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DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD FOR QUANTIFICATION OF 15 NON-TRICYCLIC ANTIDEPRESSANTS IN SERUM WITH UPLC-MS/MS

Dissertação apresentada para provas de Mestrado em Química Forense

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"We may march to victory or we march to defeat, but we go forward, only forward"

George Martin

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Contents

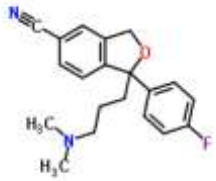
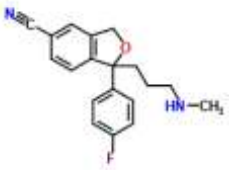
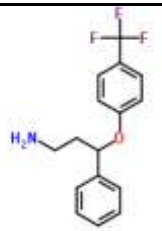
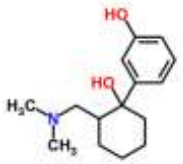
JUSTIFICATION OF RESEARCH

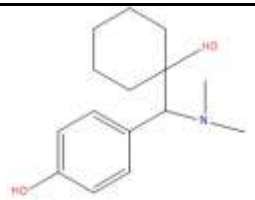
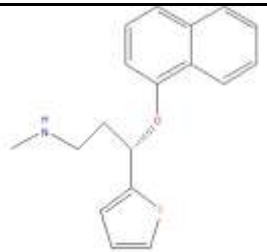
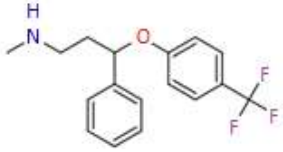
Mental disorders are on the rise in the European Union. Major depression is a commonly occurring, serious, recurrent disorder linked to a diminished role functioning and quality of life, which can even lead to suicide. In a recently survey made by World Mental Health Organization, 49.4% of the respondents claim already had a time lasting several days when they were sad, depressed, or lost interest in their usual activities [1]. Usually, the treatment for these cases is performed with the use of antidepressants and antipsychotics [2]. These drugs are often associated with deliberate self-poisoning (DSP) and it is one of the most common reasons to visit the emergency department. In 2012, 72 out of 312 DSP cases were due to antidepressants in Belgium [3].

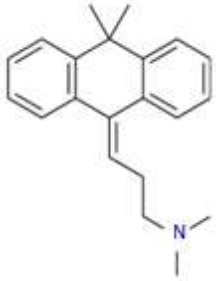
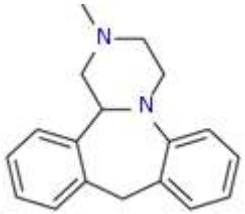
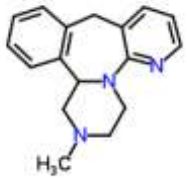
Determining the presence of various drugs is an important facet of toxicology, to establish their use and possible contribution to the cause of poisoning or even death. Liquid Chromatography-Mass Spectrometry (LC-MS) has established itself as the clear leader in the quantification of the psychotropic drugs in biological samples. There are numerous reports of using LC-MS methods for determination of these compounds in biological matrices, such as plasma, serum or whole blood [2, 4, 5, 6, 7, 8, 9, 10, 11]. The pharmacologic effects of most drugs have a direct correlation with their concentrations in plasma, which can serve as a basis for therapeutic monitoring. Therefore, the plasma is preferred for quantitative analysis when interpretation of the concentrations and effects are required [4].

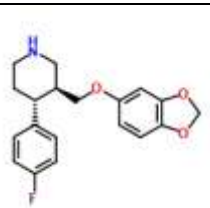
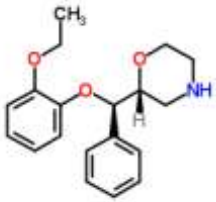
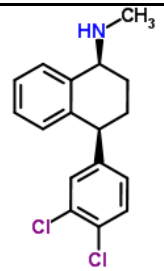
So, it was developed developed and validated an ultra-performance liquid chromatography-tandem mass spectrometry method for the quantification of 15 common non-tricyclic antidepressants in serum: citalopram, desmethylcitalopram, desmethylfluoxetine, desmethylvenlafaxine, duloxetine, fluoxetine, fluvoxamine, melitracen, mianserin, mirtazapine, paroxetine, reboxetine, sertraline, trazodone and venlafaxine. The selected pharmaceuticals represent (nor) tramadol (added because of the desmethylvenlafaxine interference) and all the non-tricyclic antidepressants available in Belgium – table I.

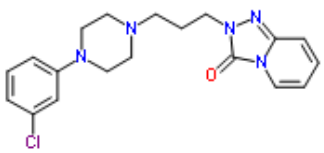
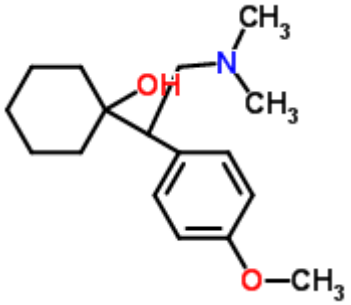
Table I - Physico-chemical characteristics of psychotropic drugs studied in this project

Compound	Chemical Structure	Chemical Name (IUPAC)	CAS	Molecular Formula	Molecular weight (g/mol)	Categories
1. Citalopram		1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile	59729-33-8	$C_{20}H_{21}FN_2O$	324.392	Antidepressant
2. Desmethyl-Citalopram (DM Citalopram)		1-(4-Fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydro-2-benzofuran-5-carbonitrile	144010-85-5	$C_{19}H_{19}FN_2O$	310.365	Metabolite
3. Desmethyl-Fluoxetine (DM Fluoxetine)		3-Phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine	83891-03-6	$C_{16}H_{16}F_3NO$	295.299	Metabolite
4. Desmethyl-Tramadol (O - DM Tramadol)		3-{2-[(Dimethylamino)methyl]-1-hydroxycyclohexyl}phenol	73986-53-5	$C_{15}H_{23}NO_2$	249.349	Metabolite-Analgesic

5.	**Desmethyl-Venlafaxine (DM Venlafaxine)**		4-[2-(dimethylamino)-1-(1-hydroxycyclohexyl)ethyl]phenol	149289-30-5	$C_{16}H_{25}NO_2$	263.375	Metabolite
6.	**Duloxetine**		(3S)-N-methyl-3-naphthalen-1-yloxy-3-thiophen-2-ylpropan-1-amine	116539-59-4	$C_{18}H_{19}NOS$	297.415	Antidepressant
7.	**Fluoxetine**		N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine	56296-78-7	$C_{17}H_{19}ClF_3NO$	309.326	Antidepressant
8.	**Fluvoxamine**		2-[(E)-[5-methoxy-1-[4-(trifluoromethyl)phenyl]pentylidene]amino]oxyethanamine	54739-18-3	$C_{15}H_{21}F_3N_2O_2$	318.335	Antidepressant

9. Melitracene		3-(10,10-dimethylanthracen-9-ylidene)-N,N-dimethylpropan-1-amine	5118-29-6	$C_{21}H_{25}N$	291.4299	Antidepressant
10. Mianserine		2-Methyl-1,2,3,4,10,14b-hexahydrodibenzo[c,f]pyrazino[1,2-a]azepine	24219-97-4	$C_{18}H_{20}N_2$	264.365	Antidepressant
11. Mirtazapine		2-Methyl-1,2,3,4,10,14b-hexahydropyrazino[2,1-a]pyrido[2,3-c][2]benzazepine	85650-52-8	$C_{17}H_{19}N_3$	265.353	Antidepressant

12. Paroxetine		(3S,4R)-3-[(1,3-Benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine	61869-08-7	$C_{19}H_{20}FNO_3$	329.365	Antidepressant
13. Reboxetine		(2R)-2-[(R)-(2-Ethoxyphenoxy)(phenyl)methyl] morpholine	98769-81-4	$C_{19}H_{23}NO_3$	313.391	Antidepressant
14. Sertraline		(1S,4S)-4-(3,4-Dichlorophenyl)-N-methyl-1,2,3,4-tetrahydro-1-naphthalenamine	79617-96-2	$C_{17}H_{17}Cl_2N$	306.230	Antidepressant

15. Tramadol		(1R,2R)-2-[(Dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol	73986-53-5	$C_{16}H_{25}NO_2$	263.375	Analgesic
16. Trazodone		2-{3-[4-(3-Chlorophenyl)-1-piperazinyl]propyl}[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one	19794-93-5	$C_{19}H_{22}ClN_5O$	371.864	Antidepressant
17. Venlafaxine		1-[2-(Dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol	93413-69-5	$C_{17}H_{27}NO_2$	277.402	Antidepressant

ABBREVIATIONS

5-HT	Serotonin
ACN	Acetonitrile
ANOVA	Analysis of variance
API	Atmospheric Pressure Ionization
CE	Collision Energy
CNS	Central Nervous System
Conc.	Concentration
CV	Coefficient of Variation (frequency distribution)
CYP 1A2	Cytochrome P450 1A2 (enzyme from the Cytochrome P450 family)
CYP 2D6	Cytochrome P450 2D6 (enzyme from the Cytochrome P450 family)
CYP 3A4	Cytochrome P450 3A4 (enzyme from the Cytochrome P450 family)
DA	Dopamine
Dev. %	Deviation from nominal concentration (in percentage)
DM	Desmethyl
EMA	European Medicines Agency
ESI	Electrospray Ionization
eV	Electronvolt
FDA	Food and Drug Administration
HPLC	High-performance liquid chromatography
IS	Internal standard
KKGT	Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie
LC	Liquid Chromatography
LC-MS	Liquid Chromatography - Mass Spectrometry
LC-MS/MS	Liquid Chromatography - Tandem Mass Spectrometry
LLE	Liquid–liquid extraction
LOD	Limit Of Detection
LOQ	Limit Of Quantification
m/z	Mass-to-Charge ratio
MAOI	Monoamine Oxidase Inhibitors
MeOH	Methanol
ME	Matrix Effects

MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NaSSA	Noradrenergic/Specific Serotonergic antidepressants
NDF	Number of degrees of freedom
NE	Norepinephrine
ntads	Non-triclycil antidepressants
p0H	Tabulated value
PD	Psychotropic drugs
PP	Protein precipitation
Q1	First Quadrupole
q2	Second Quadrupole (Collision Cell)
Q3	Third Quadrupole
QC	Quality Control
R	Correlation coefficient (measure the strength of association between two variables)
R^2	Coefficient of determination (measure how well data fit a statistical model)
SARI	Serotonin Antagonists/Reuptake Inhibitors
SD	Standard deviation
SE	Standard error (uncertainty related with mean estimate)
SNRI	Serotonin Norepinephrine reuptake inhibitors
SS	Sum of squares
SPE	Solid Phase Extraction
SSRI	Selective Serotonin Reuptake Inhibitors
Std.	Standard
Std. Fit	Standard fit
SWGTOX	Scientific working group for forensic toxicology
T0	Time of origin – when studying stability of processed samples, as soon as the batch is prepared, one should be immediately analyzed to establish the time zero.
TCA	Tricyclic Antidepressants
TQD	Triple Quadropole Detector
TV	Test values

VarFit	Fitting residual variance. it is obtained by dividing the sum of squares by the respective degrees of freedom
Ym	Signal Mean Value

RESUMO

Objectivo: Antidepressivos não-tricíclicos são frequentemente encontrados em casos de overdose, e para alguns, a monitorização terapêutica tem de ser garantida. Assim sendo, foi desenvolvido e validado um método com cromatografia líquida acoplada a espectrometria de massa sequencial para a quantificação de 15 antidepressivos não tricíclicos comuns em soro: citalopram, desmetilcitalopram, desmetilfluoxetina, desmetilvenlafaxina, duloxetina, fluoxetina, fluvoxamina, melitracene, mianserina, mirtazapina, paroxetina, reboxetina, sertralina, trazodone e venlafaxina. Os fármacos selecionados também contêm (nor) tramadol (adicionado devido à sua interferência com desmetilvenlafaxine) e todos os antidepressivos não tricíclicos disponíveis na Bélgica.

Métodos: Amostras de soro (250 µL) foram precipitadas com metanol e acetonitrilo que contem 5 ng de cada padrão interno: mirtazapine-d_4, paroxetine-d_5, reboxetine-d_6 e venlafaxine-d_6. O sobrenadante foi evaporado a 56ºC sob um fluxo de nitrogénio e reconstituído em 100 µL de 50:50 metanol:água com 2 mM de acetato de amónio. 5 µL foram injectados e os analitos separados numa coluna 100 × 2.1 mm ACQUITY BEH C18 (Waters, Zelik, Bélgica), usando o sistema Waters ACQUITY Cromatografia Líquida de alta pressão. Os analitos foram eluídos em 7.5 min, usando um gradiente de 0.2% de acetato de amónio e 0.1% de ácido fórmico em água e metanol a um fluxo de 0.4 mL/min. A quantificação foi efectuada usando o equipamento Waters quadropolo triplo ACQUITY TQD, com monitorização de reacção múltipla em modo positivo (2 MRM's por analito). Uma curva de calibração de seis pontos foi usada, de modo a englobar concentrações de 10 ng/mL a 1000 ng/mL. O método foi validado baseado no guia do Scientific Working Group for Forensic Toxicology (SWGTOX).

Resultados: Não foram encontradas interferências da matriz, padrões internos ou medicamentos antidepressivos tricíclicos e o método provou ser selectivo para todos os compostos. Limites de quantificação e de detecção vão desde 10 a 85 ng/mL e 2.0 a 28 ng/mL, respectivamente. Supressão iónica dos efeitos de

matriz variam de 36 a 108% para os compostos e de 47 a 102% para os padrões internos. A eficiência do processo varia desde 22 a 106%. A imprecisão intra e inter-ensaios varia desde 3.5 a 12.3% e 3.1 a 12.3%, rescpectivamente. A inexactidão foi mais baixa que 15% para todos os compostos, excepto para as concentrações mais baixas de desmetilcitalopram, tramadol e trazodone (exactidão < 20%). Não foram observados fenómenos de arrastamento.

Conclusão: Uma boa validação foi obtida graças ao uso de padrões internos deuterados que se ajustaram a todos os compostos de modo a compensar a variabilidade global do método. Este método é então adequado tanto a monitorização terapêutica como a quantificação nos casos de suspeita de overdose.

Palavras-chave: fármacos psicotrópicos; soro; quantificação; validação; LC-MS/MS; TQD

ABSTRACT

Objective: Non-tricyclic antidepressants are often encountered in overdose cases, and for some of them therapeutic monitoring might be warranted. Therefore we developed and validated an ultra-performance liquid chromatography-tandem mass spectrometry method for the quantification of 15 common non-tricyclic antidepressants in serum: citalopram, desmethylcitalopram, desmethylfluoxetine, desmethylvenlafaxine, duloxetine, fluoxetine, fluvoxamine, melitracene, mianserin, mirtazapine, paroxetine, reboxetine, sertraline, trazodone and venlafaxine. The selected pharmaceuticals represent (nor) tramadol (added because of the desmethylvenlafaxine interference) and all the non-tricyclic antidepressants available in Belgium.

Methods: Serum samples (250 µL) were precipitated with methanol and acetonitrile containing 5 ng of the internal standards: mirtazapine-d_4, paroxetine-d_5, reboxetine-d_6 and venlafaxine-d_6. The supernatant was evaporated at 56ºC under a flow of nitrogen and reconstituted in 100 µL of 50:50 methanol:water with 2 mM ammonium acetate. Five µL was injected and the target analytes were separated on a 100 × 2.1 mm ACQUITY BEH C18 column (Waters, Zellik, Belgium) using a Waters ACQUITY Ultra-Performance Liquid Chromatography system. The analytes were eluted within 7.5 min, using a gradient of 0.2% ammonium acetate and 0.1% formic acid in water and in methanol at a flow rate of 0.4 mL/min. Quantification was performed on a Waters triple quadrupole ACQUITY TQD using multiple reaction monitoring (MRM) in positive mode (2 MRMs per analyte). A six point calibration curve was used to cover a large concentration range from 10 ng/mL to 1000 ng/mL. The method was validated based on the Scientific Working Group for Forensic Toxicology (SWGTOX) guideline.

Results: No interfering signals from matrix, internal standard or tricyclic antidepressant drugs were found. Limits of quantification and limits of detection ranged from 10 to 85 ng/mL and 2.0 to 28 ng/mL respectively. Ion suppression from matrix effects varied from 36 to 108% for the compounds and 47 to 102% for the internal standards. Process efficiency varied from 22 to 106%. Intra-

and interassay imprecision varied from 3.5 to 12.3% and 3.1 to 12.3%, respectively. The bias of the assay was lower than 15% for all the compounds, except the lowest concentrations of desmethylcitalopram, tramadol and trazodone (bias < 20%). No carryover was observed.

Conclusion: Good validation performance was obtained thanks to the use of selected deuterated internal standards that adjusted for all of the compounds, to compensate for the global method variability. This method is therefore suitable for both therapeutic drug monitoring and quantification in suspected overdose cases.

Keywords: psychotropic drug; serum; quantification; validation; LC-MS/MS; TQD

1. INTRODUCTION

1.1 Psychiatric Drugs

A psychcotropic drug (PDs) can be defined as any medication capable of affecting the mind, emotions, and behavior [12]. Psychotropic Drugs affect the functioning of the mind through pharmacological action on the Central Nervous System (CNS) by crossing the blood-brain barrier, resulting in alterations in perception, mood or behavior [13]. Examples of psychiatric drugs include tricyclic antidepressants, phenothiazines antipsychotic drugs, tetracyclic antidepressants, butyrophenones, and serotonin reuptake inhibitors [14].

PDs are divided in six main categories, such as antidepressants, antipsychotics, anxiolytics, antiepileptics, lithium and other drugs [15]. In forensic toxicology, antidepressants are of considerable interest because of their abuse potential and their involvement in intoxications and suicides, which makes the ability to reliably detect this type of drugs in human biological specimens a necessity [5, 6]. The detection of a PD is crucial in determining whether these drugs played a role in the cause of death [16].

1.1.1 Depression and Antidepressants

Depression is expected to be the second most serious illness by the year 2020 [18]. It is a serious psychiatric illness with a highly variable set of symptoms: feelings of helplessness and hopelessness, loss of interest in daily activities, appetite or weight changes, sleep changes, anger or irritability, loss of energy and poor concentration. The individual's ability to take care of his everyday responsibilities is affected and at its worst can even lead to suicide [8]. World Health Organization considered depression as number 1 in causing disability [1]. The World Mental Health made a study about the prevalence of depression episodes in the 18 countries participating in its surveys (Table 1.1).

Treatment of depression includes various forms of psychotherapy and pharmacotherapy with antidepressants and even electroconvulsive therapy [8]. The antidepressants are currently among the most frequently therapeutic agents in medicine, mainly because of their efficacy (elevation of mood, improved appetite and sleep patterns, increases physical activity, decreased feelings of guilt, among others [19]). This type of drugs can also be used to treat anxiety, obsessive-compulsive or psychosomatic disorders. However, antidepressants in general have some side effects, as insomnia, dry mouth, blurred vision, constipation, dizziness, agitation, irritability [15].

Tabel 1.1. - Prevalence of depression in the 18 countries participating in the WMH surveys. Screen+ - the proportion of respondents who reported ever having a time lasting several days when they were sad, depressed, or lost all interest in their usual activities;
SE – Standard Error.
Adapted from [9].

	Screen +	
	%	(SE)
High income		
Belgium	14.1	0.1
France	21.0	1.1
Germany	9.9	0.6
Israel	10.2	0.5
Italy	9.9	0.5
Japan	6.6	0.5
Netherlands	17.9	1.0
New Zealand	17.8	0.4
Spain	10.6	0.5
United States	19.2	0.5
Total	14.6	0.2
Low- to middle-income		
Brazil (São Paulo)	18.4	0.8
Colombia	13.3	0.6
India (Pondicherry)	9.0	0.5
Lebanon	10.9	0.9
Mexico	8.0	0.5
PRC (Shenzhen)	6.5	0.4
South Africa	9.8	0.7
Ukraine	14.6	0.7
Total	11.1	0.2

Commonly prescribed antidepressants are divided into three classes: monoamine oxidase inhibitors (MAOI), trycliclic antidepressants (TCA) and selective serotonin reuptake inhibitors (SSRI). Of these three classes, tryciclics and SRRIs work by blocking reuptake of neurotransmitters, especially norepinephrine, dopamine, and serotonin [4, 7, 10]. Therefore, they produce an increase in the concentration of neurotransmitters in the synaptic gap.

Although the underlying pathophysiology of depression has not been clearly defined, preclinical and clinical evidence suggest disturbances in serotonin (5-HT), norepinephrine (NE), and dopamine (DA) neurotransmission in the central nervous system [20]. When the balance of neurotransmitters like dopamine, norepinephrine and serotonin is disturbed, emotional regulation becomes unstable and the syndromes of depression and mania may develop. Dopamine and serotonin, for example, is popular for be contributor to feelings of well-being and happiness. Also, in the absence of serotonin, depression is easily triggered [21]. Norepinephrine affects parts of the

brain where attention and responding actions are controlled and plays a determinant role in executive functioning regulating cognition, motivation, and intellect, which are fundamental in social relationships [20].

Although the new-generation antidepressants have a low toxicity profile, analysis of forensic and clinical samples is important to investigate cases of violent crime, unknown death, drug facilitated sexual assault cases and therapeutic drug monitoring. Intoxications with these new-generation antidepressants in healthy individuals are rare and require very high concentrations (reflecting intentional overdoses), but may still be involved in overdose deaths, particularly when combined with other drugs [8].

Antidepressants can be classified according to the chemical structure or pharmacological action. The pharmacological action is currently more used [15]. The selective serotonin reuptake inhibitors (SSRI) have emerged as a major therapeutic advance in psychopharmacology [13], but serotonin norepinephrine reuptake inhibitors (SNRI), Noradrenergic/Specific Serotonergic antidepressants (NaSSA) and Serotonin Antagonists/Reuptake Inhibitors (SARI) are also used nowadays [4,10].

1.1.1.1 Selective Serotonin Reuptake Inhibitors

Serotonin is a neurotransmitter especially relevant in affective disorders, compulsive-obsessive disorder and aggressive behavior [15]. It is also linked to memory, cognition, mood regulation, sleep, pain, blood vessel regulation, anxiety and depression [21].

SSRI inhibit serotonin reuptake, increasing concentrations of serotonin in the synapse gap, which causes enhancing of serotonin neurotransmission and results in their antidepressant effects [2, 10, 12, 13]. Some SSRIs also inhibit the reuptake of norepinephrine (such as fluoxetine and paroxetine), while others inhibit the reuptake of dopamine (sertraline).

This type of antidepressant is absorbed relatively slowly and it is metabolized primarily by liver metabolic enzymes. SSRIs are potent inhibitors of several different isozymes of the cytochrome P450 family of enzymes. Cytochrome P450 2D6 (enzyme member of the cytochrome P450 mixed-function oxidase system) has received the most attention, because all of SSRIs are inhibitors of this particularly enzyme, interfering in its task: O-demethylation [23]. Their metabolites are mainly eliminated in the urine [19].

When SSRIs are ingested with other medications or in really high doses, it may lead to serotonin syndrome – excessive serotonin levels that arise from an overdose of serotonin reuptake inhibitor or by co medication of serotonin reuptake inhibitor and drugs that interfere with the metabolism of serotonin. This serotonin syndrome leads to agitation, mental status change, diaphoresis, myoclonus, diarrhea, fever, hyperreflexia, tremor, or incoordination and can eventually lead to death [8]. In general, SSRIs have milder adverse effects than older antidepressants, and their adverse effects are often dose related [19].

Citalopram, fluoxetine, fluvoxamine, paroxetine and sertraline are some of the antidepressants studied in this project that are SSRIs.

1.1.1.2. Serotonin Norepinephrine reuptake inhibitors

Norepinephrine is also hypothesized to be involved in depression (due a dysfunction of a brain area where norepinephrine is present [20]) and it is synthesized from dopamine, another mood neurotransmitter [24].

SNRI selectively inhibit the reuptake of norepinephrine, but have little effect on the reuptake of serotonin or dopamine. They have little affinity for muscarinic or cholinergic receptors and do not interact with serotonergic, dopaminergic or histaminergic receptors [15].

All SNRI work in a similar way and generally cause similar side effects. However, each SNRI varies in chemical makeup, so one may affect you differently than another does. Side effects are usually mild and go away after the first few weeks of treatment. The most common side effects of SNRI include nausea, dry mouth, dizziness, excessive sweating and other side effects may include tiredness, difficulty urinating, constipation, insomnia or loss of appetite [19].

Duloxetine, reboxetine, venlafaxine are examples of this type of antidepressants. Duloxetine is mainly metabolized by cytochrome P450 1A2 and 2D6 (enzymes members of the cytochrome P450 mixed-function oxidase system)and eliminated by urine and feces [25]; reboxetine is metabolized in liver, mainly through the 3A4 isozyme of cytochrome P450 and it is excreted by kidney; venlafaxine is extensively metabolized in the liver via the CYP 2D6 isoenzyme to desmethylvenlafaxine. The primary route of excretion of venlafaxine and its metabolites is via the kidneys [19]. It

is a potent inhibitor of the reuptake of serotonin, at higher doses inhibits the reuptake of noradrenaline and slightly inhibits the reuptake of dopamine [15].

1.1.1.3. Noradrenergic/Specific Serotonergic antidepressants

NaSSA works by blocking receptors called alpha-2 receptors that are found on nerve cells in the brain. This enhances the action of noradrenaline and serotonin in the brain [19, 26].

People who take NaSSA may have side effects like sleepiness and nausea. Other common side effects are dizziness, increased appetite high cholesterol or weight gain. Less common adverse effects include weakness, rapid heartbeats, dry mouth, tremor, confusion or vision disturbances [4, 10].

This type of antidepressants is extensively metabolized via CYP 1A2, 2D6 and 3A4 and it is excreted by the kidneys. Mirtazapine is a compound studied in this project that belongs to this category. It is well absorbed from gastrointestinal tract after oral administration and eliminated from the body mostly by the kidneys [26].

1.1.1.4. Serotonin Antagonists/Reuptake Inhibitors

SARI are another class used as antidepressants, anxiolytics and hypnotics that modulate serotonin activity [27]. They act by blocking the postsynaptic 5-HT2 receptors and the transporter, inhibiting the reuptake of serotonin [28].

SARI are completely absorbed from the GI tract and metabolized primarily by the liver [19]. Most common side effects are sedation, nausea, postural hypotension or priapism (rare, but dangerous) [15].

Since the SARI have drawn attention for their sleep-improving potential, Trazodone is now the second most commonly prescribed drug for insomnia [27]. Its mechanism of action is the modulation of serotonergic neurotransmission; it is a relatively specific inhibitor of the reuptake of serotonin [15]. Trazodone is metabolized by CYP3A4 and eliminated by urine in 72 hours [19].

The main psychochemical characteristics of the compounds of interest are in table I (Section V).

This method was also developed for detection and quantification of two analgesics: Tramadol and DM Tramadol. Since Tramadol and DM venlafaxine have the same precursor ion and similar product ions (table 1.3), we needed a method that can distinguish both. This is important because false positives results for tramadol were observed in cases where subjects were being treated with venlafaxine [29].

Table 1.3. Percursor and product ions from DM Venlafaxine, Tramadol and DM Tramadol.

	Percursor Ion	Product Ion
DM Venlafaxine	263.375	58.000 107.100 246.220
Tramadol	263.375	58.000 246.200
DM Tramadol	249.349	44.000

Tramadol is a centrally acting analgesic used for the treatment of moderate to severe pains. The drug inhibits the reuptake of norepinephrine and serotonin. Side effects from tramadol use include seizures and respiratory depression [30]. Its main active metabolite is DM Tramadol that retains activity as a norepinephrine reuptake inhibitor.

1.1.2. Drugs Intoxication

One of the most commonly used methods of self-injury worldwide is drug overdose [31]. As depression is a serious psychiatric illness, there is a high suicide rate among depressed patients, however acute intoxications with these new-generation antidepressants in healthy individuals are rare and mostly concern very high concentrations. Pharmacological treatment is essential for an adequate management of these psychiatric disorders. These highly prescribed antidepressants are frequently coadministered with other legal or illegal drugs, in case of large intentional overdoses [8, 25]. More, there are clinical studies that question the efficacy and safety of these drugs, showing a profile of toxicity that is still not well known [33]. This fact has led to the development of reliable analytical methods for their analysis [34].

Therefore, the development of more efficient analytical techniques is important in clinical toxicology, where they help in monitoring therapy (because it is difficult to interpret the relationship between plasma concentration and therapeutic and side

effects), and in forensic toxicology, given the correlation between depression and premature death [33].

Forensic science is concerned with determining the cause of death and the results of a forensic investigation may have a serious impact on lives [12]. It implies an understanding of drug use in the immediate ante mortem setting, analytical methodologies and interpretation of results [36]. Post-mortem forensic science involves analyzing body fluids and organs from death cases and interpreting that information [37].

One of the more important issues confronting the interpretation of post-mortem toxicology results the possibility of changes in drug concentration after death; also, there are several biological matrices that can be analyzed to determinate concentrations. Generally, specimens routinely collected at autopsy include fluids such as blood from peripheral sites and heart blood, urine, bile, cerebrospinal fluid, vitreous humor, gastric contents and organ tissues, particularly liver [37].

The substance itself does not make something a drug or a poison but rather the amount of it that is ingested. Even common materials and pharmaceuticals can be poison if too much is ingested; it is the dose that makes a poison. A drug is a substance characterized by having properties that is used to treat or prevent a disease or to treat symptoms of a disease or injury. A poison is a substance that is capable of causing harm to an organism, whether it is an illness, injury or death. The modern definition of term poison is essentially the same as that of a toxic substance. The more toxic or poisonous a substance is, the more harm a small amount of it can cause. Only the amount and time over which the substance is administered will allow to determine how harmful it will be [38].

It is also important to define therapeutic levels. Those levels are the steady state concentrations that need to be reached for the drug to exert a significant clinical benefit without causing unacceptable side effects. A therapeutic level implies a concentration at which a useful response is obtained free from any toxicity. And toxic levels are concentrations above which unacceptable side or toxic effects might appear. [31, 34]. A lethal concentration is the concentration that has been reported to cause death, or is so far above reported therapeutic or toxic concentrations, that one can judge that it might cause death in humans.

Table 1.4. shows therapeutic, toxic and lethal concentrations in blood for each drug that is subject of study in this project. These concentrations are calculated on plasma exposure values. However, all these values are not considered absolute, but are to be used as a guide. It's important to mentioned that these values can be affected by route of administration, absorption differences, age and sex, tolerance or pathological or disease state [40].

Table 1.4. Toxic concentrations, lethal concentrations and recommended therapeutic range for psychotropic drugs.

Compound	Therapeutic Concentration (ng/mL)	Toxic Concentration (ng/mL)	Lethal Concentration (ng/mL)	Reference
Citalopram	81 – 160	--	240 – 1300	[40]
DM Citalopram	10 – 200	--	5000 – 6000	[39]
DM Fluoxetine	180 – 466	--	900 - 5000	[41]
DM Tramadol	100 - 1000	1000	2000	[39]
DM Venlafaxine	200 – 400	1000 – 1500	6600	[39]
Duloxetine	0 - 30	--	>1000	[39]
Fluoxetine	90 – 400	1000	1300 - 6800	[40]
Fluvoxamine	150 – 250	650	2800	[39]
Melitracene	10 – 100	--	12000 - 23000	[39]
Mianserine	10 – 150	500 - 5000	3000 – 19000	[39]
Mirtazapine	4 - 40	100 - 200	2000 – 4000	[39]
Paroxetine	31- 62	--	1400 - 3400	[40]
Reboxetine	C_{max} < 300	--	--	[39]
Sertraline	55 – 250	290	1600 – 3000	[40]
Tramadol	100 - 600	1000	2000	[39]
Trazodone	800 – 1600	4000	12000 – 15000	[39]
Venlafaxine	200 – 400	1000 – 1500	6600	[39]

1.1.3. Biological Matrices

In clinical or forensic toxicology, a specific analytical method may be indicated to screen and quantify compounds (in this case, antidepressants) in biological matrices

[8]. A drug may be detected in any body fluid or tissue with which it has been in contact. The primary choice of biological specimen for drug analysis depends on issues like purpose of the sampling, time interval to study, willingness to provide a voluntary sample, cost of sample preparation and its analysis, drug concentration and drug stability. Blood, urine, oral fluid and hair are the most common matrices in toxicology [42].

To choose the biological matrix, it is important to know the stability of analytes in biological material. This is very important to ensure the reliability of analytical results, because there are gaps between sample collection, transportation, sample preparation and time of the analysis [43]. Different biological matrices are used to determine antidepressants, but blood is the most relevant matrix as it gives a direct link between the compound concentration and the effect [8].

1.1.3.1. Blood

Blood (including serum and plasma) is the only biological specimen, except for cerebrospinal fluid, which reflects the drug concentration in the brain. Blood samples must be collected by qualified personnel with vacuum tubes or syringes and usually 5-10 mL is collected [42]. Blood is use to determinate the presence of drug intoxication, inebriation or supra-therapeutic drug use [5, 37, 39].

The following table contains advantages and disadvantages about blood as biological matrix (Table 1.5).

Table 1.5. Some advantages and disadvantages of using blood as a biological matrix. Adapted from [42]

	Blood
Maximum drug detection period	**1-2 days***
Intrusive Sampling	**Yes**
Adulteration potential	**None**
Possibility for environmental contamination	**No**
Potential for negative result after drug use	**Low**
Analytical costs (Including confirmation testing)	**Medium**

* There are some exceptions. For example, fluoxetine half-life is 4 to 6 days, so it can be detected for longer time [42].

The main limitation of blood is its maximum drug detection period: blood samples are not suitable to study drug use during a wider time-frame.

There are several analytical methods for drugs of abuse in blood [45]. Blood is analyzed by gas chromatography with single or tandem mass spectrometric detection (GC-MS), and recently liquid chromatography with single or tandem mass spectrometric detection (LC-MS and LC-MS/MS) has become the technology of choice for identification and quantification of a wide range of compounds [37, 40].

1.2. Analytical methodologies for psychotropic drug analysis

An important step in the development of an analytical method is the extraction of the compounds of interest from the biological matrix as this will have implications on the overall sensitivity and selectivity of the method [8]. Sample preparation is, by definition, a method to concentrate a component of interest to adequate levels for measurement free from interfering matrix elements [46].

1.2.1. Analytical methodology for sample preparation

The main objective of sample preparation is to convert a real biological matrix into a form suitable for analysis by the desired analytical technique [39, 42 - 44]. Biological matrices, like plasma, are very complex. They contain a wide variety of matrix components such as proteins, lipids and salts. Typical preparation includes dilution, extraction, evaporation and reconstitution [50].

The point of sample preparation is to remove potential interferences such as proteins and peptides, from the sample [43, 44, 46]. Peptides and proteins are incompatible with standard chromatographic set-ups since the columns would be block and ruined. Furthermore, peptides tend to interfere with atmospheric pressure ionization, increasing the baseline and causing noise. Phospholipids have also to be removed; otherwise they can cause unspecific signals and ion suppression [48].

Another important point of sample preparation is that it can concentrate the analyte in order to obtain lower limits of quantification [49]. Concentration is usually performed by extraction methods. Removal of salts also minimizes contamination of the ion source and is thus relevant for robustness [48].

Four main methods of sample extraction are applied in liquid chromatography: Protein precipitation (PP) (by addition of organic solvents, inorganic acids and/or chaotropic salts); protein filtration; solvent extraction (liquid–liquid extraction, LLE); and solid phase extraction (SPE). The method must be chosen considering the respective analyte and the MS/MS system [43, 44].

All procedures also requires the addition of an internal standard (IS). Internal standards play critical roles in ensuring the accuracy of reported concentrations in LC-MS/MS analysis. To quantify traces amount of analytes in complex biological samples by this technique, those have to be treated. Variable losses of the analytes may occur during these sample treatment steps; also, there might be some variations during the LC-MS/MS analysis (variations in injection volume or ionization) [45 ,55].

To reduce the impact of those losses and instrumental variations, an IS is added in equal amount to both concentration-known and unknown samples (calibration standards and quality controls). IS must have the same or similar physical and chemical properties as the analyte [53].

The IS is employed as a calibrant and the concentration of a unknown sample is calculated from the ratio between analyte/IS signal ratio [53].

An IS should meet the following requirements:

- It should have the same or very similar physico-chemical properties as the analyte, so that it can mimic closely the performance of the analyte in every stage of analysis. In this way, any losses during sample preparation or variations in can be corrected [45, 55];
- It must have adequate purity. The interference of an internal standard to other cointernal standards in a multianalyte method is rare, but it should be also evaluated. Though there are no reported criteria for this, it should be at least less than 15 % of the concentration of a cointernal standard in a multianalyte method [54];
- It must be stable during sample processing and LC separation. In addition, an internal standard should not correspond to any in vivo metabolic products of the analyte [55];
- Its molecular mass should be distinct from that of the analyte.

There are three main types of IS [56, 58]. The first ones are stable isotope labeled (SIL) internal standards. They are compounds in which several atoms in the analytes are replaced by their respective stable isotopes, such as deuterium (^{2}H, D or d), ^{13}C, ^{15}N, or ^{17}O.

The second type of IS covers structural analogues with the same (e.g. an ortho and meta substituted compound) or different mass (e.g. compounds with similar structure and small differences in functional groups) [52]. Finally, there are also compounds from the same chemical family. In this project, we used the third type and then changed the method to choose the first type.

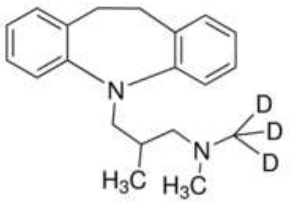


Fig. 1.1. Trimipramine-d_3

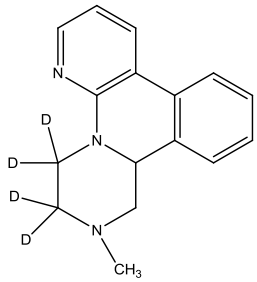


Fig. 1.2. Mirtazapine-d_4

The IS chosen was Trimipramine-d_3 (Fig. 1.1), 3-(10,11-Dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N,2-trimethyl-1-propanamine. It's the non-deuterated molecular formula is $C_{20}H_{26}N_2$ and its mass is 294.434 g/mol. It is also an antidepressant, with sleep-promoting effects. It is mainly used to treat insomnia [56].

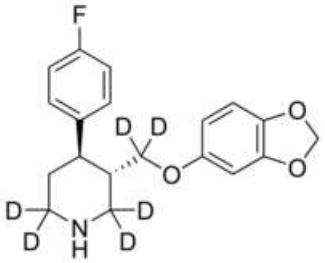


Fig. 1.3. Paroxetine-d_6

However, we didn't obtain good results with trimipramine-d_3. The Results and Discussion below explain why. New internal standards were added (and trimipramine-d_3 excluded) in order to get better results: Mirtazapine-d_4, Paroxetine-d_6, Reboxetine-d_5 and Venlafaxine-d_6 (Fig. 1.2 – 1.5).

