Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen

Departement Farmaceutische Wetenschappen

Routine forensic analysis of psychoactive substances:
In-depth assessment of instrumentation, confirmation and quantification.

Routinematige forensische analyse van psychoactieve stoffen:
Diepgaande studie m.b.t. instrumentatie, confirmatie en kwantificatie.

Proefschrift voorgelegd tot het behalen van de graad van

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LIST OF ABBREVIATIONS

#

5-HT	5-hydroxy-tryptamine, serotonin

A

ACN	Acetonitrile
AD	Antidepressant
AP	Antipsychotic
AS	Autosampler (stability)

B

BT	Benchtop (stability)
BZD	Benzodiazepine or Z-drug

C

CAL L#	Calibration level #
CE	Collision energy
Cpd	Compound of interest
CV	Coefficient of variation

D

DAD	Diode array detector
DCM	Dichloromethane
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
dMRM	Dynamic multiple reaction monitoring

E

EE	Extraction efficiency
EMA	European Medicines Agency
EPS	Extrapyramidal symptoms
EtAc	Ethyl acetate

F

FGA	First generation antipsychotic
FT	Freeze-thaw (stability)
FV	Fragmentor voltage

G

GABA	Gamma-aminobutyric acid
GC	Gas chromatography

H

HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry

I

ISTD	Labelled internal standard

L

LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LT	Long-term (stability)

M

MAO	Monoamine oxidase
MAO-I	Monoamine oxidase inhibitor
MeOH	Methanol
MF	Matrix factor
MP	Mobile phase
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl-tertiary-butyl-ether

N

NA	Noradrenaline, norepinephrine
NARI	Norepinephrine re-uptake inhibitor
NaSSA	Noradrenergic & specific serotonergic antidepressant
NDRI	Norepinephrine-dopamine re-uptake inhibitor
NMR	Nuclear magnetic resonance
NPS	New psychoactive substance(s)

P

PD	(drug) pharmacodynamics
PK	(drug) pharmacokinetics
PM	Post-mortem
PMR	Post-mortem redistribution

PP	Protein precipitation
PSA	Primary secondary amine

Q

QC	Quality control
QQQ	Triple quadrupole mass spectrometry
QTOF	Quadrupole time-of-flight mass spectrometry
QTRAP	Triple quadrupole ion trap mass spectrometry
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe

R

RT	Retention time

S

SARI	Serotonin antagonist & re-uptake inhibitor
SGA	Second generation antipsychotic
SNRI	Serotonin-norepinephrine re-uptake inhibitor
SPE	Solid-phase extraction
SSRI	Selective serotonin re-uptake inhibitor
STA	Systematic toxicological analysis
STD	Reference standard

T

TCA	Tricyclic antidepressant
TDM	Therapeutic drug monitoring
TGA	Third generation antipsychotic
tMRM	Triggered multiple reaction monitoring

U

ULOQ	Upper limit of quantification
UPLC	Ultra-performance liquid chromatography

V

VAD	Flemish expertise centre Alcohol and other Drugs

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CHAPTER 1

INTRODUCTION

1.1. ANALYSIS OF DRUGS

1.1.1. Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) is defined as the measurement of pharmaceutical drugs and their metabolites to optimise treatment efficacy whilst limiting potentially harmful side effects [1,2]. It is part of so-called precision or personalised medicine, taking into account any known information about a drug's pharmacological properties, its concentration in a patient's samples and that patient's specific characteristics to guide the treatment [3–5]. Both parent compound and metabolites (active or inactive) should be monitored [2]. TDM is particularly recommended for drugs with a narrow therapeutic index, even if symptoms of intoxication have not manifested themselves [1,2,6,7]. Another indication for TDM may be the absence of an anticipated effect or the occurrence of serious adverse effects [1,2,8]. Such unexpected findings may be related to inter-individual variability in drug pharmacokinetics (PK) [7]. The field of pharmacogenomics has identified genes responsible for poor or ultrafast drug metabolism. Additionally, liver function and renal excretion generally decrease with increased age [9–12]. Polypharmacy, e.g. in the treatment of comorbidities, could lead to inhibition or induction of metabolic enzymes or to the occurrence of unexpected symptoms from drug-drug interactions [13–15]. Besides their approved use, many drugs are prescribed to children and adolescents, but have only been tested in the adult population. Their safety data are usually extrapolated to younger patients, despite numerous publications on developmental changes in drugs' pharmacodynamics (PD) and PK [16–19]. Therefore, TDM should be standardly performed in paediatric medicine. Also during pregnancy or breastfeeding, strict control of the circulating drug levels is of the utmost importance for both mother and child [20–22]. Other indications for TDM include the need for a high prescription dose, therapy switching (even between different formulations of the same compound), and administration of drugs with non-linear PK [1,8,23,24]. Once an optimised dosing scheme has been established, TDM can be used to follow up on a patient's adherence to the therapy, next to continued monitoring for its efficacy. This could be verified by asking patients to return any empty drug packages or blisters, yet detection of an active compound in biological samples is the only objective measurement of therapy compliance [2,7,8]. Besides being in the patient's best interest, maintenance therapy adherence reduces the cost of social care by preventing relapse and subsequent re-hospitalisation [25,26]. Even more, the World Health Organisation stated that the improvement of therapy adherence would have a greater impact on the population's health than any improvement of medical treatment would [27].

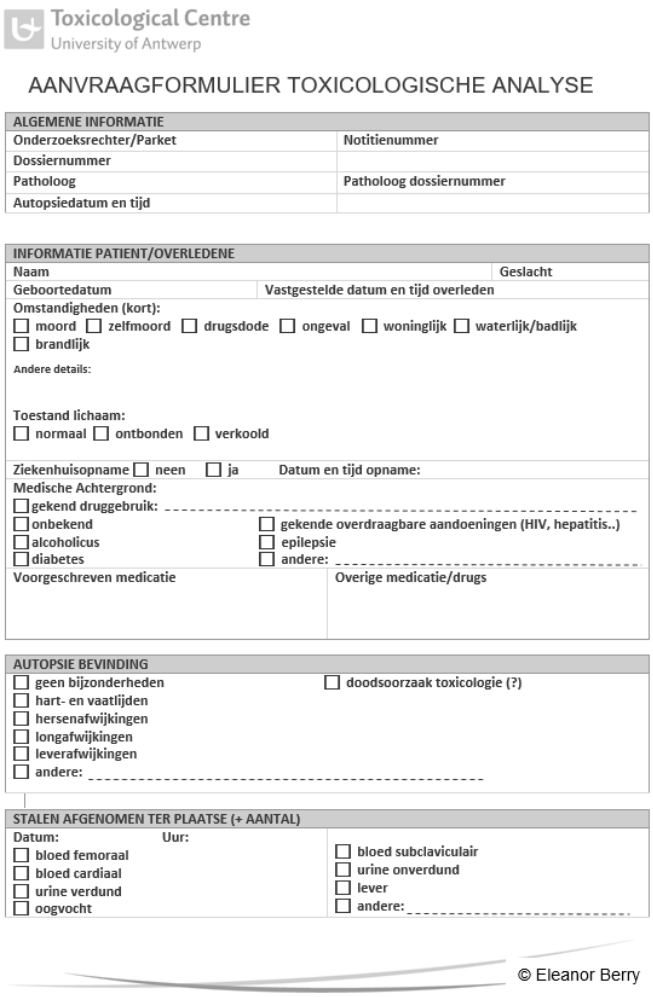
Determination of a patient's optimal drug levels should always consider the patient's age and medical history, as well as when the therapy was started, whether the patient was prescribed another drug or formulation previously and when the last dose was administered [1]. Because the presence or absence of an effect is inherently linked to the circulating drug levels in the body, blood is the preferred sample for TDM [6]. From a sample preparation and analytical point of view, plasma or serum are preferred over whole blood [7,28]. For standardisation purposes, measurement of the trough concentration (minimal concentration at the end of the longest dosing interval) at steady state concentrations (after four to six elimination half-lives) is recommended. An ideal sample would be collected in the morning before the first dose of the day. Collection before administration of the next dose suffices for therapies relying on drug depots or extended release formulations [2,7]. Sampling

through dried blood spots (for drugs with an defined blood spot-to-plasma ratio) has been proposed because of their minimally invasive collection, low volume needed, easy transport and storage, and potential for self-sampling by the patient. Standardisation of the volume collected can be achieved through the use of capillaries or volumetric absorptive microsampling devices [7,29,30]. The type of filter paper used may have an influence on the presence of interferences, on the spreading behaviour of the droplet, on the sample homogeneity, on the stability of the compounds and on the recovery of the analytes of interest from the paper [31–34]. A list of recommendations for sampling and analysis of dried blood spots is beyond the scope of this introduction, but an excellent overview can be found in a publication by Capiau et al. [29]. Few other matrices have been considered for TDM purposes. Oral fluid was investigated for its non-invasive collection. It was thought to reflect the unbound fraction of drugs, but has since been proven insufficiently reliable [35].

1.1.2. Clinical and forensic toxicology

Toxicological analyses are performed for a wide range of medical and or legal purposes, including, but not limited to, suspected drug intoxications and death investigations, road safety drug testing, workplace or correctional drug testing, drug-facilitated crimes, sports testing and environmental monitoring [36,37]. It is a multi-disciplinary field requiring knowledge of analytical chemistry (for detection, identification and quantification) and pharmacology (for interpretation). Both therapeutically prescribed, including those not registered for use in specific countries, and illicit drugs should be monitored for their potential to cause behavioural changes and/or adverse or toxic effects [36,38]. Because of its significant legal, social and economic consequences, careful, unbiased but well-informed interpretation of results is needed [39]. Any samples obtained should ideally be accompanied by an information sheet, detailing the type of samples included and their collection date/time, relevant demographic information of the donor, medical history and or autopsy findings, and findings at the place of the incident [37,40]. For good chain of custody in forensic investigations, information on the sample collector as well as their transport and storage should also be recorded [36,37]. An example of such an information sheet can be find in Figure 1 - 1.

Similar to TDM, blood is the matrix of choice due to good correlation between the measured concentrations and the observed biological effects, provided it has been sampled sufficiently close to the time of the incident [36,41]. Peripheral blood collected from adequately isolated femoral veins is preferred for quantitative purposes. Analysis of central blood should be limited to qualitative purposes only [42,43]. The use of sodium fluoride (2% w/V) containing tubes is recommended, or K_2EDTA if plasma needs to be obtained, as is storage at either 4 °C (during analysis) or -20 °C (long-term) [36,44]. However, fluoride containers could lead to an accelerated degradation of organophosphate-containing poisons [45]. Other matrices may also be used during screening assays. Urine in particular may be of interest due to its relatively high concentration of analytes and/or their metabolites, reduced amounts of endogenous compounds and non-invasive collection [39,46–48]. Its longer detection window for most drugs may be of benefit in drug facilitated sexual assault cases [37,49]. Conversely, in cases of acute overdose with rapid death, urine sample analysis may be less appropriate [41]. Hair analysis is recommended if there is a suspicion of historic or longer-term (weeks to months) continuous exposure, and should be collected from the vertex posterior area as close to the scalp as possible [50,51]. In contrast to urine, it is more likely to detect the parent compounds in hair [52]. Incorporation of compounds in hair is dependent on its colour (darker hair



Toxicological Centre
University of Antwerp

AANVRAAGFORMULIER TOXICOLOGISCHE ANALYSE

ALGEMENE INFORMATIE	
Onderzoeksrechter/Parket	Notitienummer
Dossiernummer	
Patholoog	Patholoog dossiernummer
Autopsiedatum en tijd	

INFORMATIE PATIENT/OVERLEDENE	
Naam	Geslacht
Geboortedatum	Vastgestelde datum en tijd overleden

Omstandigheden (kort):
☐ moord ☐ zelfmoord ☐ drugsdode ☐ ongeval ☐ woninglijk ☐ waterlijk/badlijk
☐ brandlijk

Andere details:

Toestand lichaam:
☐ normaal ☐ ontbonden ☐ verkoold

Ziekenhuisopname ☐ neen ☐ ja Datum en tijd opname:

Medische Achtergrond:
☐ gekend druggebruik: _____
☐ onbekend ☐ gekende overdraagbare aandoeningen (HIV, hepatitis..)
☐ alcoholicus ☐ epilepsie
☐ diabetes ☐ andere: _____

Voorgeschreven medicatie	Overige medicatie/drugs

AUTOPSIE BEVINDING
☐ geen bijzonderheden ☐ doodsoorzaak toxicologie (?)
☐ hart- en vaatlijden
☐ hersenafwijkingen
☐ longafwijkingen
☐ leverafwijkingen
☐ andere: _____

STALEN AFGENOMEN TER PLAATSE (+ AANTAL)
Datum: Uur:
☐ bloed femoraal ☐ bloed subclaviculair
☐ bloed cardiaal ☐ urine onverdund
☐ urine verdund ☐ lever
☐ oogvocht ☐ andere: _____

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Figure 1 - 1. Analytical request form used by the Toxicological Centre at the University of Antwerp. For optimised interpretation purposes, as much information as possible about the sample, the circumstances of the incident and the medical history of the donor should be acquired.

containing more melanin pigment will bind more compounds) and on the drug type (basic drugs are generally present at higher concentrations). Other sources of incorporation, or potential contamination, may – amongst several other factors – result from diffusion from sweat and sebaceous secretion into the external shaft [36,53]. Nails could provide a valuable alternative, but care should be taken regarding the more variable retention and uptake of drugs [54–56]. Once incorporated, drugs have shown excellent stability in keratinous matrices even during storage at room temperature [44]. The applicability of oral fluid samples is debated. Their main benefit is the potential for supervised collection (to avoid adulteration or substitution) with little infringement on a person's privacy and performed by non-medical personnel [36]. Additionally, recently ingested drugs or smoked compounds may be retained in high concentrations in the mucosa of the mouth. However,

secretion of drugs into saliva is highly depending on the pH in the oral cavity and on protein binding and other drug-related physicochemical characteristics, and therefore the collected oral fluid may not reflect the circulating drug levels [57,58].

At post-mortem (PM), liver may be collected as the primary solid tissue sample. It could provide additional information to blood analysis (see section 8.3.1 p. 186) if sampled from the more anatomically isolated right lobe [43,52]. Liver contains relatively high concentrations of both parent compounds and metabolites, and their respective concentrations may be used to differentiate between acute overdose and prescribed use of drugs with a narrow therapeutic index [44]. It may also serve as an alternative to PM blood (for qualitative purposes) if the latter is not available [36,37]. Brain (for lipophilic substances), lung (for inhalation or intravenous exposure), spleen (for carbon monoxide poisoning) or kidney tissue (for metal or metalloid poisoning) may also be analysed. Subcutaneous fat and muscle tissue should be considered if signs of drug injection are present. Tissue samples should always be stored under freezer conditions until analysis [37,44,59]. The presence of tablets in the stomach contents could indicate an acute overdose, thus these should always be analysed [60]. Vitreous humour is stored in an anatomically isolated and protected area, resulting in excellent stability of drugs in this matrix, as well as a higher resistance to putrefaction [37,52]. It is less affected by PM ethanol production, relatively devoid of interfering compounds and more representative of ante-mortem situations in cases with significant body decomposition [52,61]. Bile accumulates high concentrations of drugs and represents the best alternative to urine should that not be available [37,52]. However, it is rich in bile acids and other endogenous compounds, thus usually requires more laborious sample preparation techniques. It should be preserved with sodium fluoride and frozen until analysis to prevent fermentation [43,62]. Other biological samples could be collected and analysed on a case-by-case basis. Any samples taken into evidence from the scene of the incident are useful to guide the analyses, although care should be taken not to introduce any cognitive bias into the analyses or interpretation of the results [44,63].

1.1.3. Analytical strategies and procedures

Prior to extraction, sample preparation often starts with enzymatic digestion for solid tissues or hydrolysis for matrices containing phase II metabolites (e.g. urine, Figure 1 - 2) [64–66]. Cleavage of glucuronidated and sulphated conjugates will result in higher concentrations of the target analytes (parent compounds and/or phase I metabolites). Additionally, phase II metabolites tend to generate few, uncharacteristic fragment ions, hindering reliable identification [39]. Enzymatic hydrolysis is preferred over hydrolysis in strong acidic or basic environments, as it provides cleaner extracts and better analyte stability/less artefacts. β-glucuronidase from *Escherichia coli* or from *Helix pomatia* are most commonly used. The former operates under a larger pH optimum and renders cleaner extracts but needs the addition of arylsulfatase for cleavage of sulphated conjugates. The opposite is true for the latter [66].

With varying physico-chemical properties of analytes of interest, relatively non-selective extraction procedures should be used for sample preparation [66–68]. When both acidic, neutral and basic drugs need analysing, different sample preparation protocols may be required [46,69]. Direct analysis (after filtration) of biological matrices in its simplest form or following dilution (dilute-and-shoot) is usually reserved for urine samples only [39]. It may be beneficial to lower turn-around times, but suffers

from incompatibility with many analytical techniques, as well as insufficient sensitivity due to the often low concentration of the relevant compounds and high interference by endogenous substances [70,71]. Protein precipitation can be achieved by addition of inorganic acids (frequently tungstic acid or trichloroacetic acid) or organic solvents (methanol, acetonitrile) [37]. Although sensitivity issues or pronounced matrix effects may limit its stand-alone use as a sample preparation, it is commonly incorporated in solid-phase extraction (SPE) or liquid-liquid extraction (LLE) workflows to avoid blockage of cartridges or formation of emulsions, respectively [66].

LLE is based upon the partitioning of compounds between two immiscible liquid phases. It provides a clean, simple, fast and effective extraction for a wide range of analytes [70,72,73]. The polarity range of extractable analytes can be enlarged by mixing different solvents, often apolar ones such as hexane, dichloromethane or n-butyl-chloride, with more polar ones such as acetone or isopropanol [70,74,75]. The use of solvents such as ethyl acetate may lead to higher extraction efficiencies but also a higher background noise through co-extracted matrix interferences [36,70]. Adjustment of the pH by the use of buffers may also result in better recoveries, but with less matrix interferences present, and can additionally prevent formation of emulsions through high protein contents in the sample. A reduction of the pH to below 5.0 is recommended for extraction of acidic drugs, to above 6.0 for basic drugs [37,66,70]. SPE is based upon the partitioning of compounds between a liquid and a solid phase. Compared to other sample preparation techniques, SPE is more selective, allows for detection of lower analyte concentrations (due to a higher pre-concentration of the samples) and is readily coupled with automation [72,74]. It also allows for sequestration and sequential elution and/or collection of different drug classes from the same sample [66]. On the other hand, SPE procedures are more costly and more time consuming, and generate greater amounts of solvent and consumable waste [72,74]. A typical workflow consists of four steps (conditioning, loading, washing and elution), often preceded by filtration and/or centrifugation steps when analysing blood samples [41]. More recently developed sample preparation techniques commonly aim to reduce the volume of sample needed, whilst benefitting from the speed of LLE and the clean-up/selectivity of SPE. An excellent overview of such methods has been described by Borden et al. [76].QuEChERS were first reported by Anastassiades et al. and involve two main steps: salting out followed by dispersive SPE [77]. Their use in forensic and clinical toxicology is still limited, but they are said to benefit from a highly effective extraction, low amounts of sample and solvents needed, and an overall low cost [78]. Overall, the selected type of sample preparation should yield acceptable levels of sample clean-up and compound recovery, but ultimately is determined by empirical experiences of a laboratory and by its main users [36,68].

Once extracted and reconstituted, the analytes of interest can be identified and, if needed, quantified. Chromatographic techniques are employed to increase sensitivity (by reducing the number of analytes arriving at the detector at the same time) and specificity (by separating isobaric/isomeric compounds). Gas chromatography (GC) was the historical method of choice and is still widely used. When coupled to mass spectrometry (MS), electron impact sources benefit from a highly stable ionisation energy, resulting in reproducible spectra comparable with vast, commercially available libraries [79]. Separation of analytes can be achieved by varying the stationary phase and temperature program [37]. It is used to analyse a large range of compounds, but is less suitable for highly polar or less volatile substances, as well as for those with poor thermal stability [36]. Derivatisation of polar

or non-volatile substances may be needed prior to GC analysis. It may also be used to introduce electronegative moieties into a molecule if negative ion chemical ionisation mass spectrometry is used [66,80–82]. Different derivatisation techniques are available for basic drugs (acetylation, trifluoroacetylation, pentafluoropropionylation, heptafluorobutyration and trimethylsilylation) and for acidic drugs (methylation, pentafluoropropionylation, trimethylsilylation and tertiary-butyldimethylsilylation) [41,83]. Silylation reactions require strict anhydrous conditions but are particularly useful as they do not require evaporation of excess reagent prior to injection [66].

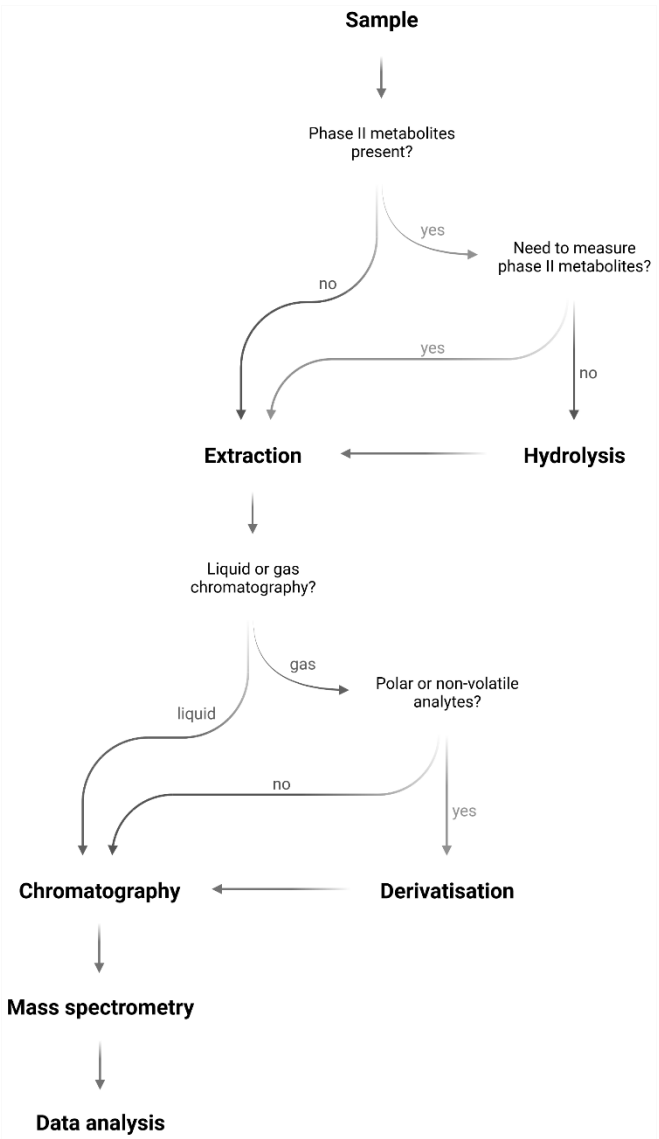


Figure 1 - 2. Generalised strategy for the forensic toxicological analysis of liquid samples. Solid, tissue samples may require enzymatic digestion prior to hydrolysis or extraction. The choice of extraction, chromatography or mass spectrometry method depends on the analytes of interest and the availability/experience of the laboratory.

Liquid chromatography (LC) is preferred for polar and thermally labile compounds, as they can be analysed without the need for derivatisation. Separation is achieved by a combination of the stationary phase, the mobile phase composition and the gradient applied [37]. A distinction can be made between high performance LC (HPLC; particle size 3 µm – 5 µm, backpressure 300 bar – 400 bar) and ultra-performance LC (UPLC; particle size < 2 µm, back pressure > 1000 bar) [39]. However, with an average peak width of one to three seconds, very short cycle times are required for acquisition of data-rich UPLC spectra, mostly restricting its use to high-resolution MS (HRMS) [68,84]. Development of the core-shell technology allowed for the same high separation efficiency, combined with lower backpressures [39]. In contrast to GC applications, no single optimal ionisation energy (for electrospray ionisation sources) exists for a wide variety of compounds, with (fragmentation) spectra depending on the nature of the eluent as well as the chromatographic conditions applied, the configuration of the LC-MS interface and the set MS conditions [79,85]. The newer development (mostly due to advancements in instrumentation and software) of 2D-chromatography allows for increased resolution of highly complex mixtures of compounds [86].

Detectors are usually based upon the principle of MS as this allows for sufficiently low limits of detection (generally in the subtherapeutic range) combined with the acquisition of compound-specific information such as m/z-value and isotopic pattern [39]. Spectra obtained from both GC and LC-coupled detectors may offer complementary information and toxicological laboratories usually benefit from the availability of both techniques. A distinction can be made between low resolution MS, which allows for the unequivocal detection of compounds differing with at least 1 unit in their m/z-value, and HRMS, with a resolution > 20000 full width at half maximum and mass accuracy < 5 ppm. HRMS detectors benefit from an increased specificity for co-eluting compounds, and allow for more wholesome screening and structural elucidation. Their application in analytical toxicology has rapidly increased over the past two decades [87–90]. Selectivity and sensitivity can be further increased by the acquisition of secondary fragment ions, a feature usually – but not exclusively – reserved for tandem-MS detectors [75,79]. Fragments may either be generated by a spatially different MS compared to the one performing precursor ion selection (tandem-in-space; e.g. triple quadrupole or quadrupole time-of-flight applications) or in the same MS also selecting the precursor ions (tandem-in-time; e.g. orbitrap applications) [39]. The latest improvements in resolution have come from the development of ion mobility, separating ions based upon their drift speed. Because the drift speed is related to an ion's cross-section area rather than its mass, this technique may allow for the distinction between co-eluting isomers [91,92].

Nuclear magnetic resonance (NMR) detectors benefit from their high reproducibility, non-destructive detection principle and generally minimally required sample preparation [93]. More so than MS-based techniques, they can be used in the elucidation of an analyte's molecular structure [94]. Moreover, NMR displays a fully linear correlation between the output signal and the concentration of the analyte, allowing for easy quantification even in the absence of a reference standard [95]. However, its usefulness to forensic toxicology is restricted by the relatively high limit of detection and as such most applications focus on the analysis of counterfeit medication or drug seizures, detection of chemical precursors or impurities in illicitly manufactured synthetic drugs, or structural elucidation of designer compounds in the absence of a reference standard [96–102].

1.2. COMPOUNDS UNDER INVESTIGATION

1.2.1. Psychoactive substances

The term 'psychoactive drug' refers to any substance that has its main site of action in the central nervous system and influences any of three psychic functions: cognition, mood or behaviour. From a pharmaceutical aspect, three major types of drugs have been defined. The analgesics are used to treat symptoms of pain and could be subdivided in narcotic (e.g. opioids) or non-narcotic analgesics (e.g. non-steroidal anti-inflammatory drugs and paracetamol). Psycholeptics suppress nervous system functions and include the neuroleptics or antipsychotics (APs) and the anxiolytics, hypnotics or sedatives, to which the benzodiazepines and Z-drugs (BZDs) belong. Psychoanaleptics stimulate the nervous system and include antidepressants (ADs) amongst other psychostimulants [103].

According to a survey in 2018 by the Flemish expertise centre Alcohol and other Drugs (VAD), 15% of the population above 15 years old had used one or more psychoactive substances in the two weeks prior to the survey, predominantly BZDs (12% of the population) [104]. The highest rates of psychoactive substance use were reported in care homes, with 42% of the elderly being prescribed BZDs, 37% ADs and 28% APs in 2016 [105]. Of the high school and higher education students, a respective 16% and 14% admitted to having used BZDs at some time in their life, with 8% having used them in the past year. A further respective 3% and 11% had already used psychoanaleptics [106,107]. A recent mental health survey across ten European countries concluded that the Belgian adult population had the second highest use (17%) of psychoactive medication [108]. This was in line with the 2018 prescription data from Farmanet, indicating that psychoactive medication, predominantly ADs, were the third most commonly prescribed drugs [103].

This thesis will focus on three classes of psychoactive substances: ADs, APs and BZDs. Opioids were not the subject of this research. For practical reasons, only those compounds for which a reference standard was available at the Toxicological Centre of the University of Antwerp will be considered. In total 40 ADs (Figure S1 - 1), 37 APs (Figure S1 - 2) and 54 BZDs (Figure S1 - 3) were included.

1.2.2. Antidepressants

Following the Diagnostic and Statistical Manual of Mental Disorders guidelines, depressive disorders can be subdivided into major depressive disorder, persistent depressive disorder and disruptive mood dysregulation disorder [109]. Diagnostic criteria for depression include irritable mood, decreased interest in pleasurable activities or ability to experience pleasure, significant weight changes, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or energy loss, feelings of worthlessness or excessive guilt, diminished ability to think or concentrate and recurrent thoughts of suicide or death [110].

Treatment of depressive disorders consists of electroconvulsive therapy, psychotherapy and/or pharmacotherapy [111]. The foundations for a pharmaceutical treatment were laid in 1965 by Dr. Schildkraut, who proposed the catecholamine hypothesis based upon previous findings by Dr. Kety: low levels of norepinephrine (NA) in the brain are involved in depressive disorders [112]. Lapin et Oxenkrug later suggested an additional role for the neurotransmitter serotonin (5-HT) [113]. Currently, most of the approved antidepressants work by increasing the levels of one or more

monoamine neurotransmitters in the synaptic cleft through either re-uptake inhibition, inactivation of breakdown enzymes, blockage of inhibitory feedback or direct postsynaptic actions (Figure 1 - 3) [114]. A more detailed overview per AD type was published by Skånland et Cieślar-Pobuda [115].

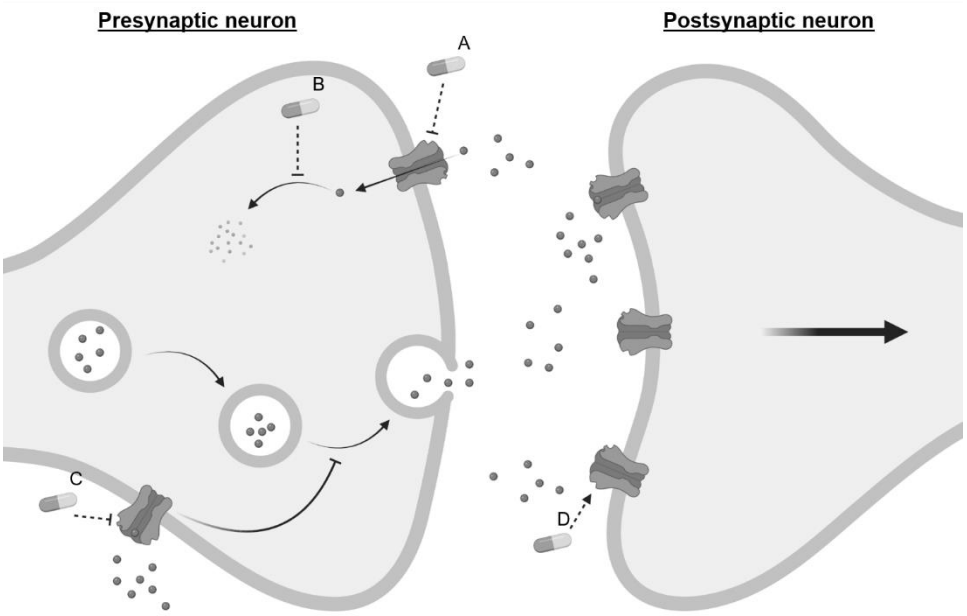


Figure 1 - 3. Most common mechanisms of action of antidepressants at the synaptic cleft. Presynaptic inhibitory actions will result in an increase of neurotransmitters in the synaptic cleft. This can be achieved by A) antagonising re-uptake receptors, B) blocking degradation by the mono-amine oxidase enzyme or C) inhibiting negative autoreceptor feedback mechanisms. They may also directly activate the postsynaptic receptors and stimulate signal transmission (D). *Created with BioRender.com.*

A large-scale meta-regression by Freemantle et al. found no significant difference in efficacy between different types of ADs [116]. The monoamine oxidase inhibitors (MAO-Is), irreversibly deactivating the similarly named enzyme, were amongst the first discovered ADs, combined with the tricyclic ADs (TCAs). MAO-Is prevent the degradation of 5-HT, NA and dopamine (DA), thus resulting in a mood-elevating effect [117–120]. Due to their poor adverse effect profile (see below), they are no longer commonly used. Moclobemide only reversibly inactivates the MAO enzyme and therefore is linked to less severe side effects [121,122]. TCAs act by antagonising the re-uptake of both 5-HT and NA, albeit with different affinities for either neurotransmitter between drugs of this class [123]. Their side effects are often severe and treatment requires close monitoring of patients [2]. Selective serotonin re-uptake inhibitors (SSRIs), also known as second generation ADs, were first introduced in 1988 (fluoxetine) and have readily replaced MAO-Is and TCAs in the treatment of depression [119]. Their discovery was based upon hypothesised alterations to the serotonin receptor sensitivity in the pathophysiology of depressive disorders [118]. SSRIs selectively block the re-uptake of 5-HT at the presynaptic nerve terminal and have an overall wide therapeutic index [2,124]. Weak effects on the re-uptake of NA and DA have also been observed [125,126]. TDM of patients throughout therapy is strongly recommended because of their non-linear PK, with e.g. fluoxetine and paroxetine inhibiting their own metabolic breakdown [2]. Atypical ADs exert their effects through a wide variety of actions on the monoamine neurotransmitters in the central nervous system. Reboxetine is known

as a norepinephrine re-uptake inhibitor (NARI), which indirectly recruits postsynaptic α- and β-adrenoreceptors through elevations of the NA levels in the brain [127,128]. Elevations in the DA levels, particularly in the frontal cortex, have also been reported [129–132]. ADs such as duloxetine and venlafaxine belong to the class of serotonin-norepinephrine re-uptake inhibitors (SNRIs). Peculiarly, specificity for either 5-HT or NA may vary with the dose administered [133,134]. Because they experience less drug-drug interactions compared to the typical ADs, SNRIs are the drugs of choice in elderly patients who usually suffer from multiple comorbidities [118].

The norepinephrine-dopamine re-uptake inhibitor (NDRI) bupropion is generally associated with a lesser occurrence of side effects, due to high specificity for both receptor types with little action on other brain receptors [135–137]. Several studies have found the drug to exhibit a positive effect on sexual desire and arousal [138–141]. It is structurally similar to the amphetamines, but no related effects have been observed at therapeutic doses [142]. Trazodone is a serotonin antagonist and re-uptake inhibitor (SARI), which may improve sleep continuity [143,144]. However, it is a modest AD at best and is, due to its postsynaptic 5-HT_2-receptor blockage, mostly used for its hypnotic and anxiolytic effects [118]. Mirtazapine and mianserin belong to the noradrenergic and specific serotonergic ADs (NaSSAs) [2]. Mirtazapine blocks α_2-adrenoreceptors and increases synaptic 5-HT through indirect neuron stimulation [145]. It may alleviate some side effects of TCAs (dry mouth, drowsiness, constipation) and SSRIs (gastro-intestinal distress, insomnia, sexual dysfunction) [118]. Mianserin on the other hand is a weak inhibitor of NA re-uptake by the α_2-adrenoreceptors [146]. As both NaSSAs antagonise the 5-HT_{2C}- and 5-HT_{2A}-receptors, they may be used to improve mood, treat sexual dysfunction and restore sleep cycles [147,148]. Agomelatine's mechanism of action is poorly understood but thought to differ from that of TCAs, SSRIs or MAO-Is as no adaptive changes in pre- or postsynaptic 5-HT_{1A}-receptors have been observed [149]. Rather, it stimulates melatonin MT_1- and MT_2-receptors, resulting in increased melatonin concentrations, which in turn antagonise the 5-HT_2-receptors [150–153]. It also displays a more beneficial side effect profile compared to traditional ADs, due to the overall absence of weight gain or serotonin syndrome, low risk of sexual dysfunction and low incidence of gastro-intestinal adverse events [124,154]. Acute discontinuation syndrome (see below) has not been observed, even after abrupt therapy discontinuation [155–157].

Besides their approved use, ADs are widely prescribed for off-label purposes, predominantly sleeping disorders. A link between depressive symptoms and chronic insomnia has been established and underlies their success [158,159]. However, AD treatment could lead to REM sleep behaviour disorder, nightmares, sleep walking, sleep-related eating disorders and hallucinations [160]. An excellent overview of the influence and underlying mechanisms of different AD types on the sleep pattern was published by Mayers et Baldwin in 2005 [123]. ADs may also be used in the treatment of chronic – often neuropathic – pain and usually exhibit an effect at lower doses than needed for their primary purpose [161]. The exact mechanism is unknown but may include increased levels of the pain inhibiting neurotransmitter NA, block of Na- and Ca-channels or activation of K-channels, inhibition of N-methyl-D-aspartate receptors and an effect on $GABA_B$-receptor functioning [162–166]. Other off-label uses include the treatment of eating disorders, as an aid for smoking cessation, in migraine prevention, as second-line treatment in the reduction of ADHD symptoms, and to prevent premature ejaculation [115].

The occurrence of adverse effects is common and related to the action on different synaptic receptors (Figure 1 - 4). At the start of therapy, most patients may experience anxiety or agitation, which may further lower a patient's already depressed mood and explain the increased suicide rates linked to ADs. However, this risk does not outweigh the benefit of treatment and close patient monitoring, including TDM to reduce unwanted symptoms, is recommended [119]. Patients prescribed MAO-Is must adhere to a strict tyramine-low diet. Under MAO-I therapy, this compound is no longer metabolised, which may lead to hypertensive crisis, including headache, sweating and vomiting in the prodromal phase, potentially followed by autonomic instability, hypertension, cardiac arrhythmia, coma and ultimately death [118,119]. Orthostatic hypertension is another frequently occurring MAO-I side effect, as are dizziness, weight gain, sedation and sexual dysfunction. Serotonin syndrome may present itself, though usually only in combination with TCAs or SSRIs. Significant drug-drug interactions have been observed with opioids (autonomic instability, delirium, death) and antihypertensive agents (hypotension) [119,124]. Side effects of TCAs include sedation, orthostatic hypotension, confusion and memory disorders, urine retention and – due to their membrane stabilising effects – lengthening of the QRS-interval and related cardiotoxicity [114,167,168]. Weight gain and sexual side effects are also commonly reported [136]. SSRIs are most commonly associated with insomnia, likely caused by the increased levels of 5-HT in combination with an inhibition of the NA re-uptake [20,169–171]. Other common side effects include sexual dysfunction (delayed ejaculation, anorgasmia or decreased libido) through stimulation of the 5-HT_2- and 5-HT_3-receptors and gastro-intestinal problems (transient nausea and diarrhoea) followed by weight gain due to desensitisation and/or down-regulation of 5-HT-receptors associated with appetite control [119,141,172–176]. Extrapyramidal symptoms (EPS) are rare but can include akathisia [136,177]. As for all drugs that increase the concentration of 5-HT, patients are at risk of developing serotonin syndrome, characterised by nausea, diarrhoea, restlessness, extreme agitation, hyperreflexia, autonomic instability, myoclonus, hyperthermia, rigidity, delirium, seizure and status epilepticus. Severe cases may also result in cardiovascular collapse, coma and death [119]. Of particular concern for their use in children and adolescents is the tendency for suicidal thoughts, though more studies are needed to establish a definite link with SSRI use [178]. Limited serious adverse effects have been described for a therapeutic dose of NARIs. Overdoses are commonly linked to sweating, tachycardia, anxiety and hypo-/hypertension, but are unlikely to result in serious sequelae or death. However, only limited *in vivo* studies on their pharmacology and toxicology have been published [142,179]. As SNRIs do not interact with histaminergic or cholinergic/adrenergic receptors, they are less linked to side effects such as hypotension and sedation, even when titrated to relatively high doses [118]. On the other hand, insomnia, sexual dysfunction, hypertension and tachycardia, decreased appetite, irritability, anxiety and restlessness are commonly observed [173,180,181]. NDRIs may cause insomnia, headache, tremors and nausea, as well as increased irritability and agitation. The occurrence of an effect on REM sleep following long-term treatment is still debated [124]. Their increased risk of seizures can be reduced by sustained-release formulations [118,179]. Most importantly, they are known to strongly inhibit CYP 2D6, and drug-drug interactions are common [2]. The adverse effects of SARIs are up for debate. Some sources report these drugs as well tolerated by patients, with priapism as the most common unwanted effect [118]. Other sources label them as effective histamine H_1-receptor blockers, causing sedation, weight gain and cardiovascular/autonomic effects. The SARI trazodone is quickly – but incompletely – metabolised into mCPP, a weak D_2- and α_2-adrenoreceptor

antagonist and 5-HT_{2C}-receptor agonist, leading to side effects such as tachycardia, anxiety and irritability [124,182,183]. NaSSAs antagonising effects on muscarinic and α_1-adrenoreceptors may lead to central, cardiovascular and autonomic side effects [147]. Particularly for mirtazapine, with its marked histamine H_1-receptor antagonism, sedation and obesity may occur [136].

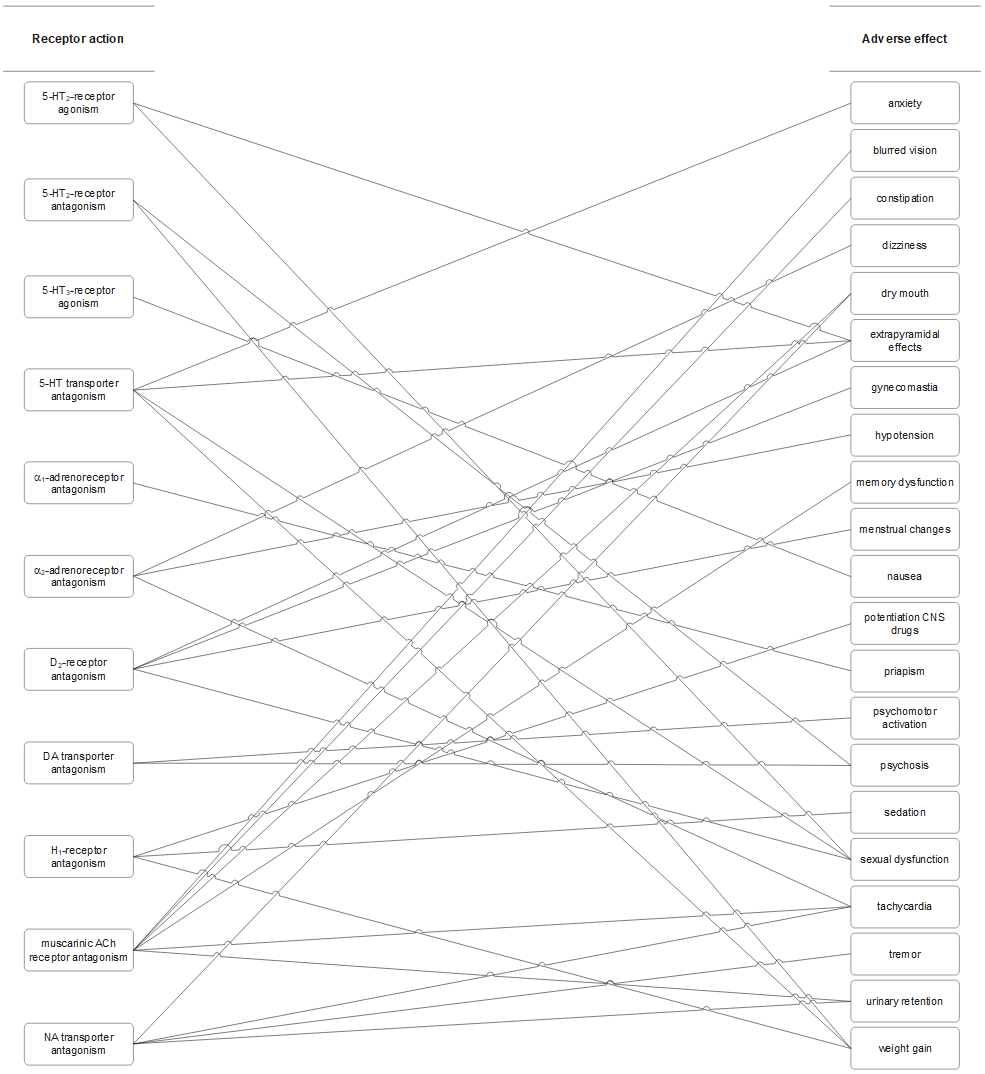


Figure 1 - 4. Relationship between receptor activation or block and observed adverse effects. Serotonin, 5-HT; central nervous system, CNS.

The occurrence of these adverse effects is the most important reason for therapy discontinuation within the first year, particularly for the TCAs (up to 50% dropout rates) and the SSRIs (up to 25% dropout rates) [184,185]. Upon discontinuation, patients may experience withdrawal syndrome, rebound phenomena or re-emergence of the primary disorder. AD withdrawal syndrome is characterised by a rapid onset (within one week following discontinuation) and spontaneous

resolution two to six weeks thereafter. Symptoms are mostly mild and non-specific (dizziness, nausea, headache, irritability, disturbed sleep) and should resolve once medication is resumed [186]. Because of its short half-life (~ 5 h), venlafaxine is particularly susceptible to AD discontinuation syndrome. Its active metabolite O-desmethyl-venlafaxine exhibits a similar efficacy and a twice as long half-life and could be used as an alternative to venlafaxine treatment [2,187–189].

In recent years, serotonergic hallucinogens such as lysergic acid diethylamide and psilocybin have been studied as new AD drug therapies. Their antidepressant, anxiolytic and anti-addictive properties were initially studied in the 1950s – 1970s, but their use was hindered by their international scheduling and related social stigma [190–194]. They act primarily on the 5-HT_{2A}- and the D_2-receptor. An additional effect on the glutamate receptors of the N-methyl-D-aspartate type has also been observed, hinting at another potential target for the development of new ADs [195]. Indeed, tianeptine, which antagonises these receptors, has been found to enhance the AD efficacy of SSRIs [196]. Similarly, ketamine and its S-enantiomer exhibit rapid AD action through non-competitive glutamate receptor antagonism [115,197]. An indirect influence on 5-HT re-uptake has also been observed [124]. Esketamine has recently been approved for treatment-resistant depression by the United States Food and Drug Administration [198]. Lastly, lowered cerebrospinal fluid and plasma concentrations of gamma-amminobutyric acid (GABA) have been observed in patients suffering from depression or suicidal thoughts, which were elevated/stabilised by AD therapy [199,200]. GABA-ergic drugs such as brexanolone, zuranolone and ganaxolone are currently being investigated for their therapeutic usefulness [201]. Overall, these findings show that, although still valid, the monoamine hypothesis explains part of the pathology of depressive disorders only. Involvement of other mechanisms could lead to the development of drugs with a superior efficacy, a better safety profile or a more rapid onset of action. Alternatively, these could be used in the treatment of therapy-resistant patients.

1.2.3. Antipsychotics

Pharmacotherapy of psychosis was based upon the hypothesis that schizophrenia is linked to increased cerebral DA activity. This idea was reinforced during initial experiments with DA antagonists, which were clinically effective against psychotic or manic symptoms, whereas agonists worsened or even induced psychosis [202]. Later on, it was discovered that increased DA activity in the mesolimbic pathway of the brain is responsible for positive symptoms of psychosis, including delusions, hallucinations and bizarre behaviour. Negative symptoms (apathy, anhedonia, alogia, avolition) are caused by reduced activity in the mesocortical pathway (Figure 1 - 5). Generally, positive symptoms are more distressing to patients, whereas negative ones are better predictors for long-term treatment outcome [203,204].

The first effective antipsychotic drug, chlorpromazine, was synthesised in 1952 by Dr. Charpentier as a new treatment for malaria. Treatment with this drug left patients in a relaxed or indifferent state without impairing consciousness, similar to the effect of a surgical lobotomy. Further successful testing on psychiatric patients and synthesis of related phenothiazine drugs, gave rise to the first generation APs (FGAs), also known as classic, conventional or typical APs. Derivatives based upon the general butyrophenone, thioxanthine, dibenzoxazepine, dihydroindolone and diphenylbutylpiperidine structure were later synthesised too [205]. The FGAs are subdivided in high-,

mid- and low-potency APs, based upon their affinity for the D_2-receptor, all of which successfully reduce the occurrence of positive symptoms [206]. Due to their unfavourable adverse effect profile (see below), a second generation of APs (SGAs) was developed. The first atypical AP was clozapine, marketed in 1989. Other SGAs were modelled after its greater antagonising affinity for the 5-HT_{2A}-receptor compared to the D_2-receptor. By specifically blocking the 5-HT_{2A}-receptor, they both reduce positive symptoms of schizophrenia and increase frontal dopamine levels, thereby also improving cognitive and negative symptoms. SGAs benefit from generally lower incidences of EPS and broader efficacy on mood and negative symptoms, but are associated with metabolic complications [205]. As a result, they tend to be better tolerated by patients compared to FGAs [207,208]. They may also induce neurogenesis or neurotropic factors and could potentially offer an additional neuroprotective benefit [205,209]. Aripiprazole, approved for use in 2002, can be considered the first of the third generation APs (TGAs). It has a high affinity but low intrinsic activity as partial presynaptic D_2-receptor agonist, postsynaptic D_2-receptor antagonist and partial 5-HT_{1A}-receptor agonist [210,211].

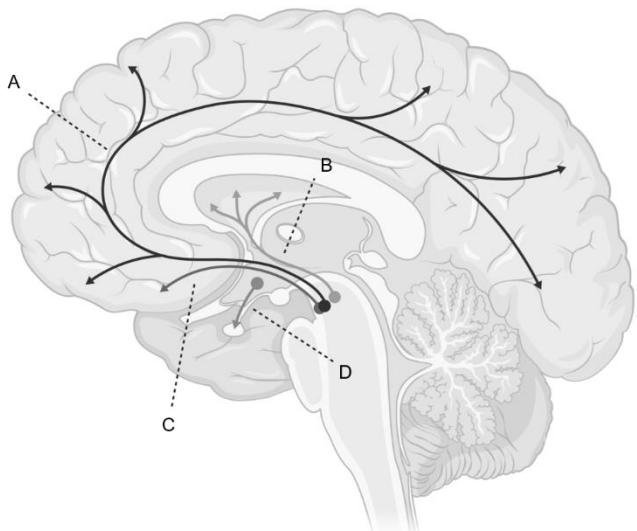


Figure 1 - 5. Dopaminergic pathways in the brain: A) mesocortical pathway, B) nigrostriatal pathway, C) tubero-infundibular pathway and D) mesolimbic pathway. Increased dopamine activity in the mesolimbic pathway has been linked to positive symptoms of psychosis, reduced activity in the mesocortical pathway to negative symptoms. *Created with BioRender.com.*

The most marked improvements in symptoms can be expected during the initial week of treatment, with more marginal changes thereafter. If no response has been noted after four to six weeks, it is recommended to switch to an AP with a different receptor-binding profile [212,213]. However, in such cases TDM is recommended to exclude non-compliance due to medication intolerability [214]. Successful AP action requires a minimum D_2-receptor occupancy of 65%. Higher percentages result in little therapy outcome improvements, but increase the risk of adverse effects such as hyperprolactinaemia (> 70% occupancy) or extrapyramidal symptoms (> 80% occupancy) [215]. FGAs, which preferentially bind DA receptors, have been used to successfully treat positive symptoms of psychosis, but due to their high receptor occupancy display an unfavourable adverse

effect profile [205]. Extrapyramidal symptoms are most commonly associated with their use, particularly for high-potency FGAs. EPS is an umbrella term for four distinctly different pathologies: pseudoparkinsonism (tremors, rigidity, bradykinesia, akinesia, hypersalivation, shuffling gait), akathisia (excessive pacing, inability to remain still), acute dystonia (sustained, spastic contractions of muscles) and tardive dyskinesia (potentially irreversible, involuntary movements of the head, face, trunk or limbs) [216–220]. High doses, rapid titration or non-parenteral administration of FGAs may lead to cardiovascular side effects including QRS- and QT_c-prolongation, torsades de pointes and ventricular fibrillation [206]. Autonomic dysregulation (dizziness, orthostatic hypotension, sedation, reflex tachycardia) stems from a block of α_1-adrenoreceptors and is mostly seen in treatments with low-potency FGAs. Anticholinergic side effects result in constipation, nausea, vomiting, dry mouth, paralytic ileus, blurred vision and open-angle glaucoma. D_2-receptor blockage has also been found to increase prolactin secretion, leading to gynecomastia, galactorrhea, amenorrhea, decreased libido, erectile dysfunction, infertility and decreased bone density. Lastly, cutaneous maculopapular rash and photosensitivity may also occur [205].

SGAs may be prescribed for a wider range of indications, including acute and long-term maintenance treatment of schizophrenia, schizoaffective disorder, acute mania and maintenance treatment of bipolar disorder [205]. Clozapine use should be restricted to treatment-resistant or refractory schizophrenia only, due to an increased risk of agranulocytosis. However, recent studies have indicated a significant reduction in mortality for patients receiving clozapine compared to other SGAs, suggesting its usefulness may need to be reconsidered [221,222]. Increasingly, atypical APs such as quetiapine and aripiprazole are used in the treatment of major depressive disorder [223–225]. Other off-label uses of SGAs include the treatment of delusional disorders (paranoia), Tourette's syndrome, psychotic episodes of Huntington's disease or personality disorders, and schizoaffective disorder [206]. They more specifically target D_2-receptors in the mesolimbic and mesocortical pathways over those in the nigrostriatal pathway, resulting in a reduced risk of EPS compared to FGAs [205]. However, several studies have demonstrated that low-potency FGAs are as safe as SGAs with regards to the risk of EPS [226–228]. Prolongation of the QT_c-interval may also be observed, although not usually to a clinically significant degree. Other unwanted side effects include orthostatic hypotension, transient elevation of liver enzymes and constipation, as well as sexual dysfunction, gynecomastia, galactorrhea and erectile dysfunction. The use of SGAs during pregnancy is strongly discouraged. Teratogenicity has thus far not been proved nor disproved. Additionally, neurological side effects to both mother and foetus have been described. Clozapine in particular has been linked with myocarditis and cardiomyopathy, which tends to be fatal in up to 20% of cases [205,229,230]. It may also increase the risk of drug-induced seizures (observed in 5% – 10% of patients) and cause fatal agranulocytosis. Weekly complete blood counts are recommended during the initial six months of treatment, followed by monthly white blood cell counts thereafter [205,231]. Aripiprazole has the lowest reported incidences of EPS and has not been linked to any significant weight gain. Nonetheless, tremor and other parkinsonism symptoms may present themselves at higher doses [232,233]. Metabolic disturbances including obesity, diabetes, glucose intolerance and dyslipidemia may occur during SGA treatment (and to a lesser extent also FGA treatment), likely linked to their additional antagonism of the serotonergic 5-HT_{2C}- and histaminergic H_1-receptors [230,234–239]. In the central nervous system, APs have been found to activate hunger centres, inhibit satiety centres and disrupt food reward pathways [240–242]. Additionally, they decrease energy expenditure by

impairing locomotion and exert an inhibiting effect on the autonomous nervous system [243]. Peripherally, potential direct effects on the liver (increased lipogenesis and glucose output), adipose tissue (increased adipogenesis, lipogenesis and level of pro-inflammatory cytokines) and skeletal muscles (decreased glucose uptake) have been proposed [244–247]. Pancreatic functioning may also be affected, leading to reduced or halted insulin secretion [243].

Combination therapy with multiple AP drugs should be reserved for treatment-resistant patients only [213,248,249]. Such multi-drug regimens risk an overall high (above licensed maximal) dose with increased burden by adverse effects, drug-drug interactions, variability in serum levels and difficulties for patients to adhere to dosing scheme [214,250,251]. Nonetheless, despite a clear lack of pharmacological rationale, it is prescribed to up to 50% of patients depending on the country in question [250,252]. The use of APs in the geriatric population is widely debated and improvements in the quality of life must be weighed against increased risks of cerebrovascular and cardiac adverse events [253–255]. Adverse events are more common in this group due to polypharmacy, age-related PK impairments and comorbidities [210,253,256]. At the same time, APs are increasingly used in paediatric patients for treatment of schizophrenia as well as bipolar disorder, obsessive compulsive disorder, tic disorders or aggressive/disruptive behaviour [257–259]. Dosing regimens based upon body weight are commonly extrapolated from adults [260,261]. However, oral bioavailability tends to be lower in children due to a more pronounced first pass effect. Additionally, activity of CYP 1A2, 2C9 and 3A4 is markedly upregulated compared to adults [262,263]. Therefore, usually higher adjusted doses are needed, increasing the risk of prolactinaemia, obesity and type 2 diabetes as well as of cardiac effects [260,264,265]. Aripiprazole therapy may be beneficial with respect to such adverse effects. In contrast to its use in adults or the paediatric use of FGAs and SGAs, higher incidences of EPS and sedation have been observed [266,267].

Overall, no differences in improvement of quality of life have been observed between FGAs and SGAs [226,228,268]. Variable benefits in the reduction of positive symptoms have been reported for SGAs versus FGAs [269–271]. An effect on negative symptoms (social withdrawal, lack of motivation) is more difficult to assess as these are linked to mood and motility, both of which can be negatively affected by APs themselves [229,271]. However, together with cognitive symptoms (deficient working memory, impaired verbal fluency), no benefit of SGAs over FGAs is thought to exist [269,272,273]. Discontinuation of AP treatment should be avoided as it has been linked to a significantly increased relapse risk within the first five years compared to maintenance therapy, as well as increased risks of rehospitalisation and suicide [274,275].

1.2.4. Benzodiazepines & Z-drugs

Benzodiazepines are amongst the most commonly prescribed psychoactive medication [276]. They were first introduced in the early 1960s as drugs with anxiolytic, hypnotic, anticonvulsant and muscle relaxant properties, and readily replaced the side-effect prone meprobamate and barbiturates [38,277,278]. All BZDs have a similar core structure, consisting of a benzene ring fused with a seven-membered diazepine ring, with the N-atoms on the 1 and 4 or 1 and 5 position on the diazepine ring (Figure 1 - 6). The latter were developed for greater receptor efficacy whilst at the same time displaying fewer adverse effects, although this is currently still debated in practice [279,280].

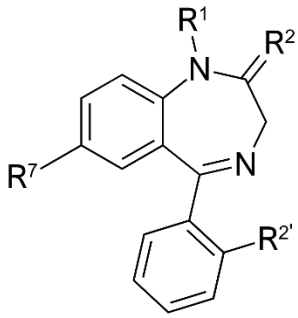


Figure 1 - 6. Generic benzodiazepine structure. Their name is derived from the fusion of a benzene ring with a seven-membered diazepine ring. The N-atoms may be on either the 1 and 4 or the 1 and 5 position of the diazepine ring.

They act on the $GABA_A$-receptor, located in the postsynaptic membranes throughout the central nervous system [281]. This receptor is made up of six protein subunits located around a central chlorine channel [282]. Seven subunit families are known to exist: $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ and $\varrho_{1\text{-}3}$. Most receptors consist of $\alpha\beta\gamma$-combinations, with the BZD binding site located at the interface of the α- and γ-subunit [281]. More recent discoveries have also identified a high affinity binding site for diazepam located at the interface of the β_2- and γ_2-subunits [283,284]. Diazepam-sensitive $GABA_A$-receptors are made up of α_1-, α_2-, α_3- or α_5-subunits, combined with either of the β-subunits and with the γ_2-subunit [285,286]. The Z-drugs display a preferential selectivity for the α_1-subunit [287]. The $\alpha_1\beta_2\gamma_2$-receptor is the major diazepam-sensitive subtype, resulting in sedation, anterograde amnesia and anticonvulsant effects. However, the role of the different subtypes is poorly understood, with α_2-subunits thought to be involved in anxiolysis, α_5-subunits in anterograde amnesia and α_2-, α_3- and α_5-subunits in muscle relaxation [280,281,288]. Rather than a direct activation, BZDs allosterically modify the $GABA_A$-receptor, increasing the frequency of the Cl-channel opening with a given GABA concentration [280,289,290]. The resulting chlorine influx hyperpolarises the neuron, moving the membrane potential away from the firing threshold and thus inactivating the neuron (Figure 1 - 7) [277]. Depending on their metabolic half-life ($t_{½}$), BZDs can be subdivided into short-acting ($t_{½}$ < 10 h), intermediate-acting ($t_{½}$ 10 h – 24 h) and long-acting drugs ($t_{½}$ > 24 h) [280].

BZDs are currently prescribed in the treatment of anxiety, insomnia and panic disorders, as well as for sedation and anterograde amnesia during surgery. They can further be used to minimise acute alcohol withdrawal symptoms, for their anticonvulsant properties in acute seizures or epilepsy, as relaxant during muscle contractions or as adjunctive treatment for schizophrenic patients [280]. Treatment of insomnia is linked to short- and intermediate-acting BZDs, of anxiety to long-acting ones [291]. Anterograde amnesia may result from interference with the formation of new memories upon short-term BZD use [292]. The exact mechanism remains unknown but is thought to involve extrasynaptic $GABA_A$-receptors containing the α_5-subunit, which mediate tonic neuronal inhibition [293,294]. Long-term use may impair memory as information is not transferred from the short-term memory [295]. Whether this effect persists after therapy discontinuation and thus whether long-term BZD use may lead to cognitive decline is highly debated [292,296,297]. New therapeutic applications such as pain relief and treatment of depression are also currently being investigated, although their use in depressive disorders is most likely limited to anxiolysis only [199,298–300]. The discovery of

$GABA_A$-receptors in the smooth muscle of the respiratory system has led to an investigation of their potential to reduce bronchospasm in asthma [301,302].

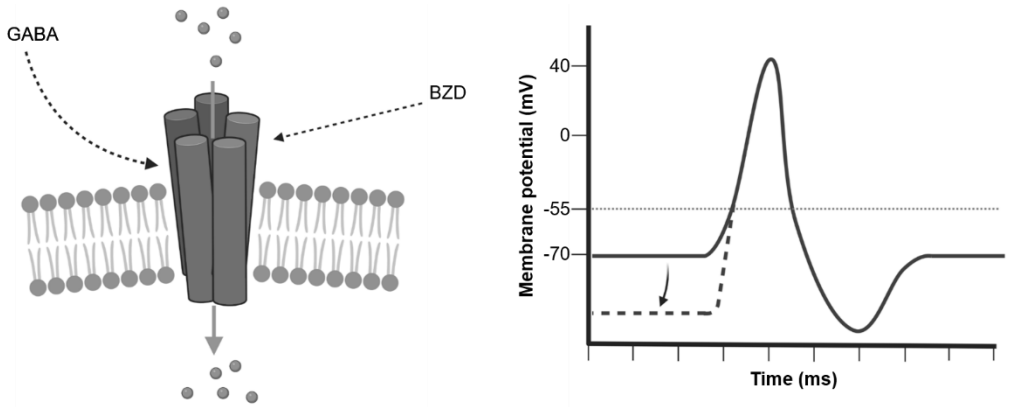


Figure 1 - 7. Visualisation of the $GABA_A$-receptor (left) and its effect on the neuronal action potential (right). Upon activation by GABA, the receptor's ion channel opens resulting in an influx of Cl^-. This hyperpolarises the membrane potential (resting state -70 mV) away from the firing threshold (-55 mV, dotted line). Benzodiazepines bind the receptor in a different site than that of GABA and increase the ion channel's opening frequency but do not directly activate the receptor. Benzodiazepine, BZD, gamma-aminobutyric acid, GABA. *Created with BioRender.com.*

BZD use is associated with a multitude of adverse effects. Common but generally less severe symptoms include psychological (anxiety, insomnia, nightmares, memory/concentration impairment, depressive symptoms, confusion, amnesia), physical (muscle tension/spasms/weakness, sedation, dizziness) and perceptual (hypersensitivity to light/sound/touch) effects, as well as derealisation or depersonalisation and occasional fits, paranoia or confusional psychosis. Life-threatening symptoms occur less frequently but may include delirium tremens, status epilepticus, self-harm, suicidal thoughts, mania and violence [38,280,303]. Their use during pregnancy has been linked to congenital malformations, preterm birth, low birth weight and neonatal morbidity [304]. The greatest risks to the infant are observed for patients treated with BZDs in the third trimester of pregnancy and include floppy infant syndrome and neonatal BZD withdrawal syndrome, both of which may persist for hours to months after birth [292,305–307]. Elderly patients may experience reduced drug clearance due to age-related changes in PKs and therefore are more prone to falls, dependence or withdrawal, and reversible (or potentially irreversible) cognitive impairment [308–310]. Their use for longer than two to four weeks or prescription of high doses is generally not recommended, as tolerance to the effects, dependence and withdrawal may occur [292,311–314]. Their use for chronic anxiety should be avoided, rather BZDs could be used short-term until long-term medication (SSRIs, SNRIs) has manifested the desired therapeutic effects [303,315,316]. Long-term use has also been linked to poorer sleep, blepharospasm and impairment of the immune system [317–319]. Dependence is defined as the loss of control of drug use, leading to key diagnostic criteria such as ineffective attempts to cut down on its use, increased time spent on seeking the drug or recovering from its effects, curtailment of activities due to continued drug use and continued use in spite of adverse consequences [320]. Withdrawal of BZDs has been associated with mostly non-specific symptoms (insomnia, anxiety, mood swings, tremor, headache, nausea, sweating, blurred vision) and sensory disturbances (hyper- or hyposensitivity to noise, light, smell, touch and/or taste). Feelings of unreality

have also been reported. In less than 10% of patients, complications such as psychosis or epileptic seizures may develop [279,312]. More severe symptoms include tachycardia or palpitations, hypotension, hyperthermia and hallucinations/delusions [321].

From a forensic point of view, paradoxical excitement may be of concern as it may lead to increased anxiety, hyperactivity, hostility or rage, and assault or rape; observed in up to 20% of patients [38]. They are also increasingly found in driving under the influence of drugs cases [322–326]. Furthermore, multi-drug poisonings involving BZDs are frequently reported and, besides their own abuse potential, BZDs may exacerbate an existing substance use disorder, which often co-occurs in patients with severe mental illnesses such as schizophrenia or bipolar disorder [276,291,314,327–329]. Particularly in combination with alcohol or opioids, respiratory depression and fatal overdoses have been reported, even at subtherapeutic doses [330,331]. Poly-drug use has also been linked to an increased risk of infectious diseases (through injection needles or risky sexual behaviour), criminality and poorer self-reported quality of life [276,332–335]. BZD abuse is exacerbated by increased availability on illicit markers and the development of potent designer compounds [276,325,336]. As of 2020, the European Monitoring Centre for Drugs and Drug Addiction is monitoring 30 designer benzodiazepines, over half of which first appeared in the previous five years [337]. These are often derived from pharmaceutical drug candidates that have not been marketed or from simple structural modifications of registered BZDs, and can be sold under their own name or as counterfeit medication [338,339]. However, their PK properties are unknown, potentially leading to overdoses (by the drug or an active metabolite) or unforeseen long-lasting effects [340].

1.3. SUPPLEMENTARY INFORMATION FOR CHAPTER 1

1.3.1. Figures

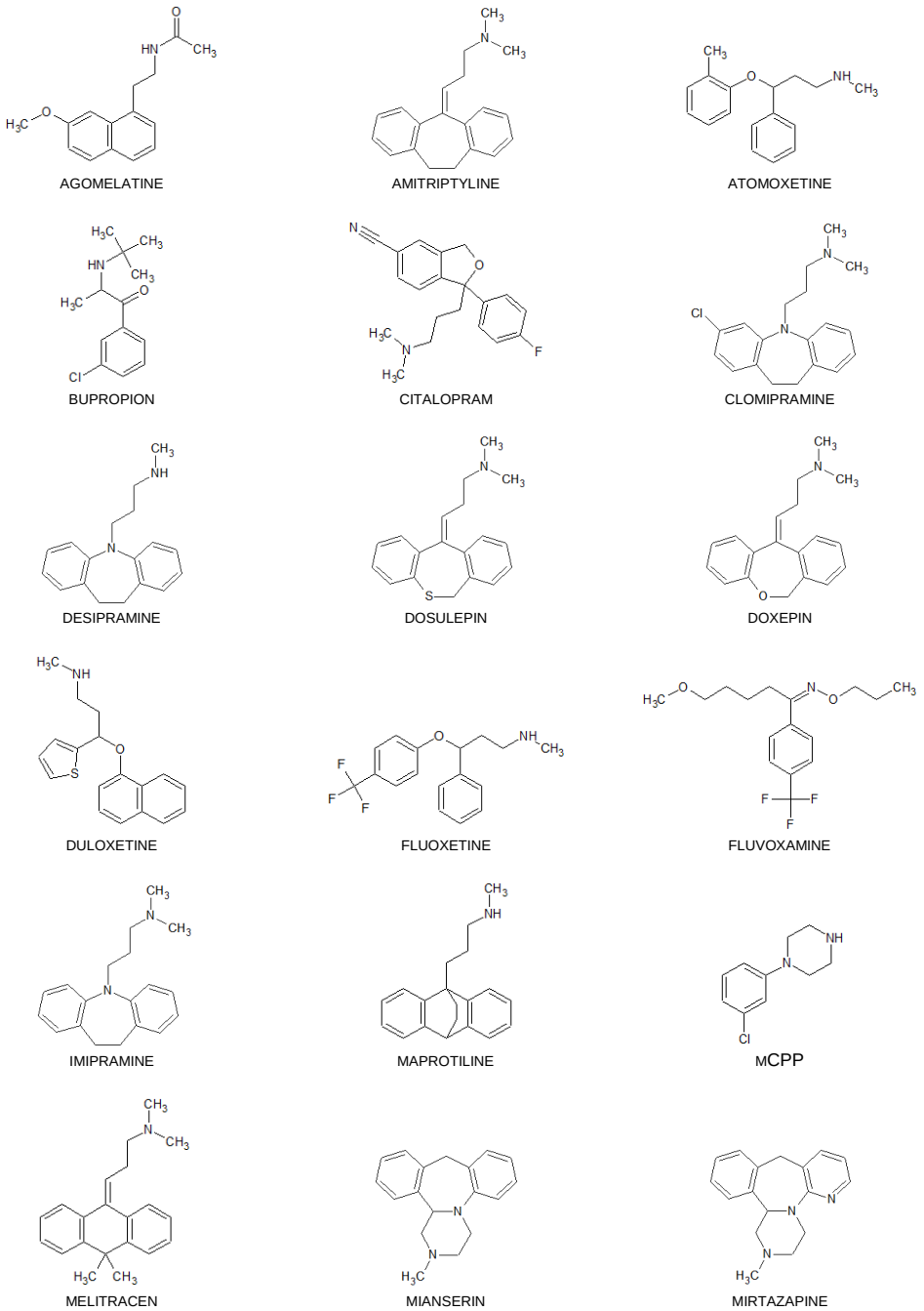
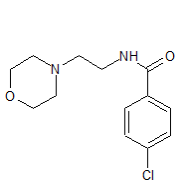


Figure S1 - 1. Analytes of interest belonging to the antidepressants.



