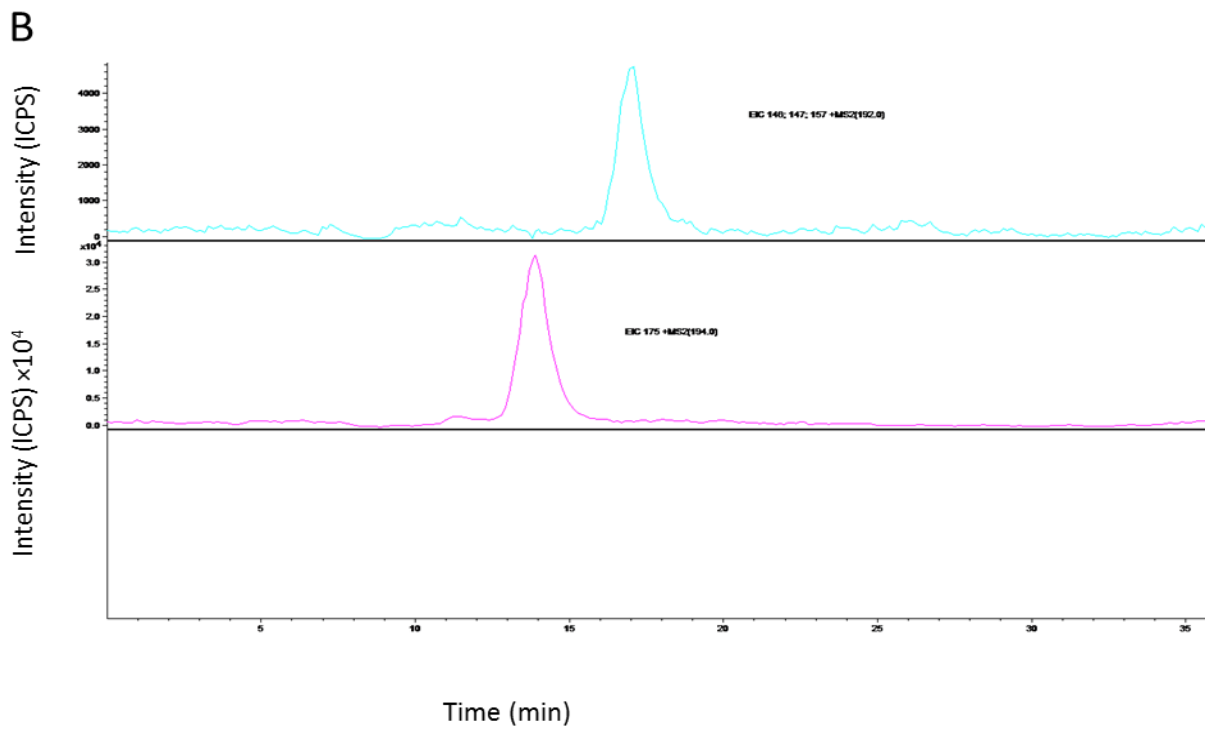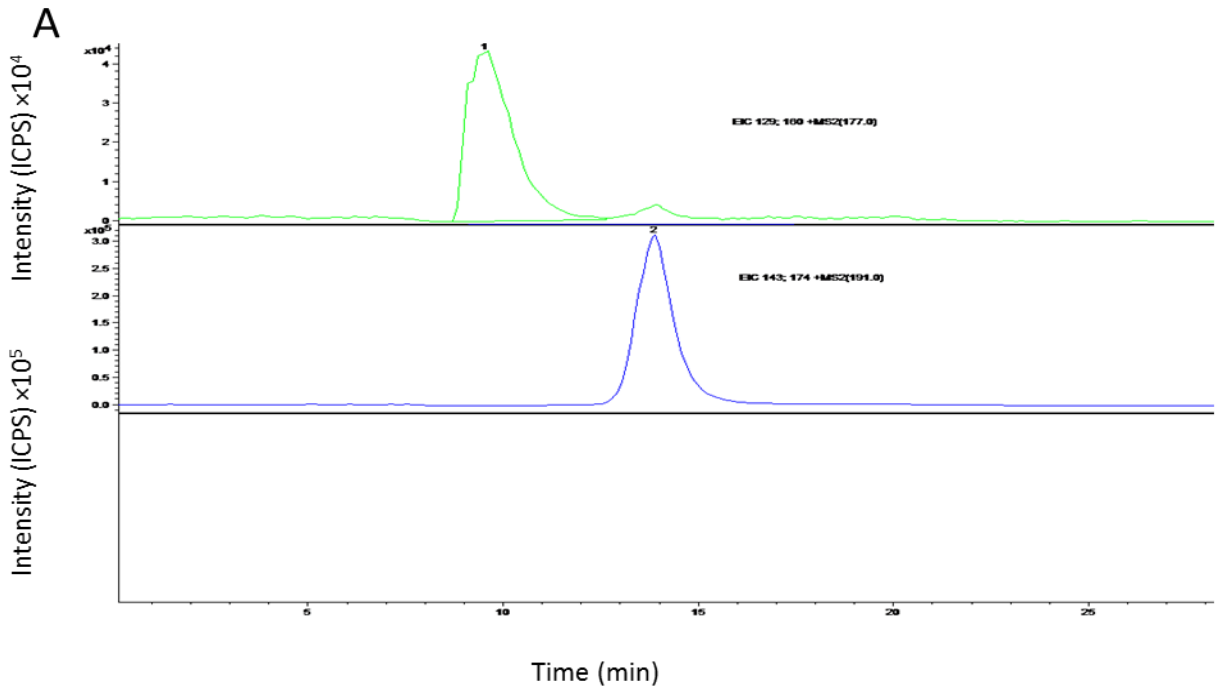
Figure 24. A. Overlay chromatogram of 5-HT and its internal standard, showing their fragment masses. B. Overlay chromatogram of 5-HIAA and its internal standard, showing their fragment masses.