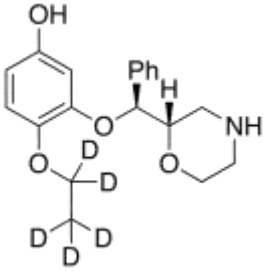


Fig. 1.4. Reboxetine-d_5

1.2.2. Sample preparation of plasma – Protein precipitation

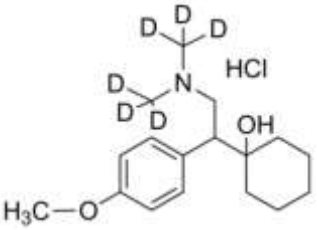


Fig. 1.5. Venlafaxine-d_6

Protein precipitation (PP) with miscible organic solvents (usually acetonitrile or methanol) is the most commonly used plasma sample preparation method because of its low cost and minimal method development requirements. This method

provides sufficient clean-up for most LC-MS analyses [57]. It is rapid and simple, and has a good recovery of non polar and analytes, compared to liquid-liquid extraction [43, 46]. However, the precipitated proteins may bind various small molecules and remove them from the solution and thus, influence quantification [49].

PP is based on the interaction between the reagent and the protein [47]. To denature the proteins, an organic solvent (typically acetonitrile, methanol or ethanol) or an acid (e.g. trichloroacetic) is added to a sample. Organic solvents are the most used because of its cost and minimal method development requirements [57].

With the addition of an IS and a treatment agent (2-5 volumes of a water mixable organic solvent such as acetonitrile, methanol, isopropanol or acetone) to the sample, the mixture is agitated to increase the aggregation speed of the proteins. The supernatant, which contains the analyte, is then separated from the protein aggregate by centrifugation [47].

PP is straightforward, but does not allow concentration of the analytes. Instead, typically a dilution of at least 1:2 is obtained [48]. PP may be performed alone or in conjunction with another extraction technique. For example, protein precipitation is commonly performed prior to (SPE) [58].

1.3. Liquid Chromatography coupled to Mass Spectrometry

Over the last two decades, there has been a growing interest in the development of methodologies for qualitative and quantitative analysis of several drugs in different biological matrices. The most common approach to detecting drugs is the analysis of biological fluids and tissues [59]. Liquid chromatography-mass spectrometry (LC-MS) has become a mature technique finding many applications in clinical chemistry and forensic toxicology. It is often used for the analysis of most of the common drugs in biological matrices [8, 22, 50]. LC-MS is the method of choice because the sensitivity, selectivity and the relatively high throughput that can be achieved and the determination of multiple groups of compounds can be performed in a single method. Some of the advantages of this technique include easier sample preparation, avoidance of derivatization procedures and short analysis time [60].

Chromatography is the concept of separating compounds based on physicochemical properties. The components can be separated by distribution between two phases: a phase that is stationary (component of the system with which the compounds in the mixture will interact), and a mobile phase that moves in a definite direction [61].

But this step is not sufficient to give us an unequivocal identification. It needs further information from an auxiliary technique – Mass spectrometry. This last one provides molecular weight of the analyte and structural information from the molecule under investigation, which is unique for every molecule [12]. It's powerful analytical features, such as sensitivity, selectivity, speed of analysis, cost and effectiveness, have continually improved, resulting in more reliable instruments and easier to use [62].

1.3.1. Liquid Chromatography (LC)

In liquid chromatography, the substances are separated based on differential solubility's in the two phases, with identification based on retention times within a column. Retention time (RT) is the time required for an analyte to elute from a chromatographic column with a particular mobile phase [61].

High-performance liquid chromatography (HPLC) is the term used to describe LC in which the liquid mobile phase is mechanically pumped through a column (with stationary phase) with high pressures [12].

The interaction of the analyte with the stationary phase may be adsorption, partition, size exclusion, affinity and ion exchange. In adsorption chromatography, the solute are in contact with both phases and when interacts with the stationary phase, the polar solutes will be retained longest by polar stationary phases and the nonpolar solutes will be retained best by nonpolar stationary phases [56, 60].

Fig. 1.6 illustrates the components of an HPLC system. A solvent reservoir and solvent delivery system or high pressure pump are coupled with a sample injection system to provide the flow of the mobile phase through the column and for the separation of the sample in the column bed. A detector detects the bands of the sample components as they exit the column. These are converted into electrical signals that can be processed by the data management system or computer associated with the system [61].

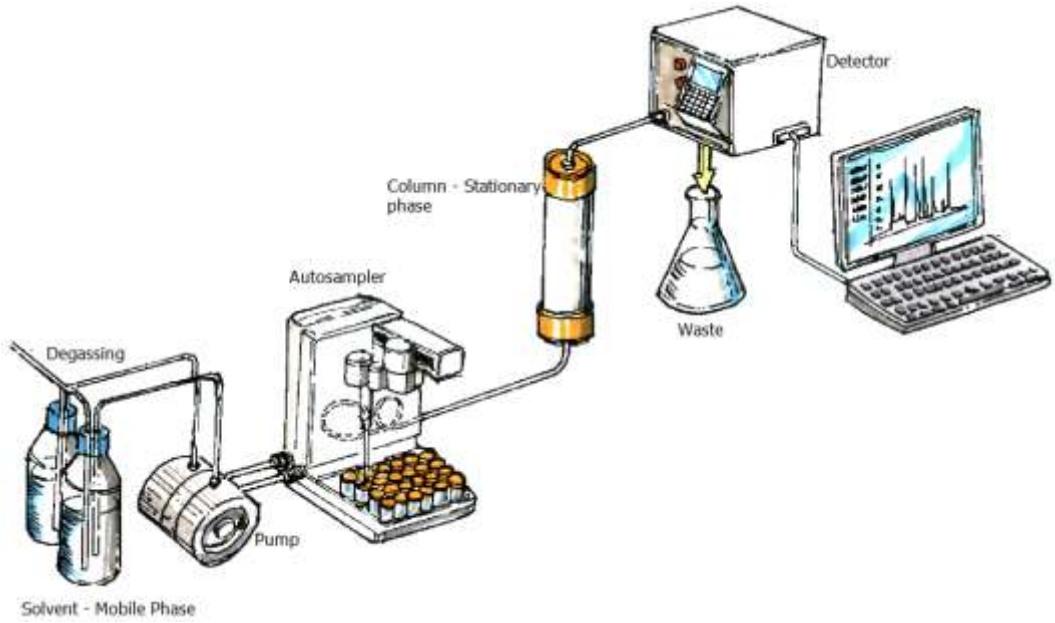


Fig.1.6. Basic Components of HPLC. Adpated from [98].

The capacity to precisely regulate pressure provides direct control over solvent velocity, which enhances the degree of separation and decreases the level of component diffusion into the column. Large molecules can be reliably analyzed and a much broader range of polarities can be handled with this technique [61].

HPLC is divided into two basic procedures: normal-phase HPLC and reverse-phase HPLC. The main difference is in the composition of column packing material, which necessitates the use of different solvents. Normal phase is the less common method and it has been replaced by almost exclusive use of reverse phase. In normal phase separations, the stationary phase is relatively polar and the mobile phase is relatively non-polar. More polar analytes will be retained on the polar stationary phase, while less polar analytes elute faster [52, 60].

Reverse phase is the most common and, typically, more efficient method. It uses a non-polar stationary phase and a polar mobile phase. An example stationary phase is long chain C18 bonded to silica. Most mobile phases for reverse phase are a combination of water and miscible organic solvents, such as acetonitrile or methanol, or a mixture with various buffer salts [61]. Thus, the more polar analytes elute more rapidly than the less polar ones and a decrease in the polarity of the mobile phase results in a decrease in solute retention.

In LC-MS certain ionic modifiers are often added to the mobile phase to influence analyte ionization. A volatile mobile phase must be used to ensure reliable analysis. Small organic acids like formic and acetic acid are among the most commonly used additives. They improve ionization and resolution of a wide range of molecules [59].

Retetion time can also be controlled by changing the polarity of the mobile phase: increasing the polarity of the mobile phase leads to longer retention times and vice-versa.

Chromatography is a purification technique that is generally considered a nonspecific form of identification: it doesn't necessarily provide definitive proof in the identification of a substance. Although retention times are characteristic for a given compound under a specific set of analytical conditions, one must have a reference for comparison and identification. The molecular weight of the analyte together with the structural information that may be generated, allows an unequivocal identification –

the mass spectrometer provides the most definitive identification of all the HPLC detectors [12].

1.3.2. Mass Spectrometry

The mass spectrometer is an analytical instrument that separates and identifies ionized analytes or fragments based on their mass/charge ratio by passing them through a magnetic field. All the instruments have an ionization source at the inlet, followed by mass selective detector that allows for the selection of particular masses and the final component is a detector that translates and amplifies the selected ion signal into an electronic signal sufficient for data processing [64].

While compounds may be multiply charged, for most toxicological applications, analytes are singly charged. This means that the z in the m/z ratio equals to 1. The purpose is to obtain a unique, reproducible fragmentation pattern of a particular molecular species [12].

The first step in the mass spectrometric analysis is the production of gas phase ions (ionization) of the sample being tested and to introduce those ions into the high-vacuum, mass selecting stage of the instrument, so they can be collected and recorded. High-vacuum environment ensures that the ions can travel unimpeded to the detector. The next step is the separation and mass analysis of the molecular ions and their charged fragments on the basis m/z ratio and finally, the ion current is measured and the result is in the form of a mass spectrum (Fig. 1.7) [53].

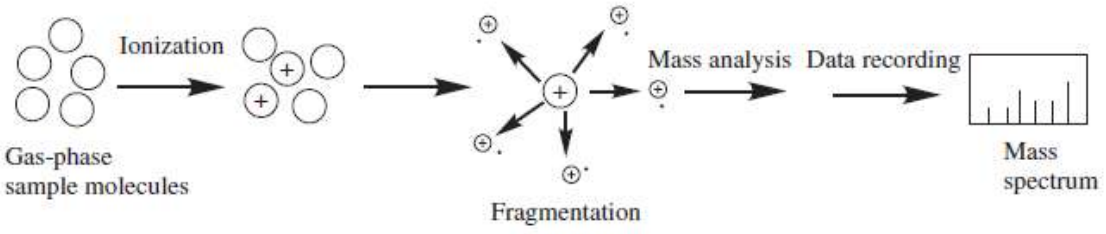


Fig. 1.7. Basic concept of mass spectrometry analysis. The excess energy transferred during an ionization event may break the molecule into characteristic fragments. Adapted from [53].

The inlet system for LC, often termed the "interface" between the two component techniques, must therefore remove as much of the unwanted mobile phases as possible while still passing the maximum amount of analyte into the mass

spectrometer. Fig. 1.8. represents the essential components of a mass spectrometer [53].

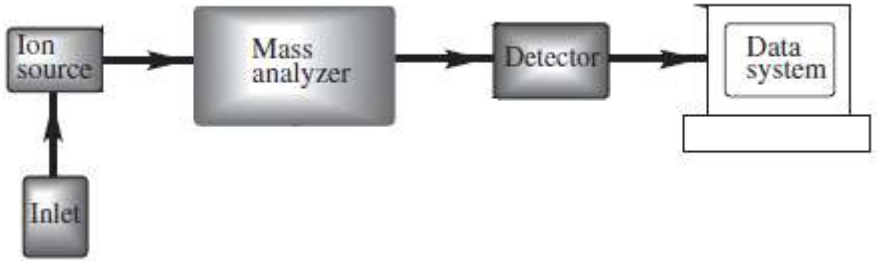


Fig.1.8. Essential components of a mass spectrometer. The inlet system transfers a sample into the ion source. The ion source coverts the neutral sample molecules into gas-phase ions. Then, the mass analyzer separates and analyze the ionic species. Here, a magnetic field is used to control the motion of ions. The detector measures and amplifies the ion current of mass-resolved ions. Finally, the data system records, processes, stores and displays data. Adapted from [53].

Liquid solutions are difficult to handle by MS vacuum systems and require some novel introduction and ionization systems. Ionization of the analyte is a very important step in the analysis of any class of compounds by MS.

1.3.3. Modes of Ionization

The choice of a particular method is mostly dictated by the nature of the sample under investigation and the type of information desired [53]. In this case, toxicology analyzes mostly smaller molecules and highly polar compounds. This makes Electrospray Ionization (ESI) the most appropriate for our experiment. ESI has been a major advancement and essentially is the technology that allows for the existence of LC-MS/MS [61]. In particular, it has made an enormous impact in the characterization of large biomolecules [53]. Electrospray analysis can be performed in positive, which typically results in protonated molecular ions, $[M+H]^+$, and negative ionization modes, which means deprotonated molecular ions $[M-H]^-$ [55]. Positive ionization mode is generally applied, because most toxicologically relevant compounds have basic properties.

There are three stages of ESI: nebulization, evaporation and ionization. Nebulization is the formation of very small charged droplets, thanks to the charged needle. Evaporation further shrinks the droplets by using a warm or dry gas (such as nitrogen) to evaporate the solvent and to increase the surface charge density. Ionization happens when the droplet reaches a critical size, at which like charges repel each other and fragment the small droplet. As the solvent further evaporates, the

droplet reaches a size such that charged molecules can no longer stay in the solution phase and are expelled into the gas phase (fig 1.9) [52 , 61].

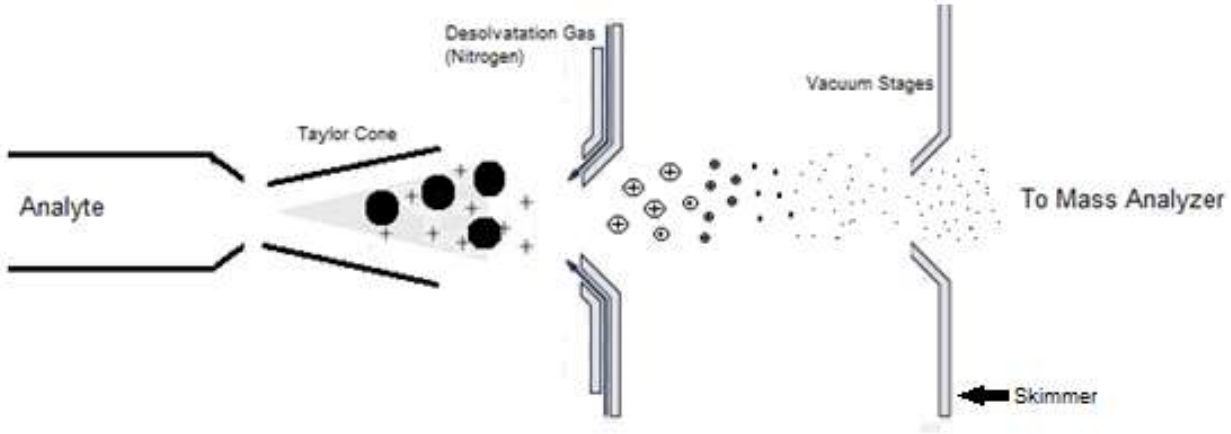


Fig. 1.9. Mechanism of ion formation in the positive ESI mode. The analyte is introduced into the ESI source throw a needle as an eluent flow from the LC. The Electrospray itself is formed as a result of a large electrostatic potential difference between the capillary and a cone electrode. Cations concentrate at the tip of the capillary (Taylor cone) and tend to migrate toward the cone electrode. The air, which is passed continuously in the region spraying, helps the evaporation of the solvent. As the size of the droplet reduces, the repulsive force between charges on the surface of the droplets overcome the cohesive forces of surface tension and causes the droplets to disintegrate (Coulombic explosion). The skimmer is used to retain these droplets and guide the ion to the analyzer region of the mass spectrometer. Adapted from [53].

ESI is most applicable to larger, more polar compounds, but, macromolecules greater than about 1 kilodalton may experience fragmentation. ESI only works for compounds that are ions in solution. This makes it solvent dependent. Only those solvents that can support the solution ions and help form the ions in solution can be used. ESI is also flow dependent. The dynamics of the flow are an integral part of droplet formation. Too much or too little flow and droplets do not form properly [53], [65].

1.3.4. Quadrupole Analyzer

Once the gas-phase ions have been produced, they need to be separated according to their masses, which must be determined. The physical property of ions that is measured by a mass analyzer is their mass-to-charge ratio (m/z).

As there are a great variety of sources, several types of mass analyzers have been developed. All mass analyzers use static or dynamic electric and magnetic fields that can be used alone or combined [55].

A quadrupole analyzer is the type of mass analyzer used in this project and is probably the most used type of mass analyser. It is an ideal detector for chromatography as it is capable of fast scanning [53].

The quadrupole analyser is a device which uses the stability of the trajectories in oscillating electric fields to separate ions according to their m/z ratios. This field is made up of four circular rods that must be perfectly parallel [53]. This allows for ions of a given mass to charge ratio to pass while others are not stable through the length of the field (fig 1.10).

In this method, ions of a specific mass to charge ratio are able to traverse the path established by the electromagnetic fields applied to the poles. Those ions that are too heavy or too light are unable to travel the entire distance to the detector and are thus not detected. So the electromagnetic conditions can be varied very rapidly to select the desired masses in the analysis [65].

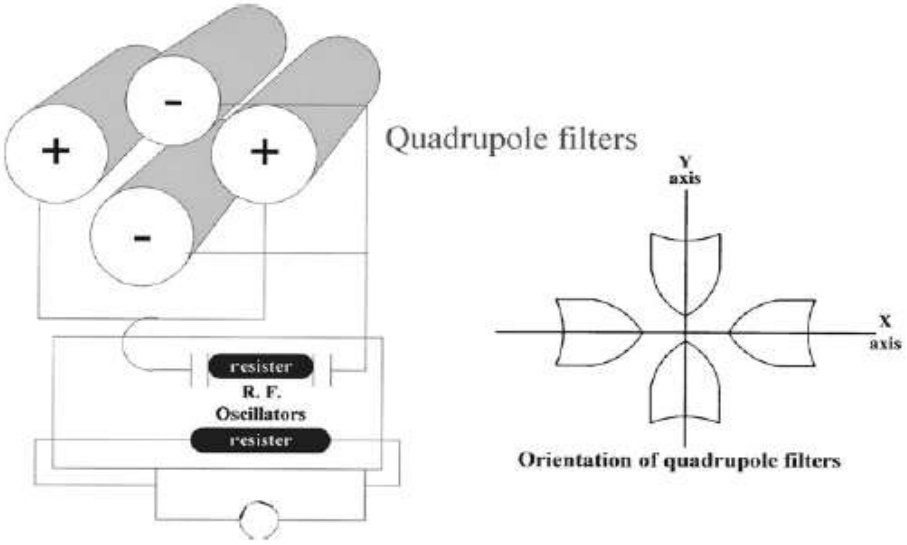


Fig. 1.10. Schematic representation of a quadropole analyzer. The quadrupole mass analyzer uses combined DC (direct current) and RF (radio frequency) potentials applied to a set of four rods. It uses a central cavity created by four rods to filter ion fragments. The potentials at each rod can be independently regulated to direct ions of a specific mass-to-charge (mass) toward the detector. Ions that do not have a stable trajectory through the quadrupole configuration will collide with the rods (are filtered) and never reach the detector [12].

Two opposite rods have the same voltage, while the perpendicular ones have a voltage with opposite signs (+ and -, respectively). The oscillating field applied to the rods alternately attracts and repels ions passing through the mass filter, inducing an ion motion that is exploited to differentiate ions on the basis of their mass. The detector will count the mass and relative abundance of each fragment and show the information in peaks [56, 60, 61].

Some things to consider with a quadrupole is that they are often the lowest cost mass selective device, especially as a single quadrupole. They typically have good sensitivity, precision, accuracy, and linearity for many forensic applications. Unfortunately, quads cannot produce exact or accurate mass information and scan speeds can limit sensitivity, and with rapid chromatography the trade of rapid scans to accommodate narrow peaks may compromise sensitivity [64].

1.3.4.1. MS vs. MS/MS

There are differences between MS mode and tandem MS/MS mode. In the MS mode, ions formed in the ionization source are separated by a single-stage mass analyzer. In tandem in space MS/MS there are three main steps in tandem mass spectrometry (Fig. 1.11): (i) ion selection, (ii) ion activation (fragmentation), and (iii) analysis of the fragments of the selected ion [55].

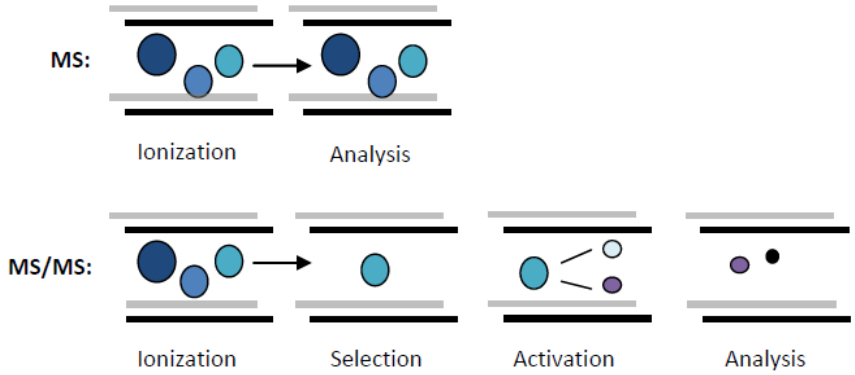


Fig. 1.11. Differences between single stage MS and tandem MS/MS. The reason MS/MS is required is because many compounds have the same intact mass. The combination of the specific parent mass and the unique fragment ion is used to selectively monitor for the compound to be quantified.Adapted from [99].

Tandem mass spectrometry is used to determine ion structure and to detect and quantify compounds in complex mixtures [12]. In this project, we used tandem MS/MS, so any individual ion can be selected and then activated to generate fragments characteristic of the selected ion. The fragments originating exclusively from the precursor ion can then be analyzed separately with another mass analyzer.

Figure 1.12 shows the general diagram of an instrument with three quadrupoles, used for MS/MS experiments. Quadrupole mass spectrometers are symbolized by upper case Q, and RF-only quadrupoles with a lower case q.

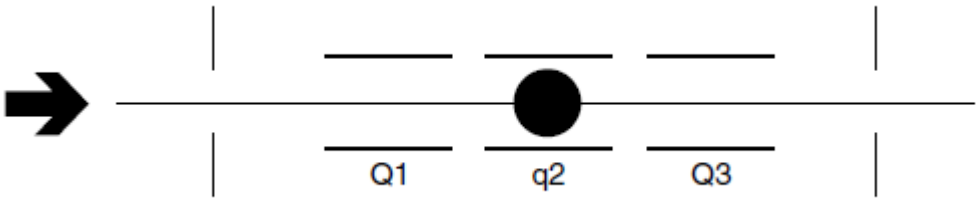


Fig. 1.12. Diagram of a triple quadrupole instrument. The first and the last (Q1 and Q3) are m/z selectors. The centre quadrupole, q2, is a collision cell made up of a quadrupole using RF only. Adapted from [55].

The first quadropole (Q1), selects a "precursor" ion with a desired mass to charge ratio from the ESI source. The second quadropole (q2) is the collision cell, where collisions with a neutral gas such as N_2 or Ar causes the ions to fragment. Ions are confined to the collision cell by a quadropole, operated with a radiofrequency voltage between the poles. The resulting fragment ions are transmitted to third quadropole (Q3). In here, only fragment ions of the desired mass to charge ratio are allowed to pass and reach the detector [64].

Typically the mass spectrometer is set to scan a specific mass range. This mass scan can be wide as in the full scan analysis or can be very narrow as in selected ion monitoring. There are many ways to acquire LC/MS/MS data, but in this experiment, we used multiple reaction monitoring.

1.3.5. Multiple Reaction Monitoring (MRM)

Liquid chromatography coupled with tandem quadropole mass spectrometry using triple quadropode mass spectrometry in multiple reaction monitoring mode is often the analytical method of choice for determination and quantification of drugs and their metabolites in biological samples [64]. So, we combined the MRM experiment with the chromatographic separations and thus, we can achieve high levels of sensitivity and specificity, by being more selective.

MRM combines three sectors (fig 1.13):

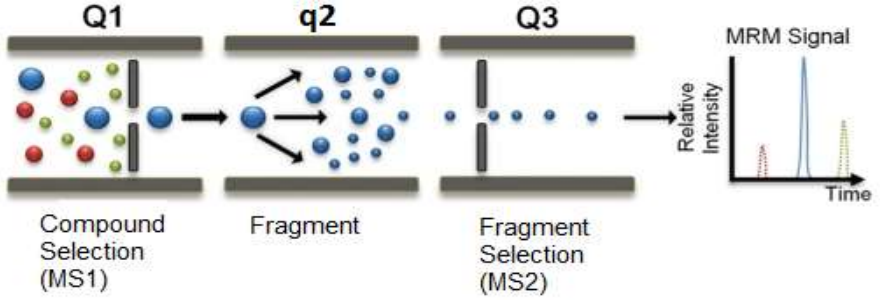


Fig. 1.13. Schematic representation of MRM. A specific m/z value is selected in Q1 and is fragment in q2. Q3 is set to transmit only ions of a selected m/z rather. Adapted from http://www.mrmproteomics.com/

The first sector is normally a quadropole, such as the second sector, but the third sector is the variable quad: can be a quad, TOF or Trap.

One typically use MRM to quantify known analytes in complex samples:

- Drug metabolite and pharmacokinetic studies;
- Pesticides and herbicides analysis;
- Screening for target drugs in toxicology and forensic studies [66].

In this project, the first step was assure that all of the antidepressants have the correct MRM's, by infusing them. An infusion pump is used to deliver a constant flow to analyte into the LC eluent at a point after the chromatographic column and before the mass spectrometer ionization source (Fig. 1.15). The analyte is injected under the desired chromatographic conditions and the response from the infused analyte recorded [64]. The results form a mass spectrum fragmentation. An example is shown in figure 1.14.

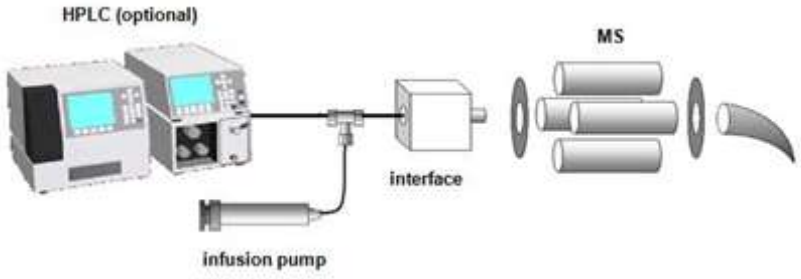


Fig 1.14. System configuration for infusing a standard into the LC-MS for tuning. This step is crucial to make sure that the instrument is working well for a particular component. The essence of making these infusions is that we want the instrument to give the maximum signal possible for our analyte. Because LC-MS relies on chemical ionization, the chemical environment in the LC-MS interface influences how ions are generated. So not only are the interface settings important (voltages, flow rates, temperatures, vacuum, etc.), but also the chemical composition of the mobile phase from the HPLC influences the analyte ionization. Adapted from http://www.sepscience.com/Techniques/LC/Articles/695-/HPLC-Solutions-5-LC-MS-Calibration-vs-Tuning

1.4. Analytical Method Validation

Method validation is the process by which it is established through laboratory studies, that the performance characteristics of the method meet the requirements for its intend purpose [67]. The role and progress of LC-MS/MS in toxicology is becoming increasingly important in routine analysis. These procedures must be validated before use to ensure their reliability and applicability for the intend purpose [64].

Bioanalysis is one of the branches of analytical science that requires method validation: for example, without proving that the results are based on a validated method, that study is nowadays meaningless and will not be recognized [68]. The international scientific community need published research results that are valid, reproducible and comparable [53, 64, 65].

Method validation is usually considered to be very closely tied to method development. Many of the method performance characteristics that are associated with method validation are usually evaluated, at least approximately, as part of method development. Indeed it is often not possible to determine exactly where method development finishes and validation begins [70].

There are legal, technical and commercial reasons for the need of implementation of validation methods [69]. Millions of tests, measurements and examinations are made every day in thousands of laboratories around the world. The cost of carrying out there measurements is high, but still it is clearly important to make a correct measurement and be able to show that the result is correct [70]. In routine daily work in clinical and forensic toxicology, unreliable analytical data might not only be contested in court, but could also lead to unjustified legal consequences for the defendant or to wrong treatment of the patient [65, 67, 68].

Organizations like International Union of Pure and Applied Chemistry (IUPAC), International Organization of Standardization (ISO), Association of Official Analytical Chemist (AOAC), EURACHEM, European Medicines Agency (EMA), Food and Drug Administration (FDA) and International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use developed several quality guides [53, 66, 68–71]. In several countries, judicial authorities impose proficiency testing and/or accreditation according to the International Organization for Standardization (ISO) - norms on laboratories performing analysis of certain samples in

a forensic or clinical setting. However, all these guidelines seldom provide a practical approach to how validation should occur in a particular laboratory setting. Laboratories sometimes struggle with the experimental set up, differences in guidelines, choice of appropriate decision criteria and statistics [69].

Due to the importance of method validation, a number of guidance documents on this subject have been issued by various international organizations or conferences [64]. In these guidelines, definitions, procedures and parameters of validation are established. However, there is no consensus on the extent of validation experiments and no acceptance criteria for validation parameters of bioanalytical methods in forensic an clinical toxicology [69]. Most analytical chemists are aware of the importance of validation, but why it should be done and when, and exactly what needs to be done, is not always clear [70].

Validation is also required when it is necessary to demonstrate the equivalence of results obtained by two methods, e.g. a newly developed method and an existing standard/regulatory method [73]. Any modification of an analytical method would require revalidation of the procedures [72, 73].

The developed method will then be validated for the "fitness for use" [65, 74]. The validation procedure includes first the validation of the analytical method: selectivity, linearity, precision (repeatability, intermediate precision), accuracy, recovery, limit of quantification (LOQ) and limit of detection (LOD); and second the validation of the stability in the biological matrix. This validation procedure needs to be performed prior to the routine use of the analytical procedure [79]. In this project, the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology will be followed [80]

2. RESULTS AND DISCUSSION

All the results shown in this part concern to citalopram and paroxetine, both antidepressives included in this method and commonly found in patient samples. The results regarding the others compounds can be found in appendices (section 6).

A. Trimipramine d_3 as Internal Standard

A. 2.1. Method Development

This section presents the results obtained during method development, more precisely, which parameters to use in the spectrometric conditions .

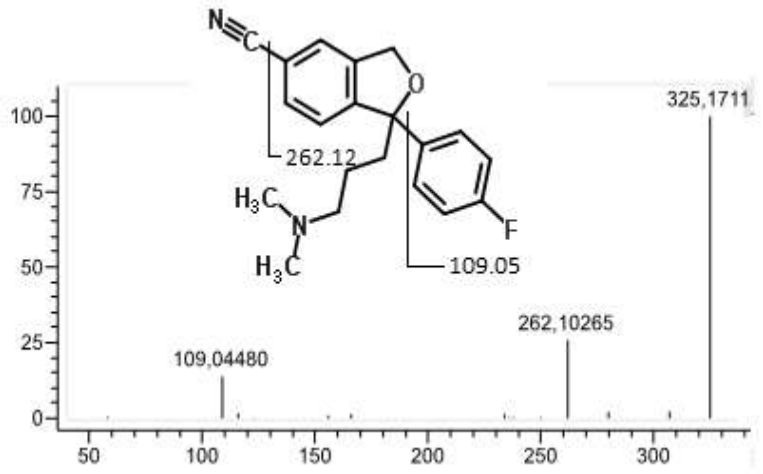
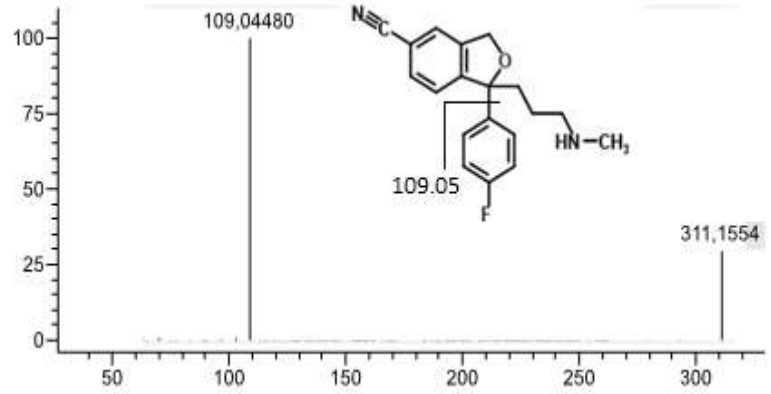
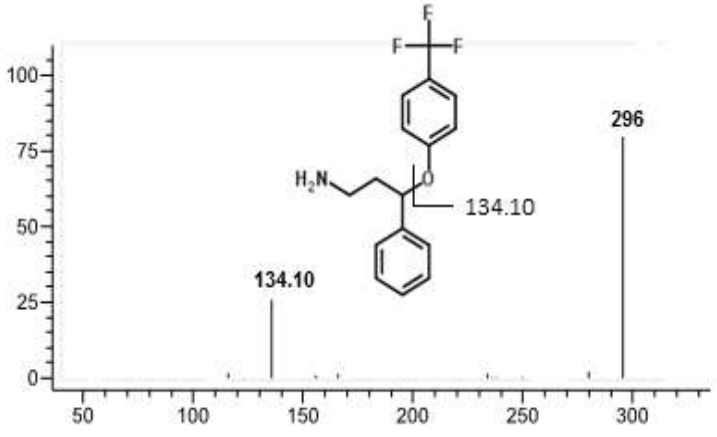
The first step was to prepare the solutions to make the infusions. This allows the optimization of some parameters, to establish optimal conditions for each of the molecules as the collision energy and cone voltage.

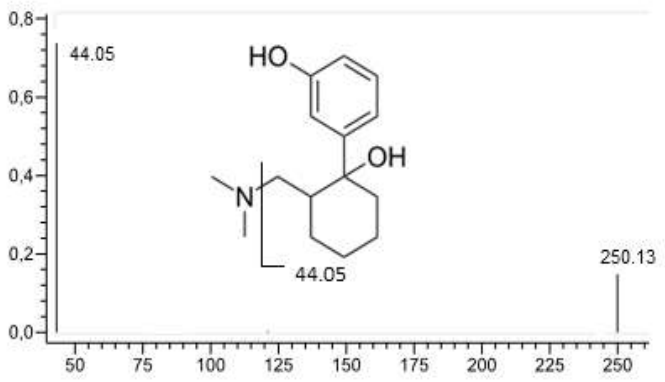
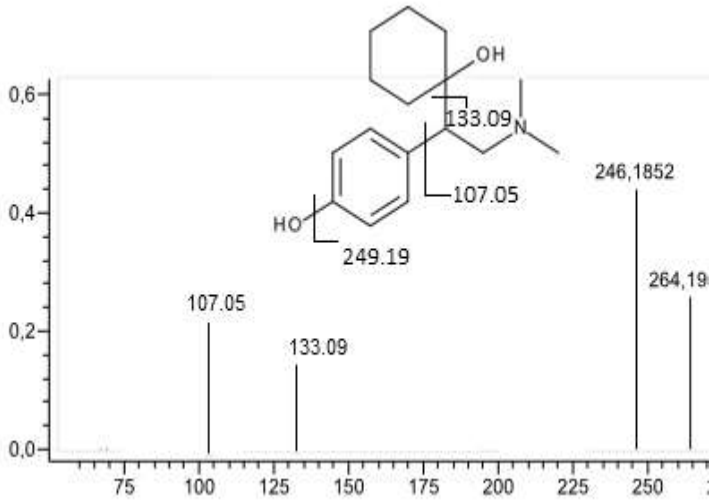
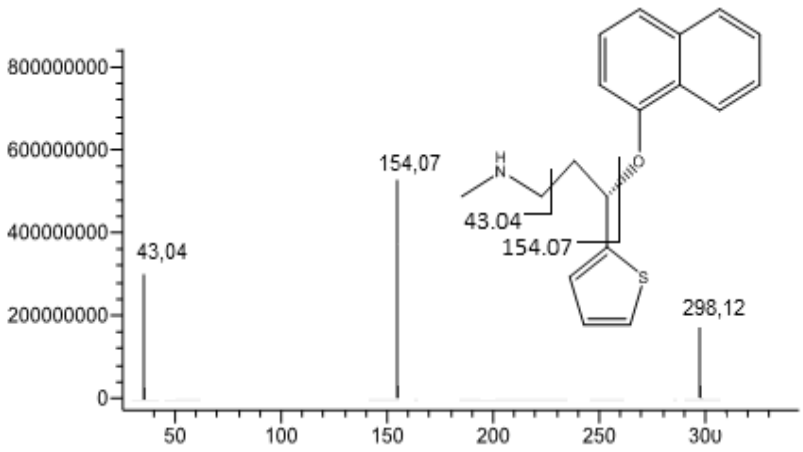
After these parameters are optimized, fragmentation mass spectra for each analyte of interest can be observed with all fragments of the analyte. With the fragmentation spectra, it is possible to choose what transitions to monitor.

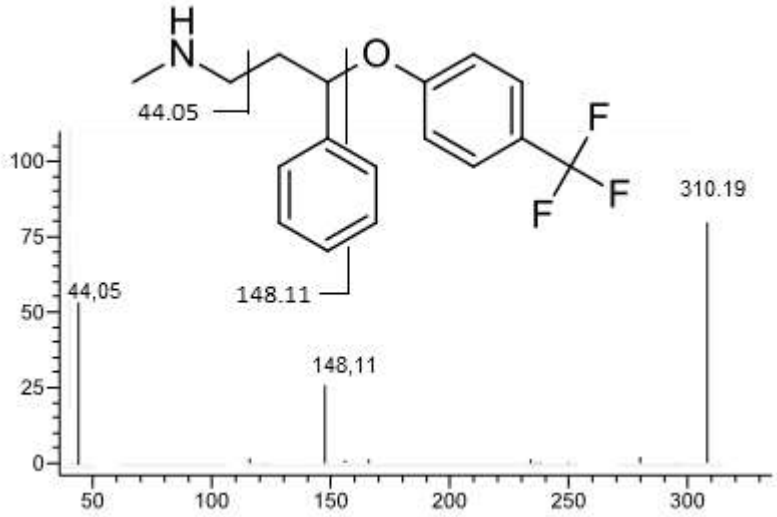
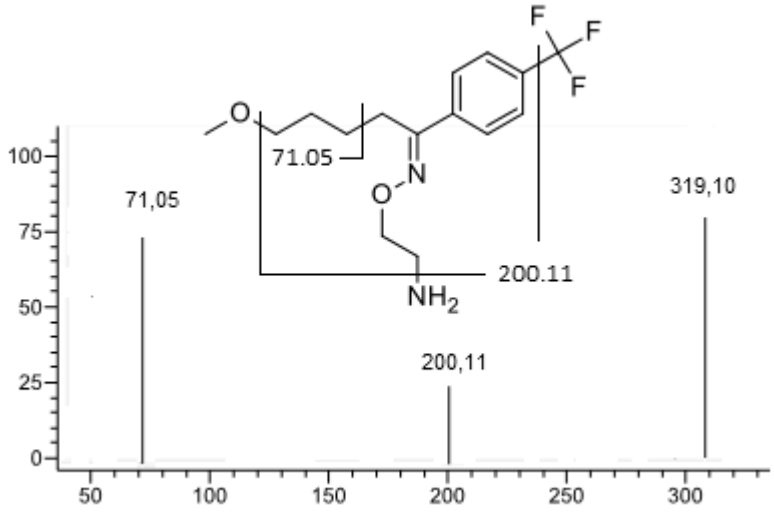
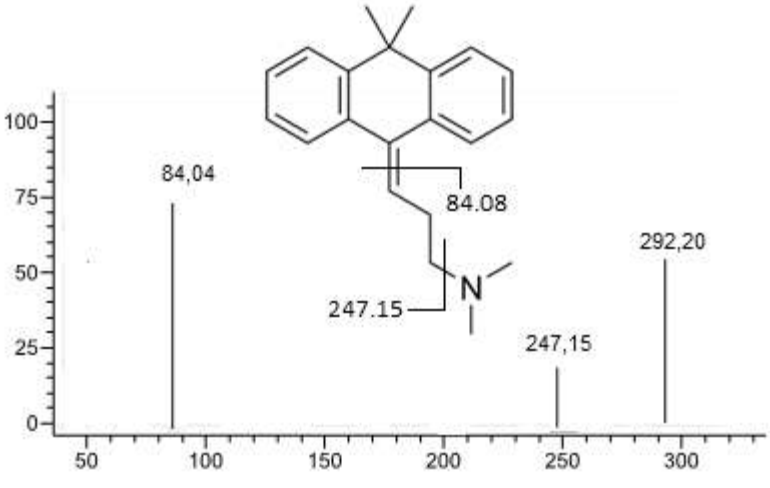
For many reasons product ions of 'low mass' can be problematic for MRM detection. One of these reasons is due to the observation that 'chemical noise' (background) is considerably more intense at lower m/z values. The ideal product ion to use in MRM method would be the one that can be observed at good relative abundance in the spectrum.