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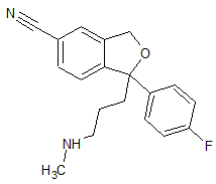

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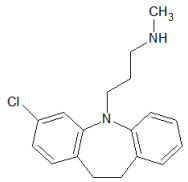

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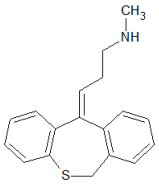

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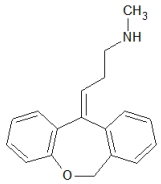

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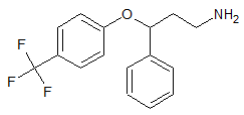

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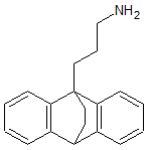

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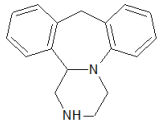

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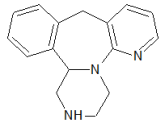

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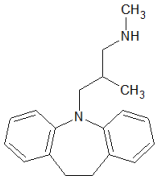

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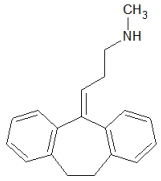

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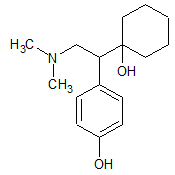

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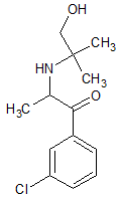

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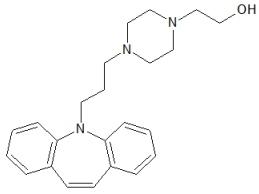

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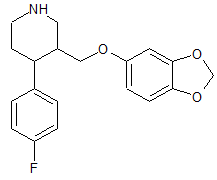

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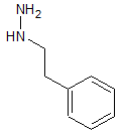

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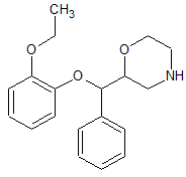

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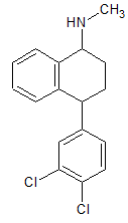
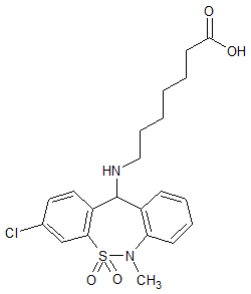


SERTRALINE

Figure S1 - 1. Analytes of interest belonging to the antidepressants. (continued)



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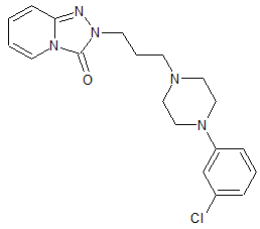

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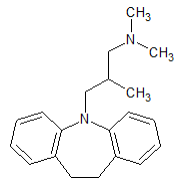

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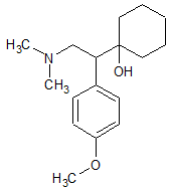
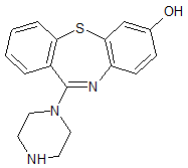


VENLAFAXINE

Figure S1 - 1. Analytes of interest belonging to the antidepressants. (continued)



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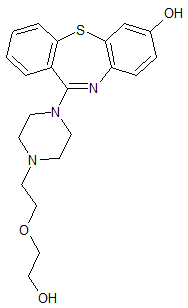

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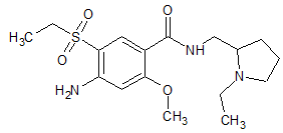

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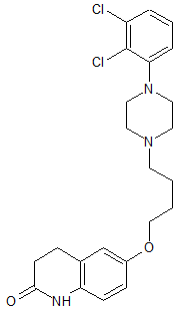

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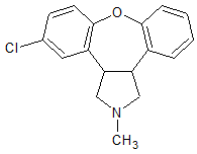

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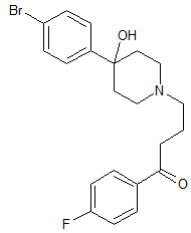

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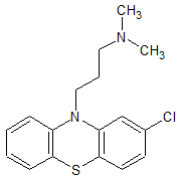

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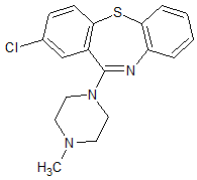

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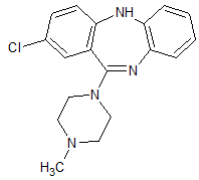

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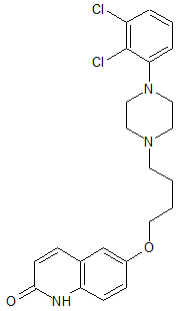

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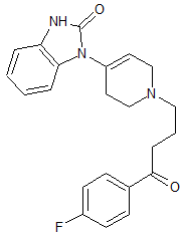
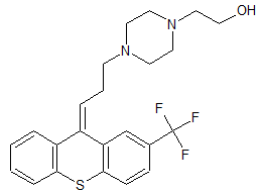
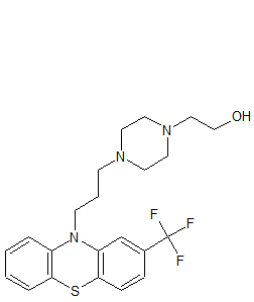


DROPERIDOL



FLUPENTIXOL

Figure S1 - 2. Analytes of interest belonging to the antipsychotics.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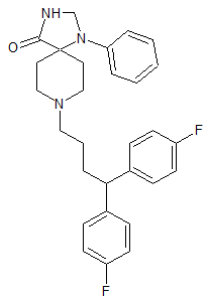
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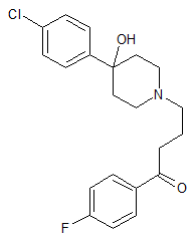
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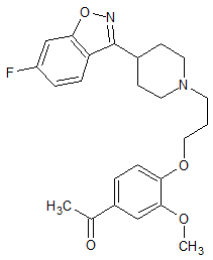
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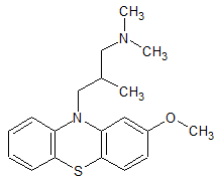
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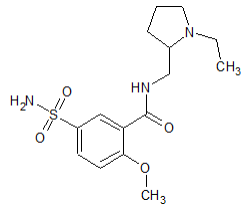
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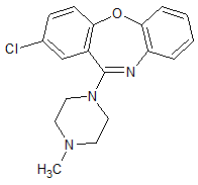
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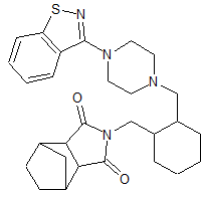
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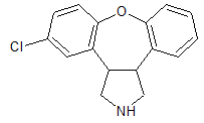
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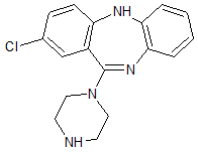
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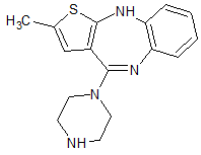
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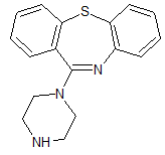
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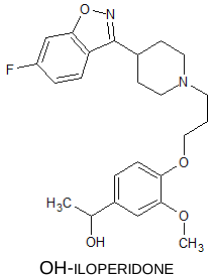
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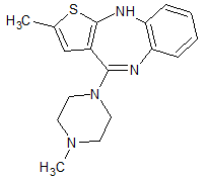
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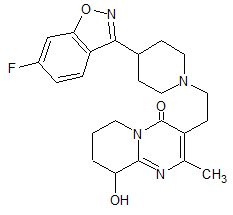
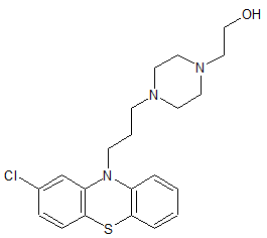

PALIPERIDONE

Figure S1 - 2. Analytes of interest belonging to the antipsychotics. (continued)



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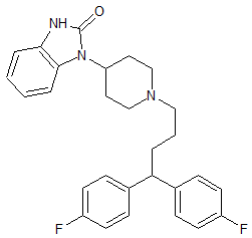

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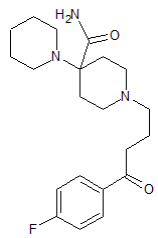

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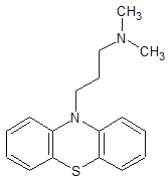

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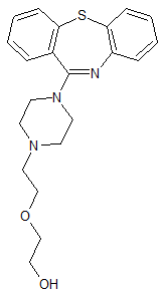

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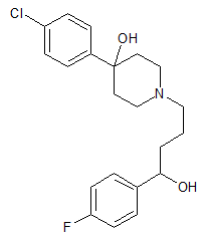
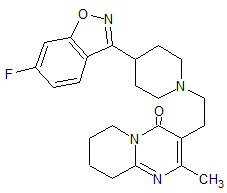
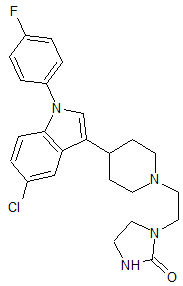


REDUCED HALOPERIDOL



RISPERIDONE



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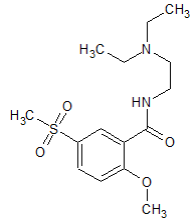

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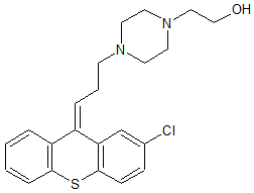
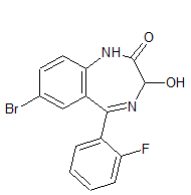
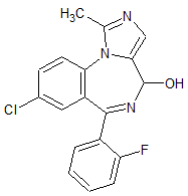


ZUCLOPENTHIXOL

Figure S1 - 2. Analytes of interest belonging to the antipsychotics. (continued)



3-OH-FLUBROMAZEPAM



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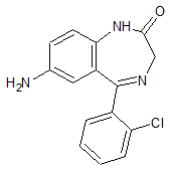

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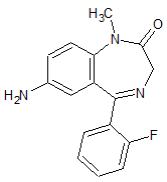

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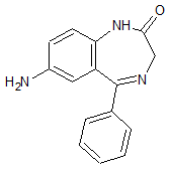

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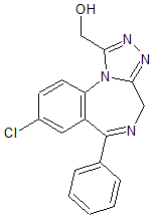
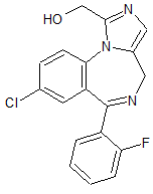


α-OH-ALPRAZOLAM



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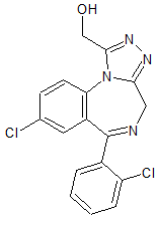

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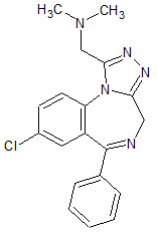

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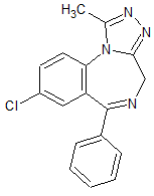
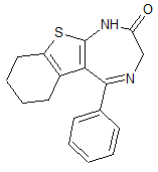


ALPRAZOLAM



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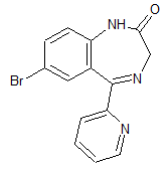

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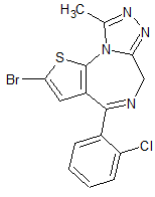

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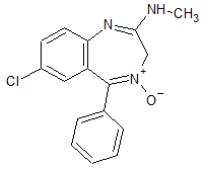

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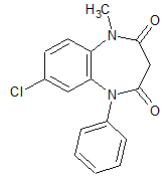

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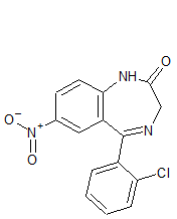
Figure S1 - 3. Analytes of interest belonging to the benzodiazepines and Z-drugs.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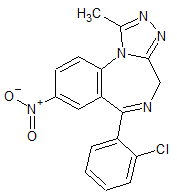
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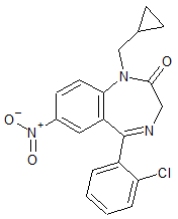
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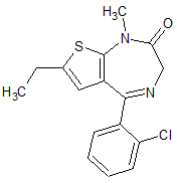
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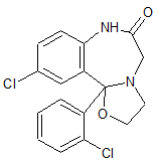
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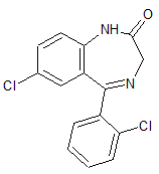
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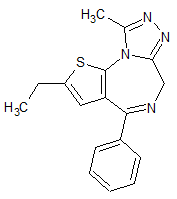
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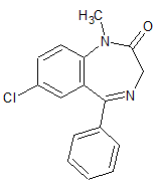
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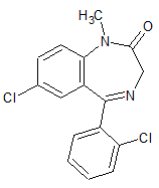
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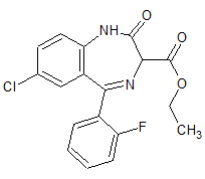
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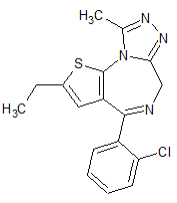
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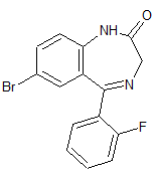
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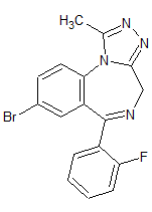
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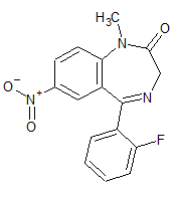
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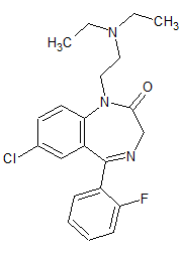
FLURAZEPAM

Figure S1 - 3. Analytes of interest belonging to the benzodiazepines and Z-drugs. (continued)

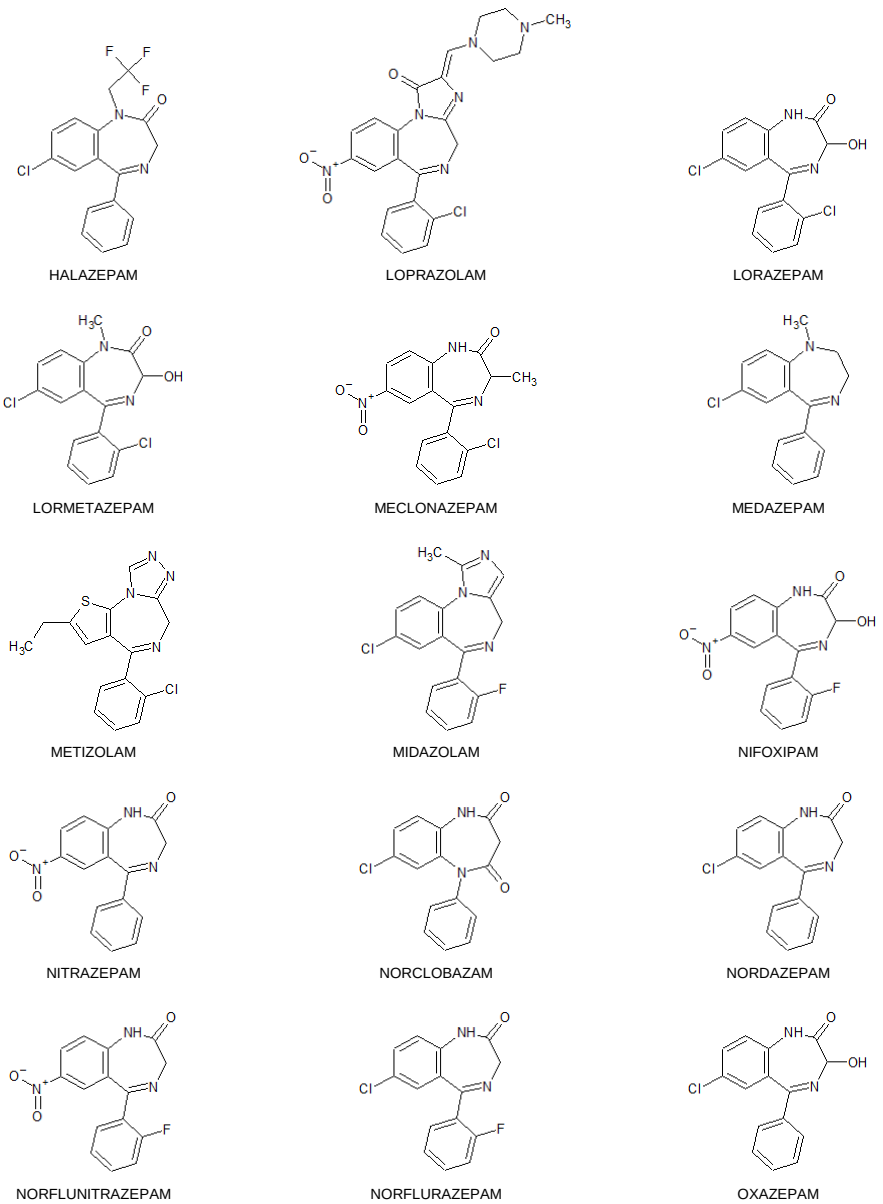

Figure S1 - 3. Analytes of interest belonging to the benzodiazepines and Z-drugs. (continued)

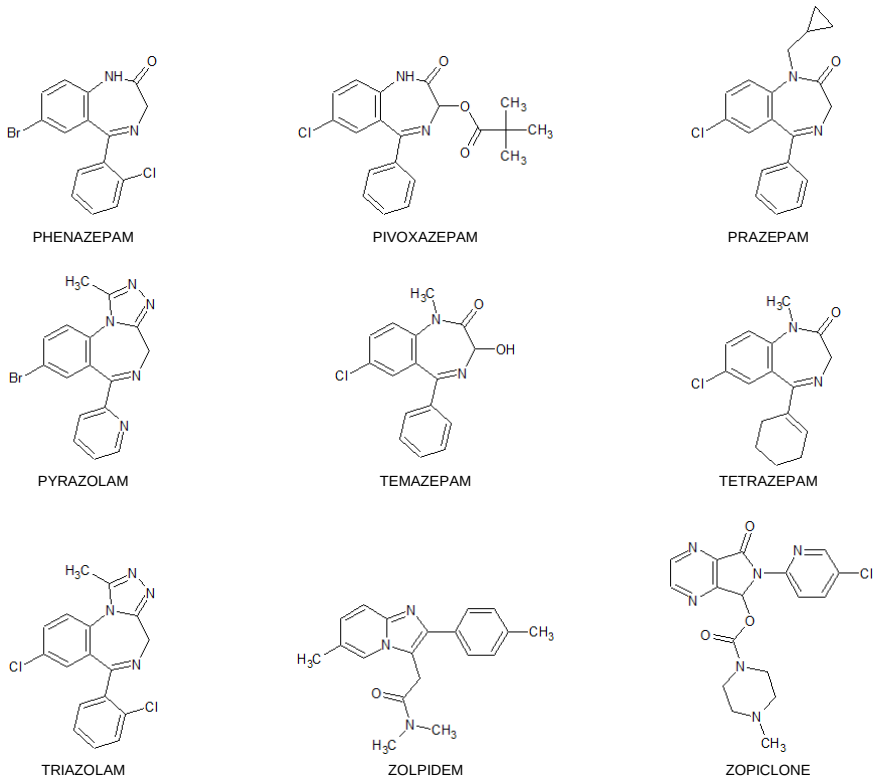

Figure S1 - 3. Analytes of interest belonging to the benzodiazepines and Z-drugs. (continued)

CHAPTER 2

OBJECTIVES & OUTLINE

This thesis will discuss the development and validation of analytical methods for the identification and quantification of psychoactive substances belonging to the classes of the antidepressants, antipsychotics and benzodiazepines/Z-drugs (Figure 2 - 1). The focus of this research is the implementation of these methods in routine forensic casework and, as such, efforts are put in limiting the time spent on sample preparation, instrumental analysis and post-acquisition data handling. For their broader applicability in therapeutic drug monitoring and clinical toxicology, the instrumentation should ideally be widely available, reliable and easy to use. Additionally, data handling should be automated requiring only simple manual checking of each batch by an analyst.

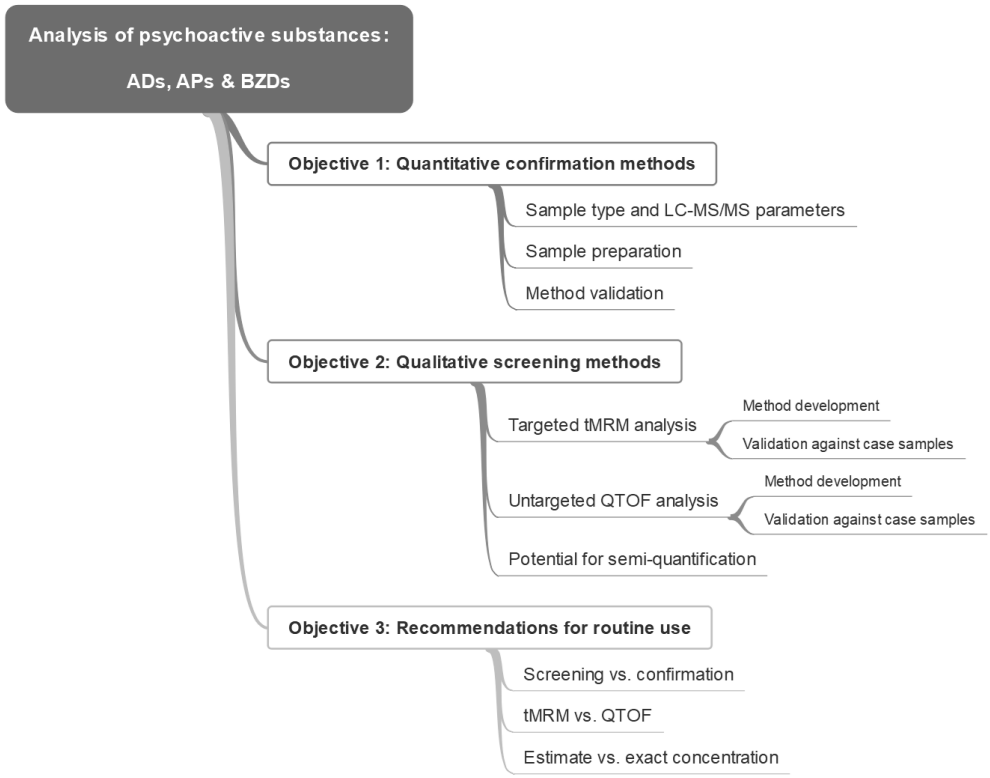

Figure 2 - 1. Objectives of the thesis. Three main objectives were defined: 1) the development and validation of fully quantitative confirmation methods using LC-QQQ, 2) the development and validation of qualitative screening methods using LC-QQQ or LC-QTOF, including an investigation of their semi-quantitative potential, and 3) a discussion on their applicability in a routine setting. Antidepressants, ADs; antipsychotics, APs; benzodiazepines & Z-drugs, BZDs; liquid chromatography, LC; quadrupole time-of-flight mass spectrometry, QTOF; tandem mass spectrometry, MS/MS; triggered multiple reaction monitoring, tMRM; triple quadrupole mass spectrometry, QQQ.

In the first part of this thesis, the development and validation of confirmation analyses will be discussed. Liquid chromatography coupled to triple quadrupole mass spectrometry was selected for its sensitivity, quantitative power and sample preparation without the need of derivatisation. **Chapter 3** will focus on the optimisation of the different analytical parameters, including the most appropriate biological matrix, a chromatographic method with an acceptable run time, and multiple reaction monitoring tandem mass spectrometry. In **Chapter 4**, the most appropriate sample preparation technique will be investigated. Selection criteria include the volume of sample needed, the

applicability to a wide group of compounds, the analyst's hands-on time and total laboratory time, and the efficiency of sample clean-up and ability to detect the intended lowest concentrations. **Chapter 5** will provide an overview of the method validation data in accordance with international guidelines, including accuracy, precision, matrix effect, extraction efficiency and stability under different conditions for each of the analytes of interest.

The second part will elaborate on the development of screening assays. Both a targeted, triggered multiple reaction monitoring method using liquid chromatography coupled to triple quadrupole mass spectrometry (**Chapter 6**) and an untargeted, liquid chromatography – quadrupole time-of-flight method (QTOF, **Chapter 7**) will be investigated. Their performance will be evaluated against that of the confirmation methods developed in the first part by the parallel analysis of medico-legal case samples. Especially for the QTOF analysis, additional time will be dedicated to the data analysis workflow and the potential automation thereof, keeping its applicability in a routine environment in mind. Part of setting up this workflow will also involve the creation of a high-resolution mass spectral database. Lastly, the semi-quantitative potential of both screening methods will be evaluated. Both pure mathematical criteria as well as the toxicologist's interpretation of the case as a whole will be taken into account.

In **Chapter 8** the applicability of the created methods in a routine setting will be analysed. Three main discussion points will be touched: 1) Is it necessary to perform confirmation for all cases or would screening only suffice? 2) Which screening method is preferred, targeted tMRM analysis or untargeted QTOF analysis? 3) Should the exact concentration always be determined or is semi-quantitative analysis more appropriate?

PART I:

QUANTITATIVE METHODS FOR PSYCHOACTIVE SUBSTANCES

CHAPTER 3

METHOD DEVELOPMENT

Based upon the following publications:

M. Degreef, A.L.N. van Nuijs, K.E. Maudens, Validation of a simple, fast liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of 40 antidepressant drugs or their metabolites in plasma, Clin. Chim. Acta. 485 (2018) 243–257. doi:10.1016/J.CCA.2018.06.047.

M. Degreef, E.M. Berry, K.E.K. Maudens, A.L.N. van Nuijs, Multi-analyte LC-MS/MS quantification of 38 antipsychotics and metabolites in plasma: Method validation & application to routine analyses, J. Chromatogr. B. 1179 (2021). doi:10.1016/J.JCHROMB.2021.122867.

M. Degreef, L. Vits, E.M. Berry, K.E.K. Maudens, A.L.N. van Nuijs, Quantification of 54 Benzodiazepines and Z-Drugs, Including 20 Designer Ones, in Plasma, J. Anal. Toxicol. 45 (2021) 141–153. doi:10.1093/jat/bkaa059.

3.1. SELECTING THE APPROPRIATE MATRIX

Generally, *in vivo* available matrices are restricted to blood, urine, hair, oral fluid and sweat [341]. Hair as a matrix benefits from its easy collection, but can be particularly susceptible to environmental contamination (e.g. personal care products). More importantly, hair reflects long-term or less recent exposure, whereas in forensic toxicology and therapeutic drug monitoring (TDM) reflection of drugs circulating in the body at the time of sample collection or of a specific incident is more preferred. Sweat was excluded as a potential matrix because of its small volume available (order of magnitude in μL), its non-homogeneity and the absence of any relationship between the administered dose and the observed concentration [341,342]. Oral fluid has gained particular interest in driving under the influence of drugs cases because of its easy, on-site collection in the absence of specialised medical personnel. However, the observed drug concentration can vary due to the pH and the oral fluid-to-blood ratio may change depending on the in-blood drug concentration. Additionally, the volume of excreted saliva can also be influenced by the circadian rhythm, hormonal changes, nutritional and hydration state and effects of drugs on the parasympathetic and sympathetic nervous system. Potential contamination by food or other exogenous substances and sample dilution/interference by the buffering solution can further affect the results. Corrective actions can be taken, but are often copious or labour-intensive [342–344]. Urine offers a relatively long detection window, is easily collected and – not unimportantly – contains most metabolites which may support the findings and reduce the number of false positive or negative results. However, detected concentrations once more offer little information on the pharmacological effects of a drug on the user at the time of sample collection [341,342]. Therefore, blood was selected as the preferred matrix. It combines an excellent *in vivo* dose-effect correlation with a reflection of circulating drug levels in the body and with a low risk of adulteration or contamination. These findings are not new and indeed, blood has been used in both forensic toxicology and TDM for several decades [345].

Detected drug concentrations in blood can vary based upon the sampling site, the sampling time and the type of blood analysed. For post-mortem forensic purposes, blood samples should ideally be collected from a suitably isolated, peripheral site such as the femoral vein, though blood drawn from the subclavian or iliac veins can be used too [342]. Ante-mortem blood collection sites are less well defined. With regards to the sampling time, it is recommended for TDM to analyse blood that has been obtained immediately prior to administration of the next dose (trough dose) once the steady-state has been reached [7]. Particularly for TDM, serum or plasma are preferred over whole blood as they require less rigorous sample preparation and generally reflect the unbound, biologically available fraction of the compounds [41,346]. Serum tends to result in lower amounts of sample volume and blood clotting during storage, whereas drugs in plasma may be affected by the anticoagulant or other additives in the collection tube. Although serum is said to contain less proteins, the difference is mostly made up by those involved in the blood clotting process, with similar amounts of drug binding ones (e.g. albumins and globulins) in both matrices. Overall, no data have been published which demonstrate substantial differences in concentration between both matrices [7,347]. The influence of anticoagulants on the sample is poorly understood. As a chelating agent, EDTA binds metallic ions and thereby may interfere with the antibody-binding in immunoassays [348]. Kulkarni et al. reported a potential overestimation of anticancer drugs using EDTA-lined collection tubes, although they acknowledged the limitations of their study [349]. The use of heparin blood tubes is discouraged

(they may interfere with plasma protein binding) as are serum-separating tubes (drugs may be absorbed into the separator gel) [347,350–353]. However, the greatest influences on the analyte concentration are expected from inappropriate sample collection and storage prior to or during the analyses.

For their applicability in both TDM and forensic toxicology, and taking into account the at the Toxicological Centre available collection devices and intended storage of the collected blank blood for multiple years, plasma was chosen as the matrix for further method development and validation. Human plasma samples from healthy, drug-free volunteers were collected in 9 mL Vacuette® K_2EDTA tubes (Greiner Bio-One GmbH, Kremsmünster, AT). Blood samples were centrifuged immediately after collection and the plasma was stored at -20 °C for the duration of the experiments. The samples were not pooled to account for inter-individual variability. The donation was approved by the ethical committee of the University Hospital Antwerp (EC/PC/avl/2018.039).

3.2. LIQUID CHROMATOGRAPHY

In line with preliminary experiments on benzodiazepine drugs, a Zorbax Eclipse Plus C8 column (2.1 x 150 mm, 3.5 µm) from Agilent Technologies (Santa Clara, California, US) was selected. The stationary phase consisted of silica with a pore size of 95 Å, double-endcapped with trimethylsilyl chains (Figure 3 - 1). Combined with C18 columns, this type of column is recommended as starting point for method development and benefits from overall good peak shapes, compatibility with most compounds (acids, bases and neutrals) and tolerability to a wide pH range (pH 2-9). In contrast to a similar C18 column, a C8 column tends to be less retentive for non-polar compounds, without loss in selectivity, and therefore allows for a reduction in the overall method run time. The internal diameter of 2.1 mm is marketed as ideal in liquid chromatography (LC) – mass spectrometry (MS) applications where limited sample volume is available, as well as best at reducing solvent use. A length of 150 mm – 250 mm, although it slightly increases analysis times, improves the resolution and is therefore especially beneficial when large numbers of analytes are comprised in one method. The chosen particle size (3.5 µm) is said to provide the highest resolution for high performance LC methods, with lower particle sizes being optimal for ultra-performance LC applications [354,355].

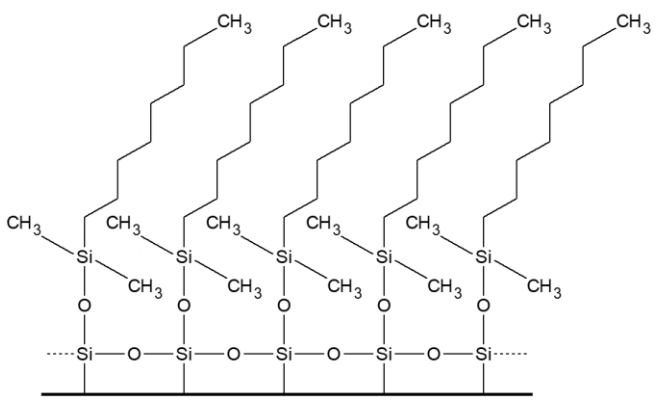

Figure 3 - 1. Stationary phase for the Agilent Zorbax Eclipse Plus C8 column. The silica are double-endcapped to avoid secondary reactions with the analytes. Analytes are retained based upon their preference for the gradually in polarity decreasing mobile phase and the apolar stationary phase.

The mobile phases (MPs) were purposely kept simple. MP A consisted of ultrapure water, in-house generated by an Elga Purelab water purification system (Veolia Water Technologies, Tienen, BE). MP B was made up of a 9:1 mixture of ACN and ultrapure water. To each of the MPs, 0.1% formic acid (V/V) was added, lowering the pH to 3.0 as measured on MP A. In such an acidic environment, the basic analytes would be predominantly ionised, increasing their affinity for the mobile phase over the stationary phase and thus shortening the overall method run time. A gradient elution going from 5% MP B to 95% MP B was applied over 9 min at a flow rate of 0.5 mL/min, followed by 3 min re-equilibration time (Table 3 - 1, Figure 3 - 2). The first analyte (levosulpiride) eluted at 1.7 min, the last (pivoxazepam) at 7.9 min. A total analysis time of 12 min from injection to injection provided the necessary separation, whilst still allowing for an acceptable throughput. To ensure stable flow rates, the system was kept at a constant temperature of 40 °C.

Table 3 - 1. Gradient applied to the dMRM methods. The flow rate was 0.5 mL/min, the total analysis time 12 min from injection to injection. Mobile phase, MP.

Time (min)	MP A (%)	MP B (%)
0.00	95	5
9.00	5	95
9.40	5	95
9.80	95	5
11.40	95	5

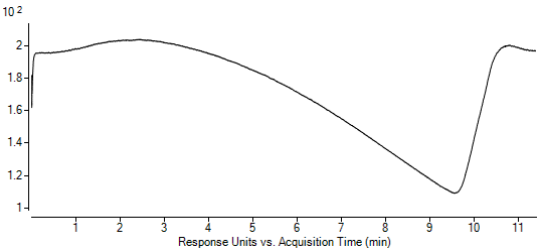

Figure 3 - 2. Pressure profile for the dMRM methods. The y-axis represents the pressure in bars. The maximum pressure was around 215 bars.

3.3. TRIPLE QUADRUPOLE MASS SPECTROMETRY

Analytes were detected by triple quadrupole mass spectrometry (QQQ), fitted with an electrospray ionisation source. Source parameters remained unchanged compared to the standardly programmed ones of the manufacturer: N_2 gas temperature 300 °C, gas flow 10 L/min, nebuliser pressure 30 psi, sheath gas heater 250 °C, sheath gas flow 11 L/min, capillary voltage 4000 V, nozzle voltage 500 V. The QQQ was operated in dynamic multiple reaction monitoring (dMRM) mode. Following ionisation in the source, MRM acquisition methods will specifically select predefined precursor ions in the first quadrupole. The second quadrupole, also named the collision cell, will cause controlled and reproducible fragmentation of these precursor ions into product ions by collision with neutral molecules such as He, N (as used in the here presented methods) or Ar. The third quadrupole will then specifically select the predefined product ions which are detected by the instrument. More than just the presence of all product ions, their relative signal abundances – the ion ratios – are highly specific for a compound. To increase the sensitivity, not all transitions are monitored throughout the entire run. Rather, dMRM modes will only acquire precursor-product ion transitions within a predefined time frame. For the developed methods, this so called retention time (RT) window was set to 0.5 min, indicating that transition monitoring will take place from 0.25 min before until 0.25 min after the set RT of the analyte. The process of selecting appropriate precursor and product ions for each analyte individually will be illustrated below for the benzodiazepine midazolam.

Compound optimisation can occur by direct, continuous infusion of a standard into the mass spectrometer, or – in our case – by repeated injections onto the LC-QQQ in the absence of a column.

Methods can be kept short (0.2 min – 0.5 min) and a simple mobile phase composition suffices, run isocratically at 50:50 mobile phases A:B. Availability of a reference standard is required. We used a standardised concentration of 1 ng/µL for easy comparison of ionisation efficiency between analytes. During the first injections, a MS-spectrum is captured in scan mode with a fragmentor voltage (FV) standardly programmed at 135 V. A first scan going from m/z 100 to m/z 1000 serves to check the purity of the reference standard: is the expected precursor ion present, are there any noteworthy breakdown products (potentially from in-source fragmentation) or are there any Na-, K- or other adducts visible? The scan range can be reduced to one closer around the m/z of the precursor ion to more accurately define that m/z if required. Additionally, the isotope pattern of the molecule is verified in comparison to one predicted by the online Isotope Distribution Calculator and Mass Spec Plotter software (Adaptas Solutions, Palmer, Massachusetts, USA) [356]. Specifically for midazolam, no significant signals were visible apart from that of the precursor ion at m/z 326.1. The typical isotope pattern for chlorine-containing molecules was clearly visible (Figure 3 - 3).

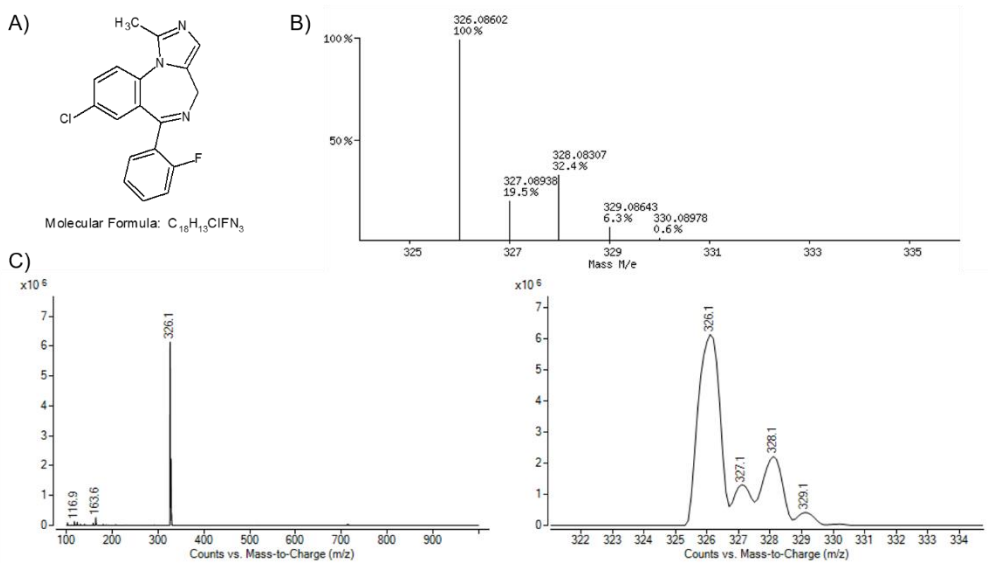

Figure 3 - 3. MS2SCAN compound optimisation for midazolam. The analyte's chemical structure (A) and predicted isotope pattern of its protonated ion (B) are displayed on top. Below chromatograms (C) represent a wide scan (left) and one zoomed in on the mass of the precursor ion (right). Apart from the molecular ion at *m/z* 326.1, no significant breakdown products or adducts are present. The predicted isotope pattern for a chlorine-containing compound can be observed.

From the next injections on, only the specific m/z of the precursor ion is looked at. To optimise the signal intensity of the precursor ion, and thereby the sensitivity of the instrument for that m/z-value, several injections are performed in single ion monitoring mode with varying fragmentor voltages. These can be ramped going from 30 V to 200 V during continuous infusion or increased in steps of 10 V to 15 V with repeated injections. Generally one would expect to see a rise in signal intensity (by evaluating the total ion chromatogram of the precursor ion) until the maximal FV is reached, after which the signal should decrease again. In the example of midazolam, the highest abundance was observed at FV 60 V.

Thus far, only parameters specific for the first quadrupole have been optimised. The next step would be the identification of the product ions. In the appropriately named product ion mode, the first quadrupole selectively monitors the m/z of the precursor ion, while the third quadrupole scans for any product ions present. Generally an m/z-range from 100 to just above that of the precursor ion suffices, though lower m/z-values may be investigated based upon literature or in the otherwise absence of high abundant product ions. In the second quadrupole, the collision energy (CE) was varied between 10 eV and 50 eV, though higher CEs may be applied if required. Higher CEs lead to more extensive fragmentation of the product ion but no relationship between the CE and the size of the fragment seems present. The most abundant or prevalent product ions (preferably a minimum of five) are retained for further investigation. For midazolam, four product ions were selected: m/z 209.1, m/z 223.1, m/z 249.1 and m/z 291.2 (Figure 3 - 4). Based upon literature, a potential product ion at m/z 244.1 was also added to that list [357].

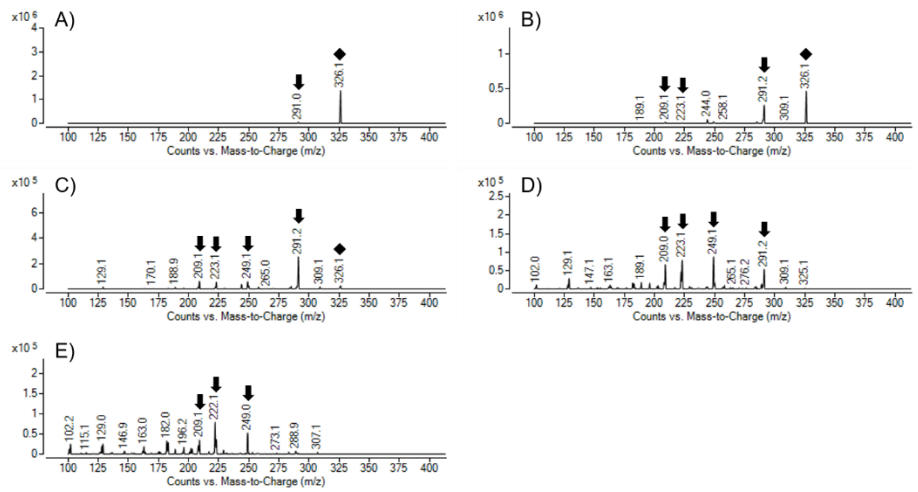

Figure 3 - 4. Product ion selection for midazolam. Scans have been acquired with increasing collision energies: A) 10 eV, B) 20 eV, C) 30 eV, D) 40 eV and E) 50 eV. The precursor ion is indicated by the diamond, selected product ions by the arrows. Based upon literature a potential product ion at *m/z* 244.1 was also selected for further optimisation.

In a final step, the method is switched to MRM mode. Rather than adding several different transitions, the same precursor-product ion pair is monitored multiple times, with varying CEs in steps of 5 eV to determine the optimal value (defined as highest abundance) for that specific product ion. Based upon these findings, the final precursor-product ion pairs can be selected per analyte. In general, the most abundant product ion would be selected as quantifier ion, the peak area of which is used to calculate the concentration of the analyte in a sample. Ions originating from the loss of water, characterised as an m/z-difference of 18 between precursor and product ion, may occur at random and should not be set as quantifier ion. Also product ions that are shared with other analytes from the same class should not be selected for quantification purposes as they might not be sufficiently specific. The next most abundant product ions serve as qualifier ions. Their ratio to the quantifier ion should be determined in the intended matrix. If this qualifier-quantifier ion ratio exceeds ± 30% of the nominal value, an analyte must not be listed as detected regardless of the

presence of all product ions. To ensure the highest confidence in the identification of the analytes, we opted to include where possible three precursor-product ion transitions per analyte (two for the internal standards). An overview of the tandem-MS settings for all selected analytes can be found in Table S3 - 1. Specifically for midazolam, the quantifier ion was *m/z* 291.2, with qualifier ions *m/z* 223.1 and *m/z* 249.1. The respective ion ratios were 100%, 23% and 23% (Figure 3 - 5). Final creation of a dMRM method requires determination of the RT with the LC column installed.

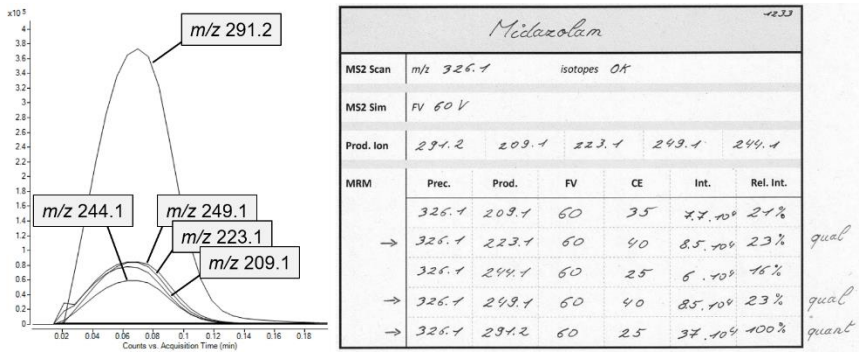

Figure 3 - 5. Optimised MRM parameters for midazolam. The transition to *m/z* 291.2 was the most predominant one and therefore was selected as the quantifier transition. Product ions at *m/z* 223.1 and *m/z* 249.1 were the next most abundant ones and will make up the qualifier ions in the final method.

Particular problems were observed during the optimisation of adinazolam. The most abundant product ion was *m/z* 58.2. Other product ions were not present in relative abundances greater than 1% (Figure 3 - 6). Therefore, it was opted to include a second precursor ion, based upon the isotope pattern of adinazolam. Indeed, the molecule contains a chlorine atom, which is naturally present as ^{35}Cl (100%) and ^{37}Cl (33%). The ion pair *m/z* 352.0 → *m/z* 58.2 was selected as quantifier transition, the ion pair *m/z* 354.0 → *m/z* 58.2 as qualifier transition.

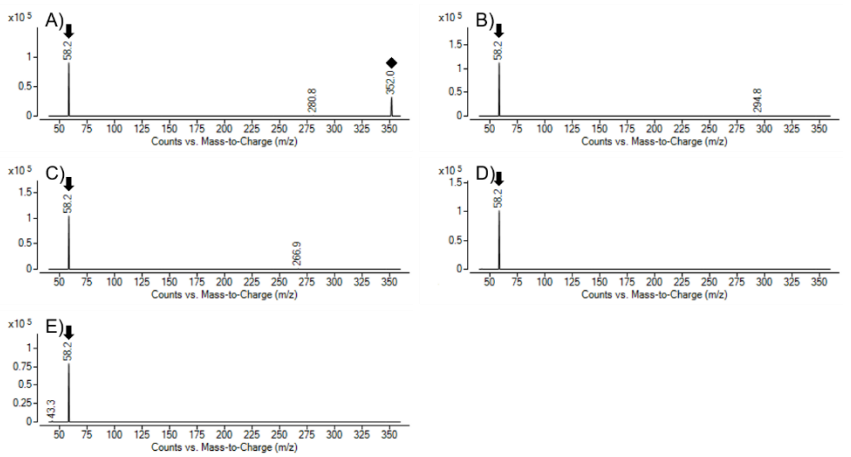

Figure 3 - 6. Product ion selection for adinazolam. Scans have been acquired with increasing collision energies: A) 10 eV, B) 20 eV, C) 30 eV, D) 40 eV and E) 50 eV. The precursor ion is indicated by the diamond, selected product ions by the arrows. Only one product ion was found (*m/z* 58.2). Therefore, it was opted to include a second precursor ion (*m/z* 354.0) based upon the Cl-related isotope pattern of adinazolam.

3.4. SUPPLEMENTARY INFORMATION FOR CHAPTER 3

3.4.1. Tables

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
			ANTIDEPRESSANTS		
agomelatine	244.1	75	185.0 (100%)	10	5.45
			170.0 (29%)	25	
			141.0 (22%)	50	
amitriptyline	278.2	95	233.1 (100%)	10	4.65
			218.1 (48%)	20	
			105.1 (140%)	22	
amitriptyline-D_3	281.1	95	233.1 (100%)	12	4.65
			105.1 (131%)	20	
atomoxetine	256.1	55	44.2 (100%)	6	4.41
			65.1 (1%)	97	
bupropion	240.1	65	184.0 (100%)	6	3.48
			166.0 (42%)	13	
			131.0 (53%)	25	
bupropion-D_9	249.1	65	185.0 (100%)	6	3.48
			167.0 (37%)	13	
citalopram	325.1	110	109.0 (100%)	25	4.11
			262.0 (29%)	15	
			83.0 (13%)	75	
citalopram-D_6	331.2	115	109.1 (100%)	25	4.12
			262.1 (31%)	15	
clomipramine	315.1	100	86.1 (100%)	13	4.95
			58.1 (54%)	45	
clomipramine-D_3	318.1	105	89.1 (100%)	13	4.95
			61.1 (50%)	45	
desipramine	267.2	90	72.2 (100%)	10	4.47
			208.2 (5%)	20	
desipramine-D_3	270.4	80	75.1 (100%)	12	4.45
			208.1 (6%)	20	
dosulepin	296.1	70	203.1 (100%)	35	4.45
			221.1 (38%)	47	
			178.1 (87%)	48	
dosulepin-D_3	299.1	60	203.1 (100%)	35	4.45
			178.1 (129%)	50	
doxepin	280.1	90	107.0 (100%)	20	4.18
			165.1 (29%)	60	
			115.0 (31%)	52	
doxepin-D_3	283.0	90	107.0 (100%)	20	4.19
			165.0 (31%)	60	
duloxetine	298.1	75	44.2 (100%)	7	4.59
			154.1 (13%)	2	
duloxetine-D_7	305.1	75	44.2 (100%)	7	4.58
			154.1 (14%)	2	
fluoxetine	310.0	65	44.2 (100%)	6	4.87
			148.0 (5%)	2	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
fluoxetine-D_6	316.1	70	44.2 (100%)	6	4.87
			154.1 (5%)	2	
fluvoxamine	319.1	80	71.1 (100%)	9	4.60
			259.0 (5%)	5	
			200.0 (8%)	15	
fluvoxamine-D_3	322.1	75	74.1 (100%)	10	4.59
			200.0 (6%)	15	
imipramine	281.1	80	58.1 (100%)	42	4.53
			86.1 (224%)	10	
imipramine-D_3	284.1	75	61.1 (100%)	43	4.53
			89.1 (199%)	12	
maprotiline	278.1	60	250.1 (100%)	13	4.64
			219.1 (55%)	22	
			169.1 (25%)	13	
maprotiline-D_5	283.2	55	255.1 (100%)	15	4.64
			221.1 (65%)	22	
mCPP	197.0	95	154.0 (100%)	18	3.09
			119.0 (52%)	25	
			118.0 (73%)	37	
mCPP-D_8	205.1	95	158.1 (100%)	17	3.08
			123.1 (70%)	25	
melitracen	292.2	75	247.1 (100%)	13	4.93
			232.1 (98%)	22	
			217.1 (108%)	35	
melitracen-D_6	298.2	73	253.1 (100%)	15	4.92
			217.1 (95%)	35	
mianserin	265.2	65	58.1 (100%)	23	4.0
			208.0 (63%)	17	
			91.0 (28%)	48	
mianserin-D_3	268.2	70	61.1 (100%)	25	4.08
			208.0 (53%)	17	
mirtazapine	266.1	80	195.0 (100%)	23	2.82
			209.1 (11%)	17	
			72.1 (68%)	15	
mirtazapine-D_3	269.2	80	195.0 (100%)	23	2.80
			75.1 (65%)	15	
moclobemide	269.0	65	182.0 (100%)	15	2.64
			139.0 (65%)	32	
			111.0 (52%)	53	
moclobemide-D_8	277.1	70	182.0 (100%)	15	2.63
			139.0 (65%)	33	
norcitalopram	311.1	60	109.0 (100%)	22	4.05
			262.1 (44%)	12	
			83.1 (19%)	70	
norcitalopram-D_3	314.1	65	109.0 (100%)	23	4.06
			262.0 (43%)	11	
norclomipramine	301.1	75	72.1 (100%)	12	4.88
			44.1 (17%)	48	
norclomipramine-D_3	304.1	75	75.1 (100%)	12	4.87
			47.1 (24%)	48	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
nordosulepin	282.1	65	223.1 (100%)	18	4.38
			218.1 (187%)	18	
			203.0 (63%)	33	
nordoxepin	266.1	80	107.0 (100%)	18	4.09
			235.1 (40%)	8	
			115.0 (18%)	50	
nordoxepin-D_3	269.3	90	107.0 (100%)	17	4.11
			235.0 (28%)	10	
norfluoxetine	296.0	60	134.0 (100%)	2	4.77
			30.0 (146%)	2	
norfluoxetine-D_6	302.2	65	140.1 (100%)	1	4.76
			30.1 (30%)	2	
normaprotiline	264.1	85	169.1 (100%)	13	4.51
			247.1 (23%)	10	
			219.1 (44%)	18	
normianserin	251.1	60	208.1 (100%)	17	4.02
			118.1 (32%)	25	
			91.1 (41%)	47	
normirtazapine	252.0	75	195.0 (100%)	20	2.65
			209.0 (22%)	20	
			194.0 (26%)	40	
normirtazapine-D_6	258.1	70	197.1 (100%)	22	2.64
			196.1 (26%)	42	
nortrimipramine	281.4	85	86.1 (100%)	10	4.67
			208.1 (8%)	18	
			193.0 (8%)	38	
nortriptyline	264.1	70	233.1 (100%)	7	4.59
			218.1 (25%)	20	
			105.1 (76%)	15	
nortriptyline-D_3	267.1	70	233.1 (100%)	8	4.59
			105.1 (88%)	15	
O-desmethyl-venlafaxine	264.2	75	58.1 (100%)	15	2.69
			246.1 (23%)	5	
			107.1 (11%)	35	
O-desmethyl-venlafaxine-D_6	270.2	75	64.1 (100%)	15	2.69
			252.2 (20%)	5	
OH-bupropion	256.1	65	238.0 (100%)	5	3.04
			167.0 (16%)	19	
			139.0 (18%)	25	
OH-bupropion-D_6	262.1	65	244.1 (100%)	5	3.03
			139.0 (19%)	25	
opipramol	364.1	85	171.1 (100%)	16	3.73
			143.1 (34%)	27	
			70.1 (25%)	45	
opipramol-D_4	368.1	95	175.1 (100%)	17	3.70
			147.1 (36%)	28	
paroxetine	330.0	110	70.1 (100%)	28	4.43
			192.1 (55%)	15	
			151.0 (13%)	20	
paroxetine-D_6	336.0	95	76.1 (100%)	30	4.43
			198.1 (87%)	17	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
reboxetine	314.1	55	176.1 (100%)	5	4.18
			91.1 (28%)	30	
			85.1 (13%)	25	
reboxetine-D_5	319.1	55	176.1 (100%)	5	4.16
			91.1 (24%)	30	
sertraline	306.1	60	159.0 (100%)	22	4.85
			275.0 (63%)	4	
			123.0 (16%)	55	
sertraline-D_3	309.1	70	159.0 (100%)	23	4.86
			275.0 (65%)	4	
tianeptine	437.3	100	291.9 (100%)	10	4.19
			228.0 (24%)	37	
			165.1 (8%)	83	
tianeptine-D_{12}	449.1	100	291.9 (100%)	10	4.17
			228.0 (36%)	38	
trazodone	372.1	100	176.0 (100%)	20	3.64
			148.0 (83%)	33	
			78.0 (62%)	65	
trazodone-D_6	378.1	110	182.0 (100%)	22	3.64
			150.0 (70%)	35	
trimipramine	295.2	90	100.1 (100%)	12	4.74
			58.1 (75%)	38	
trimipramine-D_3	298.1	85	103.1 (100%)	12	4.75
			61.1 (69%)	37	
venlafaxine	278.2	90	58.1 (100%)	15	3.53
			260.2 (25%)	5	
			121.1 (12%)	25	
venlafaxine-D_6	284.2	85	64.2 (100%)	15	3.53
			266.2 (22%)	5	
		ANTIPSYCHOTICS			
7-OH-norquetiapine	312.1	172	226.0 (100%)	26	2.00
			164.0 (82.5%)	62	
			208.0 (62%)	38	
7-OH-norquetiapine-D_8	320.2	172	226.0 (100%)	26	1.97
			164.0 (78.5%)	62	
7-OH-quetiapine	400.2	172	269.0 (100%)	18	2.30
			237.1 (16%)	42	
			295.0 (14%)	22	
7-OH-quetiapine-D_8	408.2	196	274.1 (100%)	22	2.26
			302.1 (30.5%)	26	
amisulpride	370.2	188	242.0 (100%)	26	2.52
			196.0 (46%)	42	
			112.1 (34%)	22	
amisulpride-D_5	375.2	188	242.0 (100%)	26	2.50
			196.0 (47%)	42	
aripiprazole	448.2	228	285.1 (100%)	22	4.60
			98.1 (41.5%)	38	
			176.1 (37.5%)	30	
aripiprazole-D_8	456.2	220	293.1 (100%)	26	4.59
			176.0 (46.5%)	30	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
asenapine	286.1	172	229.0 (100%)	18	4.28
			166.0 (99.5%)	34	
			215.0 (43.5%)	30	
asenapine-^{13}C-D$_3$	290.1	172	229.0 (100%)	22	4.28
			166.0 (105.5%)	34	
bromperidol	420.1	172	165.0 (100%)	22	4.42
			123.0 (46%)	46	
			402.0 (7%)	14	
chlorpromazine	319.0	95	86.1 (100%)	15	4.79
			58.1 (57%)	40	
			214.0 (3.5%)	40	
clotiapine	344.0	65	287.0 (100%)	16	4.52
			255.0 (49%)	30	
			209.0 (6.5%)	30	
clotiapine-D$_8$	352.0	60	292.0 (100%)	17	4.50
			260.0 (50.5%)	32	
clozapine	327.1	172	270.0 (100%)	18	3.49
			192.0 (52%)	46	
			164.0 (14.5%)	90	
clozapine-D$_8$	335.2	172	275.1 (100%)	22	3.45
			192.0 (59%)	50	
dehydro-aripiprazole	446.1	176	285.1 (100%)	18	4.42
			98.1 (24%)	42	
			84.1 (3.5%)	62	
dehydro-aripiprazole-D$_8$	454.2	214	293.1 (100%)	22	4.41
			102.1 (18.5%)	46	
droperidol	380.0	125	165.0 (100%)	23	3.73
			194.0 (74.5%)	10	
			123.1 (59%)	53	
flupentixol	435.2	175	305.1 (100%)	25	4.99
			265.1 (68.5%)	37	
			390.1 (59.5%)	20	
flupentixol-D$_4$	439.2	170	305.1 (100%)	27	4.98
			265.1 (76%)	40	
fluphenazine	438.0	165	171.1 (100%)	22	4.84
			143.1 (57.5%)	28	
			70.1 (31%)	50	
fluspirilene	476.1	165	98.0 (100%)	33	5.34
			371.1 (30%)	15	
			55.1 (15%)	65	
haloperidol	376.2	172	165.0 (100%)	22	4.33
			123.0 (81%)	42	
			95.1 (20%)	82	
haloperidol-D$_4$	380.2	172	165.0 (100%)	22	4.31
			123.0 (75.5%)	42	
iloperidone	427.2	196	261.1 (100%)	26	4.28
			233.1 (61%)	30	
			190.0 (57.5%)	42	
iloperidone-D$_3$	430.2	196	261.1 (100%)	26	4.27
			190.0 (55%)	42	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
levomepromazine	329.1	65	100.1 (100%)	15	4.62
			58.1 (63%)	40	
			242.0 (7.5%)	18	
levosulpiride	342.2	188	112.1 (100%)	22	1.73
			214.0 (29.5%)	30	
			110.1 (1%)	42	
loxapine	328.0	60	271.0 (100%)	18	4.24
			193.0 (26%)	48	
			164.0 (12%)	78	
loxapine-D_8	336.0	55	276.0 (100%)	20	4.22
			193.0 (23%)	50	
lurasidone	493.3	260	166.1 (100%)	42	5.12
			177.0 (33%)	46	
			120.1 (30%)	66	
lurasidone-D_8	501.3	260	166.1 (100%)	46	5.12
			120.1 (43.5%)	66	
norasenapine	272.1	90	165.0 (100%)	57	4.17
			229.0 (128%)	13	
			166.0 (74.5%)	30	
norclozapine	313.1	172	192.0 (100%)	42	3.21
			270.0 (71%)	22	
			227.0 (17%)	26	
norclozapine-D_8	321.2	172	192.0 (100%)	46	3.17
			275.1 (33%)	22	
norolanzapine	299.1	176	198.0 (100%)	38	1.88
			256.0 (98%)	22	
			213.0 (66%)	26	
norolanzapine-D_8	307.2	176	198.0 (100%)	38	1.91
			213.0 (62%)	26	
norquetiapine	296.1	110	210.0 (100%)	30	3.65
			139.0 (60%)	66	
			183.0 (47%)	42	
norquetiapine-D_8	304.2	115	210.0 (100%)	30	3.60
			139.0 (34.5%)	70	
OH-iloperidone	429.2	196	261.1 (100%)	18	4.04
			190.0 (20.5%)	42	
			233.1 (16.5%)	30	
OH-iloperidone-D_4	433.3	196	261.1 (100%)	18	4.01
			190.0 (20%)	42	
olanzapine	313.2	176	256.0 (100%)	18	1.93
			198.0 (22%)	42	
			169.0 (12.5%)	42	
olanzapine-D_3	316.2	176	256.0 (100%)	18	1.95
			198.0 (22%)	42	
paliperidone	427.2	176	207.1 (100%)	26	3.29
			110.0 (19%)	46	
			82.1 (6%)	58	
paliperidone-D_4	431.2	176	211.1 (100%)	26	3.29
			114.1 (17.5%)	46	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
perphenazine	404.1	140	171.1 (100%)	20	4.49
			143.1 (64%)	27	
			70.1 (34%)	45	
pimozide	462.1	70	109.1 (100%)	55	5.14
			328.1 (75.5%)	27	
			147.1 (71%)	38	
pipamperone	376.2	166	165.0 (100%)	26	2.34
			123.0 (62.5%)	50	
			291.1 (48%)	14	
prothipendyl	286.1	65	241.0 (100%)	10	3.99
			213.0 (48%)	26	
			181.0 (40.5%)	43	
prothipendyl-D_6	292.1	60	241.0 (100%)	10	3.98
			213.0 (45.5%)	27	
quetiapine	384.2	172	253.0 (100%)	18	3.84
			221.1 (46%)	38	
			279.1 (16.5%)	22	
quetiapine-D_8	392.2	172	226.1 (100%)	38	3.81
			257.7 (103.5%)	2	
reduced haloperidol	378.2	166	149.0 (100%)	26	4.08
			109.0 (41%)	58	
			342.1 (13.5%)	18	
reduced haloperidol-D_4	382.2	166	149.0 (100%)	26	4.07
			109.0 (45%)	54	
risperidone	411.2	188	191.1 (100%)	26	3.30
			110.0 (6%)	54	
			82.1 (4.5%)	66	
risperidone-D_4	415.3	188	195.1 (100%)	26	3.30
			114.1 (4.5%)	54	
sertindole	441.2	214	113.1 (100%)	30	5.08
			71.2 (10.5%)	54	
sertindole-D_4	445.2	214	117.1 (100%)	34	5.08
			73.2 (13%)	58	
tiapride	329.0	55	256.0 (100%)	15	1.98
			213.0 (27.5%)	32	
			212.0 (14%)	28	
zuclopenthixol	401.2	176	100.1 (100%)	26	4.68
			230.9 (100%)	38	
			221.0 (85.5%)	58	
zuclopenthixol-D_4	405.2	176	221.0 (100%)	58	4.68
			231.0 (141%)	34	
BENZODIAZEPINES & Z-DRUGS					
3-OH-flubromazepam	349.0	120	303.0 (100%)	20	5.24
			273.0 (15%)	28	
			194.0 (14%)	45	
4-OH-midazolam	342.0	65	297.0 (100%)	27	3.72
			234.0 (76%)	22	
			109.0 (56%)	30	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
7-amino-clonazepam	286.1	100	121.1 (100%)	28	2.85
			222.1 (70%)	21	
			250.1 (47%)	15	
7-amino-clonazepam-D_4	290.1	100	121.1 (100%)	28	2.82
			226.1 (68%)	22	
7-amino-flunitrazepam	284.1	75	135.1 (100%)	25	3.29
			227.1 (33%)	22	
			226.1 (26%)	30	
7-amino-flunitrazepam-D_7	291.1	75	138.1 (100%)	25	3.26
			230.1 (22%)	30	
7-amino-nitrazepam	252.0	115	121.0 (100%)	25	1.88
			146.0 (10%)	25	
			104.0 (8%)	45	
7-amino-nitrazepam-D_5	257.0	120	121.0 (100%)	25	1.85
			146.0 (9%)	25	
α-OH-alprazolam	325.1	85	297.1 (100%)	22	4.96
			216.1 (46%)	40	
			205.1 (38%)	47	
α-OH-midazolam	342.0	95	168.0 (100%)	40	3.99
			324.0 (227%)	15	
			203.0 (88%)	23	
α-OH-triazolam	359.0	150	331.0 (100%)	25	4.98
			176.0 (75%)	25	
			250.0 (38%)	42	
adinazolam	352.0	75	58.2 (100%)	17	3.63
	354.0		58.2 (33%)	15	
alprazolam	309.1	80	281.1 (100%)	23	5.34
			205.1 (83%)	42	
			274.1 (30%)	22	
alprazolam-D_5	314.1	80	286.1 (100%)	23	5.31
			210.1 (78%)	43	
bentazepam	297.1	90	166.1 (100%)	25	3.89
			269.1 (30%)	20	
			139.1 (27%)	37	
bromazepam	316.0	100	182.1 (100%)	30	4.26
			209.1 (64%)	25	
			288.1 (25%)	15	
bromazepam-D_4	322.0	110	186.1 (100%)	30	4.23
			213.1 (55%)	25	
brotizolam	395.0	140	314.1 (100%)	18	5.62
			279.0 (22%)	25	
			210.0 (16%)	43	
chlordiazepoxide	300.0	100	227.0 (100%)	20	3.55
			241.0 (28%)	10	
			255.0 (20%)	15	
clobazam	301.1	75	259.1 (100%)	15	5.94
			224.1 (27%)	31	
			153.1 (9%)	41	
clobazam-D_5	306.1	75	264.1 (100%)	15	5.91
			229.1 (18%)	32	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
clonazepam	316.0	125	270.0 (100%)	21	5.38
			214.0 (30%)	38	
			241.0 (22%)	35	
clonazepam-D_4	320.0	150	274.1 (100%)	24	5.35
			218.0 (26%)	40	
clonazolam	354.0	150	308.0 (100%)	25	5.02
			280.0 (25%)	35	
			326.0 (18%)	22	
cloniprazepam	370.0	90	316.0 (100%)	17	7.01
			270.0 (45%)	30	
			214.0 (13%)	50	
clotiazepam	319.1	80	291.1 (100%)	20	5.85
			154.1 (63%)	25	
			218.1 (37%)	25	
cloxazolam	349.0	85	305.0 (100%)	20	3.03
			140.0 (38%)	38	
			165.0 (10%)	37	
delorazepam	305.0	100	140.0 (100%)	28	5.87
			165.0 (34%)	27	
			206.1 (31%)	35	
deschloro-etizolam	309.0	60	255.0 (100%)	20	4.90
			280.0 (50%)	20	
			240.0 (27%)	40	
diazepam	285.1	80	193.1 (100%)	30	6.12
			154.0 (87%)	25	
			222.1 (51%)	25	
diazepam-D_5	290.1	75	198.1 (100%)	30	6.09
			154.0 (87%)	25	
diclazepam	319.0	75	154.0 (100%)	27	6.47
			227.0 (89%)	30	
			291.0 (27%)	20	
ethyl loflazepate	361.0	100	259.0 (100%)	28	6.55
			287.0 (43%)	15	
			289.0 (35%)	15	
etizolam	343.0	70	314.0 (100%)	23	5.67
			289.0 (80%)	23	
			274.0 (18%)	42	
flubromazepam	333.0	115	226.0 (100%)	27	5.73
			184.0 (80%)	30	
			179.0 (47%)	50	
flubromazolam	371.0	75	292.0 (100%)	25	5.35
			223.0 (87%)	45	
			343.0 (80%)	25	
flunitrazepam	314.1	125	268.1 (100%)	22	5.70
			239.1 (35%)	34	
			183.0 (23%)	57	
flurazepam	388.1	85	315.1 (100%)	18	4.11
			288.1 (11%)	20	
			134.1 (9%)	57	
flurazepam-D_{10}	398.1	85	315.1 (100%)	18	4.08
			134.1 (9%)	57	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
halazepam	353.0	90	241.0 (100%)	40	7.37
			222.0 (62%)	30	
			193.0 (50%)	35	
loprazolam	465.1	200	85.1 (100%)	25	3.94
			111.1 (92%)	25	
			408.2 (67%)	25	
lorazepam	321.0	70	275.0 (100%)	20	5.36
			303.0 (47%)	8	
			229.0 (26%)	30	
lormetazepam	335.0	100	289.0 (100%)	16	5.95
			177.0 (16%)	42	
lormetazepam-^{13}C-D$_3$	341.0	100	295.0 (100%)	16	5.92
			180.1 (3%)	42	
meclonazepam	330.0	145	284.0 (100%)	23	5.89
			214.0 (21%)	40	
			204.0 (21%)	42	
medazepam	271.0	95	91.0 (100%)	33	4.07
			207.0 (59%)	15	
			180.0 (42%)	15	
metizolam	329.0	65	275.0 (100%)	25	5.47
			300.0 (27%)	20	
			260.0 (26%)	42	
midazolam	326.1	60	291.2 (100%)	25	4.01
			249.1 (23%)	40	
			223.1 (23%)	40	
midazolam-D$_4$	330.1	60	295.2 (100%)	25	3.98
			253.1 (22%)	40	
nifoxipam	316.0	105	270.0 (100%)	15	4.59
			298.0 (73%)	10	
			224.0 (51%)	25	
nitrazepam	282.0	90	236.0 (100%)	20	5.11
			180.0 (50%)	40	
			207.0 (35%)	35	
norclobazam	287.0	65	245.0 (100%)	15	5.37
			210.0 (33%)	30	
			181.0 (20%)	55	
norclobazam-^{13}C$_6$	293.0	60	251.0 (100%)	15	5.34
			216.0 (20%)	30	
nordazepam	271.1	85	140.1 (100%)	25	5.35
			165.1 (65%)	25	
			208.1 (58%)	25	
nordazepam-D$_5$	276.1	90	140.1 (100%)	25	5.32
			165.1 (86%)	25	
norflunitrazepam	300.1	100	254.1 (100%)	21	5.15
			198.1 (48%)	40	
			225.1 (30%)	35	
norflunitrazepam-D$_4$	304.1	100	258.1 (100%)	23	5.12
			202.1 (42%)	39	
norflurazepam	289.0	75	140.1 (100%)	28	5.61
			226.0 (46%)	26	
			165.0 (25%)	27	

Table S3 - 1. Optimised dynamic multiple reaction monitoring parameters. (continued) The ion ratios (between brackets) are relative to those of the quantifier ions (underlined). Collision energy, CE; retention time RT.