4.5 Validation and analytical assessment

Plasma calibration samples containing a mixture of 5-HT and 5-HIAA in the range of 0-50µg/mL were spiked with fixed and optimal amount of the corresponding internal standards (20µg/mL of 5-CH$_3$O-HT and d$_2$-5-HIAA respectively) selected from the result of the response factor behaviour. The analytical performance parameters such as; range, linearity, selectivity, recovery, precision, limit of detection and limit of quantification were determined. The method was linear over the studied range of concentration. The correlation coefficients was r^2 = 0.9823 for serotonin and r^2 = 0.9892 for 5-hydroxyindole-acetic acid which indicates strong correlation between the studied concentration of the analytes and the signal. However the response of the metabolite, 5-HIAA was not linear when the solution was analysed after some hours of standing. This could be attributed to degradation. The linear regression graph of the two species are shown in Fig. 25A and B.

y = 0.0052x + 0.0108
R² = 0.9892
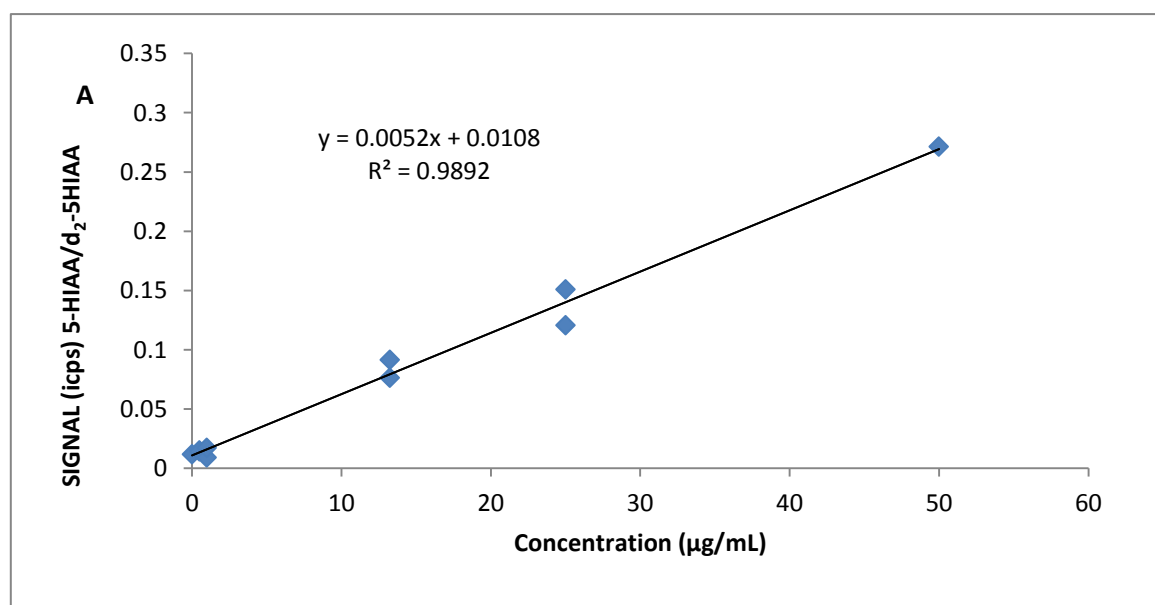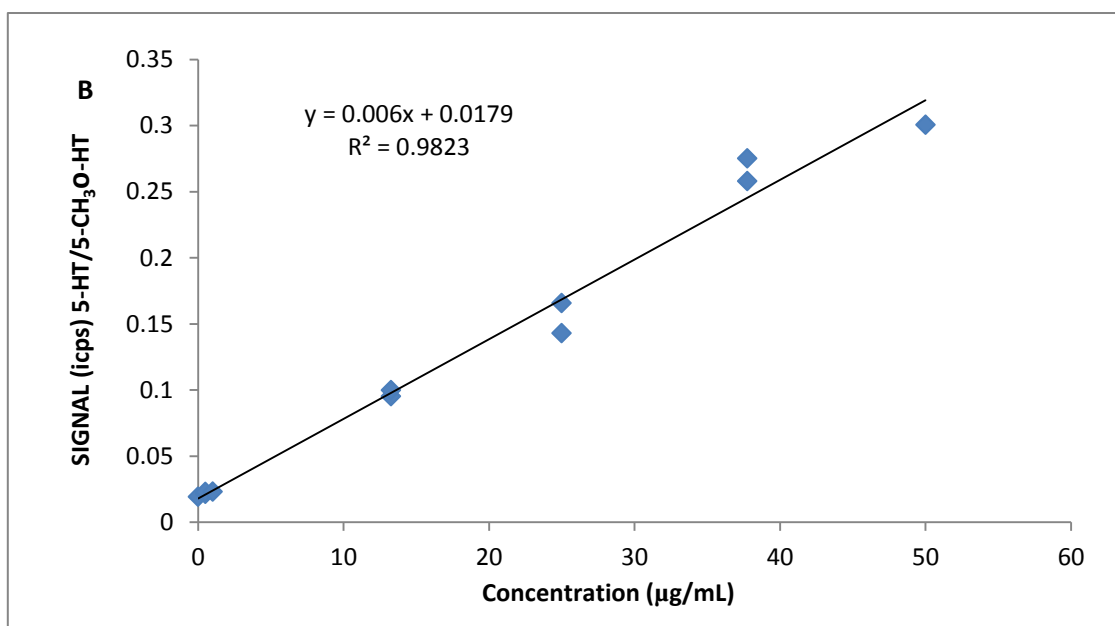
Figure 25. A. Linear regression graph of the signal of the ratio 5-HIAA and d_2-5HIAA against the concentrations.B. Linear regression graph of the signal of the ratio 5-HT and 5-CH$_3$o-HT against the concentrations.

The selectivity of the method was determined by the EIC of a sample spiked with 5-HT and 5-HIAA to the EIC from a blank plasma samples. The method was very selective towards the two analytical species as seen in the overlay chromatogram (Fig. 24A and B). The precision of the method for 5-HT and 5-HIAA was achieved based on repeatability with RSD of 3.07-7.73% and 3.93-9.99% respectively. The percentage recoveries were 83-119% and 84-116% for serotonin