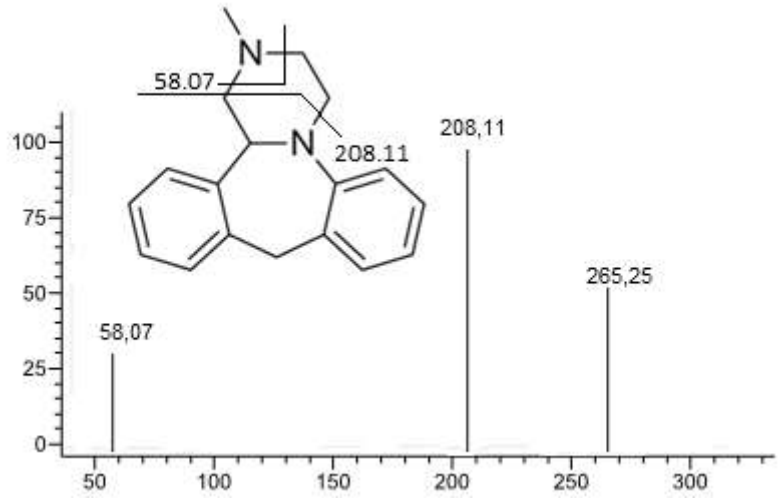
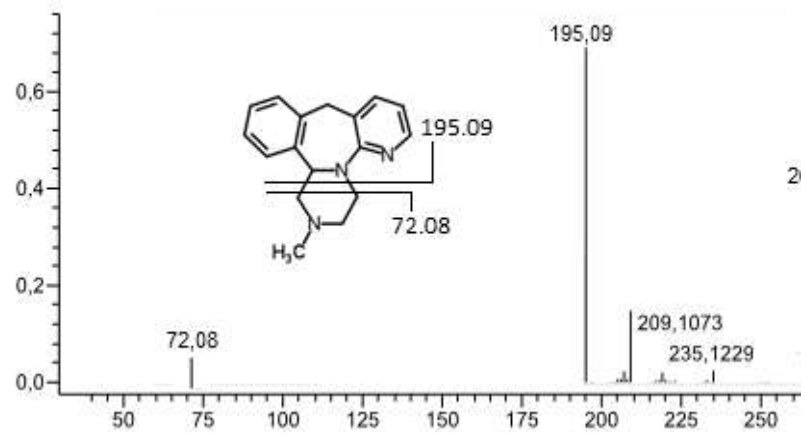
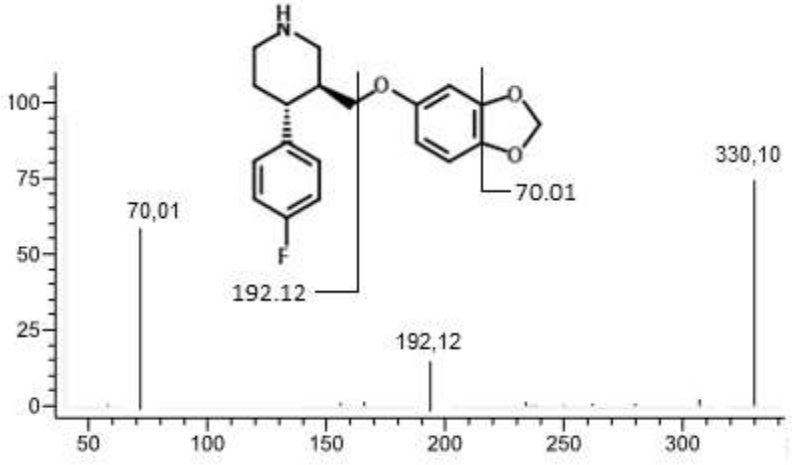
The following table (table 2.1) shows the fragmentations of each compound that was monitored in this project. The mass spectras were sorted from mzCloud® database. The highest fragment was chosen to be monitored, although both fragments were written in the method.

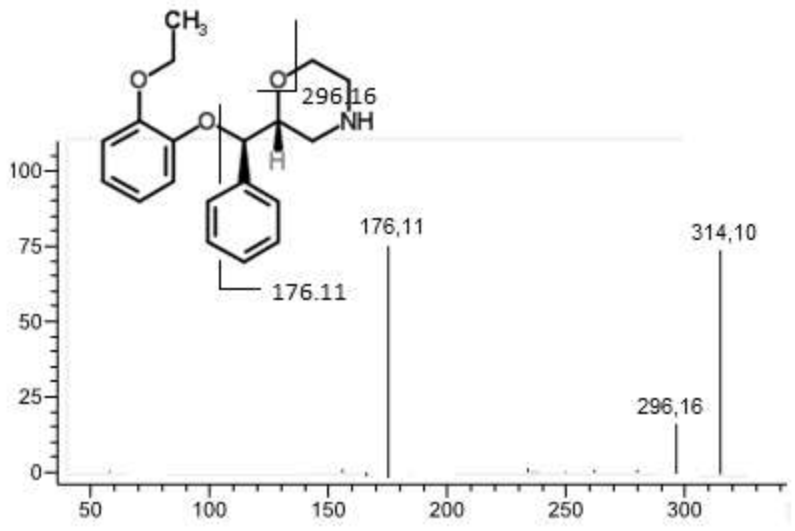
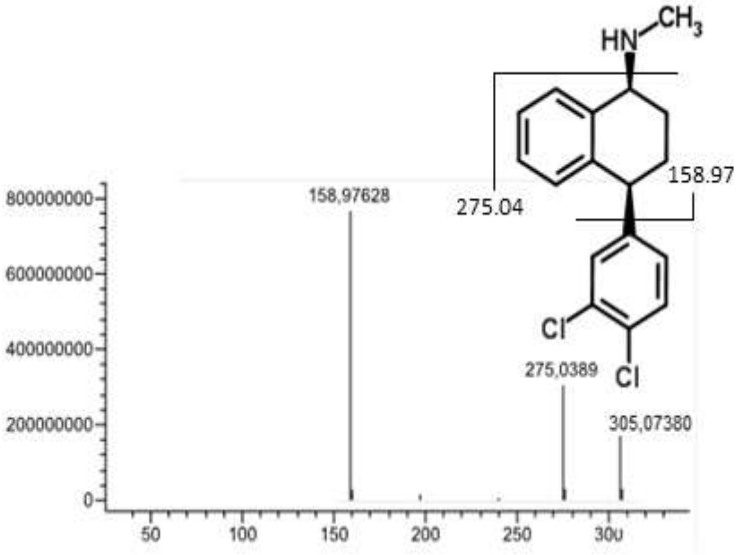
Table 2.1. Fragmentations of all compounds

Compound	Chemical Structure, Monitored Fragmentations and Mass Spectrum
Citalopram	
DM Citalopram	
DM Fluoxetine	

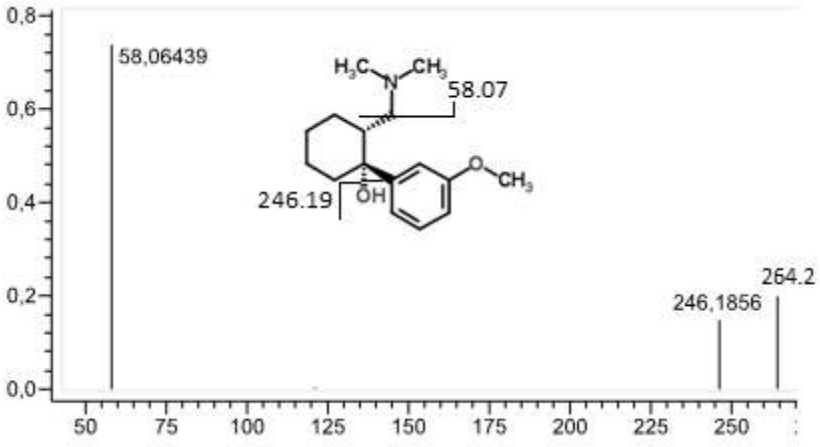
DM Tramadol	
DM Venlafaxine	
Duloxetine	

Fluoxetine	
Fluvoxamine	
Melitracene	

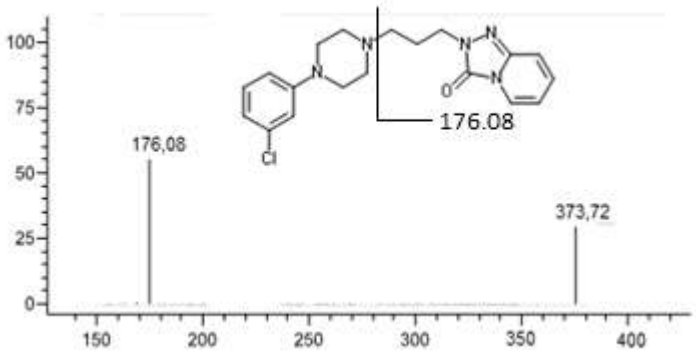
Mianserine	
Mirtazapine	
Paroxetine	

Reboxetine	
Sertraline	

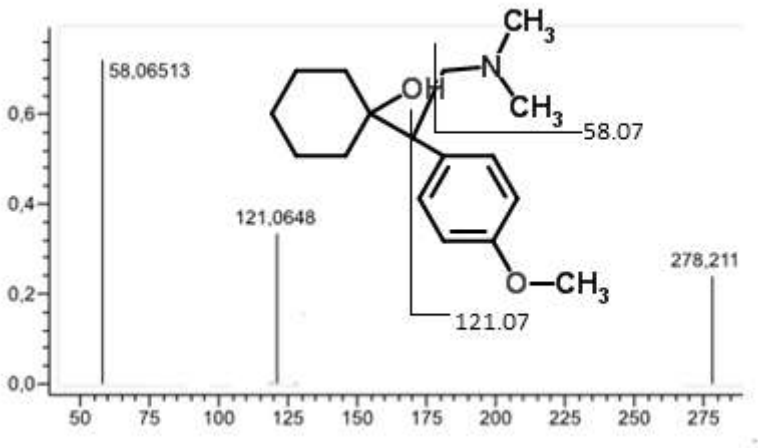
Tramadol



Trazodone



Venlafaxine



A.2.2. Compound Identification

As was mentioned before, the compounds can be identified by the retention time and ionic fragments (m/z) to be monitored in MRM mode. Not all of the transitions were monitored, just one – the transition that produces the higher fragment. Table 2.2 shows the compounds sorted by retention time and the transitions monitored.

Table 2.2. MRM's monitored and retention time for all compounds.

Compound	Retention Time (min)	Transitions (m/z)	
		Q1	Q3
DM Venlafaxine	2.04	264.20	246.10
Tramadol	2.36	264.20	58.20
Mirtazapine	2.73	266.10	72.20
DM Tramadol	2.93	250.13	44.00
Trazodone	3.70	373.72	176.05
Venlafaxine	4.03	278.10	58.00
Citalopram	4.25	325.19	109.00
DM Citalopram	4.27	311.20	109.10
Mianserine	4.31	265.25	208.00
Reboxetine	4.61	314.10	176.10
Paroxetine	4.73	330.10	70.20
Duloxetine	4.86	298.12	154.10
Trimipramine d_3	4.94	298.00	103.00
Fluvoxamine	4.97	319.19	71.00
Fluoxetine	5.02	310.19	44.30
DM Fluoxetine	5.03	296.00	134.10
Melitracene	5.10	292.20	247.20
Sertraline	5.15	306.13	159.00

A.2.3. Preliminary studies

In order to do a preliminary study, the calibration points with non-tricyclic antidepressants (ntads) at different concentrations – 1000, 800, 600, 400, 200, 100, 50, 20 and 10 ng/mL in serum were prepared and analyzed them in the LC-MS/MS with the method. It is important to mention that to achieve these results, several runs were made and the MRM's transitions, the gradient and the extraction procedure were changed in order to optimize the process.

The following results show citalopram and paroxetine's values and calibration curves (table 2.3 and fig 2.1, and table 2.2 and figure 2.3, respectively), from 1000 ng/mL calibrator to the 10 ng/mL, including a table where the peak areas are shown, internal standard area, signal/noise, response, concentration calculated and deviation from the standard concentration, and also the calibration curve type (linear).

Table 2.3. Preliminary study 1 results – citalopram

#	Name	Std. Conc. (ng/mL)	Response	Conc.	%Dev
1	ntads 100%	782	2.7471	686.6	-12.2
2	ntads 80%	626	2.2167	592.4	-5.4
3	ntads 60%	469	1.6829	499.2	6.4
4	ntads 40%	313	1.1525	408.0	30.3
5	ntads 20%	199	0.4929	185.3	-6.9
6	ntads 10%	78	0.3535	95.1	22.0
7	ntads 5%	39	0.2209	41.5	6.5
8	ntads 2%	16	0.1427	16.1	0.3
9	ntads 1%	8	0.1155	5.8	-27.0

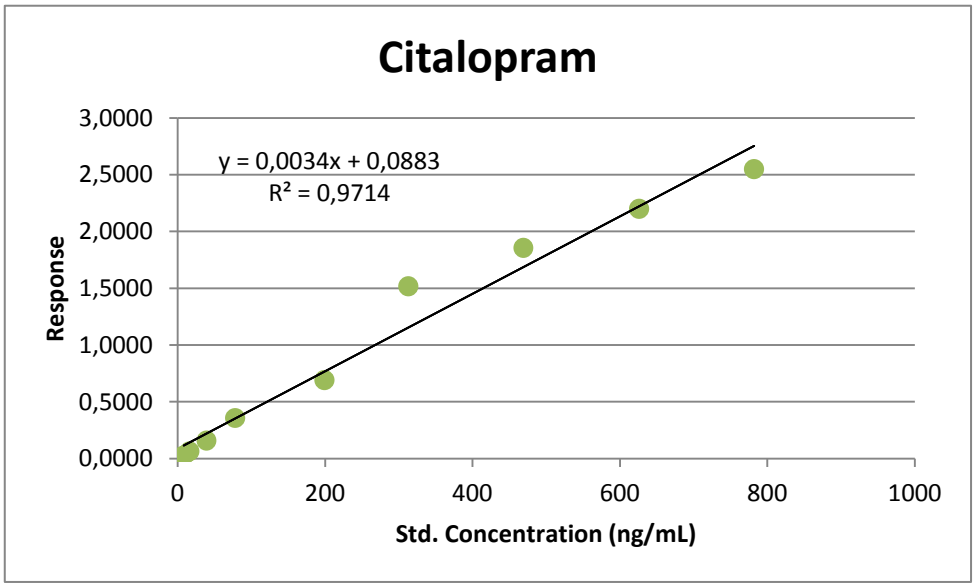


Fig.2.1. Calibration curve from preliminary study results – citalopram

Table 2.4. Preliminary study 1 results – paroxetine

#	Name	Std. Conc. (ng/mL)	Response	Conc.	%Dev
1	ntads 100% A	996	4.9421	939.8	-5.6
2	ntads 80% A	797	3.8680	735.2	-7.8
3	ntads 60% A	597	3.5163	668.2	11.9
4	ntads 40% A	398	2.1141	401.4	0.9
5	ntads 20% A	199	1.2110	229.6	15.4
6	ntads 10% A	100	0.5484	103.4	3.4
7	ntads 5% A	50	0.2980	55.7	11.5
8	ntads 2% A	20	0.1695	31.3	56.5
9	ntads 1% A	10	0.0176	2.3	-76.7

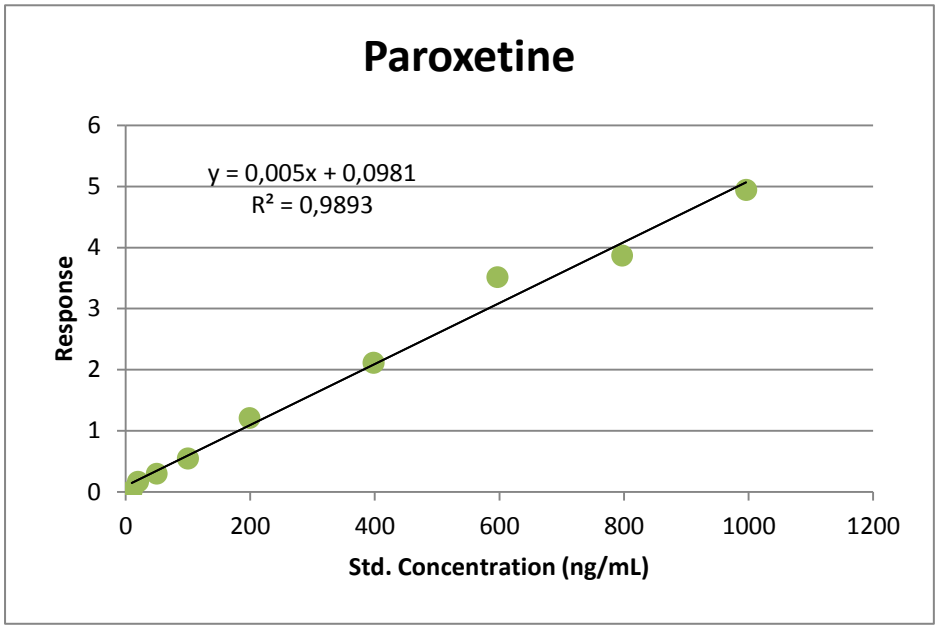


Fig.2.2. Calibration curve from preliminary study results – paroxetine.

As we can see, in the lowest calibrator, it should have been detected 10 ng/mL and the method only detected 2.3 ng/mL, which is far too different. The following figure (fig. 2.3) shows paroxetine at 10 ng/mL. The small area of the peak shows a low sensitivity of the method, explaining why such a low concentration was detected.

Fig. 2.3. Paroxetine at 10 ng/mL.

Taking a look at both calibration curves and their equations, it is clear that the regression model can't meet our objectives. The closer the R^2 value is to 1, the better the data fits the curve however, R^2 can't determine whether the coefficient estimates and predictions are biased, which is why you must assess the residual plot (Fig. 2.4 and 2.5). Residuals are the deviations of the observed values from the values predicted by the applied calibration model [64]. The residuals should not be either systematically high or low and should be centered on zero throughout the range of fitted values. In other words, the residuals should fall in a symmetrical pattern and have a constant spread throughout the range.

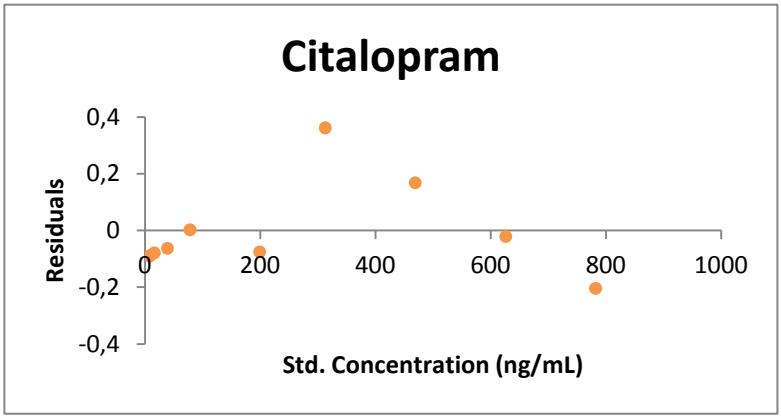


Fig. 2.4. Residuals plot of citalopram

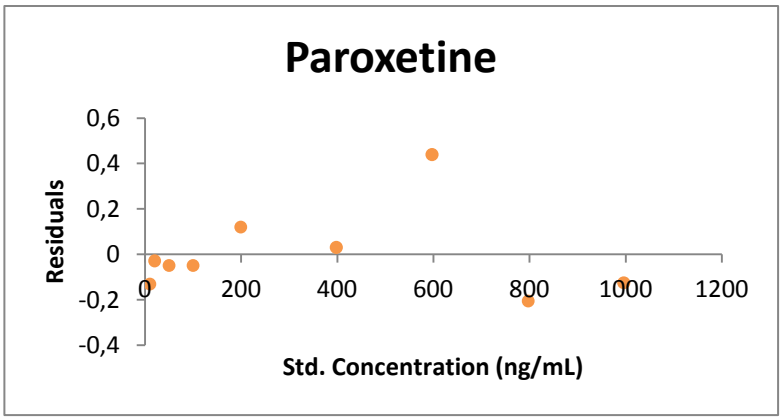


Fig. 2.5. Residuals plot for paroxetine

The residual plot in fig. 2.4 showed a non-random pattern that could indicate that the deterministic portion (predictor variables) of the model is not capturing some explanatory information that is "leaking" into the residuals. The residuals plot in fig. 2.5 shows a more-or-less random distribution, compared to fig. 2.4, who presents an inverted-u shape. However, there seems to be an uneven spreading of residuals across fitted values and an asymmetrical pattern, suggesting a non-constant variance.

Since the calibration models failed and sensitivity was an issue, it was decided to change the sample preparation. The sample preparation used (described in 4.6 Extraction Procedure) only diluted the sample, making decreasing the amount of drug injected in the system. After re-searching the literature, it was decided to test a new sample preparation, adapted from [81]. This sample preparation was choose because of its good results with a similar equipment and method.

This sample preparation (sample preparation B) consisted in precipitation of serum samples (250 µL) with methanol and acetonitrile containing 5 ng of the internal standard, trimipramine-d_3. The supernatant (900 µL) was evaporated to dryness at 56ºC under a flow of nitrogen and reconstituted in 100 µL of 50:50 methanol:water with 2 mM ammonium acetate.

Another preliminary study was made (preliminary study 2), considering sample preparation B, preparing seven calibrators. Table 2.5 shows the values for citalopram fig 2.6 shows its calibration curve. As for paroxetine, the results are in table 2.6 and the fig. 2.7 shows the calibration curve.

Table 2.5. Preliminary study 2 results with sample preparation B – citalopram

#	Name	Std. Conc. (ng/mL)	Response	Conc.	%Dev
1	ntads 80%	626	12.631	580.7	-7.2
2	ntads 40%	313	6.6064	298.4	-4.7
3	ntads 30%	235	5.8343	262.2	11.6
6	ntads 20%	156	4.1559	183.6	17.7
4	ntads 10%	78	2.0306	84.0	7.6
5	ntads 5%	39	1.0544	38.2	-2.0
7	ntads 1%	8	0.2863	2.2	-22.0

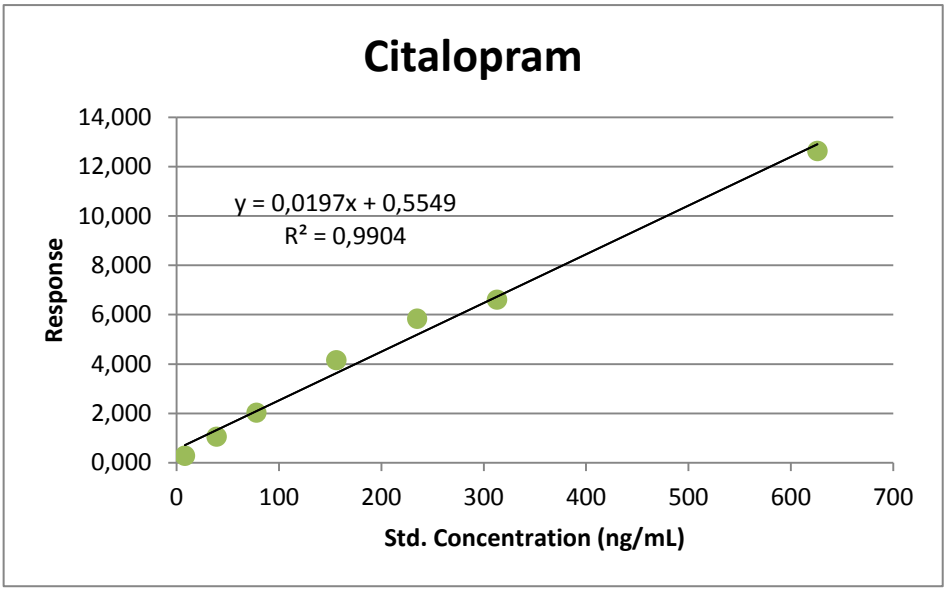


Fig.2.6. Calibration curve from preliminary study 2 results with sample preparation B – citalopram.

Table 2.6. Preliminary study 2 results with sample preparation B – paroxetine

#	Name	Std. Conc. (ng/mL)	Response	Conc.	%Dev
1	ntads 80%	797	5.3613	713.5	-10.5
2	ntads 40%	398	2.9526	385.0	-3.3
3	ntads 30%	299	2.5417	329.0	10.0
6	ntads 20%	199	2.0804	266.0	33.7
4	ntads 10%	100	0.8974	104.7	4.7
5	ntads 5%	50	0.4583	44.8	-10.3
7	ntads 1%	10	0.1698	5.5	-55.0

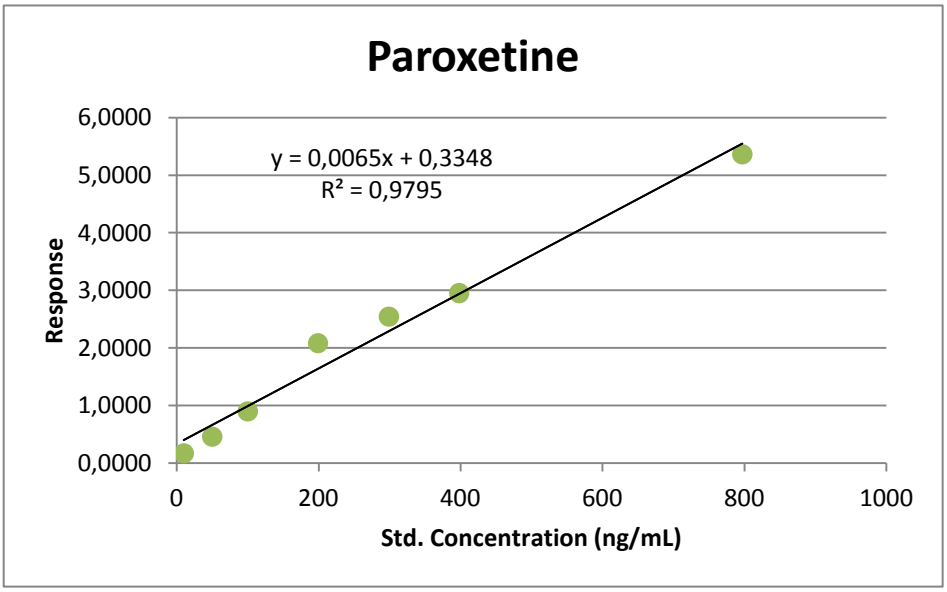


Fig.2.7. Calibration curve from preliminary study 2 results with sample preparation B – paroxetine.

For citalopram, the linearity of curves had an improvement and, as we can see for fig. 2.8 (paroxetine at 10 ng/mL), sensitivity of the method is no longer an issue with this sample preparation.

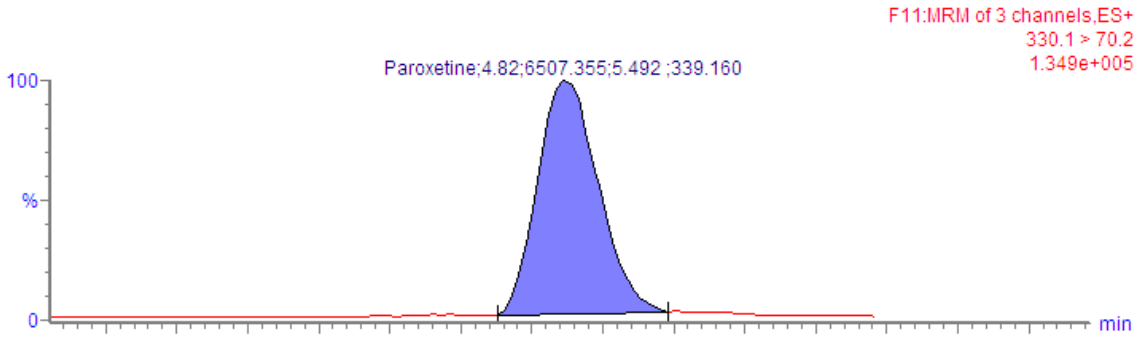


Fig.2.8. Paroxetine at 10 ng/mL, with sample preparation B.

Paroxetine's peak shape at 10 ng/mL improved significantly and the same happened to all compounds. Looking at the residuals of both compounds at fig. 2.9 and 2.10, we can see that sample preparation B proved to be better than the previous one, but the results were not good enough to start a validation procedure. Both residuals plots show an asymmetrical distribution along the x-axis. Also, the same inverted u-shaped is now in both compounds, suggesting that both calibration models failed.

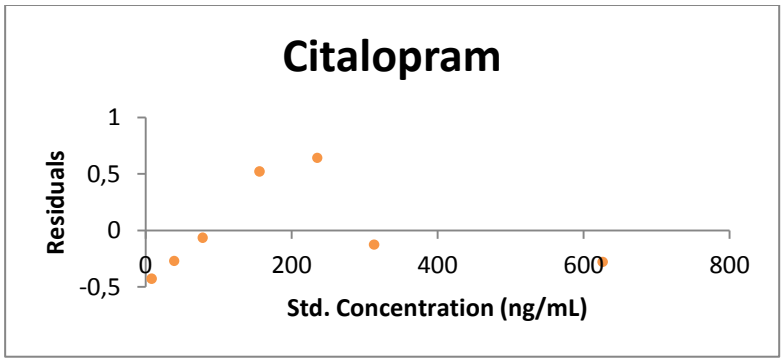


Fig. 2.9 Residual plot for citalopram

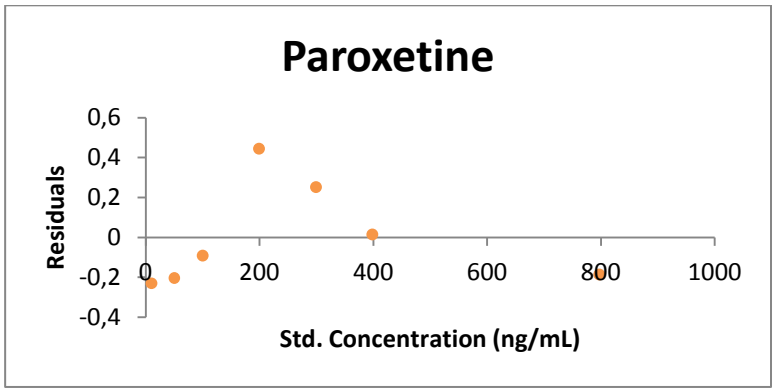


Fig. 2.10. Residual plot for paroxetine

Trimipramine-d_3 is a deuterated internal standard, based on trimipramine, a tryciclic antidepressant (and we are working with non-tryciclic and analgesics), so the physical-chemical properties are not so similar between internal standard and analytes. It was decided to test others internal standards, deuterated compounds that were present in our list of compounds: mirtazapine-d_4, paroxetine-d_6, reboxetine-d_5 and venlafaxine-d_6.

B. Mirtazapine-d_4, paroxetine-d_6, reboxetine-d_5 and venlafaxine-d_6 as Internal Standards

B.2.1. Method Development

The first step was infusions, which were made to establish optimal conditions for each of the molecules as the collision energy and cone voltage. The results from the infusions are given in table 2.7.

Table 2.7: MRM transitions used collision energies and cone voltages.

Compound	Transitions (m/z)		Collision Energy (V)	Cone Voltage (V)	Dwell time (s)
	Precursor Ion	Product Ion			
Mirtazapine-d_4	270.1	76	24	30	0.01
Paroxetine-d_5	336.1	76.2	30	29	
Reboxetine-d_6	319.2	176.1	12	22	
Venlafaxine-d_6	284.2	64.0	20	28	

To find out which concentration should be used all the internal standards were injected in different concentrations (1 µg/mL, 100 ng/mL and 20 ng/mL). The agreed concentration to all of the compounds was 100 ng/mL, so a new precipitation fluid (PF II) was made (100 ng/mL of all the internal standards in 50:50 methanol:acetonitrile).

B.2.2. Preliminary study

Preliminary study 3 consists in sample preparation B but, with the internal standards mentioned before. The results for citalopram are in table 2.8 and fig. 2.11, while the results for paroxetine are given in table 2.9 and figure 2.12.

Table 2.8. Results from the preliminary study 3 with sample preparation B and new internal standards – citalopram

#	Name	Std. Conc. (ng/mL)	Response	Conc.	%Dev
1	ntads 80%	626	9.2997	650.2	3.9
2	ntads 60%	469	7.1697	501.6	7.0
3	ntads 30%	235	3.2158	225.9	-3.9
6	ntads 20%	199	2.5995	180.4	-9.3
5	ntads 5%	39	0.5351	38.9	-0.3
5	ntads 2%	16	0.2148	16.6	3.5
7	ntads 1%	8	0.1180	9.8	-1.9

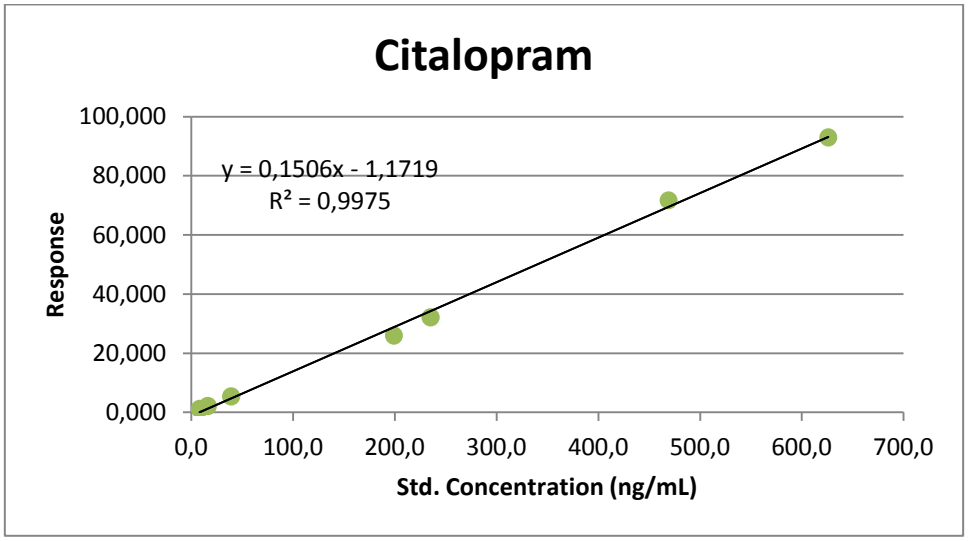


Fig.2.11. Calibration curve – citalopram

Table 2.9. Results from the preliminary study 3 with sample preparation B and new internal standards – paroxetine

#	Name	Std. Conc. (ng/mL)	Response	Conc.	%Dev
1	ntads 80%	797	51.688	826.2	3.7
2	ntads 60%	597	37.251	595.3	-0.3
3	ntads 30%	299	17.613	281.4	-6.2
6	ntads 20%	199	11.750	187.6	-5.7
5	ntads 5%	50	3.232	51.5	2.9
5	ntads 2%	20	1.303	20.5	2.6
7	ntads 1%	10	0.671	10.5	5.2

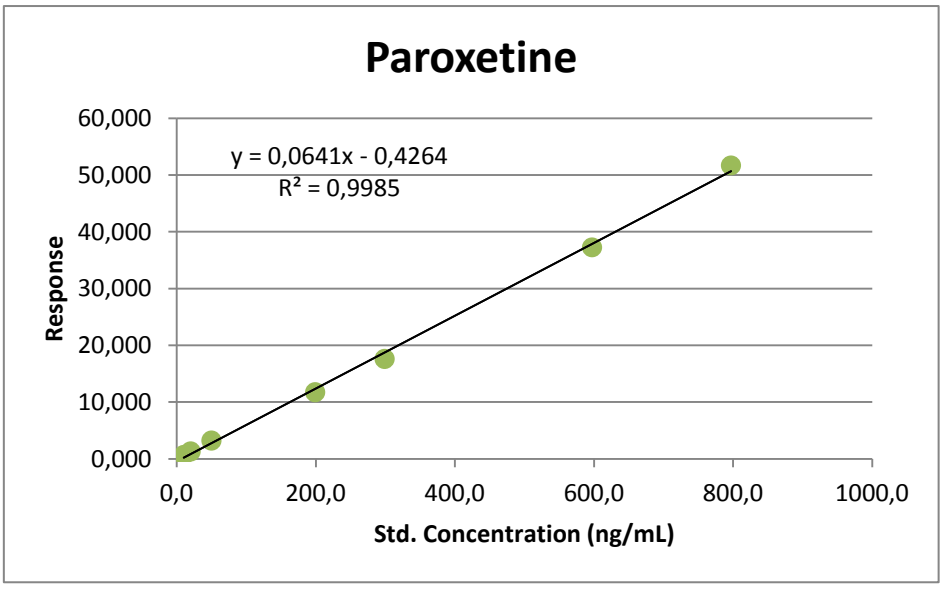


Fig.2.12. Calibration curve - paroxetine

The preliminary study 3 with the new internal standards was successful – linearity was achieved and the deviations'-issue was solved (all deviations were significantly reduced). The fact that paroxetine-d_6 was used as internal standard for paroxetine explain the excellent results, better than for citalopram. Taking a look at the residuals plots (Fig. 2.13 and 2.14), both residuals plots showed a random distribution and a more or less symmetrical pattern.

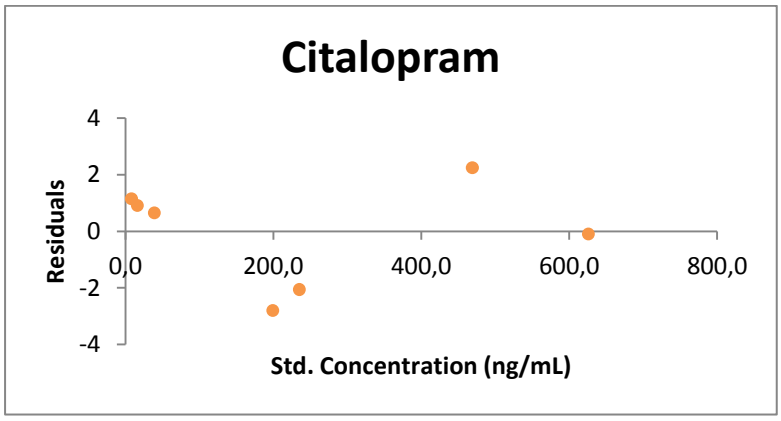


Fig. 2.13. Residuals plot – citalopram

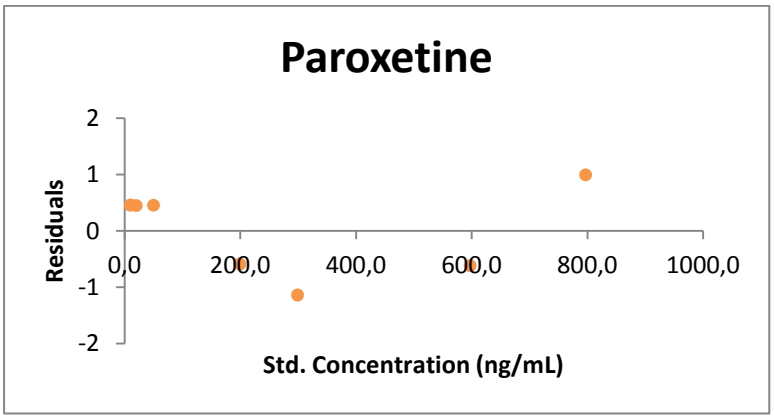


Fig. 2.14. Residuals plot – paroxetine

The following table (table 2.10) shows only the R^2 for the compounds achieved with this preliminary study and the internal standard chosen, based on this last parameter.