Compound	Precursor ion (*m/z*)	FV (V)	Product ions (*m/z*)	CE (eV)	RT (min)
norflurazepam-D_4	293.0	75	140.1 (100%)	28	5.58
			230.1 (50%)	27	
oxazepam	287.1	60	241.1 (100%)	20	5.19
			269.0 (61%)	10	
			104.1 (27%)	40	
oxazepam-D_5	292.1	60	246.1 (100%)	20	5.16
			109.1 (20%)	40	
phenazepam	349.0	140	206.0 (100%)	35	5.97
			184.0 (95%)	32	
			179.0 (86%)	50	
pivoxazepam	371.0	70	269.0 (100%)	5	7.87
			241.0 (75%)	27	
			163.0 (11%)	55	
prazepam	325.1	90	271.1 (100%)	18	7.24
			140.1 (23%)	38	
			165.1 (15%)	37	
prazepam-D_5	330.1	90	276.1 (100%)	19	7.21
			140.1 (16%)	38	
pyrazolam	354.0	135	167.0 (100%)	35	4.07
			206.0 (75%)	30	
			285.0 (25%)	20	
temazepam	301.1	75	255.1 (100%)	20	5.76
			283.1 (35%)	7	
			177.1 (16%)	40	
tetrazepam	289.1	65	225.1 (100%)	25	5.35
			253.1 (76%)	20	
			197.1 (74%)	35	
triazolam	343.0	55	308.0 (100%)	25	5.47
			315.0 (57%)	27	
			239.0 (52%)	45	
zolpidem	308.2	65	235.1 (100%)	35	3.35
			263.1 (37%)	25	
			92.1 (16%)	55	
zolpidem-D_7	315.2	50	242.1 (100%)	35	3.32
			270.1 (57%)	23	
zopiclone	389.1	70	245.1 (100%)	10	2.83
			217.1 (55%)	32	
			112.0 (47%)	63	
zopiclone-D_4	393.1	80	245.1 (100%)	10	2.80
			217.1 (55%)	32	

CHAPTER 4

OPTIMISATION SAMPLE PREPARATION

Based upon the following publication:

M. Degreef, L. Vits, E.M. Berry, K.E.K. Maudens, A.L.N. van Nuijs, Quantification of 54 Benzodiazepines and Z-Drugs, Including 20 Designer Ones, in Plasma, J. Anal. Toxicol. 45 (2021) 141–153. doi:10.1093/jat/bkaa059.

4.1. TECHNIQUES UNDER INVESTIGATION

4.1.1. Description samples

Due to difficulties in obtaining human plasma, initial experiments were performed with blank horse serum (Sigma Aldrich International GmbH, St. Gallen, CH). The class of the benzodiazepines and Z-drugs (BZDs) served as model compounds as they spanned the entire retention time range. During method validation (see Chapter 5 p. 69), the final protocol would need to be tested against the other classes as well. A mixture containing all BZDs standards was prepared at a concentration corresponding to BZD CAL L5 (Table S5 - 1 p. 85). The labelled internal standards (ISTDs) were combined into a separate mix at a five times lower concentration than that of their non-labelled counterpart. For stability reasons, all mixes were prepared in acetonitrile (ACN) and stored at -20 °C for the duration of the experiments.

4.1.2. Protein precipitation

Protein precipitation (PP) is a technique where, by addition of a precipitating agent (e.g. an organic solvent), endogenous proteins in a sample coagulate. Due to their differential solubility, target compounds remain dissolved. Strongly plasma protein bound compounds will also be freed due to denaturation of the proteins by the organic solvent. Following centrifugation, the supernatant can be transferred and directly injected onto the LC system. Usually higher volumes of solvent compared to sample are used. Organic solvents are most commonly used, eliminating up to 95% of proteins. Other endogenous molecules, including lipids, are unaffected by this technique [358,359].

The method under investigation used 200 µL sample added to an Eppendorf tube and spiked with 20 µL standard mix and 20 µL ISTD mix. The organic solvent was 800 µL ACN. The samples were vortex mixed for 2.5 min at 2000 rpm and centrifuged for 5 min at *10000 g*, after which the supernatant was transferred to an LC vial.

4.1.3. Liquid-liquid extraction

Liquid-liquid extraction (LLE) of compounds is based upon their preferential solubility for one of two immiscible liquid phases. Its ease-of-use, low cost and relatively good sample clean-up make it a frequently used sample preparation technique in many laboratories. By varying the pH of the mixture, acidic, neutral and basic compounds may be separated out in different fractions. The analytes of interest in this thesis are all basic drugs. Addition of a basic pH buffer will guarantee their presence in the unionised form, which preferentially dissolves in strong organic solvents. Selection of the appropriate organic solvent may be determined by multiple factors. Chloroform and diethyl ether are highly versatile solvents but pose a risk to the health of the analyst. Dichloromethane (DCM) has a better safety profile but is usually heavier than the aqueous samples (as is chloroform), requiring specific instrumentation to aspirate the upper layer. Lastly, highly volatile solvents risk significant loss of e.g. amphetamine-type substances. The use of ACN might be beneficial in this case, as might the addition of a small volume of acidified methanol to the volatile solvent [342,360].

Two different LLE methods were applied to the samples. Both started from 200 µL sample, spiked with 20 µL standard mix, 20 µL ISTD mix, and 65 µL 1 M carbonate buffer pH 9.5. For method LLE I, the highly frequently used solvents ethyl acetate (EtAC, 240 µL) and hexane (560 µL) were

added to the sample. Method LLE II had an extraction based upon the addition of methyl-tertiary-butyl-ether (MTBE), which was previously shown beneficial for the extraction of second generation antipsychotics [361]. The mixtures were vortex mixed for 2.5 min at 2000 rpm, rotor mixed for 10 min at 40 rpm and centrifuged for 5 min at *10000 g*. The upper layer was transferred to a new Eppendorf tube and evaporated under a gentle stream of N_2 at 40 °C. The dried fraction was reconstituted in 40 µL ACN and vortex mixed for 2.5 min at 2000 rpm. Following a second centrifugation step (2 min at *10000 g*), the supernatant was transferred to an LC vial for injection onto the instrument.

4.1.4. Solid-phase extraction

Solid-phase extraction (SPE), though laborious, is frequently used because of its higher selectivity and significant pre-concentration of analytes of interest, as well as excellent compatibility with automated sample preparation stations. Liquid phases (including the sample) are consecutively passed through a column or cartridge packed with the solid phase. Both hydrophobic/hydrophilic and electrostatic interactions may play a role in the adsorption of the analyte onto the solid phase. A combination of both interaction mechanisms (mixed-mode sorbents) is most advantageous in forensic settings due to the variety in drug classes and physicochemical properties. The first step in any SPE protocol should be the dilution of the sample to avoid obstruction of the flow pathway by any particles present. Often, buffer solutions are used for this purpose as they can influence the ionisation state of the analytes of interest and therefore their interaction with the solid phase. The second step would be the conditioning of the cartridges, to activate the functional groups on the sorbent and thereby ensure consistent interaction with the analyte, and to make them more compatible with the sample. The third step is the loading of the sample with retention onto the sorbent followed by washing of the cartridges to remove interfering and unwanted substances. Often used washing solvents include water-based solutions to remove proteins without the risk of them precipitating. Acidic solutions may be used to ionise amino functions and thus more strongly bind these compounds to the solid phase (based upon electrostatic interactions). The final step elutes the retained analytes by reversing the mechanisms of adsorption onto the sorbent. By varying the pH and solvents used, different compound classes may be eluted in different fractions [342,362].

As for the LLE, two different SPE protocols were investigated. Bond Elut Certify I mixed-mode cation exchange cartridges (Agilent Technologies, Santa Clara, California, US) were used as sorbent. For both protocols, 500 µL sample was spiked with 50 µL standard mix and 50 µL ISTD mix, diluted in 4 mL 0.1 M acetate buffer pH 4 (to ensure positive ionisation of the basic BZD class), vortex mixed and centrifuged for 10 min at 3500 rpm. Cartridges were conditioned with either 2 mL methanol (MeOH) and 2 mL 0.1 M acetate buffer pH 4.0 (SPE I) or with 3 mL MeOH, 3 mL water and 1 mL 250 mM phosphite buffer pH 6.0 (SPE II). Following loading, the cartridges were washed with 10 mL MeOH:water (1:10) and with 1 mL water + 5% NH_4OH (V/V), and dried for 15 min after each solvent addition for SPE protocol I. The washing step of SPE II consisted of 2 mL 0.1 N HCl and 3 mL MeOH, after which the cartridge was dried for 10 min at full vacuum. Elution was performed by twice 3 mL EtAc + 3% NH_4OH (V/V) for SPE I or twice 3 mL DCM:isopropanol (8:2) + 2% NH_4OH (V/V) for SPE II. All liquid phases were passed through the cartridges under vacuum to speed up the protocols. The eluates were evaporated under N_2 at 40 °C and reconstituted

in 100 µL ACN. Following vortex mixing (5 min at 2000 rpm) and centrifugation (10 min at 3500 rpm), the upper layer was transferred to an LC vial for injection onto the instrument.

4.1.5. Mini-QuEChERS

QuEChERS, pronounced as 'catchers', were developed by Anastassiades and Lehotay in 2003 [77,363]. They are said to benefit from SPE's highly efficient sample clean-up and compound extraction. Additionally, they mirror LLE properties such as a limited number of handling steps and a reduced overall cost as no SPE cartridges or highly specialised instrumentation is needed. The more recently developed miniaturised version in Eppendorf tubes requires significantly smaller volumes of solvents and is therefore also safer in use. In fact, all of their advantages are taken up in the technique's name: **Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged and **S**afe. Two main steps can be distinguished: salting out and dispersive SPE (Figure 4 - 1). Salting out involves mixing of the sample, an extraction solvent and a salt in an Eppendorf tube. At high enough concentrations, the salt will denature the proteins in the sample, causing them to aggregate and separate out of the sample-solvent mixture. Additionally, binding of the aqueous sample with the salt molecules promotes partitioning of analytes into the non-polar solvent. Buffers can be added to further facilitate this process (see section 4.1.3 above). Lastly, if a water-miscible extraction solvent is used, addition of salt can induce separation between the sample and the solvent. Commonly used salts include magnesium sulphate, sodium chloride, sodium acetate, sodium citrate tribasic dehydrate and sodium citrate dibasic sesquihydrate. Salting out can be followed by dispersive SPE. This technique uses dissolvable, porous sorbents to capture remaining interfering substances. Additionally, more salt can be added as a drying agent (reducing residual water present in the mixture). Prior to analysis the mixture is centrifuged to remove the salts and sorbents (if used) [78,342,364,365].

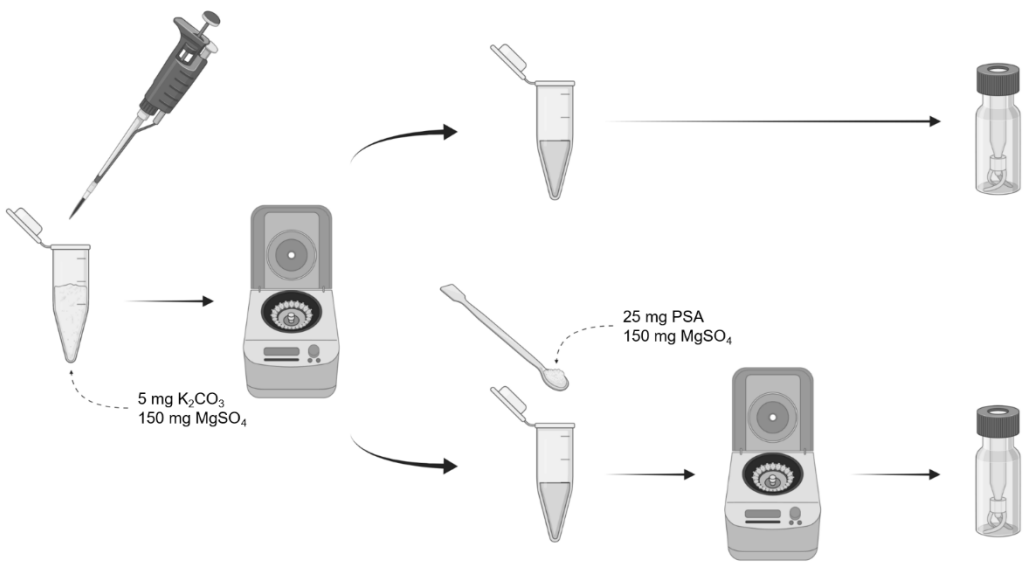

Figure 4 - 1. Simplified protocol for the mini-QuEChERS extraction. In a first step (salting out), the sample is mixed with an extraction solvent and salt in an Eppendorf tube. Following centrifugation and evaporation/reconstitution this mixture can be directly injected onto the instrument (upper fork). Alternatively, a second dispersive solid-phase extraction step can be executed by adding porous sorbents such as primary secondary amine (PSA, lower fork). *Created with BioRender.com.*

Based upon literature, four different QuEChERS protocols were set up, varying in the water solubility and extraction efficiency of the non-polar solvent [365,366]. $MgSO_4$ (150 mg) and K_2CO_3 (5 mg) were added to an Eppendorf tube, along with 100 µL sample, 10 µL standard mix and 10 µL ISTD mix. $MgSO_4$ is preferable over other salts for its reported higher capacity of water removal, promoting partitioning of analytes into the non-polar solvents [367]. The extraction solvents (600 µL) were ACN (method Q I), DCM (method Q II), MTBE (method Q III) or hexane (method Q IV). These mixtures were vortex mixed (5 min at 2000 rpm) and centrifuged (10 min at *10000 g*). The supernatant was transferred to a new Eppendorf tube, evaporated under N_2 at 40 °C and reconstituted in 40 µL ACN. Following another vortex mixing (2.5 min at 2000 rpm) and centrifugation step (2 min at *10000 g*), the supernatant was transferred to an LC vial and injected onto the instrument. To investigate the potentially beneficial effect of dispersive SPE, all analyses were repeated with inclusion of a secondary clean-up step (methods Q I_{dSPE} – Q IV_{dSPE}). Rather than evaporating the supernatant after the first mixing and centrifugation step, it was transferred to a new Eppendorf tube containing 150 mg $MgSO_4$ and 25 mg primary secondary amine (PSA). The latter belongs to the most commonly used sorbents for its high efficiency in removing sugars and fatty acids [367]. This mixture was again vortex mixed (5 min at 2000 rpm) and centrifuged (10 min at *10000 g*), after which the sample preparation continued as described above.

4.2. COMPARISON DIFFERENT TECHNIQUES

4.2.1. Reducing the number of methods

A total of 13 methods were up for investigation: PP, LLE I, LLE II, SPE I, SPE II, Q I, Q II, Q III, Q IV, Q I_{dSPE}, Q II_{dSPE}, Q III_{dSPE} and Q IV_{dSPE}. In a first step, it was sought to reduce this number to ideally four methods, one per sample preparation technique. For each of the analytes in the standard and ISTD mixes, the peak shape and peak areas were compared using the Agilent MassHunter Quantitative Analysis 10.0 (for QQQ) software (Agilent Technologies, Santa Clara, California, US). More preferable sample preparation techniques resulted in Gaussian peak shapes and higher peak areas.

Due to an overall issue for the PP (poor peak shapes and markedly reduced areas), this technique was excluded from further investigations (Figure 4 - 2). Likely, PP-extracted samples suffered from insufficient sample clean-up in combination with an additional five times dilution. It should be noted that a form of protein precipitation is present in the final steps of each of the other techniques. Indeed, reconstitution in ACN – though not its purpose – may cause precipitation of the remaining proteins in the extracts. LLE methods I (EtAc + hexane) and II (MTBE) did not result in significantly different outcomes. In general, method II seemed to give marginally better results for most analytes. However, a slight observer bias cannot be excluded as this method had already proven its usefulness in the extraction of antipsychotics from blood [361]. Similarly, only moderately better results were seen for SPE method I compared to method II. These could potentially be explained by the difference in preconditioning of the cartridges, with SPE I being conditioned to a lower pH (pH 4.0, the same as the samples themselves) than SPE II (pH 6.0).

Looking at the results for the mini-QuEChERS, hexane (method Q IV) showed a markedly reduced extraction efficiency (Figure 4 - 2). This solvent has previously been recommended as excellent

starting point for the optimisation of LLE methods. However, it is significantly less miscible with water compared to the other solvents, which might limit its use in dispersive SPE applications [342]. ACN (method Q I), DCM (method Q II) and MTBE (method Q III) all had comparable outcomes. As DCM is less desirable from an occupational health perspective, this solvent was also dropped from further investigations. An additional clean-up step with PSA gave no improved results, at the cost of more labour-intensive sample preparation and a reduction in signal intensities. Keeping the intended low concentrations of the lower limits of quantification in mind, methods Q I_{dSPE} – Q IV_{dSPE} were also excluded from further analyses.

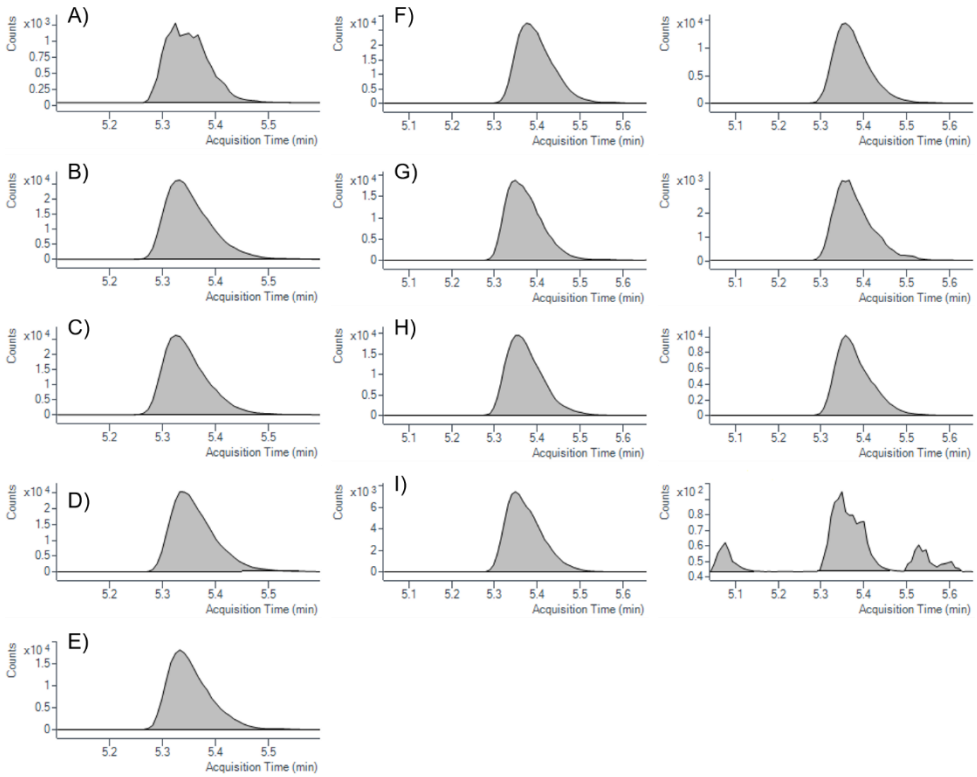

Figure 4 - 2. Visual analysis of the extraction efficiency for nordazepam. All analytes were spiked at a concentration of 1000 ng/mL. Protein precipitation (A) did not result in satisfactory results and was excluded from further analysis. Regarding liquid-liquid extraction, better results were obtained using MTBE (LLE II, C) compared to EtAc + hexane (LLE I, B) as extraction solvent. Similarly, solid-phase extraction method I (D) gave marginally better results than method II (E). Chromatograms F – I show the QuEChERS extraction of nordazepam without (left) and with additional clean-up step (right). The extraction solvents were ACN (F), DCM (G), MTBE (H) or hexane (I). Hexane less efficiently extracted the analyte compared to the other solvents; DCM was excluded for safety reasons. Clean-up with primary secondary amine (right) was more labour-intensive and did not result in better a better signal.

4.2.2. In-detail analysis

Four protocols were selected for a more in-depth analysis: a LLE with MTBE as extraction solvent (LLE II), a SPE using EtAc + 3% NH_4OH (V/V) as elution solvent (SPE I) and two QuEChERS methods using either ACN (Q I) or MTBE (Q III) as extraction solvents. To allow for direct comparison between the methods, each of the protocols was down- and/or upscaled to a sample

volume of 100 µL, 200 µL and 500 µL. The effectiveness of each method was evaluated using the extraction efficiency: a comparison of samples spiked prior to extraction versus those spiked after extraction (the ISTD mix is always spiked after extraction; see also section 5.4 p. 74) [368]. Samples were analysed in quadruplicate for each of the protocols and each of the sample volumes. The prodrug ethyl loflazepate exhibited poor extraction efficiencies for all tested protocols. This analyte was later shown to demonstrate significant instability in blood at both ambient temperature and at -20 °C (see section 5.5 p. 76) and was excluded from the data analysis.

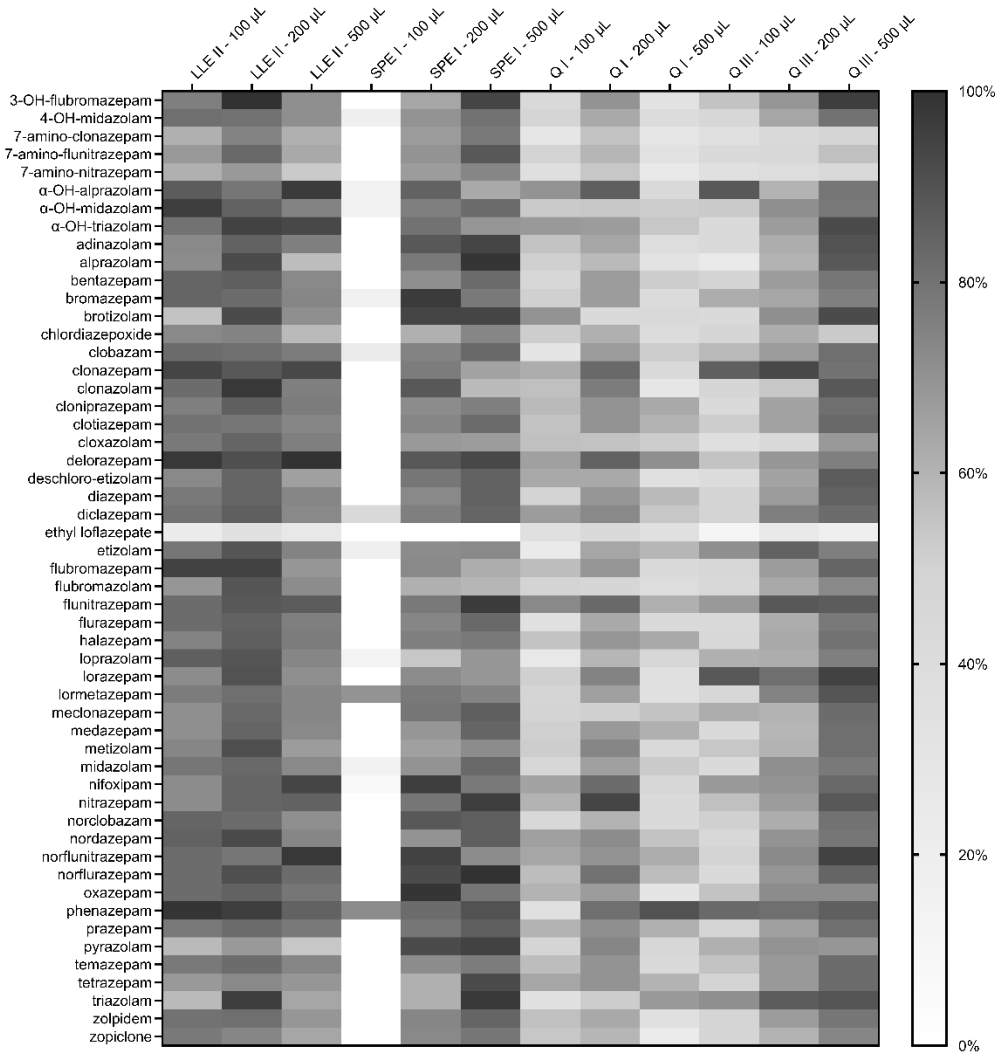

Figure 4 - 3. Extraction efficiencies of the selected sample preparation methods. Results for LLE method II, SPE method I and two different mini-QuEChERS protocols (Q I and Q III) were compared for three different sample volumes (100 µL, 200 µL and 500 µL). Extraction efficiencies were calculated based upon four repeats and plotted on a scale from 0% (compound not extracted from the sample) to 100% (no compound loss during extraction). The best recoveries were observed for the LLE with 200 µL sample volume and the SPE with 500 µL sample volume. Overall poor results were seen for the mini-QuEChERS methods.

For LLE method II, high recoveries with excellent reproducibility were seen for all sample volumes (Figure 4 - 3). The previously validated method using 200 µL sample had the highest recovery (85% ± 8%), with the lowest extraction efficiency found for a sample volume of 500 µL (75% ± 14%) [361]. As LLEs are known for their non-specific extraction of substances from the samples, it could be assumed that too many interfering matrix compounds may be present at higher sample volumes, suppressing the signals of the analytes of interest [342,360]. However, matrix effects were not evaluated at this step so no definite conclusions could be drawn for this observation. With regards to the SPE, a sample volume of 100 µL proved too low, resulting in overall low, highly variable extraction efficiencies (6 ± 246%). Substantial loss of analyte during consecutive washing and drying steps likely underlies this finding. Indeed, recoveries increased with increasing sample volume: 76% ± 13% for 200 µL and 81% ± 13% for 500 µL. QuEChERS extraction did not return satisfactory results. Using ACN (Q I) the recoveries were 53% ± 22%, 67% ± 15% and 48% ± 27%, respectively 100 µL, 200 µL and 500 µL sample volume. For MTBE as extraction solvent (Q III), no differences were observed for sample volumes 100 µL (52% ± 25%) and 200 µL (66% ± 16%). In contrast, excellent recoveries similar to SPE were found for a sample volume of 500 µL (80% ± 13%). Such reduced extraction efficiencies were also seen by Anzillotti et al. (30% – 70% at low concentrations) and by Famiglini et al. (50% – 60%) [369,370]. On the other hand, Kusano et al. and Usui et al. reported excellent recoveries for selected benzodiazepines in biological matrices [371,372].

4.3. OPTIMAL SAMPLE PREPARATION

Based upon the combined findings, a LLE using 200 µL of sample and MTBE as extraction solvent was selected for the quantitative methods. Similar recoveries were seen for SPE method I and QuEChERS method III, both using 500 µL sample volume, but the LLE offered several advantages over the other techniques. First and foremost, a limited number of handling steps are involved, reducing analyst workloads and increasing turn-around times. Compared to SPE, more samples could also be analysed per batch (24 for LLE, 16 for SPE). With regards to the mini-QuEChERS, it must be noted that the majority of the added sample preparation time was spent in weighing off the required amounts of salt into the Eppendorf tubes. Commercial kits are available with the salts coated onto the sides of the tubes, however these would significantly increase the cost of the analysis. PP had even faster turn-around times, but suffered from insufficient sample clean-up, as well as expected problems at lower concentrations due to a dilution of the sample (vs. pre-concentration with the other techniques). A second benefit would be the use of lower sample volumes for LLE (200 µL) compared to the optimal SPE and QuEChERS protocols (500 µL). This may be of particular importance for forensic samples, where often limited amounts of blood are available and multiple analytes of different drug classes might need to be quantified. Lastly, by working in Eppendorf tubes, the LLE protocol uses small volumes of solvents only, further reducing the cost of the analysis whilst also minimising exposure of the analysts to these solvents and being beneficial from an environmental point of view.

It needs pointing out that SPE can readily be combined with automated sample preparation workflows, which – after an initial cost – could markedly reduce analyst time and exposure to dangerous substances, and increase throughput and overall cost-effectiveness. Should such technique be(come) available in a laboratory, it is worth re-evaluating the optimal sample preparation method.

CHAPTER 5

METHOD VALIDATION

Based upon the following publications:

M. Degreef, A.L.N. van Nuijs, K.E. Maudens, Validation of a simple, fast liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of 40 antidepressant drugs or their metabolites in plasma, Clin. Chim. Acta. 485 (2018) 243–257. doi:10.1016/J.CCA.2018.06.047.

M. Degreef, E.M. Berry, K.E.K. Maudens, A.L.N. van Nuijs, Multi-analyte LC-MS/MS quantification of 38 antipsychotics and metabolites in plasma: Method validation & application to routine analyses, J. Chromatogr. B. 1179 (2021). doi:10.1016/J.JCHROMB.2021.122867.

M. Degreef, L. Vits, E.M. Berry, K.E.K. Maudens, A.L.N. van Nuijs, Quantification of 54 Benzodiazepines and Z-Drugs, Including 20 Designer Ones, in Plasma, J. Anal. Toxicol. 45 (2021) 141–153. doi:10.1093/jat/bkaa059.

The methods described in Chapter 3 and Chapter 4 were fully validated according to the Guidelines on Bioanalytical Method Validation, as published by the European Medicines Agency (EMA) and added recommendations for forensic toxicology [368,373–375]. An in-depth, theoretical discussion on each of the validation parameters is beyond the scope of this chapter but can be found in excellent review articles elsewhere [373–379]. For those compounds where no labelled analogue was available as the internal standard, the different validation parameters were investigated using both a structurally related and a retention time matched labelled internal standard (ISTD). The reported results are for the ISTDs showing the highest accuracies and lowest variations in the combined experiments.

5.1. SELECTIVITY

Compounds of interest (cpds) should ideally unambiguously be identified in all samples. Interference from endogenous compounds must be ruled out by running extracted blank samples from at least six individual sources. Other potential sources of interference that should be investigated may stem from metabolites and degradation products, from the ISTDs or from co-administered medication. Absence of interference is defined as a response < 20% of that in the lowest calibrator for cpds and < 5% for ISTDs.

Based upon the injection of analytical standards, the three (where possible, two for ISTDs) most relevant transitions were selected per compound of interest. The most abundant yet unique transition was selected as the quantifier ion. For the antidepressants (ADs), nortrimipramine (m/z 281.4, retention time 4.67 min) shared all tested transitions with imipramine (m/z 281.1, retention time 4.53 min). The most abundant transition (to m/z 86.1) was selected as quantifier ion. Imipramine has a unique transition to m/z 58.1, which was used for quantification. Co-elution with imipramine-D_3 allows for further distinction between the two cpds. Stock solutions containing 39 ADs, 32 AD ISTDs, 37 APs, 25 AP ISTDS, 54 BZDs or 20 BZD ISTDs were run against all methods, indicating that the chosen quantifier transitions were free of interference. Qualifier ions were ideally unique for that cpd, although this was not a limiting requirement as long as the determined qualifier/quantifier ratios (Table S3 - 1 p. 47) did not exceed ± 20% of the expected value (standardly programmed in the MassHunter software). The potential interference between O-desmethylvenlafaxine and tramadol has previously been described [380]. It was found that tramadol could be distinguished from O-desmethylvenlafaxine based upon a retention time shift of 0.25 min (2.69 min vs. 2.93 min) and the absence of a unique product ion at m/z 107.1. Nonetheless, it is advised to take particular caution in interpreting the data from samples containing tramadol.

5.2. CALIBRATION CURVE

5.2.1. Calibration levels

Calibration curves should be prepared in the same matrix as that of the intended case samples. For each curve, a minimum of six levels (CAL L1 – L6) should be included, as well as a blank sample (extracted blank matrix) and a zero sample (blank matrix spiked with the ISTD solution prior to extraction). The lower limit of quantification (LLOQ) is represented by the lowest calibrator (CAL L1). A linear relationship between the levels is not required, rather the most simple yet adequate relationship, determined on minimally three CAL curves, should be described. Although the

coefficient of determination is often used to express linearity, such evaluation tends to be biased in favour of an analyst's expectations or preferences [381,382]. Rather, adequate relationships can be characterised by back calculated concentrations within ± 15% (± 20% at LLOQ) of the nominal ones for each of the levels.

The concentrations ranges were chosen to include at least one subtherapeutic level and one level within the generally accepted toxic range, based upon available literature and in-house experience [7,8,383,384]. The calibration range of the designer BZDs was based upon those reported in other scientific publications or by analogy with their non-designer counterpart. For each of the ADs, a calibration curve consisting of ten levels was prepared (Table S5 - 1 & Table S5 - 2). For flupentixol, the recommended dose range was updated during the validation; the LLOQ is now equal to the lower limit of the therapeutic range. Linear and quadratic curves, and unweighted and weighted ($1/x$ and $1/x^2$) curve fittings were evaluated. The wide calibration range favoured a quadratic curve with $1/x^2$ weighing for the majority of the cpds. Because of its prominent quadratic curve, LLOQ values for atomoxetine were skewed when CAL L10 was included, which was therefore treated as two different calibration curves: one ranging from CAL L1 to L9 and one ranging from CAL L2 to L10. AP CAL curves were made up of seven levels (Table S5 - 1 & Table S5 - 2). CAL L1 had to be excluded for asenapine and its demethylated metabolite due to a previously reported sensitivity issue [361]. A linear curve fit was found for all but 10 cpds, with a weighing factor of $1/x^2$ for most. Where multiple ISTDs were investigated, the method seemed to favour a structurally related over a retention time matched one, although no significant differences were noted. With regards to the BZDs, quadratic curves (existing of six levels, Table S5 - 1 & Table S5 - 2) were favoured for the majority of cpds. Linear curve fittings could be used for those cpds that have a labelled analogue as the internal standard. Additionally, for 33 out of 54 cpds a weighing factor of $1/x^2$ resulted in the best accuracies over the entire calibration range.

5.2.2. Carry-over

Carry-over by high concentrated samples should be assessed by injecting blank samples following the highest calibrator. Absence of carry-over is defined as a response < 20% of that at the LLOQ for cpds and < 5% for ISTDs.

The high concentration of CAL L10 caused carry-over in the next two or more blank samples for the majority of the ADs. To avoid any interference with the other validation steps, it was decided to run these calibration curves at the end of each batch. However, for routine use, such a wide calibration range might not be needed, in which case dropping of CAL L9 and L10 would resolve the issues. Carry-over was not observed for any of the APs or BZDs. Nonetheless, should a highly concentrated sample be present in a batch, it is recommended to dilute the concerning sample one in ten times and rerunning this sample along with the consecutive two samples of that batch.

5.3. ACCURACY & PRECISION

Also known as trueness, accuracy expresses the closeness of a calculated concentration to the actual one in a sample. It is calculated according to Equation 5.1, with perfect accuracies equalling 100% and lower or higher values representing under- or overestimations of the concentration, respectively. Precision expresses the closeness of agreement between a series of measurements with the same

actual concentration and obtained under similar conditions. Rather than using the standard deviation, it is represented by the coefficient of variation (CV), which can be calculated with Equation 5.2.

$$\text{accuracy (\%)} = \frac{\text{calculated concentration}}{\text{actual concentration}} \cdot 100\% \qquad \textbf{Equation 5.1}$$

$$\text{CV (\%)} = \frac{\text{standard deviation of accuracy}}{\text{average accuracy}} \cdot 100\% \qquad \textbf{Equation 5.2}$$

5.3.1. Within-batch

Accuracy and precision within the same batch should be determined on a minimum of five samples per concentration level. Values for the average accuracy and the CV should be within ± 15% (± 20% at LLOQ) of the nominal ones. Four concentration levels need to be investigated: LLOQ (same concentration as CAL L1), quality control (QC) low (concentration not greater than three times CAL L1), QC mid (concentration around 30% – 50% of the CAL curve) and QC high (concentration not lower than 75% of the highest calibrator).

None of the cpds exceeded set limits (Table S5 - 3). The average accuracies and precisions for the ADs were: 99% ± 4% at LLOQ, 99% ± 3% at QC low, 99% ± 3% at QC mid and 101% ± 3% at QC high. The average accuracies and precisions for the APs were: 111% ± 4% at LLOQ, 100% ± 3% at QC low, 103% ± 3% at QC mid and 102% ± 3% at QC high. The average accuracies and precisions for the BZDs were: 105% ± 8% at LLOQ, 100% ± 7% at QC low, 102% ± 6% at QC mid and 103% ± 4% at QC high.

5.3.2. Between-batch

Accuracy and precision between batches should be determined on a minimum of five samples per concentration level, run over minimally three runs on at least two different days. Values for the average accuracy and for the CV should be within ± 15% (± 20% at LLOQ) of the nominal ones. Four concentration levels need to be investigated: LLOQ, QC low, QC mid and QC high.

As had been observed for the within-batch accuracy and precision, none of the cpds exceeded the requirements (Table S5 - 3). Between batches, the average accuracies and precisions for the ADs were: 102% ± 7% at LLOQ, 99% ± 4% at QC low, 97% ± 3% at QC mid and 98% ± 4% at QC high. The average accuracies and precisions for the APs were: 111% ± 9% at LLOQ, 100% ± 7% at QC low, 103% ± 6% at QC mid and 101% ± 6% at QC high. The average accuracies and precisions for the BZDs were: 103% ± 7% at LLOQ, 98% ± 6% at QC low, 98% ± 4% at QC mid and 101% ± 8% at QC high.

5.3.3. Dilution integrity

Samples with an estimated concentration above that of the calibration curve may be diluted with blank matrix. Appropriate dilution factors should be chosen based upon expected concentrations in case samples. Each dilution factor must be investigated on a minimum of five samples, spiked at a concentration greater than the highest calibrator, and the above defined accuracy and precision limits must not be exceeded. Because of the wide calibration ranges, it was opted to only investigate a ten times dilution for highly concentrated samples.

Based upon the results from six replicates, all but two ADs demonstrated good dilution integrity. Accuracies ranged between 93% and 113% with most cpds showing a slight positive bias. Atomoxetine and norfluoxetine fell with respectively 84% and 121% outside of the set criteria. However with a upper limit of quantification two to five times higher than the reported toxic doses, one could argue whether a deviation of 20% (vs. the accepted 15%) from the actual concentration would cause any noteworthy changes in the interpretation [7,384]. Precision was high with CVs lower than 8% for all cpds. For the APs, a dilution factor of 10 could also be reliably applied to samples with a concentration higher than that of CAL L7 (average accuracy and precision 98% ± 4%). Olanzapine, with a back calculated concentration of 128% ± 5% exceeded the set limits. Lastly, back calculated concentrations of the BZDs were within ± 15% of the spiked ones for all but four compounds: cloxazolam (77%), ethyl loflazepate (70%), flunitrazepam (82%) and medazepam (83%). All four compounds had non-linear calibration curves. CVs were 5% or less for all BZDs.

5.4. PROCESS EFFICIENCY

Process efficiency can be defined as the difference between the measured signal of an analyte following its extraction from the intended sample matrix versus that in neat solvent. It can be subdivided into the matrix effect and the extraction efficiency [385,386]. To calculate the process efficiency, a distinction is made between A-, B- and C-samples (Figure 5 - 1). A-samples consist of pure cpd and ISTD mix and represent the maximal signal that can be obtained independent of interference by the used matrix or of loss due to sample preparation. B-samples are made up by blank matrix spiked with cpd and ISTD mix after extraction. C-samples are spiked with cpd mix prior to sample preparation, but ISTD mix after sample preparation.

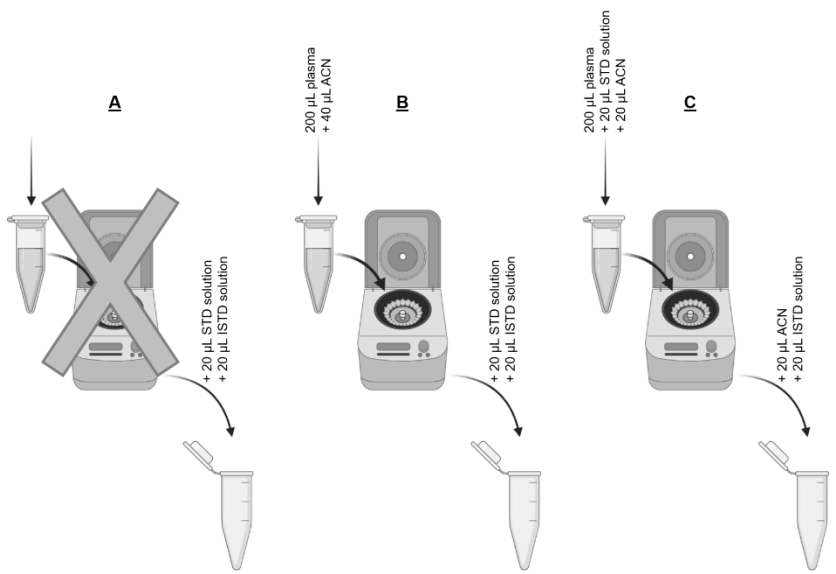

Figure 5 - 1. Composition of A- (left), B- (middle) and C-samples (right). A-samples consist of pure STD and ISTD solution. B- and C-samples consist of extracted blank matrix, spiked with the ISTD solution after extraction. The STD solution is spiked after and before the extraction, respectively. The centrifuge represents the optimised LLE extraction as described in section 4.3 p. 67. Acetonitrile, ACN; Labelled internal standard, ISTD; liquid-liquid extraction, LLE; reference standard, STD. *Created with BioRender.com.*

5.4.1. Matrix effect

Matrix effects are overall poorly understood but may result in ion enhancement or suppression. They should be assessed on a minimum of six samples from an individual source for both a low and a high concentration level. Expressed as matrix factor (MF), they can be calculated with Equation 5.3. Both the absolute (no correction for the cpds' signal by the ISTD) and ISTD-corrected MFs should be evaluated. CVs should not exceed ± 15%.

$$\mathrm{MF} = \frac{\text{B-sample}}{\text{A-sample}} \qquad \textbf{Equation 5.3}$$

The sample preparation and chromatographic settings were highly efficient at removing matrix interferences, as can be derived from the overall absence of ion suppression or enhancement (Figure 5 - 3). For the ADs, absolute MFs showed no significant changes in the peak areas compared to those of the unextracted standards. Average AP MFs were 0.98 ± 0.07 and 0.97 ± 0.04 for QC low and QC high respectively. The aberrant values for asenapine (0.83 ± 0.20 for QC low) and norasenapine (0.99 ± 0.26 for QC low and 0.56 ± 0.22 for QC high) are likely linked to a stability issue rather than being related to the sample preparation (see section 5.5 below). Also for the BZDs, no significant ion enhancement or suppression were seen. Further reductions in potential matrix effects, though not significant, were observed when correcting for the ISTDs.

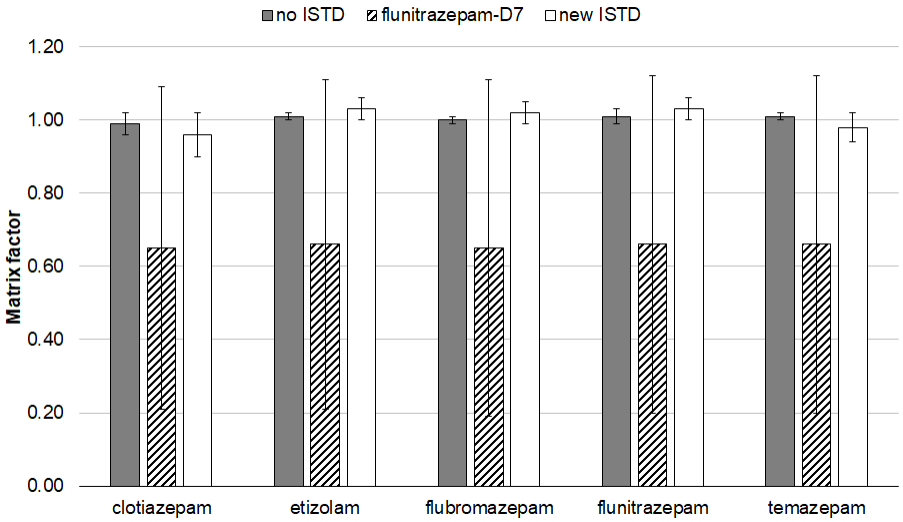

Figure 5 - 2. Matrix factor (MF) for compounds linked to the ISTD flunitrazepam-D_7. Significant ion suppression and poor reproducibility (as indicated by the error bars) were observed when the signal was corrected by the ISTD flunitrazepam-D_7. Co-elution with lorazepam is thought to underlie these findings and a new ISTD (Table S5 - 2) was allocated to the compounds.

Similar to the effect of matrix components on the cpds, the ISTDs may also be affected in their ionisation efficiency by co-eluting compounds. The effect is thought to be more pronounced with electrospray ionisation compared to atmospheric pressure chemical ionisation. It is also influenced by the structure, molecular weight, concentration and chromatographic behaviour of the molecules [387–390]. Calibration samples will have all cpds spiked into them; on the other hand routine samples are unlikely to contain all cpds. If enhancement or suppression by co-eluting compounds occurs, and

if this is not adequately corrected for by a similarly affected ISTD, the results for case samples could be affected. Should such problems arise, it is advised to change the ion source or LC parameters, which might not be feasible for routine laboratories [389,391]. When using a high number of ISTDs, such as the methods presented here, an alternative option could be to change the internal standard for the affected compounds from a structurally related one to one that elutes at the same retention time. With regards to the BZDs method, co-elution with lorazepam (m/z 321.0) was found to influence the flunitrazepam-D_7 (m/z 321.1) peak (Figure 5 - 2). Both compounds share similar transitions to m/z 275.0 and m/z 275.1, respectively. Therefore flunitrazepam-D_7 was removed from the ISTD mix. No other influences have been observed during method validation or in the analysis of externally sourced QC samples and case samples (see section 5.6). It should further be mentioned that, as a standard good practice, the ISTD response should always be evaluated for consistency throughout the batch.

5.4.2. Extraction efficiency

Based upon the authors' preference, extraction efficiency may also be called recovery. It gives an indication of the loss of analyte due to the sample preparation, irrespective of the matrix effects, and can be calculated with Equation 5.4.

$$\text{recovery (\%)} = \frac{\text{C-sample}}{\text{B-sample}} \cdot 100\% \qquad \textbf{Equation 5.4}$$

The average recovery of the ADs was calculated to be within 55% – 65% (± 9%) for most cpds, with the recoveries of the ISTDs compensating for the minimal variations (Figure 5 - 4). Tianeptine demonstrated a particularly reduced, but reproducible, recovery (37% ± 5%). This could be attributed to its amphoteric nature, with the carboxyl function being predominantly negatively charged at pH 9.5. The average AP extraction efficiencies were 76% ± 9%. Only levosulpiride (49% ± 9%), olanzapine (50% ± 12%) and norolanzapine (41% ± 12%) experienced lower but reproducible recoveries, none of which affected the LLOQ. Lastly BZD recoveries averaged 78% ± 7% (range 63% – 92%) at QC low and 79% ± 7% (range 63% – 95%) at QC high. Pyrazolam showed decreased but reproducible extraction efficiencies of 42% ± 11% and 41% ± 12%, QC low and QC high respectively. Once more, this had no influence on the LLOQ or any of the other validation parameters.

5.5. STABILITY

Stability of the cpds in the studied matrix should be investigated under different pre-analytical conditions. Accuracy and precision must be recorded for both a low and a high concentration, determined against freshly prepared calibration curves, and must not exceed ± 15% of the nominal concentration. No recommended number of samples must be analysed. The results are visualised in Figure S5 - 1 (ADs), Figure S5 - 2 (APs) and Figure S5 - 3 (BZDs).

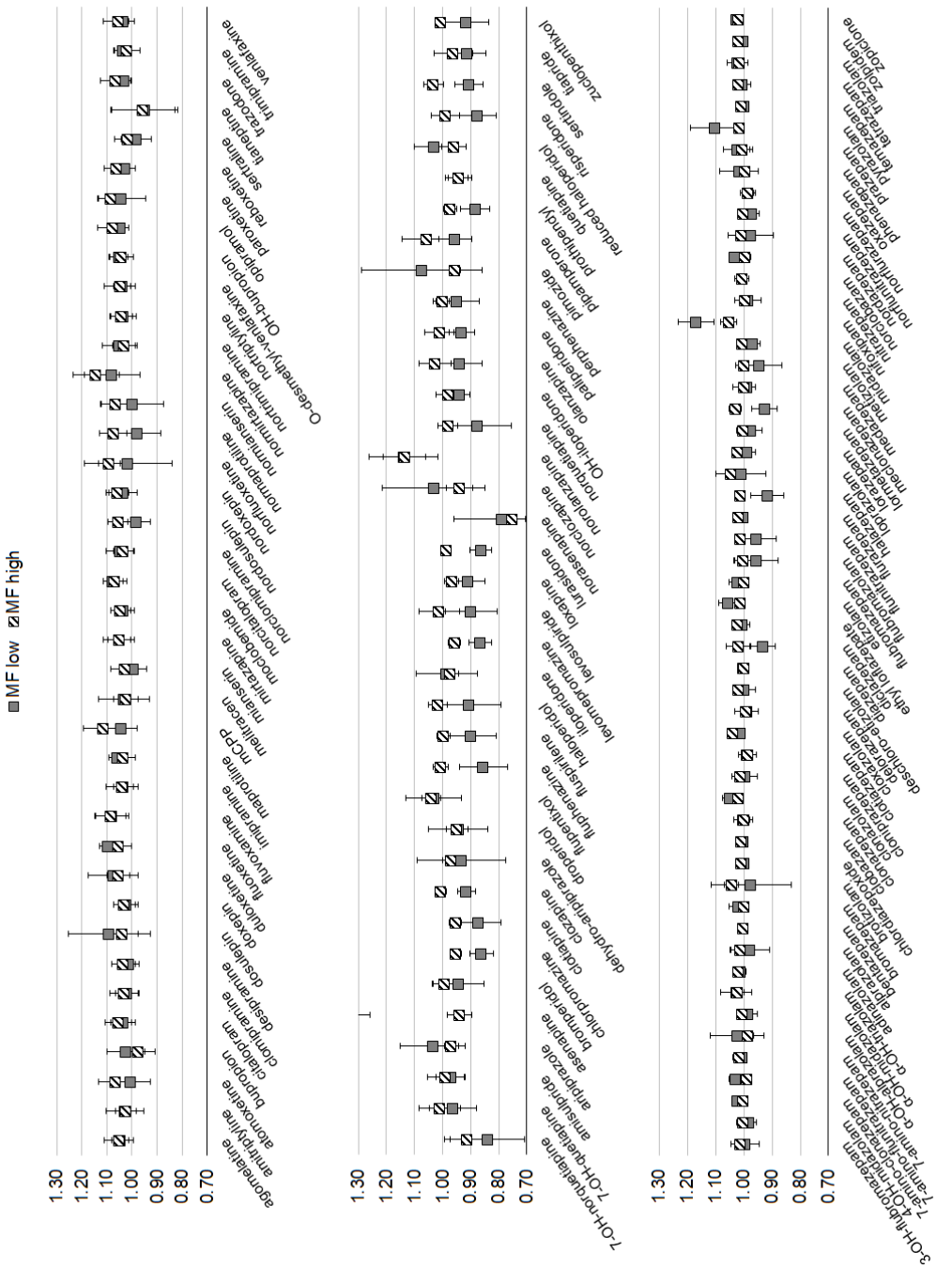

Figure 5 - 3. Absolute matrix factors (MFs) for the antidepressants (left), antipsychotics (middle) and benzodiazepines and Z-drugs (right). The mean value (n = 6) is plotted with its standard deviation (error bars) for a low and high concentration. Values lower than 1.00 indicate ion suppression, higher values indicate ion enhancement.

5.5.1. Benchtop stability

The benchtop (BT) stability reflects the changes in analyte concentrations for fresh, unextracted samples at ambient laboratory temperature. Four samples per concentration level were analysed. Per sample, double the amount of blank matrix was spiked with double amounts of QC solution, vortex mixed and split out in two aliquots (I and II). Aliquots I were extracted and analysed immediately, whereas aliquots II were left to stand for 3 h at ambient temperature prior to extraction and analysis.

ADs showed excellent BT stability for all but one cpd. The -18% difference in concentration at the LLOQ for normianserin was expected given the higher variation in back calculated concentration at such low levels. APs were stable for at least 3 hours at room temperature (average BT stability 99% ± 6% for QC low, 99% ± 5% for QC high). High stabilities (99% ± 6% at QC low and 98% ± 3% at QC high) were also found for all but two BZDs. Cloxazolam concentrations dropped to 62% ± 7% and 73% ± 3% of the initial ones, for QC low and QC high respectively. De Boeck et al. previously reported good stability for this compound (88% and 91% at their QC low and QC high concentrations respectively) in whole blood [392]. Biotransformation of cloxazolam to its active metabolite delorazepam seemed unlikely, as the concentration of the latter had not changed significantly (QC low 98% ± 7%, QC high 100% ± 3%) [393,394]. Similarly De Boeck et al. found excellent stability for ethyl loflazepate (95% at both QCs low and high) [392]. Results from our analyses were highly variable, yet all pointed towards a significant drop in concentration (QC low 17% ± 32%, QC high 12% ± 58%). The pro-drug ethyl loflazepate undergoes extensive first pass hydrolysis to form loflazepate (not included in the method), followed by decarboxylation to norflurazepam [395–397]. The minor increase from nominal concentrations for norflurazepam (QC low 113% ± 6%, QC high 112% ± 1%) suggests that the ethyl loflazepate in-sample transformation (if any) had not yet reached its conclusion. The inclusion of loflazepate in the method could give a better insight in the stability of ethyl loflazepate in plasma.

5.5.2. Autosampler stability

The autosampler (AS) stability reflects the changes in concentration for fresh, extracted samples on the instrument prior to injection. Two samples per concentration level were analysed. Six to eight replicates per sample were prepared, pooled after extraction and divided over six to eight LC vials. Samples were injected every three hours for 72 h straight. The autosampler was kept at ambient temperature.

Once extracted and reconstituted in acetonitrile, the majority of APs and BZDs were stable for 72 hours on the autosampler at ambient temperature, except for clotiapine which dropped to 77% of the nominal concentration after 72 hours. After 24 h, 48 h and 72 h on the autosampler, asenapine QC low and QC high concentrations respectively averaged 48% and 93%, 48% and 89%, and 79% and 80% compared to their nominal concentrations. Due to a programming error in the worklist, the AS stability of the ADs was investigated for a time period of 17.5 h only. All cpds showed excellent stability, apart from agomelatine for which an accurate measurement could be guaranteed for up to 10 h.

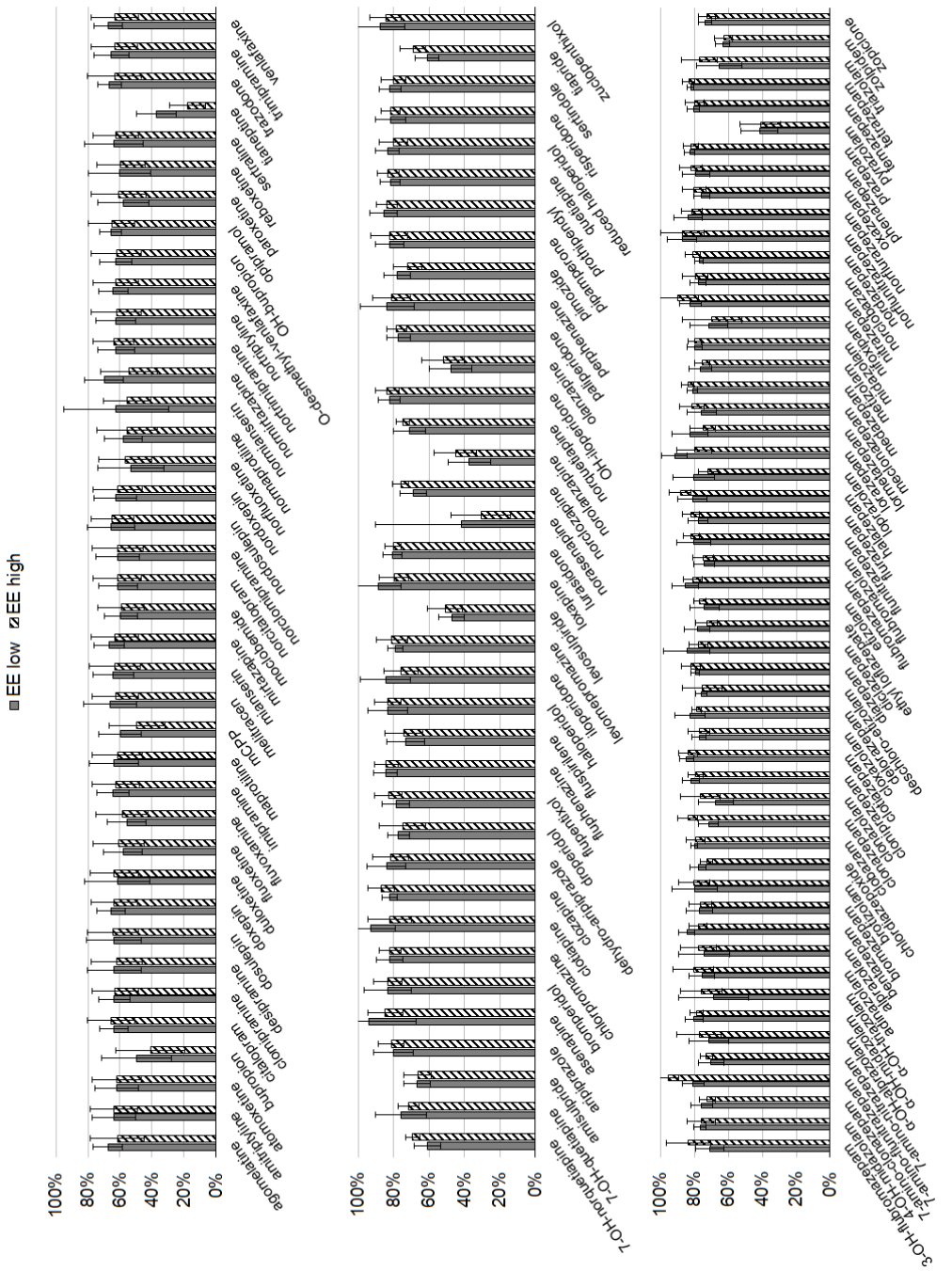

Figure 5 - 4. Extraction efficiencies (EEs) for the antidepressants (left), antipsychotics (middle) and benzodiazepines and Z-drugs (right). The mean value (n = 6) is plotted with its standard deviation (error bars) for a low and high concentration. Values lower than 100% indicate loss of analyte during sample preparation.

5.5.3. Freeze-thaw stability

The freeze-thaw (FT) stability reflects the changes in concentration for fresh, unextracted samples due to consecutive thawing and refreezing steps. Each freezing step should last for at least 12 h. Four samples per concentration level were analysed. Per sample, four times the amount of blank matrix was spiked with four times the amount of QC solution, vortex mixed and split out in four aliquots (I-IV). Aliquots I were extracted and analysed immediately, aliquots II-IV were frozen. The next day aliquots II-IV were allowed to come to room temperature, aliquots II extracted and analysed, and aliquots III and IV refrozen. This was repeated for the remaining aliquots to give a total of three freeze-thaw cycles.

None of the ADs showed an average change of more than 15% in concentration compared to aliquot I, with the exception of fluoxetine after 1 cycle and normianserin after 2 cycles when spiked at the LLOQ (these data were excluded). Bupropion showed diminished freezer stability (see section 5.5.4 below). AP-positive samples could also be confidently thawed and re-frozen three times (average freeze-thaw stability 106% ± 10% for QC low and 96% ± 4% for QC high), with the exception of two times for lurasidone (average relative concentration 123% ± 63% for QC low after the third thawing cycle). Consecutive freeze-thaw cycles further increased asenapine's instability, resulting in relative respective average QC low and QC high concentrations of 127% ± 14% and 100% ± 7% after one cycle, 111% ± 6% and 90% ± 12% after two cycles and 421% ± 17% and 37% ± 14% after 3 cycles. When norasenapine was spiked at QC low, these results were 88% ± 41%, 121% ± 35% and 322% ± 43%, respectively, and at QC high 83% ± 9%, 85% ± 11% and 8% ± 18%, respectively. Lastly, criteria of acceptability could not be met for ten BZDs. Accurate concentrations of adinazolam, brotizolam, ethyl loflazepate, etizolam, loprazolam, medazepam, nifoxipam and nitrazepam could be guaranteed for up to two freeze-thaw cycles only. Bentazepam showed diminished stability (reliable up to one cycle only). No articles were found addressing the freeze-thaw stability of this drug, however Tomková et al. describe excellent in-serum bench-top stability for up to 10 h [398]. An inexplicable drop to 70% in the concentration of cloxazolam was observed after one cycle at QC low. However, at QC high, excellent freeze-thaw stability was seen for three cycles. De Boeck et al. have reported good stabilities at both high and low concentrations for three freeze-thaw cycles for this compound [392].

5.5.4. Long-term stability

The long-term (LT) stability reflects the changes in concentration for fresh, unextracted samples due to prolonged in-freezer storage. The freezer temperature was -20 °C, as is true for case samples. Four samples per concentration level were analysed. Per sample, four times the amount of blank matrix was spiked with four times the amount of QC solution, vortex mixed and split out in four aliquots (I-IV). Aliquots I were extracted and analysed immediately, aliquots II-IV were frozen. The latter were thawed, extracted and analysed after approximately 1 week, 1 month and 3 months, respectively aliquots II, III and IV.

The storage capability of the ADs at -20 °C was investigated and deemed to be acceptable for a minimum of 1.5 months for all but six cpds (at the LLOQ levels). Atomoxetine, dosulepin, fluvoxamine, normirtazapine and OH-bupropion were stable for up to a week at LLOQ concentration levels. All five were previously reported stable for at least one month in plasma

at -20 °C. This is reflected in the good stability up to 1.5 months of the QC mid samples [399–404]. Bupropion showed considerable losses in concentration during storage due to its extensive metabolism, which should be considered if a sample tests positive for OH-bupropion rather than for bupropion [405]. In their frozen state, unextracted AP-positive samples can be kept for a minimum of three months (average long-term stability 99% ± 12% for QC low, 97% ± 7% for QC high), with the exception of iloperidone (77% ± 29% for QC low at 3 months), norolanzapine (54% ± 61% for QC low and 83% ± 9% for QC high at 3 months) and perphenazine (84% ± 6% for QC low and 80% ± 9% for QC high at 3 months). The concentrations of the latter three compounds are guaranteed for up to one month. Once more asenapine and norasenapine concentrations showed significant aberrations in accuracy and precision For asenapine the calculated concentrations for QC low and QC high respectively averaged 124% ± 39% and 99% ± 8% after 1 week, 112% ± 15% and 112% ± 9% after 1 month, and 203% ± 47% and 125% ± 29% after 3 months. LT storage of norasenapine over one month is not advised as the average relative concentrations increased to 153% ± 53% for QC low and to 119% ± 40% for QC high. Besides these both compounds' significant deviations of the calculated concentrations from those expected, the high CVs indicate an additional issue besides in-sample degradation. Indeed, preliminary data by Feng et al. suggest that neutral and basic conditions (the pH was raised to 9.5 in our sample preparation) may poorly affect the stability of asenapine. Their hypothesis is supported by Ansermot et al., who report excellent stability of asenapine under all conditions after reconstitution in an acidified mobile phase (pH 3.0) [406,407]. These analytical problems are paralleled by the drug's poor, non-reproducible oral bioavailability, even after sublingual dosing [408,409]. It is recommended to analyse suspected positive samples at the earliest convenience.

The majority of BZDs were stable for a minimum of three months at -20 °C. Accurate concentrations for α-OH-alprazolam, adinazolam and brotizolam could be guaranteed for up to one month of freezer storage. Although the concentration of the QC low sample for brotizolam at time point 1 week had fallen to 78% ± 7% and therefore was outside of the acceptability criterion, this decrease was not noted at time point 1 month (100% ± 7%) nor for the QC high samples at time point 1 week (97% ± 5%), suggesting an analytical rather than a stability issue for those samples. Cloxazolam showed decreased in-freezer stability, dropping to 77% ± 3% of the original concentration after one month of storage and further to 43% ± 2% after three months. The long-term in-freezer stability results for nifoxipam were highly variable, dropping to 50% – 60% after one month, yet rising to 125% – 150% after three months of freezer storage. In contrast, Pettersson Bergstrand et al. reported a minor drop in concentration to 75% after seven months at -20 °C for this drug [410].