and 5-HIAA respectively, which shows good accuracy of the developed method. The limit of detection and limit of quanitification for both analytes were determined to be 0.5 µg/mL and 1 µg/mL respectively suggesting good sensitivity of the method. Furthermore, in comparison of the present study to previous research. Several analytical methods [70-165] have been employed in the study of serotonin in plasma. LC-MS/MS has been used; 4.6 nmol/L was reported by De Jong et al. [53], 18.2 nmol/L by Moriarty et el. [52], 1.5 nmol/L by Monaghan et al. [60]. HPLC with different detections have been employed as well and varying concentrations have been reported, 114 nmol/L [72], 44 nmol/L [83], 141 nmol/L [108] etc. Radioenzymatic assay has also been employed and different concentrations such as

38 nmol/L [78], 22.6 nmol/L [82], 2 nmol/L [100], and 0.6 nmol/L [122] have been reported. As seen in Table 1, mass spectrometric [53, 115] and radioenzymatic methods reported lower estimate of serotonin in plasma compared to HPLC methods. The present study has reported LOD of 0.5µg/mL (0.003 nmol/L) and LOQ of 1 µg/mL (0.006 nmol/L) which has given it superiority over the previous methods. A random search using key words such as "normal plasma levels of serotonin" and "plasma concentration of serotonin" revealed varying and conflicting values. However, majority of the search results seems to be within the range of 0.4-5 µg/mL (0.002-0.030 nmol/L). Also the levels of serotonin and its associated metabolite is age dependent thus the reported values accounts for normal adult, since the level is increased in children and younger age groups [8]. Therefore, supposing that the above range is correct, it can be said that some of the previous methods reported values that may give false positives.