Table 2.10. Internal standards used for every compound and R^2 achieved on this last preliminary study

Compound	IS	R^2
Citalopram	Reboxetine-d_5	0.992
DM Citalopram	Paroxetine-d_6	0.994
DM Fluoxetine	Reboxetine-d_5	0.997
DM Tramadol	Venlafaxine-d_6	0.992
DM Venlafaxine	Venlafaxine-d_6	0.998
Duloxetine	Reboxetine-d_5	0.999
Fluoxetine	Paroxetine-d_6	0.998
Fluvoxamine	Venlafaxine-d_6	0.998
Melitracene	Paroxetine-d_6	0.993
Mianserine	Reboxetine-d_5	0.998
Mirtazapine	Mirtazapine-d_4	0.999
Paroxetine	Paroxetine-d_6	0.999
Reboxetine	Reboxetine-d_5	0.998
Sertraline	Venlafaxine-d_6	0.998
Tramadol	Venlafaxine-d_6	0.992
Trazodone	Paroxetine-d_6	0.992
Venlafaxine	Venlafaxine-d_6	0.999

Since the results from the preliminary study were good enough, it was decided to proceed for the validation of the method, following the Scientific Working Group for Forensic Toxicology (SWGTOX) guideline.

B.2.3. Analytical method validation

B.2.3.1. Selectivity

It is very important to obtain a signal free from the influence of other species contained in the sample and this signal should be unequivocally assigned to the analyte of interest. Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, medication and other exogenous compounds [74].

Ten independent sources of blank whole blood were chosen, from previously analyzed cases in the Toxicology Lab of Ghent Universitary Hospital, to evaluate matrix interferences. The blank matrix samples were extracted without the addition of internal standard and analyzed using the new developed method. No interferences were noted after analysis of the blank plasma samples. An example is shown in the next figure (fig. 2.15). The 3rd MRM represents mirtazapine-d_4, the 6th, venlafaxine-d_6, the 8th, reboxetine-d_5 and the 11th, paroxetine-d_6.

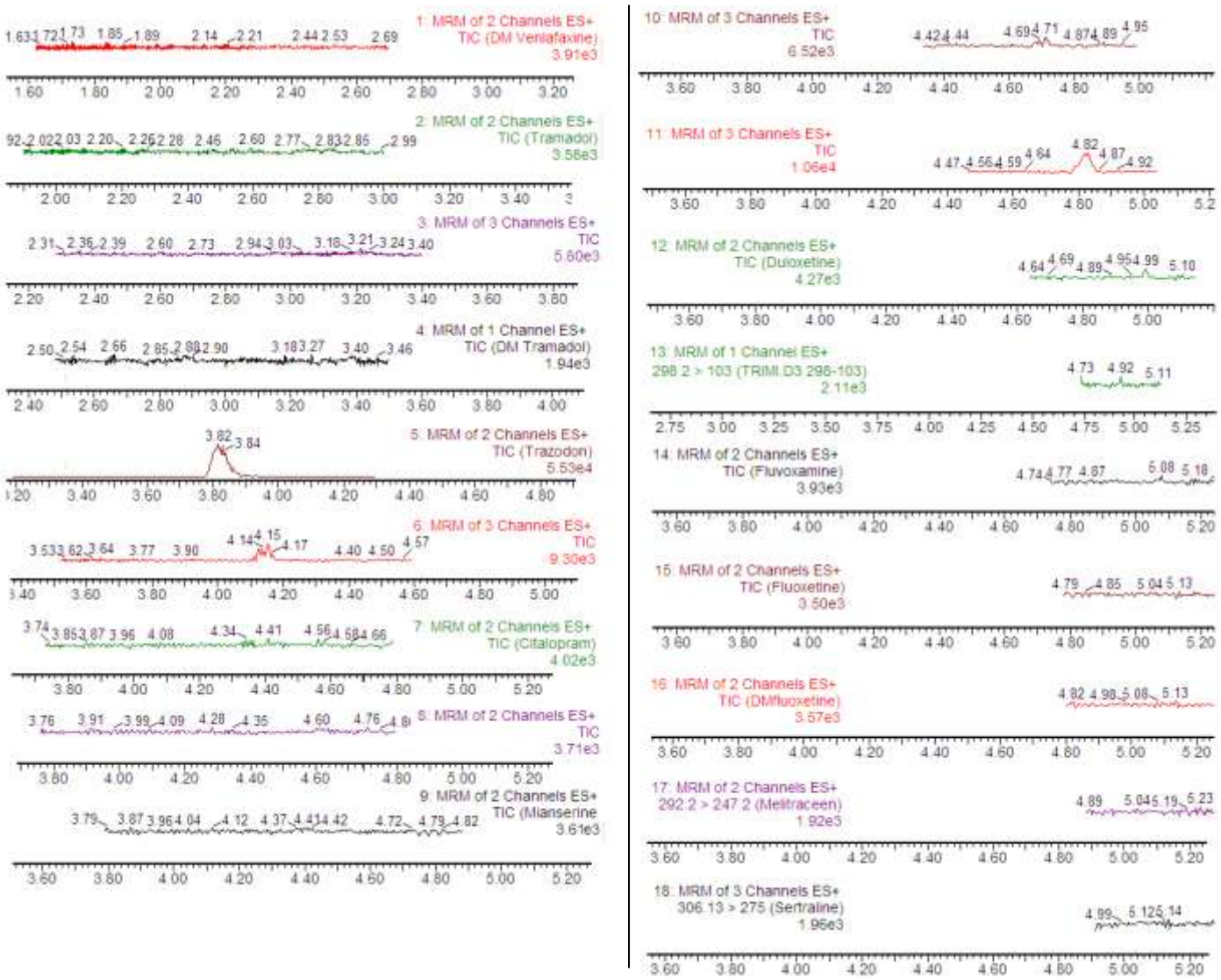


Fig. 2.15. Random patient sample taken from the Toxicology Lab, previously analyzed and the results were negative regarding non-tricyclic antidepressants. As we can see from the chromatograms, none of the compounds were found, as it was expected. There is a peak for trazodone, however, the peak area is only 1737.924, while the peak area for the lowest calibrator, at 38 ng/mL has the area of 34463.969. This peak is not considered as interference, because is less that 20% of the lowest calibrator.

One of the blank matrix samples was selected and IS was added (5 ng/mL). This was to demonstrate that the internal standard would not interfere with the signal for the other compounds. As we can see for fig. 2.16, all detected peaks represent the internal standards, which mean that there is no interference with the other compounds or their non-deuterated forms.

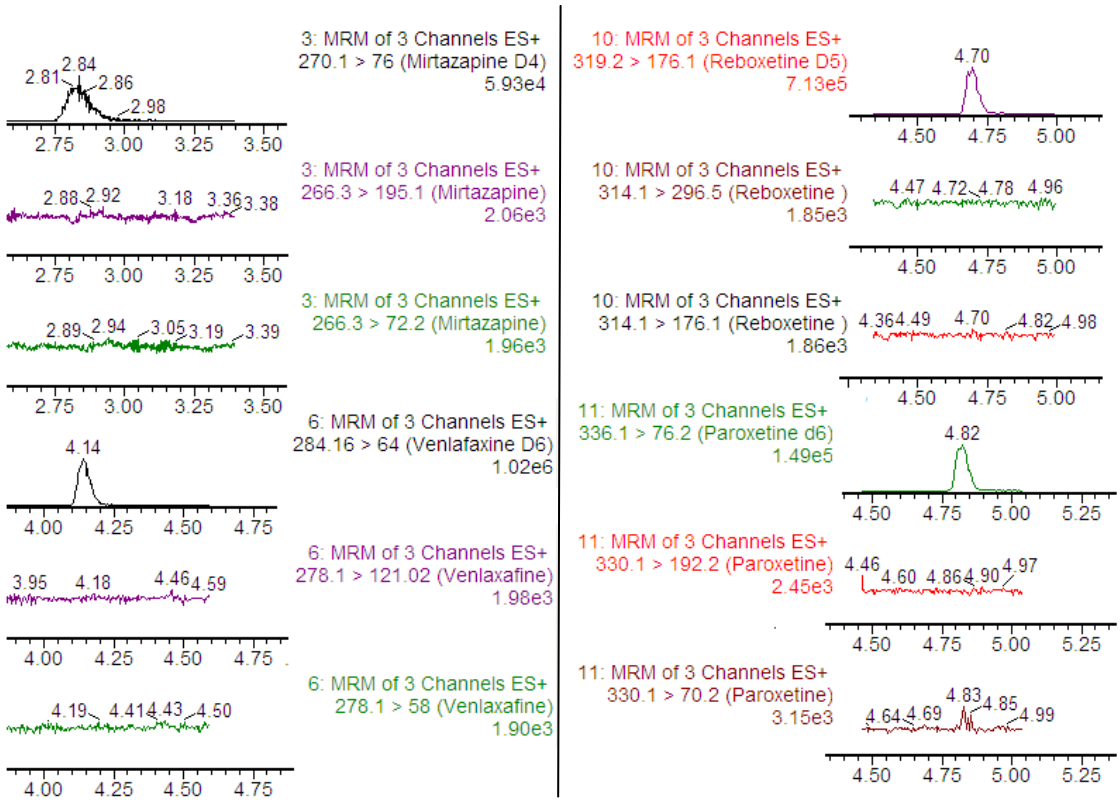


Fig. 2.16. Chromatograms of a blank sample matrix with only internal standards. The results show peaks for the deuterated forms, but all the others chromatograms were clean.

Another blank matrix sample was fortified with antidepressants (200 ng/mL) and analyzed without IS. This was to evaluate whether the unlabeled analyte ions interfere with the signal for the other compounds. The results demonstrated no interferences between the analyte and the internal standard. All analytes were detected; however, it is necessary to confirm if mirtazapine, paroxetine, reboxetine and venlafaxine are in the sample or if the signal is due to their deuterated forms (fig. 2.17).

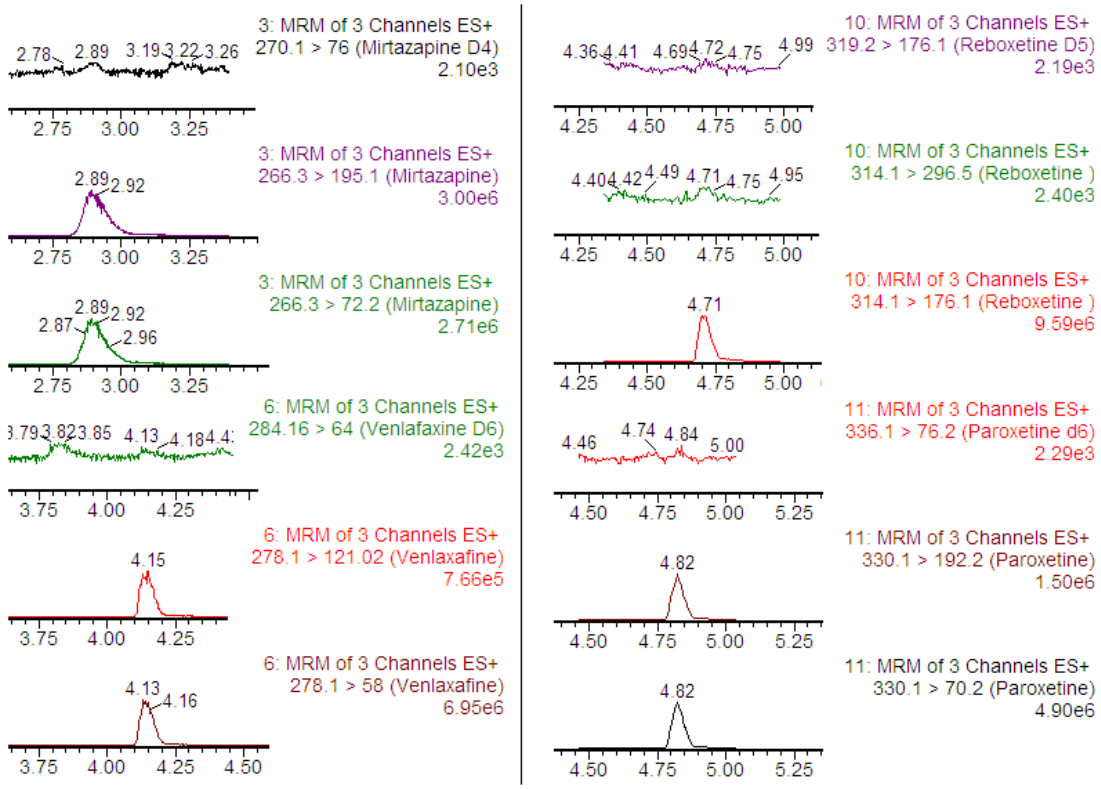
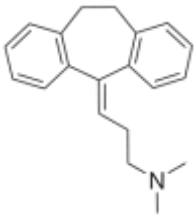
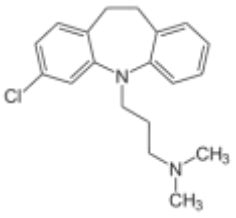
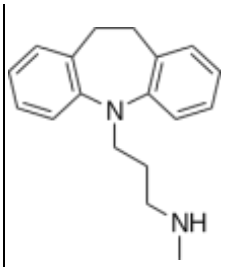
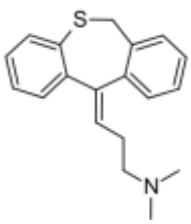
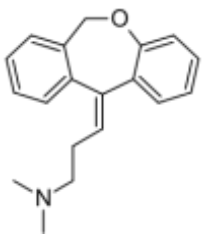
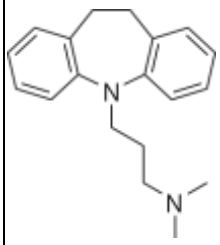
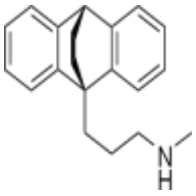
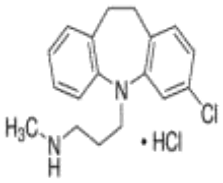
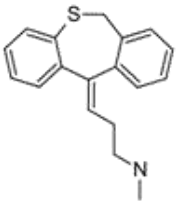
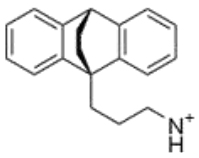
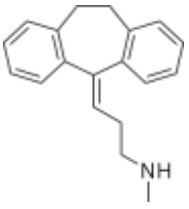
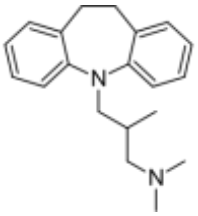


Fig. 2.17. Chromatograms of a blank sample matrix with only antidepressants. The results don't show peaks for the deuterated forms, so we can conclude that there are no interferences between IS and ntads.

Interference from possible co-administrated medications is also a very important test – many patients don't only take non-tricyclic antidepressants, they can also be taking tricyclic ones. To evaluate the interferences between these two similar categories, a solution was injected with tryciclic antidepressants diluted in serum to a concentration of 200 ng/mL for all the compounds. The tryciclic antidepressants present in the sample are described in table 2.11 and the results are in fig. 2.18.

Table 2.11. Name, molecular formula and weight (MW in g/mol) of tricyclic antidepressants used for checking interference.

					
Amitriptyline MW: 277.403	Clomipramine MW: 314.900	Desipramine MW: 266.381	Dosulepine MW: 295.450	Doxepin MW: 279.376	Imipramine MW: 280.407
					
Maprotiline MW: 277.403	Norclomipramine MW: 337.290	Nordosulepine MW: 282.450	Normaprotiline MW: 264.403	Nortriptyline MW: 263.380	Trimipramine MW: 294.434

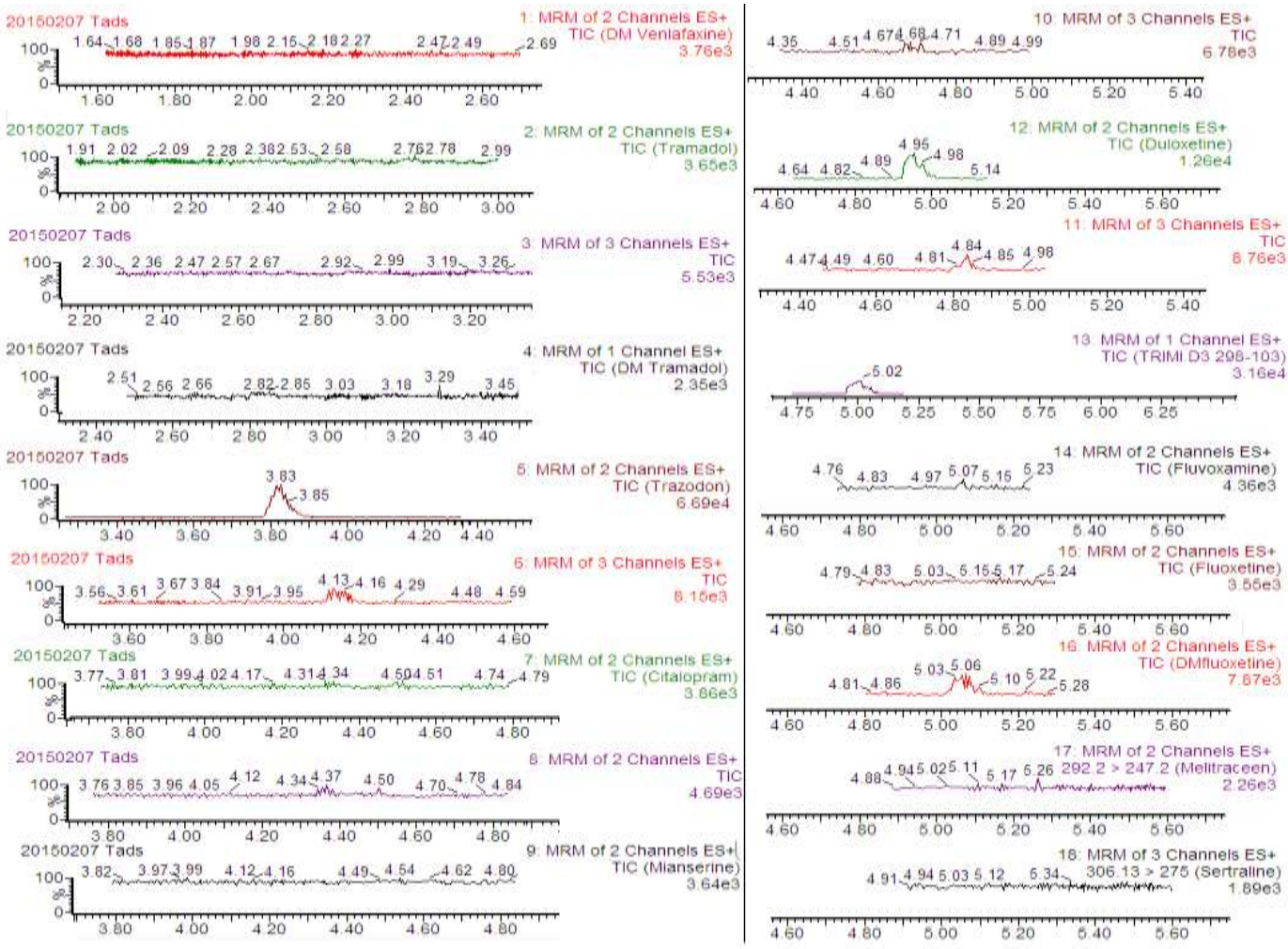


Fig. 2.18. Chromatograms from the tricyclic antidepressants sample. Again, trazodone shows a peak, however, like before, this peak doesn't have enough area to be considered interference. All other chromatograms were considered noise.

All interference studies were successful, which means the method only detects the seventeen analytes it is supposed to. Although the figures don't show, both transitions were always used to make sure about the compound detected, except for the internal standards, where only one transition was used. Since the selectivity part was completed with success, the calibration model comes next in the validation plan.

B.2.3.2. Calibration Model

It is important to know the response of the instrument with regard to the concentration of analyte over a specified concentration range. The calibration range is defined as "the region between the limits within which a quantity is measured, received or transmitted, expressed by stating the lower and upper range values"[82]. This can be performed by the analysis of spiked calibration samples and plotting the resulting responses versus the corresponding concentrations [83].

All calibration curves had a R^2 = 0.99 or higher, except for fluoxetine and tramadol. The calibrations curves for citalopram and paroxetine are represented in the following figures (fig. 2.19 (a) and 2.20 (a)).

However, the calibration model cannot be evaluated simply via its correlation coefficient (R^2). Indeed, a significant proportion of errors at the lower end of the calibration curve can coexist with acceptable R and R^2 values. Therefore, other parameters, such as visual inspection of plots of residuals versus concentration allows us to determine if the variances appear to be equal across the calibration range with a similar degree of scatter at each concentration - fig. 2.19 (b) and 2.20 (b).

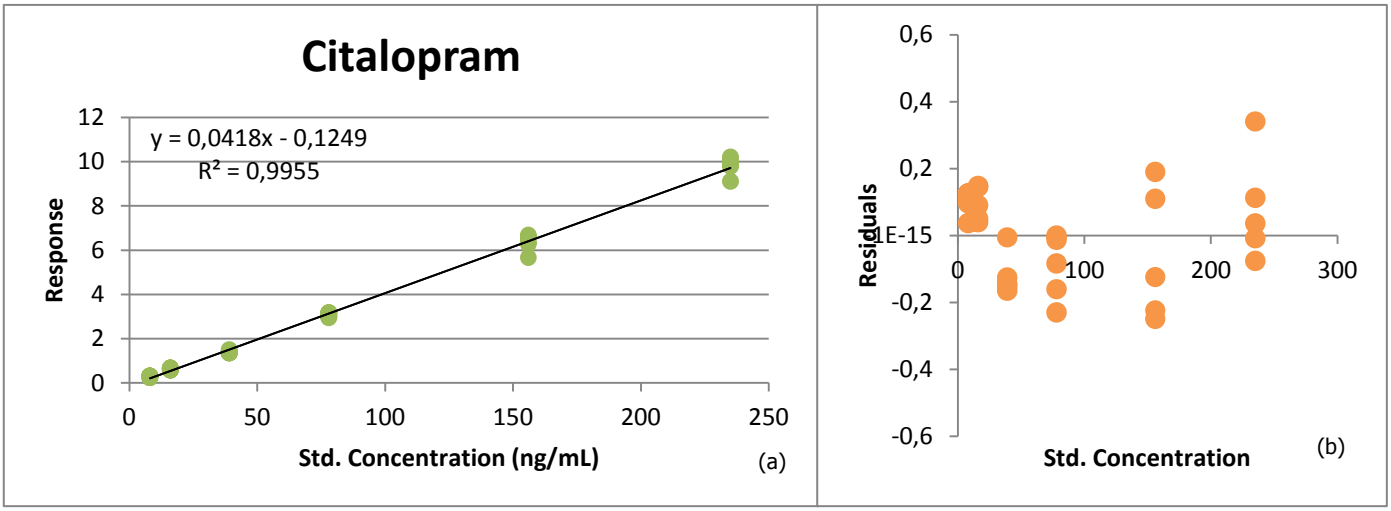


Fig. 2.19. Calibration curve (a) and plot of residuals vs. concentration (b) for citalopram

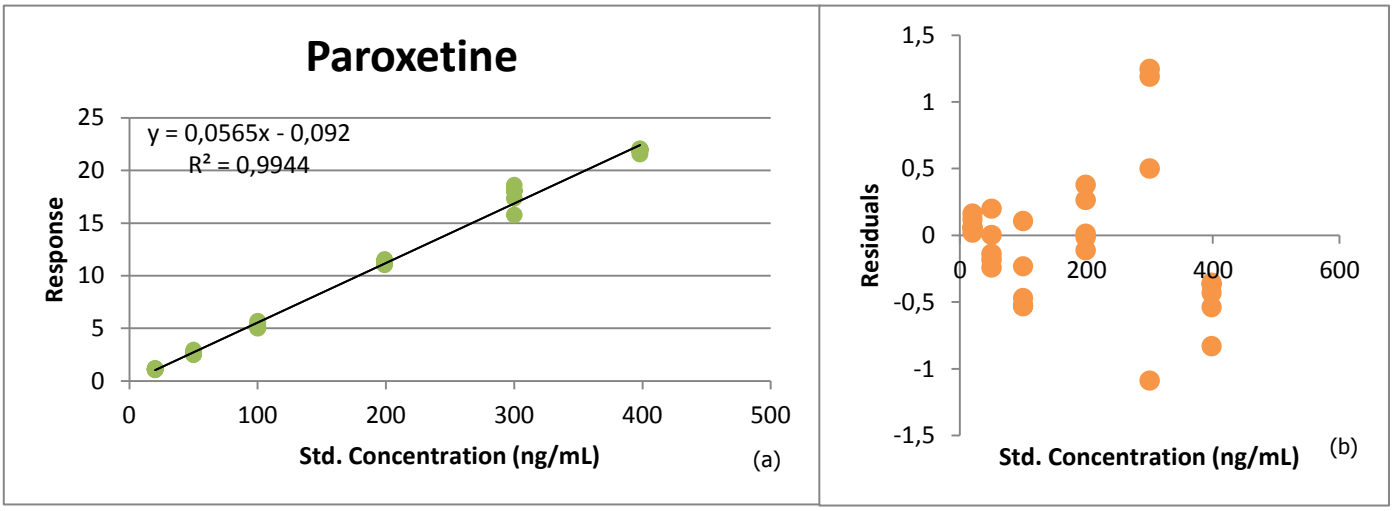


Fig. 2.20. Calibration curve (a) and plot of residuals vs. concentration (b) for paroxetine

B. 2.3.2.1. Grubbs Test

Grubbs test was used to detect outliers in a data set that follows an approximately normal distribution. More than two outliers in a complete data-set of a calibration model experiment may indicate serious problems with the method, which requires further investigation [64]. In following statistical tests, we only present the results for citalopram, but the same calculations were made for all compounds. In table 2.12, the results for citalopram show an outlier, because G_{max} in calibrator 5% is higher than 1.71, the critical value. For future analysis, the highest response (1.51) was eliminated, and without this value, G_{max} = 1.28, below the critical value.

Table 2.12. Grubbs test for citalopram values

Name	Response					G Min	G Max
ntads 30%	9.11	10.20	9.86	9.79	9.98	1.65	1.02
ntads 20%	6.37	6.60	6.28	6.69	5.67	1.63	0.91
ntads 10%	3.09	3.17	2.95	3.02	3.16	1.35	0.99
ntads 5%	1.39	1.36	1.37	**1.51**	1.34	0.72	**1.74**
ntads 2%	0.57	0.68	0.59	0.68	0.62	1.08	1.05
ntads 1%	0.32	0.29	0.30	0.23	0.32	1.67	0.76

B. 2.3.2.2. Fisher Test

The next step is to test for homogeneity of variances (Fisher-Snedecor Distribution). This test is used to compare two variances and to see if the distribution is similar in lowest and highest concentration.

Table 2.13 shows the standard concentrations, the mean, standard deviation and coefficient of variance of responses obtained (responses are already shown in table 2.12). With this data, it's possible to calculate TV and if it's higher than pH0,there is inhomogeneous variances (heteroscedasticity) over the calibration range.

Table 2.13. Results obtained in the test of homogeneity of variances for citalopram

Std. Concentration (ng/mL)	Ym	SD	%CV
8	0.29	0.04	12.5
16	0.63	0.05	8.10
39	1.40	0.06	4.60
78	3.08	0.10	3.20
156	6.32	0.40	6.40
235	9.79	0.41	4.20

According to table 2.13, $TV = \frac{0.41^2}{0.04^2} = 126.47$, while $pH0 = 0$. The H_1 hypothesis in section 4.7.2.2 is accepted and data suggest inhomogeneous variances. The next step is to do a Linearity test to check if linear is the best model to fit the data.

B. 2.3.2.3. Mandel test

There are no outliers and inhomogeneous was proven in the section before; one must evaluate the linearity and check which model (linear or quadratic) fitted better in the calibration data.

The possibility of both models have similar performance is 64% (pH0 = 0.064). Thus, we decided to choose the linear adjustment, since it has more degrees of freedom (table 2.14).

Table 2.14. Results obtained with linearity test

Linear		**Quadratic**	
SS	0.043	SS	0.011
ndf	4	ndf	3
VarFit	0.011	VarFit	0.004
StdFit	0.103	StdFit	0.060

$$F_{crit} = 8.23$$

$$pH0 = 0.064$$

B. 2.3.2.4. Weighted Least Squares

In case of heteroscedasticity data set, weighted least squares should be applied. Slops and intercepts of the calibration lines were calculated using weighted linear regression: $\frac{1}{x}$, $\frac{1}{x^2}$, $\frac{1}{y}$ and $\frac{1}{y^2}$ [83]. The best weighting factor is the one which presents the lowest value of the sum of the relative errors ($\sum|\%\ \mathrm{RE}|$) in the working range.

To test Weighted Least Squares (WLS), six calibrators were analyzed, repeating the same experiment for three different days. Evaluation of response variability was checked and it was shown that statistical dispersion is in direct proportion to the concentration (fig. 2.21) and %CV is more or less constant throughout the working range (fig. 2.22), giving more emphasis to WLS analysis.

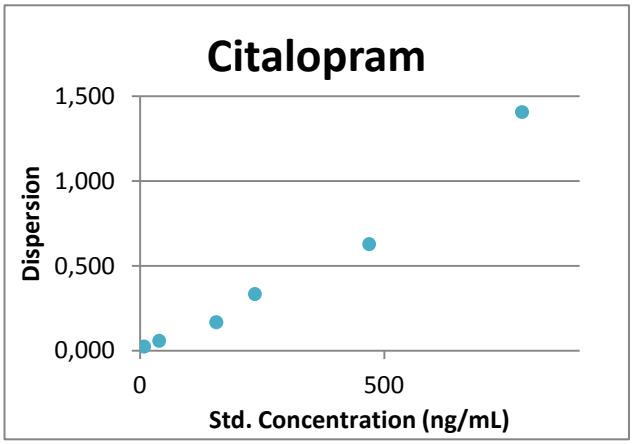


Fig. 2.21. Evaluation of dispersion and standard concentration

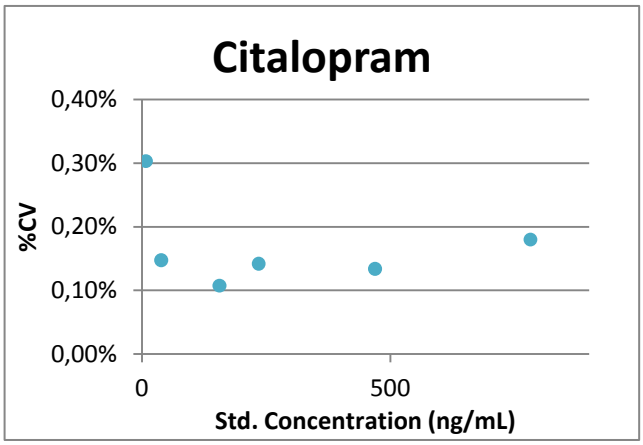


Fig. 2.22. Evaluation of %CV and standard concentration

As a measure to choose the weighting factor, it was used the percentage of relative error (%RE), which compares the estimated concentration, from the regression equation obtained for each w_i, with nominal standard concentration in the sample. The $\sum|\%\ \mathrm{RE}|$ of the different weighting factors were compared, and in citalopram case, the model chosen is $\frac{1}{x^2}$, since the error associated with this model is the lowest (table 2.15). Plots of %RE versus concentration for the four models of citalopram obtained in the study are shown in figure 2.23. For all the other compounds, the analysis is presented in appendices 6.1 and 6.2 and a summary of calibration model is in table 2.16.

Table 2.15. Relative errors (%RE) and the respective sum generated by the use of simple linear regression and weighted linear regression for each weighting factor w_i for the intra-assay data

Citalopram nominal concentration (ng/mL)	Model			
	$\frac{1}{x}$	$\frac{1}{x^2}$	$\frac{1}{y}$	$\frac{1}{y^2}$
782	12.1	14.1	12.2	12
469	18.6	12.1	19.8	16.2
235	28.9	35.4	28	30.9
156	15.9	12.3	16.4	14.8
39	11.2	9.1	9.1	10.7
8	22.8	26.4	22.7	27.3
$\sum \lvert\%\ \mathrm{RE}\rvert$	109.5	**109.4**	108.2	111.9

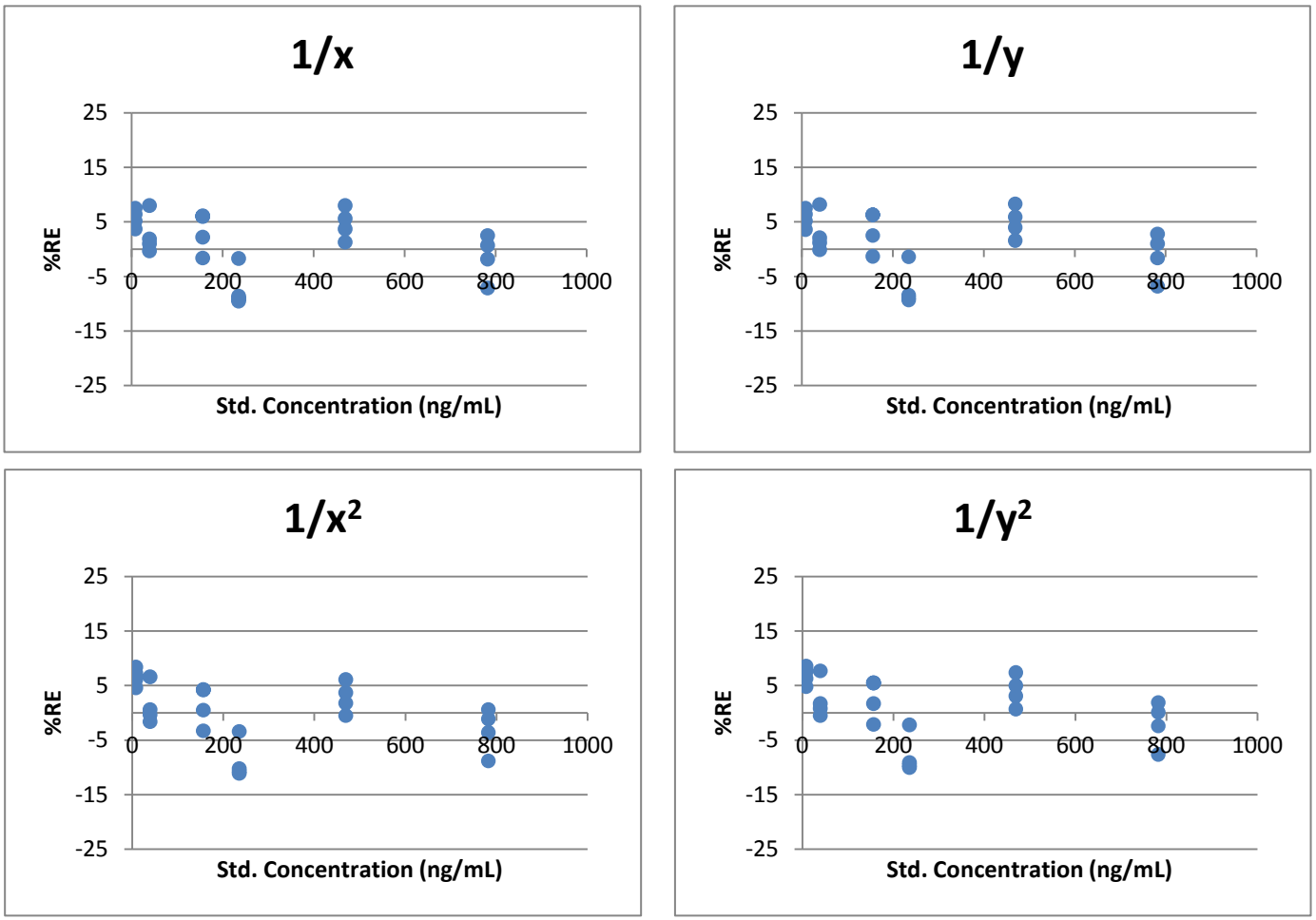


Fig. 2.23. Percentage of relative error (%RE) versus standard concentration obtained for models $\frac{1}{x}$, $\frac{1}{x^2}$, $\frac{1}{y}$ and $\frac{1}{y^2}$. All models seem to behave equally but $\frac{1}{x^2}$ has the lowest $\sum \lvert\%\ \mathrm{RE}\rvert$.

Table 2.16. Summary of calibration model for all compounds

Compound	Working range (ng/mL)	Calibration curve and errors	R^2	Regression	Interval of confidence		Weighting factor
					Lower limit (95%)	Upper limit (95%)	
Citalopram	8-235	Y=0.04 (0.2).X - 0.14 (2.5)	0.995	Linear	-0.257	0.007	$\frac{1}{x^2}$
DM Citalopram	20-400	Y=-9e-5X^2 (0.7) + 0.14X – 1.10 (3.7)	0.994	Quadratic	-0.163	1.924	$\frac{1}{y}$
DM Fluoxetine	50-600	Y=0.01 (0.0).X + 0.19 (0.7)	0.991	Linear	0.287	0.101	$\frac{1}{x}$
DM Tramadol	10-300	Y=0.02 (0.4).X - 0.04 (2.2)	0.993	Linear	-0.151	0.073	$\frac{1}{x^2}$
DM Venlafaxine	39-469	Y=0.002 (0.0).X + 0.02 (0.4)	0.991	Linear	-0.007	0.050	$\frac{1}{y^2}$
Duloxetine	20-400	Y=0.02 (0.2).X+0.12 (1.5)	0.994	Linear	-0.012	0.249	$\frac{1}{x^2}$
Fluoxetine	50-600	Y=0.01 (0.0) X - 0.33 (0.9)	0.982	Linear	-0.461	0.193	$\frac{1}{y^2}$
Fluvoxamine	50-600	Y=0.01 (0.1) X + 0.17 (2.0)	0.993	Linear	-0.033	0.306	$\frac{1}{x}$
Melitracene	10-800	Y=0.05 (0.5) X - 1.64 (1.6)	0.994	Linear	-2.381	0.907	$\frac{1}{y^2}$
Mianserine	10-300	Y=0.01 (0.0) X - 0.02 (0.5)	0.995	Linear	-0.047	0.001	$\frac{1}{x^2}$
Mirtazapine	10-300	Y=0.10(0.5)X-0.40(2.7)	0.996	Linear	-0.782	0.023	$\frac{1}{x^2}$
Paroxetine	20-400	Y=0.06 (0.6).X – 0.14 (2.1)	0.995	Linear	-0.461	0.277	$\frac{1}{x^2}$
Reboxetine	20-400	Y=0.02 (0.1).X + 0.03 (1.8)	0.991	Linear	-0.175	0.236	$\frac{1}{x^2}$
Sertraline	50-600	Y=0.01 (0.1) X + 0.05 (0.3)	0.992	Linear	-0.019	0.112	$\frac{1}{y}$
Tramadol	10-800	y = -1e-7X^2 (0.0) + 0,002X - 0.04 (0.5)	0.985	Quadratic	-0.059	0.004	$\frac{1}{x^2}$
Trazodone	213-2560	y = -3e-6 (0.1).X^2 + 0.02X + 0.69 (3.1)	0.994	Quadratic	-0.015	0.012	$\frac{1}{y^2}$
Venlafaxine	50-600	Y=0.01 (0.2).X - 0.04 (2.5)	0.990	Linear	-0.020	0.125	$\frac{1}{y}$

B.2.3.3. Imprecision and accuracy (Bias)

To establish the method's bias and precision, three pools of fortified matrix samples were prepared at the following concentrations: low (50 ng/mL for all compounds, except citalopram and desmethylvenlafaxine, 39 ng/mL and trazodone, 213 ng/mL), medium (400 ng/mL for all, citalopram and desmethylvenlafaxine, 313 ng/mL and trazodone, 1707 ng/mL) and high (1000 ng/mL for all, citalopram and desmethylvenlafaxine, 782 ng/mL and trazodone, 4267 ng/mL). Each concentration pool of fortified samples was analyzed in triplicate (A, B and C) on five separate days along with a freshly prepared calibration curve (6 calibrators).