5.6. PROOF OF CONCEPT

5.6.1. Quality control samples

External QC samples were purchased from either Chromsystems Instruments & Chemicals GmbH (Gräfelfing, DE) or ACQ Science GmbH (Rottenburg-Hailfingen, DE). The following ADs were successfully quantified: amitriptyline, citalopram, clomipramine, desipramine, doxepin, duloxetine, fluoxetine, fluvoxamine, imipramine, maprotiline, mirtazapine, norcitalopram, norclomipramine, nordoxepin, norfluoxetine, normirtazapine, nortriptyline, O-desmethyl-venlafaxine, paroxetine,

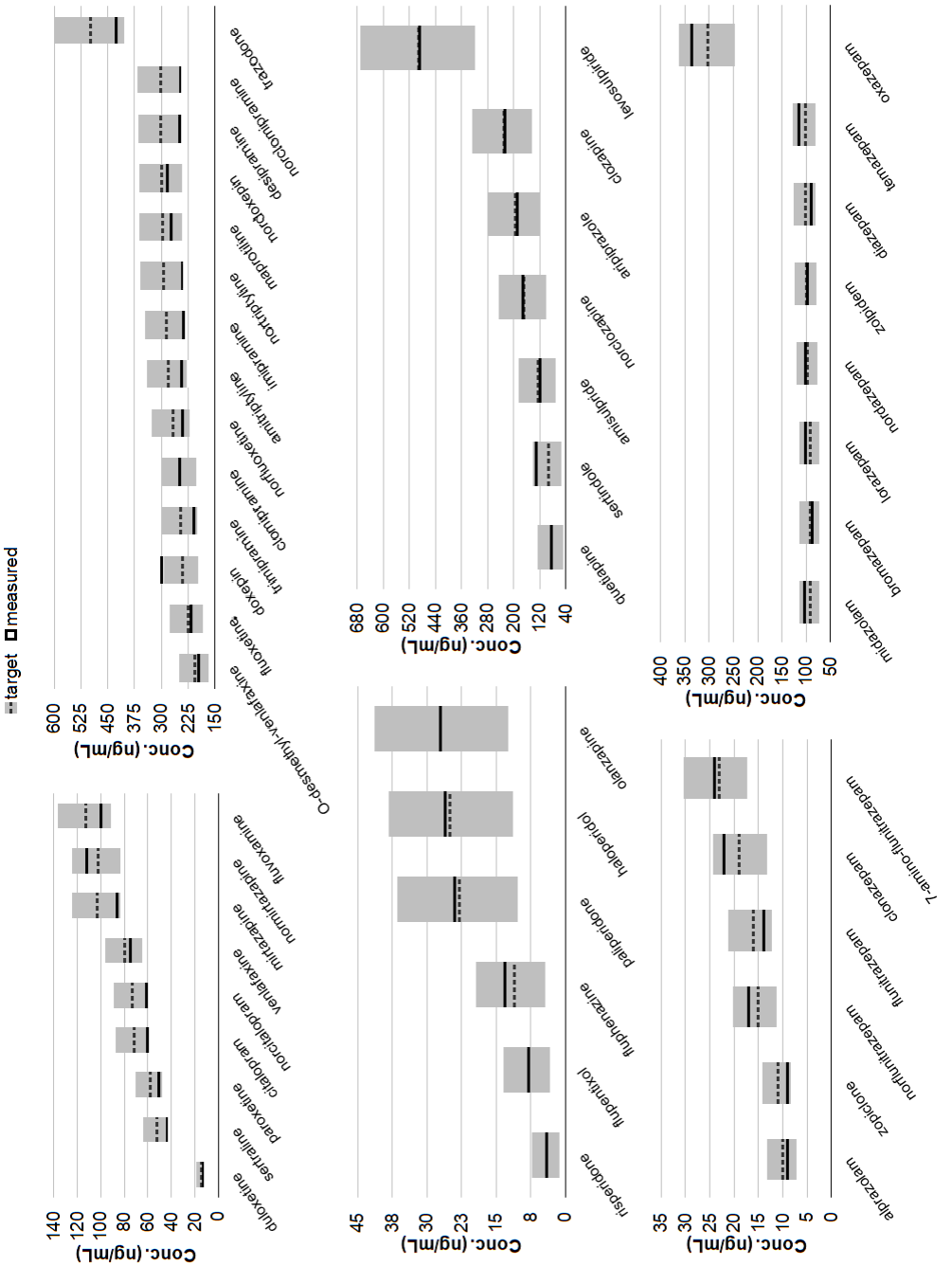

Figure 5 - 5. Analysis of external quality control samples for the antidepressants (left), antipsychotics (middle) and benzodiazepines and Z-drugs (right). The measured concentrations are represented by the full line. Target concentrations are indicated by the dotted line, with the acceptable concentration range as defined by the manufacturer in grey.

sertraline, trazodone, trimipramine and venlafaxine. For the APs, these were: amisulpride, aripiprazole, clozapine, flupentixol, fluphenazine, haloperidol, levosulpiride, norclozapine, olanzapine, paliperidone, quetiapine, risperidone and sertindole. Lastly, BZD QC samples contained 7-amino-flunitrazepam, alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nordazepam, norflunitrazepam, oxazepam, temazepam, zolpidem and zopiclone (Figure 5 - 5). Confidence in the AP results was also continuously evaluated by participating in proficiency testing schemes by the Gesellschaft für Toxikologische und Forensische Chemie [Society of Toxicological and Forensic Chemistry] (Arvecon GmbH, Walldorf, DE). 157 cpds were successfully identified and, apart from one clozapine result, quantified with acceptable z-scores.

5.6.2. Case samples

The ISO 17025 accredited Algemeen Medisch Laboratorium, division of Toxicology and Occupational Medicine, in Antwerp and the Hospital Association of North Antwerp generously donated 33 blood samples, in which 80 ADs were detected. The most prevalent cpds were the tricyclic ADs nortriptyline (n = 9), clomipramine (n = 6) and amitriptyline (n = 5), together with trazodone (n = 6) and mirtazapine (n = 5). Metabolites, when included in the method, were found for all cpds. In 46 medico-legal samples submitted to the University of Antwerp Toxicological Centre, 175 APs were detected. The majority of cases were positive for quetiapine (and its metabolites), the most commonly prescribed antipsychotic drug throughout Europe. The next most detected compound was olanzapine, which could be quantified in 7 cases, together with its demethylated metabolite. With only 1 and 2 samples positive for risperidone/paliperidone and aripiprazole, respectively, these drugs seemed less prevalent than expected from the literature. However, these compounds are mainly prescribed for paediatric use, an age group usually underrepresented in medico-legal cases [411–413]. Haloperidol (6 positive samples) was the most detected FGA. Other FGAs present were flupentixol (n = 1), zuclopenthixol (n = 2) and levomepromazine (n = 1), all of which are currently approved for use in Belgium. Lastly, 393 BZDs were detected in 120 archived medico-legal samples of the University of Antwerp Toxicological Centre. Diazepam (n = 45) and its metabolites nordazepam (n = 62), temazepam (n = 35) and oxazepam (n = 46) were the most prevalent cpds, followed by lorazepam (n = 26), alprazolam (n = 25) and bromazepam (n = 25). Additionally, in Belgium currently not prescribed BZDs bentazepam (n = 2), delorazepam (n = 4), diclazepam (n = 2), etizolam (n = 5), flubromazolam (n = 1) and medazepam (n = 5) were also detected.

5.7. CONCLUSIONS

The described methods allow for the simultaneous detection and quantification of 39 ADs or their metabolites, 37 APs or their metabolites and 54 BZDs or their metabolites in plasma. They were fully validated according to the EMA guidelines. As they benefit from a simple and rapid sample clean-up with LLE, a relatively short chromatographic run, a wide calibration range, and the use of the widely available LC-QQQ instrumentation, they can be readily implemented in both clinical and forensic toxicological laboratories. Additionally, having kept the sample preparation and LC-QQQ settings identical for all three drug classes allows for easy transitioning between or merging of methods.

5.8. SUPPLEMENTARY INFORMATION FOR CHAPTER 5

5.8.1. Tables

Table S5 - 1. Calibration levels for the analytes of interest. Calibration curves consisted of 10 levels for the antidepressants, 7 for the antipsychotics and 6 for the benzodiazepines and Z-drugs. All concentrations are expressed in ng/mL. L1 served as the lower limit of quantification, except for asenapine and norasenapine.

Compound	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
					ANTIDEPRESSANTS					
agomelatine	2	4	10	20	40	100	200	400	1000	2000
amitriptyline	10	20	50	100	200	500	1000	2000	5000	10000
atomoxetine	10	20	50	100	200	500	1000	2000	5000	10000
bupropion	5	10	25	50	100	250	500	1000	2500	5000
citalopram	5	10	25	50	100	250	500	1000	2500	5000
clomipramine	5	10	25	50	100	250	500	1000	2500	5000
desipramine	5	10	25	50	100	250	500	1000	2500	5000
dosulepin	5	10	25	50	100	250	500	1000	2500	5000
doxepin	5	10	25	50	100	250	500	1000	2500	5000
duloxetine	5	10	25	50	100	250	500	1000	2500	5000
fluoxetine	10	20	50	100	200	500	1000	2000	5000	10000
fluvoxamine	5	10	25	50	100	250	500	1000	2500	5000
imipramine	10	20	50	100	200	500	1000	2000	5000	10000
maprotiline	10	20	50	100	200	500	1000	2000	5000	10000
mCPP	2	4	10	20	40	100	200	400	1000	2000
melitracen	0.5	1	2.5	5	10	25	50	100	250	500
mianserin	2	4	10	20	40	100	200	400	1000	2000
mirtazapine	2	4	10	20	40	100	200	400	1000	2000
moclobemide	10	20	50	100	200	500	1000	2000	5000	10000
norcitalopram	2	4	10	20	40	100	200	400	1000	2000
norclomipramine	5	10	25	50	100	250	500	1000	2500	5000
nordosulepin	10	20	50	100	200	500	1000	2000	5000	10000
nordoxepin	5	10	25	50	100	250	500	1000	2500	5000
norfluoxetine	10	20	50	100	200	500	1000	2000	5000	10000
normaprotiline	10	20	50	100	200	500	1000	2000	5000	10000
normianserin	0.5	1	2.5	5	10	25	50	100	250	500
normirtazapine	0.5	1	2.5	5	10	25	50	100	250	500
nortrimipramine	10	20	50	100	200	500	1000	2000	5000	10000
nortriptyline	10	20	50	100	200	500	1000	2000	5000	10000
O-desmethyl-venlafaxine	10	20	50	100	200	500	1000	2000	5000	10000
OH-bupropion	5	10	25	50	100	250	500	1000	2500	5000
opipramol	10	20	50	100	200	500	1000	2000	5000	10000
paroxetine	5	10	25	50	100	250	500	1000	2500	5000
reboxetine	5	10	25	50	100	250	500	1000	2500	5000
sertraline	5	10	25	50	100	250	500	1000	2500	5000
tianeptine	2	4	10	20	40	100	200	400	1000	2000
trazodone	25	50	125	250	500	1250	2500	5000	12500	25000
trimipramine	5	10	25	50	100	250	500	1000	2500	5000
venlafaxine	10	20	50	100	200	500	1000	2000	5000	10000
					ANTIPSYCHOTICS					
7-OH-norquetiapine	1	2	8	16	64	128	512			
7-OH-quetiapine	1	2	8	16	64	128	512			
amisulpride	10	20	80	160	640	1280	5120			
aripiprazole	10	20	80	160	640	1280	5120			

Table S5 - 1. Calibration levels for the analytes of interest. (continued) Calibration curves consisted of 10 levels for the antidepressants, 7 for the antipsychotics and 6 for the benzodiazepines and Z-drugs. All concentrations are expressed in ng/mL. L1 served as the lower limit of quantification, except for asenapine and norasenapine.

Compound	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
asenapine	-	1	4	8	32	64	256			
bromperidol	0.5	1	4	8	32	64	256			
chlorpromazine	4	8	32	64	256	512	2048			
clotiapine	1	2	8	16	64	128	512			
clozapine	10	20	80	160	640	1280	5120			
dehydro-aripiprazole	4	8	32	64	256	512	2048			
droperidol	1	2	8	16	64	128	512			
flupentixol	0.5	1	4	8	32	64	256			
fluphenazine	0.5	1	4	8	32	64	256			
fluspirilene	0.5	1	4	8	32	64	256			
haloperidol	0.5	1	4	8	32	64	256			
iloperidone	0.5	1	4	8	32	64	256			
levomepromazine	2	4	16	32	128	256	1024			
levosulpiride	10	20	80	160	640	1280	5120			
loxapine	1	2	8	16	64	128	512			
lurasidone	4	8	32	64	256	512	2048			
norasenapine	-	1	4	8	32	64	256			
norclozapine	10	20	80	160	640	1280	5120			
norolanzapine	1	2	8	16	64	128	512			
norquetiapine	3	6	24	48	192	384	1536			
OH-iloperidone	0.5	1	4	8	32	64	256			
olanzapine	1	2	8	16	64	128	512			
paliperidone	1	2	8	16	64	128	512			
perphenazine	0.5	1	4	8	32	64	256			
pimozide	2	4	16	32	128	256	1024			
pipamperone	4	8	32	64	256	512	2048			
prothipendyl	4	8	32	64	256	512	2048			
quetiapine	10	20	80	160	640	1280	5120			
reduced haloperidol	0.5	1	4	8	32	64	256			
risperidone	1	2	8	16	64	128	512			
sertindole	2	4	16	32	128	256	1024			
tiapride	20	40	160	320	1280	2560	10240			
zuclopenthixol	1	2	8	16	64	128	512			
				BENZODIAZEPINES & Z-DRUGS						
3-OH-flubromazepam	2.5	10	25	100	250	1000				
4-OH-midazolam	0.5	2	5	20	50	200				
7-amino-clonazepam	2	8	20	80	200	800				
7-amino-flunitrazepam	0.5	2	5	20	50	200				
7-amino-nitrazepam	1	4	10	40	100	400				
α-OH-alprazolam	0.625	2.5	6.25	25	62.5	250				
α-OH-midazolam	1.25	5	12.5	50	125	500				
α-OH-triazolam	0.5	2	5	20	50	200				
adinazolam	2.5	10	25	100	250	1000				
alprazolam	1	4	10	40	100	400				
bentazepam	2.5	10	25	100	250	1000				
bromazepam	2.5	10	25	100	250	1000				
brotizolam	0.5	2	5	20	50	200				
chlordiazepoxide	25	100	250	1000	2500	10000				
clobazam	6.25	25	62.5	250	625	2500				

Table S5 - 1. Calibration levels for the analytes of interest. (continued) Calibration curves consisted of 10 levels for the antidepressants, 7 for the antipsychotics and 6 for the benzodiazepines and Z-drugs. All concentrations are expressed in ng/mL. L1 served as the lower limit of quantification, except for asenapine and norasenapine.

Compound	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
clonazepam	2	8	20	80	200	800				
clonazolam	2	8	20	80	200	800				
cloniprazepam	0.5	2	5	20	50	200				
clotiazepam	10	40	100	400	1000	4000				
cloxazolam	2.5	10	25	100	250	1000				
delorazepam	2	8	20	80	200	800				
deschloro-etizolam	1.25	5	12.5	50	125	500				
diazepam	5	20	50	200	500	2000				
diclazepam	1	4	10	40	100	400				
ethyl loflazepate	2.5	10	25	100	250	1000				
etizolam	1.25	5	12.5	50	125	500				
flubromazepam	2.5	10	25	100	250	1000				
flubromazolam	2	8	20	80	200	800				
flunitrazepam	0.5	2	5	20	50	200				
flurazepam	1	4	10	40	100	400				
halazepam	10	40	100	400	1000	4000				
loprazolam	2	8	20	80	200	800				
lorazepam	2	8	20	80	200	800				
lormetazepam	0.5	2	5	20	50	200				
meclonazepam	1	4	10	40	100	400				
medazepam	10	40	100	400	1000	4000				
metizolam	1	4	10	40	100	400				
midazolam	2.5	10	25	100	250	1000				
nifoxipam	2.5	10	25	100	250	1000				
nitrazepam	1	4	10	40	100	400				
norclobazam	25	100	250	1000	2500	10000				
nordazepam	10	40	100	400	1000	4000				
norflunitrazepam	0.5	2	5	20	50	200				
norflurazepam	5	20	50	200	500	2000				
oxazepam	10	40	100	400	1000	4000				
phenazepam	2	8	20	80	200	800				
pivoxazepam	2.5	10	25	100	250	1000				
prazepam	0.5	2	5	20	50	200				
pyrazolam	2.5	10	25	100	250	1000				
temazepam	10	40	100	400	1000	4000				
tetrazepam	6.25	25	62.5	250	625	2500				
triazolam	0.5	2	5	20	50	200				
zolpidem	5	20	50	200	500	2000				
zopiclone	1	4	10	40	100	400				

Table S5 - 2. Calibration curve settings for the analytes of interest.

Compound	Curve fitting	Weighing factor	Internal standard
		ANTIDEPRESSANTS	
agomelatine	quadratic	$1/x^2$	duloxetine-D_7
amitriptyline	quadratic	$1/x$	amitriptyline-D_3
atomoxetine	quadratic	$1/x$	reboxetine-D_5
bupropion	quadratic	$1/x^2$	bupropion-D_9
citalopram	linear	$1/x^2$	citalopram-D_6
clomipramine	linear	$1/x$	clomipramine-D_3
desipramine	linear	$1/x^2$	desipramine-D_3
dosulepin	quadratic	$1/x$	dosulepin-D_3
doxepin	quadratic	$1/x^2$	doxepin-D_3
duloxetine	linear	$1/x$	duloxetine-D_7
fluoxetine	linear	$1/x$	fluoxetine-D_6
fluvoxamine	linear	$1/x$	fluvoxamine-D_3
imipramine	linear	$1/x$	imipramine-D_3
maprotiline	quadratic	$1/x^2$	maprotiline-D_5
mCPP	quadratic	$1/x^2$	mCPP-D_8
melitracen	quadratic	$1/x^2$	melitracen-D_6
mianserin	quadratic	$1/x^2$	mianserin-D_3
mirtazapine	linear	$1/x^2$	mirtazapine-D_3
moclobemide	quadratic	$1/x^2$	moclobemide-D_8
norcitalopram	linear	$1/x$	norcitalopram-D_3
norclomipramine	linear	$1/x^2$	norclomipramine-D_3
nordosulepin	quadratic	$1/x^2$	dosulepin-D_3
nordoxepin	linear	$1/x^2$	nordoxepin-D_3
norfluoxetine	quadratic	$1/x^2$	norfluoxetine-D_5
normaprotiline	linear	$1/x$	maprotiline-D_5
normianserin	quadratic	$1/x^2$	mianserin-D_3
normirtazapine	quadratic	$1/x^2$	normirtazapine-D_6
nortrimipramine	quadratic	$1/x^2$	trimipramine-D_3
nortriptyline	linear	$1/x$	nortriptyline-D_3
O-desmethyl-venlafaxine	quadratic	$1/x$	O-desmethyl-venlafaxine-D_6
OH-bupropion	linear	$1/x$	OH-bupropion-D_6
opipramol	linear	$1/x^2$	opipramol-D_4
paroxetine	linear	$1/x^2$	paroxetine-D_6
reboxetine	quadratic	$1/x$	reboxetine-D_5
sertraline	quadratic	$1/x$	sertraline-D_3
tianeptine	quadratic	$1/x^2$	tianeptine-D_{12}
trazodone	quadratic	$1/x$	trazodone-D_6
trimipramine	quadratic	$1/x$	trimipramine-D_3
venlafaxine	quadratic	$1/x$	venlafaxine-D_6
		ANTIPSYCHOTICS	
7-OH-norquetiapine	linear	$1/x^2$	7-OH-norquetiapine-D_8
7-OH-quetiapine	linear	$1/x^2$	7-OH-quetiapine-D_8
amisulpride	linear	$1/x^2$	amisulpride-D_5
aripiprazole	linear	$1/x^2$	aripiprazole-D_8
asenapine	linear	$1/x^2$	asenapine-^{13}C-D_3
bromperidol	linear	$1/x$	haloperidol-D_4
chlorpromazine	linear	$1/x^2$	prothipendyl-D_6
clotiapine	linear	$1/x^2$	clotiapine-D_8
clozapine	quadratic	$1/x^2$	clozapine-D_8
dehydro-aripiprazole	linear	$1/x^2$	dehydro-aripiprazole-D_8

Table S5 - 2. Calibration curve settings for the analytes of interest. (continued)

Compound	Curve fitting	Weighing factor	Internal standard
droperidol	quadratic	$1/x^2$	haloperidol-D_4
flupentixol	linear	$1/x$	flupentixol-D_4
fluphenazine	quadratic	$1/x^2$	flupentixol-D_4
fluspirilene	linear	$1/x^2$	lurasidone-D_8
haloperidol	linear	$1/x^2$	haloperidol-D_4
iloperidone	linear	$1/x$	iloperidone-D_3
levomepromazine	quadratic	$1/x^2$	prothipendyl-D_6
levosulpiride	linear	$1/x^2$	amisulpride-D_5
loxapine	linear	$1/x^2$	loxapine-D_8
lurasidone	linear	$1/x^2$	lurasidone-D_8
norasenapine	quadratic	$1/x^2$	asenapine-^{13}C-D_3
norclozapine	quadratic	$1/x$	norclozapine-D_8
norolanzapine	linear	$1/x$	norolanzapine-D_8
norquetiapine	linear	$1/x^2$	norquetiapine-D_8
OH-iloperidone	linear	$1/x^2$	OH-iloperidone-D_4
olanzapine	linear	$1/x^2$	olanzapine-D_3
paliperidone	linear	$1/x$	paliperidone-D_4
perphenazine	linear	$1/x^2$	zuclopenthixol-D_4
pimozide	quadratic	$1/x$	lurasidone-D_8
pipamperone	quadratic	$1/x^2$	haloperidol-D_4
prothipendyl	linear	$1/x$	prothipendyl-D_6
quetiapine	quadratic	$1/x^2$	quetiapine-D_8
reduced haloperidol	linear	$1/x$	reduced haloperidol-D_4
risperidone	linear	$1/x$	risperidone-D_4
sertindole	linear	$1/x$	sertindole-D_4
tiapride	linear	$1/x^2$	amisulpride-D_5
zuclopenthixol	linear	$1/x^2$	zuclopenthixol-D_4
	BENZODIAZEPINES & Z-DRUGS		
3-OH-flubromazepam	quadratic	$1/x^2$	alprazolam-D_5
4-OH-midazolam	linear	$1/x^2$	zolpidem-D_7
7-amino-clonazepam	linear	$1/x$	7-amino-clonazepam-D_4
7-amino-flunitrazepam	quadratic	$1/x$	7-amino-flunitrazepam-D_7
7-amino-nitrazepam	quadratic	$1/x$	7-amino-nitrazepam-D_5
α-OH-alprazolam	quadratic	$1/x$	norflunitrazepam-D_4
α-OH-midazolam	quadratic	$1/x^2$	midazolam-D_4
α-OH-triazolam	quadratic	$1/x^2$	norflunitrazepam-D_4
adinazolam	quadratic	$1/x^2$	alprazolam-D_5
alprazolam	quadratic	$1/x$	alprazolam-D_5
bentazepam	linear	$1/x^2$	7-amino-nitrazepam-D_5
bromazepam	linear	$1/x^2$	bromazepam-D_4
brotizolam	quadratic	$1/x^2$	alprazolam-D_5
chlordiazepoxide	linear	$1/x$	zolpidem-D_7
clobazam	quadratic	$1/x$	clobazam-D_5
clonazepam	linear	$1/x$	clonazepam-D_4
clonazolam	quadratic	$1/x$	norflunitrazepam-D_4
cloniprazepam	quadratic	$1/x$	prazepam-D_5
clotiazepam	quadratic	$1/x^2$	clobazam-D_5
cloxazolam	quadratic	$1/x^2$	norflurazepam-D_4
delorazepam	quadratic	$1/x$	nordazepam-D_5
deschloro-etizolam	quadratic	$1/x$	alprazolam-D_5
diazepam	linear	$1/x^2$	diazepam-D_5

Table S5 - 2. Calibration curve settings for the analytes of interest. (continued)

Compound	Curve fitting	Weighing factor	Internal standard
diclazepam	quadratic	$1/x^2$	diazepam-D_5
ethyl loflazepate	quadratic	$1/x^2$	diazepam-D_5
etizolam	quadratic	$1/x^2$	midazolam-D_4
flubromazepam	quadratic	$1/x^2$	norflurazepam-D_4
flubromazolam	quadratic	$1/x^2$	midazolam-D_4
flunitrazepam	quadratic	$1/x^2$	norflurazepam-D_4
flurazepam	linear	$1/x$	flurazepam-D_{10}
halazepam	quadratic	$1/x^2$	prazepam-D_5
loprazolam	quadratic	$1/x^2$	midazolam-D_4
lorazepam	quadratic	$1/x^2$	lormetazepam-^{13}C-D_3
lormetazepam	linear	$1/x^2$	lormetazepam-^{13}C-D_3
meclonazepam	linear	$1/x^2$	clonazepam-D_4
medazepam	quadratic	$1/x$	diazepam-D_5
metizolam	quadratic	$1/x^2$	midazolam-D_4
midazolam	linear	$1/x^2$	midazolam-D_4
nifoxipam	quadratic	$1/x^2$	norflunitrazepam-D_4
nitrazepam	quadratic	$1/x$	norflunitrazepam-D_4
norclobazam	quadratic	$1/x$	norclobazam-$^{13}C_6$
nordazepam	linear	$1/x^2$	nordazepam-D_5
norflunitrazepam	linear	$1/x^2$	norflunitrazepam-D_4
norflurazepam	linear	$1/x$	norflurazepam-D_4
oxazepam	linear	$1/x$	oxazepam-D_5
phenazepam	linear	$1/x^2$	bromazepam-D_4
pivoxazepam	quadratic	$1/x^2$	nordazepam-D_5
prazepam	linear	$1/x$	prazepam-D_5
pyrazolam	quadratic	$1/x^2$	alprazolam-D_5
temazepam	quadratic	$1/x^2$	clobazam-D_5
tetrazepam	quadratic	$1/x^2$	diazepam-D_5
triazolam	quadratic	$1/x^2$	alprazolam-D_5
zolpidem	linear	$1/x$	zolpidem-D_7
zopiclone	quadratic	$1/x$	zopiclone-D_4

Table S5 - 3. Accuracy and precision for the analytes of interest. Mean accuracy ± coefficient of variation, based upon six replicates per concentration level. Lower limit of quantification, LLOQ.

Compound	Within-batch (%)				Between-batch (%)			
	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High
ANTIDEPRESSANTS								
agomelatine	105 ± 2	95 ± 2	98 ± 2	101 ± 1	101 ± 4	95 ± 4	95 ± 5	97 ± 4
amitriptyline	92 ± 4	102 ± 2	99 ± 3	100 ± 2	93 ± 8	101 ± 5	98 ± 1	98 ± 4
atomoxetine	95 ± 8	109 ± 4	112 ± 5	95 ± 14	89 ± 5	107 ± 7	110 ± 8	92 ± 7
bupropion	92 ± 3	99 ± 2	95 ± 2	101 ± 2	103 ± 10	103 ± 5	98 ± 2	97 ± 4
citalopram	99 ± 2	99 ± 2	98 ± 2	105 ± 2	101 ± 3	101 ± 2	99 ± 1	103 ± 3
clomipramine	105 ± 1	100 ± 1	94 ± 3	101 ± 2	110 ± 12	101 ± 4	92 ± 1	98 ± 3
desipramine	91 ± 1	97 ± 2	98 ± 2	105 ± 1	97 ± 3	100 ± 3	98 ± 2	101 ± 6
dosulepin	109 ± 7	102 ± 5	104 ± 3	101 ± 2	101 ± 7	102 ± 3	102 ± 4	99 ± 4
doxepin	92 ± 4	99 ± 3	100 ± 2	102 ± 2	95 ± 2	100 ± 3	100 ± 1	99 ± 2
duloxetine	111 ± 6	99 ± 2	98 ± 2	99 ± 2	104 ± 5	101 ± 6	97 ± 7	99 ± 5
fluoxetine	99 ± 3	101 ± 1	97 ± 3	101 ± 3	108 ± 9	100 ± 3	96 ± 2	99 ± 4
fluvoxamine	112 ± 2	100 ± 2	96 ± 3	100 ± 1	107 ± 9	98 ± 4	94 ± 1	98 ± 2
imipramine	94 ± 2	100 ± 1	97 ± 2	101 ± 1	102 ± 10	101 ± 2	95 ± 1	99 ± 3
maprotiline	96 ± 3	95 ± 4	101 ± 2	99 ± 2	93 ± 8	95 ± 3	97 ± 4	97 ± 3
mCPP	98 ± 3	89 ± 2	100 ± 2	100 ± 1	105 ± 7	93 ± 6	98 ± 6	101 ± 3
melitracen	101 ± 8	99 ± 5	100 ± 4	99 ± 2	106 ± 10	95 ± 8	97 ± 4	95 ± 3
mianserin	95 ± 5	104 ± 2	101 ± 3	99 ± 1	102 ± 5	104 ± 4	100 ± 4	96 ± 2
mirtazapine	97 ± 3	96 ± 4	98 ± 2	101 ± 3	98 ± 6	96 ± 4	99 ± 2	101 ± 5
moclobemide	97 ± 3	103 ± 1	101 ± 2	103 ± 3	100 ± 1	104 ± 3	101 ± 2	99 ± 3
norcitalopram	97 ± 3	97 ± 2	98 ± 3	102 ± 1	103 ± 6	95 ± 2	95 ± 1	99 ± 2
norclomipramine	95 ± 3	96 ± 3	97 ± 3	108 ± 1	101 ± 5	99 ± 2	98 ± 3	105 ± 1
nordosulepin	95 ± 6	107 ± 3	104 ± 3	91 ± 3	96 ± 6	112 ± 5	104 ± 5	96 ± 9
nordoxepin	102 ± 2	98 ± 2	100 ± 2	104 ± 1	103 ± 6	99 ± 3	99 ± 1	101 ± 5
norfluoxetine	81 ± 14	87 ± 3	100 ± 2	100 ± 4	97 ± 7	90 ± 7	96 ± 5	97 ± 4
normaprotiline	113 ± 5	94 ± 3	96 ± 3	99 ± 2	116 ± 9	86 ± 5	87 ± 2	97 ± 3
normianserin	86 ± 13	93 ± 5	101 ± 2	101 ± 1	104 ± 11	100 ± 7	102 ± 5	99 ± 2
normirtazapine	84 ± 11	86 ± 7	101 ± 3	99 ± 6	98 ± 11	86 ± 10	95 ± 5	98 ± 5
nortrimipramine	89 ± 5	103 ± 2	103 ± 3	110 ± 6	99 ± 10	107 ± 11	102 ± 6	93 ± 8
nortriptyline	100 ± 4	100 ± 3	98 ± 3	103 ± 2	107 ± 6	99 ± 3	96 ± 3	101 ± 2
O-desmethyl-venlafaxine	115 ± 1	99 ± 1	94 ± 2	100 ± 2	114 ± 2	101 ± 2	95 ± 3	97 ± 2
OH-bupropion	97 ± 1	102 ± 1	97 ± 2	100 ± 2	106 ± 13	102 ± 3	96 ± 1	99 ± 3
opipramol	82 ± 1	91 ± 1	105 ± 2	97 ± 1	87 ± 11	91 ± 3	102 ± 5	94 ± 3
paroxetine	90 ± 7	89 ± 3	98 ± 2	108 ± 2	98 ± 7	89 ± 3	95 ± 2	106 ± 5
reboxetine	102 ± 3	101 ± 2	98 ± 3	101 ± 1	105 ± 4	101 ± 5	97 ± 3	97 ± 5
sertraline	101 ± 4	101 ± 4	97 ± 3	99 ± 3	103 ± 7	101 ± 2	97 ± 1	96 ± 3
tianeptine	99 ± 11	99 ± 10	96 ± 7	98 ± 4	105 ± 17	93 ± 10	98 ± 7	97 ± 4
trazodone	116 ± 1	101 ± 1	94 ± 4	103 ± 4	115 ± 3	102 ± 2	95 ± 3	95 ± 6
trimipramine	119 ± 1	103 ± 1	94 ± 2	99 ± 2	113 ± 6	102 ± 3	94 ± 5	97 ± 3
venlafaxine	110 ± 1	101 ± 1	94 ± 2	100 ± 2	116 ± 2	102 ± 2	94 ± 3	97 ± 5
ANTIPSYCHOTICS								
7-OH-norquetiapine	114 ± 4	105 ± 3	113 ± 3	88 ± 4	110 ± 8	105 ± 3	111 ± 6	88 ± 5
7-OH-quetiapine	108 ± 1	111 ± 2	111 ± 1	94 ± 3	107 ± 8	99 ± 8	103 ± 6	88 ± 8
amisulpride	112 ± 1	99 ± 0	102 ± 1	104 ± 1	113 ± 2	98 ± 2	101 ± 3	108 ± 3
aripiprazole	116 ± 2	101 ± 2	111 ± 1	109 ± 1	117 ± 4	104 ± 7	111 ± 4	114 ± 4
asenapine	118 ± 10	108 ± 11	95 ± 3	109 ± 3	111 ± 8	107 ± 14	98 ± 10	100 ± 10
bromperidol	118 ± 3	93 ± 2	91 ± 3	98 ± 2	108 ± 11	103 ± 7	109 ± 8	104 ± 6
chlorpromazine	117 ± 2	99 ± 1	99 ± 5	108 ± 3	114 ± 6	99 ± 7	98 ± 2	114 ± 4
clotiapine	105 ± 7	88 ± 6	92 ± 3	107 ± 4	108 ± 9	102 ± 7	101 ± 6	103 ± 5

Table S5 - 3. Accuracy and precision for the analytes of interest. (continued) Mean accuracy ± coefficient of variation, based upon six replicates per concentration level. Lower limit of quantification, LLOQ.

Compound	Within-batch (%)				Between-batch (%)			
	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High
clozapine	82 ± 1	108 ± 1	107 ± 1	92 ± 3	86 ± 13	108 ± 6	108 ± 3	93 ± 6
dehydro-aripiprazole	118 ± 4	95 ± 2	100 ± 2	103 ± 1	115 ± 11	93 ± 5	100 ± 6	106 ± 5
droperidol	101 ± 3	85 ± 3	93 ± 3	97 ± 4	110 ± 11	91 ± 8	96 ± 9	100 ± 4
flupentixol	106 ± 7	95 ± 7	108 ± 3	104 ± 1	117 ± 9	106 ± 7	100 ± 5	105 ± 4
fluphenazine	118 ± 4	113 ± 2	115 ± 1	106 ± 2	116 ± 5	113 ± 2	112 ± 1	106 ± 8
fluspirilene	112 ± 2	88 ± 3	100 ± 3	98 ± 1	114 ± 6	92 ± 6	97 ± 4	107 ± 7
haloperidol	110 ± 5	97 ± 3	103 ± 1	103 ± 2	114 ± 9	94 ± 4	100 ± 5	110 ± 6
iloperidone	119 ± 3	97 ± 3	93 ± 2	103 ± 6	107 ± 13	107 ± 8	102 ± 10	102 ± 12
levomepromazine	112 ± 5	92 ± 2	99 ± 4	103 ± 2	114 ± 5	93 ± 6	101 ± 6	106 ± 4
levosulpiride	103 ± 7	91 ± 5	100 ± 5	96 ± 3	111 ± 4	100 ± 7	101 ± 7	105 ± 6
loxapine	106 ± 2	86 ± 4	91 ± 3	103 ± 3	118 ± 16	93 ± 11	91 ± 8	102 ± 3
lurasidone	117 ± 2	106 ± 2	111 ± 2	110 ± 1	115 ± 5	110 ± 6	111 ± 2	93 ± 10
norasenapine	108 ± 11	113 ± 10	112 ± 13	98 ± 12	107 ± 13	100 ± 18	99 ± 15	101 ± 6
norclozapine	89 ± 3	106 ± 2	115 ± 1	87 ± 1	101 ± 11	100 ± 4	112 ± 5	98 ± 13
norolanzapine	110 ± 5	97 ± 5	101 ± 2	106 ± 3	109 ± 15	101 ± 8	104 ± 11	108 ± 6
norquetiapine	108 ± 2	94 ± 1	102 ± 1	96 ± 1	114 ± 9	99 ± 8	107 ± 4	95 ± 6
OH-iloperidone	113 ± 4	96 ± 3	103 ± 2	110 ± 3	102 ± 13	93 ± 6	98 ± 3	114 ± 4
olanzapine	119 ± 2	103 ± 3	105 ± 3	110 ± 4	107 ± 14	99 ± 4	102 ± 8	107 ± 5
paliperidone	115 ± 2	99 ± 2	104 ± 1	102 ± 1	111 ± 5	98 ± 7	104 ± 2	106 ± 6
perphenazine	114 ± 3	89 ± 4	102 ± 2	109 ± 1	108 ± 9	100 ± 8	99 ± 4	106 ± 8
pimozide	111 ± 4	89 ± 4	93 ± 5	97 ± 2	115 ± 5	97 ± 7	100 ± 5	101 ± 4
pipamperone	111 ± 3	113 ± 3	112 ± 5	100 ± 5	104 ± 16	99 ± 12	101 ± 12	91 ± 11
prothipendyl	113 ± 1	96 ± 1	100 ± 1	106 ± 2	119 ± 7	100 ± 9	102 ± 8	102 ± 2
quetiapine	113 ± 1	110 ± 2	109 ± 1	98 ± 1	116 ± 3	109 ± 4	111 ± 6	99 ± 7
reduced haloperidol	114 ± 3	85 ± 4	98 ± 0	102 ± 2	116 ± 4	85 ± 5	94 ± 3	103 ± 3
risperidone	118 ± 1	101 ± 1	98 ± 1	102 ± 1	115 ± 5	99 ± 3	99 ± 4	103 ± 4
sertindole	117 ± 1	103 ± 2	109 ± 2	107 ± 2	109 ± 8	99 ± 3	112 ± 6	102 ± 4
tiapride	106 ± 10	114 ± 2	110 ± 6	102 ± 4	113 ± 5	110 ± 8	111 ± 2	90 ± 2
zuclopenthixol	111 ± 3	93 ± 5	104 ± 2	111 ± 2	116 ± 13	104 ± 9	101 ± 7	91 ± 11
BENZODIAZEPINES & Z-DRUGS								
3-OH-flubromazepam	107 ± 9	97 ± 9	93 ± 9	103 ± 9	105 ± 7	101 ± 6	101 ± 9	104 ± 8
4-OH-midazolam	95 ± 13	93 ± 5	94 ± 3	98 ± 4	109 ± 6	98 ± 12	102 ± 7	110 ± 4
7-amino-clonazepam	103 ± 2	101 ± 4	105 ± 2	103 ± 2	97 ± 14	100 ± 7	112 ± 3	100 ± 3
7-amino-flunitrazepam	90 ± 10	111 ± 5	111 ± 1	90 ± 5	97 ± 9	108 ± 4	111 ± 7	91 ± 9
7-amino-nitrazepam	106 ± 4	101 ± 4	98 ± 1	107 ± 5	106 ± 6	99 ± 5	104 ± 5	100 ± 3
adinazolam	105 ± 4	91 ± 3	96 ± 3	109 ± 2	108 ± 5	88 ± 8	102 ± 4	106 ± 5
alprazolam	87 ± 7	88 ± 9	102 ± 2	96 ± 5	103 ± 14	102 ± 6	99 ± 9	102 ± 5
α-OH-alprazolam	103 ± 6	99 ± 8	105 ± 4	98 ± 7	98 ± 10	92 ± 7	101 ± 9	105 ± 5
α-OH-midazolam	98 ± 8	95 ± 5	98 ± 3	105 ± 5	105 ± 13	104 ± 6	107 ± 7	105 ± 5
α-OH-triazolam	92 ± 14	89 ± 12	101 ± 10	98 ± 7	107 ± 10	102 ± 11	103 ± 6	104 ± 6
bentazepam	101 ± 12	100 ± 9	94 ± 6	107 ± 6	108 ± 6	107 ± 7	106 ± 6	101 ± 10
bromazepam	108 ± 7	97 ± 1	92 ± 2	114 ± 3	106 ± 7	90 ± 5	95 ± 4	113 ± 3
brotizolam	109 ± 4	97 ± 9	103 ± 5	107 ± 2	107 ± 11	104 ± 12	101 ± 5	101 ± 5
chlordiazepoxide	113 ± 9	105 ± 5	100 ± 3	105 ± 3	106 ± 6	104 ± 6	109 ± 5	104 ± 3
clobazam	90 ± 6	94 ± 6	99 ± 2	100 ± 2	87 ± 7	93 ± 4	107 ± 5	106 ± 3
clonazepam	96 ± 5	90 ± 8	85 ± 4	96 ± 5	115 ± 5	98 ± 10	102 ± 5	99 ± 5
clonazolam	98 ± 9	95 ± 9	102 ± 4	98 ± 5	112 ± 4	102 ± 13	94 ± 8	103 ± 7
cloniprazepam	103 ± 7	92 ± 6	94 ± 3	105 ± 2	116 ± 2	110 ± 9	109 ± 10	106 ± 4
clotiazepam	99 ± 9	108 ± 4	93 ± 5	86 ± 2	98 ± 7	103 ± 6	102 ± 8	98 ± 6

Table S5 - 3. Accuracy and precision for the analytes of interest. (continued) Mean accuracy ± coefficient of variation, based upon six replicates per concentration level. Lower limit of quantification, LLOQ.

Compound	Within-batch (%)				Between-batch (%)			
	LLOQ	Low	Mid	High	LLOQ	Low	Mid	High
cloxazolam	98 ± 8	110 ± 5	97 ± 4	86 ± 3	104 ± 7	104 ± 4	105 ± 6	104 ± 6
delorazepam	113 ± 4	94 ± 3	90 ± 2	103 ± 2	107 ± 8	99 ± 6	98 ± 5	104 ± 3
deschloro-etizolam	117 ± 4	107 ± 5	101 ± 3	108 ± 3	117 ± 2	96 ± 11	94 ± 5	104 ± 6
diazepam	107 ± 5	103 ± 6	102 ± 4	102 ± 3	106 ± 3	106 ± 3	106 ± 3	97 ± 2
diclazepam	109 ± 4	100 ± 8	99 ± 6	109 ± 4	102 ± 11	89 ± 9	94 ± 7	100 ± 2
ethyl loflazepate	103 ± 7	85 ± 7	93 ± 7	95 ± 8	102 ± 8	95 ± 7	103 ± 8	101 ± 9
etizolam	117 ± 4	86 ± 7	96 ± 2	108 ± 4	114 ± 5	87 ± 3	99 ± 5	105 ± 2
flubromazepam	94 ± 14	96 ± 10	98 ± 1	99 ± 4	102 ± 12	111 ± 8	105 ± 4	106 ± 2
flubromazolam	112 ± 6	88 ± 7	94 ± 3	100 ± 4	102 ± 9	98 ± 8	101 ± 7	104 ± 1
flunitrazepam	98 ± 5	108 ± 8	95 ± 9	102 ± 7	97 ± 11	102 ± 10	101 ± 11	98 ± 8
flurazepam	107 ± 4	95 ± 3	91 ± 1	100 ± 2	110 ± 5	95 ± 3	92 ± 7	99 ± 2
halazepam	91 ± 9	88 ± 3	89 ± 2	94 ± 6	105 ± 8	99 ± 8	101 ± 9	98 ± 6
loprazolam	106 ± 8	93 ± 8	97 ± 4	103 ± 4	113 ± 6	106 ± 13	103 ± 7	102 ± 3
lorazepam	104 ± 9	95 ± 11	103 ± 10	88 ± 12	98 ± 12	95 ± 11	98 ± 7	96 ± 10
lormetazepam	116 ± 2	104 ± 6	94 ± 6	101 ± 2	100 ± 8	92 ± 4	97 ± 6	111 ± 3
meclonazepam	101 ± 13	92 ± 6	92 ± 4	103 ± 5	96 ± 11	105 ± 11	103 ± 6	95 ± 5
medazepam	91 ± 10	112 ± 6	112 ± 4	87 ± 2	100 ± 13	99 ± 4	104 ± 7	100 ± 5
metizolam	113 ± 6	93 ± 6	97 ± 2	107 ± 3	110 ± 5	87 ± 7	99 ± 8	105 ± 3
midazolam	102 ± 5	102 ± 6	98 ± 3	99 ± 6	101 ± 6	101 ± 3	104 ± 5	101 ± 3
nifoxipam	102 ± 7	102 ± 5	106 ± 6	99 ± 9	100 ± 9	106 ± 7	111 ± 6	114 ± 4
nitrazepam	86 ± 15	94 ± 9	104 ± 5	98 ± 7	112 ± 6	103 ± 10	99 ± 8	104 ± 7
norclobazam	117 ± 2	106 ± 4	108 ± 2	112 ± 2	118 ± 2	108 ± 5	113 ± 1	113 ± 2
nordazepam	104 ± 7	93 ± 7	94 ± 4	102 ± 3	103 ± 6	99 ± 3	101 ± 3	103 ± 3
norflunitrazepam	106 ± 17	101 ± 9	105 ± 4	105 ± 7	103 ± 15	106 ± 9	100 ± 6	104 ± 5
norflurazepam	101 ± 11	100 ± 6	98 ± 5	105 ± 4	108 ± 9	101 ± 6	104 ± 3	106 ± 3
oxazepam	106 ± 4	97 ± 5	99 ± 3	99 ± 2	99 ± 12	98 ± 5	98 ± 7	102 ± 2
phenazepam	104 ± 4	99 ± 10	94 ± 5	109 ± 2	105 ± 11	101 ± 11	107 ± 4	94 ± 4
prazepam	103 ± 3	96 ± 3	99 ± 2	103 ± 1	104 ± 11	98 ± 4	104 ± 5	101 ± 2
pyrazolam	103 ± 7	91 ± 12	97 ± 5	102 ± 1	101 ± 13	100 ± 11	108 ± 7	102 ± 8
temazepam	105 ± 5	104 ± 4	97 ± 2	95 ± 4	102 ± 5	104 ± 6	102 ± 8	104 ± 5
tetrazepam	118 ± 9	114 ± 3	102 ± 4	93 ± 7	107 ± 7	110 ± 6	105 ± 5	99 ± 3
triazolam	115 ± 11	101 ± 7	92 ± 8	101 ± 3	97 ± 11	98 ± 6	106 ± 4	105 ± 6
zolpidem	105 ± 7	96 ± 3	95 ± 1	98 ± 2	109 ± 4	96 ± 1	93 ± 2	98 ± 2
zopiclone	111 ± 4	99 ± 4	99 ± 2	103 ± 1	113 ± 3	99 ± 5	101 ± 6	103 ± 2

5.8.2. Figures

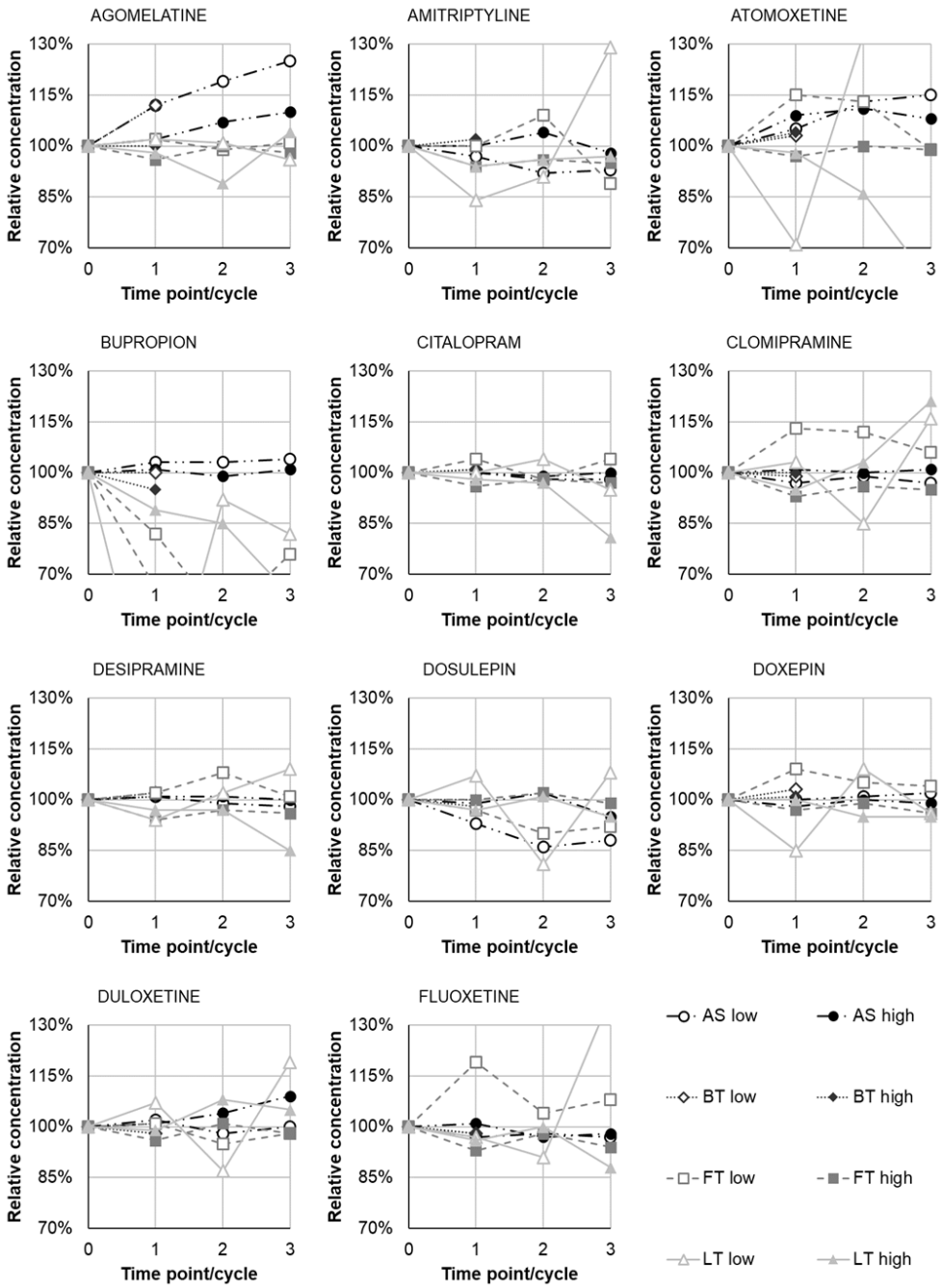

Figure S5 - 1. Stability data for the antidepressants at a low and high concentration. The data points represent 0 hours, 7.5 hours, 12.5 hours and 17.5 hours for the autosampler (AS) stability; 0 hours and 3 hours for the benchtop (BT) stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw (FT) stability; 0 hours, 1 week, 1 month and 3 months for the long-term (LT) stability.

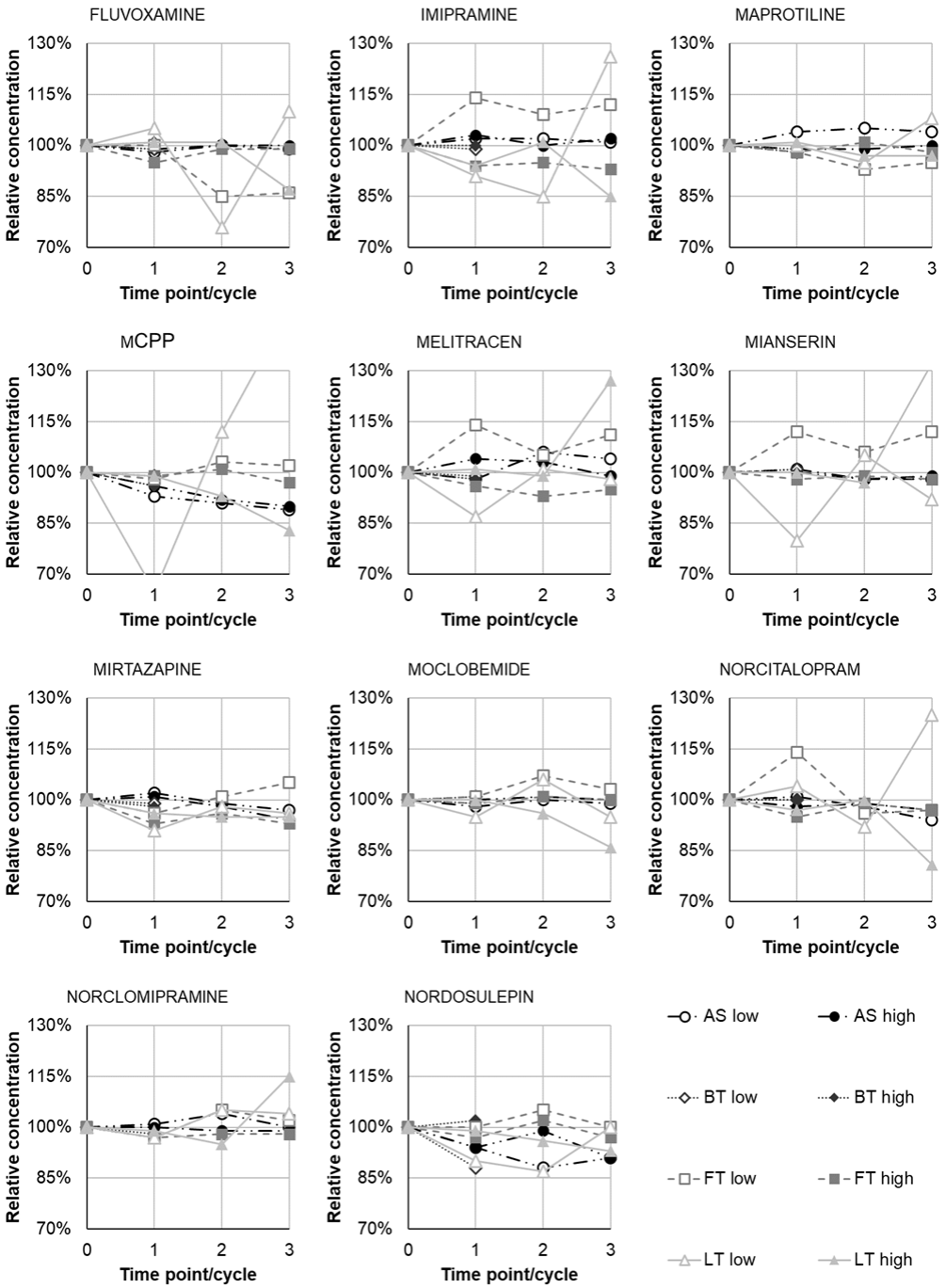

Figure S5 - 1. Stability data for the antidepressants at a low and high concentration. (continued) The data points represent 0 hours, 7.5 hours, 12.5 hours and 17.5 hours for the autosampler (AS) stability; 0 hours and 3 hours for the benchtop (BT) stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw (FT) stability; 0 hours, 1 week, 1 month and 3 months for the long-term (LT) stability.

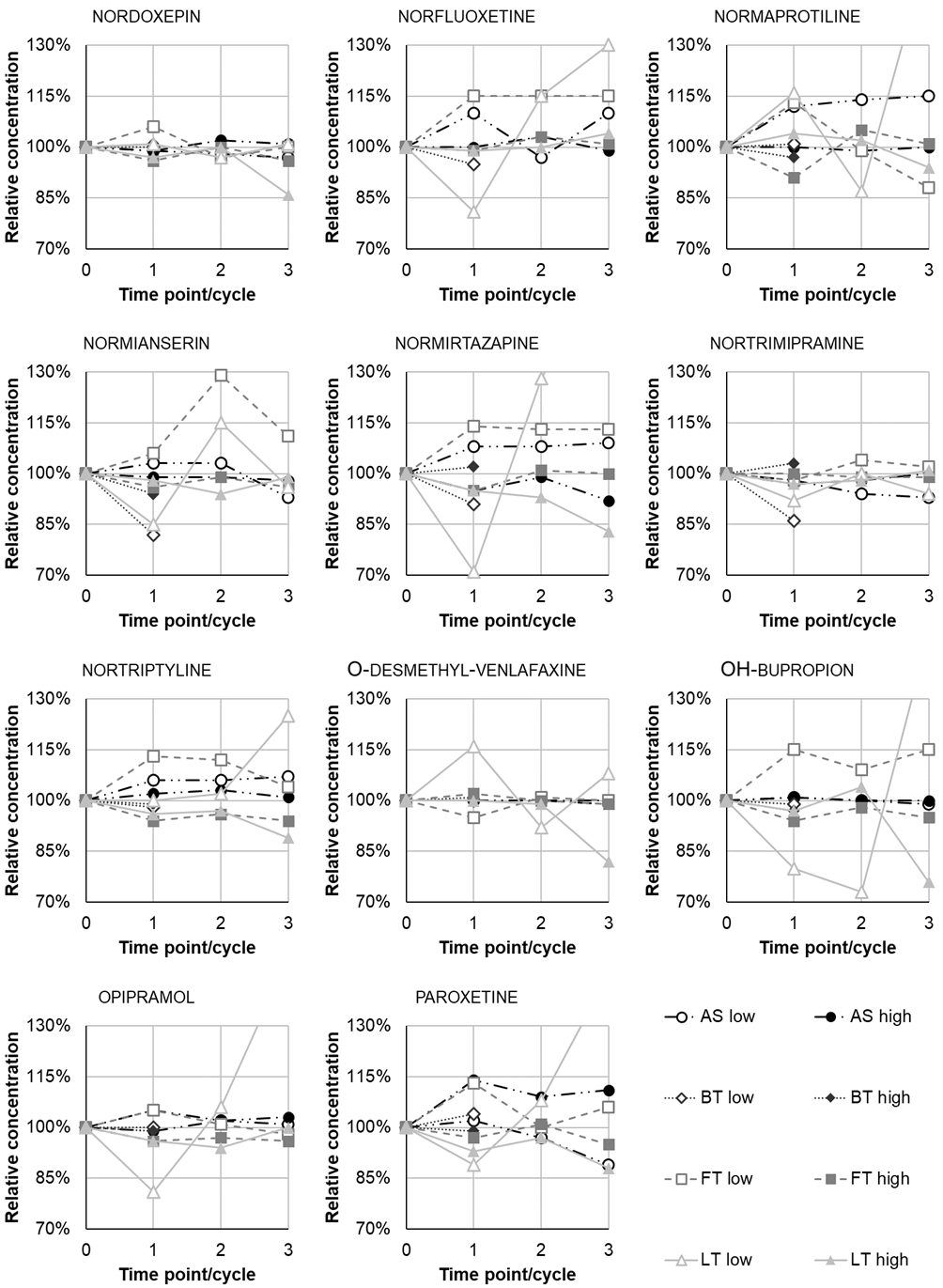

Figure S5 - 1. Stability data for the antidepressants at a low and high concentration. (continued) The data points represent 0 hours, 7.5 hours, 12.5 hours and 17.5 hours for the autosampler (AS) stability; 0 hours and 3 hours for the benchtop (BT) stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw (FT) stability; 0 hours, 1 week, 1 month and 3 months for the long-term (LT) stability.

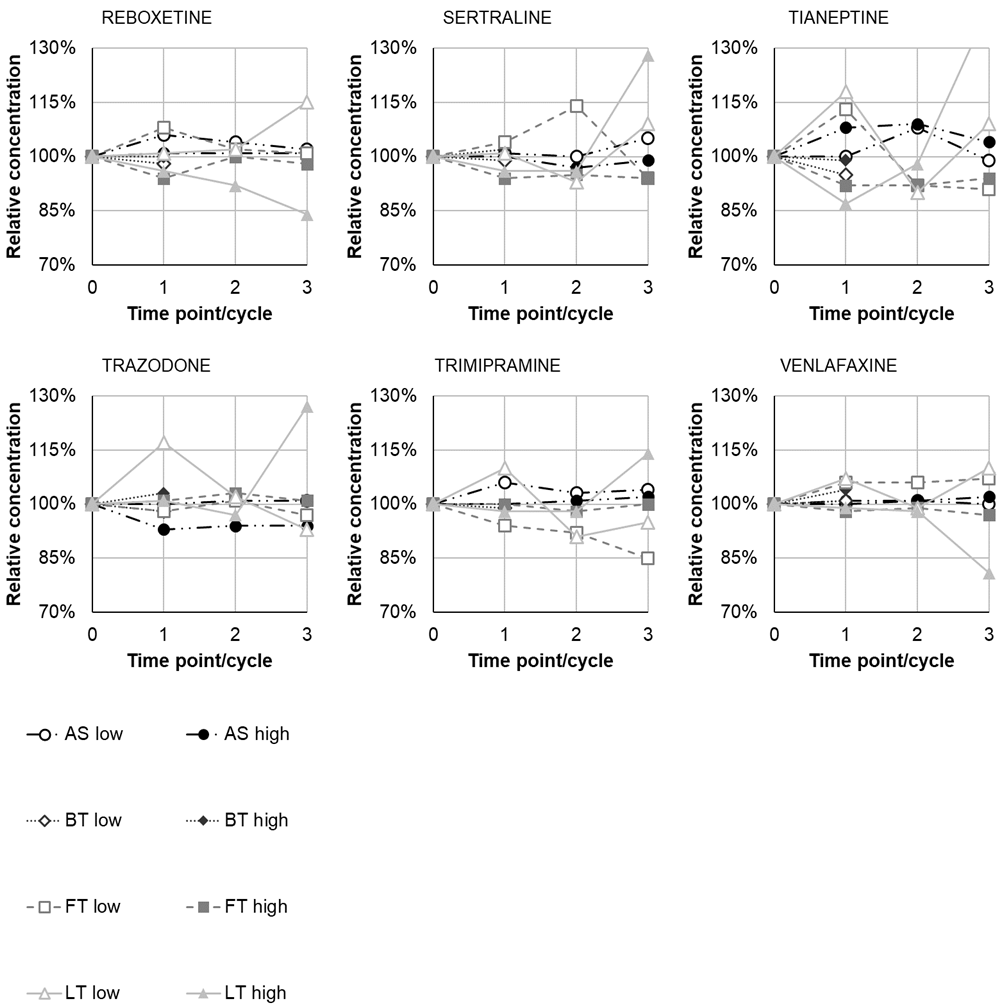

Figure S5 - 1. Stability data for the antidepressants at a low and high concentration. (continued) The data points represent 0 hours, 7.5 hours, 12.5 hours and 17.5 hours for the autosampler (AS) stability; 0 hours and 3 hours for the benchtop (BT) stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw (FT) stability; 0 hours, 1 week, 1 month and 3 months for the long-term (LT) stability.