4.6 Method application on plasma samples from a research study

Ten human plasma samples from a project related to the determination of serotonin and associated metabolites in plasma from pathological patients subjected to a diet rich in vegetables were examined by using the developed method under optimal conditions.

The quantitative analyses (Table 5) revealed that in the majority of the samples the serotonin levels were lower than the 5-hydroxyindole-acetic acid levels.

Table 5. Plasma concentration of serotonin and its acid metabolite in plasma samples from 10 vegetarian patients.

No	5-HT (µg/ml)	5-HIAA (µg/ml)
1	10.21±1.08	16.03±1.48
2	Below LOD	2.36±0.27
3	13.26±1.17	16.31±1.03
4	8.60±0.57	11.35±0.96
5	11.60±1.10	13.46±1.23
6	Below LOD	Below LOQ
7	11.99±0.56	15.35±0.87
8	Below LOD	below LOQ
9	7.69±0.72	9.30±0.69
10	12.60±1.36	15.75±1.47

The results are expressed as mean±standard deviation of triplicate values; LOD means limit of detection (0.5 µg/mL), and LOQ means limit of quanitification (1 µg/mL).

This observation supports the earlier report [18], that intake of foods which are rich in tryptophan (precursor of serotonin) increases the synthesis of serotonin. Thus, there will be surplus serotonin in circulation, which will result to proportional increase in plasma levels of 5-HIAA since more serotonin will be available for degradation by the liver to 5-HIAA. Also when the serotonergic activity is high, more serotonin is released in the synapses, and consequently, metabolized to 5-HIAA. Biochemically, in certain health conditions such as carcinoid tumour, the carcinoid causes abnormal of tryptophan metabolism, in which as much as 60 %, other than the normal 1 %, of tryptophan may follow the serotonergic pathway [13]. Moreover, elevated plasma serotonin levels are hazardous, rapid elimination mechanisms evolve to clear the excess 5-HT thereby converting it to its acid metabolite.

4.7 Concluding remarks

5-hydroxyindole-acetic acid has been utilised as a biomarker for carcinoid tumours and other pathological diseases and serotonin has been used as an index for assessment of gastrointestinal disorders (such as irretable bowel syndrome) as well as emotional related complications (including depression). We have developed a method that proposes a simple

and rapid protocol for the simultaneous extraction and subsequent determination of serotonin and 5-hydroxyindole-acetic acid in human plasma by liquid chromatogaphy tandem mass spectrometry. The extraction protocols and other sample preparation steps are quite simple and do not require much downstream preparation protocols. Nonethless, there were dificulties encountered during the method development process most notably in the development of the extraction protocols. Such set backs includes the clogging of the LC-MS/MS by protein precipitates, however, the set back was subsquently circumvented by reevaluating the sample preparation procedures.

The method may be suitable to be applied as a biomarker for determination of 5-HT and 5-HIAA in plasma in patients suffering from several neuronal and pathological diseases such as carcinoid tumour (in which elevated levels of these compunds may occur) and depression related complications where both compounds are implicated and thus may be adequate to be applied in routine analysis of 5-HT and 5-HIAA. The physiological concentration of 5-HT in plasma is several times lower than compared to whole blood concentration, thus making the measurement of serotonin in plasma very demanding and require a very sensitive technique as the method reported here. The developd method proved to be quite sensitive, selective and relatively no background noise in the chromatogram as shown in Fig. 24A and B.

In addition, to the best of our knowledge this is the first reported method that used a Doehlert design to optimize multiple amounts of internal standard for the simultaneous determination of serotonin and 5-hydroxyindole-acetic acid in plasma samples. This approach is advantageous in that it helps to simulate different concentrations of internal standards across several concentrations of the analytes as if it were in real terms thus avoiding the preparation of several solutions. The developed method has reproducible retention times with acceptable variations of less than 0.5% between samples.

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