The bias was calculated by first determining the mean for each concentration. An example (citalopram) of how it was calculated for the lowest concentration is given in table 2.17.

Table 2.17. Results for citlopram at low concentration (39 ng/mL)

	Run 1	Run 2	Run 3	Run 4	Run 5
A	41.6	42.7	34.1	45.7	44.3
B	42.9	45.1	34.2	42.6	43.8
C	41.8	36.8	34.7	45.2	43.4
Mean	42.1	44.9	34.3	44.5	43.8
SD	0.7	4.3	0.3	1.7	0.5
CV	1.7%	10.3%	0.9%	3.7%	1.0%
%Bias	2.04%	0.66%	-16.79%	7.85%	6.24%

B. 2.3.3.1. Cochran Test

One of the assumptions for the usage of ANOVA is the homogeneity of variances. Following section 4.7.3.1., if C_{cal} is higher than the C_{crit}, we concluded that the differences between the variances are statically significant (table 2.18).

Table 2.18. Results obtained with Cochran test.

Compound	Concentration (ng/mL)	C_{cal}	C_{crit}
Citalopram	39	0.836	0.789

In table 2.17, the higher bias belongs to day 3. If removed from the analysis and eliminate the outlier from run 2 (36.8 ng/mL), C_{cal} = 0.447 and there is no

statistical significance between variances. To calculate if there is a significant difference between days with ANOVA analysis:

$$TV = \frac{MS_{bd}}{MS_{wd}} = 1.09 \text{ and } pH0 = 0.408$$

There is a possibility of 40.8% of no difference between the concentrations of the four runs and the dispersion estimates are:

$$dispersion\ within\ days = \sqrt{MS_{wd}} = 2.3$$

$$total\ dispersion = \sqrt{MS_{total}} = 2.4$$

Using the same procedure for all the compounds, by checking first if there is homogeneity of variances with Cochran test and, if there's not, removing the day with the higher bias, results in table 2.19 and 2.20 were achieved.

Table 2.19. Results for BIAS and imprecision for citalopram, desmethylcitalopram, desmethylfluoxetine, desmethylvenlafaxine, duloxetine, fluoxetine, fluvoxamine and melitracen

		% BIAS	% Imprecision	
Compound	Conc. level		Within Run CV	Between Run CV
1. Citalopram	Low	5.8	5.8	3.2
	Medium	1.4	4.2	4.3
	High	5.7	4.2	3.4
2. DM Citalopram	Low	10	14.3	7.1
	Medium	9.9	5.1	5.6
	High	-3.4	5.4	4.9
3. DM Fluoxetine	Low	12.3	5.2	9.3
	Medium	8.5	5.4	5.2
	High	-9.0	5.6	4.2
4. DM Tramadol	Low	2.2	10.6	9.4
	Medium	0.0	4.8	5.4
	High	4.0	7.1	4.6
5. DM Venlafaxine	Low	9.6	7.3	12.3
	Medium	4.1	3.9	8.2
	High	11.4	5.2	5.0
6. Duloxetine	Low	8.1	8.7	5.8
	Medium	6.9	5.6	5.3
	High	3.7	4.8	4.4
7. Fluoxetine	Low	0.9	9.3	4.8
	Medium	10.7	11.8	7.2
	High	-2.2	8.9	7.1
8. Fluvoxamine	Low	11.9	6.3	10.2
	Medium	10.2	5.1	5.5
	High	-2.4	5.0	4.9
9. Melitracene	Low	1.4	8.7	8.1
	Medium	-2.2	9.8	4.8
	High	7.8	8.0	5.9

Table 2.20. Results for BIAS and imprecision for mianserin, mirtazapine, paroxetine, reboxetine, sertraline, trazodone and venlafaxine

Compound	Conc. level	% BIAS	% Imprecision	
			Within Run CV	Between Run CV
10. Mianserine	Low	9.8	7.9	6.5
	Medium	6.2	3.5	4.7
	High	0.4	5.1	3.6
11. Mirtazapine	Low	4.9	8.1	4.9
	Medium	-1.3	4.6	7.8
	High	0.2	6.5	7.1
12. Paroxetine	Low	1.0	10.1	8.0
	Medium	3.2	7.1	8.2
	High	1.4	6.7	5.2
13. Reboxetine	Low	-0.9	3.5	7.0
	Medium	1.8	3.3	4.7
	High	4.5	4.8	3.6
14. Sertraline	Low	8.8	7.9	10.8
	Medium	6.7	6.5	4.5
	High	-0.1	5.5	6.3
15. Tramadol	Low	7.5	12.3	7.0
	Medium	15.7	8.7	5.8
	High	4.7	9.2	7.5
16. Trazodone	Low	18.4	5.8	6.3
	Medium	7.2	5.0	4.9
	High	-6.9	4.4	3.1
17. Venlafaxine	Low	10.1	6.1	5.1
	Medium	3.1	2.9	5.1
	High	5.4	5.2	4.4

The Scientific working group for forensic toxicology (SWGTOX) says that both imprecision and bias shouldn't exceed 20%, and, as we can see, all of the compounds fit in that criterion.

B.2.3.4. Carry-Over

As mentioned above, carry-over is the amount of the analyte retained in an LC system from a preceding sample that carries over into the next injected sample; also, there are certain compounds with mutual affinity and lead to the formation of dimmers. These dimmers can manage variations and abnormal results [84]. Carry-over phenomenon can be measured by the response of the blank sample or the lowest calibrator after the injection of a preceding sample at high concentration.

Following section 4.7.5, if the Δlow is calculated and if it exceeds 2 × SD low unaffected, carry-over is present. Table 2.21 shows the results for all compounds:

Table 2.21. Results for carry-over

Compound	Δlow	2 x SD low
Citalopram	1.4	1.8
DM Citalopram	4.2	4.8
DM Fluoxetine	8.7	8.8
DM Tramadol	0.8	1.0
DM Venlafaxine	0.8	1.1
Duloxetine	6.3	7.3
Fluoxetine	4.0	4.5
Fluvoxamine	4.9	5.1
Melitracene	1.9	2.4
Mianserine	2.5	2.6
Mirtazapine	0.2	1.2
Paroxetine	4.6	5.8
Reboxetine	1.0	1.6
Sertraline	4.3	5.7
Tramadol	0.9	1.3
Trazodone	17.9	22.7
Venlafaxine	0.8	1.3

Sample carry-over is a major problem that can influence the accuracy and precision of the method, with the consequences being more pronounced at lower concentrations. As we can see in table 2.21, carry-over is absent in all compounds (the criterion fits).

B.2.3.5. Matrix Effects

Matrix effect (ME) causes a compound's response to differ when analyzed in a biological matrix compared to a standard solution. Molecules originating from the sample matrix that coelute with the compounds of interest can interfere with the ionization process in the mass spectrometer, causing ionization suppression or enhancement [85]. As a result, depending on the environment in which the ionization and ion evaporation processes takes place, there is a decrease in analyte ionization (ion suppression) or an increase in this ionization (ion enhancement) [86].

The origin and mechanism of matrix effects are still not fully understood. It is generally accepted that matrix effect evaluation should be a mandatory part of the validation procedure of all LC–MS based methods. Consensus on how these matrix effects should be evaluated is obviously needed. [85]. Ionization suppression/enhancement was evaluated using the description in section 4.7.6 Extraction efficiency (RE) and process efficiency (PE) were also calculated.

Ion suppression may adversely affect both the sensitivity and the reproducibility of a particular assay [87]. All the data are described in appendix 6.5. and final results for low and high concentrations are in table 2.22 and 2.23, respectively.

Table 2.22. Results for matrix effects at low concentrations

	Low Concentration		
	% Matrix effects	% Extraction efficiency	% Process efficiency
Citalopram	82	85	70
DM Citalopram	78	81	63
DM Fluoxetine	45	71	32
DM Tramadol	99	99	95
DM Venlafaxine	97	109	106
Duloxetine	42	59	25
Fluoxetine	49	46	22
Fluvoxamine	77	72	56
Melitracene	36	64	23
Mianserine	54	64	35
Mirtazapine	95	96	91
Paroxetine	45	62	28
Reboxetine	87	87	76
Sertraline	27	83	22
Tramadol	93	82	77
Trazodone	88	84	73
Venlafaxine	100	96	96
Internal Standards			
Mirtazapine-d_4	91	98	89
Paroxetine-d_6	47	73	34
Reboxetine-d_5	89	92	81
Venlafaxine-d_6	96	100	96

For example, in citalopram's case, the matrix effect was 82%, which means there was 18% of ion suppression. However, some of the results were really low, considered table 2.22. Fluoxetine, melitracene and paroxetine have low values in ME and PE. The internal standard used to measure these compounds is paroxetine-d_6, which also has low percentages in ME and PE. So, the internal standard is compensating for the low percentages.

As for DM fluoxetine, duloxetine and mianserine, both have reboxetine-d_5 as an internal standard, which doesn't compensate much for the low values. For example, for DM fluoxetine, if the process efficiency is 32%, this means that 68% of the signal is

lost. One of the possible explanations is that the extraction procedure is not appropriate for this specific compound (extraction efficiency equal to 71%). This is also the case for duloxetine and mianserine, due to its very low process efficiency (25 and 35%, respectively).

Sertraline has the biggest loss in signal, with 78% lost. Not much is lost due to ion suppression (only 27%) and the extraction efficiency is quite high (83%). Sertraline is the last compound to be eluted, with the highest retention time. Many compounds are eluted at the same time, causing interference with each other and affecting the ionization.

Table 2.23. Results for matrix effects at high concentrations

	High Concentration		
	% Matrix effects	% Extraction efficiency	% Process efficiency
Citalopram	99	78	78
DM Citalopram	93	80	74
DM Fluoxetine	70	51	36
DM Tramadol	94	92	87
DM Venlafaxine	94	102	96
Duloxetine	64	42	27
Fluoxetine	63	36	23
Fluvoxamine	90	64	58
Melitracene	49	45	22
Mianserine	66	52	70
Mirtazapine	97	84	81
Paroxetine	68	45	31
Reboxetine	98	78	77
Sertraline	43	50	22
Tramadol	98	84	82
Trazodone	97	81	78
Venlafaxine	108	87	93
Internal Standards			
Mirtazapine-d_4	97	80	77
Paroxetine-d_6	72	48	34
Reboxetine-d_5	95	78	74
Venlafaxine-d_6	102	91	93

For high concentrations, we have the same compounds giving low percentages. Fluoxetine, melitracene and paroxetine have low values regarding for process and extraction efficiency, just like paroxetine-d_6. So, once again, the internal standard is compensating the low percentages.

The same happens to DM fluoxetine and duloxetine, with low values to process and extraction efficiency. The high value in matrix effects suggests that the loss of the signal is due to ion suppression. Sertraline has low values in the three parameters, which can indicate that the loss of the signal is caused both by ion suppression and extraction procedure.

The ion suppression that both low and high concentrations show can be caused by the co-elution of several compounds, causing interference in ionization and so, losing the signal of the analytes. In figure 2.24., it is possible to see that there is a significant decrease of matrix effects from 4 min. This corresponds to the co-elution of paroxetine, duloxetine, fluvoxamine, fluoxetine, DM fluoxetine, melitracene and sertraline (in this order), the compounds that presented the highest lost of signals.

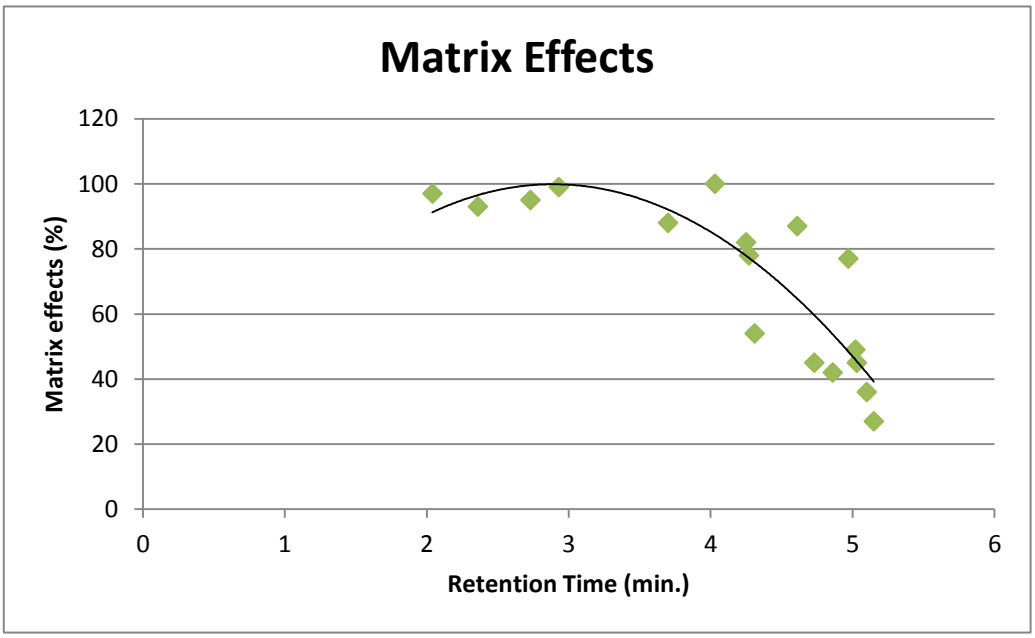


Fig. 2.24. Matrix effects vs. retention time of the analytes at high concentrations.

B.2.3.6. Limits

B.2.3.6.1 Limit of Detection

There are different approaches for the determination of LOD: the precision and accuracy of the data, the signal to noise ratio (S/N), and the parameters of the analytical curve. The estimation method based on parameters of the analytical curve shows greater statistical reliability and is useful for any quantitative method that follows a linear calibration model. So, the LOD was estimated based on the equations in Section 4.7.7.

B.2.3.6.2. Limit of Quantification

As for the LOQ, three runs were made (Run 1, 2 and 3) with six calibrators (1000, 600, 300, 200, 50 and 10 ng/mL) and three quality controls (QC) in duplicate (with 50, 20 and 10 ng/mL). From these QC's, LOQ is chosen. It is important to check that the lowest concentration should be able to achieve acceptable detection, identification and also, the bias and imprecision should not be more that ± 20%. The following table (table 2.24) shows the results and all the data.

Table 2.24: Results for LOD and LOQ in ng/mL

Compound	LOD (ng/mL)	LOQ (ng/mL)
Citalopram	3	10
DM Citalopram	1	20
DM Fluoxetine	3	10
DM Tramadol	4	20
DM Venlafaxine	4	10
Duloxetine	2	20
Fluoxetine	7	20
Fluvoxamine	5	10
Melitracene	1	20
Mianserine	4	20
Mirtazapine	1	10
Paroxetine	1	20
Reboxetine	3	20
Sertraline	2	20
Tramadol	8	20
Trazodone	6	85
Venlafaxine	2	20

B.2.3.7. Stability

As the samples may not always be analyzed immediately after extraction due to large batches or unforeseen delay, it is important to evaluate the impact of room temperature storage of processed samples sitting on the autosampler.

B.2.3.7.1. Processed Samples

To analyze stability in processes samples, twelve aliquots of fortified matrix samples at two concentrations were extracted (20 and 800 ng/mL). The concentration pool was then divided into 12 autosampler vials and placed in the autosampler. The first vials of each level was injected three times to represent the time zero (T0). The remaining vials of each concentration were analyzed in triplicate every six hours up to 66 hours. Analyte signals from the triplicate analyses were averaged and compared to the T0 signals.

By plotting the average peak areas for each compound and internal standards, it is possible to evaluate the processed samples while they were stored on the autosampler. As the required bias is $\pm$20%, two lines were made, the red one which correspond to an increase of 20% in signal, compared to T0, and a green one which correspond to a 20% decrease in signal.

These data appear to suggest all the compounds are not stable after 6h in the autosampler (Fig. 2.25 – 2.28). It was noted that at the 6-hour mark, stability seemed to have dropped very close to the "instability" point, except for DM Tramadol in the highest concentration. The same happens for the internal standards, showing some instability after 12h (in appendice 6.6).

If the areas of compounds and internal standards are decreasing throughout time, a compensation is very clear, since what makes the calibration curve is the ration between these areas. In fig. 2.29, area ratio between citalopram and reboxetine-d_5 at 20 ng/mL vs. time is shown and, if both area decrease, the response will have more or less the same values. However, there's instability 18h after extraction, so based on this study, it was decided that samples remaining in the autosampler more than 18h should be re-extracted.

Figure 2.30 shows the calculated concentration of Citalopram at 20 ng/mL vs. time. As one can see, the calculated concentration never exceeds or is inferior 20% of the calculated concentration in T0. The same happens with the other compounds at low and high concentrations, but the laboratory maintains the decision mentioned before, of not analyzing samples after 18h of extraction.

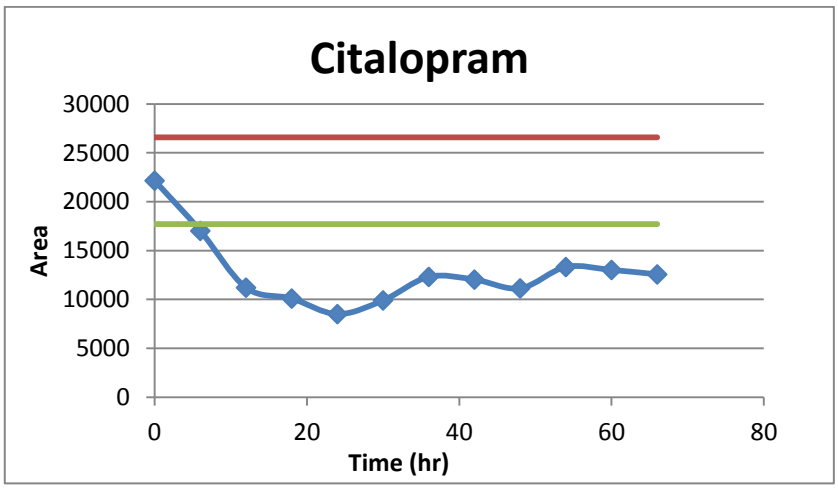


Fig. 2.25. Citalopram at 20 ng/mL. The green line represents the decrease of the peak areas, while the red line represents 20% peak area increase, compared to T0 and the blue line represents 20% peak area decrease.

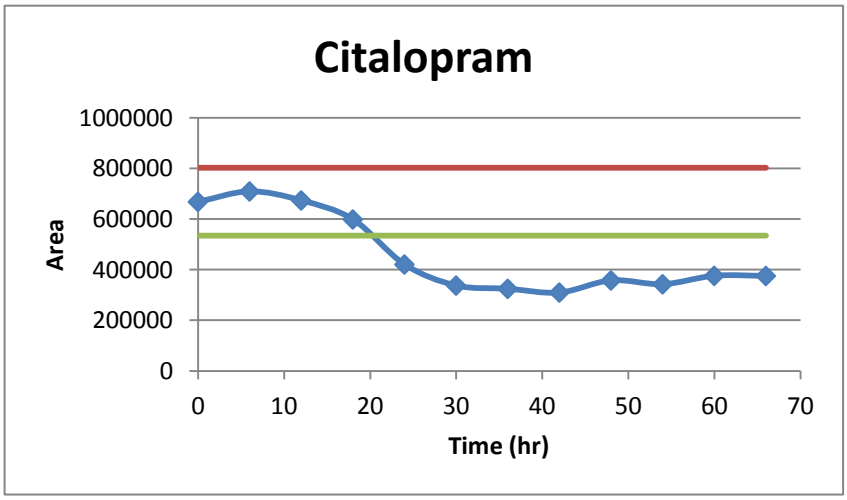


Fig. 2.26. Citalopram at 800 ng/mL

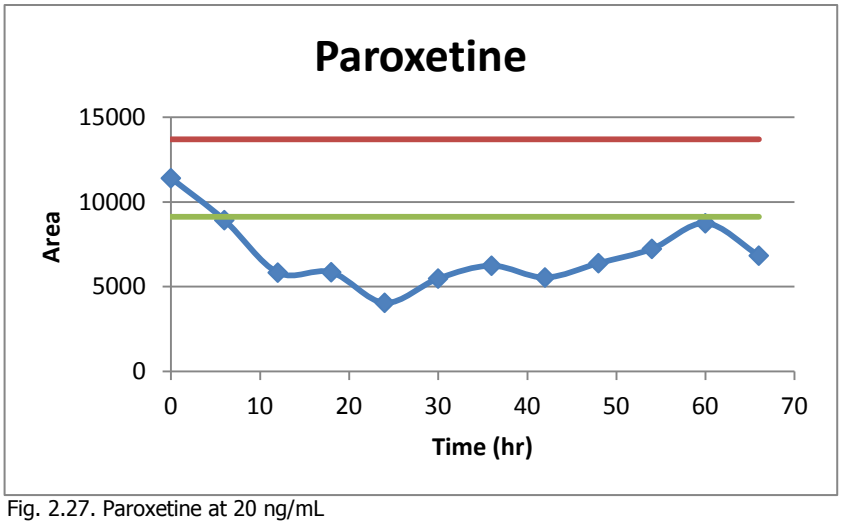


Fig. 2.27. Paroxetine at 20 ng/mL

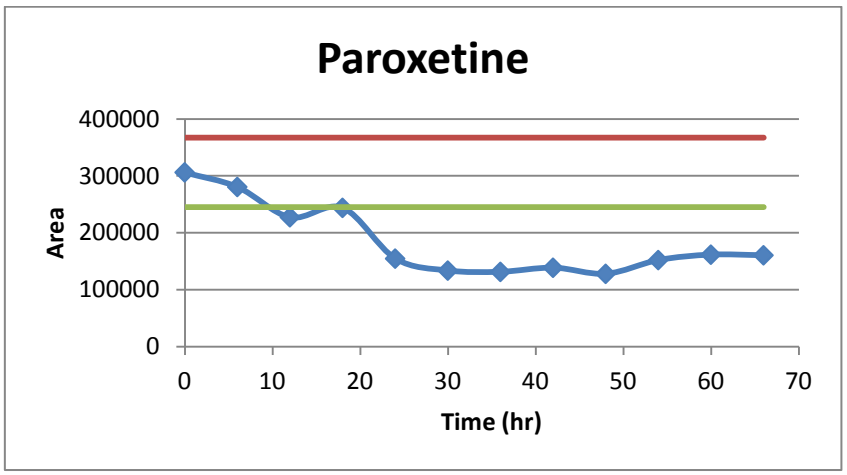


Fig. 2.28. Paroxetine at 800 ng/mL

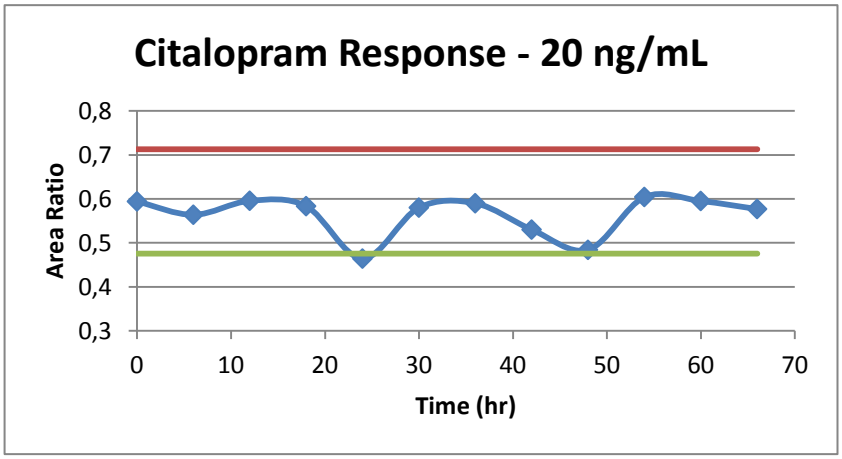


Fig. 2.29. Area ratio was calculated with citalopram and reboxetine-d_5 results. The blue line represents the response, while the red line represents 20% response increase, compared to T0 and the green line represents 20% response decrease. Since both compounds show a decrease on areas, the response seems to show more stability and so, the samples can remain in the autosampler until 18h after extraction.

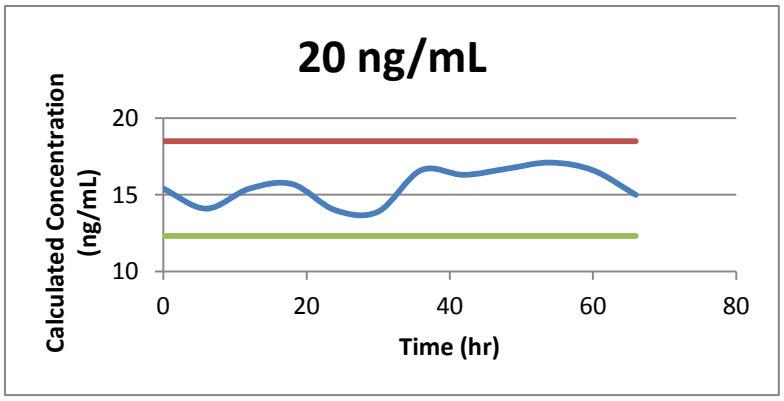


Fig. 2.30. Calculated concentration in ng/mL vs time in citalopram's case at 20 ng/mL.

B.2.4. Application of the developed analytical method to real samples

An analytical method was developed and validated for the determination of 15 antidepressants and 2 analgesics in serum by LC-MS/MS. To complete this study even more, it is important to use the method developed in patients' samples where these drugs are indeed present.

B.2.4.1. Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology (KKGT) Samples

The first analyzed samples were provided by Association for Quality Assessment in Therapeutic Drug Monitoring and Clinical Toxicology (KKGT). The objective of the association is to stimulate the quality of Therapeutic Drug Monitoring, Clinical Toxicology and the analysis of other substances in laboratories. Every year, KKGT releases a quality control samples for psychotherapeutic drugs (table 2.25). According to the report from 2015 [88], psychotherapeutic drugs III, IV and V are indicated the test with the new method.

Table 2.25. KKGT 2015 – compounds and concentrations presented in psychopharmaceuticals samples

Compound	Concentration (µg/l)
Psychopharmaceuticals III	
Fluoxetine	299.1
DM Fluoxetine	353.6
Fluvoxamine	151
Mirtazapine	75.85
Paroxetine	46.59
Psychopharmaceuticals IV	
Citalopram	90.70
DM Citalopram	36.27
Psychopharmaceuticals V	
Sertraline	59.12
Venlafaxine	61.40
DM Venlafaxine	204.7

Fluoxetine, DM Fluoxetine, Fluvoxamine, Mirtazapine, Paroxetine, Citalopram, DM Citalopram, Sertraline, Venlafaxine and DM Venlafaxine were analyzed by doing a five point's calibration curve (100%, 60%, 20%, 10% and 0%). The results for the compounds of interest are given in the following tables (table 2.26).

Table 2.26. Psychotherapeutic drugs results

Name	Type	Std. Conc	Conc.	%Dev
ntads 100%	Standard			
ntads 60%	Standard			
ntads 20%	Standard			
ntads 10%	Standard			
Blank 0%	Standard			
Psychotherapeutic Drugs III				
DM Fluoxetine	QC	353.6	303.5	-14.2
Fluoxetine	QC	299.1	218.0	-27.1
Fluvoxamine	QC	151.0	197.2	30.6
Mirtazapine	QC	75.9	74.8	-1.4
Paroxetine	QC	46.6	38.8	-16.7
Psychotherapeutic Drugs IV				
Citalopram	QC	90.7	75.0	-17.3
DM Citalopram	QC	36.3	32.9	-9.3
Psychotherapeutic Drugs V				
Sertraline	QC	59.1	54.9	-7.1
DM Venlafaxine	QC	204.7	244.8	19.6
Venlafaxine	QC	61.4	58.7	-4.4

As we can see, all the compounds have deviation above 20%, except for fluoxetine (-27.1%) and fluvoxamine (30.6%). This could be due the degradation or instability of some compounds when one is working with frequently used samples. Also, a previsouly studiy proved that fluoxetine is not stable after 8 weeks [89], while KKGT samples used for the study were analyzed in the end of March and arrived in the Toxicology Lab in the beginning of January.

B.4.2.2. Patients' samples from the Toxicology Laboratory

Patients' samples, provided by the Toxicology Laboratory in Ghent Hospital University, were previously analyzed with API 2000 LC-MS/MS Turbo Ion Spray from

ABSCIEX. This instrument has been analyzing non-tricycles antidepressants since 2003. So, positive samples already analyzed, were processed according to the protocol defined before. A five point's calibration curve was made, including the blank sample and the quality control samples from the KKGT were also analyzed (in this case, the quality control samples analyzed were from 2015).

February and March

Ten patients' samples, from February and March 2015 were analyzed and compared to the results (reported concentrantion in ng/mL) from LC-MS/MS API 2000 – C264, C313, C367, C403, C048, C034, B996, B796, B793 and B708. The results are in the following table (table 2.27) for the compounds that were found in the samples. It was also calculated the Bias, having as a nominal concentration, the value found with the API 2000, except for the quality controls, where nominal concentration is in table 2.25.

Table 2.27. Patients' samples analyzed with the new method and the method in API 2000 equipment. Concentrations achieved with both methods are shown, as the bias.

Sample	Type	Compound	Concentration LC-MS/MS TQD	Concentration LC-MS/MS API 2000	% Bias
ntads 100%	Standard				
ntads 60%	Standard				
ntads 20%	Standard				
ntads 10%	Standard				
ntads 0%	Standard				
KKGT III	QC	Citalopram	77.9		-14.1
C264	Analyte	Citalopram	276.7	400	-30.8
C313	Analyte	Citalopram	22.8	24	-5.0
C367	Analyte	Citalopram	141.9	155.9	-9.0
C403	Analyte	Citalopram	43.9	55	-20.2
KKGT IV	QC	DM Citalopram	34.970		-18.4
C264	Analyte	DM Citalopram	33.8	40	-15.5
C313	Analyte	DM Citalopram	7.2	6.1	-18.3
C367	Analyte	DM Citalopram	30.5	33	-7.6
C403	Analyte	DM Citalopram	8.1	8.6	-5.8
KKGT V	QC	DM Venlafaxine	348.5		11.4
C034	Analyte	DM Venlafaxine	68.5	108	-37
KKGT V	QC	Sertraline	54.7		-10.9
C403	Analyte	Sertraline	17.6	15.5	13.5
C367	Analyte	Trazodone	5586.7	5893	-5.2
C403	Analyte	Trazodone	974.5	1190	-18.1
C048	Analyte	Trazodone	109.2	156	-30.0
C034	Analyte	Trazodone	424.3	428	-0.9
B796	Analyte	Trazodone	299	189	58.2
B793	Analyte	Trazodone	1448.3	1460	-0.8
KKGT V	QC	Venlafaxine	57.0		-6.3
C034	Analyte	Venlafaxine	13	28	-53.6

All the values from the analysis with the new method seem to be close to the values from the API 2000, except for sample number C034, with DM Venlafaxine. However, the API 2000 doesn't have a quality control sample for DM Venlafaxine, so this result may not be right and the same applied to venlafaxine case (%bias = -53.6).

Sample number B796 also has a difference between the concentration found and the reported one. However, these concentrations are not in the expected range and trazodone doesn't have either a quality control in neither the methods, which is difficult to prove if this value is correct.

3. CONCLUSIONS AND FUTURE PERSPECTIVES

In this project, a method was developed for the identification and quantification of 15 non-tricyclic drugs in serum with LC-MS/MS.

Chromatographic conditions were created and optimized, with an efficient separation and a running time for 7.5 min. The mass spectrometer was operated in MRM mode, allowing the selection of the transitions to be monitored for each antidepressant and also the internal standard used. Each compound had 2 MRM's to allow a more reliable identification.

Since no method is complete without validation, after the preliminary studies, the method was validated according to the Scientific Working Group for Forensic Toxicology (SWGTOX) guideline. The analytical parameters studied included selectivity, calibration model (linearity), precision and accuracy, carry-over, matrix effects, limits and stability of processed samples.

The method was selective for all the compounds and no interferences were found with tricyclic antidepressants.

Calibration model test proved that the methodology was linear for most of compounds over the concentration range studied, with $R^2 > 0.99$ (except for fluoxetine and tramadol) and the residuals were also evaluated. However, it was observed a heteroscedastic distribution of the residuals, so it was used a weighted linear regression, with empirical weighting factors of $\frac{1}{x}$, $\frac{1}{x^2}$, $\frac{1}{y}$ and $\frac{1}{y^2}$.

Limits of detection ranged from 1.0 to 8 ng/mL and limits of quantification were lower than 20 ng/mL (except for trazodone).

Precision and accuracy were studied throw five runs in five different days. Intra and interassay imprecsion varied from 3.5 to 12.3% and 3.2 and 10.8%, respectively. Bias was also lower than 15%, except for the lowest values of desmethylcitalopram, tramadol and trazodone, where bias was lower than 20%. Also, some compounds at specific concentrations (mostly the lowest concentration) don't show homogeneity of variances when statistical test are performed.

Carry-over was also evaluated and all the compounds do not show any significant values for this parameter. Nevertheless, it advice to introduced wash injections (blanks with methanol 100%) in order to avoid possible contamination from the previous run performed with the equipment.

Matrix effects were evaluated, showing very different values, from 36 to 108% for the compounds and 47 to 102% for the internal standards. The low values could possibly be explained by the close retention time that paroxetine, duloxetine, fluoxetine, melitracene and sertraline had. Since ion suppression demonstrated a high value, process efficiency varied from 22 to 106%, clarifying a problem with signal loss. However, the internal standards also showed a low value, from 36 to 93% or process efficiency. This can compensate in terms of response the previous problem.

Compounds proved not stable 18h after the extraction. The temperature of the autosampler where the samples were reserved was 15ºC. In the future, it is proposed to re-study this parameter with the autosampler at 4ºC and see how the temperature affects the stability of these non-tricyclic antidepressants.

The developed method was applied to Quality Controls from KKGT and patient samples from the Toxicology Lab, previously analyzed with the equipment responsible for non-tricyclic antidepressants analysis. KKGT results proved to be acceptable, except for fluoxetine and fluvoxamine. These two compounds are not found very often in patient samples. However, patient samples proved to be good, with the concentration found with this methodology closed to the concentration reported.

This method can thus replace the method existing in the laboratory so far, since the volume of serum samples were optimized (from 500 to 250 µL) and the distinction between desmethylvenlafaxine and tramadol is clear now. Due to the increased use of antidepressants and their involvement in intoxications, the ability to reliably detect this class in biological specimens is very important, being serum a relevant biological matrix. This method is therefore suitable for both therapeutic drug monitoring and quantification in suspected overdose cases.

4. MATERIALS AND METHODS

4.1. Equipment

- Liquid Chromatography system coupled to mass spectrometer, with the following components:
  - Liquid Chromatography, Waters Acquity ® Ultra Performance LC ;
  - ESI source, Zpray® and Multi-Mode ESCi;
  - Hybrid triple quadropole/linear ion trap mass spectrometer, Waters TQD Detector;
  - Software MassLynx® for MS System;
  - Sofwtare Acquity® UPLC Console;
- Thermomixer comfort Eppendorf®;
- Vortex Genie 2 Scientific Industries;
- Centrifuge Microfuge® 16 Beckman Coutler;
- Bransonic Ultrasonic cleaner (Branson);
- Roller mixer (Greiner labortechnik).

4.2. Material

- Acquity UPLC BEH C18 1.7 µm 2.1 x 100mm Column (from Waters);
- Micropipettes® Research Plus (Eppendorf®);
- Transfer pipettes (SARSTEDT®);
- Test-tubes heavy-walled, 40 x 8 mm (NOVOLAB);
- Vials 1mL from Waters Acquity ®;
- Protein LoBind Tube 1,5 mL from Eppendorf®.

4.3. Standards

- nTads solution containing:
  - Citalopram.HBr, from Cerilliant®;
  - DM Citalopram.HCl, from Cerilliant®;
  - DM Venlafaxine.HCl, from Cerilliant®;
  - Duloxetine.HCL, from Cerilliant®;
  - Fluoxetine.HCl, from Cerilliant®;

- Fluvoxamine, from Cerilliant®;
- Melitracene.HCl, from Lipomed®;
- Mianserine.HCl, from Cerilliant®;
- Mirtazapine, from Cerilliant®;
- DM Tramadol.HCl, from Cerilliant®;
- Norfluoxetine, from Cerilliant®;
- Paroxetine, from Cerilliant®;
- Reboxetine, from Cerilliant®;
- Sertraline.HCl, from Cerilliant®;
- Tramadol.HCl, from Cerilliant®;
- Trazodone.HCl, from Cerilliant®;
- Venlafaxine.HCl, from Cerilliant®.

- Internal Standard:
  - Mirtazapine-d_4, powder from Alsachim ®;
  - Paroxetine-d_6, solution in methanol from Cerilliant ®;
  - Reboxetine-d_6, powder from SantaCruz Biotech ®;
  - Trimipramine-d_3, solution in methanol from Cerilliant ®;
  - Venlafaxine-d_6, solution in methanol from Cerilliant ®.

4.4. Reagents

- Acetonitrile (LC Grade, Biosolve) - ACN;
- Dichloromethane (Sigma ≥ 99.9%);
- Formic Acid (LC Grade, Sigma Aldrich) - FA;
- Methanol (LC Grade, Biosolve) – MeOH;
- Water (LC Grade, Veolia Water)

4.5. Method Development

The development of a LC-MS method requires the optimization of several parameters. This optimization refers to an adjustment of instrumental parameters in order to optimize performance characteristics of each compound.

To set the conditions, an infusion of every standard solution was performed with a syringe pump with a concentration of 1 µg/mL in 50:50 mobile phase A and B for all analytes. Each solution was injected one by one with a flow rate of 5 µL/min.

With this procedure, the conditions to the ESI source to apply to every substance, including the ideal collision energy (CE) for the fragmentation were optimized.

4.5.1. Instrumental Conditions

4.5.1.1. Liquid Chromatography

The separation in the chromatography was achieved with Acquity® UPLC BEH C18 1.7 µm 2.1 x 100mm Column (from Waters).