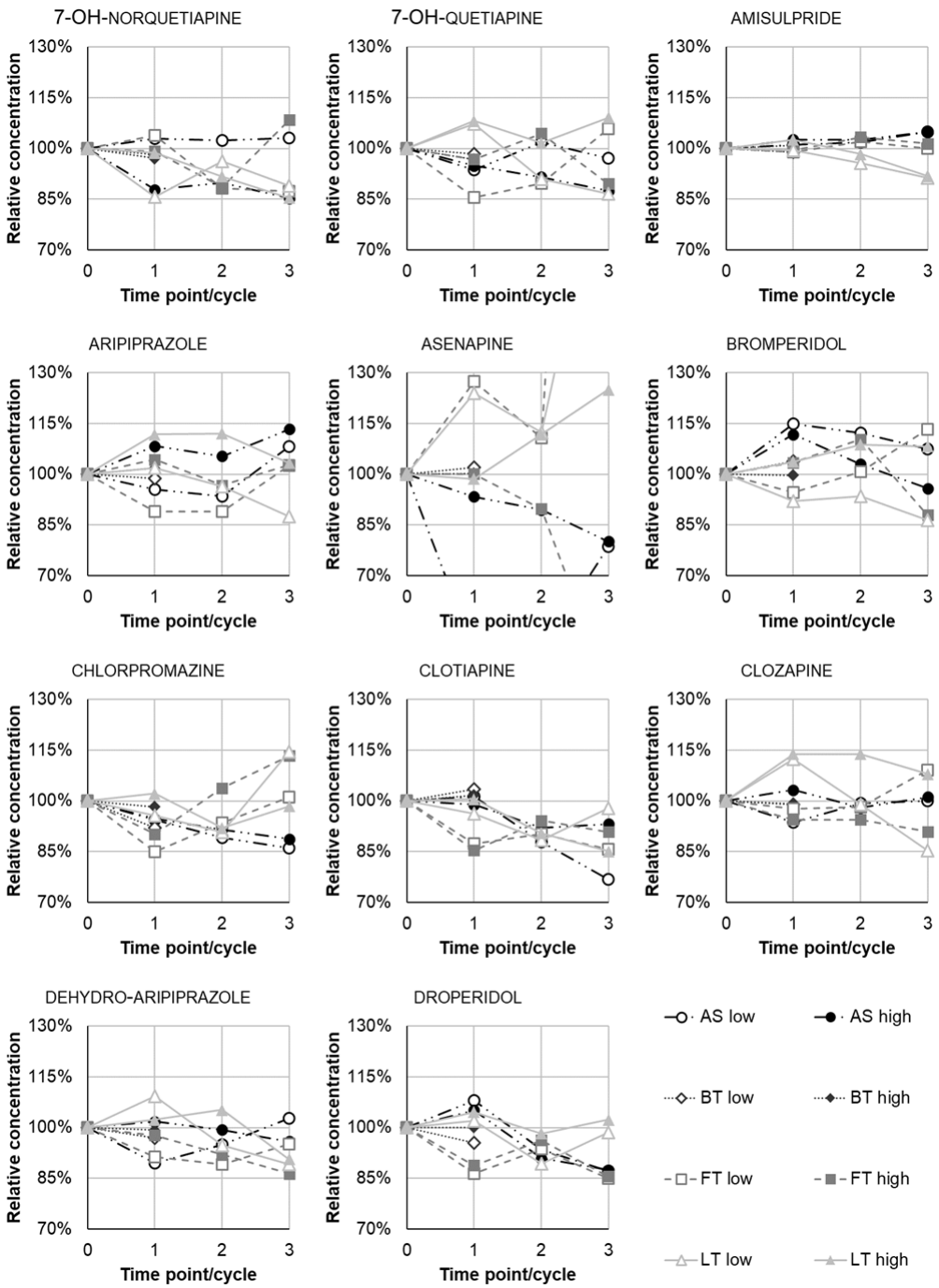

Figure S5 - 2. Stability data for the antipsychotics at a low and high concentration. The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

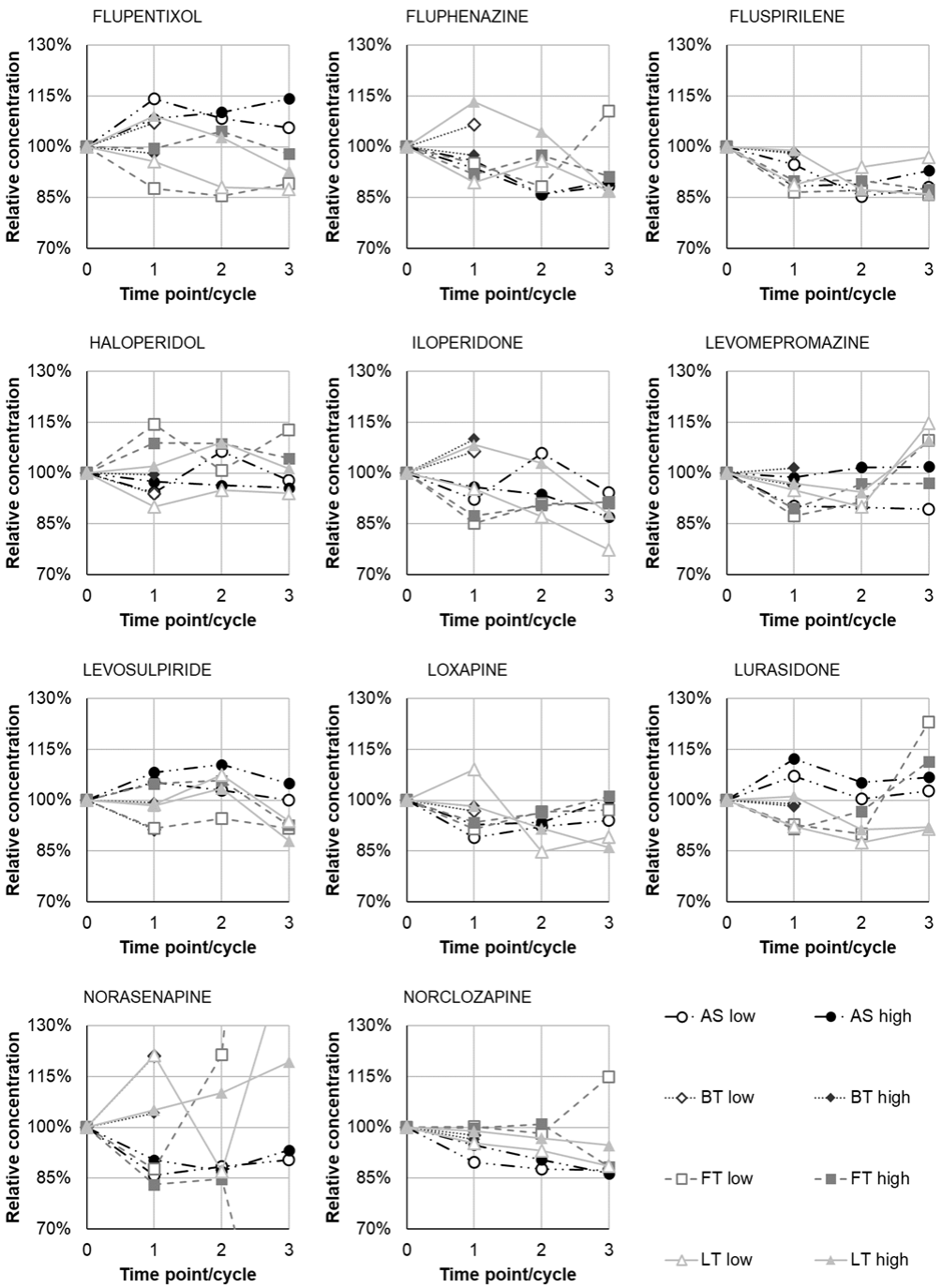

Figure S5 - 2. Stability data for the antipsychotics at a low and high concentration. (continued) The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

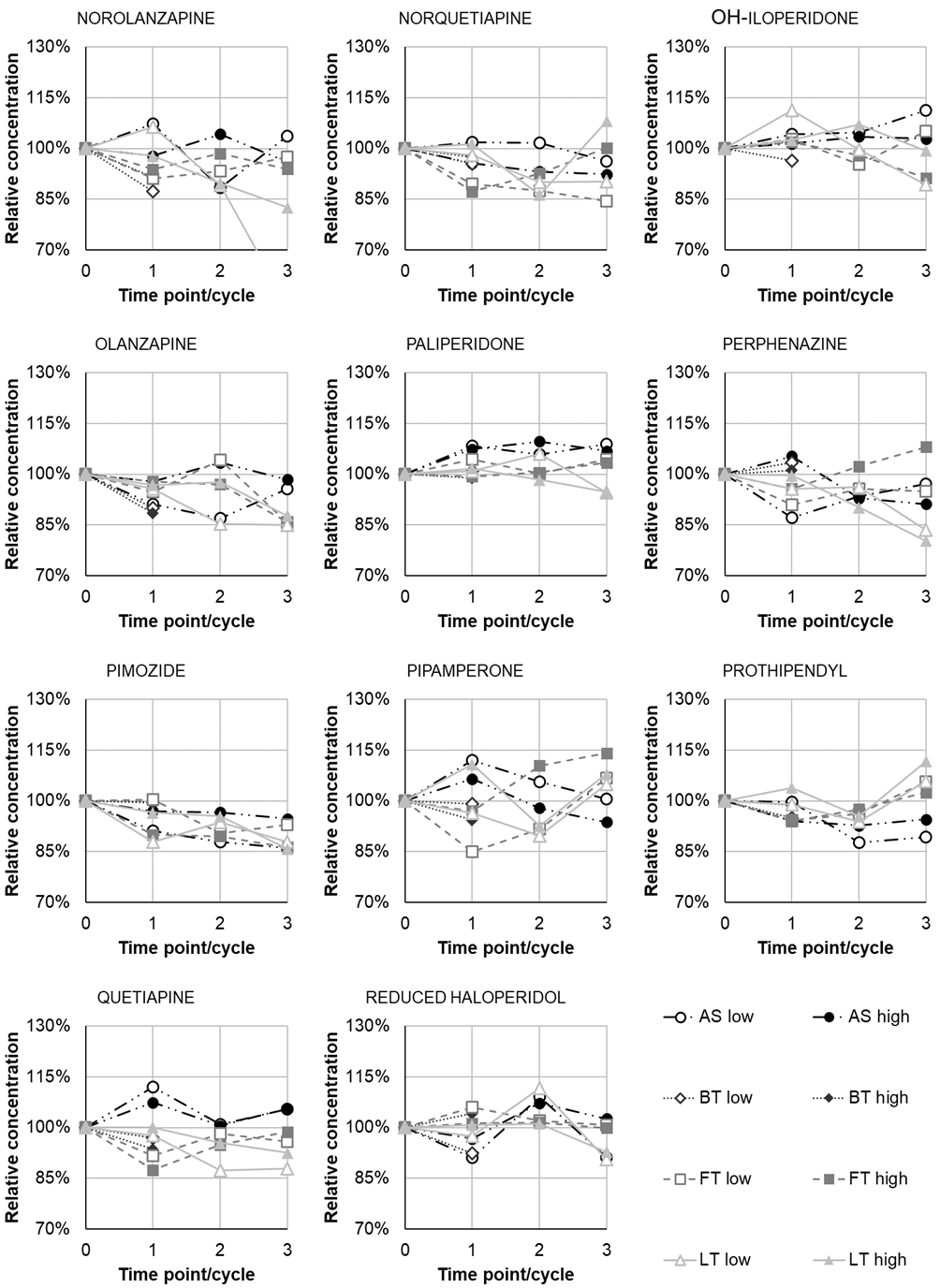

Figure S5 - 2. Stability data for the antipsychotics at a low and high concentration. (continued) The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

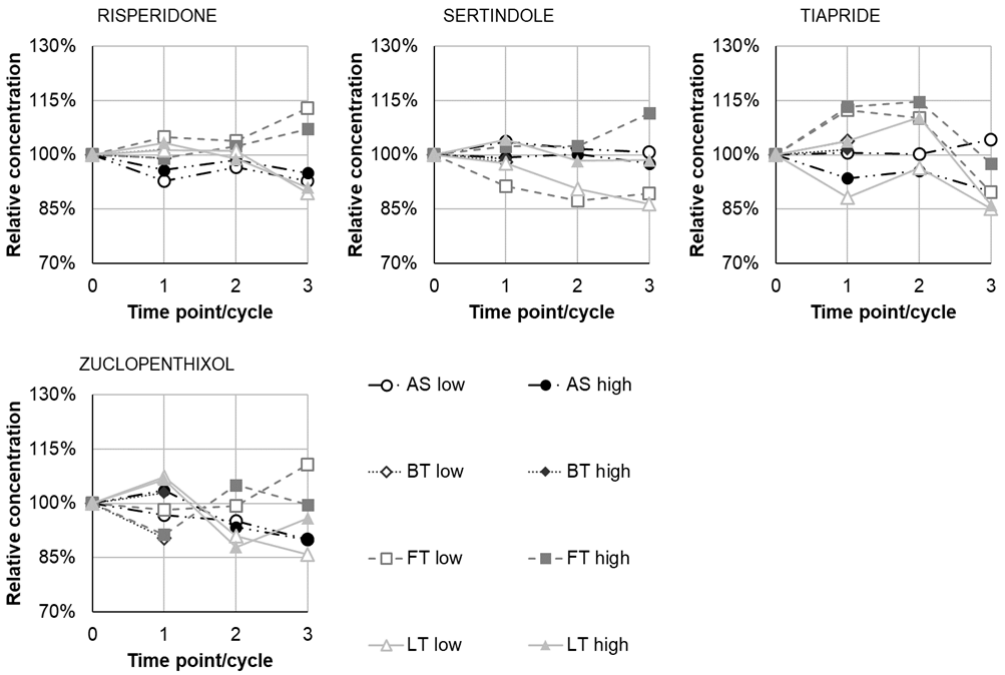

Figure S5 - 2. Stability data for the antipsychotics at a low and high concentration. (continued) The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

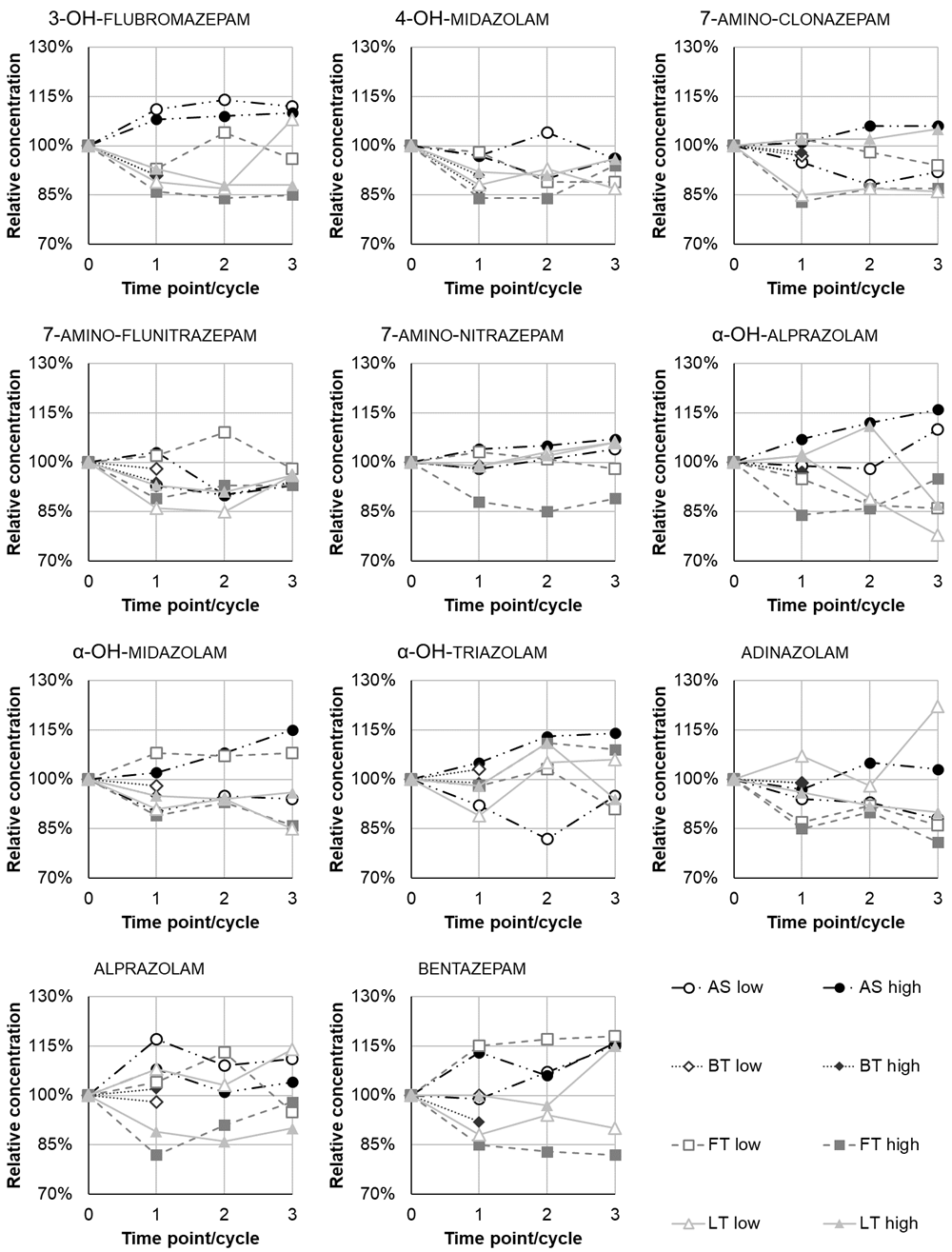

Figure S5 - 3. Stability data for the benzodiazepines and Z-drugs at a low and high concentration. The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

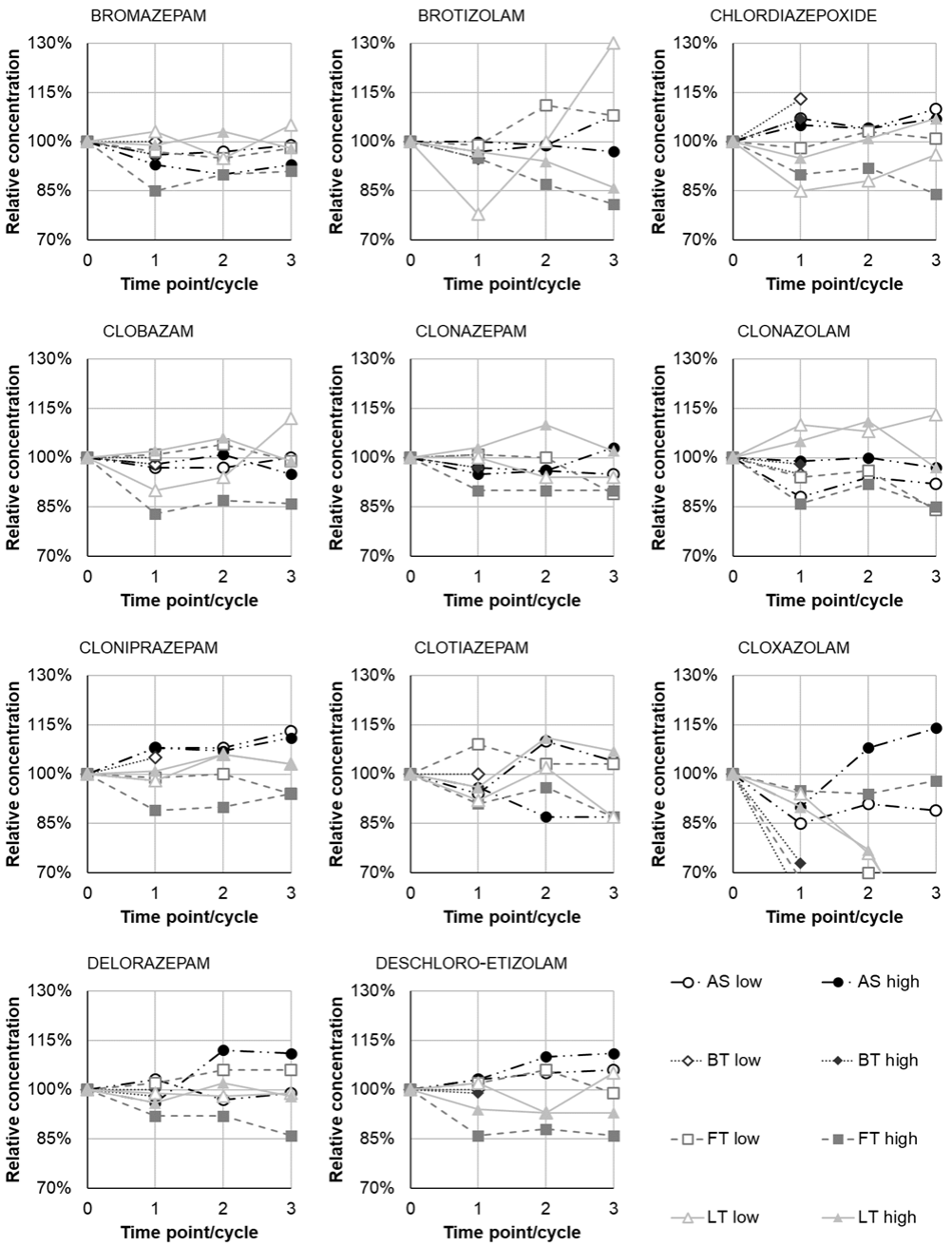

Figure S5 - 3. Stability data for the benzodiazepines and Z-drugs at a low and high concentration. (continued) The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

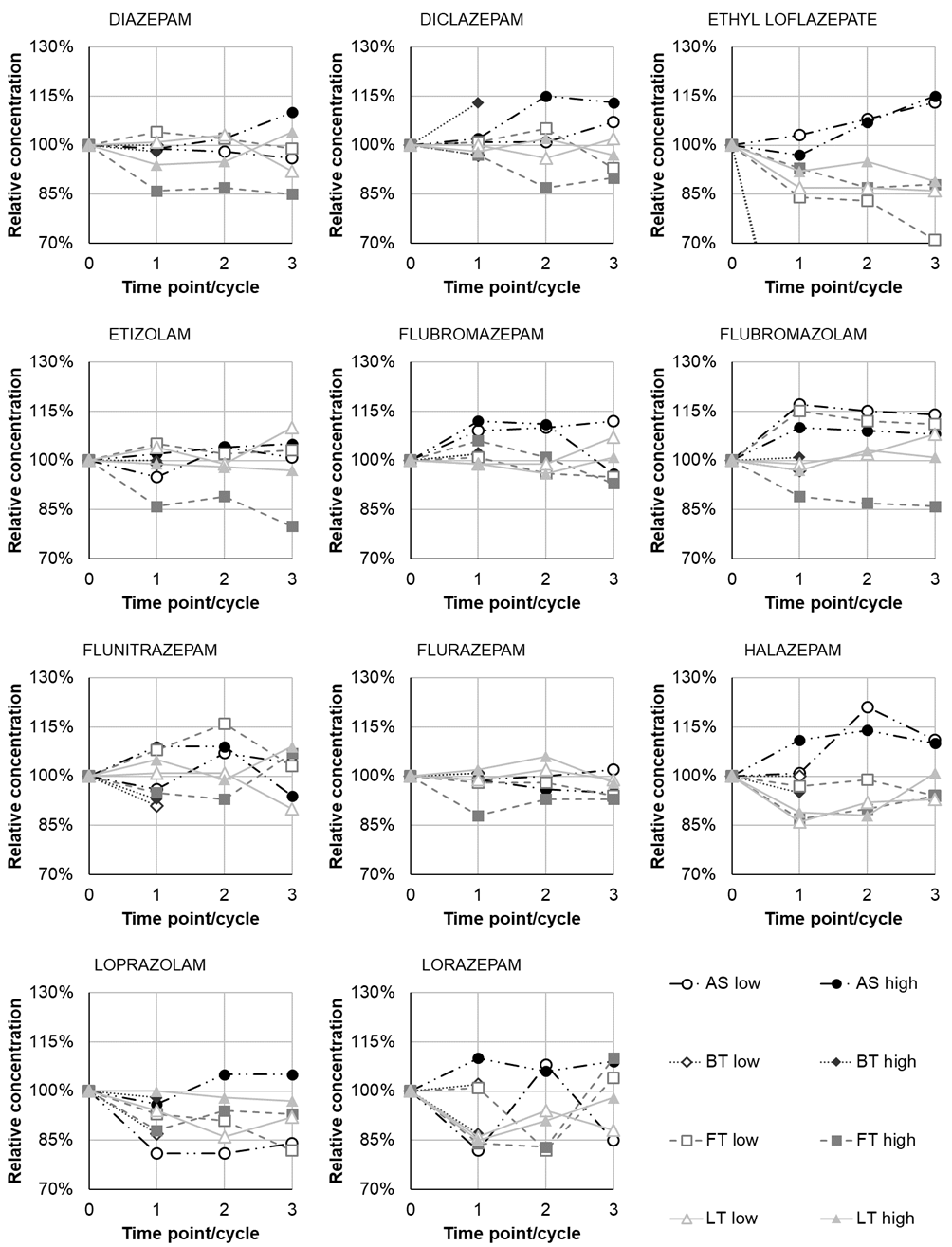

Figure S5 - 3. Stability data for the benzodiazepines and Z-drugs at a low and high concentration. (continued) The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

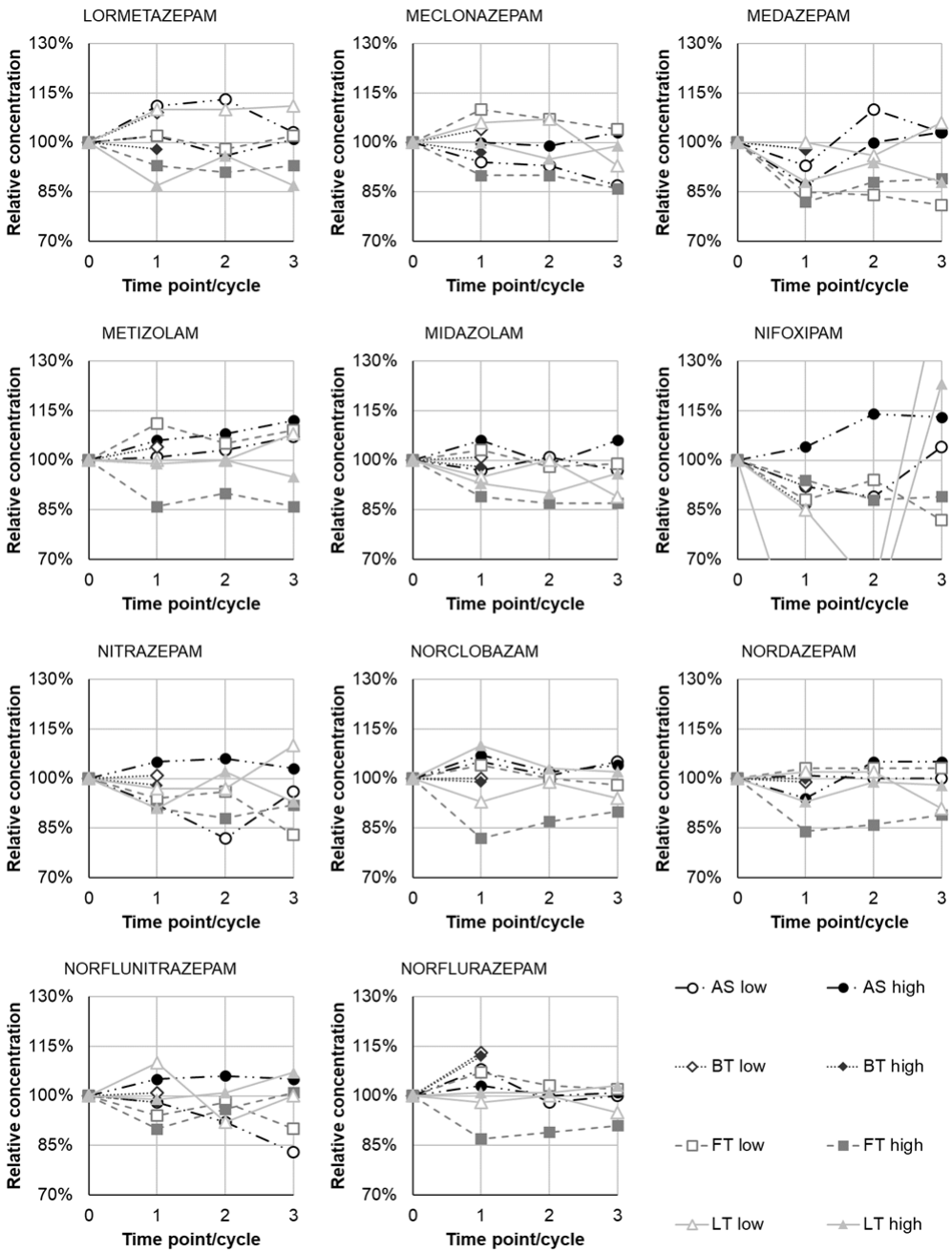

Figure S5 - 3. Stability data for the benzodiazepines and Z-drugs at a low and high concentration. (continued) The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

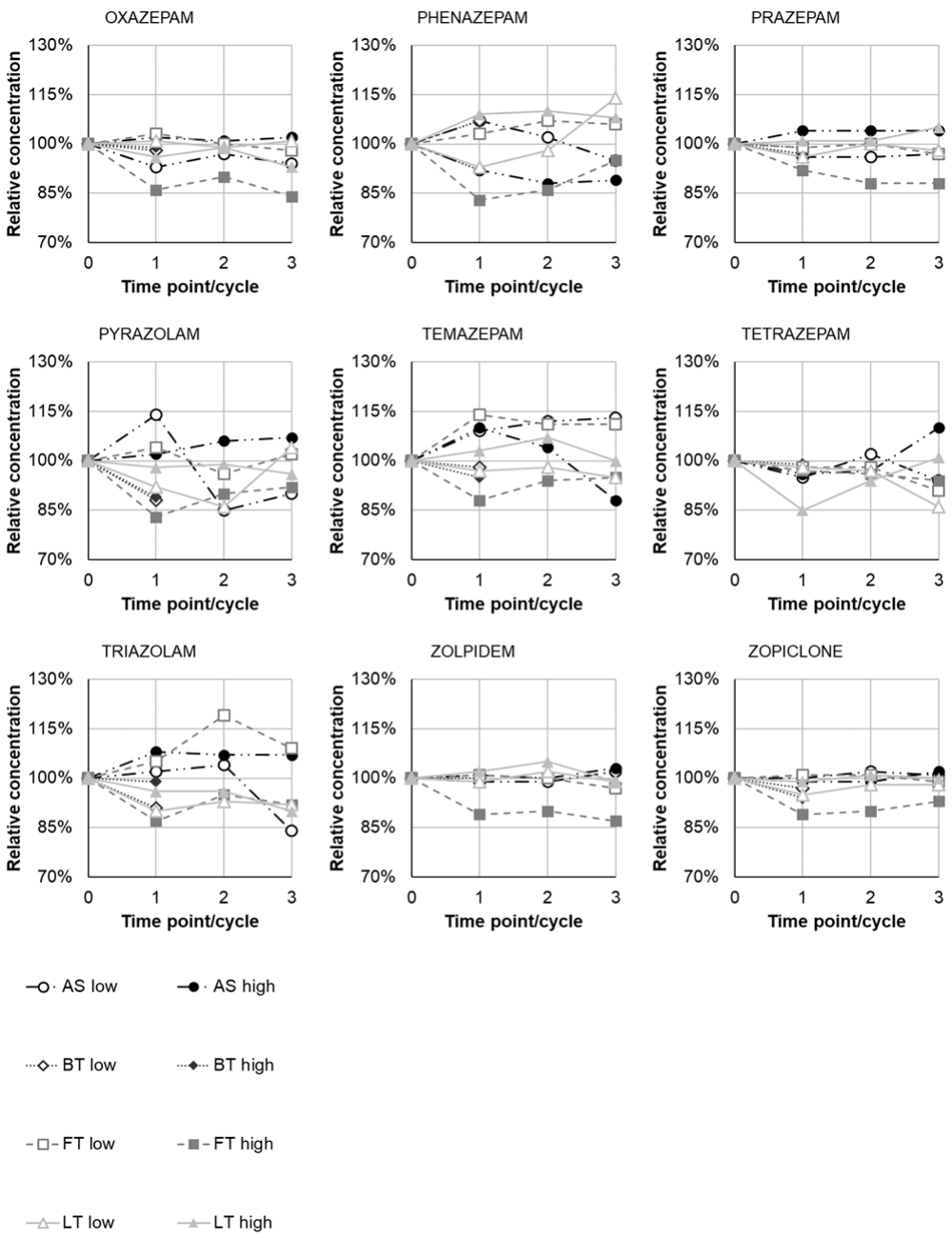

Figure S5 - 3. Stability data for the benzodiazepines and Z-drugs at a low and high concentration. (continued) The data points represent 0 hours, 24 hours, 48 hours and 72 hours for the autosampler stability; 0 hours and 3 hours for the benchtop stability; 0 cycles, 1 cycle, 2 cycles and 3 cycles for the freeze-thaw stability; 0 hours, 1 week, 1 month and 3 months for the long-term stability.

PART II:

SCREENING METHODS FOR PSYCHOACTIVE SUBSTANCES

CHAPTER 6

TRIGGERED MULTIPLE REACTION MONITORING

Based upon the following publication:

M. Degreef, E.M. Berry, A. Covaci, K.E.K. Maudens, A.L.N. van Nuijs, Qualitative and semi-quantitative screening of selected psychoactive substances in blood: Usefulness of liquid chromatography – triple quadrupole and quadrupole time-of-flight mass spectrometry in routine toxicological analyses. *(in submission).*

6.1. METHOD DEVELOPMENT

Triggered multiple reaction monitoring (tMRM) acquisition can be seen as a variation on the above described dynamic multiple reaction monitoring (dMRM) methods, meant to increase sample throughput by benefitting from faster cycle times, increased sensitivity and larger amounts of cpds that can be added in one analytical method. Similar to dMRM, the operator is required to program experimentally determined precursor-product ion transitions, including fragmentor voltage, collision energy and retention time (window), for each of the desired analytes. However, whereas all transitions are monitored continuously within their respective retention time windows for dMRM, tMRM methods make a distinction between primary and secondary transitions. Per analyte, at least one primary transition must be defined, which acts like a dMRM transition and therefore is continuously monitored. If the abundance of the primary transition exceeds a self-determined threshold, the instrument will start acquiring the secondary transitions. Multiple secondary transitions may be defined per analyte, as long as the total number of transitions is not greater than 10. When and how to monitor each transition is an essential part of the method development and will be discussed in the following sections.

6.1.1. Mass spectrometric optimisation

6.1.1.1. Selection acquisition parameters

Initially, 72 different tMRM methods were created, which differed in the tMRM-specific parameters trigger window, trigger entrance, trigger delay and repeats. The trigger threshold was set to 0 (no triggering of secondary transition acquisition needed) in order to better evaluate the effect of each change in parameters independent of potential noise, ionisation efficiency or other confounding factors. Precursor-product ion transitions from the previously optimised dMRM methods (Table S3 - 1 p. 47) for antidepressants (ADs), antipsychotics (APs) and benzodiazepines or Z-drugs (BZDs) were used. Quantifier ions were set as primary transitions and qualifier ions as secondary transitions. For convenience during data analysis, a limited number of compounds were investigated at this stage, using a reference mixture containing citalopram, mirtazapine, prazepam, quetiapine and zopiclone and a labelled internal standard (ISTD) mixture containing citalopram-D_6, prazepam-D_5 and quetiapine-D_8. Mixtures were prepared in acetonitrile (ACN), containing each of the compounds at a concentration of 200 ng/mL. Blank horse serum (500 µL) was spiked with 50 µL of each mixture and subsequently extracted using the above described liquid-liquid extraction (see section 4.3 p. 67). Reconstituted in 40 µL ACN, the samples were analysed in duplicate per method and the results visually compared using the MassHunter Qualitative Analysis 10.0 software (Agilent Technologies, Santa Clara, California, US).

The trigger window is defined as the time range within which secondary transitions may be acquired, provided the trigger threshold has been exceeded. Although standardly set to the same time range as the retention time window, it can be narrowed to ensure secondary transition acquisition closer to the apex of the peak. Investigated trigger windows were varied between 0.05 min, 0.1 min, 0.2 min and 0.3 min. The retention time window was set to 0.5 min in all experiments. Larger trigger windows resulted in more Gaussian peak shapes and more acquisition cycles for the secondary transitions. Noteworthy differences were found between trigger windows of 0.2 min and 0.3 min on the one hand and 0.05 min and 0.1 min on the other hand (Figure 6 - 1).

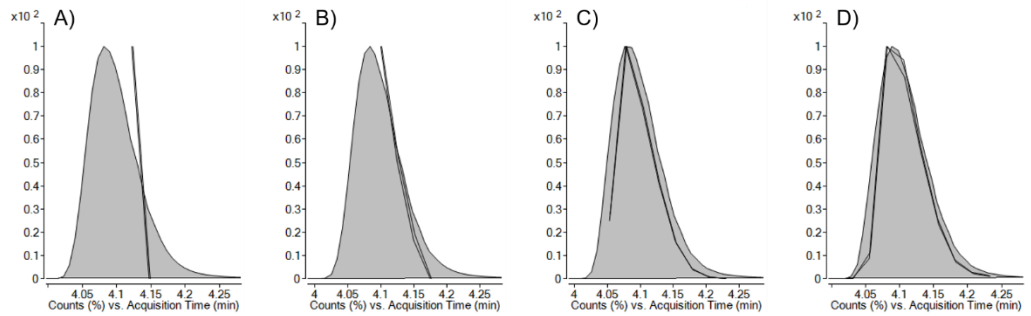

Figure 6 - 1. Influence of the trigger window on the tMRM acquisition of citalopram. The trigger window was varied between A) 0.05 min, B) 0.1 min, C) 0.2 min and D) 0.3 min. The trigger entrance and delay were 0 and 2 cycles, respectively. More data points have been acquired for secondary transitions using higher trigger windows.

The trigger entrance determines the number of cycles that will be skipped before the acquisition of secondary transitions starts. For example, if a trigger entrance of 2 is chosen, the instrument will not acquire secondary transitions in the first 2 cycles after the trigger threshold has been reached, but will obtain them from the third cycle on only. Values of 0, 5 and 10 cycles were set. A trigger entrance value of 10 significantly delayed the onset of secondary transitions acquisition, potentially missing the peak apex. Minimal differences were seen for a delay of 0 and 5 cycles (Figure 6 - 2).

Not to be confused with trigger entrance, trigger delay is the number of cycles that will be skipped between each acquisition cycle of secondary transitions. For example if a trigger delay of 1 is chosen, secondary transitions will be acquired at every other cycle. Delays of 2, 5 and 10 cycles were investigated. Smaller trigger delays resulted in more data points acquired, increasing confidence in the detection of the analyte in question (Figure 6 - 3).

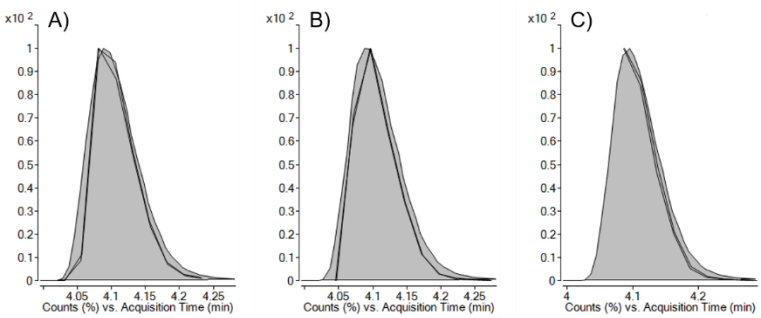

Figure 6 - 2. Influence of the trigger entrance on the tMRM acquisition of citalopram. The trigger entrance was varied between A) 0 cycles, B) 5 cycles and C) 10 cycles. The trigger window and delay were 0.3 min and 2 cycles, respectively. A trigger entrance of 10 cycles pushed the onset of secondary transitions acquisition too far back, potentially missing the peak apex.

Lastly, the number of repeats were set to either 10 or 20, indicating the maximum number of cycles during which secondary transitions could be acquired. This value excludes non-acquisition cycles (as defined by the combination of trigger entrance and delay) and is reset once the signal of the primary transition drops below the trigger threshold. No differences in the number of data points acquired over the peaks were observed when varying this parameter for each of the methods.

Overall, methods with a trigger window of 0.2 min or 0.3 min, a trigger entrance of 0 or 5 cycles and a trigger delay of 2 or 5 cycles showed the most promising results. It should however be noted that slight retention time shifts had been observed for some compounds, which may have influenced the findings, particularly for the trigger window.

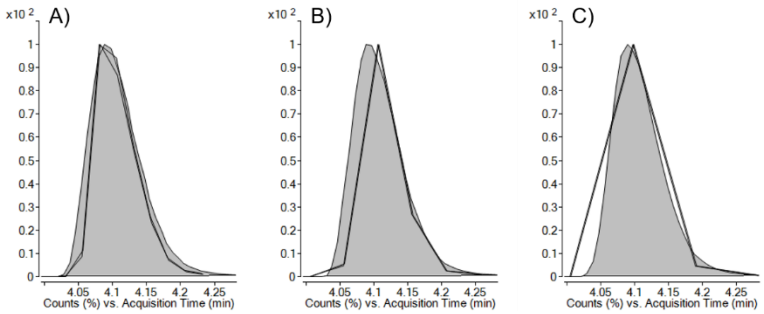

Figure 6 - 3. Influence of the trigger delay on the tMRM acquisition of citalopram. The trigger delay was varied between A) 2 cycles, B) 5 cycles and C) 10 cycles. The trigger window and entrance were 0.3 min and 0 cycles, respectively. Smaller delays increased the number of data points acquired and thereby confidence in the detection of the analyte.

6.1.1.2. Fine-tuning acquisition parameters

Based upon the findings in section 6.1.1.1, five acquisition methods were retained for further investigation (Table 6 - 1). From a list of ten reference standards (ADs citalopram, melitracen and mirtazapine; APs flupentixol, olanzapine, quetiapine and risperidone; BZDs midazolam, prazepam, zopiclone) six new reference mixtures were prepared, each containing five randomly selected compounds at a concentration of 1 µg/mL. These mixes were diluted to 20 ng/mL with ACN, containing 50 ng/mL prazepam-D_5 and injected as such onto the instrument. Similarly to section 6.1.1.1 above, the results were inspected using the MassHunter Qualitative Analysis 10.0 software. Methods were scored on overall (Gaussian) peak shape for the primary transition and on the number of acquisition cycles and acquisition near to the peak apex for secondary transitions.

Table 6 - 1. tMRM-specific parameters for the selected methods.

Method	Trigger window	Trigger entrance	Trigger delay
A	0.1 min	0 cycles	5 cycles
B	0.1 min	0 cycles	2 cycles
C	0.2 min	0 cycles	5 cycles
D	0.2 min	0 cycles	2 cycles
E	0.3 min	0 cycles	2 cycles

Primary transitions had been acquired for all compounds of interest (cpds), showing near-Gaussian peak shapes. As the spiked concentration was equal to that of the previous experiments, such results were expected and served more as a control for proper instrument functioning rather than as a method evaluation tool. With regards to the secondary transitions, the outcome was positively linked to wider trigger windows and shorter trigger delays (Figure 6 - 4). Methods A and B, both with the narrowest trigger window of only 0.1 min, showed the worst secondary transition profile with a respective average of 3 and 4 cycles acquired only. All data points were acquired on the downslope of the peaks, past the apex. Method C had a better distribution profile of the secondary transition

data points over the peaks, but still only averaged 5 out of 10 possible cycles acquired. With a respective average of 8 and 9 acquisition cycles, distributed over the width of the peaks, methods D and E performed better than the other methods.

Method E, with a trigger window of 0.3 min, trigger entrance of 0 cycles and trigger delay of 2 cycles performed most optimal for all investigated compounds and was retained for further testing.

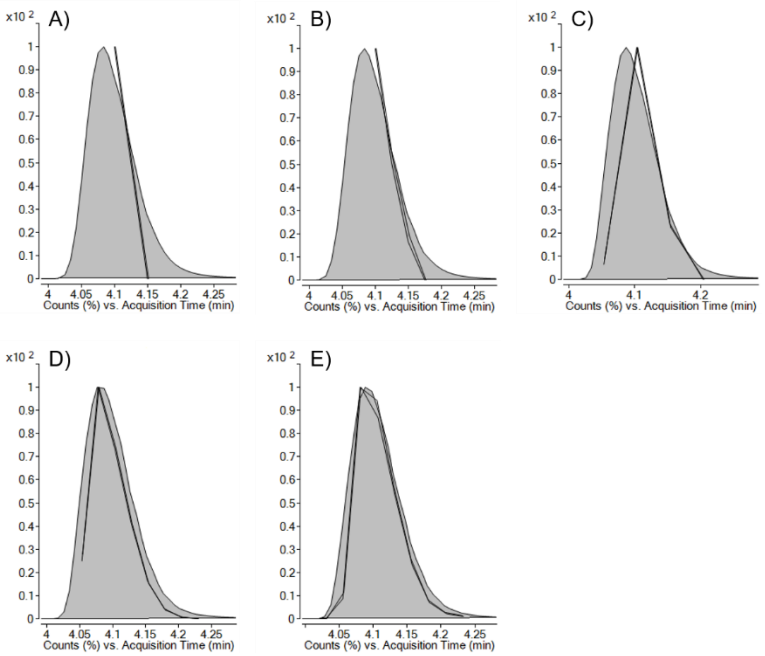

Figure 6 - 4. tMRM acquisition of citalopram for the selected methods. The trigger windows were varied between A/B) 0.1 min, C/D) 0.2 min and E) 0.3 min. Similarly, trigger delays were set to either B/D/E) 2 cycles or A/C) 5 cycles. Generally wider trigger windows and smaller trigger delays improved the outcome (a greater number of acquisition cycles and acquisition nearer to the peak apex), with method E performing the overall best.

6.1.1.3. Selection trigger threshold

To reduce the number of false positive results, further method optimisation focused on the determination of an appropriate threshold below which no secondary transitions would be acquired. A further 7 methods (I – VII) were created based upon the above mentioned method E (Table 6 - 1), for which the trigger thresholds were set to 0, 100, 200, 500, 1000, 2000 or 5000 counts, respectively. Blank horse serum was spiked with mixtures containing all included ADs, APs and BZDs at their respective CAL L1 or CAL L2 (Table S6 - 1). Additionally, serum samples spiked with CAL L1 were diluted 1/2, 1/5, 1/10, 1/20 and 1/50 times. The samples were extracted as described earlier and 1 µL was injected onto the liquid chromatography – triple quadrupole mass spectrometer (LC-QQQ).

A total of 131 cpds were spiked to each sample. The results were visually evaluated in the MassHunter Qualitative Analysis 10.0 software. If both primary and secondary acquisitions were acquired, the cpd was listed as detected. If one or more transitions were missing, the cpd was considered not detected (Figure 6 - 5). A clear distinction was observed between thresholds 0, 100, 200 and 500 (respective

percentages of false negative results at CAL L1 2%, 4%, 8% and 8%) and thresholds 1000, 2000 and 5000 (respective percentages of false negative results at CAL L1 31%, 38%, 52%). Thus, the latter thresholds were excluded from further analyses. Thresholds of 0 and 100 gave the best results, but increase the risk of false positive results, as simple baseline variation could already trigger acquisition of secondary transitions. Thresholds 200 and 500 were therefore retained for further testing. Concentrations two and five times lower than CAL L1 could be detected for 75% or more of the cpds. Greater dilutions remained generally undetected as they became indistinguishable from the background noise.

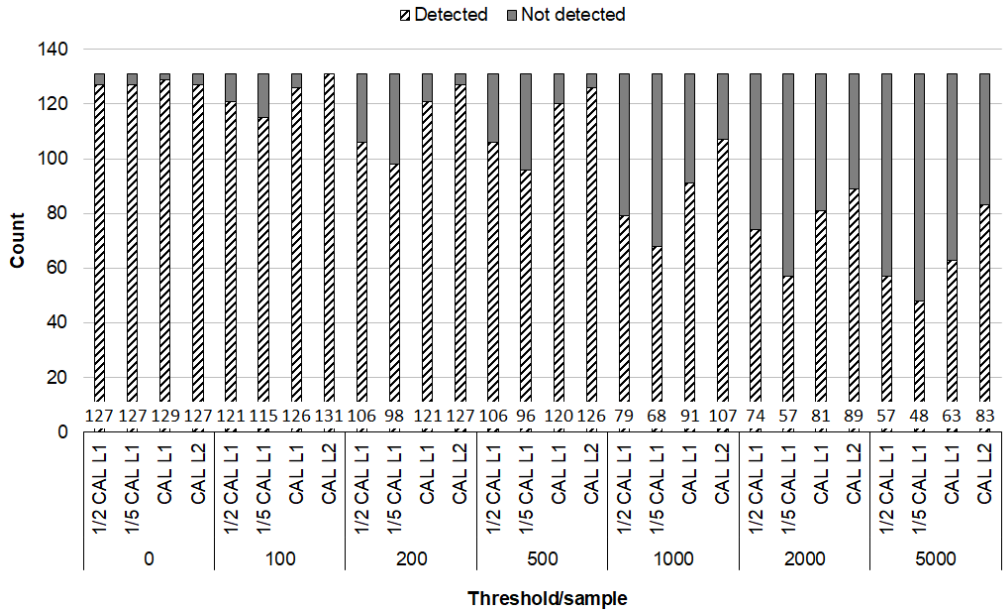

Figure 6 - 5. Investigation of the appropriate trigger threshold. To each sample 131 cpds were spiked. Detected cpds are those for which both primary and secondary transitions have been acquired. The values at the bottom of each bar mention the number of detected cpds. Thresholds greater than 500 risked not detecting a significant number of low concentrated cpds. Thresholds lower than 200 were excluded to reduce the number of false positive results.

6.1.2. Selection best method

6.1.2.1. Samples & reagents

Fresh calibrator mixes (7 levels, CAL L1-7) containing all ADs, APs and BZDs were prepared in ACN. For ease of use, the AD calibration curve was reduced from 10 to 7 levels. An overview of the different concentrations can be found in Table S6 - 1. Based upon the results of gas chromatography – mass spectrometry and liquid chromatography – diode array detection screening, 105 archived cases were selected. All samples had been submitted to the Toxicological Centre in the framework of their medico-legal analyses and had previously screened positive for the presence of at least one AD, AP or BZD, consisted of whole blood, plasma or serum as a matrix, and preferably contained a minimum of 1 mL of sample volume. Of these, 25 samples were selected at random using the RANDOM.ORG online tool [414]. A 200 µL aliquot of each sample was spiked with 20 µL ACN and 20 µL ISTD mix (see section 6.3.1 below) and extracted as described previously with 800 µL methyl-tertiary-butyl-ether. The dried extract was reconstituted in 20 µL ACN and injected onto the

LC-QQQ. Analytes of interest were acquired in tMRM mode using methods III (trigger threshold 200) and IV (trigger threshold 500) as described in section 6.1.1.3. Additionally, all samples were analysed using the validated dMRM methods (see Chapter 5 p. 69) to acquire reference values.

6.1.2.2. Outcomes & method performance

Data analysis was performed using the Agilent MassHunter Quantitative Analysis 10.0 (for QQQ) software (Agilent Technologies, Santa Clara, California, US). The dMRM data analysis methods for the ADs, APs and BZDs were combined into one new quantitative method and the calibration levels were updated where needed. As secondary transitions are not monitored continuously and may only be triggered near to the apex of the peak (especially for lower concentrated samples), relying on ion ratios for identification, as used for dMRM, is generally not recommended. An ion spectrum library was therefore generated using a sufficiently high calibration level (here CAL L5), against which each compound in the other samples was be scored (Figure 6 - 6). Generally, library match scores ≥ 90 corresponded well with the true positive identification of a compound.

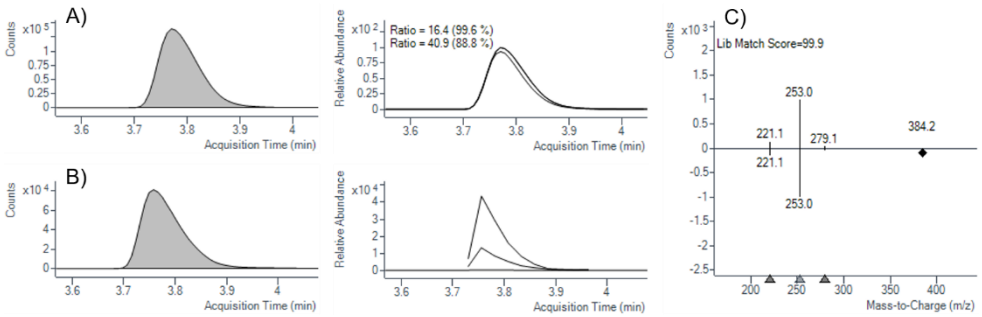

Figure 6 - 6. Comparison of the dMRM (A) and tMRM (B) spectra for quetiapine. Spectra of calibration level 3 (80 ng/mL) are visualised using the Agilent MassHunter Quantitative Analysis 10.0 (for QQQ). Qualifier-quantifier ion ratios validate the detection of a compound for dMRM methods. For tMRM methods, the acquired transitions are compared to and scored against a library spectrum (C).

Dynamic multiple reaction monitoring mode picked up 125 cpds with a concentration above the lower limit of quantification (LLOQ): 43 ADs, 24 APs and 58 BZDs. Using a respective trigger threshold of 200 and 500, 105 and 103 of these cpds had a library match score of ≥ 90 (Figure 6 - 7). On the other hand, 20 cpds were not detected using a trigger threshold of 200, 22 cpds for a threshold of 500. The most commonly missed cpd (n = 8) was mCPP, an active metabolite of trazodone as well as an abused designer drug [415]. Its precursor trazodone was detected in all but 1 sample, where the calculated dMRM concentration was lower than the LLOQ and therefore excluded from the data analysis. mCPP concentrations that were not detected ranged from just above the LLOQ of 2 ng/mL to 45 ng/mL. Norquetiapine and 7-OH-norquetiapine gave false negative results for two different samples each. Those negative for 7-OH-norquetiapine were all positive for norquetiapine, as well as quetiapine and 7-OH-quetiapine. In the samples negative for norquetiapine, both dMRM and tMRM detected quetiapine in 1 sample only (dMRM concentration 26 ng/mL) but none of its metabolites. All calculated norquetiapine concentrations were close to the LLOQ of 3 ng/mL. A further five cpds (7-amino-clonazepam, amitriptyline, diazepam, flupentixol, nordazepam) were present in dMRM analysis at concentrations around their LLOQ and were subsequently missed in the tMRM methods. Norcitalopram (11 ng/mL), normirtazapine (5 ng/mL & 12 ng/mL) and nortriptyline (25 ng/mL)

could not be detected at concentrations significantly higher than their respective LLOQs (2 ng/mL, 0.5 ng/mL & 10 ng/mL). Once more, their precursors citalopram, mirtazapine and amitriptyline had been detected in each of the blood samples. Lastly, amisulpride was missed in 1 case at a concentration of 22 ng/mL, well above its LLOQ of 10 ng/mL.

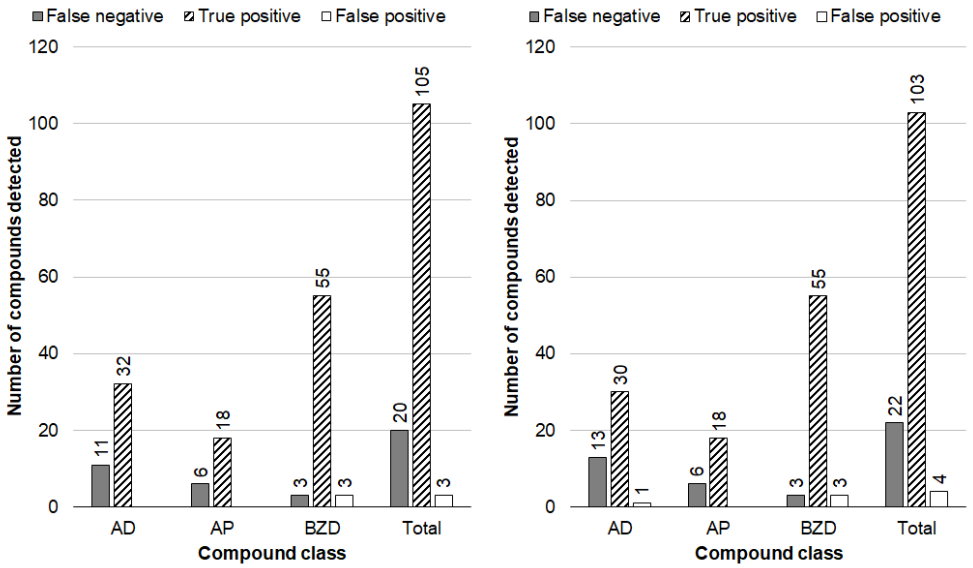

Figure 6 - 7. Results of the tMRM screening using a trigger threshold of 200 (left) or 500 (right). Results were compared to those obtained with validated dMRM quantification methods. The majority of compounds was correctly identified. False negative results were predominantly observed for mCPP and quetiapine metabolites, though their precursors could be detected in these instances, or for compounds with concentrations near their lower limits of quantification.

A BZD was erroneously identified in three samples, two of which were said to contain etizolam. This cpd shares its precursor ion and some of the product ions with triazolam, which elutes 0.2 min earlier. However, dMRM analysis did not reveal the presence of either cpd in the samples. The third sample was false positive for diazepam. Although this cpd could not be detected using dMRM analysis, nordazepam and oxazepam were present (temazepam was not identified). An additional false positive result was found for fluoxetine when running with a trigger threshold of 500. dMRM analysis revealed a highly doubtful peak, at an estimated concentration far below the LLOQ. This hit was not found using the tMRM method with threshold 200, likely indicating a random rather than systemic false positive hit.

Although barely any differences could be noted, a threshold of 200 correctly identified two additional samples compared to a threshold of 500 and was therefore retained in the final method.

6.2. VALIDATION QUALITATIVE SCREENING

6.2.1. Description optimised method

Sample preparation and LC settings remained unchanged compared to the dMRM methods. QQQ source parameters were gas (N_2) temperature 300 °C, gas flow 10 L/min, nebuliser 30 psi, sheath gas

heater 250 °C, sheath gas flow 11 L/min, capillary voltage 4000 V, voltage charging 500. 143 primary transitions were monitored (131 cpds and 12 ISTDs). Retention time windows were set to 0.5 min. Secondary transitions (n = 262) were triggered once the abundance of the primary ones surpassed 200 counts. The trigger window was set to 0.3 min, trigger entrance to 0 cycles and trigger delay to 2 cycles. Mass-to-charge values, fragmentor voltage and collision energy were copied from the dMRM methods (Table S3 - 1 p. 47). Retention times were updated following injection of a high concentrated calibration sample.

6.2.2. Validation against case samples

As no strict guidelines have been described for qualitative methods, the trends seen during method optimisation (section 6.1.2.2) needed to be confirmed on a larger number of samples prior to implementation in routine analysis. From the previously selected samples, those that were not used for method optimisation served to validate the final method (n = 80). Of the cpds detected with dMRM, 85% (n = 265) were picked up by the tMRM method (Figure 6 - 8). As seen during method development, BZDs made up the majority of detected cpds as well as of false positive results.

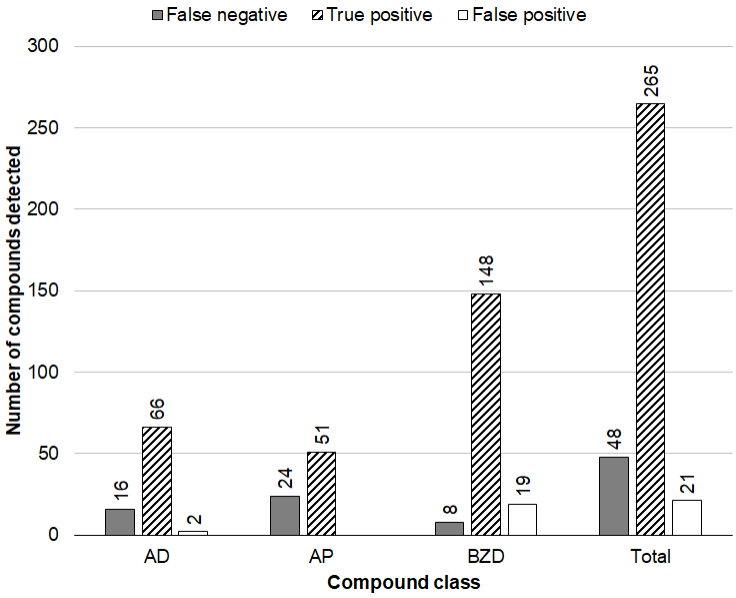

Figure 6 - 8. Validation of the optimised tMRM method. Results were compared to those obtained with validated dMRM quantification methods. The majority of compounds was correctly identified. False negative results were predominantly observed for mCPP and quetiapine metabolites, though their precursors could be detected in these instances, or for compounds with concentrations near their lower limits of quantification.

Similar to what was observed during method development, mCPP and quetiapine metabolites were most often missed. With regards to mCPP, concentrations varying between the LLOQ (2 ng/mL) and 45 ng/mL were not detected, however, the precursor trazodone could be positively identified in all samples. Norquetiapine and 7-OH-norquetiapine gave false negative results in twelve samples, in two of which neither cpd could be detected and in one of which also 7-OH-quetiapine gave a false negative result. In samples falsely negative for 7-OH-norquetiapine (n = 9), quetiapine and its other

metabolites could usually be detected (n = 6). Norquetiapine but not quetiapine was identified in one of the remaining three samples. The other two samples contained 7-OH-norquetiapine, although at a concentration only marginally different from the LLOQ (1.1 ng/mL and 1.5 ng/mL vs. LLOQ 1.0 ng/mL). They also contained norquetiapine at a concentration four times that of the LLOQ of 3 ng/mL, which was not detected in tMRM mode. Quetiapine or 7-OH-quetiapine were absent for both tMRM and dMRM analyses. Three more samples were false negative for norquetiapine, however neither quetiapine nor any other metabolite had been detected by either acquisition method.

As described before, norcitalopram (16 ng/mL), normirtazapine (1 ng/mL & 27 ng/mL) and nortriptyline (35 ng/mL & 42 ng/mL) were missed at concentrations significantly higher than their respective LLOQs (2 ng/mL, 0.5 ng/mL & 10 ng/mL), but their precursors citalopram, mirtazapine and amitriptyline could be detected in all samples. Conversely, amitriptyline was missed in one sample (concentration 11.5 ng/mL vs. LLOQ 10 ng/mL) but nortriptyline had been identified. Norolanzapine could not be identified in 6 samples, ranging from concentrations at LLOQ (1 ng/mL) to as high as 180 ng/mL, although olanzapine was identified in all. No olanzapine-positive samples were present in the optimisation data set to compare these findings with. Of the remaining false negative samples, only four contained cpds present at a concentration significantly different from the LLOQ and had no related compounds detected: amisulpride (47 ng/mL vs. LLOQ 10 ng/mL), etizolam (5 ng/mL vs. LLOQ 1.25 ng/mL), norfluoxetine (32 ng/mL vs. LLOQ 10 ng/mL) and prothipendyl (706 ng/mL vs. LLOQ 4 ng/mL). For the latter, even though the primary transition had been picked up and two secondary acquisition cycles were triggered, no positive match was made. As only one sample tested positive for prothipendyl in dMRM, further samples are needed to indicate whether this is a recurring trend or a one-off miss.

Table 6 - 2. Selected false positive tMRM results. The reported dMRM values are estimated, below LLOQ concentrations only and have therefore been excluded from the analysis. The additional confirmed presence of related compounds suggests these might have been true positive identifications by tMRM. Dynamic multiple reaction monitoring, dMRM, lower limit of quantification, LLOQ.

Compound	dMRM (ng/mL)	LLOQ (ng/mL)	Confirmed related compounds
α-OH-midazolam	1.22	1.25	midazolam
α-OH-midazolam	1.11	1.25	midazolam
oxazepam	8.93	10	nordazepam
oxazepam	9.15	10	diazepam, nordazepam, temazepam
oxazepam	6.68	10	diazepam, nordazepam
temazepam	7.09	10	diazepam, nordazepam
temazepam	8.50	10	diazepam, nordazepam
temazepam	9.61	10	diazepam, nordazepam, oxazepam
venlafaxine	9.36	10	O-desmethyl-venlafaxine

As seen during method optimisation, the majority of false positive identifications were for etizolam (10 out of 21 cases). Interference by triazolam was once more excluded as none of the samples contained this cpd. Since neither primary nor secondary transitions could be detected in blank matrix samples (neat and spiked with the ISTD mix), currently unknown endogenous or other substances may explain these false positive results (Figure 6 - 9). There was also an unexplained false positive result for brotizolam compared to dMRM. Mirtazapine was erroneously identified in one sample. Unlike truly positive samples, its demethylated metabolite was not detected. Whether or not this would be an indicator for a potential false hit, requires analysis of more samples as only five samples

(including those from the method optimisation data set) contained mirtazapine. The remaining 9 cases are unlikely to be true false positive hits. dMRM analysis picked up precursor and product ions for all cpds, but as their calculated concentrations were close to but below the LLOQ, these hits were excluded from the analyses. Additionally, pharmacokinetically related cpds were identified and confirmed by dMRM in all samples (Table 6 - 2).

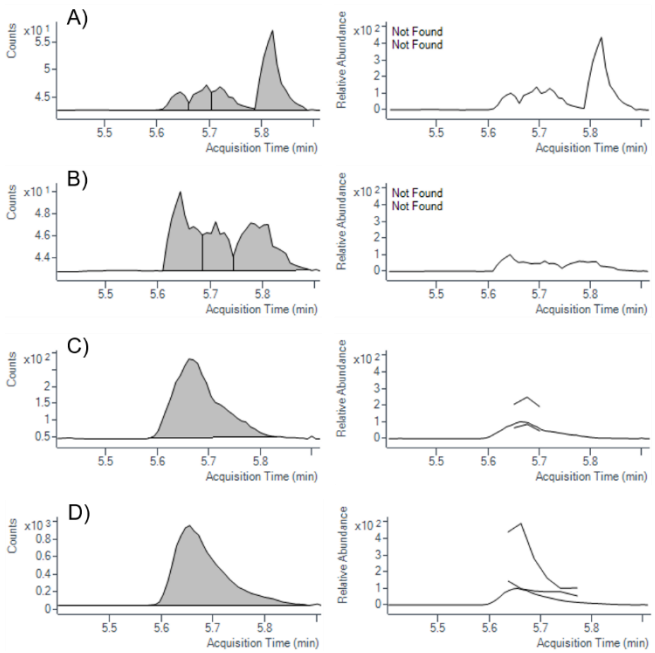

Figure 6 - 9. tMRM spectra for etizolam. Blank samples (A) and blank samples spiked with the ISTD mixture (B) show no interference for etizolam. False positive samples (D) show similar spectra to those of the lowest calibrator (CAL L1, C). The nature of the interference, whether by endogenous or exogenous compounds, is currently unknown.

6.3. VALIDATION SEMI-QUANTITATIVE SCREENING

6.3.1. Internal standard mix & samples analysed

In contrast to the validated dMRM methods, inclusion of all labelled internal standards in screening methods would not be cost-effective. Furthermore, this would risk the instrument spending considerable time on acquisition of these ISTDs thereby potentially losing out on sensitivity. It was therefore opted to work with a reduced number (n = 12) of ISTDs based upon the following criteria: I) a minimum of 3 ISTDs per cpd class should be present, II) per cpd class the ISTDs included should span the retention time range of that class, III) ideally, labelled analogues of cpds expected to be highly prevalent in case samples or of cpds representing the basic core structure of a class should be included. Table 6 - 3 below gives an overview of the composition of the new ISTD mix.

The new ISTD mix was spiked to all samples described in section 6.1.2.1 prior to extraction. Calibration curves were run at the start, middle and end of every batch. Data analysis was performed using the Agilent MassHunter Quantitative Analysis 10.0 (for QQQ) software (Agilent Technologies, Santa Clara, California, US). The best curve fits and weighing factors were chosen based upon the

back calculated concentrations of the CALs. The influence of both a structurally and a retention time (RT) related ISTD on the calculated concentrations was investigated (Table S6 - 2). RT-based ISTDs might differ in cpd class from those of the actual analytes themselves. Cpds that had their labelled analogues present were only linked to these as ISTD.

Table 6 - 3. Composition of the ISTD mix for screening and semi-quantitative purposes. Compounds were dissolved in acetonitrile at ten times the here reported intended in-plasma concentration (conc.). Antidepressant, AD; antipsychotic, AP; benzodiazepine or Z-drug, BZD; retention time, RT.

Compound	Class	RT (min)	Conc. (ng/mL)
olanzapine-D_3	AP	1.95	20
mirtazapine-D_3	AD	2.80	60
zopiclone-D_4	BZD	2.83	20
trazodone-D_6	AD	3.64	750
quetiapine-D_8	AP	3.81	200
citalopram-D_6	AD	4.12	150
bromazepam-D_4	BZD	4.23	50
melitracen-D_6	AD	4.92	15
flupentixol-D_4	AP	4.98	10
alprazolam-D_5	BZD	5.32	20
diazepam-D_5	BZD	6.08	100
prazepam-D_5	BZD	7.22	10

A total of 367 entries quantified by tMRM were included in the analysis (Table S6 - 3), 129 (35%) of which were for cpds that had their labelled analogues present in the ISTD mix. A further 111 entries (30%) were for direct or downstream metabolites of the former. The remaining 127 entries (35%) had no immediate relationship with the ISTDs. Whereas accuracy criteria for dMRM methods require calculated concentrations to be within ± 15% of nominal ones (± 20% at LLOQ), we reasoned that a maximal deviation of 30% should be allowed for semi-quantitative purposes. Results below are expressed as the accuracy compared to dMRM ± standard deviation.

6.3.2. Compounds directly related to the internal standards

The compounds discussed here are those with a direct relationship to the ISTDs, defined as either the unlabelled analogues or a downstream metabolite of those. Variable results were seen for cpds belonging to the ADs. Out of 15 positive hits for citalopram, 14 had calculated concentrations within ± 30% of those found with dMRM (average accuracy 105% ± 10%). Concentrations ranged from the LLOQ to above the upper limit of quantification. The aberrant sample had a calculated concentration of 16 ng/mL versus 8 ng/mL by dMRM. Although a significant deviation, no difference in interpretation is expected within the remits of medico-legal or therapeutic drug monitoring (TDM) casework, as the *in vivo* recommended therapeutic reference range for the latter starts from 50 ng/mL [7]. Its metabolite norcitalopram could not be reliably semi-quantified, with the accuracy of the calculated concentrations averaging 22% ± 13% compared to the true value (as determined by dMRM). Highly reliable results were also found for trazodone (n = 16, 90% ± 9%) apart from for two samples with calculated concentrations 49 ng/mL (vs. 73 ng/mL via dMRM) and 140 ng/mL (vs. 85 ng/mL via dMRM). As was true for citalopram, these deviations are unlikely to affect the case interpretation. mCPP could not be reliably detected (see sections 6.1.2.2 & 6.2.2) or semi-quantified (average concentration accuracy 11% ± 11%). Mirtazapine concentrations were accurate (98% ± 7%), however there were problems with the detection and semi-quantification of normirtazapine. Lastly,

a quadratic calibration curve fitting and $1/x^2$ weighing was previously found to result in excellent accuracies and precisions for melitracen, both within and between batches (98% ± 6%). Also the comparison with results from the General Medical Laboratory of Antwerp showed a less than 15% difference between the calculated concentrations [416]. In tMRM mode (n = 3) significant accuracy deviations (55% ± 7%) versus dMRM were seen regardless of the curve fitting or weighing factor.

For the APs, quetiapine and metabolites were the predominant cpds in the samples. Quetiapine could be reliably quantified in all cases (n = 11, 103% ± 10%) apart from in one sample where a concentration of 4534 ng/mL was found versus a dMRM concentration of > 5120 ng/mL. Either value would be significantly higher than what is expected from normal therapeutic use and thus not change the interpretation. 7-OH-quetiapine, although usually detected, showed more variability in the accuracy of the calculated concentrations. Of the ten positive hits, six had concentrations that deviated by less than 30% from the dMRM ones (100% ± 17%), while the others showed larger discrepancies (116% ± 37%). tMRM concentrations ranged between 3 ng/mL and 383 ng/mL for concentrations deviating more than 30% from the dMRM ones. Norquetiapine (34% ± 23%) and 7-OH-norquetiapine (18%) were less reliably detected in samples and coincidentally showed poor semi-quantification. Olanzapine (n = 7, 106% ± 16%) and flupentixol (n = 1, 6 ng/mL vs. 5 ng/mL) did not show major differences in their concentrations.

Diazepam and its metabolites were the most detected analytes of the benzodiazepine class (88 out of 141 hits). 26 cases were positive for diazepam, all of which resulted in acceptable semi-quantitative results (97% ± 13%). Concentrations ranged from 5 ng/mL to 1013 ng/mL. Temazepam was detected in 5 samples with concentrations between 14 ng/mL and 346 ng/mL. Average accuracies were 103% ± 17%. In two samples, concentrations exceeded the deviation limit of ± 30% compared to dMRM, resulting in calculated concentrations of 26 ng/mL (vs. 14 ng/mL) and 479 ng/mL (vs. 345 ng/mL). Oxazepam was present in 16 samples, all but one of which could be accurately semi-quantified (92% ± 12%). For the latter, the concentration difference of 31 ng/mL (tMRM) vs. 23 ng/mL (dMRM) is unlikely to result in an alternative interpretation for either TDM or medico-legal purposes. Only nordazepam had a less reliable outcome, with only 32% of cases (13 out of 41) showing acceptable semi-quantitative results. In most samples, significantly elevated concentrations were observed via tMRM (average accuracy 145% ± 33%). On the other hand, 80% of cases could be reliable quantified (104% ± 12%) when the ISTD was swapped from the structurally related diazepam-D_5 to the RT related alprazolam-D_5, hinting at a potential ion suppressive matrix effect in the calibration samples (see section 6.4). Other BZDs performed equally well. Alprazolam was detected and quantified in 20 cases (89% ± 14%), with two minor exceedances of the ± 30% deviation limit (-35% for a concentration of 36 ng/mL and -37% for a concentration of 60 ng/mL). Similar results were found for α-OH-alprazolam (n = 8, 88% ± 14%) with the tMRM calculated concentration of one sample deviating by -39% of the dMRM calculated concentration. Two hits were found for either prazepam and zopiclone, all of which had accurate semi-quantitative results. Lastly, 21 concentrations were determined for bromazepam with an average accuracy of 93% ± 16%. In one sample the concentration at the lower end of the calibration range fell with 135% (6 ng/mL vs. 4 ng/mL) just outside of the set criteria, though no interpretative differences are expected for such low concentrations.

6.3.3. Compounds not directly related to the internal standards

In the absence of their labelled analogues as ISTD, the accuracies of the measured AD tMRM concentrations were noticeably reduced compared to those discussed in section 6.3.2. Bupropion and its hydroxylated metabolite were consistently underestimated by 45% or more compared to dMRM. Although of little importance at lower concentrations, interpretative differences may be anticipated for samples with high concentrations. Especially for TDM purposes, a tMRM blood concentration of e.g. 1227 ng/mL may be interpreted as a high therapeutic concentration (recommended range 850 ng/mL – 1500 ng/mL), whereas the dMRM concentration (> 2500 ng/mL) exceeds the recommended laboratory alert level of 2000 ng/mL, warning the treating physician that the dose may be too high and potentially harmful [7]. Venlafaxine (n = 8) and O-desmethyl-venlafaxine (n = 10) displayed equally poor outcomes. The ISTD citalopram-D_6 was chosen for its structural similarity, though still poor at best, and resulted in respective accuracies of 61% ± 32% and 28% ± 9%. RT-related ISTDs trazodone-D_6 and mirtazapine-D_3 did not noticeably improve the outcomes, resulting in accuracies of 73% ± 36% and 31% ± 21% for venlafaxine and its metabolite, respectively. Significant reductions of 50% or more (up to -99%) in concentration were also observed for duloxetine, fluoxetine, norfluoxetine and sertraline, regardless of the type of ISTD used. Amitriptyline was linked to melitracen-D_6 as ISTD for both structural and RT purposes and was the only AD that performed well. Based upon four samples, the average accuracy was 106% ± 8%. Substantial deviations were found for nortriptyline with concentrations equalling 15% of those calculated by dMRM. Potential matrix interferences (see section 6.4 below) may underlie these discrepancies, as well as nortriptyline's poor detection rate.

The variable semi-quantitative results for APs in section 6.3.2 were also observed here. Haloperidol performed excellently, with a slight preference for the structurally related flupentixol-D_4 (86% ± 9%) over RT-related melitracen-D_6 as ISTD (84% ± 12%). Reduced haloperidol was preferentially quantified with a structurally related ISTD as well, although satisfactory results were obtained for only 50% of cases. In contrast, calculated clotiapine concentrations were three to five times higher than those calculated by dMRM for both sets of ISTDs. However, both dMRM and tMRM concentrations reflected the findings in limited case reports of chronic administration therapy [405]. Pipamperone concentrations varied highly, with accuracies of 23% to 105% when linked to the RT-related ISTD mirtazapine-D_3 and even lower ones with the structurally related ISTD flupentixol-D_4. Higher variations in the peak area of mirtazapine-D_3 may account for the deviations though no issues were seen with the back calculated concentrations of the calibration curves. With just two positive cases, no real conclusions could be drawn for prothipendyl. However, initial findings suggest better quantitative results for the lower concentrations, which coincides with the relatively low doses administered to patients (recommended therapeutic in-blood reference range 20 ng/mL – 80 ng/mL) [7]. The RT-related ISTD citalopram-D_6 should not be used, rather the structurally related flupentixol-D_4 is preferred. For the remaining APs, initial results once more indicated a generally better accuracy using a structurally related vs. RT-based ISTD, although none of the concentrations fell within the set deviation criteria of ± 30%. However, none of these cpds were quantified in more than one case and more data are needed to confirm these results.

As had been observed for the benzodiazepines and Z-drugs (see section 6.3.2), the overall semi-quantitative performance of the BZDs was markedly better than for the other compound classes.