A gradient was developed (Table 4.1.) with a flow rate of 0.4 mL/min and 7.5 min of running time for each sample to have an efficient separation, with the description of mobile phase A and B.

The volume that was injected for each sample was 5 µL.

Table 4.1. Gradient used in this method

	Mobile phase (%v/v)	
Time (min)	**% A Water + 0.1% Formic Acid + 2 mM Ammonium Acid**	**% B Methanol + 0.1% Formic Acid + 2 mM Ammonium Acid**
2	75	25
5	20	80
6	20	80
6.5	10	90
7	10	90
7.5	75	25

4.5.2. Mass Spectrometry

The mass spectrometer is equipped with an ESI source, which was operated in positive ion mode. All values of the source dependent parameters were optimized and described in table 4.2.:

Table 4.2. Values of the ESI source

Parameter	Value
Voltages	
- Capillary (kV)	3
- Cone (V)	35
- Extractor (V)	1
- RF Lens (V)	1.0
Temperatures	
- Source Temperature (ºC)	120
- Desolvation Temperature (ºC)	400

All values of the analyzer are described in table 4.3:

Table 4.3. Values for the analyzer

Parameter	Value
LM Resolution 1	14
HM Resolution 1	14.1
Ion Energy	0.3
Collision	10
LM2 Resolution 2	14
HM Resolution 2	14.4
Ion energy 2	1.8
Gain	1
Collision Gas Flow (mL/min)	0.15

To monitor the precursor and product ions of each analyte, the mass spectrometer was operated in MRM mode. To establish the appropriate MRM conditions for the individual compounds, the different compounds, in mobile phase (50:50 A:B, vol/vol), were infused into the mass spectrometer and the cone voltage was optimized to maximize the intensity of the protonated molecular species [M+H]+. Collision-induced dissociation of each protonated molecule was performed. The collision energy was adjusted to optimize the signal for the most abundant product ions, which were subsequently used for MRM analysis. The transitions monitored are given in table 4.4. Every compound has two product ions to increase specificity.

Tabel 4.4. Transitions monitored, with collision energies and cone voltages.

Compound	Transitions (m/z)		Collision Energy (V)	Cone Voltage
	Precursor Ion	Product Ion		
Citalopram	325.2	109	30	30
		262.1	20	30
DM Citalopram	311.2	109.1	30	30
		262.2	25	30
DM Fluoxetine	296.0	134.1	5	15
DM Tramadol	250.1	44.0	20	30
DM Venlafaxine	264.2	107.1	20	30
		121.0	20	30
		246.1	10	37
Duloxetine	298.1	43.9	5	10
		154.1	5	10
Fluoxetine	310.2	44.3	12	20
		148.1	10	20
Fluvoxamine	319.2	71.0	15	25
		87.2	15	30
Melitracene	292.2	84.05	50	15
		247.20	50	36
Mianserine	265.3	117.8	35	39
		208.0	25	39
Mirtazapine	266.1	72.2	25	35
		195.4	25	40
Paroxetine	330.1	70.2	28	40
		192.2	20	40
Reboxetine	314.1	131.2	20	22
		176.1	5	21
Sertraline	306.1	159.0	30	20
		275.0	15	20
Tramadol	264.2	58.2	5	30
		121.0	40	30
Trazodone	373.7	176.05	15	30
Venlafaxine	278.1	58	10	25
		121.02	15	25
Trimipramine-d_3	298.0	103.0	30	35
Mirtazapine-d_4	270.1	72.2	35	30
Paroxetine-d_5	336.1	76.2	29	29
Reboxetine-d_6	319.2	176.1	20	22
Venlafaxine-d_6	284.2	266.2	25	28

4.6. Extraction Procedure

4.6.1. Protein Precipitation

First, a solution with all the analytes (called ntads) was prepared. The concentrations and volumes of each compound to make the ntads solutions are given in table 4.5. Citalopram and DM venlafaxine have a different initial concentration from the others and trazodone has the highest final concentration because of its expected range.

Table 4.5. Volumes and final concentrations of compounds in the stock solution used for the calibrators

	Inicial Concentration (µg/mL)	Inicial Volume (µL)	Final Concentration (µg/mL)	Final Volume (µL)
Citalopram	100	200	7.824	
DM Citalopram	1000	104	9.957	
DM Fluoxetine	1000	104	9.957	
DM Tramadol	1000	104	9.957	
DM Venlafaxine	100	200	7.824	
Duloxetine	1000	104	9.957	
Fluoxetine	1000	104	9.957	
Fluvoxamine	1000	104	9.957	1406
Melitracene	1000	104	9.957	
Mianserine	1000	104	9.957	
Mirtazapine	1000	104	9.957	
Paroxetine	1000	104	9.957	
Reboxetine	1000	104	9.957	
Sertraline	1000	104	9.957	
Tramadol	1000	105	9.957	
Trazodone	1000	150	42.674	
Venlafaxine	1000	104	9.957	

The calibration points – 100%, 80%, 60%, 40%, 20%, 10%, 5%, 2% and 1% were made in serum. The volume information of what was added are in table 4.6.

Table 4.6. Calibrators' concentration, volume of stock solution used and volume of serum and water added to achieve the final volume of 1 mL.

Calibrator	Concentration (ng/mL)			Volume of the stock solution (µL)	Volume of Serum (µL)	Volume of water (µL)
	Most of the compounds	Citalopram and DM venlafaxine	Trazodone			
100%	996	782	4267	100	800	100
80%	797	626	3414	80		120
60%	597	469	2560	60		140
40%	398	313	1707	40		160
30%	299	265	1280	30		170
20%	199	156	853	20		180
10%	100	78	427	10		190
5%	50	39	213	5		195
2%	20	16	85	2		198
1%	10	8	43	1		199

After preparing the calibration points, 50 µL of each one of them were added to 50 µL of water and 200 µL of precipitation fluid (containing 20 ng/mL of Trimipramine in MeOH) in an Eppendorf cup. The samples were mixed by vortex and by continuous agitation for 5 minutes at 1000rpm in the thermomixer. To help the proteins to aggregate, they were centrifuged at 14,000×*g* for 5 minutes. 50 µL of each supernatant were collected to a vial and 50 µL of water were added (to make a dilution 1:2).

The previous sample preparation was not satisfactory, so a second sample preparation was designed – serum samples (250 µL) were precipitated with methanol and acetonitrile containing 5 ng of the internal standards: mirtazapine-d_4, paroxetine-d_5, reboxetine-d_6 and venlafaxine-d_6. The supernatant was evaporated at 56ºC under a flow of nitrogen and reconstituted in 100 µL of 50:50 methanol:water with 2 mM ammonium acetate. Five µL of each vial was injected.

4.7. Analytical Method Validation

Validation is required for any new method; for a particular type of material and a particular operating range of concentrations, the method must be able to solve a particular analytical problem [78]. The following steps ensure that the minimum

standards of practice for validating analytical methods in forensic toxicology have been performed.

4.7.1. Selectivity

Selectivity is the ability of the bioanalytical method to measure unequivocally and to differentiate the analytes in the presence of components, which may be expected to be present [77]. In other words, is the ability to determine the analyte in presence of other compounds [68].

To evaluate selectivity, 10 different sources of each matrix without IS, 1 blank sample with IS and 1 fortified sample with high analyte concentration and without IS were selected, along with samples containing potentially interfering compounds or metabolites but no analyte. Normally, absence of interfering components is accepted where the response is less than 20% of the lower limit of quantification for the analysis and 5% for the internal standard [73].

4.7.2. Calibration Model (Linearity)

The choice of an appropriate calibration model is necessary for reliable quantification. Therefore, the relationship between the concentration of analyte in the sample and the corresponding response must be investigated [64]. It is the ability of the method to obtain test results which are proportional to the concentration in the sample [68]. It is necessary to use a sufficient number of calibrators to define adequately the relationship between concentration and response [79]. The Scientific Working Group for Forensic Toxicology recommends 6 calibrators at 6 concentration levels, with 5 replicates each [80]. At least, a minimum of five concentrations is recommended [67].

It is necessary to determinate the range of analyte concentrations over which the method shall be used, sometimes called the working range. Within this range, there will be a correlation between signal response and analyte concentration in the sample [80].

The choice of an appropriate calibration model (linear, quadratic…) is necessary for accurate and reliable quantitative results. The most often used calibration model is the simple linear regression model using least squares method [80]. This model represented the relationship between two variables by a straight line, mathematically

expressed by the equation 4.1, where **y** is the instrumental response and **x** is the concentration of the compound [83].

$$y = b0 + b_1.x \qquad \text{Eq. [4.1]}$$

Where, **y** (response) and **x** (concentration) are independent and dependent variable, **b** and **m** are the calibration parameters, the y-intercept and the slope, respectively.

Although it has become widespread practice, it is emphasized that a calibration model cannot be evaluated simply via its correlation coefficient (R) and coefficient of determination (R^2) [80], that should be above 0.99 in both cases [90]. So, statistic provides a fundamental tool in the qualitative and quantitative analysis of a specific analyte. Statistic distribution, like Grubbs, Fisher or Mandel tests are related with testing hypothesis, since they can provide a basis for comparison between the test values (TV) and the critical values.

4.7.2.1. Grubbs Test

The initial step of studying the calibration function is to check for outliers [64]. This can be checked by appropriate statistical procedures like the Grubbs test and eliminated if found to be significant.

Grubbs test is defined by the hypothesis:

- H_0 = There is no outliers in the data set;
- H_1 = There is outliers in the data set.

There are two sides of the test: the test whether checks if the minimum value is an outlier, or the maximum value is an outlier, following this two equations (Eq. 4.2 and 4.3):

$$G\ min = \frac{Ymin - Ymean}{s} \qquad \text{Eq. 4.2}$$

$$G\ max = \frac{Ymax - Ymean}{s} \qquad \text{Eq. 4.3}$$

So, Grubbs' test can be used to answer the following questions: is the maximum value an outlier? Or the minimum value is an outlier? For the two-sided test, the hypothesis of no outliers is rejected if (Eq. 4.4):

$$G > \frac{(N-1)}{\sqrt{N}} \sqrt{\frac{(\frac{t\,\alpha}{(2N),N-2})^2}{N-2+(\frac{t\,\alpha}{(2N),N-2})^2}} \qquad \text{Eq. 4.4}$$

With tα/(2N),N−2 denoting the critical value of the t-distribution with (N-2) degrees of freedom and a significance level of α/(2N).

The critical value for α=0.05 is 1.71. If any value from G_{min} and G_{max} is superior, it will be considered an outlier and H_1 is accepted. More than two outliers in a complete data-set of a calibration model indicate problems with the method.

4.7.2.2. Fisher Test

The next step is to check for homogeneity of variances (homoscedasticity). Again, this can first be done visually using residuals plots. The plots of residuals versus concentration appear to show residuals randomly distributed around the x-axis. In fact, the variances tend to increase as the concentration increases, which usually points to the hypothesis of others models of calibration beyond the simple linear regression. In such cases, homoscedasticity should be tested by an appropriate statistical procedure.

To compare de variances of a normal distribution, the Fisher test should be used. This test follows the equation 4.5:

$$TV = \frac{\sigma_1^2}{\sigma_2^2} \qquad \text{Eq. 4.5}$$

Where σ_1^2 stands for the standard deviation at the highest calibrator and σ_2^2 stands for the standard deviation at the lowest calibrator. Then, it is compared the value with the tabulated value (pH0) of the F distribution at a confidence level of 95% and 5 degrees of freedom, given by the equation 4.6:

$$pH0 = fdist\,(TV; 5; 5) \qquad \text{Eq. 4.6}$$

The criteria of these results are:

- H_o = $TV \leq pH0$ – the difference in variances is not statistically significant and thus, there is homoscedasticity, so the ordinary least squares regression models are applicable.
- H_1 = $TV > pH0$ – scatter of the replicates increases with concentration indicating inhomogeneous variances (heteroscedasticity) over the calibration range, so weighted least squares regression models are applicable.

The evaluation of the behavior of variance is very important for the choice of the correct regression model.

4.7.2.3. Mandel Test

The test is based on the assumption that relatively large deviations of measured values from a straight line are caused by nonlinearity and may be reduced through the selection of a "better" regression model, in this case, a quadratic model. The first step is to adjust a first degree polynomial (P1) and a second degree polynomial (P2) and calculate for each the sum of squares. Six calibrators and the mean of response were used to calculate the variance (Var) of linear correlation and quadratic correlation (eq. 4.7 and 4.8):

$$Var_{Linear} = \frac{\sum(y_{observed} - y_{predicted})^2}{N-2} \quad \text{Eq. 4.7}$$

$$Var_{Quadratic} = \frac{\sum(y_{observed} - y_{predicted})^2}{N-3} \quad \text{Eq. 4.8}$$

N is the number of calibration standards used to construct the curve. From this, it is possible to calculate the fit standard error with equation 4.9:

$$Stdfit = \sqrt{Var_{Linear}} \text{ or } Stdfit = \sqrt{Var_{Quadratic}} \quad \text{Eq. 4.9}$$

From this, is possible to calculate the significance of this variance (F_{cal}) through the equation 4.10:

$$F_{cal} = \frac{\Delta\sigma^2}{\sigma_{pe}^2} = \frac{(\frac{\Delta SS}{\sigma v})}{\sigma_{pe}^2} \quad \text{Eq. 4.10}$$

Then, F_{crit} ($F_{0.01}$ (σv;Δpe)) is calculated, where σv represents the difference between degrees of freedom (in this case, one). If F_{crit} presents a good

possibility of both models have similar performance (for example, more than 50% chances), one should choose P1. If it presents a very low value, P2 should be chosen.

4.7.2.4. Weighted Least Squares

The constant variance over the whole range is not always observed. Large deviations at larger concentrations tend to influence (weight) the regression line. Thus, if the data are heteroscedastic ($F_{cal} > F_{crit}$), the use of weighted least squares (WLS) is the simplest and the most effective way to harmonize the differences of variances of the line points [83].

For this study, calibration curves for all the analytes were prepared with six calibrators each - 100%, 60%, 30%, 20%, 5%, 1%, with 5 ng of IS. This procedure was repeated in three different days.

Since there are evidences of the heteroscedastic, the following step should be the choice of the weighting factores (w_i). As it is not suitable to calculate the inverse of variance in laboratory routine, taking the account the fact that it requires several determinations for each calibration point and a fresh calibration line each time the method is used, so other empirical weights should be studied: $\frac{1}{x}$, $\frac{1}{x^2}$, $\frac{1}{y}$ and $\frac{1}{y^2}$ [83].

The best weighting factor is chosen according to a percentage relative erros (%RE), which compares the regressed concentration (C_{found}) computed from the regression equation obtained for each w_i, with the nominal standard concentration, C_{nom} (eq. 4.11).

$$\%RE = \frac{C_{found} - C_{nom}}{C_{nom}} \times 100 \qquad \text{Eq. 4.11}$$

Plots of %RE versus concentration were performed for the analytes in order to choose the best weighing factor, along with the sum of %RE ($\sum \%RE$) The w_i more adequate will be the one which gives rise to a slight horizontal band of randomly distributed %RE around the x-axis and presents the smallest value of across the whole concentration range [83].

In the simple linear regression model, the relationship between variables is established by a straight line, mathematically expressed by the equation (4.1). But,

since it is used a WLSLR, the model parameters (*b1* and *b0*) of the weighted straight line equation need to be estimated using the term according to the following equations (eq. 4.12 and 4.13), before being calculate the $C_{calculated}$ [83].

$$b_1 = \frac{\sum w_i \times \sum w_i x_i y_i - \sum w_i x_i \times \sum w_i y_i}{\sum w_i \times \sum w_i x_i^2 \times (\sum w_i y_i)^2} \quad \text{Eq. 4.12}$$

$$b_0 = \frac{\sum w_i x_i^2 \times \sum w_i y_i - \sum w_i x_i \times \sum w_i x_i y_i}{\sum w_i \times \sum w_i x_i^2 \times (\sum w_i y_i)^2} \quad \text{Eq. 4.13}$$

Where x_i and y_i is the i[th] data pair of n total data pairs and wi is the weighting factor chose. Finally, the correlation coefficient (R) of the weighted straight line equation can be obtained with equation 4.14.

$$R = \frac{\sum w_i \times \sum w_i x_i y_i - \sum w_i x_i \times \sum w_i y_i}{\sqrt{[\sum w_i \times \sum w_i x_i^2 - (\sum w_i x_i)^2] \cdot [\sum w_i \times \sum w_i y_i^2 - (\sum w_i y_i)^2]}} \quad \text{Eq. 4.14}$$

4.7.3. Precision

Precision is the degree of agreement among individual test results when an analytical method is used repeatedly to multiple samplings of a homogeneous sample, under the prescribed conditions [64], [67], [68]. Precision may be considered in three levels: repeatability, intermediate precision and reproducibility.

Precision can be expressed as coefficient of variation (CV), variance (S^2), standard deviation (S) [75]. In this project, it will be expressed as coefficient of variation.

Repeatability is a measure of the variability in results when a measurement is performed by a single analyst using the same equipment over a short timescale. Intermediate precision expresses within laboratories variations (different analysts, different equipment, etc) [64], [70]. Reproducibility is a measure of the variability in results between laboratories. It has to be studied only if a method is supposed to be used in different laboratories [64]. In this project, the reproducibility of the method was not studied.

The study of intermediate precision and the repeatability consisted of analyzing a test sample in several different runs. Within each run, the sample was analyzed 5 times under repeatability conditions [64], [70], [91]. Three pools of fortified matrix samples at the concentrations 50 ng/mL, 400 ng/mL and 1000 ng/mL were prepared. Each concentration pool of fortified samples was analyzed in triplicate on five separate days along with a freshly prepared calibration curve (6 calibrators). The precision is first calculated by determining the mean for each concentration. From these values, it was calculated the within-run and between-run using the one-way ANOVA approach, expressed in terms of % CV, through the following equations (eq. 4.15 and 4.16) [80]:

$$Within-run\ CV\ (\%) = \frac{\sqrt{MS_{wg}}}{grand\ mean\ for\ each\ concentration} \times 100 \quad \text{Eq. 4.15}$$

$$Between-Run\ CV\ (\%) = \frac{\sqrt{\frac{MS_{bg}+(n-1)\times MS_{wg}}{n}}}{grand\ mean\ for\ each\ concentration} \times 100 \quad \text{Eq. 4.16}$$

The % CV shall not exceed 20% at each concentration [80].

4.7.3.1. Cochran Test

One of the assumptions for the usage of ANOVA is the homogeneity of variances. Cochran test is used in this case, so one can decide if a single estimate of variance is significantly larger than a group of variances. This test follows the equation (eq 4.17), where C detects one exceptionally large variance value.

$$C_{cal} = \frac{s_{max}^2}{\sum_{i=1}^{n} s_i^2} \quad \text{Eq. 4.17}$$

Where s_{max}^2 is the maximum variance and $\sum_{i=1}^{n} s_i^2$ is the sum of all variances. The value of C_{cal} is compared with tabled value, C_{crit}, (α=0.01) for the Cochran test, where the number of replicas is 3 and the number of runs is 5. The criteria used for these results are:

- If $C \leq C_{crit}$, there is homogeneity of variances and thus, ANOVA can be used;

- If C > C_{crit}, the differences between the variances are statistically significant, and therefore, ANOVA can't be used.

4.8.4. Accuracy (bias)

Accuracy is the difference between the expectation of the test results and an accepted reference value [92]. It can be measured as a percentage deviation from the accepted reference value [64].

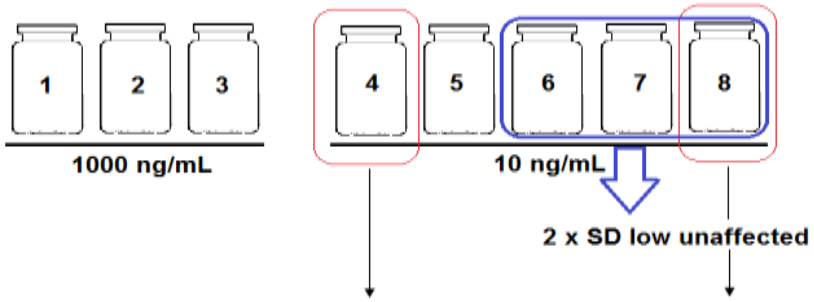
A practical determination of bias relies on comparison of the mean of the results from the candidate method with a suitable reference value [70]. It shall be measured in pooled fortified matrix samples using a minimum of three separate samples per concentration at three different concentration pools (low, medium, and high) over five different runs. The following equation should be used (eq. 4.18) [80]:

$$Bias\ (\%) at\ Concentration = \frac{Grand\ mean\ of\ calculated\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100 \quad \text{Eq. 4.18}$$

The maximum acceptable bias is 20% at each concentration [73], [74], [80].

4.7.5. Carry-Over

Carryover is the contamination of a sample by the analyte of interest coming from a previous sample injection. The autosampler is often assumed to be the main source of carryover due to the high concentration of analyte exposed to a large variety of surface materials and large number of interrupted flow paths [93]. To evaluate carryover as part of the method validation, it is recommended that following an injection of a high concentration sample, it should be injected the lowest calibrator. Eight samples were prepared: three with 1000 ng/mL and five with 10 ng/mL (Fig 4.1) and concentration analyzed with equation 4.19.



$$\Delta low = Concentrantion\ from\ 4 - Concentration\ from\ 8 \quad \text{Eq. 4.19}$$

Fig.4.1. Illustration of how carry-over was studied. This batch was repeated three times.

Then, the Δlow is calculated and if it exceeds 2 × SD low unaffected, the carry-over is present. This concentration shall be confirmed using triplicate analyses [80].

4.7.6. Matrix Effects

Matrix effect is the effect on an analytical method caused by all other components of the sample except the specific compound to be quantified [94]. Matrix effects result from co-eluting matrix components that affect the ionization of the target analyte, resulting either in ion suppression, or, in some cases, ion enhancement. Matrix effects can be highly variable and can be difficult to control or predict [95]. It is a well-known phenomenon in LC-MS/MS analysis, mainly depending on the sample matrix, sample preparation procedure, quality of chromatography separation, mobile-phase additives and ionization type [64], [86].

There are two common methods to assess matrix effects: the post-column infusion method and the post-extraction spike method [95]. In this project, post-extraction was used. The post-extraction spike method quantitatively assesses matrix effects by comparing the response of an analyte in neat solution to the response of the analyte spiked into a blank matrix sample that has been carried through the sample preparation process [95].

In this case, three different sets of samples are prepared: one, consisting on neat standards (set A), one prepared in blank matrix extracts from different sources and spiked before extraction (set B) and one prepared in blank matrix from the same sources but spiked after extraction (set C).

From these data, one can calculate the ME (ion suppression/enhancement) RE (extraction efficiency) and PE (process efficiency), according to the equations 4.20, 4.21 and 4.22. Two ionization suppression or enhancement percentages will be established – one at low concentration (50 ng/mL) and one at the high concentration (500 ng/mL).

$$ME\ (\%) = \left(\frac{Peak\ Area\ in\ sample\ from\ set\ C}{Peak\ Area\ in\ sample\ from\ set\ A}\right) \times 100 \quad \text{Eq. 4.20}$$

$$RE\ (\%) = \left(\frac{Peak\ Area\ in\ sample\ from\ set\ B}{Peak\ Area\ in\ sample\ from\ set\ C}\right) \times 100 \quad \text{Eq. 4.21}$$

$$PE\ (\%) = \left(\frac{Peak\ Area\ in\ sample\ from\ set\ B}{Peak\ Area\ in\ sample\ from\ set\ A}\right) \times 100 \quad \text{Eq. 4.22}$$

4.7.7. Limits

4.7.7.1. Limit of Detection (LOD)

The LOD must be differentiated from the LOQ. LOD is the smallest concentration that can be distinguished from the noise level. The LOQ should be at least twice the response of the LOD. The LOQ should serve as the lowest concentration on the calibration curve [79].

Five calibrations curves are constructed across the working range of the analytical method over different runs. The LOD can be estimated from the standard error of the y intercept ($s_{(b0)}$) and the average slope (Avg_{b1}) with equation 4.23 [80]:

$$LOD = \frac{3.3\ s(b0)}{\mathrm{Avg}_{\mathrm{b1}}} \quad \text{Eq. 4.23}$$

4.7.7.2. Limit of quantification (LOQ)

Limit of quantification is the lowest sample concentration that can be quantified with suitable accuracy and precision [64], [72], [96], [97]. There are a number of different approaches for determining a method's LOQ. A method's LOQ incorporates instrumental performance, as well as the sample matrix and inherent procedural limitations [80].

In this project, LOQ was determined with three quality control samples at 10, 20 and 50 ng/mL are analyzed in duplicate (two separate samples) over a minimum of five runs. The concentrations used for this approach must remain within the previously established calibration curve.

The lowest concentration that was capable of reproducibly providing symmetrical peaks and the minimum mass spectral identification ratios, while maintaining a bias of ±20% and a % CV of <20%, is considered the estimated LOQ [80].

4.7.8. Stability

Stability is the absence of an influence of time on the concentration of the analyte in a sample [72]. It is the chemical stability of an analyte in a given matrix under specific conditions for given time intervals [47]. Stability of an analyte during the whole analytical procedure is a prerequisite for reliable quantification [64].

Analyte stability may be affected by a number of variables, including storage conditions and sample processing. All stability determinations shall include a set of samples prepared from reference materials. The reference materials are used to prepare fortified samples of the analyte(s) at both low and high concentrations (20 and 800 ng/mL) in each matrix that will be analyzed in the method.

4.7.8.1. Processed samples

Sometimes, it may be necessary to run the samples the following day or later. In these instances, it is important to evaluate the length of time a processed sample

can be maintained before it undergoes unacceptable changes, preventing reliable analyte detection, identification and quantification. Typically processed fortified samples are combined per concentration and then divided into different autosampler vials. The first vials of each concentration are immediately analyzed in triplicate to establish the time zero responses (T0). All remaining vials are stored in a manner that they would typically be stored during routine analysis (in our case, the autosampler). The remaining vials are then analyzed in triplicate at different times intervals. Average responses at each time interval are compared to the time zero responses. The processed samples in different autosampler vials are analyzed repeatedly up to 72hours. [80].

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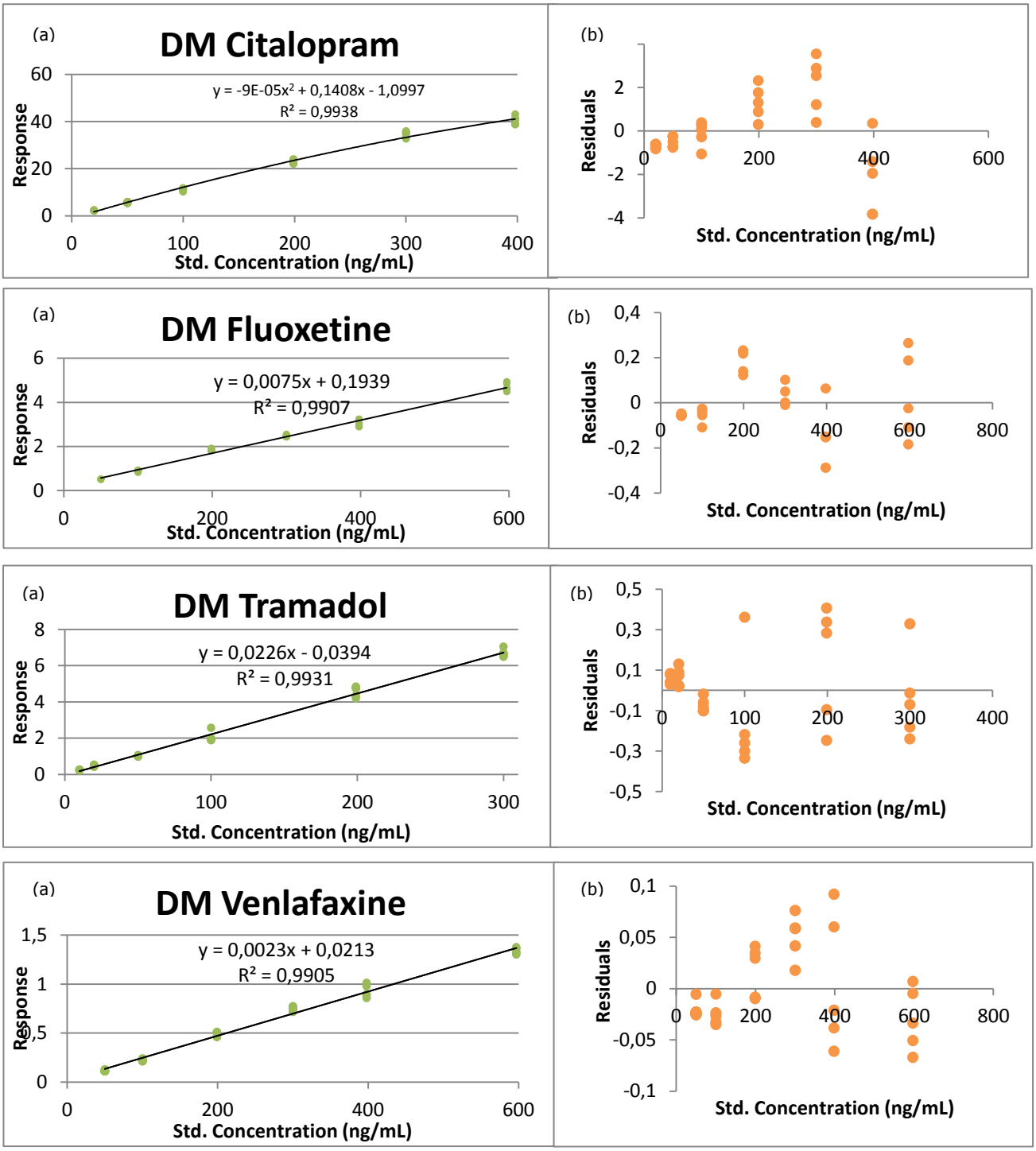
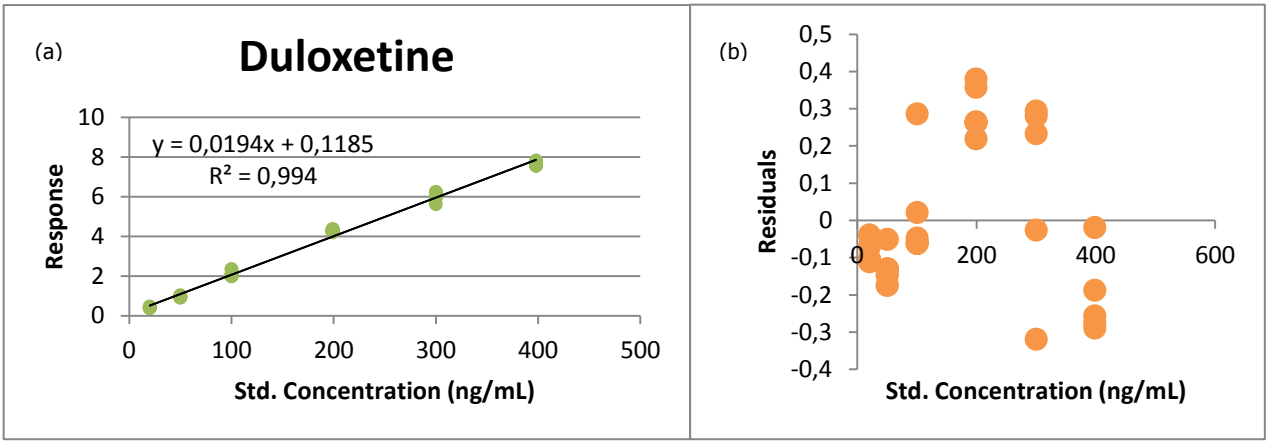
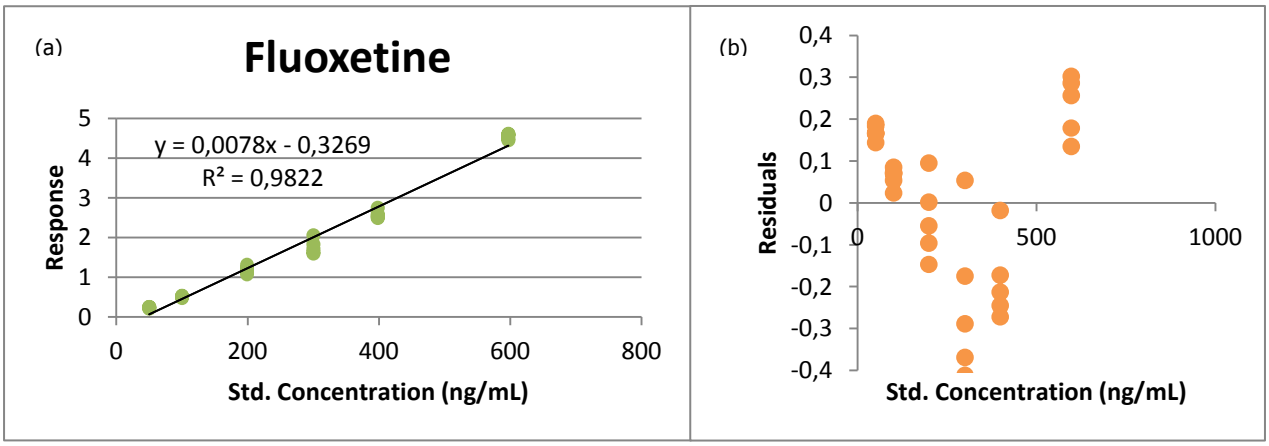
6. APPENDIX

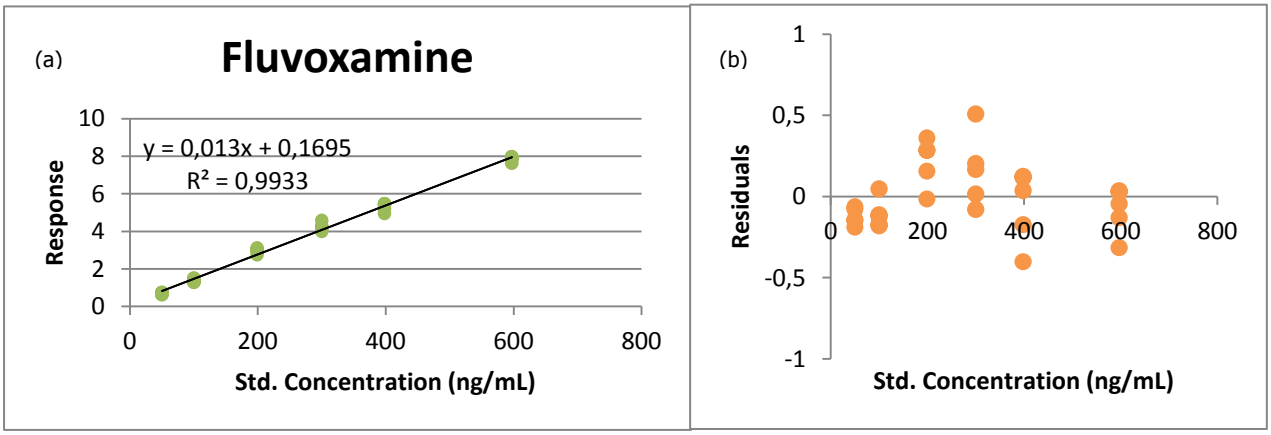
Appendix 6.1: Calibration Model

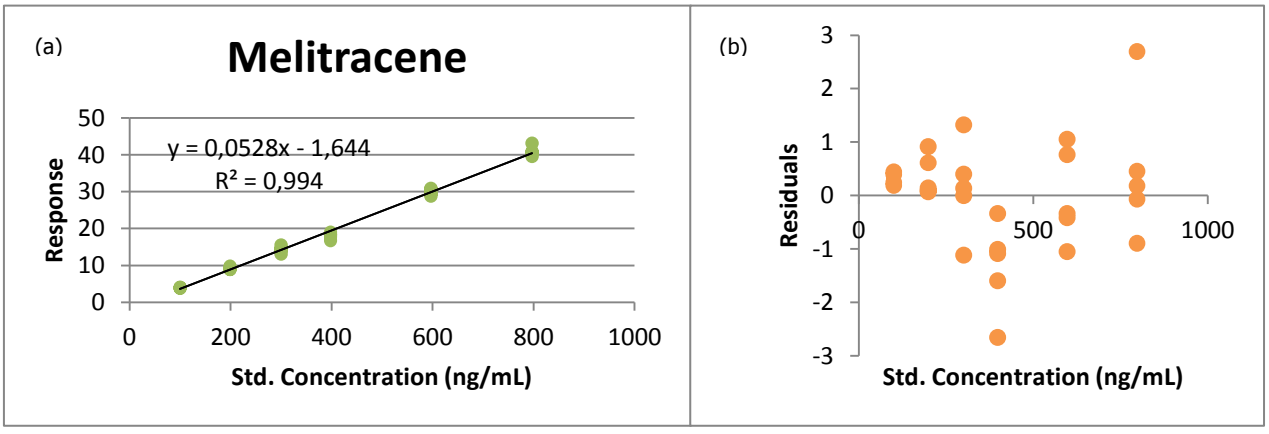
Calibration curves (a) and residuals plots (b) for desmethylcitalopram, desmethylfluoxetine, desmethyltramadol, desmethylvenlafaxine, duloxetine, fluoxetine, fluvoxamine, melitracene, mianserin, mirtazapine, reboxetine, sertraline, trazodone and venlafaxine.