Lorazepam was the most detected cpd in the samples (n = 17). Linked with either diazepam-D_5 (a structurally related ISTD) or alprazolam-D_5 (a RT-based ISTD), excellent respective average accuracies of 111% ± 19% and 86% ± 22% were found. For those few cases with a deviation > 30%, their overall low dMRM and tMRM concentrations are unlikely to have had an influence on the interpretation of medico-legal cases or in relation to the recommended therapeutic range (30 ng/mL – 100 ng/mL) [7]. For its metabolite lormetazepam, a significant proportion deviated by > 30% (3 out of 5 vs. 4 out of 17 for lorazepam), although again because of the low concentrations in the samples, these deviations are likely to have little interpretative significance. Concentrations for midazolam and its α-hydroxylated metabolite were also highly accurately determined by tMRM. Respective average accuracies were 109% ± 16% and 97% ± 17% when linked to the structural ISTD alprazolam-D_5, and 112% ± 12% and 105% ± 12% when linked to the RT ISTD citalopram-D_6. For one sample containing α-OH-midazolam, the tMRM concentration with either ISTD was around 50% lower than that determined via dMRM. On the other hand, that sample's ISTD had a peak area ~50% lower than the other samples in the dMRM batch, which could explain the discrepancy with the tMRM findings. For two samples a discrepancy was found between the results of the structural and the RT ISTD, with the former not detecting the cpds. Upon closer inspection, the dMRM concentration differed by less than 20% from the LLOQ (0.625 ng/mL). Using a structurally related ISTD, an excellent database match was obtained but the calculated concentrations were < LLOQ and therefore considered not detected, whereas a concentration > LLOQ was obtained with a RT-based ISTD. Such variations can be expected from mass spectrometric methods and are reflected in the accuracy requirements set by the European Medicines Agency guidelines [368]. Flurazepam or norflurazepam accounted for 10 quantifiable results, 9 of which were accurately quantified using either type of ISTD. For the last result, a slight overestimation of a near-LLOQ concentration of flurazepam using a structurally related ISTD (8 ng/mL vs. 6 ng/mL) was preferred over the overestimation of a higher flurazepam concentration by the RT-based ISTD (> 400 ng/mL vs. 263 ng/mL). Three samples contained both clonazepam and 7-amino-clonazepam. The latter could not be accurately quantified (44% ± 10%), however clonazepam results were more reliable (84% ± 13%). 7-amino-flunitrazepam was preferably linked to the RT-based ISTD trazodone-D_6 (103% ± 14%), although no significant discrepancies were noted for the structurally related ISTD diazepam-D_5 (124% ± 10%). Lastly, the Z-drug zolpidem accounted for 7 positive hits. Overall better results were found for the structurally related ISTD zopiclone-D_4 compared to trazodone-D_6 (94% ± 23% vs. 75% ± 15%), though this would, as seen for other BZDs, not result in any expected interpretation differences.

6.4. OVERALL METHOD PERFORMANCE

Because of the minimal differences between the dMRM and tMRM acquisition modes, any compound optimisation data can readily be transferred from one method to another. On the condition that they are free from interference, quantifier ions can be listed as primary transitions, with qualifier ions making up the secondary ones. Particular care must be taken in choosing tMRM-specific parameters such as trigger window, trigger entrance and trigger delay [417–419]. Standardly set to the same range as the RT window, opting for a more narrow trigger window may increase the chance of acquiring secondary transitions near the apex of a compound's peak. On the other hand, a too narrow window limits the number of acquisition cycles for secondary transitions and thereby

confidence in the findings. In combination with a RT window of 0.5 min and an average peak width of 0.2 min, a trigger window of 0.3 min resulted in sufficient secondary transition data points, spread over the entire peak. Higher trigger entrance and delay values negatively influenced the number of acquired secondary transition cycles by respectively pushing back the start of data acquisition to predominantly the downslope of the peak or by waiting too long in between acquisition cycles. Overall, an entrance value of 0 cycles, combined with a delay of 2 cycles (average cycle time < 50 ms), proved the most beneficial. To avoid interference by baseline fluctuations, a threshold of 200 counts was set above which acquisition of secondary transitions was permitted. Such threshold allowed reproducible secondary transition monitoring at the level of CAL L1 without triggering an abundance of false positive results. The remainder of false positive hits can be filtered out using the tMRM library function post-analysis. Here, the ion spectrum of each cpd in a sample is compared to those obtained in a high concentrated calibrator. A match score of 90 served as the cut-off value below which the cpd was considered not detected.

Looking at the cpds detected using dMRM and tMRM, excellent overall agreements were found. Out of the 438 cpds identified at a concentration > LLOQ with traditional dMRM methods, 85% were also identified with this tMRM method. The cpds that were missed could be grouped in two categories. On the one hand, cpds such as mCPP, norcitalopram, normirtazapine, norolanzapine, norquetiapine and nortriptyline tended to be missed at concentrations significantly different from their respective LLOQs. A lower ionisation efficiency for olanzapine could partially explain why this cpd was systematically missed, as the signal for CAL L1 and L2 did not reach above 200 counts thus not triggering secondary transitions. No apparent explanation was available for the other cpds. However, all of the above listed cpds are metabolites of therapeutically administered active drugs and the parent substances were successfully identified in these cases. On a side note, it is worth mentioning that mCPP rather than just a metabolite of trazodone can also be consumed as a designer drug itself for its weak stimulant and hallucinogenic, MDMA-like effects [415,420,421]. The second category of cpds were only occasionally missed, usually at concentrations minimally different from their respective LLOQs. As the calibration ranges were chosen to include (where possible) at least one subtherapeutic level, one may question the importance of the missed cpds. For TDM purposes, not detecting a compound that is known to be prescribed should always prompt further investigation, regardless as to whether it concerns an undetected subtherapeutic dose requiring therapy adjustment, or a true negative result indicating an issue with therapy compliance. For forensic purposes, relying on therapeutic plasma concentration ranges often determined *in vivo* on healthy volunteers is strongly discouraged. Besides interpretative difficulties relating to tolerance and often multidrug use, post-mortem samples in particular pose additional challenges such as post-mortem redistribution (see section 8.3.1 p. 186), bacterial metabolism and post-mortem interval. Reliance on detailed case reports is of more value than reference ranges and each result requires interpretation on an individual, case-specific basis [59,422–424]. However, overall the LLOQs were chosen to represent expected concentrations observed from subtherapeutic or very low therapeutic doses. Therefore, the not detected low concentrations of ADs and APs would not be expected to cause any major (adverse) effects and therefore not have impacted case interpretation. The central nervous system suppressant effects of the BZDs, especially in combination with alcohol or opioid-like drugs, and the availability of a multitude of designer BZDs with unknown pharmacology, may require their detection even at the lowest levels. For these cpds, the presented tMRM method showed excellent sensitivity with a

true positive detection rate of 95% compared to the dMRM methods. Furthermore, 24 false positive hits were found, most of which were for etizolam. The hypothesis that a portion of these were misidentifications of triazolam was previously investigated and found not to be true [425]. dMRM analysis of the samples confirmed that no samples were positive for either cpd. Interference by the ISTD mix or any of the reagents used during sample extraction was excluded based upon the results of blank samples run in the same batch. More research into this interference is required. If needed, two primary transitions can be set for etizolam. Similar to the quantifier-qualifier ratio for dMRM methods, the ratio between these two primary transitions could potentially filter out false positive results. The remainder of the false positive hits was predominantly made up of cpds where all transitions could be picked up during dMRM analysis but were excluded from the final dataset as their estimated concentration fell just below the LLOQ, which coincidentally was also set as the LOD. These findings highlight the need for critical, manual evaluation of hits by the analyst, rather than blind reliance on database findings and automatic reporting.

To reduce time and money spent on often unnecessary quantification of all compounds in any sample, screening methods ideally hold a semi-quantitative aspect. However, whereas validated methods for accurate quantification should display accuracies within ± 15% of the true concentration, such requirements are poorly defined for semi-quantitative purposes in international guidelines [368,375,378]. Rather, acceptable accuracy criteria should be set depending on the intended application of the method. Based upon the ± 20% criterion set by the European Medicines Agency (for confirmation methods) and the slightly wider allowed range described by Paterson et al., we therefore deemed a deviation of ± 30% from the concentration determined by validated dMRM methods acceptable [368,426,427]. The choice of internal standard significantly influenced the outcome of the results, with cpds that had their labelled analogues present consistently scoring better in their accuracies compared to the other cpds, even those with a high degree of structural similarity such as direct metabolites. Potential differences in ionisation efficiencies might underlie these findings. The most notorious are matrix effects, predominantly ion suppression when using electrospray ionisation, originating from competition for a droplet's surface charge and from droplet precipitation due to the presence of non-volatile additives [388,426,428,429]. Previous publications have shown that for the current sample preparation and LC settings, no significant matrix effects arise [416,425,430]. In addition, calibration samples spiked with all cpds are more likely to experience ion suppression, in which case an overestimation rather than the here more prevalent underestimation of concentrations is expected. It has also been shown that ionisation efficiency can vary based upon mobile phase composition, with a lower pH and a higher percentage organic solvent being advantageous for positive ionisation modes [426,431]. This may explain the better results for the BZDs, which are more spread out over the entire RT range and (on average) eluting at higher mobile phase B percentages than the ADs and APs.

Besides from a pure mathematical approach, accuracy could also be evaluated from an interpretation point of view. Especially for most post-mortem forensic toxicology casework, knowledge of the approximate rather than exact concentration can be sufficient for AD and AP drugs. These compounds are known for their substantial inter-individual variation in blood concentrations and effects when given in an equally high dose, and dosages are therefore tailored to a patient's needs. Therefore, results are often interpreted as low or expected from therapeutic use vs. a potentially too

high concentration in line with the medical history of the deceased [7,177,205,214,432]. Provided the deceased was prescribed the drugs, only around 10% of cases would have been wrongfully interpreted regardless of a deviation above or below ± 30% of the dMRM concentrations. Particularly cases positive for venlafaxine or O-desmethylvenlafaxine, norquetiapine or 7-OH-norquetiapine and clotiapine should always be confirmed using dMRM methods (Figure S6 - 1 & Figure S6 - 2). Benzodiazepines are more difficult to interpret as their effects are dependent on tolerance and are often aggravated by co-ingestion of alcohol or other central nervous system depressant drugs. Additionally, effects of the designer BZDs are largely unknown and difficult to predict [276,433,434]. Therefore, even though their semi-quantitative concentrations showed high accuracies (Figure S6 - 3), they should be interpreted with caution and never outside of the full scope of substances detected in a sample or of the case circumstances.

6.5. SUPPLEMENTARY INFORMATION FOR CHAPTER 6

6.5.1. Tables

Table S6 - 1. Calibration levels for the tMRM and QTOF methods. Values are expressed in ng/mL and represent the in-sample concentrations. Seven levels were included for antidepressants and antipsychotics, six for benzodiazepines & Z-drugs. L1 was excluded for asenapine and norasenapine.

Compound	L1	L2	L3	L4	L5	L6	L7
			ANTIDEPRESSANTS				
agomelatine	2	4	20	40	80	200	1000
amitriptyline	10	20	100	200	400	1000	5000
atomoxetine	10	20	100	200	400	1000	5000
bupropion	5	10	50	100	200	500	2500
citalopram	5	10	50	100	200	500	2500
clomipramine	5	10	50	100	200	500	2500
desipramine	5	10	50	100	200	500	2500
dosulepin	5	10	50	100	200	500	2500
doxepin	5	10	50	100	200	500	2500
duloxetine	5	10	50	100	200	500	2500
fluoxetine	10	20	100	200	400	1000	5000
fluvoxamine	5	10	50	100	200	500	2500
imipramine	10	20	100	200	400	1000	5000
maprotiline	10	20	100	200	400	1000	5000
mCPP	2	4	20	40	80	200	1000
melitracen	0.5	1	5	10	20	50	250
mianserin	2	4	20	40	80	200	1000
mirtazapine	2	4	20	40	80	200	1000
moclobemide	10	20	100	200	400	1000	5000
norcitalopram	2	4	20	40	80	200	1000
norclomipramine	5	10	50	100	200	500	2500
nordosulepin	10	20	100	200	400	1000	5000
nordoxepin	5	10	50	100	200	500	2500
norfluoxetine	10	20	100	200	400	1000	5000
normaprotiline	10	20	100	200	400	1000	5000
normianserin	0.5	1	5	10	20	50	250
normirtazapine	0.5	1	5	10	20	50	250
nortrimipramine	10	20	100	200	400	1000	5000
nortriptyline	10	20	100	200	400	1000	5000
O-desmethyl-venlafaxine	10	20	100	200	400	1000	5000
OH-bupropion	5	10	50	100	200	500	2500
opipramol	10	20	100	200	400	1000	5000
paroxetine	5	10	50	100	200	500	2500
reboxetine	5	10	50	100	200	500	2500
sertraline	5	10	50	100	200	500	2500
tianeptine	2	4	20	40	80	200	1000
trazodone	25	50	250	500	1000	2500	12500
trimipramine	5	10	50	100	200	500	2500
venlafaxine	10	20	100	200	400	1000	5000
			ANTIPSYCHOTICS				
7-OH-norquetiapine	1	2	8	16	64	128	512
7-OH-quetiapine	1	2	8	16	64	128	512
amisulpride	10	20	80	160	640	1280	5120
aripiprazole	10	20	80	160	640	1280	5120

Table S6 - 1. Calibration levels for the tMRM and QTOF methods. (continued) Values are expressed in ng/mL and represent the in-sample concentrations. Seven levels were included for antidepressants and antipsychotics, six for benzodiazepines & Z-drugs. L1 was excluded for asenapine and norasenapine.

Compound	L1	L2	L3	L4	L5	L6	L7
asenapine		1	4	8	32	64	256
bromperidol	0.5	1	4	8	32	64	256
chlorpromazine	4	8	32	64	256	512	2048
clotiapine	1	2	8	16	64	128	512
clozapine	10	20	80	160	640	1280	5120
dehydro-aripiprazole	4	8	32	64	256	512	2048
droperidol	1	2	8	16	64	128	512
flupentixol	0.5	1	4	8	32	64	256
fluphenazine	0.5	1	4	8	32	64	256
fluspirilene	0.5	1	4	8	32	64	256
haloperidol	0.5	1	4	8	32	64	256
iloperidone	0.5	1	4	8	32	64	256
levomepromazine	2	4	16	32	128	256	1024
levosulpiride	10	20	80	160	640	1280	5120
loxapine	1	2	8	16	64	128	512
lurasidone	4	8	32	64	256	512	2048
norasenapine		1	4	8	32	64	256
norclozapine	10	20	80	160	640	1280	5120
norolanzapine	1	2	8	16	64	128	512
norquetiapine	3	6	24	48	192	384	1536
OH-iloperidone	0.5	1	4	8	32	64	256
olanzapine	1	2	8	16	64	128	512
paliperidone	1	2	8	16	64	128	512
perphenazine	0.5	1	4	8	32	64	256
pimozide	2	4	16	32	128	256	1024
pipamperone	4	8	32	64	256	512	2048
prothipendyl	4	8	32	64	256	512	2048
quetiapine	10	20	80	160	640	1280	5120
reduced haloperidol	0.5	1	4	8	32	64	256
risperidone	1	2	8	16	64	128	512
sertindole	2	4	16	32	128	256	1024
tiapride	20	40	160	320	1280	2560	10240
zuclopenthixol	1	2	8	16	64	128	512
			BENZODIAZEPINES & Z-DRUGS				
3-OH-flubromazepam	2.5	10	25	100	250	1000	
4-OH-midazolam	0.5	2	5	20	50	200	
7-amino-clonazepam	2	8	20	80	200	800	
7-amino-flunitrazepam	0.5	2	5	20	50	200	
7-amino-nitrazepam	1	4	10	40	100	400	
α-OH-alprazolam	0.625	2.5	6.25	25	62.5	250	
α-OH-midazolam	1.25	5	12.5	50	125	500	
α-OH-triazolam	0.5	2	5	20	50	200	
adinazolam	2.5	10	25	100	250	1000	
alprazolam	1	4	10	40	100	400	
bentazepam	2.5	10	25	100	250	1000	
bromazepam	2.5	10	25	100	250	1000	
brotizolam	0.5	2	5	20	50	200	
chlordiazepoxide	25	100	250	1000	2500	10000	
clobazam	6.25	25	62.5	250	625	2500	

Table S6 - 1. Calibration levels for the tMRM and QTOF methods. (continued) Values are expressed in ng/mL and represent the in-sample concentrations. Seven levels were included for antidepressants and antipsychotics, six for benzodiazepines & Z-drugs. L1 was excluded for asenapine and norasenapine.

Compound	L1	L2	L3	L4	L5	L6	L7
clonazepam	2	8	20	80	200	800	
clonazolam	2	8	20	80	200	800	
cloniprazepam	0.5	2	5	20	50	200	
clotiazepam	10	40	100	400	1000	4000	
cloxazolam	2.5	10	25	100	250	1000	
delorazepam	2	8	20	80	200	800	
deschloro-etizolam	1.25	5	12.5	50	125	500	
diazepam	5	20	50	200	500	2000	
diclazepam	1	4	10	40	100	400	
ethyl loflazepate	2.5	10	25	100	250	1000	
etizolam	1.25	5	12.5	50	125	500	
flubromazepam	2.5	10	25	100	250	1000	
flubromazolam	2	8	20	80	200	800	
flunitrazepam	0.5	2	5	20	50	200	
flurazepam	1	4	10	40	100	400	
halazepam	10	40	100	400	1000	4000	
loprazolam	2	8	20	80	200	800	
lorazepam	2	8	20	80	200	800	
lormetazepam	0.5	2	5	20	50	200	
meclonazepam	1	4	10	40	100	400	
medazepam	10	40	100	400	1000	4000	
metizolam	1	4	10	40	100	400	
midazolam	2.5	10	25	100	250	1000	
nifoxipam	2.5	10	25	100	250	1000	
nitrazepam	1	4	10	40	100	400	
norclobazam	25	100	250	1000	2500	10000	
nordazepam	10	40	100	400	1000	4000	
norflunitrazepam	0.5	2	5	20	50	200	
norflurazepam	5	20	50	200	500	2000	
oxazepam	10	40	100	400	1000	4000	
phenazepam	2	8	20	80	200	800	
prazepam	0.5	2	5	20	50	200	
pyrazolam	2.5	10	25	100	250	1000	
temazepam	10	40	100	400	1000	4000	
tetrazepam	6.25	25	62.5	250	625	2500	
triazolam	0.5	2	5	20	50	200	
zolpidem	5	20	50	200	500	2000	
zopiclone	1	4	10	40	100	400	

Table S6 - 2. Calibration curves for semi-quantitative analysis. Compounds that did not have a labelled analogue available as ISTD were matched with both a structurally related one and a RT-based one.

Compound	Structural ISTD			RT-based ISTD		
	Fit	Weight	Name	Fit	Weight	Name
			ANTIDEPRESSANTS			
agomelatine	quadratic	1/x	melitracen-D_6	quadratic	1/x	diazepam-D_5
amitriptyline	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
atomoxetine	linear	1/x	citalopram-D_6	quadratic	1/x	melitracen-D_6
bupropion	quadratic	1/x	trazodone-D_6	quadratic	1/x	trazodone-D_6
citalopram	linear	1/x	citalopram-D_6	linear	1/x	citalopram-D_6
clomipramine	linear	1/x	melitracen-D_6	linear	1/x	flupentixol-D_4
desipramine	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
dosulepin	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
doxepin	linear	1/x	melitracen-D_6	quadratic	1/x	bromazepam-D_4
duloxetine	quadratic	1/x	citalopram-D_6	quadratic	1/x	melitracen-D_6
fluoxetine	quadratic	1/x	melitracen-D_6	linear	1/x	melitracen-D_6
fluvoxamine	linear	1/x	citalopram-D_6	quadratic	1/x	melitracen-D_6
imipramine	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
maprotiline	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
mCPP	linear	1/x	trazodone-D_6	linear	1/x	trazodone-D_6
melitracen	linear	1/x	melitracen-D_6	linear	1/x	melitracen-D_6
mianserin	linear	1/x	mirtazapine-D_3	linear	1/x	citalopram-D_6
mirtazapine	linear	1/x	mirtazapine-D_3	linear	1/x	mirtazapine-D_3
moclobemide	quadratic	1/x	trazodone-D_6	quadratic	1/x	mirtazapine-D_3
norcitalopram	linear	1/x	citalopram-D_6	quadratic	1/x	citalopram-D_6
norclomipramine	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
nordosulepin	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
nordoxepin	linear	1/x	melitracen-D_6	linear	1/x	citalopram-D_6
norfluoxetine	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
normaprotiline	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
normianserin	linear	1/x	mirtazapine-D_3	quadratic	1/x	citalopram-D_6
normirtazapine	quadratic	$1/x^2$	mirtazapine-D_3	quadratic	1/x	mirtazapine-D_3
nortrimipramine	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
nortriptyline	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
O-desmethyl-venlafaxine	linear	1/x	citalopram-D_6	quadratic	1/x	mirtazapine-D_3
OH-bupropion	linear	1/x	trazodone-D_6	linear	1/x	trazodone-D_6
opipramol	linear	1/x	melitracen-D_6	quadratic	1/x	quetiapine-D_8
paroxetine	quadratic	1/x	trazodone-D_6	quadratic	1/x	melitracen-D_6
reboxetine	linear	1/x	citalopram-D_6	quadratic	1/x	bromazepam-D_4
sertraline	quadratic	1/x	trazodone-D_6	quadratic	1/x	melitracen-D_6
tianeptine	linear	1/x	melitracen-D_6	quadratic	1/x	bromazepam-D_4
trazodone	linear	1/x	trazodone-D_6	linear	1/x	trazodone-D_6
trimipramine	quadratic	1/x	melitracen-D_6	quadratic	1/x	melitracen-D_6
venlafaxine	linear	1/x	citalopram-D_6	linear	1/x	trazodone-D_6
			ANTIPSYCHOTICS			
7-OH-norquetiapine	quadratic	1/x	quetiapine-D_8	quadratic	1/x	mirtazapine-D_3
7-OH-quetiapine	linear	1/x	quetiapine-D_8	linear	1/x	mirtazapine-D_3
amisulpride	linear	1/x	olanzapine-D_3	linear	1/x	mirtazapine-D_3
aripiprazole	quadratic	1/x	flupentixol-D_4	linear	1/x	melitracen-D_6
asenapine	linear	1/x	olanzapine-D_3	quadratic	1/x	bromazepam-D_4
bromperidol	quadratic	1/x	flupentixol-D_4	linear	1/x	melitracen-D_6
chlorpromazine	quadratic	1/x	flupentixol-D_4	quadratic	1/x	melitracen-D_6
clotiapine	quadratic	1/x	quetiapine-D_8	quadratic	1/x	melitracen-D_6

Table S6 - 2. Calibration curves for semi-quantitative analysis. (continued) Compounds that did not have a labelled analogue available as ISTD were matched with both a structurally related one and a RT-based one.

Compound	Structural ISTD			RT-based ISTD		
	Fit	Weight	Name	Fit	Weight	Name
clozapine	quadratic	1/x	quetiapine-D_8	linear	1/x	trazodone-D_6
dehydro-aripiprazole	linear	1/x	flupentixol-D_4	linear	1/x	melitracen-D_6
droperidol	linear	1/x	flupentixol-D_4	linear	1/x	quetiapine-D_8
flupentixol	linear	1/x	flupentixol-D_4	linear	1/x	flupentixol-D_4
fluphenazine	linear	1/x	flupentixol-D_4	linear	1/x	melitracen-D_6
fluspirilene	linear	1/x	flupentixol-D_4	quadratic	1/x	alprazolam-D_5
haloperidol	linear	1/x	flupentixol-D_4	linear	1/x	melitracen-D_6
iloperidone	linear	1/x	flupentixol-D_4	linear	1/x	bromazepam-D_4
levomepromazine	quadratic	1/x	flupentixol-D_4	linear	1/x	melitracen-D_6
levosulpiride	linear	1/x	olanzapine-D_3	linear	1/x	olanzapine-D_3
loxapine	linear	1/x	quetiapine-D_8	quadratic	1/x	bromazepam-D_4
lurasidone	linear	1/x	olanzapine-D_3	quadratic	1/x	alprazolam-D_5
norasenapine	quadratic	1/x	olanzapine-D_3	linear	1/x	bromazepam-D_4
norclozapine	linear	1/x	quetiapine-D_8	linear	1/x	trazodone-D_6
norolanzapine	linear	1/x	olanzapine-D_3	linear	1/x	olanzapine-D_3
norquetiapine	quadratic	$1/x^2$	quetiapine-D_8	linear	1/x	trazodone-D_6
OH-iloperidone	quadratic	1/x	flupentixol-D_4	quadratic	1/x	citalopram-D_6
olanzapine	linear	1/x	olanzapine-D_3	linear	1/x	olanzapine-D_3
paliperidone	linear	1/x	flupentixol-D_4	quadratic	1/x	trazodone-D_6
perphenazine	linear	1/x	flupentixol-D_4	linear	1/x	melitracen-D_6
pimozide	linear	1/x	flupentixol-D_4	quadratic	1/x	alprazolam-D_5
pipamperone	quadratic	1/x	flupentixol-D_4	quadratic	1/x	mirtazapine-D_3
prothipendyl	linear	1/x	flupentixol-D_4	quadratic	1/x	citalopram-D_6
quetiapine	linear	1/x	quetiapine-D_8	linear	1/x	quetiapine-D_8
reduced haloperidol	linear	1/x	flupentixol-D_4	quadratic	1/x	citalopram-D_6
risperidone	linear	1/x	flupentixol-D_4	quadratic	1/x	trazodone-D_6
sertindole	linear	1/x	flupentixol-D_4	quadratic	1/x	alprazolam-D_5
tiapride	linear	1/x	olanzapine-D_3	quadratic	1/x	mirtazapine-D_3
zuclopenthixol	quadratic	1/x	flupentixol-D_4	quadratic	1/x	melitracen-D_6
			BENZODIAZEPINES & Z-DRUGS			
3-OH-flubromazepam	linear	1/x	bromazepam-D_4	linear	1/x	alprazolam-D_5
4-OH-midazolam	linear	1/x	alprazolam-D_5	quadratic	1/x	quetiapine-D_8
7-amino-clonazepam	linear	1/x	diazepam-D_5	linear	1/x	trazodone-D_6
7-amino-flunitrazepam	quadratic	1/x	diazepam-D_5	quadratic	1/x	trazodone-D_6
7-amino-nitrazepam	linear	1/x	bromazepam-D_4	linear	1/x	olanzapine-D_3
α-OH-alprazolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	alprazolam-D_5
α-OH-midazolam	linear	1/x	alprazolam-D_5	linear	1/x	citalopram-D_6
α-OH-triazolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	alprazolam-D_5
adinazolam	linear	1/x	alprazolam-D_5	linear	1/x	trazodone-D_6
alprazolam	linear	1/x	alprazolam-D_5	linear	1/x	alprazolam-D_5
bentazepam	linear	1/x	diazepam-D_5	linear	1/x	citalopram-D_6
bromazepam	linear	1/x	bromazepam-D_4	linear	1/x	bromazepam-D_4
brotizolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	diazepam-D_5
chlordiazepoxide	quadratic	1/x	prazepam-D_5	linear	1/x	trazodone-D_6
clobazam	linear	1/x	diazepam-D_5	linear	1/x	diazepam-D_5
clonazepam	linear	1/x	diazepam-D_5	linear	1/x	diazepam-D_5
clonazolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	alprazolam-D_5
cloniprazepam	linear	1/x	prazepam-D_5	linear	1/x	prazepam-D_5
clotiazepam	quadratic	1/x	diazepam-D_5	quadratic	1/x	diazepam-D_5

Table S6 - 2. Calibration curves for semi-quantitative analysis. (continued) Compounds that did not have a labelled analogue available as ISTD were matched with both a structurally related one and a RT-based one.

Compound	Structural ISTD			RT-based ISTD		
	Fit	Weight	Name	Fit	Weight	Name
cloxazolam	linear	1/x	alprazolam-D_5	linear	1/x	trazodone-D_6
delorazepam	linear	1/x	diazepam-D_5	linear	1/x	diazepam-D_5
deschloro-etizolam	quadratic	1/x	alprazolam-D_5	linear	1/x	melitracen-D_6
diazepam	linear	1/x	diazepam-D_5	linear	1/x	diazepam-D_5
diclazepam	linear	1/x	diazepam-D_5	linear	1/x	prazepam-D_5
ethyl loflazepate	linear	1/x	prazepam-D_5	linear	1/x	prazepam-D_5
etizolam	quadratic	1/x	alprazolam-D_5	linear	1/x	diazepam-D_5
flubromazepam	linear	1/x	bromazepam-D_4	linear	1/x	diazepam-D_5
flubromazolam	linear	1/x	alprazolam-D_5	linear	1/x	diazepam-D_5
flunitrazepam	linear	1/x	diazepam-D_5	linear	1/x	diazepam-D_5
flurazepam	linear	1/x	diazepam-D_5	linear	1/x	citalopram-D_6
halazepam	linear	1/x	diazepam-D_5	quadratic	1/x	prazepam-D_5
loprazolam	linear	1/x	alprazolam-D_5	linear	1/x	citalopram-D_6
lorazepam	linear	1/x	diazepam-D_5	linear	1/x	alprazolam-D_5
lormetazepam	quadratic	1/x	diazepam-D_5	quadratic	1/x	diazepam-D_5
meclonazepam	linear	1/x	diazepam-D_5	linear	1/x	diazepam-D_5
medazepam	linear	1/x	diazepam-D_5	linear	1/x	citalopram-D_6
metizolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	diazepam-D_5
midazolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	citalopram-D_6
nifoxipam	quadratic	1/x	bromazepam-D_4	quadratic	1/x	melitracen-D_6
nitrazepam	linear	1/x	bromazepam-D_4	quadratic	1/x	alprazolam-D_5
norclobazam	linear	1/x	diazepam-D_5	quadratic	1/x	diazepam-D_5
nordazepam	linear	1/x	diazepam-D_5	linear	1/x	alprazolam-D_5
norflunitrazepam	linear	1/x	diazepam-D_5	quadratic	1/x	alprazolam-D_5
norflurazepam	linear	1/x	diazepam-D_5	linear	1/x	diazepam-D_5
oxazepam	linear	1/x	diazepam-D_5	linear	1/x	alprazolam-D_5
phenazepam	quadratic	1/x	bromazepam-D_4	quadratic	1/x	diazepam-D_5
prazepam	linear	1/x	prazepam-D_5	linear	1/x	prazepam-D_5
pyrazolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	citalopram-D_6
temazepam	quadratic	1/x	diazepam-D_5	quadratic	1/x	diazepam-D_5
tetrazepam	quadratic	1/x	diazepam-D_5	linear	1/x	alprazolam-D_5
triazolam	quadratic	1/x	alprazolam-D_5	quadratic	1/x	diazepam-D_5
zolpidem	quadratic	1/x	zopiclone-D_4	linear	1/x	trazodone-D_6
zopiclone	linear	1/x	zopiclone-D_4	linear	1/x	zopiclone-D_4

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
		ANTIDEPRESSANTS			
amitriptyline	69	75	108%	75	108%
amitriptyline	80	94	117%	94	117%
amitriptyline	228	232	102%	232	102%
amitriptyline	254	246	97%	246	97%
bupropion	> 2500	1227		1227	
citalopram	7	7	97%	7	97%
citalopram	8	16	196%	16	196%
citalopram	15	14	89%	14	89%
citalopram	16	19	124%	19	124%
citalopram	32	36	114%	36	114%
citalopram	34	32	96%	32	96%
citalopram	40	43	107%	43	107%
citalopram	47	44	92%	44	92%
citalopram	81	79	97%	79	97%
citalopram	84	97	115%	97	115%
citalopram	84	83	98%	83	98%
citalopram	132	146	111%	146	111%
citalopram	183	204	112%	204	112%
citalopram	219	253	116%	253	116%
citalopram	> 2500	> 2500		> 2500	
duloxetine	44	12	27%	10	23%
duloxetine	89	20	23%	19	21%
duloxetine	242	37	15%	58	24%
duloxetine	350	149	43%	171	49%
fluoxetine	267	104	39%	129	48%
fluoxetine	1177	374	32%	486	41%
mCPP	8	3	30%	3	30%
mCPP	43	2	6%	2	6%
mCPP	45	2	5%	2	5%
mCPP	180	8	4%	8	4%
melitracen	11	6	56%	6	56%
melitracen	64	40	62%	40	62%
melitracen	194	88	46%	88	46%
mirtazapine	2	2	97%	2	97%
mirtazapine	11	12	108%	12	108%
mirtazapine	11	19	173%	19	173%
mirtazapine	20	20	99%	20	99%
mirtazapine	76	67	88%	67	88%
moclobemide	> 5000	> 5000		> 5000	
norcitalopram	5	2	46%	2	46%
norcitalopram	12	3	22%	3	22%
norcitalopram	15	7	49%	7	49%
norcitalopram	16	4	25%	4	25%
norcitalopram	22	3	16%	3	16%
norcitalopram	23	7	30%	7	30%
norcitalopram	25	4	16%	4	16%
norcitalopram	26	3	11%	3	11%
norcitalopram	27	3	10%	3	10%

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. (continued) tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
norcitalopram	70	6	9%	6	9%
norcitalopram	216	33	15%	33	15%
norfluoxetine	154	10	7%	10	7%
normirtazapine	85	1	1%	1	1%
nortriptyline	163	28	17%	28	17%
nortriptyline	225	12	5%	12	5%
nortriptyline	2306	420	18%	420	18%
O-desmethyl-venlafaxine	46	13	28%	3	6%
O-desmethyl-venlafaxine	102	25	25%	24	24%
O-desmethyl-venlafaxine	117	36	31%	34	29%
O-desmethyl-venlafaxine	142	44	31%	31	22%
O-desmethyl-venlafaxine	263	92	35%	140	53%
O-desmethyl-venlafaxine	285	135	47%	227	80%
O-desmethyl-venlafaxine	301	37	12%	30	10%
O-desmethyl-venlafaxine	490	114	23%	150	31%
O-desmethyl-venlafaxine	803	173	22%	226	28%
O-desmethyl-venlafaxine	> 5000	1246		> 5000	
OH-bupropion	9	5	55%	5	55%
OH-bupropion	71	43	60%	43	60%
OH-bupropion	> 2500	1636		1636	
sertraline	182	37	20%	49	27%
sertraline	249	118	47%	153	62%
sertraline	978	11	1%	31	3%
sertraline	1207	430	36%	532	44%
sertraline	> 2500	993		1618	
trazodone	35	31	89%	31	89%
trazodone	56	48	86%	48	86%
trazodone	61	47	76%	47	76%
trazodone	73	49	67%	49	67%
trazodone	85	71	83%	71	83%
trazodone	85	140	164%	140	164%
trazodone	157	119	76%	119	76%
trazodone	161	143	89%	143	89%
trazodone	224	196	88%	196	88%
trazodone	255	224	88%	224	88%
trazodone	296	264	89%	264	89%
trazodone	303	299	99%	299	99%
trazodone	461	494	107%	494	107%
trazodone	586	549	94%	549	94%
trazodone	835	851	102%	851	102%
trazodone	984	1003	102%	1003	102%
venlafaxine	48	62	129%	64	132%
venlafaxine	79	59	75%	79	101%
venlafaxine	230	150	65%	209	91%
venlafaxine	276	166	60%	183	66%
venlafaxine	315	215	68%	290	92%
venlafaxine	986	397	40%	483	49%
venlafaxine	1940	382	20%	505	26%
venlafaxine	2796	790	28%	688	25%

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. (continued) tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
		ANTIPSYCHOTICS			
7-OH-norquetiapine	21	4	20%	4	20%
7-OH-norquetiapine	24	4	15%	4	15%
7-OH-quetiapine	6	3	53%	3	53%
7-OH-quetiapine	9	10	109%	10	109%
7-OH-quetiapine	18	23	125%	23	125%
7-OH-quetiapine	30	26	84%	26	84%
7-OH-quetiapine	34	47	137%	47	137%
7-OH-quetiapine	55	71	131%	71	131%
7-OH-quetiapine	83	65	79%	65	79%
7-OH-quetiapine	96	100	104%	100	104%
7-OH-quetiapine	266	383	144%	383	144%
7-OH-quetiapine	> 512	> 512		> 512	
amisulpride	30	11	39%	0	
aripiprazole	144	249	173%	278	193%
clotiapine	7	31	460%	25	369%
clotiapine	14	87	642%	69	512%
clotiapine	18	78	435%	77	427%
dehydro-aripiprazole	21	27	132%	0	
flupentixol	5	6	120%	6	120%
haloperidol	3	2	96%	3	99%
haloperidol	4	3	78%	3	77%
haloperidol	5	4	77%	3	69%
haloperidol	5	5	95%	4	89%
levosulpiride	37	51	139%	51	139%
norquetiapine	5	3	52%	3	52%
norquetiapine	79	4	5%	4	5%
norquetiapine	113	24	21%	24	21%
norquetiapine	120	85	71%	85	71%
norquetiapine	128	16	12%	16	12%
norquetiapine	147	34	23%	34	23%
norquetiapine	164	64	39%	64	39%
norquetiapine	185	14	8%	14	8%
norquetiapine	242	31	13%	31	13%
norquetiapine	724	427	59%	427	59%
norquetiapine	724	312	43%	312	43%
norquetiapine	726	138	19%	138	19%
norquetiapine	1277	936	73%	936	73%
olanzapine	2	2	125%	2	125%
olanzapine	18	18	101%	18	101%
olanzapine	58	46	79%	46	79%
olanzapine	97	98	101%	98	101%
olanzapine	152	176	116%	176	116%
olanzapine	155	192	123%	192	123%
olanzapine	216	205	95%	205	95%
paliperidone	18	8	43%	7	37%
pipamperone	19	9	47%	4	23%
pipamperone	68	155	229%	41	60%
pipamperone	312	311	100%	328	105%

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. (continued) tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
prothipendyl	31	29	93%	56	183%
prothipendyl	> 2048	1310		1108	
quetiapine	26	28	109%	28	109%
quetiapine	49	51	104%	51	104%
quetiapine	85	86	101%	86	101%
quetiapine	243	268	110%	268	110%
quetiapine	298	347	117%	347	117%
quetiapine	482	539	112%	539	112%
quetiapine	503	466	93%	466	93%
quetiapine	1099	1082	98%	1082	98%
quetiapine	1154	1196	104%	1196	104%
quetiapine	4368	3602	82%	3602	82%
quetiapine	> 5120	4534		4534	
reduced haloperidol	8	14	174%	21	257%
reduced haloperidol	30	44	145%	54	179%
reduced haloperidol	36	45	123%	48	134%
reduced haloperidol	162	138	85%	122	75%
zuclopenthixol	96	52	55%	21	21%
		BENZODIAZEPINES & Z-DRUGS			
7-amino-clonazepam	61	38	62%	32	52%
7-amino-clonazepam	69	28	40%	23	33%
7-amino-clonazepam	397	162	41%	148	37%
7-amino-flunitrazepam	1	1	151%	1	125%
7-amino-flunitrazepam	5	7	130%	5	96%
7-amino-flunitrazepam	5	7	126%	6	104%
7-amino-flunitrazepam	13	11	88%	11	88%
α-OH-alprazolam	1	1	79%	1	79%
α-OH-alprazolam	1	1	110%	1	110%
α-OH-alprazolam	2	1	61%	1	61%
α-OH-alprazolam	2	2	93%	2	93%
α-OH-alprazolam	3	3	104%	3	104%
α-OH-alprazolam	3	2	83%	2	83%
α-OH-alprazolam	4	4	88%	4	88%
α-OH-alprazolam	5	4	86%	4	86%
α-OH-midazolam	2	0		1	90%
α-OH-midazolam	3	4	112%	4	120%
α-OH-midazolam	6	6	114%	6	109%
α-OH-midazolam	16	15	89%	19	114%
α-OH-midazolam	21	15	73%	19	91%
α-OH-midazolam	114	62	54%	51	44%
alprazolam	3	2	96%	2	96%
alprazolam	6	6	97%	6	97%
alprazolam	7	6	89%	6	89%
alprazolam	18	17	94%	17	94%
alprazolam	19	16	81%	16	81%
alprazolam	20	18	94%	18	94%
alprazolam	24	26	105%	26	105%
alprazolam	25	19	78%	19	78%

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. (continued) tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
alprazolam	26	22	86%	22	86%
alprazolam	32	29	91%	29	91%
alprazolam	33	42	125%	42	125%
alprazolam	36	37	103%	37	103%
alprazolam	36	24	65%	24	65%
alprazolam	48	41	87%	41	87%
alprazolam	60	58	97%	58	97%
alprazolam	60	38	63%	38	63%
alprazolam	66	49	75%	49	75%
alprazolam	77	70	90%	70	90%
alprazolam	152	114	75%	114	75%
alprazolam	156	147	94%	147	94%
bromazepam	3	3	125%	3	125%
bromazepam	4	6	135%	6	135%
bromazepam	25	25	97%	25	97%
bromazepam	30	30	98%	30	98%
bromazepam	38	32	84%	32	84%
bromazepam	38	37	95%	37	95%
bromazepam	65	53	82%	53	82%
bromazepam	66	58	88%	58	88%
bromazepam	68	51	75%	51	75%
bromazepam	71	57	81%	57	81%
bromazepam	86	82	95%	82	95%
bromazepam	99	86	87%	86	87%
bromazepam	114	94	82%	94	82%
bromazepam	116	110	94%	110	94%
bromazepam	142	175	124%	175	124%
bromazepam	252	196	78%	196	78%
bromazepam	279	249	89%	249	89%
bromazepam	366	338	93%	338	93%
bromazepam	380	288	76%	288	76%
bromazepam	481	423	88%	423	88%
bromazepam	904	847	94%	847	94%
clonazepam	16	11	69%	11	69%
clonazepam	21	21	101%	21	101%
clonazepam	592	490	83%	490	83%
delorazepam	42	38	91%	38	91%
diazepam	5	5	103%	5	103%
diazepam	7	8	117%	8	117%
diazepam	7	5	79%	5	79%
diazepam	8	7	85%	7	85%
diazepam	11	14	122%	14	122%
diazepam	14	14	102%	14	102%
diazepam	24	29	122%	29	122%
diazepam	26	22	84%	22	84%
diazepam	31	31	99%	31	99%
diazepam	31	31	97%	31	97%
diazepam	39	45	114%	45	114%
diazepam	49	45	91%	45	91%

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. (continued) tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
diazepam	60	60	100%	60	100%
diazepam	66	59	89%	59	89%
diazepam	78	73	94%	73	94%
diazepam	82	95	115%	95	115%
diazepam	85	70	83%	70	83%
diazepam	86	71	83%	71	83%
diazepam	89	82	92%	82	92%
diazepam	101	81	80%	81	80%
diazepam	122	120	98%	120	98%
diazepam	176	177	101%	177	101%
diazepam	189	171	90%	171	90%
diazepam	207	206	100%	206	100%
diazepam	374	372	100%	372	100%
diazepam	1013	861	85%	861	85%
flurazepam	4	6	130%	5	127%
flurazepam	6	8	150%	6	109%
flurazepam	263	247	94%	> 400	
lorazepam	2	3	130%	3	143%
lorazepam	4	5	130%	4	107%
lorazepam	4	3	68%	2	63%
lorazepam	6	14	229%	12	197%
lorazepam	8	10	127%	8	99%
lorazepam	14	13	90%	9	64%
lorazepam	15	17	117%	13	87%
lorazepam	15	14	93%	12	75%
lorazepam	19	19	98%	15	79%
lorazepam	19	18	94%	11	60%
lorazepam	19	20	105%	15	81%
lorazepam	20	26	132%	23	119%
lorazepam	24	28	115%	18	75%
lorazepam	27	36	132%	21	77%
lorazepam	42	55	130%	34	81%
lorazepam	121	119	99%	92	76%
lorazepam	> 800	655		550	
lormetazepam	2	3	184%	3	184%
lormetazepam	6	7	135%	7	135%
lormetazepam	9	11	133%	11	133%
lormetazepam	19	17	90%	17	90%
lormetazepam	41	37	92%	37	92%
midazolam	3	0		4	124%
midazolam	7	6	88%	7	101%
midazolam	10	12	120%	11	110%
midazolam	30	37	124%	33	110%
midazolam	130	120	92%	124	95%
midazolam	183	220	121%	236	129%
midazolam	> 1000	> 1000		849	
nordazepam	16	10	63%	10	63%
nordazepam	17	11	64%	11	64%
nordazepam	18	10	59%	10	59%

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. (continued) tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
nordazepam	19	18	97%	17	92%
nordazepam	29	42	146%	49	168%
nordazepam	32	25	80%	18	57%
nordazepam	36	45	125%	30	85%
nordazepam	54	71	132%	54	101%
nordazepam	65	95	146%	83	128%
nordazepam	66	104	157%	86	129%
nordazepam	71	107	151%	65	93%
nordazepam	75	120	160%	105	140%
nordazepam	79	106	134%	94	119%
nordazepam	83	152	183%	81	97%
nordazepam	92	101	110%	75	82%
nordazepam	96	130	136%	81	85%
nordazepam	111	144	129%	106	96%
nordazepam	116	163	141%	130	113%
nordazepam	121	224	185%	176	145%
nordazepam	125	180	143%	128	102%
nordazepam	130	148	114%	167	128%
nordazepam	143	205	143%	155	108%
nordazepam	145	219	151%	142	98%
nordazepam	151	310	205%	220	145%
nordazepam	170	284	167%	226	133%
nordazepam	209	296	142%	222	106%
nordazepam	214	357	167%	250	117%
nordazepam	220	311	141%	219	100%
nordazepam	226	389	172%	231	103%
nordazepam	240	360	150%	282	117%
nordazepam	241	378	157%	336	139%
nordazepam	308	544	177%	317	103%
nordazepam	347	433	125%	339	98%
nordazepam	347	473	136%	366	105%
nordazepam	617	776	126%	618	100%
nordazepam	652	1025	157%	647	99%
nordazepam	804	935	116%	892	111%
nordazepam	869	1132	130%	929	107%
nordazepam	994	992	100%	1044	105%
nordazepam	1254	1345	107%	1275	102%
nordazepam	2003	2032	101%	1959	98%
norflurazepam	5	7	121%	7	121%
norflurazepam	10	10	107%	10	107%
norflurazepam	12	15	124%	15	124%
norflurazepam	73	55	75%	55	75%
norflurazepam	75	61	81%	61	81%
norflurazepam	161	146	91%	146	91%
norflurazepam	502	419	84%	419	84%
oxazepam	11	10	98%	13	118%
oxazepam	12	12	98%	14	116%
oxazepam	13	11	82%	12	94%
oxazepam	14	16	112%	16	119%

Table S6 - 3. Compounds detected in case samples and their (semi-)quantitative concentrations. (continued) tMRM concentrations are calculated with both a structurally and a RT-related ISTD. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values above the upper limit of quantification. Labelled internal standard, ISTD.

Compound	dMRM	tMRM structural ISTD		tMRM RT-based ISTD	
	Conc.	Conc.	Accuracy	Conc.	Accuracy
oxazepam	18	17	93%	18	97%
oxazepam	20	17	83%	15	74%
oxazepam	20	21	104%	21	101%
oxazepam	23	31	137%	21	95%
oxazepam	23	23	99%	24	106%
oxazepam	33	28	85%	26	79%
oxazepam	33	33	98%	27	82%
oxazepam	35	28	80%	31	87%
oxazepam	77	66	85%	66	85%
oxazepam	89	74	83%	74	83%
oxazepam	96	67	70%	57	60%
oxazepam	109	122	112%	81	74%
prazepam	2	2	103%	2	103%
prazepam	7	6	92%	6	92%
temazepam	14	17	116%	17	116%
temazepam	18	26	143%	26	143%
temazepam	22	29	129%	29	129%
temazepam	27	28	103%	28	103%
temazepam	346	479	139%	479	139%
zolpidem	7	6	86%	0	
zolpidem	9	6	74%	5	61%
zolpidem	11	7	63%	6	58%
zolpidem	18	25	135%	19	102%
zolpidem	22	21	93%	17	75%
zolpidem	24	21	89%	18	74%
zolpidem	54	65	120%	44	81%
zopiclone	1	1	80%	1	80%
zopiclone	> 400	> 400		> 400	

6.5.2. Figures

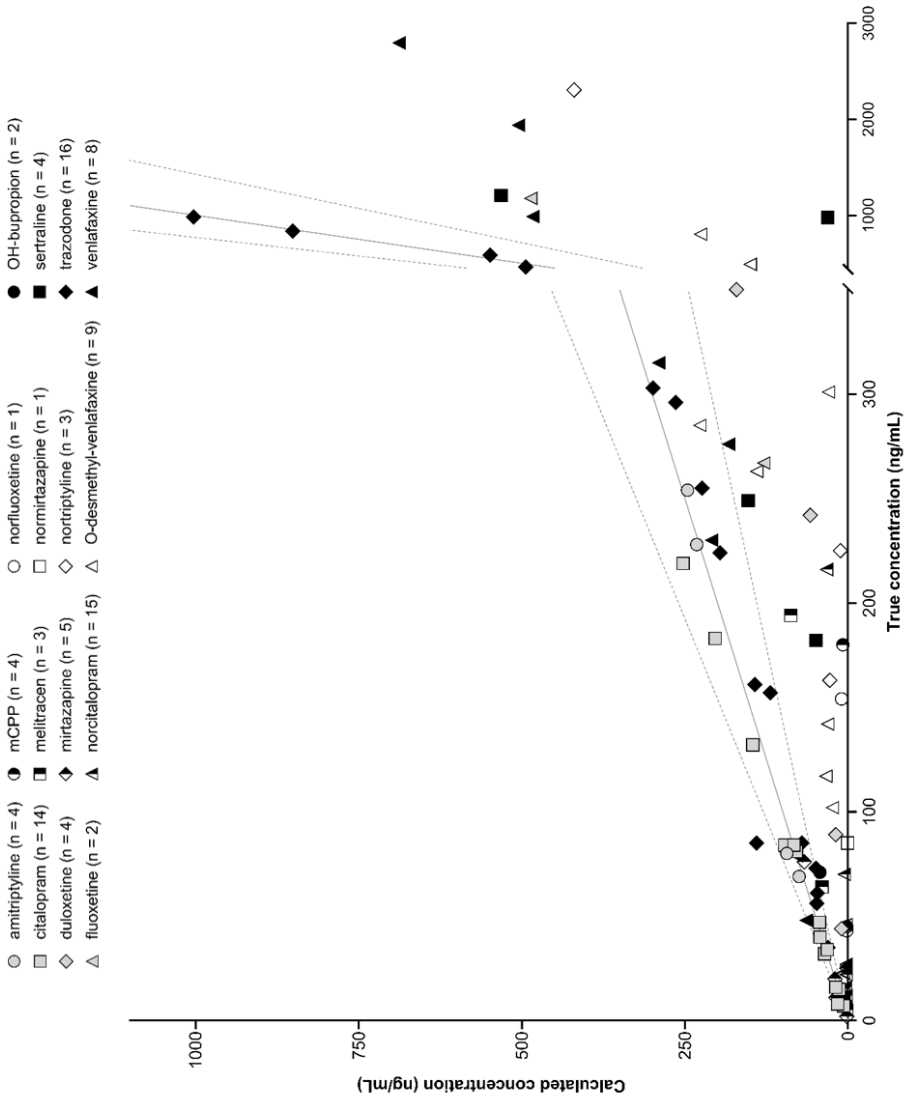

Figure S6 - 1. Semi-quantitative concentrations of the antidepressants as determined by tMRM screening. The true concentration was obtained from validated confirmation methods. The dotted lines represent a spread of ± 30% of the accuracy compared to the true concentration (full line). The concentrations of compounds above the upper limit of quantification could not be accurately determined and therefore are not plotted.

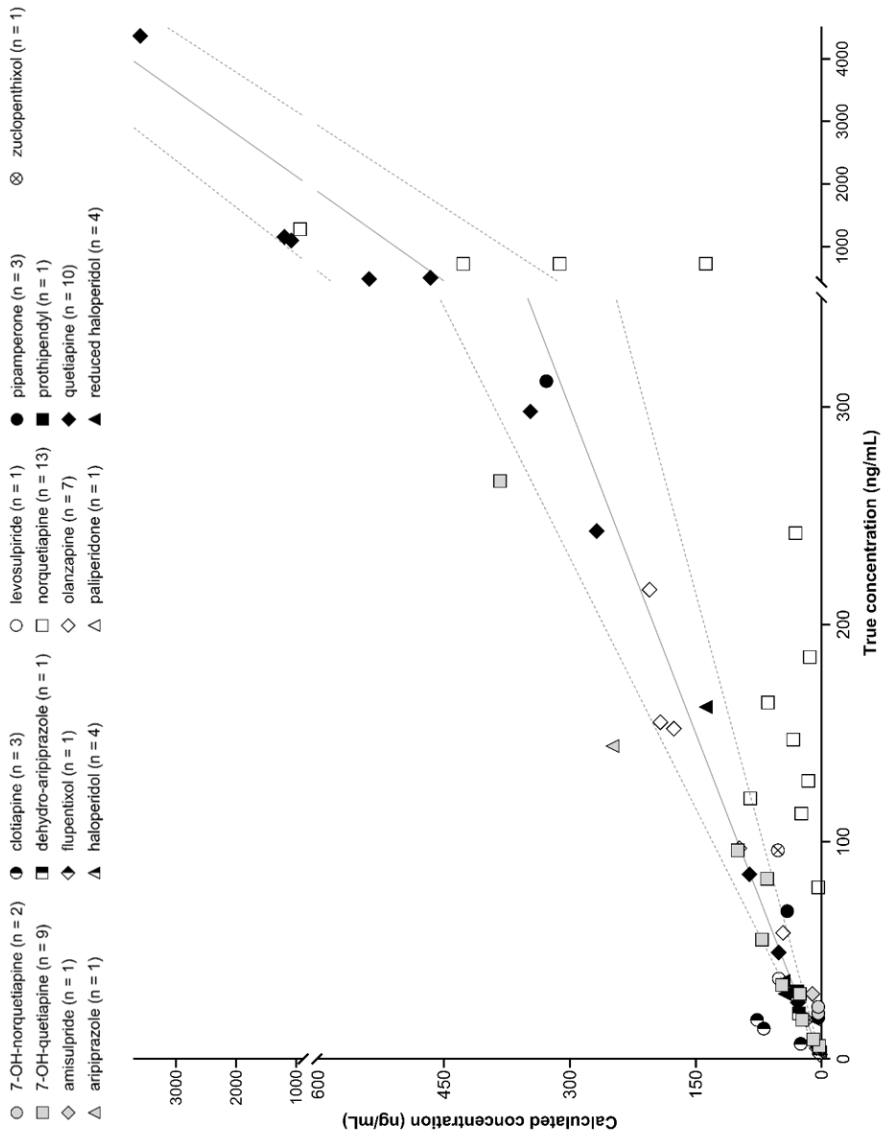

Figure S6 - 2. Semi-quantitative concentrations of the antipsychotics as determined by tMRM screening. The true concentration was obtained from validated confirmation methods. The dotted lines represent a spread of ± 30% of the accuracy compared to the true concentration (full line). The concentrations of compounds above the upper limit of quantification could not be accurately determined and therefore are not plotted.

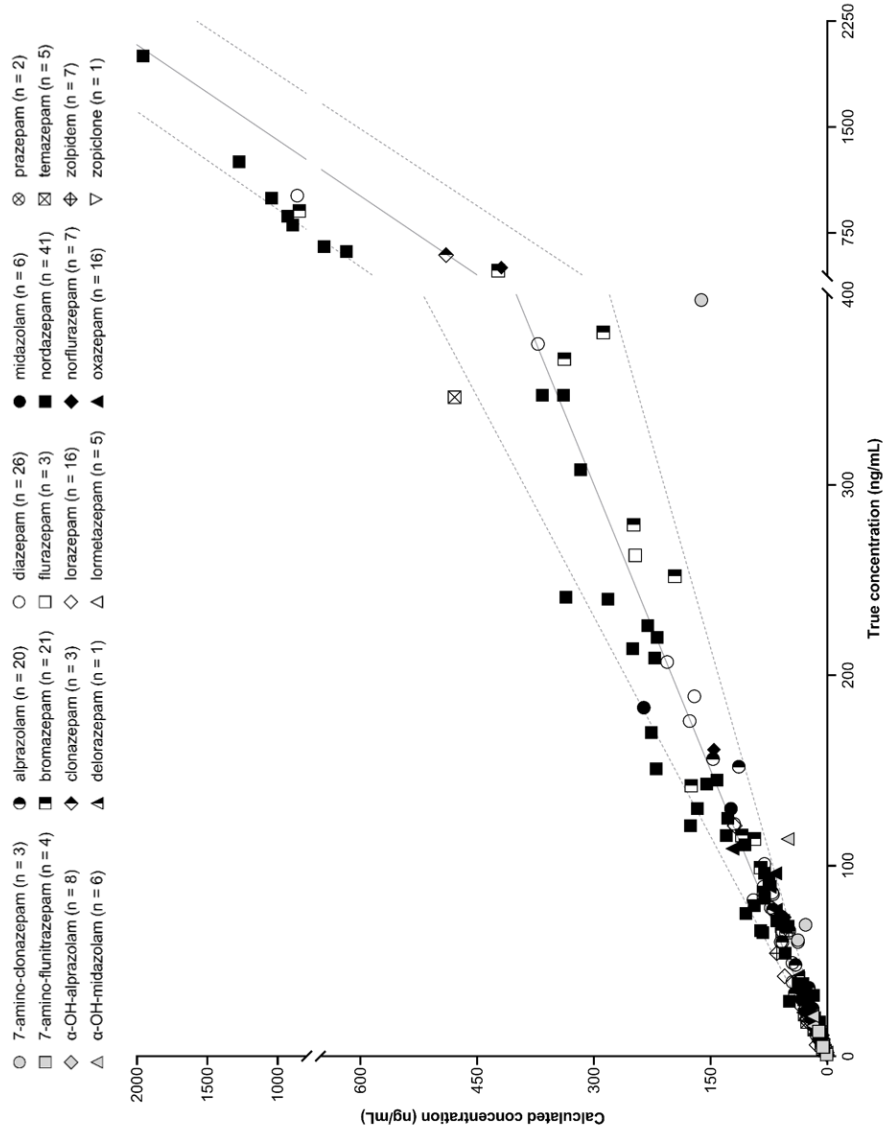

Figure S6 - 3. Semi-quantitative concentrations of the benzodiazepines and Z-drugs as determined by tMRM screening. The true concentration was obtained from validated confirmation methods. The dotted lines represent a spread of ± 30% of the accuracy compared to the true concentration (full line). The concentrations of compounds above the upper limit of quantification could not be accurately determined and therefore are not plotted.

CHAPTER 7

QUADRUPOLE TIME-OF-FLIGHT

Based upon the following publication:

M. Degreef, E.M. Berry, A. Covaci, K.E.K. Maudens, A.L.N. van Nuijs, Qualitative and semi-quantitative screening of selected psychoactive substances in blood: Usefulness of liquid chromatography – triple quadrupole and quadrupole time-of-flight mass spectrometry in routine toxicological analyses. *(in submission).*

7.1. METHOD DEVELOPMENT

7.1.1. Analytical optimisation

7.1.1.1. Data-dependent vs. data-independent acquisition

With high resolution mass analysers such as quadrupole time-of-flight mass spectrometers (QTOF), data can be gathered by either data-dependent (DDA) or data-independent acquisition (DIA). The latter is also sometimes referred to as MS^e (Waters) or All-ions (Agilent Technologies) and involves the instrument continuously scanning for any ion in a specific m/z-range, followed by fragmentation of each ion and subsequent detection of the product ions. Although this technique has been reported to be beneficial in terms of sensitivity over other acquisition methods, product ions of co-eluting precursors are combined into one spectrum and the post-analytical workflow relies strongly on appropriate data deconvolution and the use of high-resolution, multi-analyte spectral databases [435–437]. Such DIA method was successfully applied by our laboratory to the qualitative screening of new psychoactive substances in serum and urine samples, however considerable time had to be spent on post-analytical data mining, requiring highly qualified and experienced personnel [438]. Therefore, aiming to increase the sample turn-around time, it was decided for this thesis to work with a DDA method, the main advantage being a reduction in the number of potential hits as only ions above a certain threshold are acquired and/or selected for further fragmentation. Liquid chromatography (LC) parameters were kept similar to those determined for targeted analysis (see section 3.2 p. 42). In short, a gradient elution going from 95% mobile phase A (ultrapure water + 0.1% formic acid V/V) to 95% mobile phase B (9:1 acetonitrile:ultrapure water + 0.1% formic acid V/V) in 9 min was applied. Separation was achieved using an Agilent Zorbax Eclipse Plus C8 column. Starting from the published method by Kinyua et al. [438], the QTOF was operated in positive electrospray ionisation mode, with a fragmentor voltage (FV) of 75 V. A threshold of 1000 counts was set for DDA of tandem mass spectrometry (MS/MS)spectra. The different collision energies were updated to match those of commercially available Agilent high-resolution mass spectrometry databases (10 eV, 20 eV and 40 eV).

7.1.1.2. Selection extraction method

Using the above mentioned basic method parameters, the most appropriate sample preparation method was re-evaluated. As the finalised QTOF application would serve as a screening tool for psychoactive substances, sample preparation methods under investigation should ideally be quick to perform and non-selective with regards to the analytes they extract from the matrix. Hence, solid-phase extraction and mini-QuEChERS were not selected for being too laborious; only liquid-liquid extraction (LLE) with methyl-tertiary-butyl-ether (MTBE) and protein precipitation (PP) were re-evaluated. A full description of these protocols can be found in sections 4.1.2 and 4.1.3 p. 61. In short, 200 µL horse serum was spiked with 20 µL of both reference mixture and labelled internal standard (ISTD) mixture (see section 6.1.1.1 p. 111). For the PP, 800 µL acetonitrile (ACN) was added, the aggregated proteins centrifuged off and the supernatant transferred to an LC vial for injection. For the LLE, 65 µL 1 M carbonate buffer pH 9.5 and 800 µL MTBE were mixed with the horse serum and separated again by centrifugation. The supernatant was evaporated to dryness, reconstituted in ACN and injected onto the instrument. Next to serum, urine was also included as a matrix under investigation. It is the historical matrix of choice for screening purposes as it is usually

available in larger volumes compared to blood and contains most metabolites, in addition to (some of) the parent compounds [405,436]. However, as part of the pharmacokinetic pathways in the body, many drugs are conjugated to either glucuronic acid or sulphate (phase II metabolism) prior to excretion in urine [439]. Therefore, the urine sample preparation started by combining 500 µL urine with 50 µL reference solution and 50 µL ISTD solution, which was buffered to pH 4.5 with 200 µL sodium acetate buffer. After the addition of 10 µL β-glucuronidase – aryl sulphatase for deconjugation, the sample was vortex mixed for 90 s at 2000 rpm, rotor mixed for 5 min at 40 rpm and incubated for 1 h at 50 °C. A 200 µL aliquot was transferred to a new Eppendorf tube and the analytes extracted using the above described LLE and PP.

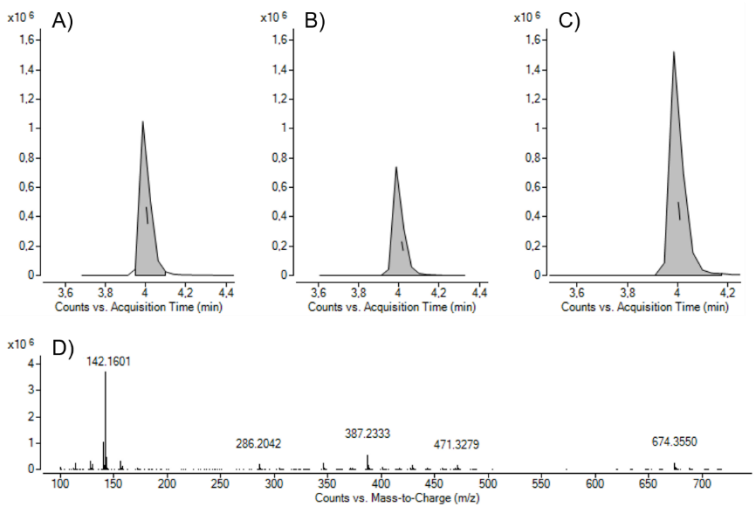

Figure 7 - 1. Extraction of quetiapine from spiked A/B) serum and C/D) urine samples. A liquid-liquid extraction using MTBE (A,C) resulted in consistently higher signal abundances for each of the matrices based upon three replicates. Protein precipitation of serum samples (B) gave acceptable results. Looking at the ion scan for urine samples (D) no signal for quetiapine (*m/z* 384.17062) could be detected following protein precipitation.

Similar to what had been found before (section 4.2.2 p. 65), when analysing serum samples the LLE yielded better results for all investigated analytes compared to PP. Observed peak abundances were about 70% of those obtained by LLE (Figure 7 - 1). Likely, the latter suffered from insufficient sample clean-up in combination with an additional five times dilution. Results for the urine analysis indicated a similar trend, albeit more pronounced. Whereas LLE peak abundances for urine samples were about 30% higher than those of serum samples, no signal for any of the analytes had been detected for PP of urine samples. No explanation is currently available for this absence of any analyte signal. It seems unlikely that the slightly increased dilution factor (five times for blood samples vs. seven times for urine samples) would be the cause as the samples were spiked at a high concentration. A potential matrix suppressant effect might play a role (fresh blank horse serum is likely a more clean matrix than pooled human urine) although still is not expected to result in complete absence of any signal. An instrumental issue was ruled out as each sample was extracted and analysed in triplicate and samples injected before and after the urine PP samples did not show any issues. Overall, the findings seen for the targeted methods were confirmed and a LLE on 200 µL serum using 800 µL MTBE proved superior over other investigated sample preparation techniques.

7.1.1.3. Optimisation fragmentor voltage

As previously discussed in section 3.3 p. 43, selecting the appropriate FV will increase the signal intensity of the precursor ion and thereby the sensitivity of the instrument for that analyte. Although the best FV is unique to every compound, one generalised value needs to be set for QTOF applications. Standardly, the FV is programmed to 135 V by the software, but a range between 120 V and 395 V (going in steps of 25 V) was investigated, based upon the observations of more experienced colleagues at the Toxicological Centre. Blank horse serum (200 µL) was spiked with the above described reference and ISTD mixtures (section 6.1.1.1 p. 111) and submitted to the LLE procedure. The reconstituted extract was injected onto the LC-QTOF for analysis and the signal abundance visually compared using the MassHunter Qualitative Analysis 10.0 software. Samples were prepared and analysed in duplicate.

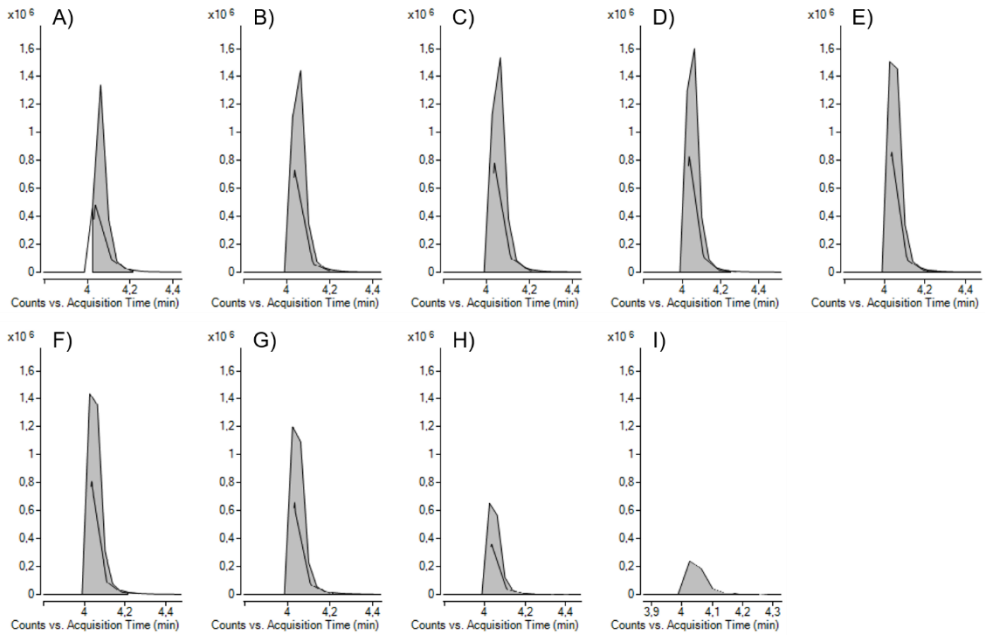

Figure 7 - 2. Effect of different fragmentor voltages (FV) on the signal for quetiapine. FVs visualised are A) 120 V, B) 145 V, C) 170 V, D) 195 V, E) 220 V, F) 245 V, G) 270 V, H) 295 V and I) 320 V. No significant differences were observed for the lower FVs, with the signal of the precursor ion markedly decreasing at the higher FV values.

Ranging between 120 V and 245 V, no marked differences were seen for each of the FVs investigated. On the other hand, a significant drop in signal intensity was seen for the higher voltages, with the signal of the precursor ion being too low to be selected for fragmentation from a FV value greater than 320 V (Figure 7 - 2). Overall, the highest signal abundances were observed for FVs 170 V and 195 V. An arbitrary value of 175 V was selected as the most appropriate FV for the current set of analytes.