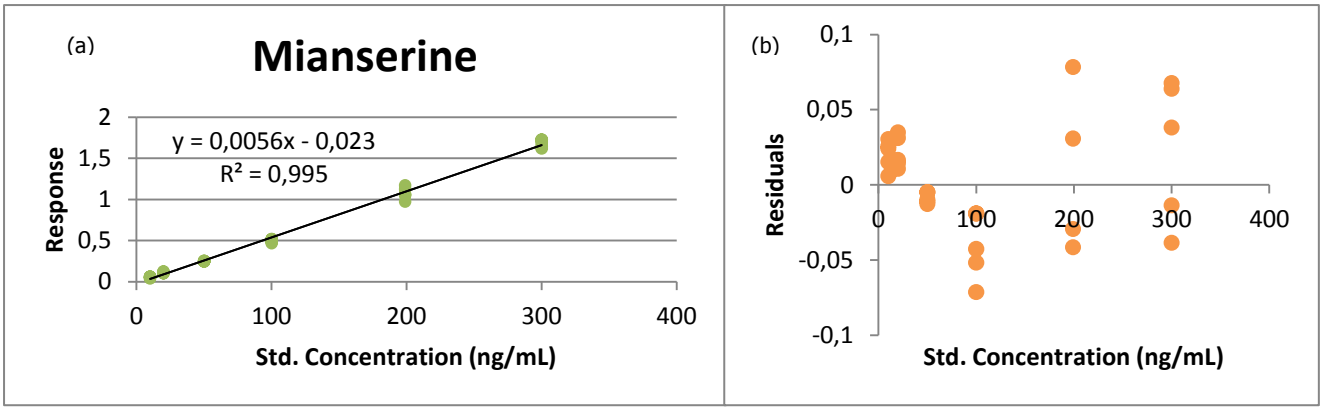


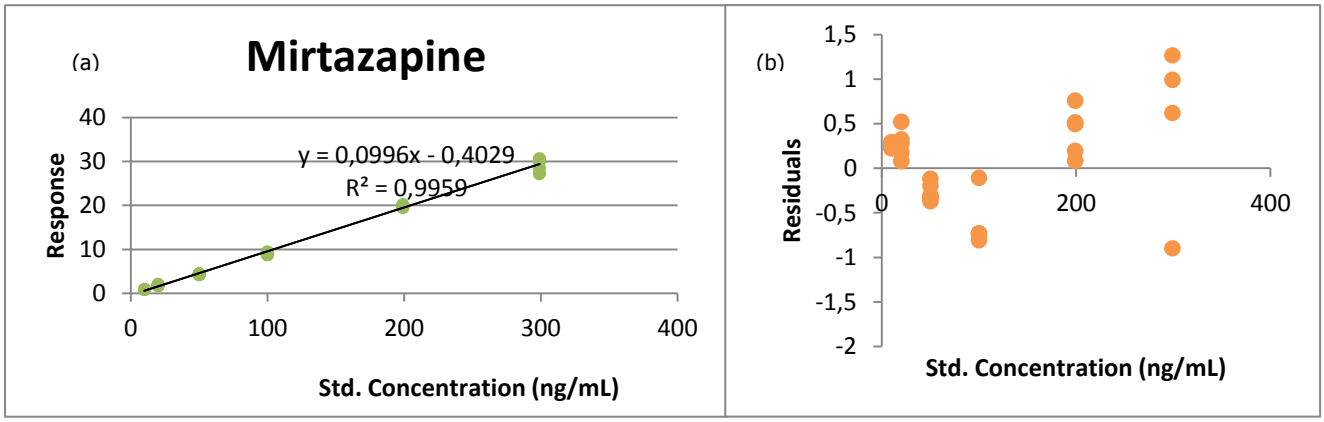

(a)
Duloxetine
y = 0,0194x + 0,1185
R² = 0,994
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)

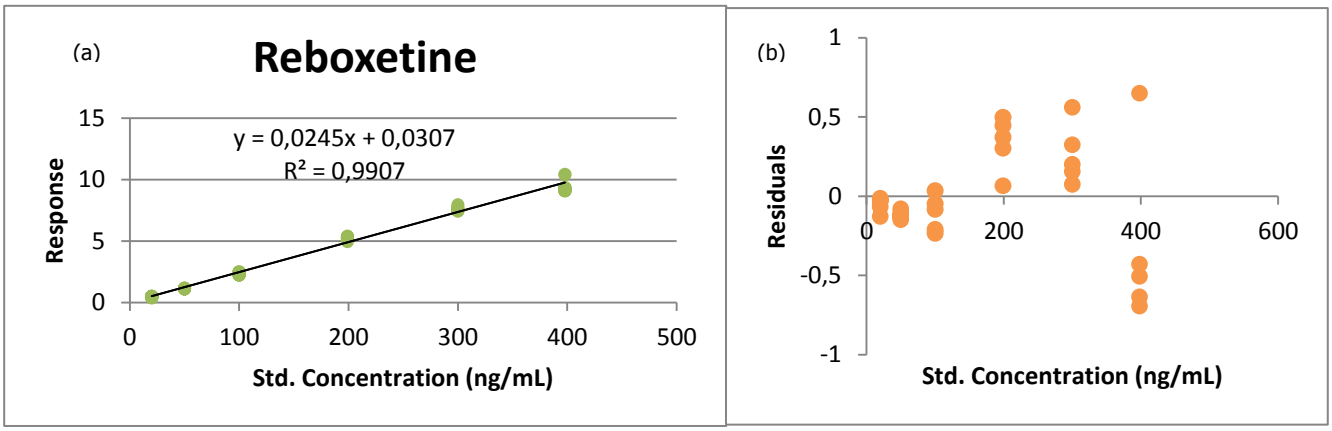

(a)
Fluoxetine
y = 0,0078x - 0,3269
R² = 0,9822
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)

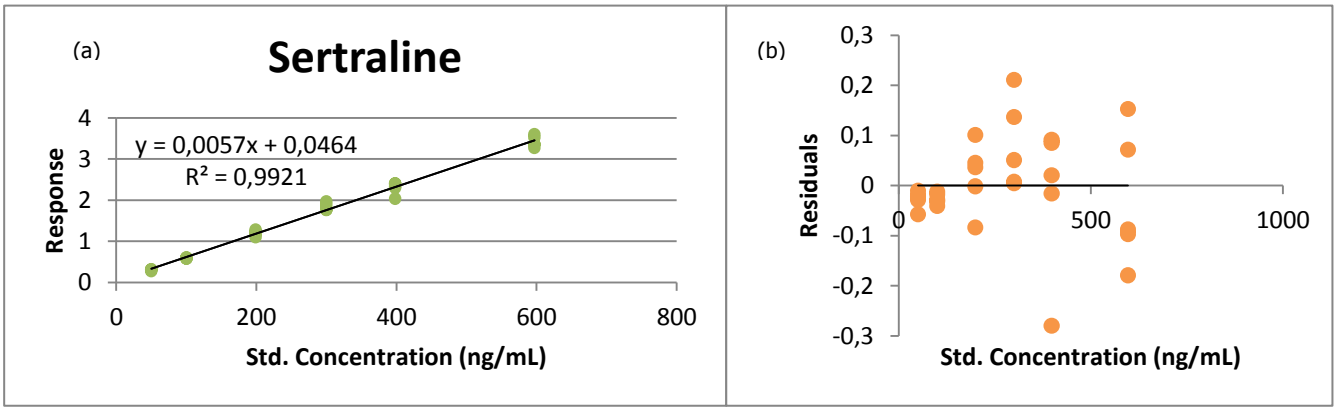

(a)
Fluvoxamine
y = 0,013x + 0,1695
R² = 0,9933
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)

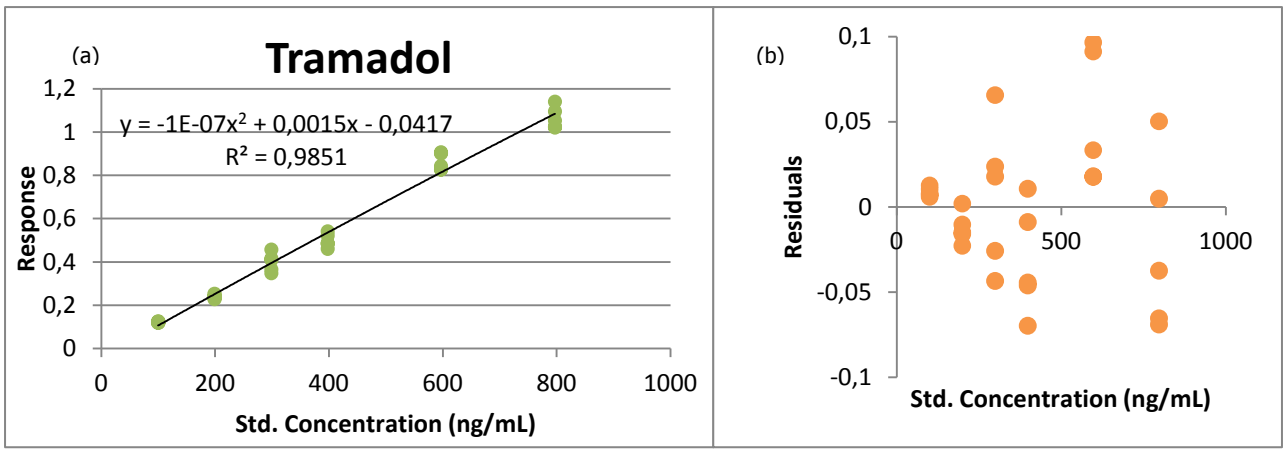

(a)
Melitracene
y = 0,0528x - 1,644
R² = 0,994
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)

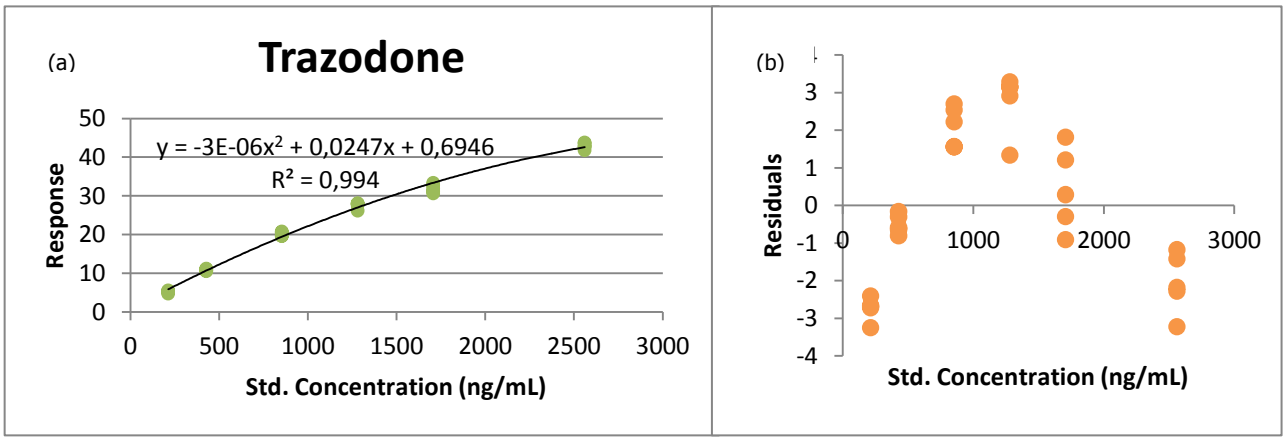

(a)
Mianserine
y = 0,0056x - 0,023
R² = 0,995
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)

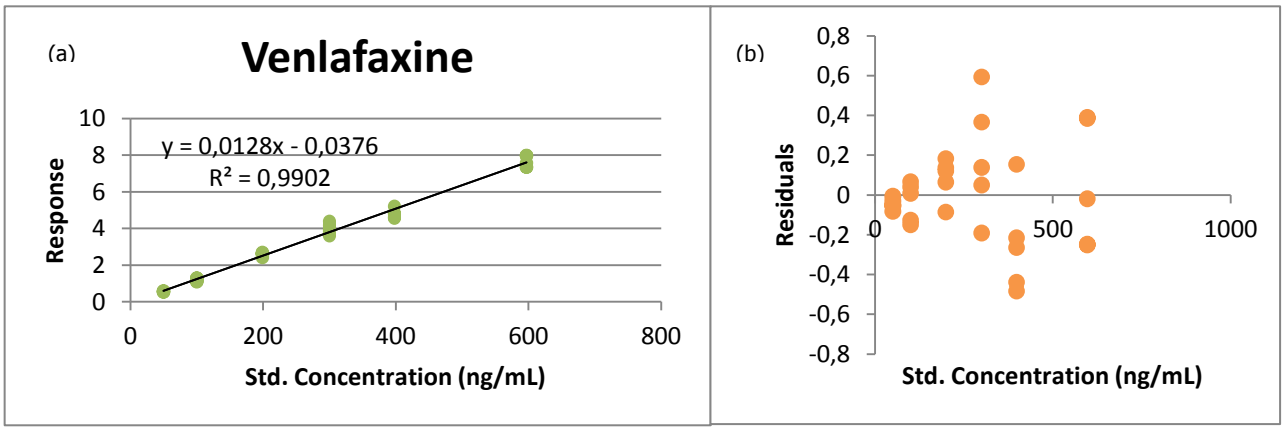

(a)
Mirtazapine
y = 0,0996x - 0,4029
R² = 0,9959
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)


(a)
Reboxetine
y = 0,0245x + 0,0307
R² = 0,9907
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)


(a)
Sertraline
y = 0,0057x + 0,0464
R² = 0,9921
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)


(a)
Tramadol
y = -1E-07x² + 0,0015x - 0,0417
R² = 0,9851
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)


(a)
Trazodone
y = -3E-06x² + 0,0247x + 0,6946
R² = 0,994
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)


(a)
Venlafaxine
y = 0,0128x - 0,0376
R² = 0,9902
Response
Std. Concentration (ng/mL)
(b)
Residuals
Std. Concentration (ng/mL)

Appendix 6.2: Data of the study of weighted least squares linear regression

DM Citalopram

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	23.6	25.9	25.9	23.9
597	20.9	18.6	18.6	22.3
300	36.1	47	47	39.5
199	19.3	7.1	7.1	14.8
50	24.7	23.8	23.8	24.3
10	35.3	33.8	33.8	38.8
$\sum \%RE$	159.9	**156.2**	**156.2**	163.6

Duloxetine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	20.1	44.1	18.4	35.8
597	21.6	6	23.4	9.7
300	10	27.3	9.3	18.8
199	30.6	10.1	31.9	15.8
50	50.3	35.9	49.2	45.5
10	44.7	39.3	52.4	43.1
$\sum \%RE$	177.3	**162.7**	184.6	168.7

DM Fluoxetine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	26.9	66.6	23.6	50
597	15.9	29.5	17.5	14.9
300	14.9	32.6	15.2	14.6
199	60.9	16.9	63.4	37.4
50	93.9	66	90.5	87.4
10	56.7	63	84.9	71.2
$\sum \%RE$	**269.2**	274.6	295.1	275.5

Fluoxetine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	16	25.5	16.9	29
597	20.2	15.8	19.2	14.2
300	9.1	9.2	9.1	9.7
199	16	15.5	15.3	15.7
50	40.4	41	37.7	36.9
10	57.6	34.4	67.9	34.7
$\sum \%RE$	159.3	141.4	166.1	**140.2**

DM Tramadol

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	9.2	6.1	10.2	10
597	12.9	12.6	13	13
300	42.1	47	40.9	41.3
199	10.4	10.3	10.4	10.4
50	29	23.3	31.2	30
10	38.5	36.2	42.5	39.8
$\sum \%RE$	142.1	**135.5**	148.2	144.5

Fluvoxamine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	9.7	31.6	9.1	16.3
597	9.5	24.3	10.4	9
300	34.9	58.5	33.5	37.7
199	44.7	16.8	46.3	32.8
50	75.4	57	75.3	67.5
10	43.4	41.7	48.8	60.3
$\sum \%RE$	**217.6**	229.9	223.4	223.6

DM Venlafaxine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	19.3	29.7	21.1	30.8
597	17.9	18.4	18	20.8
300	47.1	39.7	45.3	26.1
199	22.6	17	20.3	23.7
50	24	24.5	24	31
10	65.9	28.4	79.7	4.8
$\sum \%RE$	196.8	157.7	208.4	**137.2**

Melitracene

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	22.9	28.7	17.4	28.9
597	18.2	29	26.6	34.7
300	49.7	41.7	39.8	35.1
199	19.1	11.2	20.5	11.6
50	16.1	16.3	11.9	10.1
10	60.2	22.4	55.7	21.7
$\sum \%RE$	186.2	149.3	171.9	**142.1**

Mianserine

Std. Conc..	1/x	$1/x^2$	1/y	$1/y^2$
996	8.3	16.7	16.7	16.7
597	22.8	13.6	13.6	13.6
300	27.8	35.6	35.6	35.6
199	12.6	11.1	11.1	11.1
50	17	14.1	14.1	14.1
10	47.4	49	49	49
$\sum \%RE$	**135.9**	140.1	140.1	140.1

Tramadol

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	5.9	4.2	5.9	5.9
597	13.6	13.8	17.7	17.7
299	21	24.3	22	22
199	14.7	14.6	16.4	16.4
50	30	21.1	16.4	16.4
10	39.3	32.5	32.1	32.1
$\sum \%RE$	124.5	**110.5**	**110.5**	**110.5**

Mirtazapine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	16.8	16.7	16.9	16.8
597	12.4	10.4	12.9	12.8
299	28.6	32.2	27.5	27.8
199	20.5	20.5	20.6	20.6
50	16.4	14.2	17.3	16.6
10	30.9	25.1	35.3	28.8
$\sum \%RE$	125.6	**119.1**	130.5	123.4

Trazodone

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
4267	19.3	19.3	18.9	18.2
2560	27	27	24.4	24
1280	34.3	34.3	69.9	25.9
853	12.3	12.3	30.9	37.8
213	74.4	74.4	30.3	67.7
43	33.2	33.2	35.4	21.4
$\sum \%RE$	200.5	200.5	209.8	**195**

Paroxetine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	16.4	16.3	17.3	20.2
597	15	14.9	15.9	18.7
300	51.1	51.7	49.5	45.6
199	7.9	8.4	7.3	7.4
50	16.2	15.8	18.2	18.2
10	51.1	41.1	62.7	45.9
$\sum \%RE$	157.7	**148.2**	170.9	156.0

Venlafaxine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	16.3	16.3	18.2	25.8
597	20.5	20.5	22.4	27
300	58	58	55.8	48.8
199	13	13	12	11.9
50	26.5	26.5	25.3	29.9
10	61	61	38.2	43.1
$\sum \%RE$	195.3	195.3	**171.9**	186.5

Reboxetine

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	11.4	11.4	11.8	11.8
597	9.6	8.9	10.6	10.6
300	46.4	47.8	45.1	45.1
199	8.1	7.2	8.9	8.9
50	9.8	12	9.6	9.6
10	51.8	36.2	62.3	62.3
$\sum \%RE$	137.1	**123.5**	148.3	148.3

Sertraline

Std. Conc.	1/x	$1/x^2$	1/y	$1/y^2$
996	21.2	34.7	9.7	14.9
597	13.9	32.4	16.9	25.3
300	12	30.1	15	17.2
199	36.8	20.1	28.3	27.6
50	96.3	62.9	67.8	55.8
10	56.5	44.3	62	71.7
$\sum \%RE$	236.7	224.5	**199.7**	212.5

Appendix 6.3: Imprecision and accuracy (bias)

Concentrations of the five runs made in five different days. All the samples were made in triplicate (A, B and C) at low, medium and high concentrations.

DM Citalopram

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	57.3	50.7	55.8	68.2	69.4
B	55.0	65.9	48.7	64.2	66.8
C	62.6	47.6	51.6	68.3	65.3
Medium					
A	458.3	432.3	452.5	493.9	398.1
B	471.8	412.8	432.6	504.2	402.3
C	426.8	357.4	416	488.2	416.5
High					
A	844.0	967	948.8	962.5	980.8
B	863.7	857.6	1001.6	956.8	1038.2
C	873.6	1002.7	1016.8	990.9	1131.3

	Low	Medium	High
Mean	59.8	437.5	962.4
SD	7.7	40.4	77.6
CV	13.0%	9.2%	8.1%
Final Bias	19.7%	9.9%	-3.4%

DM Fluoxetine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	49.5	61.9	43.3	58.8	67.5
B	53.9	59.1	46.4	53.4	69.5
C	52.7	52.4	42.9	57.2	67.1
Medium					
A	436.3	419.6	454.6	454.4	478.9
B	419.1	357.9	413.7	461.3	476
C	405.5	366.4	428	478.3	428.7
High					
A	870.3	1015.1	837.6	847.5	935.7
B	858.4	858.6	927.7	876.6	1010.6
C	906.9	877.6	878.7	867.7	1029.8

	Low	Medium	High
Mean	55.7	431.9	906.5
SD	8.4	37.1	63.9
CV	15.1%	8.6%	7.1%
Final Bias	11.4%	8.5%	-9.0%

DM Tramadol

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	46.5	47	42.8	47.5	54.5
B	48.6	45.7	44	49.7	55.2
C	52.9	50.2	42.3	50.4	62.7
Medium					
A	429.4	356.5	457.2	370.9	422.9
B	444.9	341	407.1	366.4	427.4
C	395.6	372.2	402.7	361.2	417.3
High					
A	924.1	1194.6	987.1	1022.2	1037.3
B	961.1	977.1	1033.2	1039.8	1218.2
C	1004.2	1069.7	1059.4	950.4	1060.5

	Low	Medium	High
Mean	49.3	398.2	1035.9
SD	5.3	35.2	81.5
CV	10.9%	8.8%	7.9%
Final Bias	-1.3%	0.0%	4.0%

DM Venlafaxine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	45.5	41.1	33	58.6	47.2
B	38.3	42.6	33.4	53.6	41.8
C	37.2	40.9	34.2	56.7	43
Medium					
A	304.3	275.9	319	396.7	307.7
B	292.5	301.4	324.5	397.3	302.2
C	302.5	307.3	305.3	420	328.6
High					
A	767.6	938	784.7	862	884.6
B	765.4	856.7	810.4	880.3	967.8
C	815.5	989.4	874	921.3	948.3

	Low	Medium	High
Mean	43.1	325.6	871.0
SD	8.0	43.0	72.2
CV	18.6%	13.2%	8.3%
Final Bias	10.6%	4.1%	11.4%

Duloxetine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	48.9	55.6	37.9	54	61.3
B	56.3	56.5	40.9	51.1	60.3
C	48.4	43.3	38.7	52.6	60.1
Medium					
A	416.7	451.5	419.5	459.4	468.3
B	391.6	391	387.1	475	446.6
C	389.8	378.2	395	491.1	420.5
High					
A	949.2	1059.6	956.8	979	1069
B	920.2	1006.6	1078	1042	1168.1
C	1001.5	1090.5	989	995.7	1180.2

	Low	Medium	High
Mean	51.1	425.4	1032.3
SD	7.8	37.1	75.9
CV	15.4%	8.7%	7.4%
Final Bias	2.1%	6.9%	3.7%

Fluoxetine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	49.4	55.2	50.9	49.4	65.7
B	51.4	59.8	48.3	47.1	60.4
C	49.8	43.9	53.4	47	61.6
Medium					
A	444.4	464.9	518.2	479.9	508.3
B	432.9	318.6	429	462	520.8
C	398.1	390	411.6	412.9	419
High					
A	793.1	1036.8	987.4	875.7	1006.7
B	969	877.6	1090.3	985.4	1133.9
C	896.8	820.8	1002.8	922.5	1213.3

	Low	Medium	High
Mean	52.9	440.7	974.1
SD	6.3	54.2	115.3
CV	11.8%	12.3%	11.8%
Final Bias	5.8%	10.7%	-2.2%

Fluvoxamine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	57.1	57.1	43	57.8	68.6
B	51.6	49.6	44.3	51.9	68.9
C	48.7	57.9	43	60.6	70.4
Medium					
A	409.9	384.7	466.3	481.9	428.5
B	432.2	391.1	435.1	499.1	484.5
C	374.9	414	432.1	474.4	472.2
High					
A	911.3	1103.5	915.1	875.3	1018
B	872.3	973.9	1000.7	991.2	1080.6
C	897.4	1041.6	952.7	883	1068.3
	Low		Medium		High
Mean	55.3		438.7		972.3
SD	9.1		39.3		78.6
CV	16.5%		9.0%		8.1%
Final Bias	10.7%		10.2%		-2.4%

Melitracene

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	55.7	47.5	41.8	61.8	61.3
B	54	55	41.9	58.1	57.4
C	55.1	40.7	42.8	53.9	60.4
Medium					
A	401	441.8	423.3	465.5	380
B	400.5	362.9	377.1	468	399.4
C	381.5	320.7	368.5	490.7	412.1
High					
A	916.4	1157.1	1025.8	1066.9	1011.6
B	965.3	946.7	1090.9	1097.7	1250.2
C	942.9	1101.6	1137	1138.7	1263
	Low		Medium		High
Mean	52.4		406.2		1074.1
SD	7.5		45.5		106.8
CV	14.3%		11.2%		9.9%
Final Bias	5.0%		2.1%		7.8%

Mianserine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	48.9	57.3	48	66.8	63.2
B	52.6	63.4	47.9	61.7	63.5
C	54.6	49.1	50.3	62.5	59.8
Medium					
A	431.7	405.2	410.6	469.2	436.5
B	431.7	375.8	418.8	483	422.9
C	398.4	362.7	413.8	463.2	415.2
High					
A	900.4	1059.4	972.9	984.4	999.7
B	949.2	912.1	1053.9	980.2	1072.5
C	964.1	1011.1	994.4	1028	1123.9
	Low		Medium		High
Mean	56.6		422.6		1000.4
SD	6.7		32.5		60.2
CV	11.9%		7.7%		6.0%
Final Bias	13.3%		6.2%		0.4%

Mirtazapine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	48.7	49.3	41.2	63.4	54
B	51.3	52.7	41.7	54.9	52.7
C	48.4	47.9	44.1	49.4	56.7
Medium					
A	417.3	372.1	316.9	445.6	374.8
B	432.3	354.6	347.6	492.7	411.4
C	423.5	364.6	322.1	440.3	376.1
High					
A	1017.6	1215.5	811.3	1034.5	1021.9
B	947.7	1025.9	864.8	903.2	1089.2
C	995.8	1084.9	798.8	1023.3	1142.1
	Low		Medium		High
Mean	50.4		392.8		998.4
SD	5.8		49.6		116.9
CV	11.4%		12.6%		11.7%
Final Bias	0.9%		-1.3%		0.2%

Reboxetine

	Run 1	Run 2	Run 3	Run 4	Run 5
Super Low					
A	44.7	48.8	41.5	55.1	55.1
B	47.8	51.7	42.5	52.1	55.9
C	46.1	46.9	41.9	57.1	56.4
Medium					
A	408.9	373.5	418.1	424.4	429.6
B	409.6	350.7	400.5	447.7	408.5
C	395.8	349.5	393.3	460.4	407.1
High					
A	953.3	1154.2	1013	1028.9	1069.8
B	963.1	978.2	1066.5	1005.1	1092.6
C	1016.1	1053.7	1028.2	1016.8	1166.5
	Low		Medium		High
Mean	49.5		405.1		1040.4
SD	5.6		30.9		62.2
CV	11.3%		7.6%		6.0%
Final Bias	-0.9%		1.8%		4.5%

Paroxetine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	52.3	44.7	44.5	56.5	58.2
B	52.8	55.5	40.8	53.3	62.2
C	55.6	38.4	41.4	58	59.5
Medium					
A	421.3	408.9	424.1	486.9	372.6
B	433.3	361.2	383.9	509.7	397.1
C	378.4	312	370.9	506.1	394.6
High					
A	907	981.4	920.9	1032.9	1018
B	932.9	894.9	1010.5	1014.2	1118.2
C	946.6	1080.6	1003.6	1066.3	1224.6
	Low		Medium		High
Mean	51.6		410.1		1010.2
SD	7.6		55.4		88.3
CV	14.8%		13.5%		8.7%
Final Bias	3.2%		3.2%		1.4%

Sertraline

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	48.7	62.9	35.4	66.2	63.2
B	55.1	56	41.5	54.8	61.0
C	47.3	53.8	40.9	58.4	65.9
Medium					
A	411	422.4	431.9	514.8	415.1
B	401	383.6	403.2	534.2	484.0
C	405.8	415.1	425.6	545.4	498.9
High					
A	902.0	1039.9	923.3	930.1	1069.7
B	857.4	972.9	932.4	999.2	1261.1
C	913.4	1063.2	975.6	973.6	1113.8
	Low		Medium		High
Mean	54.1		446.1		995.2
SD	9.5		53.7		102.2
CV	17.7%		12.0%		10.3%
Final Bias	8.1%		12.1%		-0.1%

Tramadol

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	47.2	50.6	49.4	46.5	50.7
B	45.1	47.3	49.4	49.8	59.4
C	66.1	54.4	46.7	53.5	63.3
Medium					
A	450.6	441.4	512.4	476.3	454.4
B	441.1	392.4	469.4	549.6	486.7
C	359	490.2	465	473.6	443.5
High					
A	851.4	1186.7	1139.4	1016.1	1003.5
B	871.3	962.3	1098.1	950	1344.6
C	933.4	1109.8	1086.8	965.7	1130.4
	Low		Medium		High
Mean	52.0		460.3		1043.3
SD	6.3		45.3		130.8
CV	12.2%		9.9%		12.5%
Final Bias	3.9%		15.7%		4.7%

Trazodone

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	237.1	258.4	219.4	271.8	289.7
B	267.1	257.9	221	258.1	263.3
C	255.3	245	214.2	260.5	265
Medium					
A	1967.9	1694.4	1791.3	1887.6	1936.6
B	1862.7	1717.2	1784.1	2112.8	1786.4
C	1849.5	1512.4	1779.9	2041.3	1727.5
High					
A	3942.1	3987.3	3811.9	3767.7	4026.2
B	3850.7	3655.4	4161.5	3649	4264.7
C	3956.8	4068.5	4063.9	4001.4	4388.9

	Low	Medium	High
Mean	252.2	1830.1	3973.1
SD	21.1	148.9	207.9
CV	8.3%	8.1%	5.2%
Final Bias	18.4%	7.2%	-6.9%

Venlafaxine

	Run 1	Run 2	Run 3	Run 4	Run 5
Low					
A	58.7	52.5	40.5	54.8	61.9
B	48.8	50.5	41.3	49.5	61.2
C	58	50.6	40	52.5	61.3
Medium					
A	395.3	367.1	426	469.7	401.8
B	401.8	380.8	416.8	456.7	388.7
C	383.2	376.4	395.1	475.5	419.8
High					
A	962.9	1177.7	1006.7	1011.8	1080.9
B	948.2	999	1033.8	1003.1	1197.6
C	1017	1134.3	1069.9	985.4	1124.8

	Low	Medium	High
Mean	52.1	410.3	1050.2
SD	7.4	33.8	77.3
CV	13.7%	8.2%	7.4%
Final Bias	4.3%	3.1%	5.4%

Appendix 6.4: Carry-over

To perform carry-over study, three samples in high concentrations (1000 ng/mL) , followed by five samples in very low concentrations (10 ng/mL) were analyzed in three runs (1st, 2nd and 3rd) . The results for all the compounds are on the tables below. Std. Conc. stands for the nominal concentration and Conc. is the value calculated by the method. %Dev. shows the deviation from the nominal and the calculated concentration.

Citalopram

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	782	775 .8	-0 .8
Cal 100% B 1st	782	860 .9	10 .1
Cal 100% C 1st	782	778 .9	-0 .4
Cal 1% A 1st	8	9 .7	21 .5
Cal 1% B 1st	8	8 .1	0 .6
Cal 1% C 1st	8	7 .6	-5 .3
Cal 1% D 1st	8	7 .9	-1 .6
Cal 1% E 1st	8	8	-0 .3
Cal 100% A 2nd	782	775	-0 .9
Cal 100% B 2nd	782	812 .6	3 .9
Cal 100% C 2nd	782	736 .6	-5 .8
Cal 1% A 2nd	8	10 .4	30 .3
Cal 1% B 2nd	8	8 .7	9 .1
Cal 1% C 2nd	8	7 .6	-5 .4
Cal 1% D 2nd	8	8 .1	1 .2
Cal 1% E 2nd	8	7 .7	-3 .9
Cal 100% A 3rd	782	743 .8	-4 .9
Cal 100% B 3rd	782	794 .5	1 .6
Cal 100% C 3rd	782	759	-2 .9
Cal 1% A 3rd	8	7 .7	-3 .5
Cal 1% B 3rd	8	7 .4	-8
Cal 1% C 3rd	8	6 .9	-13 .7
Cal 1% D 3rd	8	7 .3	-8 .7
Cal 1% E 3rd	8	7 .8	-2 .1

DM Citalopram

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	1012	1 .6
Cal 100% B 1st	996	1108 .6	11 .3
Cal 100% C 1st	996	1001 .2	0 .5
Cal 1% A 1st	10	15 .5	54 .5
Cal 1% B 1st	10	10 .4	4 .5
Cal 1% C 1st	10	9 .3	-6 .8
Cal 1% D 1st	10	9 .3	-7
Cal 1% E 1st	10	10	-0 .2
Cal 100% A 2nd	996	893 .2	-10 .3
Cal 100% B 2nd	996	910 .4	-8 .6
Cal 100% C 2nd	996	840 .1	-15 .7
Cal 1% A 2nd	10	16 .9	69 .4
Cal 1% B 2nd	10	11 .7	17 .5
Cal 1% C 2nd	10	9 .1	-8 .7
Cal 1% D 2nd	10	9 .6	-3 .9
Cal 1% E 2nd	10	10 .2	2 .5
Cal 100% A 3rd	996	1033 .9	3 .8
Cal 100% B 3rd	996	1080 .5	8 .5
Cal 100% C 3rd	996	1075 .6	8
Cal 1% A 3rd	10	10	0 .2
Cal 1% B 3rd	10	9 .5	-5 .3
Cal 1% C 3rd	10	8 .6	-14 .2
Cal 1% D 3rd	10	8 .8	-12
Cal 1% E 3rd	10	9 .6	-4 .1

DM Fluoxetine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	985	-1 .1
Cal 100% B 1st	996	1074 .9	7 .9
Cal 100% C 1st	996	1000 .9	0 .5
Cal 1% A 1st	10	16 .8	68 .3
Cal 1% B 1st	10	10 .8	7 .6
Cal 1% C 1st	10	10 .8	7 .6
Cal 1% D 1st	10	8 .1	-18 .6
Cal 1% E 1st	10	8	-20 .4
Cal 100% A 2nd	996	914 .9	-8 .1
Cal 100% B 2nd	996	1000 .6	0 .5
Cal 100% C 2nd	996	923 .7	-7 .3
Cal 1% A 2nd	10	23 .1	131 .3
Cal 1% B 2nd	10	12 .7	27 .1
Cal 1% C 2nd	10	10 .7	6 .6
Cal 1% D 2nd	10	9 .9	-1 .1
Cal 1% E 2nd	10	9 .2	-8 .4
Cal 100% A 3rd	996	980 .9	-1 .5
Cal 100% B 3rd	996	1057 .2	6 .1
Cal 100% C 3rd	996	1018 .7	2 .3
Cal 1% A 3rd	10	9 .8	-2 .1
Cal 1% B 3rd	10	7 .4	-26 .3
Cal 1% C 3rd	10	7	-30 .2
Cal 1% D 3rd	10	6 .5	-35 .1
Cal 1% E 3rd	10	6 .5	-35

DM Tramadol

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	848 .2	-14 .8
Cal 100% B 1st	996	889 .4	-10 .7
Cal 100% C 1st	996	840 .6	-15 .6
Cal 1% A 1st	10	10 .9	8 .6
Cal 1% B 1st	10	9 .9	-1 .5
Cal 1% C 1st	10	9 .3	-6 .8
Cal 1% D 1st	10	9 .6	-3 .8
Cal 1% E 1st	10	9 .7	-2 .6
Cal 100% A 2nd	996	1035 .2	3 .9
Cal 100% B 2nd	996	1260 .8	26 .6
Cal 100% C 2nd	996	1237 .4	24 .2
Cal 1% A 2nd	10	10 .6	5 .7
Cal 1% B 2nd	10	9 .8	-1 .8
Cal 1% C 2nd	10	9 .1	-8 .5
Cal 1% D 2nd	10	9 .6	-4 .1
Cal 1% E 2nd	10	9 .5	-4 .7
Cal 100% A 3rd	996	851 .2	-14 .5
Cal 100% B 3rd	996	1018 .9	2 .3
Cal 100% C 3rd	996	984 .2	-1 .2
Cal 1% A 3rd	10	10 .3	2 .5
Cal 1% B 3rd	10	9 .4	-5 .6
Cal 1% C 3rd	10	9 .8	-2
Cal 1% D 3rd	10	10 .4	4 .3
Cal 1% E 3rd	10	10 .2	2 .4

DM Venlafaxine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	782	755 .3	-3 .4
Cal 100% B 1st	782	728 .5	-6 .8
Cal 100% C 1st	782	737 .1	-5 .7
Cal 1% A 1st	8	8 .4	4 .9
Cal 1% B 1st	8	7 .2	-9 .8
Cal 1% C 1st	8	8	0 .5
Cal 1% D 1st	8	7 .7	-3 .5
Cal 1% E 1st	8	8 .2	3 .1
Cal 100% A 2nd	782	793 .8	1 .5
Cal 100% B 2nd	782	854 .7	9 .3
Cal 100% C 2nd	782	848 .2	8 .5
Cal 1% A 2nd	8	9 .6	20 .1
Cal 1% B 2nd	8	7 .9	-1 .8
Cal 1% C 2nd	8	7 .6	-4 .4
Cal 1% D 2nd	8	7 .8	-3
Cal 1% E 2nd	8	7 .8	-2 .8
Cal 100% A 3rd	782	748 .1	-4 .3
Cal 100% B 3rd	782	809 .8	3 .6
Cal 100% C 3rd	782	762 .9	-2 .4
Cal 1% A 3rd	8	8 .3	3 .5
Cal 1% B 3rd	8	7 .6	-5
Cal 1% C 3rd	8	7 .9	-0 .8
Cal 1% D 3rd	8	7 .6	-4 .5
Cal 1% E 3rd	8	7 .9	-1 .5

Duloxetine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	943 .7	-5 .2
Cal 100% B 1st	996	1123 .1	12 .8
Cal 100% C 1st	996	984 .5	-1 .2
Cal 1% A 1st	10	15 .6	55 .9
Cal 1% B 1st	10	9 .4	-6 .3
Cal 1% C 1st	10	9 .2	-7 .5
Cal 1% D 1st	10	9 .5	-5 .1
Cal 1% E 1st	10	8 .6	-14 .2
Cal 100% A 2nd	996	937 .9	-5 .8
Cal 100% B 2nd	996	1001 .3	0 .5
Cal 100% C 2nd	996	909	-8 .7
Cal 1% A 2nd	10	21 .3	112 .7
Cal 1% B 2nd	10	11 .5	15
Cal 1% C 2nd	10	9	-9 .6
Cal 1% D 2nd	10	9 .7	-2 .6
Cal 1% E 2nd	10	9 .5	-5
Cal 100% A 3rd	996	960 .6	-3 .6
Cal 100% B 3rd	996	1055 .4	6
Cal 100% C 3rd	996	1045 .6	5
Cal 1% A 3rd	10	7 .9	-21 .1
Cal 1% B 3rd	10	8 .4	-15 .7
Cal 1% C 3rd	10	7 .6	-24 .5
Cal 1% D 3rd	10	8	-20 .4
Cal 1% E 3rd	10	7 .7	-22 .8

Fluoxetine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	799 .1	-19 .8
Cal 100% B 1st	996	1073 .9	7 .8
Cal 100% C 1st	996	937	-5 .9
Cal 1% A 1st	10	13 .2	31 .8
Cal 1% B 1st	10	10 .4	4 .1
Cal 1% C 1st	10	10 .6	5 .8
Cal 1% D 1st	10	8 .8	-11 .6
Cal 1% E 1st	10	8 .8	-12 .2
Cal 100% A 2nd	996	930 .4	-6 .6
Cal 100% B 2nd	996	1059 .2	6 .4
Cal 100% C 2nd	996	944 .7	-5 .1
Cal 1% A 2nd	10	16 .1	61
Cal 1% B 2nd	10	12	19 .8
Cal 1% C 2nd	10	10 .2	1 .6
Cal 1% D 2nd	10	9 .5	-4 .6
Cal 1% E 2nd	10	7 .9	-21 .5
Cal 100% A 3rd	996	977 .6	-1 .8
Cal 100% B 3rd	996	1117 .3	12 .2
Cal 100% C 3rd	996	1124 .2	12 .9
Cal 1% A 3rd	10	8 .8	-12 .5
Cal 1% B 3rd	10	7 .4	-25 .6
Cal 1% C 3rd	10	9 .3	-7 .4
Cal 1% D 3rd	10	8 .5	-14 .9
Cal 1% E 3rd	10	9 .3	-7 .2