7.1.1.4. Building a post-analysis library

To aid in the data analysis, a reference library was built using the MassHunter PCDL Manager B.08.00 software (Agilent Technologies, Santa Clara, California, US). Each of the compounds of interest and

the ISTDs was prepared individually at a 1 µg/mL concentration in ACN and injected onto the instrument. LC settings were kept similar to what has been described previously. The autosampler was equipped with a cooling element and kept at a constant 4 °C. All spectra were acquired in positive mode, scanning for all ions between m/z 100 and m/z 950. In line with commercially available libraries, precursor ions were fragmented at collision energies 10 eV, 20 eV and 40 eV (Figure 7 - 3).

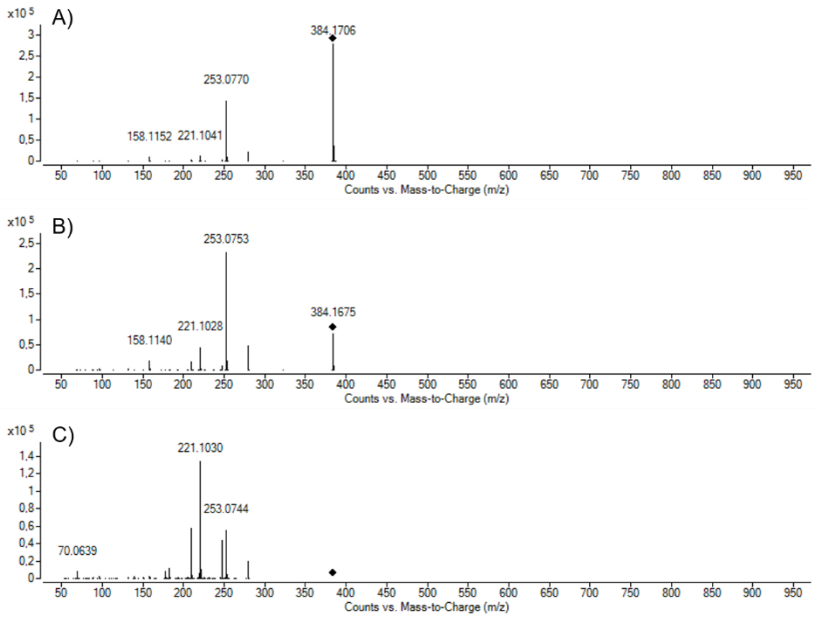

Figure 7 - 3. Quadrupole time-of-flight reference spectra for quetiapine. Spectra have been acquired at collision energies A) 10 eV, B) 20 eV and C) 40 eV. The precursor ion is indicated by the diamond. Product ions at *m/z* 279.1, *m/z* 253.0 and *m/z* 221.1 had previously been selected for the targeted triple quadrupole methods.

Using the MassHunter Qualitative Analysis 10.0 software, the MS/MS spectra were extracted, allocated to the right compound and transferred to the reference library. Any ions present at an abundancy < 5% compared to the most abundant one were excluded. Retention times (RT) were automatically copied in as well and all entries were manually updated with the chemical formula (used to calculated the exact mass). A total of 144 compounds populated the library: 132 analytes and 12 ISTDs. Caffeine, with a RT of 2.59 min, was included for quality control reasons. In healthy adults, this compound is (almost) always detected in blood and its absence – together with the absence of any of the ISTDs – could indicate an issue with the sample preparation or injection of the sample in question.

7.1.2. Selection post-analytical workflow

In contrast to triggered multiple reaction monitoring (tMRM methods), where only specified compounds are monitored, QTOF methods require careful post-analytical extraction of analytes of interest. Several strategies have been investigated and published by our laboratory. Cuykx et al. described a molecular feature extraction with the Agilent MassHunter software followed by statistical principal component analysis using the Umetrics' EZinfo software for metabolic profiling of steatosis progression in HepaRG® cells [440]. Vervliet et al. reported a comprehensive three-workflow data

analysis process for the identification of *in vitro* generated metabolites of the synthetic cannabinoid 5Cl-THJ-018 [441]. Lastly, Iturrospe et al. developed an R-based workflow in combination with in-house and online public libraries for the detection of polar, endogenous metabolites in different human matrices [442]. Here, the selection of the most appropriate workflow focussed on user-friendliness and time-efficiency, combining analyte extraction with identification based upon the afore-mentioned in-house library (see section 7.1.1.4). As described for tMRM, the best performing workflow was selected based upon a subset of 25 archived medico-legal samples (section 6.1.2.1 p.115). A 200 µL aliquot of each sample was spiked with 20 µL ACN and 20 µL ISTD mix (see section 6.3.1 p. 120) and extracted as described previously with 800 µL MTBE. The dried extract was reconstituted in 20 µL ACN and injected onto the LC-QTOF. The mass spectrometer was operated in auto-MS/MS mode, whereby the instrument continuously scans for any analytes present over a set m/z-range (here m/z 100 – m/z 950). If any of the detected ions exceeded 2000 counts, these were selected for fragmentation at the above defined collision energies. Each sample was injected twice. For the first injection, an inclusion list was added to the method, which defined the masses and RTs of the analytes that should preferably but not exclusively be selected for fragmentation (method I). For the second injection no preferred ions were defined, rather the signals for purine and hexakis phosphazine (components of the calibrant solution) were excluded from fragmentation to increase the time spent on the MS/MS acquisition of analytes of interest (method II).

7.1.2.1. Workflow 1: MassHunter Profinder software

A first workflow uses the MassHunter Profinder 10.0 software (Agilent Technologies, Santa Clara, California, US), specifically developed for batch feature extraction of time-of-flight based data files. Five different extraction algorithms are available: batch molecular feature extraction, batch recursive feature extraction for either small molecules and peptides or for large molecules, batch targeted feature extraction and batch isotopologue extraction. Using the 'Find compound by formula' algorithm based upon a reference library, batch targeted feature extraction combined analyte extraction with identification and was therefore selected. The previously created reference library (section 7.1.1.4) was set as the source for formula targets, with both a mass and retention time match as requirements for positive identification. Mass tolerances were set to 20 ppm, RT tolerances to 0.5 min. The overall match score was made up by a mass score, isotope abundance score, isotope spacing score and retention time score, weighing in at respective factors 75, 50, 50 and 75. Any peaks with an absolute area < 10000 counts were excluded. The outcome results are presented in an easy-to-interpret format and allow for further filtering if required.

To reduce the number of potential false positive results, the influence of the peak area, the peak score and an analyst's judgement as exclusion criteria were investigated (Table 7 - 1). Cut-off values for peak area were set to 10000 counts, 50000 counts or the average amount of counts of the lower limit of quantification (LLOQ; based upon four injections of CAL L1); for peak score these were 50 and 75. The analyst's judgement criteria consisted of exclusion of all peaks with an error flag (e.g. 'no H adduct' or 'no EIC peaks'), exclusion of peaks not co-eluting with those of the calibration samples and exclusion of any peak with a significant non-symmetrical shape or less Gaussian shape. The findings were compared to those of targeted dynamic multiple reaction monitoring (dMRM) analysis, which positively identified 123 analytes in the samples. Similar outcome results were observed for acquisition methods I (preferred fragmentation list) and II (exclusion reference masses). A minimum

of 15% – 20% of the compounds identified by dMRM were missed by QTOF analysis, irrespective of the cut-off values applied. This number was found to increase with around 10% when only analytes with a peak score ≥ 75 (vs. one ≥ 50) were included (Figure S7 - 1). An additional influence is seen for the analyst's judgement. Only removing those with error flags vs. also excluding peaks that do not co-elute with CAL solutions run in the same batch did not result in any significant difference in the number of false negative results. A more noticeable drop in correctly identified analytes was observed when non-Gaussian peak shapes were also excluded. However, the latter is a less strictly defined criterion and therefore more prone to observer bias. Indeed, visual evaluation of appropriate peak shape might depend on the analyst's experience with chromatography and mass spectrometry based applications, but can even be skewed when comparing peak shapes between targeted triple quadrupole mass spectrometry (QQQ) and untargeted QTOF methods, as less data points per peak may be acquired by the latter. The influence of peak area as a criterion is most noticeable in the number of false positive results, which decreased from 104 hits (method A, ≥ 10000 counts) to 56 hits (method G, ≥ 50000 counts) and even further to 19 hits (method M, ≥ LLOQ) as the requirements became more strict.

Table 7 - 1. Inclusion criteria under investigation for compounds identified by the MassHunter Profinder software. The influence of a cut-off value based upon peak area, peak score and/or the analyst's judgement was investigated. The peak area of the LLOQ was determined from four injections of CAL L1. Calibrator, CAL; lower limit of quantification, LLOQ.

Method	Peak area	Peak score	Analyst's judgement
A	≥ 10000	≥ 50	no error flags
B	≥ 10000	≥ 50	no error flags & co-elution with CALs
C	≥ 10000	≥ 50	no error flags & co-elution with CALs & Gaussian shape
D	≥ 10000	≥ 75	no error flags
E	≥ 10000	≥ 75	no error flags & co-elution with CALs
F	≥ 10000	≥ 75	no error flags & co-elution with CALs & Gaussian shape
G	≥ 50000	≥ 50	no error flags
H	≥ 50000	≥ 50	no error flags & co-elution with CALs
I	≥ 50000	≥ 50	no error flags & co-elution with CALs & Gaussian shape
J	≥ 50000	≥ 75	no error flags
K	≥ 50000	≥ 75	no error flags & co-elution with CALs
L	≥ 50000	≥ 75	no error flags & co-elution with CALs & Gaussian shape
M	≥ LLOQ	≥ 50	no error flags
N	≥ LLOQ	≥ 50	no error flags & co-elution with CALs
O	≥ LLOQ	≥ 50	no error flags & co-elution with CALs & Gaussian shape
P	≥ LLOQ	≥ 75	no error flags
Q	≥ LLOQ	≥ 75	no error flags & co-elution with CALs
R	≥ LLOQ	≥ 75	no error flags & co-elution with CALs & Gaussian shape

Overall, method N, which excluded analytes with a score < 50, a peak area smaller than that of the LLOQ, and a RT not perfectly agreeing with that of CAL samples in the same batch, provided the best combination of minimal amounts of false positive results without markedly increasing the number of false negative results. No marked differences were observed between acquisition methods I and II, but as method II (exclusion reference masses) had less missed analytes, this acquisition method was kept for a more in-depth data investigation. Out of 123 analytes identified using targeted dMRM methods, 75% was correctly identified by the QTOF method (Figure 7 - 4A). Bupropion and its metabolite OH-bupropion were both missed in the same sample, in which dMRM analysis showed their presence in above upper limit of quantification (ULOQ) concentrations. Whereas

OH-bupropion was not acquired by the instrument, bupropion itself showed a peak at a slight forward RT-shift and was therefore deemed not present. In true routine analyses, it would be recommended to re-inject the sample or analyse it using another detection technique for confirmation purposes. Similarly, trazodone was missed in two cases at high concentrations (984 ng/mL and 835 ng/mL), one of which where the software extracted two peaks but identified the wrong one due to a RT mismatch between the reference library and the CALs. In both samples, mCPP was positively identified. Conversely, in six samples where low concentrations of mCPP were missed (range 2 ng/mL – 31 ng/mL), trazodone had been detected. Mirtazapine was missed in one sample at a concentration five times that of the LLOQ (2 ng/mL). Normirtazapine had been detected in all dMRM-positive cases, but was excluded based upon its peak area three times lower than that of the LLOQ. For the same reason, norcitalopram was missed in one sample (dMRM concentration 11 ng/mL) but citalopram had been detected. Also amitriptyline and nortriptyline were missed for this reason in the same sample. Both were present at a low to near-LLOQ concentration during dMRM analysis and no other samples positive for these analytes were available to compare these results to. For the antipsychotics (APs), quetiapine metabolites were the most missed compounds. Norquetiapine was missed in one out of seven cases, although at the LLOQ concentration of 3 ng/mL. On the other hand, 7-OH-norquetiapine could not be detected in any of the cases (n = 3, dMRM concentration range 5 ng/mL – 107 ng/mL). Quetiapine and 7-OH-quetiapine were detected in all cases. Flupentixol, reduced haloperidol and amisulpride were both missed at a low concentration. Whether this is an analyte-specific (as for 7-OH-norquetiapine) or concentration-specific (as for norquetiapine) problem could not be determined as no other samples were positive for these compounds. As had been seen for 7-OH-norquetiapine, the benzodiazepine (BZD) midazolam had not been acquired in any of the samples (n = 2), regardless of the concentration as determined by dMRM analysis (7 ng/mL and 130 ng/mL). On the other hand, its α-hydroxylated metabolite was correctly identified in all cases. Similarly, α-OH-alprazolam was missed in both positive cases, although twice at a near-LLOQ concentration, but its precursor alprazolam had been picked up. 7-amino-clonazepam was not picked up in one sample (dMRM concentration 3 ng/mL vs. LLOQ 2 ng/mL), clonazepam itself could not be detected by either QQQ or QTOF in that sample. Temazepam gave a false negative signal due to a lower than LLOQ peak area in one sample, but diazepam, nordazepam and oxazepam had been found in that sample. Lastly, prazepam and zopiclone were both missed in one sample at a near-LLOQ concentration, though no other positive samples were present to confirm these findings.

In addition to these findings, 10 false positive results were found, the majority of which indicated the presence of a BZD in the sample. Clobazam was wrongfully identified in two samples, as was lorazepam. Clobazam shares its exact molecular weight with temazepam (300.066570 u, $C_{16}H_{13}ClN_2O_2$) and differs from it with only 0.2 min in RT. For both cases, temazepam as well as diazepam, nordazepam and oxazepam were present potentially explaining this false positive result. The presence of lorazepam could not be explained at this time, however the structurally related compounds delorazepam or lormetazepam were present in the samples as well. Diazepam, nordazepam and oxazepam all gave a false negative result in the same sample. dMRM analysis could not confirm these findings, nor did either method detect temazepam in that same sample. The antidepressant (AD) bupropion was identified in one case that was also positive for OH-bupropion. Targeted QQQ did pick up a signal for all bupropion transitions, though it was dismissed based upon

the poor peak shape. A false positive result was also found for trazodone. The analyte had been identified with dMRM although it was omitted for having a concentration lower than that of the LLOQ. The estimated concentration was 35 ng/mL, the LLOQ 50 ng/mL. (The LLOQ of trazodone had been determined at 25 ng/mL but CAL L1 had to be discarded due to a problem in that specific batch.) Finally, a likely true false positive identification was found for the AP olanzapine.

7.1.2.2. Workflow 2: MassHunter Qualitative Analysis software

The second workflow uses the Agilent MassHunter Qualitative Analysis 10.0 software (Agilent Technologies, Santa Clara, California, US). The compound mining algorithm extracted all ions that had been selected for auto-MS/MS. Potential chemical formulas were generated for all features and could contain up to 60 C-atoms, 120 H-atoms, 30 O-atoms, 30 N-atoms (all standard settings) as well as up to 5 S-atoms and 3 halogens. Additionally, ISTD formulas containing up to 20 D-atoms were allowed. A feature's exact mass, isotope abundance and spacing, RT and fragmentation pattern were compared to the previously created database. The mass tolerance was standardly set to 20 ppm, the RT window to 0.5 min. These both parameters weighed heavier in the final match score. A positive result was returned if a match score greater than 50 was obtained.

Table 7 - 2. Inclusion criteria under investigation for compounds identified by the MassHunter Qualitative Analysis software. The influence of a cut-off value based upon peak score, retention time difference with calibrator samples and peak height was investigated. Lower limit of quantification, LLOQ; retention time, RT.

Method	Peak score	RT difference	Peak height
A	≥ 50	≤ 0.5 min	≥ 1000
B	≥ 50	≤ 0.5 min	≥ 10000
C	≥ 50	≤ 0.5 min	≥ 25000
D	≥ 50	≤ 0.5 min	≥ LLOQ
E	≥ 50	≤ 0.2 min	≥ 1000
F	≥ 50	≤ 0.2 min	≥ 10000
G	≥ 50	≤ 0.2 min	≥ 25000
H	≥ 50	≤ 0.2 min	≥ LLOQ
I	≥ 75	≤ 0.5 min	≥ 1000
J	≥ 75	≤ 0.5 min	≥ 10000
K	≥ 75	≤ 0.5 min	≥ 25000
L	≥ 75	≤ 0.5 min	≥ LLOQ
M	≥ 75	≤ 0.2 min	≥ 1000
N	≥ 75	≤ 0.2 min	≥ 10000
O	≥ 75	≤ 0.2 min	≥ 25000
P	≥ 75	≤ 0.2 min	≥ LLOQ

Features where no match was found were filtered out by the software. The influence of additional filters on peak score, RT difference compared to CAL samples run in the same batch, and peak height were investigated (Table 7 - 2). In contrast to what had been observed for the Profinder software, more pronounced differences were observed between methods I (preferred fragmentation list) and II (exclusion reference masses), with either performing better than the other depending on the inclusion criteria used. The most marked influence on the number of correctly identified results was seen when the minimum peak score was increased from 50 to 75, particularly for acquisition method I (Figure S7 - 2). The effect of varying the RT difference or peak height was more obvious in a reduction in the number of false positive results, whereby narrowing the RT window lowered

the number of false positive results from 82 to 69, setting a height limit ≥ LLOQ vs. ≥ 1000 from 82 to 30, and a combination of both from 82 to 22.

Overall, method B, which excluded analytes with a score < 50, a peak height smaller than that of the LLOQ, and a RT not perfectly agreeing with that of CAL samples, provided the best combination of minimal amounts of false positive results without markedly increasing the number of false negative results. These inclusion criteria are identical to Profinder data analysis method N, which also gave the best results (section 7.1.2.1). Out of 123 analytes picked up using targeted dMRM methods, 73% was correctly identified by the QTOF method (Figure 7 - 4B). As seen using the Profinder software, bupropion was missed in one sample, in which dMRM analysis showed its presence in an above ULOQ concentration. However, OH-bupropion, also present at a concentration > ULOQ, had been detected in this instance. Trazodone was also missed at a high concentration (984 ng/mL) but its metabolite mCPP was detected in that sample. Conversely, mCPP had not been selected for fragmentation in five cases, all at low to near-LLOQ concentrations, but trazodone had in all but one. Mirtazapine and normirtazapine were both missed in the same two cases, although again at low concentrations: twice 11 ng/mL for mirtazapine, and 5 ng/mL and 12 ng/mL for normirtazapine. In both cases, normirtazapine had been excluded as its peak height was about two to three times lower than that of the LLOQ. For the same reason, norcitalopram was excluded in one sample (dMRM concentration 11 ng/mL vs. LLOQ 2 ng/mL), but citalopram had been detected. As seen during data analysis with the Profinder software, amitriptyline and nortriptyline gave one false negative result, both in the same sample where they were present at near-LLOQ concentrations according to dMRM analysis. Regarding the APs, 7-OH-norquetiapine and norquetiapine were missed in one and two samples, respectively, each at concentrations less than 10 ng/mL. Quetiapine and 7-OH-quetiapine were correctly identified in all samples where dMRM analysis showed their presence. Flupentixol, haloperidol and reduced haloperidol were not picked up in one sample each, once more at low to near-LLOQ concentrations. Amisulpride had been identified in the only sample containing this cpd (22 ng/mL), but was excluded based upon a four times lower peak height compared to the LLOQ. In contrast to the Profinder workflow, the BZD midazolam was detected in all samples. On the other hand, oxazepam had not been selected for fragmentation in four out of five positive cases (dMRM concentration ranging from 12 ng/mL to 109 ng/mL). Its precursors nordazepam and/or temazepam could be detected in all cases, as could diazepam. Similarly, α-OH-alprazolam was missed in both positive cases, although twice at near-LLOQ concentrations, but its precursor alprazolam had been picked up. 7-amino-clonazepam was not picked up in one sample (dMRM concentration 3 ng/mL vs. LLOQ 2 ng/mL), clonazepam itself could not be detected by either QQQ or QTOF analysis in that sample. Lorazepam and the structurally related lormetazepam were missed in one sample each where dMRM quantified them at a near-LLOQ concentration. Additionally, lorazepam gave a false negative result in one sample (dMRM concentration 24 ng/mL). Lastly, prazepam and zopiclone were both missed in one sample at a near-LLOQ concentration, though no other positive samples were present to confirm these findings.

In addition to these findings, 22 false positive results were found, the majority of which were either for phenelzine (n = 5) or prothipendyl (n = 9). Reboxetine gave a false hit in one sample. It shares its mass (3 ppm difference) and RT (0.1 min difference) with norcitalopram-D_3, however as this ISTD had not been included in the ISTD mix, the nature of this interference remains unknown. The

AP olanzapine was wrongfully identified in the same sample as with the Profinder workflow. 7-OH-quetiapine gave a false positive result for a sample in which dMRM analysis had detected all transitions but at a final concentration less than that of the LLOQ. For the BZDs, false positive results were found for nitrazepam (n = 1) and clonazepam (n = 2), neither of which could be explained by interference from another database compound. Finally, diazepam and nordazepam were wrongfully identified in the same sample, though temazepam and oxazepam could not be detected, nor could any of the analytes with dMRM analysis.

7.1.2.3. A two-software approach?

Both workflow 1 (MassHunter Profinder) method N (Table 7 - 1) and workflow 2 (MassHunter Qualitative Analysis) method H (Table 7 - 2) performed adequately, but with around 30 positive results not identified and 10 and 22 false positive hits, respectively, they could be improved upon. Therefore, it was investigated if a combination of both workflows would be beneficial (Figure 7 - 4C/D). Two simulations were run: simulation I where both workflows had to identify a feature for it to be considered positive, and simulation II where at least one workflow had to identify a feature for it to be considered positive. Under these conditions, it was hypothesised that simulation I would significantly reduce the number of false positive results, whilst not markedly increasing the number of false negative hits, and vice versa for simulation II (a reduction in false negative results, no increase in false positive ones).

Simulation I successfully reduced the number of false positive hits from 10 (workflow 1) or 22 (workflow 2) to 3, incorrectly identifying diazepam and nordazepam in one case and olanzapine in another. The related metabolites temazepam/oxazepam and norolanzapine, respectively, had not been identified. The number of false negative results rose from around 30 (25% of all analytes detected by dMRM) to around 40 (30% of all analytes detected by dMRM), the majority of which consisted of analytes present at low or near-LLOQ concentrations. Compared to data analysis using the MassHunter Qualitative Analysis software (workflow 2) alone, a potentially important positive result for oxazepam (109 ng/mL) was missed, although diazepam, nordazepam and temazepam were correctly identified in that same sample. When comparing simulation I with results obtained through only the MassHunter Profinder software (workflow 1), four additional analytes were missed at concentrations significantly higher than the LLOQ. 7-OH-norquetiapine (dMRM concentration 107 ng/mL) was not picked up in one sample, though quetiapine and its other metabolites were. Similarly, midazolam (130 ng/mL) and trazodone (834 ng/mL) were missed in one sample each, but α-OH-midazolam and mCPP had been detected, respectively. Although ideally no single compound would be missed, the presence of related analytes indicated that a specific substance had likely been used and its presence would have been looked for in other screening analyses. Of greater concern would be the false negative result for OH-bupropion (> ULOQ with dMRM), as the Profinder workflow had also missed the above ULOQ concentration of bupropion in that sample.

Simulation II achieved the hoped reduction in false negative results to below 20% of the analytes found by targeted QQQ analysis. None of the results differed markedly from the LLOQ, with the exception of one bupropion result (dMRM concentration > ULOQ) and one hit for trazodone (984 ng/mL). For both samples, the respective metabolites OH-bupropion and mCPP had been detected. The number of false positive results rose to 29. The majority of false positive cases were for phenelzine and prothipendyl (n = 14). Additionally, a hit for 7-OH-quetiapine (not detected by

the Profinder workflow) and one for trazodone (not detected by the MassHunter Qualitative Analysis workflow) had been detected by dMRM though were excluded from further analysis as their concentration fell below the respective LLOQs. Compared to using just workflow 1 (MassHunter Profinder), additional false positive results were obtained for clonazepam (n = 2), nitrazepam (n = 1) and reboxetine (n = 1). Compared to workflow 2 (MassHunter Qualitative) on its own, these were bupropion (n = 1), clobazam (n = 2), lorazepam (n = 2) and oxazepam (n = 1).

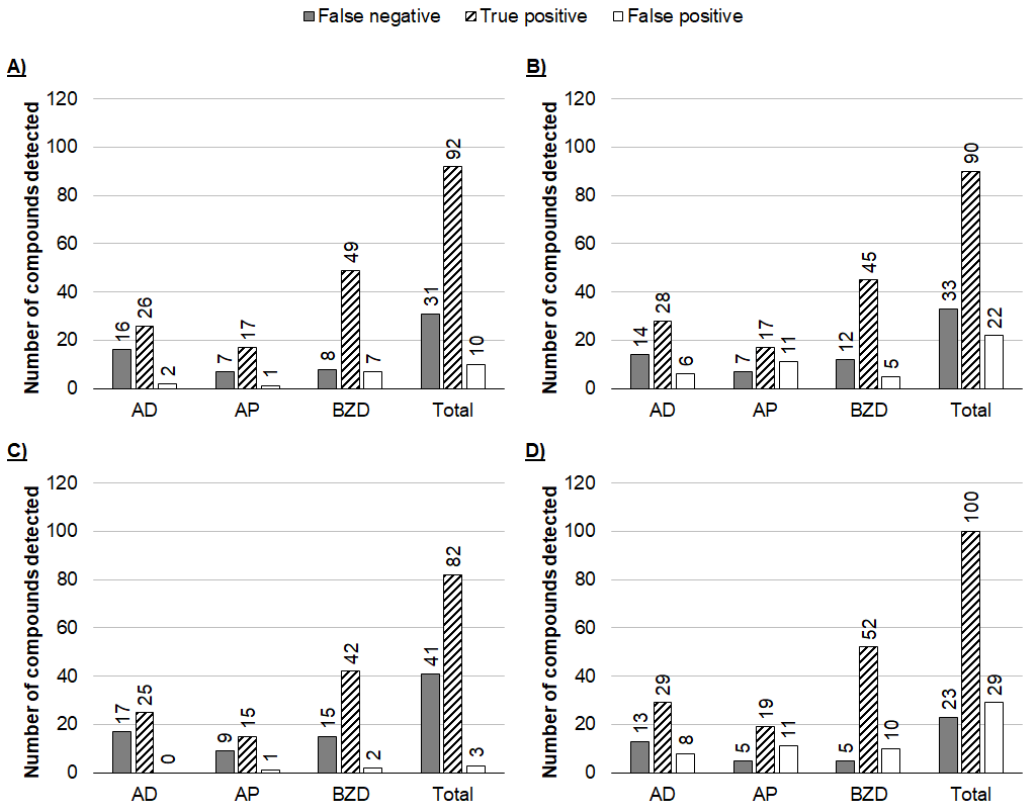

Figure 7 - 4. Results of the QTOF screening using the optimised workflows. Spectra have been acquired in auto-MS/MS mode with exclusion of the reference masses. Using only the MassHunter Profinder software (A, workflow 1), less false positive or negative results were obtained compared to the MassHunter Qualitative Analysis software (B, workflow 2). Including only analytes identified with both workflows (C) significantly reduced the number of false positives, but also that of true positive identifications. The best results were obtained when analytes detected by at least one workflow were included (D). The higher amount of false positives could be reduced when taking into account a systematic bias for phenelzine and prothipendyl.

Although simulation I markedly reduced the number of false positive results, several moderate to high concentrated analytes were additionally missed. Therefore, it was decided not to continue with this model. On the other hand, simulation II reduced the number of false negative results, whilst only moderately increasing the number of false positive results. If a systematic problem for phenelzine and prothipendyl, as well as the identification of two by dMRM analysis detected but below-LLOQ concentrated analytes, were not taken into account, no significant differences in the number of false positive results compared to either workflows 1 or 2 were seen.

7.2. VALIDATION QUALITATIVE SCREENING

7.2.1. Description optimised method

As for tMRM, sample preparation and LC settings remained unchanged compared to the dMRM methods. The mass spectrometer was equipped with an electrospray ionisation source operated in positive mode. Other source parameters were: gas (N_2) temperature 300 °C, gas flow 8 L/min, nebuliser 40 psi, sheath gas heater 350 °C, sheath gas flow 11 L/min, capillary voltage 3500 V and nozzle voltage 500 V. The fragmentor voltage was set to 175 V throughout the run. Analytes were acquired in data-dependent auto-MS/MS mode (mass range m/z 50 – m/z 950) at a rate of 5 spectra/s (200 ms/spectrum). Fragmentation was performed using collision energies 10 eV, 20 eV and 40 eV. An abundance threshold of 2000 counts was set for precursor selection, with any masses in the range m/z 120 – m/z 122 and m/z 921 – m/z 923 excluded at all times. A calibrant solution containing purine (m/z 121.0508) and hexakis phosphazine (m/z 922.0098) was continuously infused for in-run mass recalibration.

The acquired features were extracted using the Agilent MassHunter Profinder software 10.0 and compared to an in-house database containing the MS/MS spectra of the 132 psychoactive substances and 12 ISTDs under investigation. A match was returned if the exact mass of the precursor ion differed with less than 20 ppm from that of the predicted one. The RT window was set to 0.5 min. Any features with a peak area < 10000 counts were excluded from further analysis. The resulting list was additionally filtered on match score (≥ 50), RT (≤ 0.2 min difference with that of the calibrators run in the same batch) and absolute peak area (greater than or equal to that of the lowest calibrator run in the same batch). To reduce the number of false negative hits, the data files were also analysed by the Agilent MassHunter Qualitative Analysis 10.0 software. The automated workflow extracted all features selected by auto-MS/MS and compared these with the previously mentioned database. This time, the generated product ions were also taken into account when determining a match score. The identified features were additionally filtered on match score (≥ 50), RT (≤ 0.2 min difference with that of the calibrators run in the same batch) and absolute peak height (greater than or equal to that of the lowest calibrator run in the same batch). The findings of both workflows were combined and any features resulting in a match for at least one workflow were included in the final list of identified analytes.

Additionally, optimal performance of the analytical and post-acquisition methods was verified in each sample using three criteria. First, the masses of purine and hexakis phosphazine were extracted and their continuous presence throughout each run checked. Second, the positive identification of the 12 ISTDs in every sample was an absolute requirement. Third, caffeine would preferably have been identified in every sample. This compound is expected to be present in the blood (and urine) of the majority of adults or anyone drinking coffee or tea, although it might be absent in drinkers of decaffeinated coffee or herbal infusions. Hence, fulfilment of the third criterion was not obligatory.

7.2.2. Validation against case samples

Performance of the finalised acquisition and data analysis workflow was verified against those selected samples not used for workflow optimisation in section 7.1.2 above. A total of 309 analytes in 78 blood samples were detected by dMRM analysis, 80% of which were also acquired using QTOF

analysis (Figure 7 - 5). In contrast to previous findings, bupropion and OH-bupropion were correctly identified by the software, even at a low concentration (around 10 ng/mL). mCPP could not be reliably detected, with only one out of seven cases (concentration range 4 ng/mL – 40 ng/mL) correctly identified. These results correspond with previous findings, as did the positive identification of its precursor trazodone in each of the cases. Once more, mirtazapine and normirtazapine, if missed, tended to result in a false negative result in the same cases, although at concentrations expected from sub- to low therapeutic doses (2 ng/mL – 27 ng/mL). Similarly, amitriptyline and nortriptyline were negative in a single, but the same, sample. dMRM analysis had shown amitriptyline to be present at a concentration below the LLOQ. However, its metabolite nortriptyline (also prescribed on its own) had a concentration of 2306 ng/mL. Citalopram had not been picked up in one out of 31 samples, where dMRM analysis found it at an above ULOQ concentration. Norcitalopram had been detected in that same sample. Conversely, this metabolite resulted in a false negative result in three out of ten samples, although it was present at low concentrations only and citalopram itself was detected in all of them. Additional false negative results were found for the ADs duloxetine, fluoxetine and norfluoxetine, and moclobemide, none of which were detected in the optimisation data set. They tended to be missed in the majority of samples positive for these compounds (with the exception of duloxetine) irrespective of their concentration.

With regards to the APs, the previously mentioned detection issues at low concentrations for 7-OH-norquetiapine and norquetiapine could also be observed in the current samples. Quetiapine and 7-OH-quetiapine had been detected in all samples. False negative results for amisulpride could be attributed to a lower peak height/area compared to the LLOQ rather than an acquisition or database match problem. The same was true for the only false negative result for levosulpiride. Olanzapine and its metabolite tended to result in wrongful negative results at near-LLOQ concentrations, apart from one sample where norolanzapine was present at a concentration of 123 ng/mL, although generally at least one of both analytes would be detected in the relevant samples. Prothipendyl had been detected using dMRM in one sample only (concentration 706 ng/mL), but could not be picked up by QTOF analysis.

α-OH-alprazolam was the most missed BZD. It could not be identified in any of the nine positive samples, although it was present at near-LLOQ concentrations only. Sensitivity problems may underlie these findings, as α-OH-alprazolam is reported to be present in blood at a concentration less than 10% of that of its parent compound alprazolam, which had been correctly identified in all but one of fifteen cases (dMRM concentration 3 ng/mL) [405]. Oxazepam identification was markedly better than had been observed during method optimisation, with only two out of twelve cases leading to a false negative result, both at low concentrations. Similarly, diazepam resulted in two false negative findings (out of twenty cases), twice at low to near-LLOQ concentrations, and its metabolites could be detected in both cases. Nordazepam was false negative in one case only, where oxazepam was negative too. No diazepam or temazepam could be detected using either instrument or post-analysis method. Midazolam and α-OH-midazolam were both missed in the same sample, where their signal was lower than that of the LLOQ. dMRM analysis had also shown their presence at LLOQ concentrations. Lastly, 7-amino-clonazepam and lormetazepam were false negative in one case each only, both at LLOQ concentrations, as were delorazepam, etizolam and lorazepam at near-

LLOQ concentrations. In contrast to the optimisation findings, prazepam and zopiclone were correctly identified in all cases.

False positive results were found in half of the analysed samples. As had been noticed previously, prothipendyl was wrongly identified in the majority of samples. In these samples, a peak was detected eluting just under 0.2 min earlier than that of the calibrators. Based upon solely the exact mass and RT, no match is found with the database when using the MassHunter Profinder workflow. However, the MassHunter Qualitative Analysis workflow, which also takes the fragmentation spectrum into account, positively identifies this peak as prothipendyl, with a match score greater than 95. Further investigation into the nature of a potentially interfering analyte is needed, as might an update of its entry into the database using a freshly prepared reference standard solution. A more strict criterion for co-elution with the CALs (e.g. set to 0.1 min difference in RT) could also be set for this compound. In addition, false positive findings for temazepam (n = 4), oxazepam (n = 2) and 7-OH-quetiapine (n = 1) could be attributed to analytes that had been detected by dMRM analysis, but were excluded based upon their calculated concentrations which were lower than those of the respective LLOQs (chosen to represent blood concentrations following subtherapeutic use). Discarding the above mentioned compounds effectively reduced the number of false positive results from 57 to 24. The ADs maprotiline and normaprotiline resulted in a combined six false positive findings, which could be attributed to respective interference by amitriptyline and nortriptyline with which they share their exact mass and RT. Other false positive results seemed to occur at random (each in one or two samples only) and could not be attributed to interference by any known analyte or ISTD. This was observed for the ADs duloxetine, norfluoxetine, normirtazapine, O-desmethyl-venlafaxine and reboxetine, the APs 7-OH-quetiapine, levomepromazine, olanzapine and quetiapine, and the BZDs brotizolam, clonazepam and lorazepam.

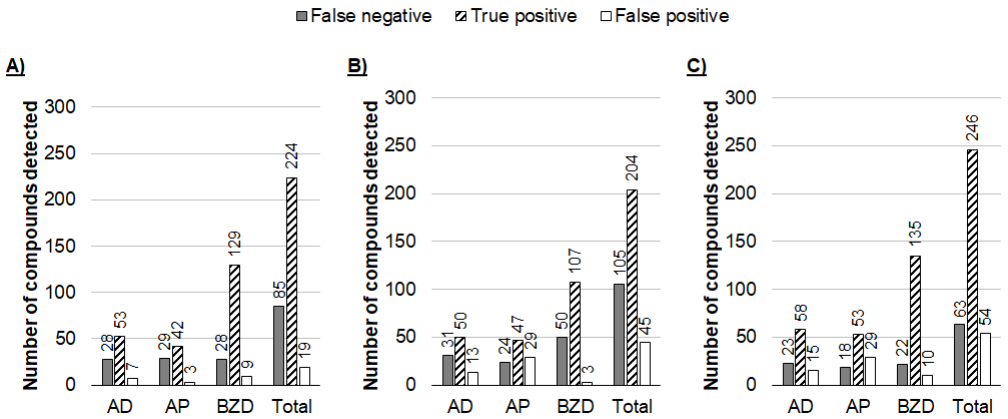

Figure 7 - 5. Validation of the QTOF screening using the optimised workflows. Spectra have been acquired in auto-MS/MS mode with exclusion of the reference masses. Using only the MassHunter Profinder software (A), less false positive or negative results were obtained compared to the MassHunter Qualitative Analysis software (B). The best results were obtained when all analytes detected in at least one workflow were included (C). The higher amount of false positives could be significantly reduced when taking into account a systematic bias for prothipendyl.

7.3. VALIDATION SEMI-QUANTITATIVE SCREENING

Although the Agilent MassHunter software offers a Quantitative module similar to that for QQQ instruments, data should ideally be acquired in targeted mode in order to acquire sufficient data points for quantification purposes. Generally, a minimum of 10 data points is recommended for reproducible quantification. For multi-analyte methods where the chance of co-elution or other interferences increases, the number of recommended data points is 20. The untargeted data files as obtained with the here presented method were not compatible with the quantitative software, nor were enough data points acquired over each peak (on average around 7 data points for CAL L5). However, to estimate the potential for a future semi-quantitative application, calibration curves for each of the analytes with a labelled analogue present in the ISTD mix were drafted using the Excel spreadsheet software (Microsoft, Redmond, Washington, US). Calibration samples had been injected with every batch and consisted of blank horse serum spiked at the concentrations defined in Table S6 - 1 p. 129. As peak areas were required rather than peak heights, only compounds that were identified using the Profinder workflow were included, and their calculated concentrations compared to those obtained using targeted QQQ analysis.

CAL curves consisted of seven levels for the ADs and the APs, six for the BZDs. The data points were based upon the average of five different samples per concentration level, except for those few cases where the analyte had not been acquired or had been wrongfully identified in a sample. From a practical point of view, only linear trend lines were fitted to the data points (Figure 7 - 6). The highest calibration levels for diazepam and trazodone significantly skewed the results and therefore were not included. Zopiclone could not reliably be identified in the majority of calibration samples and was omitted from further investigation. As for the tMRM methods, concentrations within ± 30% of those calculated by dMRM (considered the true value) were deemed acceptable (see section 6.3.1 p 120).

Citalopram, melitracen, mirtazapine and trazodone were the ADs under investigation. Out of 14 citalopram positive samples, 7 corresponded well in concentration with those found by dMRM. For a further five samples, the QTOF analysis overestimated the concentration, with accuracies ranging from 136% to 173%. The calculated concentrations of the last two samples were more than 30% lower compared to dMRM, although these deviations were negligible due to the low amounts of analyte present (8 ng/mL vs. 15 ng/mL and < 5 ng/mL vs. 7 ng/mL, respectively QTOF vs. dMRM analysis). Only three samples were positive for melitracen, all but one of which had markedly reduced accuracies (on average 49% compared to dMRM). For mirtazapine, two samples returned a positive result. The first had an accuracy of 105%, the second deviated more but would have likely been interpreted similarly regardless (calculated concentration < 2 ng/mL vs. 11 ng/mL). Overall acceptable results were also found for trazodone. Out of 9 positive samples, 5 were accurately quantified. A further two had calculated concentrations below the LLOQ of 25 ng/mL, whereas dMRM analysis revealed these to be around CAL L2 (50 ng/mL). Larger, and inconsistent, deviations of -66% and +82% were found for two samples, both with a concentration around 85 ng/mL.

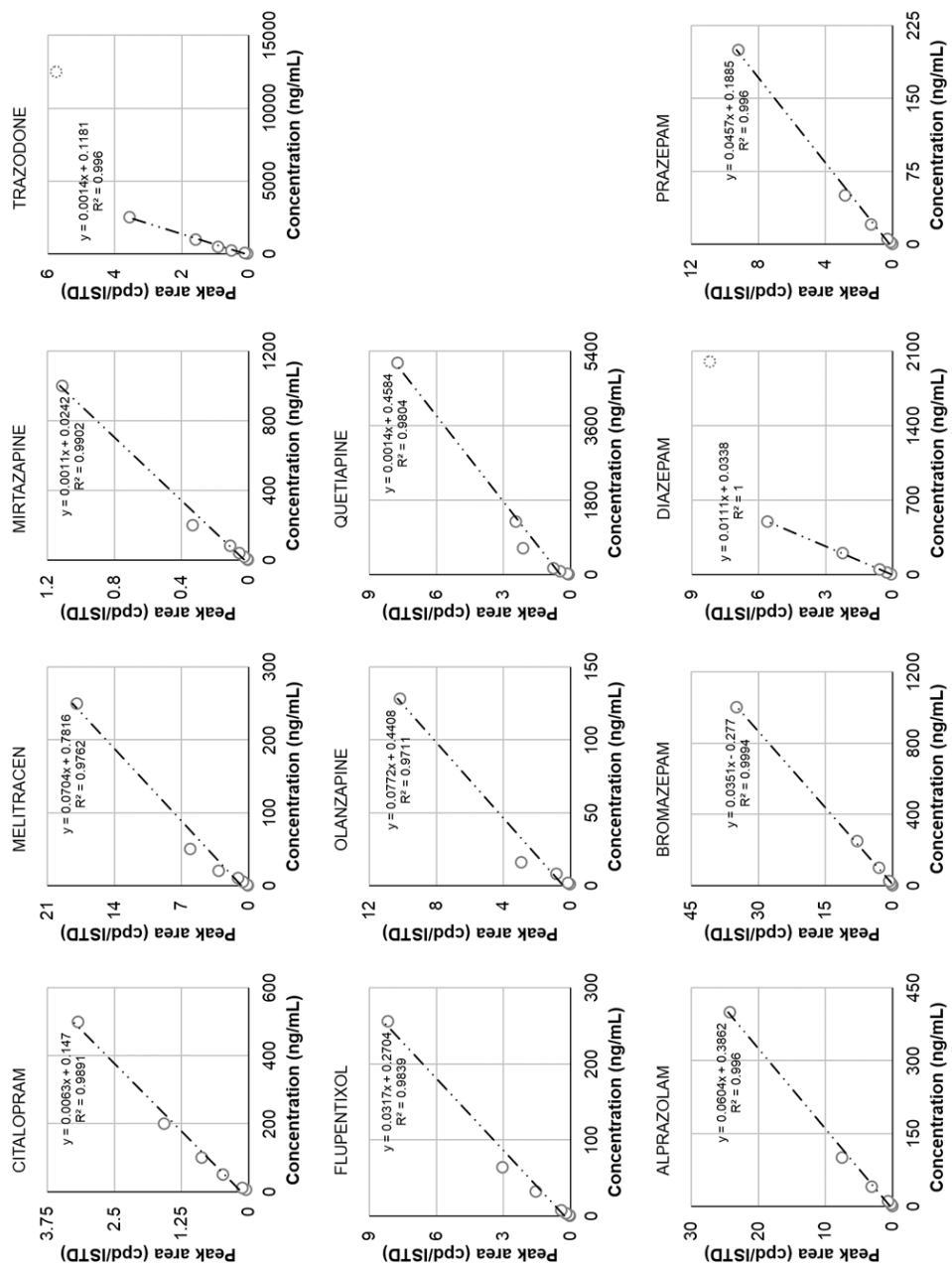

Figure 7 - 6. Excel calibration curves for the semi-quantitative analysis of selected antidepressants (ADs, left), antipsychotics (APs, middle) and benzodiazepines (BZDs, right). The highest calibrator had to be dropped for trazodone and diazepam. The most reliable results (compared to dMRM findings) were for the BZDs, followed by the ADs and the APs.

For the APs, the investigated analytes were flupentixol, olanzapine and quetiapine. In contrast to what had been observed for tMRM-based semi-quantification (Table S6 - 2 p. 132), all three analytes would likely benefit from quadratic curve fittings rather than linear ones. This was reflected in the

calculated concentrations, which differed significantly from those found with dMRM. Flupentixol and olanzapine were both identified in one sample only, at respective concentrations < 0.5 ng/mL (vs. 5 ng/mL by dMRM) and 41 ng/mL (vs. 97 ng/mL). However, neither findings are likely to influence the interpretation of the result in a forensic setting. Quetiapine had been identified in 11 samples, only three of which fell within the ± 30% deviation criterion, all at high concentrations. Three more samples with dMRM concentrations 25 ng/mL, 49 ng/mL and 85 ng/mL were classified as < LLOQ (10 ng/mL), which might have influenced the case interpretation. The concentrations of the remaining samples (range 243 ng/mL – 1099 ng/mL) were increased with around 60%, none of which were significant for interpretative purposes.

As had been noted for the tMRM method, semi-quantification of the BZDs was markedly better than for the other classes. Here, alprazolam, bromazepam, diazepam and prazepam were investigated. Of the alprazolam positive cases (n = 19), 60% had an acceptable accuracy (on average 82%). Two low-concentrated samples (6 ng/mL and 7 ng/mL according to dMRM) were wrongfully classified as < 1 ng/mL. The remaining samples had on average 40% lower concentrations compared to targeted QQQ analysis, none of which are expected to impact on their interpretation in each of the respective cases as a whole. For bromazepam, 75% of cases had acceptable semi-quantitative results. Other samples showed minor exceedances from the ± 30% criterion (on average -39%), with the exception of two near-LLOQ (2.5 ng/mL) samples, which showed a two- to three-fold increased concentration. Although the highest calibrator had to be omitted from the diazepam calibration curve, none of the samples showed unacceptable deviations from the dMRM-calculated concentration. Based upon 25 samples, the average accuracy was 93%. Lastly, prazepam showed a non-significant reduction in concentration from 7 ng/mL (dMRM) to 4 ng/mL (QTOF) in its only positive sample.

7.4. OVERALL PERFORMANCE OF THE QTOF METHOD

Under ideal circumstances, quadrupole time-of-flight based mass spectrometers would scan all ions over a broad range of m/z-values followed by fragmentation of every single ion that has been picked up. Such DIA applications have been described in literature and offer the advantage of full retrospective analysis, including fragmentation spectra, for any potential compound [435,437,443–445]. However, acquired spectra are often composed of fragments ions from multiple precursors and data analysis relies strongly on deconvolution of spectra. DDA methods may simplify post-analytical data processing and reduce the turn-around time per sample by selective acquisition of ions above certain thresholds only [446,447]. Based upon previous experience at the Toxicological Centre of the University of Antwerp, it was opted to work with an untargeted DDA method, whereby any ions in the mass range m/z 50 – m/z 950 and an abundance > 2000 counts were fragmented using collision energies 10 eV, 20 eV and 40 eV. Acquired features were compared to an in-house database containing the exact masses, RTs and fragmentation spectra of 132 psychoactive substances. Both the Agilent MassHunter Profinder software 10.0 and the Agilent MassHunter Qualitative Analysis software 10.0 were used for this purpose, and the results from both were combined into a positive identification list. Interference by matrix compounds could be reduced by filtering the results for a match score ≥ 50, co-elution with subsequently injected calibration samples, and a peak height/area greater than that of the LLOQ sample run in the same batch. Purine and hexakis phosphazine were

continuously infused for in-run mass recalibration, and their presence throughout each acquired spectrum served as quality control (QC) within each batch. Additional QC criteria were the positive identification of all 12 ISTDs in each of the samples and – although not mandatory – the presence of caffeine in biological samples.

The final workflow was tested against archived medico-legal blood samples (n = 105) analysed in parallel by validated LC-QQQ methods. Out of 432 compounds, QTOF analysis correctly identified 346 (80%). As had been seen for the tMRM method, the majority of false negative results are for compounds at low to near-LLOQ concentrations. If known, the lowest calibration levels for each of the analytes had been chosen to represent those concentrations typically seen from subtherapeutic doses in patients/ante-mortem samples. If these samples would have been analysed in the framework of therapeutic drug monitoring (TDM), it is generally known which drugs should be present. Failure to detect these in the blood of a patient may indicate either a sub-optimal dosing scheme or poor therapy compliance, both of which should be further investigated using more targeted analyses. If the samples would have been analysed for forensic purposes, the importance of the missed result would have been determined by all analytes detected, as well as the case circumstances, medical history of the person involved, etc. [416,445–447]. ADs and APs are carefully dosed to the individual's need and are usually only of concern in significant overdoses. BZDs may interact with other central nervous system depressant drugs in which case even low concentrations could potentially be of harm. For the majority of false negative results, the precursor or metabolically related compounds had been detected. A number of analytes have systematically been missed, regardless of their concentration in the sample. The trazodone metabolite mCPP could not be detected in 11 out of 16 positive cases, but its precursor could. Similar findings were observed for the metabolites 7-OH-norquetiapine, norolanzapine and α-OH-alprazolam. As their parent compounds had been detected, these false negative results would have had only minor implications on the respective cases as a whole. Of more concern are the false negative results for amisulpride, fluoxetine and its metabolite, and mirtazapine and normirtazapine. These analytes were missed in most to all positive cases by both the MassHunter Profinder or MassHunter Qualitative Analysis workflow. Amisulpride, mirtazapine and normirtazapine were filtered out of the positives list due to their generally poor match scores (< 50) and peak signals lower than those of the LLOQs, although a signal had been detected. The fluoxetine and norfluoxetine ions had not been acquired in any of the calibrators or case samples and will need looking into.

A further 83 false positive hits were found, 49 of which could be attributed to a systematic bias for (nor)maprotiline, phenelzine, prothipendyl or reboxetine. The ADs maprotiline and normaprotiline share their exact mass and RT with amitriptyline and nortriptyline, respectively, which could be identified in those same cases. Targeted analysis could distinguish between the analytes based upon the m/z-values of and ratios between the fragment ions. However, their untargeted fragmentation spectra show some degree of overlap (Figure 7 - 7). The presumed prothipendyl peak eluted at an average RT difference of -0.18 min compared to the CALs and therefore this error could be solved by applying a more strict RT inclusion criterion for this compound. Due to known extraction issues for phenelzine, no CALs were run for this compound to compare the RT or LLOQ signal to [416]. The nature of the reboxetine interference is currently unknown. A further nine false positive results were for analytes where all transitions could be picked up with targeted QQQ analysis, but which

were excluded from the final dataset as their estimated concentration fell below the LLOQ, which coincidentally was also set as the LOD. Diazepam, nordazepam and oxazepam resulted in a false positive hit in the same sample and, although dMRM analysis did not pick up on any of these analytes in the sample, therefore shed reasonable doubt on their alleged absence.

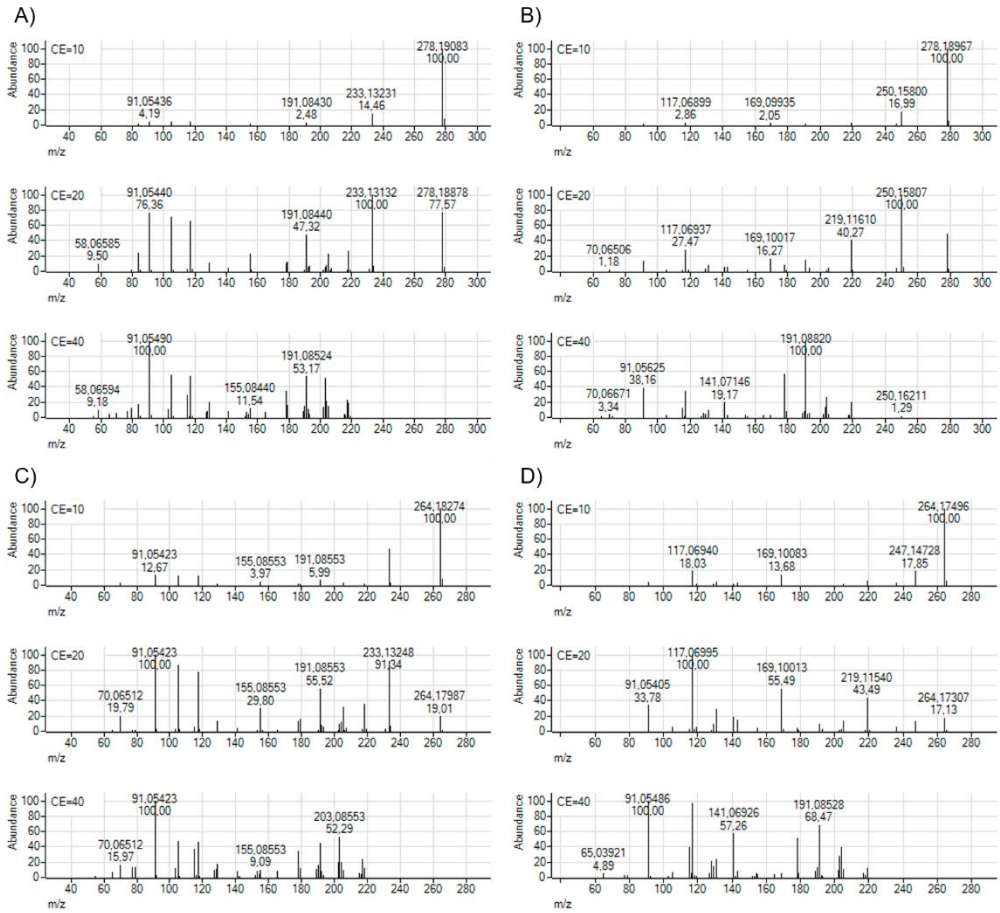

Figure 7 - 7. High resolution database entries for A) amitriptyline, B) maprotiline, C) nortriptyline and D) normaprotiline. A combination of highly similar exact masses, retention times and similar fragmentation spectra resulted in false negative hits for maprotiline and normaprotiline in samples positive for amitriptyline and nortriptyline, respectively.

Semi-quantification using the MassHunter Quantitative Analysis software proved more difficult, as this required data to be acquired in targeted mode. Nonetheless, an estimation of an analyte's concentration in the sample may already provide the necessary information for case interpretation, without the need for accurate quantification of all results and thereby the unnecessary prolongation of turn-around times or use of expensive, multi-analyte calibration mixes. Further research into an alternative quantitative software is needed, but a first investigation of the semi-quantitative power of the method was performed for selected compounds using Microsoft Excel. Each of the selected analytes had their labelled analogue present in the ISTD mix and linear curves could be drawn for all of them. An accuracy of ± 30% compared to the dMRM concentration was deemed acceptable. On

the other hand, forensic cases (especially post-mortem ones) often only require an estimated concentration as their interpretation should always be formulated in line with the medical history and other drugs present. Moreover, the influence on analyte levels by e.g. the post-mortem interval, post-mortem redistribution and microbial metabolism has been widely documented, in which case greater deviations in accuracy may be allowed [36,37,448]. The best results were obtained for the BZDs, where the order of magnitude corresponded well with that of dMRM analysis for all four compounds (alprazolam, bromazepam, diazepam and prazepam) in all positive samples (Figure S7 - 3). The ADs showed greater variations in their accuracies, and particular care should be taken when interpreting trazodone concentrations. Lastly the APs experienced the largest deviations and semi-quantitative analysis might require further investigation, in part focussing on the use of quadratic calibration curves. Additional improvements may arise from the inclusion of more or different ISTDs, as well as a different gradient spreading their elution out over the entire RT range. Indeed, it has been shown that ionisation efficiency can vary based upon mobile phase composition, with a lower pH and a higher percentage organic solvent being advantageous for positive ionisation modes [426,431]. This may explain the worse results for the APs, which all tend to elute concentrated together from the column (RT span of 3.5 min) at higher mobile phase A percentages than the ADs and BZDs.

7.5. SUPPLEMENTARY INFORMATION FOR CHAPTER 7

7.5.1. Tables

Table S7 - 1. Selected compounds detected in case samples and their (semi-)quantitative concentrations. All concentrations are expressed in ng/mL. Accuracies could not be calculated for values below the lower or above the upper limit of quantification.

Compound	dMRM conc.	QTOF conc.	Accuracy
	ANTIDEPRESSANTS		
citalopram	7	< 5	
citalopram	8	8	95%
citalopram	15	8	54%
citalopram	16	22	143%
citalopram	32	37	116%
citalopram	34	42	123%
citalopram	40	50	124%
citalopram	47	77	162%
citalopram	81	70	87%
citalopram	84	125	148%
citalopram	84	91	108%
citalopram	132	227	173%
citalopram	183	250	136%
citalopram	219	271	124%
melitracen	11	2	20%
melitracen	64	47	73%
melitracen	194	102	53%
mirtazapine	11	< 2	
mirtazapine	76	79	105%
trazodone	56	< 25	
trazodone	61	< 25	
trazodone	85	29	34%
trazodone	85	155	182%
trazodone	161	116	72%
trazodone	224	194	87%
trazodone	255	232	91%
trazodone	296	297	100%
trazodone	586	570	97%
	ANTIPSYCHOTICS		
flupentixol	5	< 0.5	
olanzapine	97	41	43%
quetiapine	26	< 10	
quetiapine	49	< 10	
quetiapine	85	< 10	
quetiapine	243	383	157%
quetiapine	298	469	158%
quetiapine	482	873	181%
quetiapine	503	359	71%
quetiapine	1099	1664	151%
quetiapine	1154	1350	117%
quetiapine	4368	2254	52%
quetiapine	> 5120	> 5120	
	BENZODIAZEPINES & Z-DRUGS		
alprazolam	6	< 1	

Table S7 - 1. Selected compounds detected in case samples and their (semi-)quantitative concentrations. (continued)
All concentrations are expressed in ng/mL. Accuracies could not be calculated for values below the lower or above the upper limit of quantification.

Compound	dMRM conc.	QTOF conc.	Accuracy
alprazolam	7	< 1	
alprazolam	18	12	69%
alprazolam	19	10	52%
alprazolam	20	14	72%
alprazolam	24	19	76%
alprazolam	25	15	61%
alprazolam	26	15	59%
alprazolam	32	26	81%
alprazolam	33	33	99%
alprazolam	36	30	84%
alprazolam	36	19	51%
alprazolam	48	38	79%
alprazolam	60	39	66%
alprazolam	60	54	89%
alprazolam	66	42	64%
alprazolam	77	66	85%
alprazolam	152	125	82%
alprazolam	156	129	83%
bromazepam	3	10	345%
bromazepam	4	11	238%
bromazepam	25	24	94%
bromazepam	30	31	102%
bromazepam	38	39	103%
bromazepam	38	35	91%
bromazepam	65	65	100%
bromazepam	66	58	88%
bromazepam	68	46	67%
bromazepam	71	61	86%
bromazepam	86	70	81%
bromazepam	99	86	87%
bromazepam	114	79	69%
bromazepam	116	110	95%
bromazepam	142	119	84%
bromazepam	252	155	62%
bromazepam	279	209	75%
bromazepam	366	256	70%
bromazepam	380	212	56%
bromazepam	481	368	76%
bromazepam	904	713	79%
diazepam	5	< 5	
diazepam	7	5	78%
diazepam	7	< 5	
diazepam	8	6	73%
diazepam	11	12	110%
diazepam	14	14	102%
diazepam	24	20	82%
diazepam	26	24	90%
diazepam	31	29	92%
diazepam	39	39	99%
diazepam	49	44	90%

Table S7 - 1. Selected compounds detected in case samples and their (semi-)quantitative concentrations. (continued)
All concentrations are expressed in ng/mL. Accuracies could not be calculated for values below the lower or above the upper limit of quantification.

Compound	dMRM conc.	QTOF conc.	Accuracy
diazepam	60	60	100%
diazepam	66	65	98%
diazepam	78	83	107%
diazepam	82	99	120%
diazepam	85	77	90%
diazepam	86	65	76%
diazepam	89	110	124%
diazepam	101	82	82%
diazepam	122	115	94%
diazepam	176	175	99%
diazepam	189	162	85%
diazepam	207	188	91%
diazepam	374	264	71%
diazepam	1013	> 500	
prazepam	7	4	67%

7.5.2. Figures

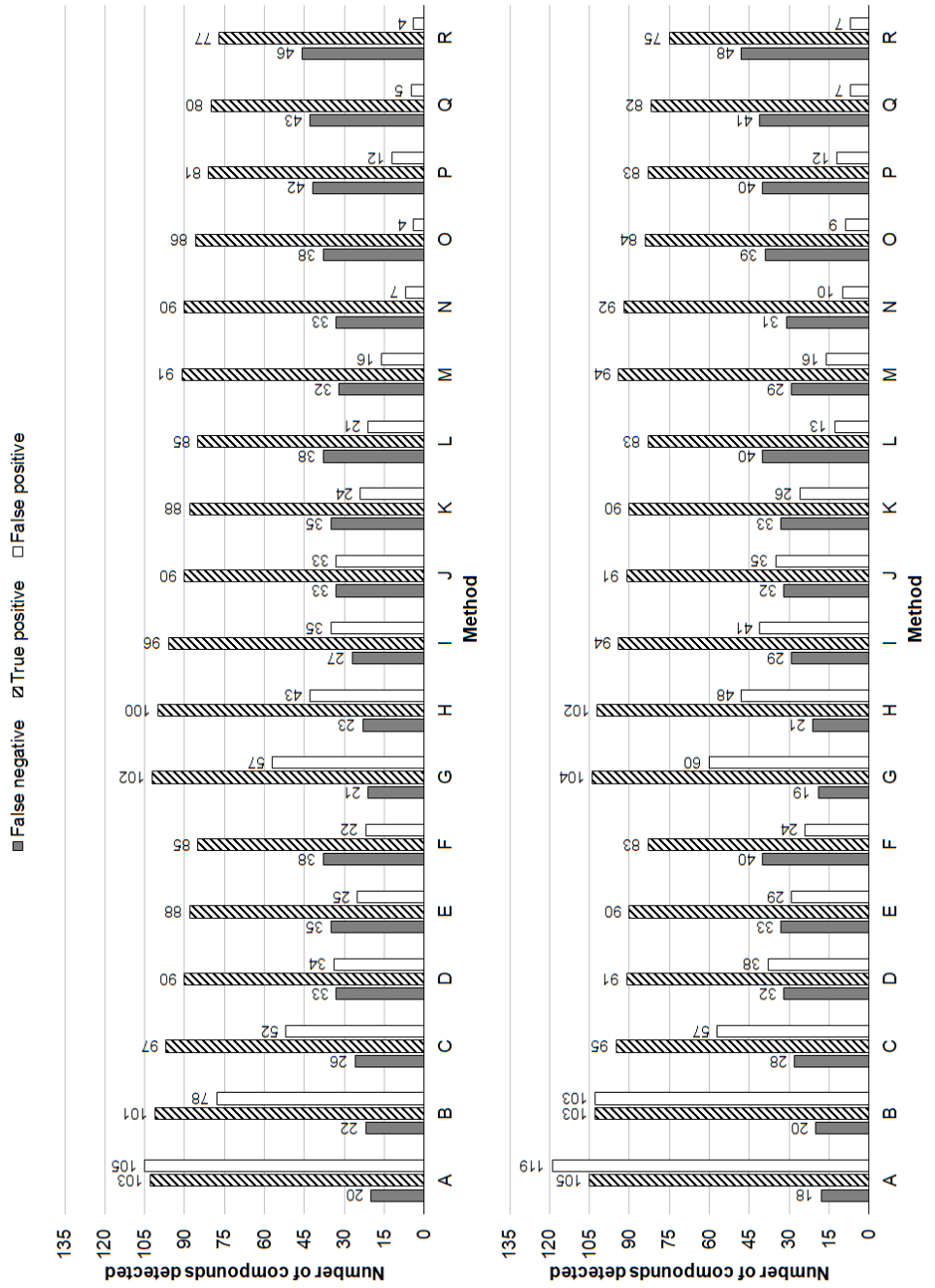

Figure S7 - 1. Results of the QTOF screening using the MassHunter Profinder software. Spectra were acquired in auto-MS/MS mode using a preferred list (left) or by exclusion of the reference masses (right). The latter resulted in greater numbers of true positive identifications. The number of false positive results could be reduced by applying thresholds on the peak area and RT window. Table 7 - 1 gives an overview of the different thresholds set in each of the methods (A-N).

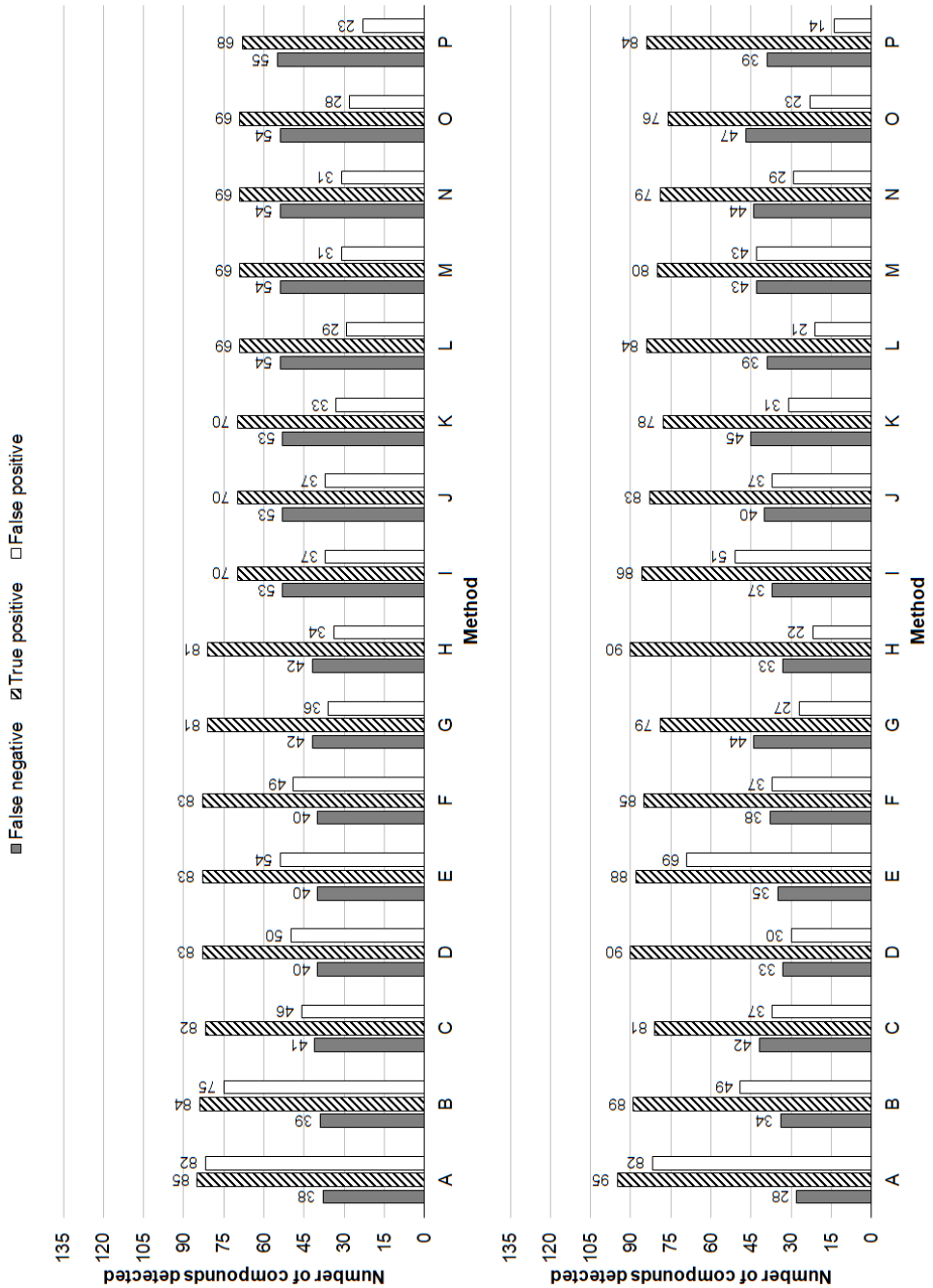

Figure S7 - 2. Results of the QTOF screening using the MassHunter Qualitative Analysis software. Spectra were acquired in auto-MS/MS mode using a preferred list (left) or by exclusion of the reference masses (right). The latter resulted in greater numbers of true positive identifications. The number of false positive results could be reduced by applying thresholds on the peak area and RT window. Table 7 - 2 gives an overview of the different thresholds set in each of the methods (A-P).

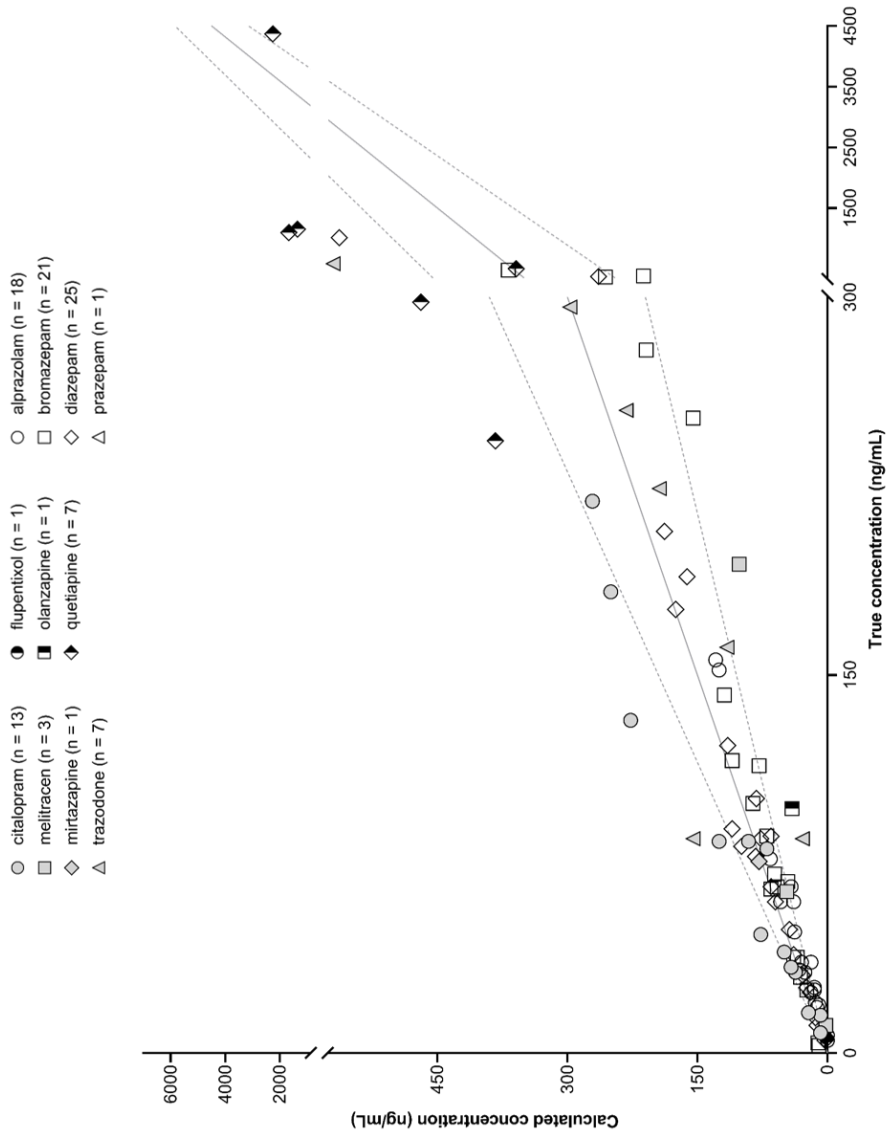

Figure S7 - 3. Semi-quantitative concentrations of selected compounds as determined by QTOF screening. The true concentration was obtained from validated confirmation methods. The dotted lines represent a spread of ± 30% of the accuracy compared to the true concentration (full line). The concentrations of compounds above the upper limit of quantification could not be accurately determined and therefore are not plotted.

CHAPTER 8

GENERAL DISCUSSION

8.1. SCREENING VS. CONFIRMATION METHODS

8.1.1. Systematic toxicological analysis

With an estimated 8000+ relevant substances, toxicological laboratories must rely heavily on screening methods in their investigations, to exclude the involvement of the majority of compounds, while accurately detecting (and quantifying) the ones that are present [70,79]. Systematic toxicological analysis (STA), sometimes also called general unknown screening, is the first step in any toxicological workflow and refers to all methods that allow for the detection of a large range of relevant compounds in biological or other case-related samples [39,68,449]. Additionally, these methods should ideally be rapid, easy to perform, flexible, available around the clock and – most importantly – straightforward, comprehensive and specific in their outcome results [450,451]. Other factors to consider are cost per analysis, workload per sample and degree of experience required for analysts. Immunoassays are commonly used as an initial screening tool but are restricted in the number of compounds that can be identified with one test. The antigen-antibody binding also suffers from an inherent biological variability in its efficiency as well as cross-reactivity between structurally related compounds. Although the latter can be an advantage when screening for unknowns, it does not comply with the specificity requirements of true STA [39,52,452]. A variation on immunoassay screening was developed by Cannaert et al., who could detect a drug's pharmacological effect based upon receptor binding and subsequent activation [453].

Gas chromatography (GC) coupled to mass spectrometry (MS) tends to be one of the methods of choice for STA due to its high-resolution separation on capillary columns, combined with high selectivity and sensitivity of the MS detection [449,454]. Such methods are robust, moderate in cost and, due to the standardised electron impact ionisation energy of 70 eV, allow for inter-laboratory exchange of highly reproducible reference mass spectra [39,86]. Currently, multi-analyte databases containing several thousand spectra are commercially available [455–457]. The introduction of the user-friendly AMDIS software (Automated Mass Spectral Deconvolution and Identification System), for purification and matching of complex spectra, has greatly improved their significance [68,69,458]. A study by Meyer et al. compared the AMDIS software against manual identification by an experienced toxicologist, recommending it for its superior identification results. However, they did warn that it was inherently limited by the number of database entries, potentially missing highly abundant or relevant peaks [459]. The major disadvantage of GC-MS based screening lies in the poor detector sensitivity for polar, non-volatile and thermally labile compounds, often requiring lengthy derivatisation steps which can in turn introduce variability to the results [39,70,449,460]. Therefore, it is often recommended to combine GC-MS screening with at least one other method, based upon a different physical or chemical principle, for unambiguous identification of a substance [461].

Liquid chromatography (LC) offers a wider flexibility through variations in the mobile and stationary phases, and a more general applicability [451]. It can be readily coupled with diode array detectors (DADs), which benefit from the availability of spectral libraries, a long-term reproducible concentration-absorption relationship, low cost and easy handling [462–465]. Due to the non-destructive nature of the detector, LC-DAD instruments can be coupled with MS detection, although the type of mobile phase needs careful consideration as e.g. formic acid based mobile phases may cause high noise levels at wavelengths below 240 nm [454]. As mentioned for immuno-assays, cross-

reactivity between structurally similar compounds may occur with DAD detectors (if the difference is not located in a group directly conjugated with the chromophore), reducing its specificity yet at the same time allowing for the detection of metabolites or related substances not present in a library [449,454]. LC-MS techniques have long been overlooked for STA applications given the inability to generate reproducible spectra between instruments and laboratories and the resulting absence of large, commercially available reference libraries [449,466]. In the last two decades, advancements in the technology, aided by better post-analytical algorithms, have improved spectrum robustness and matching between laboratories with the same instruments and between instruments of the same vendor [449,467–469]. To increase confidence in the findings, fragment ions could be generated either in-source by applying multiple fragmentor voltages (for MS instruments) or in a specifically designed collision cell by varying the collision energy (for MS/MS instruments) [68,470,471]. It should be noted that the relative intensity and nature of the fragments remains less reproducible between vendors [469].

Limited targeted LC-MS/MS screening assays have been developed. Dresen et al. describe a multi-analyte method for the identification of 700 drugs and metabolites using LC coupled to a hybrid triple quadrupole – ion trap mass spectrometer (QTRAP). Their sample preparation was a dilute-and-shoot method using urine as a matrix [472]. Similarly, Viette et al. developed a LC-QTRAP method for the detection of 300 substances in serum samples. Extraction of the analytes of interest was achieved by solid-phase extraction (SPE) [473]. Gergov et al. published a qualitative screening method for 238 analytes in blood. The sample preparation consisted of liquid-liquid extraction (LLE), followed by LC-triple quadrupole (QQQ) analysis [474]. In all methods, the instruments were operated in positive ionisation mode and used targeted, multiple reaction monitoring (MRM) mode for data acquisition. The triggered MRM (tMRM) method described in Chapter 6 (p. 109) sought to improve on these methods. The total run time was reduced from around 20 min to 12 min, increasing sample throughput. The LLE further reduced turn-around times (vs. SPE by Viette et al.) and used only 200 µL sample (vs. 1 mL for Gregov et al. and Viette et al.). Lastly, confidence in the findings was increased significantly by the acquisition of 3 rather than 1 MRM transition (vs. Dresen et al. and Gregov et al.). Currently set up for 130 psychoactive substances and metabolites, its simple sample preparation and LC parameters allow for easy and fast incorporation of other drug classes, as illustrated by unpublished experience with fentanyl analogues and antiarrhythmic drugs. Sensitivity and specificity might need to be re-evaluated if more targets are added.

A drawback of targeted methods in STA is their limitation to a priori known compounds only, which can be overcome with high-resolution mass spectrometric (HRMS) applications using quadrupole time-of-flight (QTOF) mass analysers [458,470,475]. Both data-independent (DIA) and data-dependent acquisition (DDA) methods have been studied, as both allow for true unknown screening and – if needed – retrospective data-mining [476]. DDA screening suffers from limited numbers and types of ions that will be fragmented, potentially excluding relevant but low intensity ions. Iterative injections with automatically generated exclusion lists may increase the sensitivity of the instrument but also the sample's turn-around time [477,478]. Therefore, DIA methods which fragment all ions are said to have lower limits of detection [39,435]. However, DIA suffers from complicated data interpretation as fragments from co-eluting analytes/endogenous compounds will make up the same spectrum [476]. Identification of acquired analytes is based upon their accurate mass, isotope pattern

and relative isotope abundance [88,479]. Reference libraries for compound identification can easily be created by importing molecular formulas from which the accurate mass and isotope pattern can be calculated, leading to tentative identifications without the need for certified reference standards [39]. Such approach could also lead to false positive results, predominantly for isobaric and isomeric compounds, and unequivocal identification requires analyte-specific parameters, such as RT (with full separation of isobars/isomers) and fragmentation pattern [480–483]. *In silico* fragmentation tools are available and – for some vendors – built into the database software, although their reliability is still debatable [458,484–486]. Schymanski et al. proposed a five-level system for confidence in HRMS findings (Figure 8 - 1), also concluding that for the highest level of confidence (level 1 – confirmed structure) knowledge of the RT and fragment ions is mandatory [487].

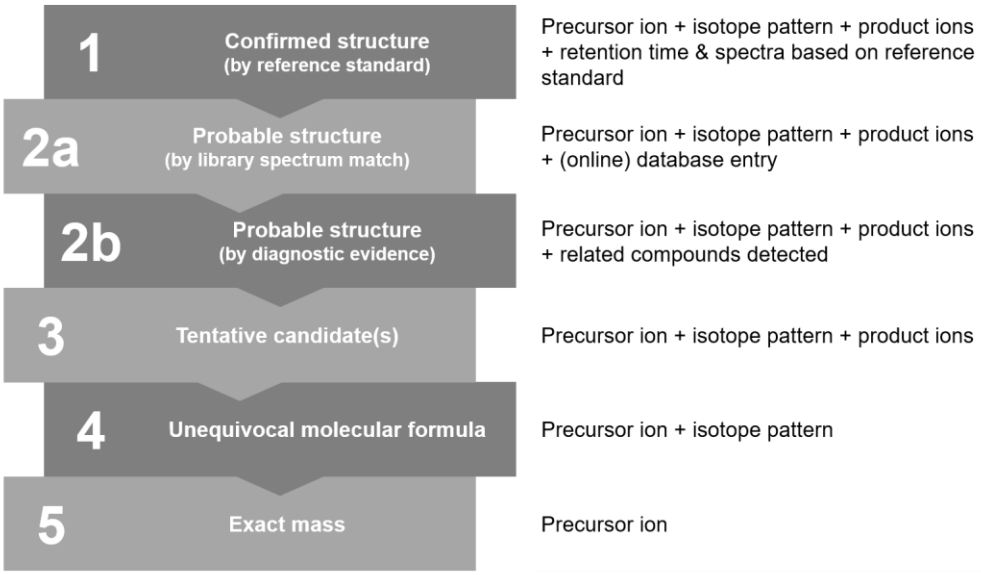

Figure 8 - 1. Confidence levels for high-resolution mass spectrometric analyte identification. The highest confidence (level 1) can only be reached in the presence of a reference standard. For screening purposes, levels 2a (probable identification based upon in-house or publically available MS/MS-libraries) and 2b (probable identification based upon presence of metabolites and other related compounds in the same sample) may suffice. The system was proposed by Schymanski et al. [487].

Table 8 - 1 provides an overview of untargeted HRMS methods using blood as a matrix, that have been published in the past two decades. DIA methods are more commonly used. This could potentially be explained by the complex composition of blood as a matrix, giving rise to a higher and more variable background signal, therefore excluding relevant low-concentrated analytes from DDA-based fragmentation [449]. Our own in-house experience had seen a considerable increase in turn-around time for DIA data analysis, thus in Chapter 7 (p. 147) it was opted to investigate the suitability of a DDA method, whilst also considering its applicability for routine use [438]. Compared to other published DDA methods, its strong point is the sample preparation. Decaestecker et al. and Oberacher et al. both used time-consuming SPE methods and required 1 mL and 2 mL of sample for their analyses, respectively [488,489]. Broecker et al. and Partridge et al. used the more simple LLE but still required 500 µL blood [490,491]. We managed to reduce the sample volume to 200 µL

blood, whilst still achieving a sensitivity in the sub- to low therapeutic range for the majority of analytes. Additionally, the sample preparation is fully compatible with the semi-quantitative tMRM screening and – if no quantification is needed – targeted LC-QQQ confirmation methods. Especially for blood samples with their usually limited available volume, analysis on multiple instruments without the need for re-extraction seems highly advantageous. The excellent stability of all analytes for up to three days at ambient temperature after extraction is discussed in section 5.5.2 p. 78. The turn-around time was further improved compared to other published methods by reducing the total run time from around 21 min on average to 12 min only. Limited by the availability of certified reference standards, the in-house database contained 144 entries (each with 3 fragmentation spectra) only. However, if needed it can easily be combined with commercially available ones or populated with other compounds (see section 8.2.2).

Table 8 - 1. Selected publications on untargeted, high-resolution mass spectrometric methods. All methods were developed for the qualitative and/or semi-quantitative analysis of blood on LC-(Q)TOF instruments. The assigned confidence levels follow the recommendations by Schymanski et al. [487]. Data-dependent acquisition, DDA; data-independent acquisition, DIA; mass spectrometry, MS; not applicable, N/A; (quadrupole) time-of-flight, (Q)TOF.

Acquisition		Data analysis		Quantification	Article
MS	**DDA/DIA**	**n° analytes**	**Confidence**		
TOF	N/A[a]	175	L3	no	Dalsgaard et al., 2012 [492]
TOF	N/A[a]	227	L3	no	Sundström et al., 2013 [445]
TOF	N/A[a]	50500	L4	no	Polettini et al., 2008 [483]
QTOF	**DDA**	**144**	**L1**	**yes[d]**	**This thesis**
QTOF	DDA	> 300	L1	yes	Decaestecker et al., 2004 [488]
QTOF	DDA	320	L1	yes[d]	Partridge et al., 2018 [491]
QTOF	DDA	1208	L2a	no	Oberacher et al., 2013 [489]
QTOF	DDA	> 7500	L1 - L2a[c]	no	Broecker et al., 2010 [490]
QTOF	DIA	30	L1	no	Dalsgaard et al., 2013 [493]
QTOF	DIA	37	L1	yes	Pasin et al., 2015 [494]
QTOF	DIA	39	L1	yes	Roemmelt et al., 2015 [495]
TOF	DIA	151	L1	no	Teng et al., 2015 [496]
QTOF	DIA	> 185	L2a	yes[d]	Bidny et al., 2017 [497]
QTOF	DIA	> 250	L1	no	Krotulski et al., 2020 [498]
QTOF	DIA	256	L1	no	Pedersen et al., 2013 [493]
QTOF	DIA	> 950	L1	yes[d]	Rosano et al., 2014 [499]
QTOF	DIA	1353	L1	no	Grapp et al., 2018 [458]
QTOF	DIA	1500	L1 - L2a[c]	no	Kinyua et al., 2015 [438]
TOF	DIA	> 2000	L2a	no	Lung et al., 2016 [500]
QTOF	Both[b]	82	L1	no	Marin et al., 2015 [501]
QTOF	Both[b]	1208	L2a	no	Arnhard et al., 2015 [39]
QTOF	Both[b]	1326	L2a	no	Roemmelt et al., 2014 [437]

[a] Fragmentation ions were not acquired. [b] Both acquisition methods were compared with each other. [c] Retention times needed for confidence level 1 were available for selected analytes only. [d] Quantification was investigated for selected analytes only.

8.1.2. Confirmation of findings

Confirmation methods aim to indicate the presence of an analyte 'beyond any reasonable doubt', meaning they can distinguish it from all known interferences in the relevant matrix. At least two different analytical techniques should be used for a substance to be reported as positive in a sample [37]. Additionally, during each analysis the substance must exhibit identical physical and chemical behaviour to that of its corresponding reference substance [79,502]. As mentioned before, RT and fragmentation pattern are mandatory requirements for unequivocal analyte identification. For

confirmation purposes, it is recommended to monitor between two and four transitions, preferably with a relative intensity ≥ 5% compared to the most abundant one (100%). Product ions resulting from a non-specific neutral loss (e.g. H_2O, NH_3) or solvent adducts should ideally be avoided as well. Transitions representing common fragments for a class of compounds are useful for screening purposes but may limit unequivocal identification. Relative ion ratios can vary significantly based upon the analyte's concentration and day-to-day instrumental variability, and can be derived from calibrators run within the same batch [79,86,503]. Furthermore, stability of the analytes during all stages of the workflow, including storage conditions, should be known to rule out false negative or positive results. These could also arise from matrix-specific ion suppression or enhancement and carry-over following a high concentrated sample, all of which must be investigated [86,504,505].

Due to the often low concentrations of toxicologically relevant substances in samples, many STA findings are tentative or probable at best and require confirmation by means of more sensitive, multi-analyte, targeted methods [40,450]. Strict guidelines ensuring the highest reliability of these methods have been published by the European Medicines Agency (EMA), the Scientific Working Group for Forensic Toxicology and the United States Food and Drug Administration [368,378,506]. A more in-depth description of the method validation can be found Chapter 5 (p. 69). Each of the developed methods was fully validated according to the EMA guidelines and adhered to the above mentioned parameters: RTs were known and reproducible, three (or if not available two) fragment ions were monitored and their relative intensities verified for consistency with those of calibration samples, and matrix effects and carry-over were generally absent over the tested concentration range. Stability issues were described for some compounds in unextracted samples and should be taken into account [416,425,430]. When available as a reference standard, the major metabolites were also added to the methods as they can provide a strong indication of the potentially missed acquisition of a parent drug, depending on its pharmacokinetic profile [39,86,450]. In preserving the sample preparation and LC-QQQ parameters between the tMRM and dMRM methods, both screening and confirmation can be performed on the same instrument, avoiding column and mobile phase switching and re-equilibrating, and on the same samples, avoiding more time and (already limited) sample volume spent on re-extraction.

8.2. QUALITATIVE SCREENING METHODS

8.2.1. tMRM vs. QTOF

The ultimate goal for the development of both screening methods was their implementation in a routine toxicological setting. As such, the evaluation criteria for their performance should not only cover the analytes identified or not, but also the practicality of each step prior to and after instrumental analysis.

With the exception of immuno-assays, sample preparation is an essential first step in all analyses. It can range from simple, quick but unselective methods such as protein precipitation, to very stringent, highly selective but time-consuming SPE workflows [41]. Different sample preparation techniques were investigated in Chapter 4 (p. 59) and Chapter 7 (p. 147). Both the LC-QQQ and the LC-QTOF analyses showed the best results for the investigated compound of interest when using a LLE with methyl-tertiary-butyl-ether. The LLE provided sufficient extraction power and sample clean-up to

allow detection of analytes in the for toxicological purposes relevant low to sub-ng/mL concentration ranges. It further benefited from a low amount of sample volume used – of particular importance for the usually limited in volume blood samples – and minimal hands-on sample preparation time for analysts. Because the same sample preparation could be used for both types of analysis, samples could simply be transferred from one instrument to the other, increasing their throughput.

Once ready for analysis, liquid chromatography can be used to distribute analytes of interest over the retention time range and thereby avoid loss of sensitivity at the detector, as well as separate out highly similar or interfering substances in a sample to increase the specificity of the method. On both instruments, identical mobile and stationary phases and gradient elution were used. As discussed in Chapter 3 (p. 39), the finalised method was purposely kept simple for compatibility with a large number and variety of drugs. A gradient elution of less than 12 min allowed for fast turn-around times whilst still providing the necessary separation.

The difference between tMRM and QTOF methods lies in their data acquisition and post-acquisition workflow. tMRM mass spectrometry is a variation on dMRM acquisition. Once a dMRM method has been set up, it can be converted into a tMRM one without much effort. Particular care should be taken when deciding on an appropriate threshold for secondary ion triggering (see section 6.1.1.1 p. 111). An abundancy threshold of 200 counts provided the most reliable results in terms of reduction of false negative and false positive results. Data analysis could be performed using the MassHunter Quantitative Analysis 10.0 software, profiting from its user-friendliness, clear visualisation of quality control criteria (detection/absence of an analyte in samples, RT shift, …), easy filtration of results and vast experience of the analysts with the software. Additionally, if calibration samples have been run, a concentration can be calculated for each analyte and reports can be generated automatically. The untargeted nature of the QTOF-analysis – and previous experience/efforts from other PhD candidates at the Toxicological Centre – significantly reduced time spent on optimising the analytical method. Extraction and interpretation of the data requires more work than for targeted LC-QQQ analysis. Two different programs were investigated for the data analysis: the MassHunter Profinder 10.0 software and the MassHunter Qualitative Analysis 10.0 software (see section 7.1.2 p. 152). The former is straightforward in its use and requires little training of the analysts. Results can easily be sorted or filtered and different error flags can be set, visualising any problem instantaneously. The use of the MassHunter Qualitative Analysis 10.0 software requires more input and data transformation by the analysts. The process can be accelerated by exporting the extracted results to Microsoft Excel. In contrast to the MassHunter Profinder 10.0 software, it also takes the fragmentation pattern into account, increasing confidence in the identifications. However, the most reliable results were obtained when both data analysis workflows were combined, a for now laborious task requiring manual comparison of the findings for each sample individually.

Both the tMRM and QTOF methods had similar rates of false negative results (15% and 18% based upon 427 findings, respectively) but the latter resulted in higher numbers of false positive identifications (Figure 8 - 2). Half of the false negative results had not been identified by either method. Particular problems were seen for mCPP, where any sample with a concentration below ~40 ng/mL was consistently reported as negative, and norfluoxetine, which was missed regardless of its concentration. Additional problems were seen for the quetiapine metabolites norquetiapine (detection limit > ~15 ng/mL) and 7-OH-norquetiapine (detection limit > ~10 ng/mL for QTOF

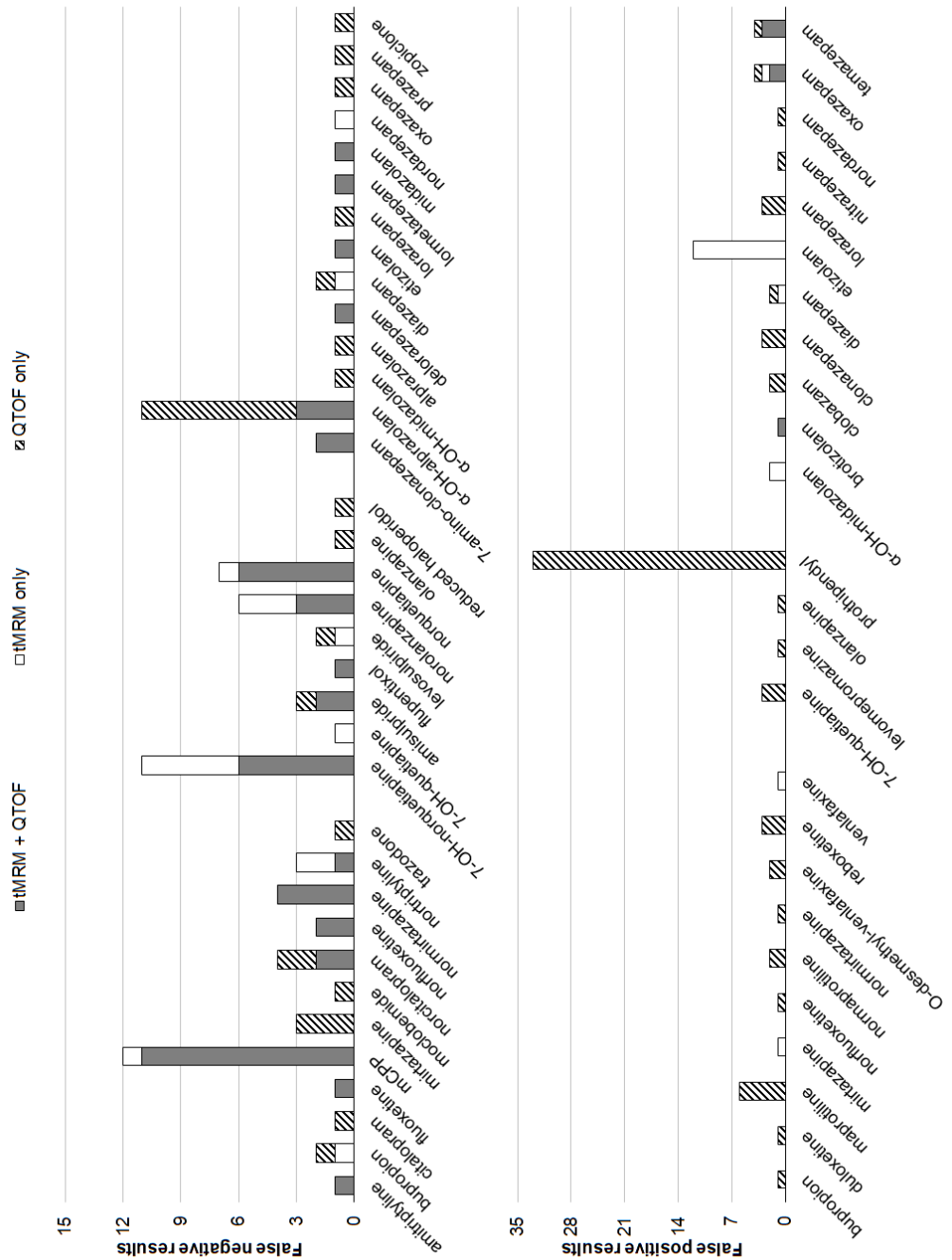

Figure 8 - 2. False negative (left) and false positive (right) findings for the screening methods. Results were compared to findings of targeted, validated LC-QQQ analyses. False negative results tended to represent mostly low-concentrated samples. False positive findings could be filtered out by the absence of metabolites in the samples.

screening, not detected by tMRM screening). Specifically for the tMRM workflow, nortriptyline tended to be missed at concentrations < 45 ng/mL and norolanzapine could not be identified regardless of its concentration. α-OH-alprazolam could not readily be identified by the QTOF workflow. All concentrations (n = 11) were lower than 5 ng/mL, thus it could not be determined if this was due to the limit of detection or a systematic error. Random misses distributed over the concentration range were seen for samples containing mirtazapine, citalopram and norcitalopram with the QTOF analysis. The importance of the missed analytes is difficult to evaluate out of the context of the case as a whole (see section 8.3.1). With the exception of norolanzapine, the above mentioned compounds all exhibit some activity and may have had an influence in that specific case. On the other hand, the concentrations of these false negative results were generally less than the respective L2 calibrator (CAL L2, Table S5 - 1 p. 85), with the lower calibrators chosen to reflect sub- to low therapeutic *in vivo* concentrations from which no relevant effect is expected, and related substances such as their metabolic precursors could be detected in those cases (e.g. identification of trazodone in cases where mCPP was not acquired).

False positive results could be categorised in three groups. On the one hand, there were those compounds that led to systematic wrong identifications. Such results were found for etizolam after tMRM analysis and for (nor)maprotiline and prothipendyl following QTOF analysis. No explanation is currently available for the etizolam false positives. Maprotiline and normaprotiline hits were derived from an overlap in exact mass, RT and fragmentation with amitriptyline and nortriptyline, respectively, which were also identified in those samples. Prothipendyl could be filtered out by applying a more strict RT-window. A second group represented molecules that had not been picked up by targeted dMRM analysis but were likely to be present in the sample (at concentrations below the limit of detection). For example, oxazepam and temazepam were positively identified in cases that were confirmed to contain diazepam and nordazepam, thus likely were no true false positive hits. A third group were any other false positive hit. These could not be explained by the presence of up- or downstream metabolites (as was true for group two) nor could their potential false identification be anticipated (in contrast to group one).

Overall, both screening methods benefited from the same, simple and time-efficient sample preparation. LC-conditions were also standardised between instruments, allowing for the analysis of the same extract on multiple instruments. The time spent on data analysis was more favourable for the targeted tMRM method, but needs to be outweighed against the benefits of untargeted data acquisition (see section 8.2.2). Sensitivity of both assays was in the low to sub-ng/mL concentration ranges, with findings of both methods being complementary to each other. False positive results were more common for the untargeted QTOF method compared to the tMRM method. If an analyte is identified in isolation (no related substances such as metabolites or precursors present), a confirmation analysis should be performed. Additional information on potential false positive results might be derived from the findings of other screening techniques such as LC-DAD or GC-MS.

8.2.2. Benefits of untargeted high-resolution mass spectrometry

Modern TOF-based applications are able to distinguish masses with an accuracy below 2 ppm. Thus, for a theoretical m/z-value of 100.000000, any ions outside of the m/z-range 99.999800 – 100.000200 would be recognised as a different species. This in contrast to the confirmation method of choice,

LC-QQQ, which typically operates at a unit mass resolution (distinction m/z 100.0 from m/z 101.0, 10000 ppm). As a result, HRMS applications are less reliant on LC separation for the unequivocal identification of isobaric compounds [88,89].

Potentially the biggest advantage of untargeted methods is their adaptability towards new analytes. This may be of particularly importance in the detection of new psychoactive substances (NPS). Reference standards needed for the development of targeted NPS methods are often not available (or affordable) to routine clinical and forensic laboratories. Furthermore, if they were, considerable time would need to be invested in their implementation (and validation) in a targeted method [507,508]. For untargeted HRMS methods, any known information could be entered into the database (used during post-analysis) without influencing the performance or quality of the data acquisition. Moreover, the high mass accuracy allows for exact, unequivocal determination of an analyte's chemical composition and tentative identification (Schymanski levels 3 or 2b, Figure 8 - 1) even in the absence of a reference standard. Therefore, once a method has been established, new analytes can be added in a time- and cost-efficient manner [89,326,508].

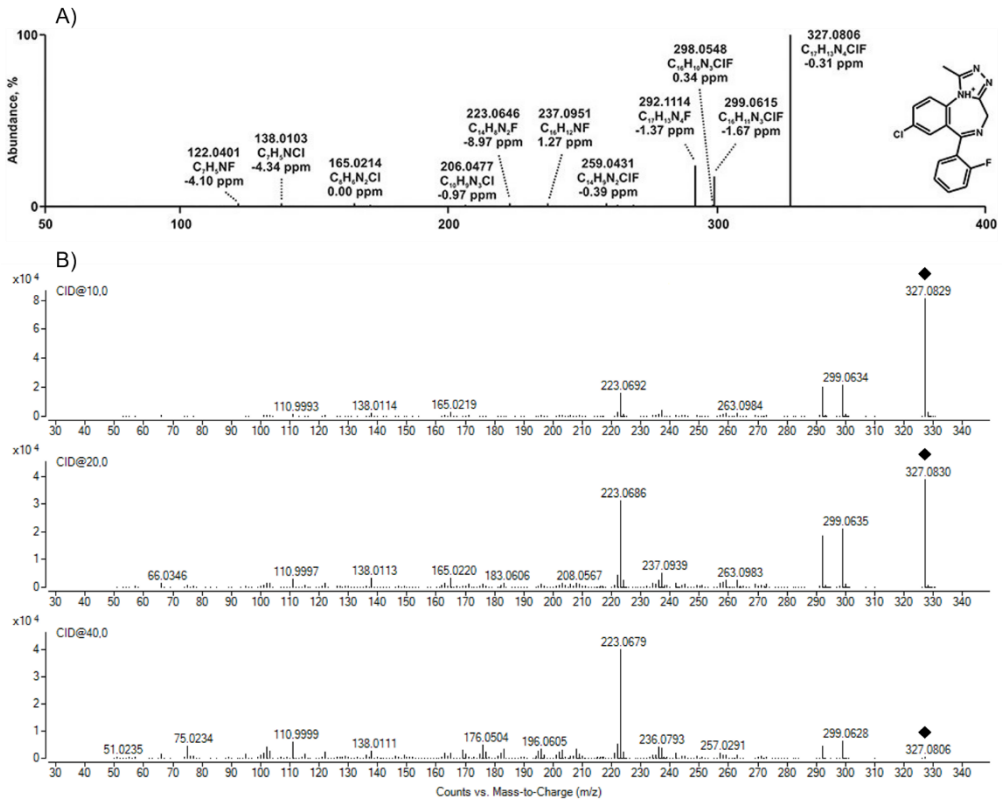

Figure 8 - 3. High-resolution MS/MS spectra for flualprazolam. The published reference spectrum (A) was acquired following analysis of online bought flualprazolam on a Thermo Fisher Scientific LC-quadrupole-Orbitrap at CEs 17.5 eV, 35 eV and 52.5 eV [509]. Our analysis (B) was performed on an Agilent LC-QTOF at CEs 10 eV, 20 eV and 40 eV. The precursor ion is indicated by the diamond. The nature of the product ions was highly reproducible and could be used for compound identification. The exact mass and isotope pattern also matched with those predicted from the molecular formula. Collision energy, CE.

As virtually all analytes are screened for, identification of additional substances in previously analysed samples does not require re-extraction and -analysis, rather the post-acquisition workflow with updated database can simply be re-run on the originally acquired data [326,508,510]. This can be illustrated for the analysis of flualprazolam, a NPS benzodiazepine first discovered in 2018 and since associated with multiple poisonings or deaths due to drug overdoses [337]. Several urine samples had been received from the Ghent University Hospital and were analysed for designer benzodiazepines using the previously described dMRM and QTOF methods (Chapter 5 p. 69 and Chapter 7 p. 147). Overall the findings agreed with (or surpassed) those of their LC-TOF method, but flualprazolam had not been picked up as it was initially not included in the database. Its molecular formula as well as those of the OH- and di-OH-metabolites were obtained from literature and added to the database, which calculated the exact mass and isotope pattern and abundance, and the data were reprocessed. Based upon the database entries, flualprazolam and di-OH-flualprazolam could be identified. MS/MS spectra had been obtained during the initial analysis and matched those published in literature (confidence level 2a, Figure 8 - 3) [509]. Overall, identification of this NPS in samples, following being notified about it, took less than half a day, did not require re-extraction or -analysis of the samples, and could be done without having purchased a reference standard.

8.3. (SEMI-)QUANTITATIVE SCREENING METHODS

8.3.1. Interpretation of a measured concentration

When assigned a case, a toxicologist's primary role is to interpret the findings and formulate an answer to the question if one or more substances may have had an influence at the time of the incident or death. However, such question requires a nuanced answer based upon clear communication between the toxicologist and the clinician or pathologist [41]. The concentration of an analyte may give an indication, but should always be interpreted with care. Long delays between the time of the incident and the sample collection may lead to low concentrations due to continued metabolic breakdown and clearance. For post-mortem (PM) samples, anaerobic, bacterial metabolism may also occur [36,342]. If a person has been hospitalised during that period, medical interventions may have eliminated the original toxicant and introduced other impairing compounds, in which case the analysis of samples collected at the time of hospital admission may be of greater value [37,511]. Inter-individual variability in pharmacokinetics will also have an influence. Metabolism is known to slow down with age, but genetic polymorphism of metabolic enzymes, liver or kidney impairment, body mass and gender can cause differences as well. Additional influences may stem from drug-drug and food-drug interactions and from tolerance towards the effects of a drug. Thus, a measured concentration cannot be related to an administered dose nor directly to the intensity of an effect [342,448,512,513]. Comorbidities or autopsy findings could also influence the interpretation of a given concentration. Different pathologies may cause symptoms reminiscent of drug toxicity or impairment [41]. On the other hand, a theoretical drug overdose may not have contributed to death, depending on the case circumstances (e.g. an intoxicated victim that died in a shooting incident) [36]. The type of sample should also be taken into account when interpreting drug concentrations. Plasma concentrations may not reflect those in whole blood due to uneven distribution of drugs between plasma and erythrocytes [342,514,515]. The sampling site can cause additional variations, with differences between venous and arterial concentrations described for therapeutically administered doses [516].

Particularly for PM samples, the potential for post-mortem redistribution (PMR) should always be considered. PMR is defined as 'the physiological process of drug release and/or mixing of drugs from one compartment (tissues) into another (blood) after death' [448]. Although currently poorly understood, different mechanisms are thought to underlie the elevated concentrations of some drugs in post-mortem blood over time. Most commonly referred to is the distribution from drug depots (e.g. liver, gastric contents) into the blood [517,518]. In the hours prior to death as well as post-mortem, normal cellular metabolism will slow down and halt completely, with anaerobic metabolism taking over. The resulting intracellular lactic acid accumulation will lead to a change in the pH (a drop of 2 units within 24 h has been reported) and progressive degradation of the cell membrane. Due to the increased permeability, drugs may passively diffuse along the concentration gradient from the intracellular to the extracellular space and subsequently to the blood vessels [52,448]. Additional mechanisms could be the aspiration of stomach contents into the lungs or continued release after death from drug delivery systems (e.g. transdermal fentanyl patches) [342,519].

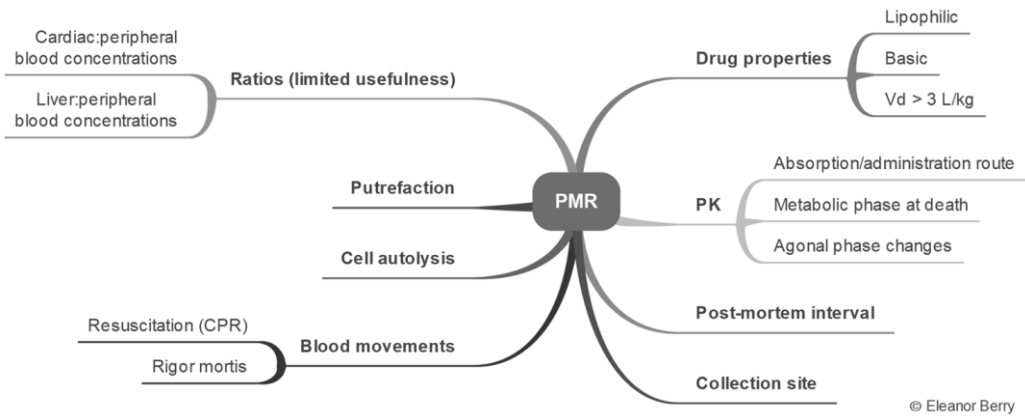

Figure 8 - 4. Non-exclusive list of factors associated with post-mortem redistribution (PMR). Although each of these factors is known to increase the risk of PMR, the extent of PMR in a sample – if any – can at best be estimated only and measured concentrations should be reported alongside the assumptions made. Cardiopulmonary resuscitation, CPR; pharmacokinetics, PK; volume of distribution, Vd.

An exact prediction of the extent of PMR – even when investigated it remains highly variable – and of drugs that are prone to PMR is challenging, but certain case-specific characteristics could indicate its occurrence (Figure 8 - 4) [36,448]. The longer the delay between the time of death and of sample collection (post-mortem interval), the more likely PMR will have occurred. This is partially but not exclusively linked to inadequate storage conditions of the body. Movement of the body from the site of the incident to the site of the autopsy can also cause drugs to be redistributed throughout the body [52,448]. Highly lipophilic drugs tend to be more prone to PMR, which may explain the usually lower observed changes for benzodiazepines [520]. Other factors to consider are the volume of distribution and degree of protein binding, the pKa, the tissue affinity, the body's degree of putrefaction, etc. Although it has been investigated more thoroughly for cardiac or central blood, PMR from skeletal muscles into peripheral blood vessels may also occur. Therefore, the general assumption should be that, unless proven otherwise, PMR may have had an influence on the sample [36,448]. Analysis of alternative matrices may be useful in such case. For example, tricyclic antidepressants are known to accumulate in the liver. Combined parent and active metabolite concentrations (analysed on the more

anatomically isolated right lobe) < 15 mg/kg likely indicate absence of a toxicity, those > 30 mg/kg toxicity. A parent to metabolite ratio > 1.0 would indicate recent or acute exposure [521]. However, a reliable interpretation of a drug's concentration and its effect on a person can only be derived from blood measurements [36,41].

Overall, when drawing conclusions from a measured concentration, one must always interpret these in line with the case history and other findings. Generalised reference concentrations, such as available for therapeutic drug monitoring, should not be trusted. Rather, case reports clearly describing the type of sample, the route of ingestion and any medical or case-related history are to be used. Any assumptions made, e.g. if the duration of the post-mortem interval is unknown, must clearly be stated [36,37,41,342,405].

8.3.2. Accurate quantification vs. semi-quantitative screening

Given the above-mentioned uncertainties, determination of an estimated concentration may be sufficient in forensic analyses. Both screening methods were investigated for their semi-quantitative potential. Previously developed quantitative data analysis methods for dMRM confirmation could readily be converted into a single method compatible with tMRM analysis. As concentrations were calculated in parallel with qualitative analysis in the MassHunter Quantitative Analysis 10.0 software, quick and simple semi-quantitative results were obtained. Accuracies were better for the unlabelled analogues of the ISTDs compared to other substances. However, even for the latter analytes it is unlikely that a different interpretation would have been reached. Only those samples positive for venlafaxine/O-desmethyl-venlafaxine and sertraline should be quantified by another method, even for screening purposes. The QTOF screening posed more issues, predominantly because the corresponding software required targeted analyses for quantitative purposes. Eleven analytes were selected for exploratory investigation of the method's semi-quantitative power using the Microsoft Excel software. Excellent results were obtained for the benzodiazepines and antidepressants, which all displayed linear calibration curves. Antipsychotics needed quadratic curve fittings and were therefore less reliable in their result.

Overall, semi-quantitative results were more reliable and easier to obtain for tMRM methods and could be a valuable addition to the method in routine screening. The untargeted QTOF method as it stands to date should be used for qualitative purposes only. Nonetheless, depending on the reason for analysis (e.g. therapeutic drug monitoring), exact concentrations – obtained with dMRM methods – may be required. Accuracy and precision of the measured concentrations was evaluated for each of the confirmation methods (Chapter 5 p. 69). Reliable quantitative results could be guaranteed over a range from subtherapeutic to potentially toxic concentrations. A ten times dilution could be applied if needed. The samples would need to be re-extracted as more labelled internal standards (ISTDs) are needed for determination of the exact concentration.

CHAPTER 9

CONCLUSIONS & FUTURE PERSPECTIVES

9.1. CONCLUSIONS

When performing routine analyses, the nature of the substances present in a sample is a priori unknown but might range anywhere from endogenous compounds over therapeutically prescribed medication to illicit drugs. A distinction between what may have contributed to the incident in question and what has not, can only be made if the full range of compounds is screened for during systematic toxicological analysis (STA). In this thesis, two screening methods were discussed: targeted, triggered multiple reaction monitoring mode (tMRM) screening and untargeted, quadrupole time-of-flight mass spectrometry (QTOF) screening. A pre-defined set of compounds, belonging to the antidepressants, antipsychotics and benzodiazepines and Z-drugs, was used to validate the methods. This number should ideally be expanded prior to implementation in routine casework. The performance of both methods (compared to validated, confirmation methods) was comparable with 15% false negative results for the tMRM screening and 18% false negative results for the QTOF screening, generally for compounds present at low to sub-ng/mL concentrations. More false positive results were obtained for the QTOF (n = 83) versus the tMRM (n = 24) method. Exclusion of a known bias for phenelzine and prothipendyl following QTOF analysis significantly reduced these numbers. Overall, the untargeted acquisition of QTOF instruments is preferred for STA, but improvements to the data-analysis workflow (user-friendliness, time spent per sample, need for skilled personnel) are needed.

The targeted tMRM method may provide complementary data to the QTOF screening. The good accuracy of its semi-quantitative results often suffices for interpretation of case findings, particularly given the limitations associated with measured concentrations in post-mortem samples. The use of a limited number of labelled internal standards (ISTDs) significantly reduces costs compared to validated confirmation methods, whilst still providing highly similar concentrations. The sample preparation has been preserved between both screening methods and stability of the analytes after extraction was investigated for up to 72 h. Thus, positive findings of the QTOF analysis could be verified and semi-quantified by tMRM without the need for additional sample preparation or further use of often limited sample volume.

A final decision on the presence or absence of a substance should never be made solely based upon the findings of one analytical method. A combination of two or more methods using different physical or chemical principles is preferred. Confirmation using validated, targeted analyses is recommended for conflicting findings between methods (taking into account their limitations). When decisions are based upon screening methods alone, knowledge of the exact mass, fragmentation pattern and retention time (RT) is required. For most of the compounds under investigation, their major or active metabolites have also been included in the methods. The presence or absence of such related substances might further increase confidence in the findings.

9.2. FUTURE PERSPECTIVES

Little improvements seem necessary for the validated dMRM methods as these have already been successfully implemented in routine analysis and proficiency testing schemes. Even shorter liquid chromatography (LC) run times could be investigated (Patteet et al. reported a 6 min method for the analysis of 24 antipsychotics), but the added value might not outweigh the drawbacks such as a potential loss of sensitivity and increased matrix effects [361]. For the same reason, an update of the LC parameters for the tMRM method seems unnecessary. On the other hand, a longer gradient elution might be beneficial for the LC-QTOF analysis. The database currently contains 144 entries, but for true untargeted STA purposes should be expanded to several hundreds to thousands of compounds. Examples of such large databases have been published by Broecker et al., Grapp et al. and Lung et al. [458,490,500]. However, these would risk overcrowding the chromatogram. Especially in data-dependent acquisition (DDA) mode, where fragmentation is based upon intensity and only a limited number of ions are fragmented, this might lead to loss of sensitivity [491]. Switching to data-independent acquisition (DIA) mode may partially overcome this problem but could still lead to false negative results if too many ions need to be acquired in the same cycle. Thus, separation in time by LC is needed. Additionally, the increased resolution might separate isobaric or isomeric compounds that otherwise may not have been unambiguously identified [480]. In literature, methods of 30 min or longer have been published. The increased analysis time reflects that of gas chromatography coupled to mass spectrometry (MS) screening and is outweighed by the number of analytes screened for in one run [69,459,522]. Further improvements in resolution may be achieved by coupling LC-MS to ion mobility, although the cost of such instruments and the complexity of the data-analysis currently limit their widespread routine use [92,477].

The tMRM analysis as is uses one primary ion, which is continuously monitored and used to trigger the acquisition of secondary ions. As a result, and due to the applied trigger window and trigger delay settings, ion ratios as determined for dynamic multiple reaction monitoring (dMRM) analysis are not reliable. The inclusion of a second primary ion and subsequent inclusion of the fixed ion ratio between both as identification criterion might further increase confidence in the findings and reduce the (low) number of false positive results. However, if the method were to be expanded to include different drug classes, this could lead to instrumental limitations as too many transitions would need to be monitored in one cycle. With regards to the QTOF method, the data acquisition and analysis could also be fine-tuned. As mentioned above, development of a DIA method might improve the sensitivity as any ion would be fragmented irrespective of its intensity. This could also provide an added advantage during retrospective analysis for missed or previously unknown substances [447]. Whereas some published articles report on its application in routine settings, our in-house experience with DIA methods found them too tedious for use on every sample. Nonetheless it is important to acknowledge that next to instrumental performance, the software has improved as well, resulting in more user-friendly data analysis workflows and optimised deconvolution algorithms in the newest instruments. When running in DDA mode, the fragmentation likelihood of low abundant ions may be improved by iterative MS/MS acquisition. A feature in the newest generation of QTOF instruments, users can choose between sample analysis by iterative exclusion (ions fragmented during one run are excluded from fragmentation during subsequent injections) or iterative inclusion (a preferred fragmentation list is generated based upon one or more initial scans). From an untargeted

point of view, iterative exclusion seems the preferred workflow, although repeated injections could markedly increase sample turn-around times [477,523]. The use of tailor-made R scripts could speed up the data analysis and reduce analyst hands-on time [524]. A workflow for automated data extraction, including distinction between matrix ions (based upon blank injections) and ions of interest, was published by Vervliet et al. [441]. Other currently undefined vendor-specific programs may facilitate similar features whilst tying in seamlessly with the MassHunter Qualitative Analysis or PCDL software. Further improvements in data analysis workflows are expected from machine learning approaches. Streun et al. reported on the development of a feedforward, artificial neural network able to quickly distinguish between negative and positive blood samples following LC-QTOF analysis in DIA mode [525]. However, the authors acknowledged that manual data conversion (ProteoWizard software) and preprocessing (R-software) is required, as is considerable time spent on training the algorithm during method development.

If the LC and/or MS parameters would be changed or optimised, validity of the method would need to be re-evaluated. Any of three methods can be used for this: 1) determination of the limits of detection, matrix effects and recoveries for each compound, 2) application of method 1 to a representative subset of analytes, or 3) verification against established methods [491]. The current methods were evaluated according to method 3, however, due to the limited number of compounds included in targeted methods, this approach would not be suitable for a database of several thousand compounds. Further evaluation of the qualitative and quantitative performance of the tMRM method by analysis of large amounts of routine samples is also recommended. Operating both mass spectrometers in negative ionisation mode should also be considered for true STA purposes. Research by Kinyua et al. reported on the applicability of a similar sample preparation (liquid-liquid extraction using ethyl acetate and n-hexane) and LC parameters (mobile phases consisting of water (A) and 8:2 acetonitrile:water (B), both with formic acid added) coupled to negative ionisation QTOF analysis [438]. The formation of formic acid adducts should be taken into consideration.

Semi-quantitative analysis by LC-QTOF (in Microsoft Excel) seems promising but labour-intensive. Although our attempts at using the MassHunter Quantitative Analysis software were unsuccessful, Partridge et al. published an untargeted, semi-quantitative method for selected compounds using an Agilent 6545 QTOF in DDA mode. Alternatively, R-based or other scripts/algorithms may be investigated for quantification of QTOF data. Additionally, further improvements towards the calibration range and ISTD mix might be made. The current ISTD mix was chosen based upon distribution of the analytes over the whole chromatographic run and their structural similarity with (anticipated) frequently occurring substances (see section 6.3.1 p. 120). tMRM analysis confirmed the notion that better similarity between analyte (either structural or in RT) and ISTD would lead to more accurately calculated concentrations [41,526]. For those analytes with less acceptable accuracies, the effect of a different ISTD may be investigated. Additionally, mirtazapine and olanzapine were less prevalent in samples than anticipated. Therefore, their labelled analogue could be omitted from the ISTD mix (saving on the cost of reference standards) or exchanged for another one. Conversely, for highly problematic analytes, the labelled analogue could be included in the ISTD mix to improve the accuracy of their semi-quantified concentrations. The concentration range of the calibration curves was rather wide and based upon those of validated dMRM methods. Limiting this range could lead to linear correlations for all curves, facilitating extrapolation for higher or lower concentrated

samples. Such extrapolations should only occur for semi-quantitative purposes, never for confirmation analyses, and their validity should be evaluated thoroughly against samples with a known concentration. The concentration of one or more calibration levels could also be changed to reflect concentrations of interest, e.g. cut-off values for driving under the influence of drugs cases.

Because of the legal or medical consequences associated with toxicological findings, system suitability and day-to-day quality control (QC) should be performed. QC samples should be made up in the same matrix as case samples to account for matrix-specific interferences or recovery differences, and should represent all possible matrices in a given batch. Both positive (spiked blank matrix) and negative (blank matrix) QC samples should be run, both in an open (labelled as QC sample) and blinded (labelled as case sample, contents known to the quality manager only) manner [36,526,527]. Thus far, the screening methods been evaluated against case samples. Once implemented in routine screening, QC samples should be included in each batch and participation in proficiency tests is also encouraged. Analysis of endogenous compounds could indicate performance issues, for these must be identified in all samples. Currently, caffeine was included as internal QC. Other screening methods also regularly pick up its metabolites paraxanthine (84%), theobromine (12%) and theophylline (4%). The latter two are naturally present in tea leaves and cocoa-containing food products as well [528,529]. Despite their widespread presence in the general population, these substances are not truly endogenous and absence in a sample does not unequivocally indicate an analytical problem. The anti-inflammatory and immunosuppressive glucocorticoid cortisol might be a true endogenous alternative to caffeine. It is secreted in high concentrations from the adrenal cortex following a circadian rhythm. Under the name hydrocortisone, it is also used to treat a large variety of illnesses [530–532]. Creatinine, a metabolite of creatine, is excreted in blood from skeletal muscle tissue at a constant rate and could also be included as internal QC. However, the reported low and unspecific m/z 47 of its main product ion might hinder its applicability [533]. Regardless, prior to implementation of these screening methods in routine analyses, a quality assurance protocol will need to be drafted, thoroughly tested and validated.

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SUMMARY

Routine toxicological analysis requires broad screening for an undefined number of therapeutically prescribed and other compounds, and/or their metabolites. This thesis specifically focused on three classes of psychoactive substances: antidepressants (ADs), antipsychotics (APs) and benzodiazepines and Z-drugs (BZDs). They are increasingly prescribed for often long-term use (sometimes against recommendations for best practice), frequently encountered in routine forensic samples and require close therapeutic drug monitoring due to their potential for serious adverse events. Particularly with regards to the BZDs, abuse in combination with other drugs is of particular concern and an ever growing number of designer compounds has become available.

Part I of this thesis describes the development of targeted liquid chromatography (LC) – triple quadrupole mass spectrometry (QQQ) methods for unequivocal identification and accurate quantification of 39 ADs, 37 APs and 54 BZDs. Plasma was selected as the sample matrix for its reflection of the analytes circulating in the body at the time of a given incident – provided sampling occurred close to the time of said incident – and therefore its excellent correlation with the observed effects (**Chapter 3**). For these reasons, it is currently the matrix of choice for most therapeutic drug monitoring or human toxicology investigations. Using the BZDs as example analytes, different extraction methods were investigated (**Chapter 4**). Protein precipitation and mini-QuEChERS resulted in insufficient sample clean-up and extraction efficiency, respectively, and were omitted from further experiments. The results for the tested liquid-liquid extraction (LLE) and solid-phase extraction protocols were comparable. Due to its better time-efficiency and safety profile and the use of less sample volume, LLE was retained as the preferred method. Extracted analytes of interest were separated during a 12 min LC-run in gradient elution (**Chapter 3**). The stationary phase consisted of a Zorbax Eclipse Plus C8 column (2.1 x 150 mm, 3.5 μm), the mobile phases of A) ultrapure water + 0.1% formic acid (V/V) and B) 9:1 acetonitrile:ultrapure water + 0.1% formic acid (V/V). The mass spectrometer (MS) was operated in dynamic multiple reaction monitoring mode, acquiring three ion transitions per analyte – two for labelled internal standards (ISTDs). Each of the developed methods was fully validated according to the European Medicines Agency guidelines (**Chapter 5**). Accuracies and precisions compared to spiked and external quality control samples all fell within the set criteria over a concentration range from subtherapeutic (where known) to toxic concentrations (as determined on *in vivo* patients). The previously selected LLE extraction performed well for all three classes of analytes. Stability issues in unextracted samples were observed for limited analytes only. Once extracted, all analytes showed excellent stability at ambient temperature.

In **Part II**, the development and cross-validation of screening methods is described. A first method consisted of the combination of all three above mentioned, validated methods, with the MS run in triggered multiple reaction monitoring mode (tMRM; **Chapter 6**). This acquisition mode benefits from a greater number of ion transitions that can be included without the loss of sensitivity. LC and MS parameters were kept similar to those of part I. Additional tMRM-specific parameters to be optimised were trigger window, trigger entrance, trigger delay and trigger threshold. The finalised method was tested against medico-legal case samples, previously analysed by the validated LC-QQQ

methods. Only about 15% of compounds resulted in false negative results, the majority of which were either present at sub- to low therapeutic levels or were metabolically related to other analytes detected in those samples. No differences in case interpretations would have been expected from these false negative results. The occurrence of false positive results was rare. A second screening method used the quadrupole time-of-flight mass spectrometry (QTOF) instrumentation for untargeted data acquisition (**Chapter 7**). Data analysis was facilitated by the creation of an in-house database containing fragmentation spectra and retention times for all analytes of interest. Once more the results of medico-legal samples were compared to those obtained using validated LC-QQQ methods. Two automated data extraction and identification workflows were developed: workflow 1 using the MassHunter Profinder software and workflow 2 using the MassHunter Qualitative Analysis software. Both workflows performed equally well with regards to the number of false negative results (around 25%), but workflow 2 resulted in more false positive hits. The best results were obtained by combining both workflows. When all analytes identified with either software program were included, the number of false negative results could be reduced to less than 20%. As seen for the tMRM method, either most of the missed results were for compounds at low concentrations or their presence could be derived from the identification of related analytes. More false positive results were observed due to a known bias for phenelzine and prothipendyl.

For implementation in routine analysis (**Chapter 8**), untargeted QTOF screening is recommended over targeted tMRM screening. Few differences are expected in case interpretations when one method is applied over another. However, the untargeted nature of the QTOF screening would allow for easy expansion of the number of analytes looked at – by updating the database rather than the instrumental parameters – and for retrospective data analysis should information about new substances become available. Nonetheless, a combination of multiple, complementary screening methods is recommended for adequate confidence in the findings. If such methods are not available, the use of validated, targeted methods should be considered for confirmation of the presence or absence of specific analytes. Determination of the exact concentration of a substance in a sample may only be required depending on the specific case circumstances. Particularly for long intervals between the time of an incident and of sample collection, quantification may unnecessarily increase turn-around times and cost of the analyses. Even when samples are collected close to the time of the incident, an accurate concentration might not provide relevant additional information. For this purpose, semi-quantification with each of the screening methods was investigated. Excellent results were observed using the tMRM method in combination with a small number of ISTDs. QTOF quantification was more laborious, but first results on selected compounds indicated equally adequate results.

Overall, the developed, semi-quantitative screening methods performed well and – following further validation on case samples – could be implemented in routine, forensic toxicological analysis. Confirmation of the findings and accurate concentration determination may be required depending on the case, for which purpose fully validated, targeted LC-QQQ methods have been developed.

SAMENVATTING

Tijdens routinematige, toxicologische analyses dient gescreend te worden voor een onbekend aantal stoffen en/of hun metabolieten. Deze kunnen zowel voorgeschreven als niet-therapeutisch gebruikte producten zijn. In deze thesis werd specifiek op drie klassen psychoactieve stoffen toegespitst: antidepressiva (ADs), antipsychotica (APs) en benzodiazepines en Z-drugs (BZDs). Deze drie klassen worden steeds meer voorgeschreven, vaak voor (soms tegen de richtlijnen in) langdurig gebruik. Ze worden ook frequent teruggevonden bij forensische analyses en hun potentieel voor ernstige neveneffecten vereist nauwgezette opvolging van de bloedconcentraties bij patiënten. Daarnaast is de klasse van de BZDs bijzonder gevoelig aan misbruik in combinatie met andere producten, en is een steeds groeiend aantal synthetische, designer BZDs verkrijgbaar.

Deel I van deze thesis beschrijft de ontwikkeling van vloeistofchromatografische (LC) – tripel quadrupool massaspectrometrische (QQQ) methodes voor de ondubbelzinnige identificatie en accurate concentratiebepaling van 39 ADs, 37 APs en 54 BZDs. Plasma werd gekozen als matrix voor de stalen (**Hoofdstuk 3**), omdat deze matrix de stoffen bevat die op het ogenblik van een incident in het lichaam circuleren (indien het staal kort na het incident werd afgenomen). Het weerspiegelt het beste de effecten die een persoon ondervond en wordt dan ook universeel verkozen als matrix voor therapeutische opvolging of toxicologische onderzoeken. Verschillende staalvoorbereidingsmethodes werden onderzocht voor de BZDs als voorbeeldklasse (**Hoofdstuk 4**). Proteïneprecipitatie resulteerde in onvoldoende verwijdering van interfererende stoffen, mini-QuEChERS in onvoldoende extractie van de gewenste stoffen. De resultaten voor de onderzochte vloeistof-vloeistof (LLE) en vaste fase extracties waren vergelijkbaar. LLE nam echter minder tijd in beslag, was veiliger voor laboranten en gebruikte minder staalvolume, en werd daarom weerhouden voor toekomstige analyses. Na extractie werden de analieten gescheiden door middel van een Zorbax Eclipse Plus C8 kolom (2.1 x 150 mm, 3.5 µm; **Hoofdstuk 3**). De mobiele fases (gradiëntelutie over 12 min) bestonden uit A) ultrapuur water + 0.1% mierenzuur (V/V) en B) 9:1 acetonitril:ultrapuur water + 0.1% mierenzuur (V/V). De massaspectrometer (MS) werkte in dynamische 'multiple reaction monitoring' modus. Drie transities werden opgenomen per analiet, twee voor gelabelde interne standaarden (ISTDs). Elk van de ontwikkelde methodes werd gevalideerd volgens de richtlijnen van het Europees Geneesmiddelen Agentschap (**Hoofdstuk 5**). Concentraties binnen een bereik van sub-therapeutische (indien gekend) tot toxische waarden (bepaald op *in vivo* patiënten) konden worden gemeten. Accuraatheid en precisie vielen binnen de vooropgestelde criteria voor zowel zelfbereide stalen als voor externe kwaliteitscontroles. De eerder geselecteerde LLE methode werd positief geherevalueerd voor de drie klassen van psychoactieve stoffen. Stabiliteitsproblemen voorafgaand aan extractie traden slechts bij enkele stoffen op. Na extractie werden geen problemen ondervonden, ook niet bij bewaring op kamertemperatuur.

Deel II handelt over de ontwikkeling en kruisvalidatie van screening methodes. Een eerste methode combineerde de bovenvermelde gevalideerde methodes, maar werkte met de MS in 'triggered multiple reaction monitoring' modus (tMRM; **Hoofdstuk 6**). Hierdoor kan een hoger aantal transities worden toegevoegd zonder verlies aan gevoeligheid. De overige LC en MS parameters

werden gelijk gehouden aan deze in deel I. Enkele tMRM-specifieke parameters dienden nog te worden geoptimaliseerd: 'trigger window', 'trigger entrance', 'trigger delay' en 'trigger threshold'. De finale methode werd getest op stalen van gearchiveerde zaken, die ook geanalyseerd werden met de gevalideerde LC-QQQ methodes. Vals negatieve resultaten werden voor slechts 15% van de eerder gevonden stoffen bekomen. Dit werd voornamelijk opgemerkt voor stoffen die in laag- tot sub-therapeutische concentraties aanwezig waren, of voor stoffen waarvan metabolisch gerelateerde analieten gedetecteerd konden worden. Hiervoor zou dan ook geen verschil worden verwacht bij de uiteindelijke interpretatie in de zaken. Vals positieve resultaten werden zelden teruggevonden. Een tweede methode maakte gebruik van quadrupool 'time-of-flight' massaspectrometrie (QTOF) voor niet-gerichte dataopname (**Hoofdstuk 7**). Een eigen database met fragmentatiespectra en retentietijden werd gecreëerd om de data-analyse te vergemakkelijken. De resultaten van gearchiveerde stalen werden opnieuw vergeleken met deze verkregen via gevalideerde LC-QQQ methodes. Twee geautomatiseerde workflows voor data extractie en identificatie werden ontwikkeld: workflow 1 maakte gebruik van de MassHunter Profinder software, workflow 2 van de MassHunter Qualitative Analysis software. Voor beide workflows werden gelijkaardige bevindingen bekomen met betrekking tot vals negatieve resultaten (in ongeveer 25% van de gevallen). Workflow 2 identificeerde echter meer vals positieve resultaten. Een combinatie van beide workflows bleek het meest betrouwbaar. Het aantal vals negatieve resultaten kon worden herleid tot minder dan 20% door alle stoffen geïdentificeerd in minstens een van beide workflows te includeren. De gemiste analieten waren opnieuw aanwezig in lage concentraties, of hun aanwezigheid kon worden afgeleid via de identificatie van verwante stoffen. Het hogere aantal vals positieve resultaten kon worden toegekend aan een gekende bias voor fenelzine en prothipendyl.

Voor routinematige analyses is QTOF screening te verkiezen boven tMRM screening (**Hoofdstuk 8**). Met betrekking tot interpretatie voor casussen wordt weinig verschil verwacht tussen de twee methodes. Daar bij QTOF toepassingen de stoffen niet op voorhand gedefinieerd moet worden in de analytische methode, kan het aantal onderzochte stoffen gemakkelijk worden uitgebreid door de database te updaten eerder dan de LC-MS parameters te moeten aanpassen. Voorts is het mogelijk om opgenomen data retrospectief te heranalyseren, indien nieuwe informatie beschikbaar is. Desalniettemin kan voldoende vertrouwen in de bevindingen slechts worden bekomen via de combinatie van verschillende, complementaire screeningmethodes. Indien deze niet beschikbaar zijn, dient de aan- of afwezigheid van analieten bevestigd te worden met behulp van gevalideerde, doelgerichte methodes. Bepaling van de exacte concentratie aan actieve stoffen is in vele gevallen niet nodig. Zeker indien er een groot interval bestaat tussen het tijdstip van een incident en dat van staalname, kan dit leiden tot een onnodige vertraging in het rapporteren van de resultaten en een verhoogde kost van de analyses. Ook indien dit tijdsinterval klein is, draagt de kennis van de exacte concentratie niet noodzakelijk bij aan een betere interpretatie. Met dit in het achterhoofd werd het semi-kwantitatieve potentieel van beide screeningsmethodes onderzocht. Voor de tMRM methode werden uitstekende resultaten bekomen, zeker in combinatie met het kleine aantal ISTDs. Semi-kwantitatieve QTOF screening bleek arbeidsintensiever en werd op slechts een beperkt aantal stoffen getest, wat resulteerde in vergelijkbaar betrouwbare resultaten.

Algemeen kan worden gesteld dat de ontwikkelde, semi-kwantitatieve screening methodes naar behoren werken en – na verdere validatie met behulp van stalen – kunnen worden gebruikt voor

routinematige, forensisch toxicologische analyse. Afhankelijk van de specifieke omstandigheden, kunnen deze worden gevolgd door analyse met de ontwikkelde, gevalideerde LC-QQQ methodes, al dan niet met bepaling van de exacte bloedconcentraties.

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Membership of scientific organisations

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- The Toxicological Society of Belgium and Luxembourg (BLT)
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Peer-reviewed publications

- **M. Degreef**, E.M. Berry, A. Covaci, K.E.K. Maudens, A.L.N. van Nuijs, Qualitative and semi-quantitative screening of selected psychoactive substances in blood: Usefulness of liquid chromatography – triple quadrupole and quadrupole time-of-flight mass spectrometry in routine toxicological analyses (in submission).

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Oral & poster presentations

- Oral presentation, Psychoactive medication and the forensic perspective – Analytical challenges for routine applications, Departmental Research Day of Pharmaceutical Sciences (2021). (awarded best presentation as voted for by the attendees)

- Oral presentation, Designer benzodiazepines in Belgium – A snapshot through retrospective analysis, BLT general assembly (2021).

- Oral presentation, Quantification of 54 benzodiazepines & Z-drugs, including 20 designer benzodiazepines, in plasma, BLT general assembly (2020).

- Poster presentation, Optimisation of a fast and easy quantification method for 54 benzodiazepines & Z-drugs, including 20 designer benzodiazepines, in plasma, TIAFT annual meeting (2019).

- Poster presentation, Determination of ocfentanil and W-18 in a suspicious heroin-like powder in Belgium, TIAFT annual meeting (2019).

- Poster presentation, Determination of ocfentanil and W-18 in a suspicious heroin-like powder in Belgium, Flanders (2019).

- Oral presentation, The danger of online drugs, Biotechdag (2018). (original title in Dutch)

- Oral presentation, Fast and easy quantification of 40 antidepressants or their metabolites in plasma for therapeutic drug monitoring and forensic analysis, TIAFT annual meeting (2018).

- Poster presentation, The pigeon poppy seed defence – Analysis of opiates as markers of doping use in racing pigeons, Joint SOFT-TIAFT meeting (2017).

- Oral presentation, The pigeon poppy seed defence, BLT general assembly (2017).

- Poster presentation, Comparison of the *in-vitro* metabolism of a new psychoactive compound, 4 methylamphetamine, by human S9 fraction and human microsomes, UKIAFT meeting (2014).

Dissertations supervised

- T. Vanhooydonck, Impact of the (Belgian) maximum billing system on stockpiling of psychoactive medication (2021). (original title in Dutch)
- J. Heyrman, Increasing compound coverage and identification confidence in lipidomics using ion mobility spectrometry and iterative tandem mass spectrometry (2021). (assessor)
- S. Seddiki, The use of psychoactive medication in Belgium – An analysis of the consumption data (2020). (original title in Dutch)
- G. Wauters, How does the police deal with medication in a suspicious death and what can the pharmaceutical sector do to help (2019). (original title in Dutch)
- L. Vits, Optimization of the extraction method for the detection of benzodiazepines in human plasma (2019).
- S. Ceusters, Development and validation of a liquid chromatography – tandem mass spectrometry method for the analysis of fentanyl analogues (2018). (original title in Dutch)
- J. Op de Beeck, The pharmacokinetics of opiates following oral ingestion of poppy seeds – A comparison between humans and the racing pigeon (2017). (original title in Dutch)
- S. Raats, The pharmacokinetics of opiates following poppy seed administration to pigeons (2017). (original title in Dutch)

Reviewer for

- Bioanalysis (Future Science)
  https://www.future-science.com/journal/bio
- Forensic Toxicology (Springer)
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- Journal of Chromatography B (Elsevier)
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- Journal of Pharmaceutical and Biomedical Analysis (Elsevier)
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