Fluvoxamine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	1027 .9	3 .2
Cal 100% B 1st	996	1047 .4	5 .2
Cal 100% C 1st	996	1018	2 .2
Cal 1% A 1st	10	15	50
Cal 1% B 1st	10	10	-0 .3
Cal 1% C 1st	10	9 .2	-7 .8
Cal 1% D 1st	10	8 .5	-15 .1
Cal 1% E 1st	10	8 .7	-12 .8
Cal 100% A 2nd	996	881 .5	-11 .5
Cal 100% B 2nd	996	924 .3	-7 .2
Cal 100% C 2nd	996	892 .2	-10 .4
Cal 1% A 2nd	10	16 .3	62 .9
Cal 1% B 2nd	10	12	19 .6
Cal 1% C 2nd	10	11	10 .2
Cal 1% D 2nd	10	10 .8	7 .7
Cal 1% E 2nd	10	9 .7	-2 .8
Cal 100% A 3rd	996	999 .1	0 .3
Cal 100% B 3rd	996	1115 .8	12
Cal 100% C 3rd	996	1052 .9	5 .7
Cal 1% A 3rd	10	11 .1	10 .5
Cal 1% B 3rd	10	7 .5	-25
Cal 1% C 3rd	10	8 .2	-17 .6
Cal 1% D 3rd	10	7 .7	-22 .6
Cal 1% E 3rd	10	9 .2	-8 .4

Melitracene

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	929 .9	-6 .6
Cal 100% B 1st	996	999 .1	0 .3
Cal 100% C 1st	996	970 .9	-2 .5
Cal 1% A 1st	10	12 .4	24
Cal 1% B 1st	10	9 .5	-5 .3
Cal 1% C 1st	10	10 .4	3 .9
Cal 1% D 1st	10	9 .2	-7 .6
Cal 1% E 1st	10	8 .9	-11 .2
Cal 100% A 2nd	996	999 .1	0 .3
Cal 100% B 2nd	996	1015 .3	1 .9
Cal 100% C 2nd	996	998 .5	0 .3
Cal 1% A 2nd	10	12 .6	26 .1
Cal 1% B 2nd	10	10 .3	3
Cal 1% C 2nd	10	9 .8	-1 .6
Cal 1% D 2nd	10	9 .6	-4 .3
Cal 1% E 2nd	10	9 .9	-0 .9
Cal 100% A 3rd	996	914 .9	-8 .1
Cal 100% B 3rd	996	1034 .8	3 .9
Cal 100% C 3rd	996	1102 .8	10 .7
Cal 1% A 3rd	10	9 .8	-2 .3
Cal 1% B 3rd	10	8 .8	-12 .2
Cal 1% C 3rd	10	8 .7	-13 .4
Cal 1% D 3rd	10	8 .6	-14 .2
Cal 1% E 3rd	10	10 .4	3 .9

Mianserine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	917 .1	-7 .9
Cal 100% B 1st	996	1052 .1	5 .6
Cal 100% C 1st	996	991 .8	-0 .4
Cal 1% A 1st	10	11 .6	16 .1
Cal 1% B 1st	10	9 .5	-4 .6
Cal 1% C 1st	10	9 .7	-3 .2
Cal 1% D 1st	10	10 .2	2 .2
Cal 1% E 1st	10	10 .1	0 .8
Cal 100% A 2nd	996	946 .8	-4 .9
Cal 100% B 2nd	996	1047 .2	5 .1
Cal 100% C 2nd	996	969 .9	-2 .6
Cal 1% A 2nd	10	13 .2	32 .2
Cal 1% B 2nd	10	11 .9	19 .5
Cal 1% C 2nd	10	9 .3	-7 .5
Cal 1% D 2nd	10	9 .6	-3 .6
Cal 1% E 2nd	10	8 .4	-15 .9
Cal 100% A 3rd	996	953 .3	-4 .3
Cal 100% B 3rd	996	1042 .4	4 .7
Cal 100% C 3rd	996	1042 .5	4 .7
Cal 1% A 3rd	10	10 .2	2 .4
Cal 1% B 3rd	10	9 .1	-8 .6
Cal 1% C 3rd	10	8 .9	-11
Cal 1% D 3rd	10	10	0 .2
Cal 1% E 3rd	10	9	-10 .3

Mirtazapine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	1008 .3	1 .2
Cal 100% B 1st	996	982 .9	-1 .3
Cal 100% C 1st	996	967 .6	-2 .8
Cal 1% A 1st	10	10 .7	7
Cal 1% B 1st	10	9 .8	-2 .1
Cal 1% C 1st	10	10 .3	2 .9
Cal 1% D 1st	10	10 .6	5 .8
Cal 1% E 1st	10	9 .8	-2 .5
Cal 100% A 2nd	996	902 .2	-9 .4
Cal 100% B 2nd	996	996 .3	0
Cal 100% C 2nd	996	959 .9	-3 .6
Cal 1% A 2nd	10	10 .3	2 .8
Cal 1% B 2nd	10	9 .5	-4 .7
Cal 1% C 2nd	10	10 .9	8 .5
Cal 1% D 2nd	10	8 .9	-10 .6
Cal 1% E 2nd	10	10 .8	7 .9
Cal 100% A 3rd	996	983 .5	-1 .3
Cal 100% B 3rd	996	1148 .7	15 .3
Cal 100% C 3rd	996	1013 .7	1 .8
Cal 1% A 3rd	10	10	-0 .5
Cal 1% B 3rd	10	9 .4	-5 .6
Cal 1% C 3rd	10	10 .6	6 .2
Cal 1% D 3rd	10	9 .7	-2 .6
Cal 1% E 3rd	10	9 .7	-3 .3

Paroxetine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	943 .6	-5 .3
Cal 100% B 1st	996	1024 .2	2 .8
Cal 100% C 1st	996	953 .9	-4 .2
Cal 1% A 1st	10	15 .3	53 .3
Cal 1% B 1st	10	11	9 .5
Cal 1% C 1st	10	9 .3	-7 .4
Cal 1% D 1st	10	9 .1	-9 .3
Cal 1% E 1st	10	9 .1	-8 .6
Cal 100% A 2nd	996	960 .8	-3 .5
Cal 100% B 2nd	996	1045 .8	5
Cal 100% C 2nd	996	973 .1	-2 .3
Cal 1% A 2nd	10	17 .7	76 .5
Cal 1% B 2nd	10	11 .9	19 .4
Cal 1% C 2nd	10	9 .8	-1 .8
Cal 1% D 2nd	10	10 .6	6
Cal 1% E 2nd	10	9 .4	-5 .8
Cal 100% A 3rd	996	919 .3	-7 .7
Cal 100% B 3rd	996	1055 .5	6
Cal 100% C 3rd	996	1085 .5	9
Cal 1% A 3rd	10	7 .7	-22 .7
Cal 1% B 3rd	10	7 .2	-28 .4
Cal 1% C 3rd	10	7 .4	-26
Cal 1% D 3rd	10	8 .3	-16 .7
Cal 1% E 3rd	10	8 .5	-14 .9

Reboxetine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	940	-5 .6
Cal 100% B 1st	996	1067 .1	7 .1
Cal 100% C 1st	996	981 .9	-1 .4
Cal 1% A 1st	10	10 .9	9 .3
Cal 1% B 1st	10	10 .5	4 .8
Cal 1% C 1st	10	10 .4	4 .2
Cal 1% D 1st	10	9 .3	-7 .1
Cal 1% E 1st	10	9 .5	-4 .8
Cal 100% A 2nd	996	956 .2	-4
Cal 100% B 2nd	996	1042 .4	4 .7
Cal 100% C 2nd	996	979 .5	-1 .7
Cal 1% A 2nd	10	11 .8	18 .4
Cal 1% B 2nd	10	11	10 .4
Cal 1% C 2nd	10	10 .6	5 .9
Cal 1% D 2nd	10	9 .4	-6 .1
Cal 1% E 2nd	10	10 .1	1 .4
Cal 100% A 3rd	996	938 .8	-5 .7
Cal 100% B 3rd	996	1023 .7	2 .8
Cal 100% C 3rd	996	1033 .2	3 .7
Cal 1% A 3rd	10	9 .8	-1 .5
Cal 1% B 3rd	10	9	-10 .5
Cal 1% C 3rd	10	9 .2	-8 .5
Cal 1% D 3rd	10	9 .7	-3
Cal 1% E 3rd	10	9 .8	-2 .2

Sertraline

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	1077 .6	8 .2
Cal 100% B 1st	996	1090 .4	9 .5
Cal 100% C 1st	996	1048 .2	5 .2
Cal 1% A 1st	10	17 .6	76 .1
Cal 1% B 1st	10	10 .3	3 .4
Cal 1% C 1st	10	9 .7	-2 .8
Cal 1% D 1st	10	9 .4	-6 .2
Cal 1% E 1st	10	9 .3	-7 .4
Cal 100% A 2nd	996	861 .8	-13 .5
Cal 100% B 2nd	996	833 .1	-16 .4
Cal 100% C 2nd	996	800 .8	-19 .6
Cal 1% A 2nd	10	16 .1	60 .6
Cal 1% B 2nd	10	11 .7	17
Cal 1% C 2nd	10	9 .5	-5 .1
Cal 1% D 2nd	10	8 .1	-18 .7
Cal 1% E 2nd	10	10 .4	3 .9
Cal 100% A 3rd	996	1049 .6	5 .4
Cal 100% B 3rd	996	1115 .5	12
Cal 100% C 3rd	996	1082 .1	8 .6
Cal 1% A 3rd	10	8 .4	-15 .9
Cal 1% B 3rd	10	7 .1	-28 .9
Cal 1% C 3rd	10	9 .3	-7
Cal 1% D 3rd	10	8 .4	-16 .2
Cal 1% E 3rd	10	9 .5	-5

Tramadol

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	776 .8	-22
Cal 100% B 1st	996	942 .8	-5 .3
Cal 100% C 1st	996	930 .4	-6 .6
Cal 1% A 1st	10	10 .8	8 .4
Cal 1% B 1st	10	11	9 .9
Cal 1% C 1st	10	9 .2	-8 .4
Cal 1% D 1st	10	9 .3	-7 .2
Cal 1% E 1st	10	9 .1	-8 .7
Cal 100% A 2nd	996	930 .5	-6 .6
Cal 100% B 2nd	996	1228 .1	23 .3
Cal 100% C 2nd	996	1259 .2	26 .4
Cal 1% A 2nd	10	10 .1	0 .7
Cal 1% B 2nd	10	10 .1	0 .7
Cal 1% C 2nd	10	9 .3	-7 .5
Cal 1% D 2nd	10	10 .3	3 .3
Cal 1% E 2nd	10	9	-10 .1
Cal 100% A 3rd	996	883 .5	-11 .3
Cal 100% B 3rd	996	1000 .6	0 .5
Cal 100% C 3rd	996	1014 .8	1 .9
Cal 1% A 3rd	10	9 .9	-1 .2
Cal 1% B 3rd	10	8 .8	-12 .2
Cal 1% C 3rd	10	10 .4	3 .9
Cal 1% D 3rd	10	10	-0 .2
Cal 1% E 3rd	10	10 .1	0 .6

Trazodone

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	4267	4345 .6	1 .8
Cal 100% B 1st	4267	4841 .8	13 .5
Cal 100% C 1st	4267	4235 .2	-0 .7
Cal 1% A 1st	43	73 .5	70 .9
Cal 1% B 1st	43	49 .2	14 .3
Cal 1% C 1st	43	42 .8	-0 .5
Cal 1% D 1st	43	47 .3	9 .9
Cal 1% E 1st	43	47 .3	9 .9
Cal 100% A 2nd	4267	4193 .4	-1 .7
Cal 100% B 2nd	4267	4145 .1	-2 .9
Cal 100% C 2nd	4267	3933	-7 .8
Cal 1% A 2nd	43	71 .7	66 .7
Cal 1% B 2nd	43	47 .9	11 .5
Cal 1% C 2nd	43	37 .4	-13 .1
Cal 1% D 2nd	43	43 .1	0 .1
Cal 1% E 2nd	43	42 .5	-1 .2
Cal 100% A 3rd	4267	4105 .5	-3 .8
Cal 100% B 3rd	4267	4260 .7	-0 .1
Cal 100% C 3rd	4267	4288 .4	0 .5
Cal 1% A 3rd	43	42	-2 .4
Cal 1% B 3rd	43	39 .6	-7 .9
Cal 1% C 3rd	43	35	-18 .7
Cal 1% D 3rd	43	36 .6	-14 .9
Cal 1% E 3rd	43	43 .7	1 .5

Venlafaxine

Name	Std. Conc	Conc.	%Dev
Cal 100% A 1st	996	972 .3	-2 .4
Cal 100% B 1st	996	1032 .5	3 .7
Cal 100% C 1st	996	943 .7	-5 .3
Cal 1% A 1st	10	10 .8	7 .7
Cal 1% B 1st	10	10 .8	8 .2
Cal 1% C 1st	10	9 .3	-7
Cal 1% D 1st	10	9 .5	-4 .7
Cal 1% E 1st	10	10	0 .2
Cal 100% A 2nd	996	939 .9	-5 .6
Cal 100% B 2nd	996	1051 .3	5 .6
Cal 100% C 2nd	996	1005 .3	0 .9
Cal 1% A 2nd	10	11 .1	10 .5
Cal 1% B 2nd	10	11	10 .1
Cal 1% C 2nd	10	10 .2	2 .2
Cal 1% D 2nd	10	9 .4	-6 .5
Cal 1% E 2nd	10	9 .8	-2 .2
Cal 100% A 3rd	996	967 .9	-2 .8
Cal 100% B 3rd	996	1056 .2	6
Cal 100% C 3rd	996	994 .6	-0 .1
Cal 1% A 3rd	10	10 .2	1 .8
Cal 1% B 3rd	10	9 .2	-8 .3
Cal 1% C 3rd	10	9 .6	-4 .5
Cal 1% D 3rd	10	9 .7	-2 .7
Cal 1% E 3rd	10	9 .8	-1 .8

Appendix 6.5: Matrix Effects

The following tables explain the results regarding to matrix effects. All the calculations were made with the areas of each compound.

Citalopram

Name	Area	IS Area
LOW SET A	48785	17921
LOW SET B Matrix A	31547	15568
LOW SET B Matrix B	29115	15473
LOW SET B Matric C	34589	17187
LOW SET B Matrix D	33919	16610
LOW SET B Matrix E	44020	15049
LOW SET B Matrix F	32616	14882
LOW SET C Matrix A	36024	11885
LOW SET C Matrix B	38956	13912
LOW SET C Matric C	42874	14178
LOW SET C Matrix D	43344	14328
LOW SET C Matrix E	40319	18653
LOW SET C Matrix F	39782	13466

DM Tramadol

Name	Area	IS Area
LOW SET A	51680	26626
LOW SET B Matrix A	44862	25171
LOW SET B Matrix B	38170	26234
LOW SET B Matric C	41951	27077
LOW SET B Matrix D	43837	24033
LOW SET B Matrix E	67433	24914
LOW SET B Matrix F	45292	25400
LOW SET C Matrix A	48061	20281
LOW SET C Matrix B	53059	24073
LOW SET C Matric C	51525	23254
LOW SET C Matrix D	51809	24728
LOW SET C Matrix E	51674	34891
LOW SET C Matrix F	49921	25304

DM Citalopram

Name	Area	IS Area
LOW SET A	50978	5607
LOW SET B Matrix A	27894	2008
LOW SET B Matrix B	29772	2163
LOW SET B Matric C	35653	2299
LOW SET B Matrix D	31715	2743
LOW SET B Matrix E	36145	2480
LOW SET B Matrix F	31883	2692
LOW SET C Matrix A	37444	1360
LOW SET C Matrix B	38363	1908
LOW SET C Matric C	45867	1432
LOW SET C Matrix D	40732	1845
LOW SET C Matrix E	37521	1993
LOW SET C Matrix F	37573	1948

DM Venlafaxine

Name	Area	IS Area
LOW SET A	4924	26626
LOW SET B Matrix A	5096	25171
LOW SET B Matrix B	4585	26234
LOW SET B Matric C	5031	27077
LOW SET B Matrix D	4689	24033
LOW SET B Matrix E	7106	24914
LOW SET B Matrix F	4729	25400
LOW SET C Matrix A	4817	20281
LOW SET C Matrix B	4896	24073
LOW SET C Matric C	4611	23254
LOW SET C Matrix D	4632	24728
LOW SET C Matrix E	4921	34891
LOW SET C Matrix F	4676	25304

DM Fluoxetine

Name	Area	IS Area
LOW SET	28085	18278
LOW SET B Matrix A	8174	15568
LOW SET B Matrix B	7710	15473
LOW SET B Matric C	7509	17187
LOW SET B Matrix D	9271	16610
LOW SET B Matrix E	10074	15049
LOW SET B Matrix F	10553	14882
LOW SET C Matrix A	9327	11885
LOW SET C Matrix B	12820	13912
LOW SET C Matric C	13347	14178
LOW SET C Matrix D	14522	14328
LOW SET C Matrix E	12290	18653
LOW SET C Matrix F	14338	13466

Duloxetine

Name	Area	IS Area
LOW SET A	65072	17921
LOW SET B Matrix A	13387	15568
LOW SET B Matrix B	15815	15473
LOW SET B Matric C	14433	17187
LOW SET B Matrix D	16894	16610
LOW SET B Matrix E	16696	15049
LOW SET B Matrix F	19024	14882
LOW SET C Matrix A	22094	11885
LOW SET C Matrix B	26784	13912
LOW SET C Matric C	26616	14178
LOW SET C Matrix D	31776	14328
LOW SET C Matrix E	26915	18653
LOW SET C Matrix F	29535	13466

Fluoxetine

Name	Area	IS Area
LOW SET A	16001	17921
LOW SET B Matrix A	2972	15568
LOW SET B Matrix B	2671	15473
LOW SET B Matric C	2684	17187
LOW SET B Matrix D	4057	16610
LOW SET B Matrix E	4226	15049
LOW SET B Matrix F	4875	14882
LOW SET C Matrix A	6080	11885
LOW SET C Matrix B	7645	13912
LOW SET C Matric C	7310	14178
LOW SET C Matrix D	9313	14328
LOW SET C Matrix E	7619	18653
LOW SET C Matrix F	8647	13466

Mianserine

Name	Area	IS Area
LOW SET A	9563	17921
LOW SET B Matrix A	2764	15568
LOW SET B Matrix B	3110	15473
LOW SET B Matric C	3138	17187
LOW SET B Matrix D	3775	16610
LOW SET B Matrix E	3155	15049
LOW SET B Matrix F	3965	14882
LOW SET C Matrix A	4816	11885
LOW SET C Matrix B	5165	13912
LOW SET C Matric C	5292	14178
LOW SET C Matrix D	5590	14328
LOW SET C Matrix E	4722	18653
LOW SET C Matrix F	5323	13466

Fluvoxamine

Name	Area	IS Area
LOW SET A	57607	26626
LOW SET B Matrix A	29556	25171
LOW SET B Matrix B	27025	26234
LOW SET B Matric C	29343	27077
LOW SET B Matrix D	33536	24033
LOW SET B Matrix E	41081	24914
LOW SET B Matrix F	32241	25400
LOW SET C Matrix A	42733	20281
LOW SET C Matrix B	43798	24073
LOW SET C Matric C	46473	23254
LOW SET C Matrix D	48053	24728
LOW SET C Matrix E	45770	34891
LOW SET C Matrix F	42662	25304

Mirtazapine

Name	Area	IS Area
LOW SET A	15435	1966
LOW SET B Matrix A	13424	1679
LOW SET B Matrix B	12182	1532
LOW SET B Matric C	12599	1628
LOW SET B Matrix D	13534	1782
LOW SET B Matrix E	18210	1525
LOW SET B Matrix F	14263	1824
LOW SET C Matrix A	14656	1314
LOW SET C Matrix B	14757	1550
LOW SET C Matric C	14717	1452
LOW SET C Matrix D	15592	1591
LOW SET C Matrix E	13982	2206
LOW SET C Matrix F	14405	1579

Melitracene

Name	Area	IS Area
LOW SET A	23296	5607
LOW SET B Matrix A	4478	2008
LOW SET B Matrix B	5227	2163
LOW SET B Matric C	4357	2296
LOW SET B Matrix D	6144	2743
LOW SET B Matrix E	4770	2480
LOW SET B Matrix F	6858	2692
LOW SET C Matrix A	6036	1360
LOW SET C Matrix B	8321	1908
LOW SET C Matric C	7691	1432
LOW SET C Matrix D	10290	1845
LOW SET C Matrix E	7024	1993
LOW SET C Matrix F	10792	1948

Paroxetine

Name	Area	IS Area
LOW SET A	34575	5607
LOW SET B Matrix A	8004	2008
LOW SET B Matrix B	9579	2163
LOW SET B Matric C	8071	2296
LOW SET B Matrix D	10454	2743
LOW SET B Matrix E	10663	2480
LOW SET B Matrix F	11478	2692
LOW SET C Matrix A	12751	1360
LOW SET C Matrix B	15785	1908
LOW SET C Matric C	15839	1432
LOW SET C Matrix D	18342	1845
LOW SET C Matrix E	15197	1993
LOW SET C Matrix F	16442	1948

Reboxetine

Name	Area	IS Area
LOW SET A	48160	17921
LOW SET B Matrix A	31137	15568
LOW SET B Matrix B	31772	15473
LOW SET B Matric C	39493	17187
LOW SET B Matrix D	35173	16610
LOW SET B Matrix E	48627	15049
LOW SET B Matrix F	34224	14882
LOW SET C Matrix A	41779	11885
LOW SET C Matrix B	40979	13912
LOW SET C Matric C	44871	14178
LOW SET C Matrix D	42420	14328
LOW SET C Matrix E	41902	18653
LOW SET C Matrix F	40210	13466

Trazodone

Name	Area	IS Area
LOW SET A	173941	17921
LOW SET B Matrix A	122619	15568
LOW SET B Matrix B	119188	15473
LOW SET B Matric C	124847	17187
LOW SET B Matrix D	125545	16610
LOW SET B Matrix E	149102	15049
LOW SET B Matrix F	125098	14882
LOW SET C Matrix A	154378	11885
LOW SET C Matrix B	152770	13912
LOW SET C Matric C	153811	14178
LOW SET C Matrix D	159494	14328
LOW SET C Matrix E	143182	18653
LOW SET C Matrix F	155665	13466

Sertraline

Name	Area	IS Area
LOW SET A	4992	26626
LOW SET B Matrix A	4223	25171
LOW SET B Matrix B	4571	26234
LOW SET B Matric C	4342	27077
LOW SET B Matrix D	5284	24033
LOW SET B Matrix E	5432	24914
LOW SET B Matrix F	6605	25400
LOW SET C Matrix A	3791	20281
LOW SET C Matrix B	7542	24073
LOW SET C Matric C	6065	23254
LOW SET C Matrix D	7815	24728
LOW SET C Matrix E	5497	34891
LOW SET C Matrix F	7735	25304

Venlafaxine

Name	Area	IS Area
LOW SET A	34394	26626
LOW SET B Matrix A	29995	25171
LOW SET B Matrix B	27229	26234
LOW SET B Matric C	30931	27077
LOW SET B Matrix D	29679	24033
LOW SET B Matrix E	47509	24914
LOW SET B Matrix F	33415	25400
LOW SET C Matrix A	35173	20281
LOW SET C Matrix B	34417	24073
LOW SET C Matric C	35149	23254
LOW SET C Matrix D	35020	24728
LOW SET C Matrix E	34218	34891
LOW SET C Matrix F	33213	25304

Tramadol

Name	Area	IS Area
LOW SET A	3965	26626
LOW SET B Matrix A	2768	25171
LOW SET B Matrix B	2296	26234
LOW SET B Matric C	2928	27077
LOW SET B Matrix D	2680	24033
LOW SET B Matrix E	4597	24914
LOW SET B Matrix F	3072	25400
LOW SET C Matrix A	3372	20281
LOW SET C Matrix B	3626	24073
LOW SET C Matric C	3700	23254
LOW SET C Matrix D	3941	24728
LOW SET C Matrix E	4100	34891
LOW SET C Matrix F	3421	25304

Appendix 6.6: Limits

The following tables present the data to calculate LOD's. The LOD was estimated from the standard deviation of the y-intercept and the average slope.

		Slope	Y-Intercept
Citalopram	Run 1	2 .8	8 .4
	Run 2	3 .1	8 .9
	Run 3	3 .0	7 .0
	Run 4	2 .6	3 .6
	Run 5	2 .7	3 .6
	Average	**2 .8**	**6 .3**
	SD	**0 .2**	**2 .5**

		Slope	Y-Intercept
Duloxetine	Run 1	2 .1	5 .6
	Run 2	2 .4	6 .4
	Run 3	2 .3	6 .1
	Run 4	1 .9	6 .0
	Run 5	2 .2	9 .4
	Average	**2 .2**	**6 .7**
	SD	**0 .2**	**1.5**

		Slope	Y-Intercept
DM Citalopram	Run 1	8 .8	66 .2
	Run 2	10 .1	72 .1
	Run 3	10 .0	71 .7
	Run 4	9 .0	64 .8
	Run 5	8 .8	64 .5
	Average	**9 .3**	**67 .9**
	SD	**0 .7**	**3 .7**

		Slope	Y-Intercept
Fluoxetine	Run 1	0 .5	0 .5
	Run 2	0 .4	1 .9
	Run 3	0 .4	0 .6
	Run 4	0 .4	0 .9
	Run 5	0 .4	2 .4
	Average	**0 .4**	**1 .2**
	SD	**0 .0**	**0 .9**

		Slope	Y-Intercept
DM Fluoxetine	Run 1	0 .7	3 .3
	Run 2	0 .7	4 .3
	Run 3	0 .7	3 .8
	Run 4	0 .7	3 .9
	Run 5	0 .7	5 .3
	Average	**0 .7**	**4 .1**
	SD	**0 .0**	**0 .7**

		Slope	Y-Intercept
Fluovoxamine	Run 1	1 .4	3 .8
	Run 2	1 .6	7 .1
	Run 3	1 .5	3 .6
	Run 4	1 .3	7 .1
	Run 5	1 .3	7 .7
	Average	**1 .4**	**5 .9**
	SD	**0 .1**	**2 .0**

		Slope	Y-Intercept
DM Tramadol	Run 1	1 .6	1 .4
	Run 2	1 .8	4 .6
	Run 3	1 .8	1 .1
	Run 4	1 .8	3 .1
	Run 5	2 .5	6 .3
	Average	**1 .9**	**3 .3**
	SD	**0 .4**	**2 .2**

		Slope	Y-Intercept
Melitracene	Run 1	3 .5	6 .8
	Run 2	4 .3	5 .0
	Run 3	4 .5	5 .5
	Run 4	3 .5	5 .1
	Run 5	3 .8	8 .9
	Average	**3 .9**	**6 .2**
	SD	**0 .5**	**1 .6**

		Slope	Y-Intercept
DM Venlafaxine	Run 1	0 .2	0 .3
	Run 2	0 .3	0 .7
	Run 3	0 .3	0 .0
	Run 4	0 .2	0 .4
	Run 5	0 .3	-0 .2
	Average	**0 .3**	**0 .2**
	SD	**0 .0**	**0 .4**

		Slope	Y-Intercept
Mianserine	Run 1	0 .4	1 .7
	Run 2	0 .4	1 .5
	Run 3	0 .4	0 .6
	Run 4	0 .3	0 .8
	Run 5	0 .4	1 .0
	Average	**0 .4**	**1 .1**
	SD	**0 .0**	**0 .5**

		Slope	Y-Intercept
Mirtazapine	Run 1	7 .6	17 .3
	Run 2	7 .8	18 .7
	Run 3	8 .4	16 .0
	Run 4	6 .8	16 .0
	Run 5	7 .7	12 .0
	Average	**7 .6**	**16 .8**
	SD	**0 .5**	**2 .7**

		Slope	Y-Intercept
Trazodone	Run 1	1 .8	73 .3
	Run 2	1 .9	69 .2
	Run 3	1 .9	65 .8
	Run 4	1 .6	65 .8
	Run 5	1 .7	69 .2
	Average	**1 .8**	**68 .7**
	SD	**0 .1**	**3 .1**

		Slope	Y-Intercept
Paroxetine	Run 1	5 .4	19 .6
	Run 2	6 .5	15 .2
	Run 3	6 .4	17 .2
	Run 4	5 .1	14 .5
	Run 5	5 .5	14 .6
	Average	**5 .8**	**16 .2**
	SD	**0 .6**	**2.2**

		Slope	Y-Intercept
Venlafaxine	Run 1	1 .3	2 .6
	Run 2	1 .2	3 .4
	Run 3	1 .3	2 .4
	Run 4	1 .1	3 .8
	Run 5	1 .0	2 .4
	Average	**1 .2**	**2 .9**
	SD	**0 .1**	**0 .6**

		Slope	Y-Intercept
Reboxetine	Run 1	2 .3	4 .4
	Run 2	2 .5	8 .3
	Run 3	2 .4	3 .8
	Run 4	2 .2	4 .2
	Run 5	2 .3	5 .0
	Average	**2 .3**	**5 .1**
	SD	**0 .1**	**1 .8**

		Slope	Y-Intercept
Sertraline	Run 1	0 .5	2 .0
	Run 2	0 .6	2 .8
	Run 3	0 .5	2 .3
	Run 4	0 .4	2 .0
	Run 5	0 .5	2 .3
	Average	**0 .5**	**2 .3**
	SD	**0 .1**	**0.3**

		Slope	Y-Intercept
Tramadol	Run 1	0 .1	-0 .2
	Run 2	0 .1	0 .4
	Run 3	0 .1	0 .1
	Run 4	0 .1	0 .0
	Run 5	0 .1	0 .2
	Average	**0 .1**	**0 .1**
	SD	**0 .0**	**0 .2**

Appendix 6.7: Stability

The following table shows the average peak area for processed sample stability study according time (from 0 to 66 hours). The first table shows data from the lowest concentration analysis (20 ng/mL) and the second for the highest (800 ng/mL).

Table 6.7.1. Average peak areas for 20 ng/mL

	Instability Point		Average Peak Area											
	Maximum Area	Minimum Area	T0	T6	T12	T18	T24	T30	T36	T42	T48	T54	T60	T66
Citalopram	26581 .72	17721 .15	22151 .43	17015 .31	11207 .38	10093 .74	8503 .517	9903 .442	12308 .82	12033 .07	11135 .23	13321 .4	13019 .36	12564 .25
DM Citalopram	23318 .28	15545 .52	19431 .9	14982 .31	10069 .95	9014 .758	7355 .262	9142 .396	10427 .54	10004 .82	10574 .42	12511 .1	11103 .03	10992 .6
DM Fluoxetine	9671 .708	6447 .805	8059 .757	6167 .453	4214 .056	4443 .958	2551 .667	3448 .811	4313 .845	3360 .434	3835 .355	4196 .718	5505 .451	4512 .47
DM Tramadol	20620 .31	13746 .88	17183 .6	17497 .18	18118 .15	15654 .94	11190 .43	11118 .31	15522 .39	13823 .66	11507 .24	13820 .75	13258 .4	15313 .36
DM Venlafaxine	1788 .165	1192 .11	1490 .137	1347 .688	979 .4463	974 .7313	736 .654	800 .8137	1090 .468	990 .1953	1050 .74	1255 .596	1160 .758	1111 .105
Duloxetine	24744 .85	16496 .57	20620 .71	17178 .27	11887 .76	12961 .19	7821 .983	10523 .63	12842 .71	11628 .51	12590 .69	14320 .15	15041 .39	12430 .74
Fluoxetine	4811 .413	3207 .609	4009 .511	2890 .431	2047 .681	1970 .411	1375 .511	1297 .066	1690 .061	1383 .805	1398 .568	2275 .437	1568 .476	1592 .116
Fluvoxamine	20426 .08	13617 .38	17021 .73	13926 .27	9929 .147	10567 .51	7277 .745	7574 .871	9874 .847	8776 .682	8733 .792	9593 .114	11379 .03	11138 .66
Melitraceen	9576 .307	6384 .205	7980 .256	6547 .51	4051 .092	3690 .521	3025 .383	3627 .657	4829 .949	4193 .54	3847 .216	4641 .773	4810 .993	4524 .52
Mianserine	4498 .59	2999 .06	3748 .825	2919 .988	2027 .883	1685 .69	1619 .568	1617 .681	1910 .941	1987 .177	1788 .31	2068 .725	1857 .404	2036 .559
Mirtazapine	9817 .962	6545 .308	8181 .635	6583 .567	4516 .998	4293 .145	2934 .451	3647 .245	4669 .578	4471 .055	3878 .855	4572 .269	4819 .404	5095 .087
Paroxetine	13687 .31	9124 .87	11406 .09	8933 .501	5839 .65	5861 .918	4060 .022	5488 .861	6244 .709	5550 .716	6395 .574	7238 .818	8748 .265	6840 .202
Reboxetine	23502 .86	15668 .57	19585 .72	15547 .99	10226 .24	11877 .56	7913 .837	9363 .115	12192 .33	10878 .16	9892 .778	12659 .57	12609 .84	11907 .22
Sertraline	7136 .422	4757 .615	5947 .018	4866 .885	3192 .912	2993 .949	2432 .026	2836 .068	3462 .502	3139 .47	3300 .834	3947 .258	3439 .374	3196 .26
Tramadol	1447 .51	965 .0069	1206 .259	936 .82	662 .806	559 .1203	483 .7765	327 .3823	580 .9947	439 .9947	416 .9097	625 .59	387 .362	622 .7923
Trazodone	98784 .77	65856 .51	82320 .64	62581 .99	39445 .22	36407 .51	31806 .42	36962 .67	47205 .14	44885 .14	43181 .3	50021 .1	47909 .99	46649 .12
Venlafaxine	13097 .98	8731 .985	10914 .98	8148 .258	5392 .862	4975 .127	4012 .354	3904 .453	5504 .347	4933 .955	4786 .216	6025 .324	5651 .772	5899 .967
Internal Standards														
Mirtazapine-d4	5325 .092	3550 .061	4437 .577	3509 .845	2454 .214	2158 .117	1901 .066	1701 .415	2175 .915	1774 .337	2626 .525	2562 .923	2426 .374	2471 .87
Paroxetine-d6	9877 .795	6585 .197	8231 .496	6346 .671	3909 .919	3787 .074	3617 .718	2589 .428	2562 .309	2751 .821	2458 .338	5295 .336	5321 .949	4847 .879
Reboxetine-d5	44740 .07	29826 .72	37283 .39	30179 .06	18816 .07	17303 .52	18322 .42	14898 .57	15393 .05	14800 .66	23007 .46	22047 .87	21876 .22	21780 .33
Venlafaxine-d6	47697 .15	31798 .1	39747 .62	32888 .44	23850 .74	20666 .26	19951 .89	16709 .58	19835 .59	18165 .49	23098 .89	23300 .84	23326 .84	25801 .33

Table 6.7.2. Average peak areas for 800 ng/mL

	Instability Point		Average Peak Area											
	Maximum Area	Minimum Area	T0	T6	T12	T18	T24	T30	T36	T42	T48	T54	T60	T66
Citalopram	802196 .3	534797 .5	668496 .9	709569 .8	674040 .4	598895 .4	421067 .1	337809 .2	324893 .3	310085 .6	358083	343049 .4	376342 .5	375786 .6
DM Citalopram	494933 .4	329955 .6	412444 .5	451354 .1	406515 .9	380404 .3	257870 .5	222190 .2	209240 .5	197413	216827 .2	230891 .6	242038 .2	267167 .8
DM Fluoxetine	197892 .1	131928	164910	156980 .4	115352 .9	123846 .5	87289 .58	77976 .75	70491 .04	71005 .72	70939 .38	87957 .82	81725 .35	89929 .66
DM Tramadol	531211 .1	354140 .7	442675 .9	446349 .6	625610 .2	459570 .0	501188 .6	395115	461060 .8	405743 .2	618951 .1	489617 .6	456114 .4	469060 .2
DM Venlafaxine	57556 .61	38371 .07	47963 .84	54607 .88	51018 .38	46031	35908 .01	34775 .05	37372 .37	33053 .14	39525 .03	41078 .81	41115 .64	42346 .88
Duloxetine	582047	388031 .3	485039 .1	506420 .6	392461 .2	441576 .5	319522 .6	269324 .1	252353 .4	270280 .7	268966	320263 .1	307761 .9	333652
Fluoxetine	110078 .3	73385 .55	91731 .93	71426 .3	96500 .1	56766 .35	59635 .13	35180 .9	42479 .76	37628 .37	51509 .18	47117 .67	40551 .22	76961 .35
Fluvoxamine	500329 .2	333552 .8	416941	409544 .5	399803 .8	399803 .8	300795	233680 .7	237111 .4	218481 .5	271331 .6	264906 .5	262445 .4	277700 .4
Melitraceen	306980 .4	204653 .6	255817	211218 .9	167634 .2	189690 .2	127476 .1	98900 .7	100533 .6	106608 .3	103462 .5	125914 .2	117517 .8	120700 .4
Mianserine	115643 .8	77095 .87	96369 .84	90242 .98	88617 .55	78759 .19	59192 .57	41327 .55	29819 .63	29166 .54	35181 .66	51234 .67	53494 .25	57674 .89
Mirtazapine	321641 .7	214427 .8	268034 .7	261086 .8	301102 .6	255328 .5	180818	129797 .1	148372 .8	120612 .5	171247 .6	162747 .2	166937 .5	153986 .3
Paroxetine	367368 .9	244912 .6	306140 .8	280492 .9	227292 .5	243799 .8	154581	133532 .2	131306 .3	138552 .9	127863 .7	152010 .6	161514 .1	160434
Reboxetine	661897 .7	441265 .2	551581 .4	590916 .2	576283 .9	523574 .8	367714 .6	317066 .6	319393 .3	311845 .7	331628 .7	341498 .5	361686 .2	382396 .5
Sertraline	172712 .2	115141 .5	143926 .8	130303 .3	92623 .79	113830 .5	76171 .17	66399 .38	62868 .23	65675 .68	59588 .44	81500 .58	71884 .19	85321 .49
Tramadol	38818 .93	25879 .29	32349 .11	34235 .91	50988 .44	27853 .96	27417 .79	12967 .22	20157 .17	16167 .84	26484 .3	18615 .5	15605 .46	21294 .85
Trazodone	1549929	1033286	1291607	1453151	1363496	1207912	807657 .1	685608 .6	640833 .3	646104 .6	707614 .1	718883 .5	776948 .6	805219
Venlafaxine	403028 .9	268685 .9	335857 .4	380821 .9	422533 .1	304127 .7	232632	157595 .7	181265 .3	165384 .7	213337 .1	196109 .9	194945 .1	218485 .1
Internal Standards														
Mirtazapine-d4	4758 .041	3172 .027	3965 .034	3543 .069	3324 .128	2876 .604	2196 .636	1701 .415	2175 .915	1774 .337	2467 .992	2342 .32	2313 .153	2212 .469
Paroxetine-d6	8422 .258	5614 .838	7018 .548	5984 .946	4157 .228	4229 .972	3199 .964	2589 .428	2562 .309	2751 .821	2458 .338	2974 .04	3049 .983	3335 .985
Reboxetine-d5	38529 .78	25686 .52	32108 .15	29893 .13	23992 .54	21740 .87	17849 .04	14898 .57	15393 .05	14800 .66	15873 .62	16908 .76	17518 .92	18906 .15
Venlafaxine-d6	40627 .69	27085 .13	33856 .41	32890 .71	31827 .07	25614 .44	23558 .04	16709 .58	19835 .59	18165 .49	23525 .7	20122 .23	20651 .12	21110 .74

Based on the values in table 6.7.1 and 6.7.2. and in the SWGTOX guideline, the following figures represent the stability for desmethylcitalopram, desmethylfluoxetine, desmethylvenlafaxine, duloxetine, fluoxetine, fluvoxamine, melitracene, mianserin, mirtazapine, reboxetine, sertraline, trazodone and venlafaxine. Again, the red line corresponds to an increase of 20% in signal, compared to T0, and a green one which correspond to a 20% decrease in signal.