Qualitative and semi-quantitative screening of selected psychoactive drugs in blood : usefulness of liquid chromatography – triple quadrupole and quadrupole time-of-flight mass spectrometry in routine toxicological analyses

Qualitative and semi-quantitative screening of selected psychoactive drugs in blood: Usefulness of liquid chromatography – triple quadrupole and quadrupole time-of-flight mass spectrometry in routine toxicological analyses.

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Abstract

Routine toxicological analysis requires broad screening for a large number of therapeutically prescribed and other compounds, and/or their metabolites. This article specifically focuses on three classes of psychoactive substances: antidepressants (ADs), antipsychotics (APs) and benzodiazepines and Z-drugs (BZDs). Two screening methods were compared for their ease-of-use in a routine setting, based upon the analysis of 105 medico-legal case samples. Analytes of interest were extracted using liquid-liquid extraction and separated using liquid chromatography with a total run time of 12 min per sample. A first detection method used targeted triple quadrupole mass spectrometry, operated in triggered multiple reaction monitoring mode (tMRM). False negative results were noted for 15% of the total number of detected analytes only, the majority of which were either present at sub- to low therapeutic levels or were metabolites of other analytes in the samples. The occurrence of false positive results was rare. A second screening method used quadrupole time-of-flight mass spectrometry (QTOF) for untargeted data acquisition. Data analysis was facilitated by the creation of an in-house, subset, mass spectral database. As was seen for the tMRM screening, false negative results were observed in less than 20% of the total number of detected analytes, either for compounds at low concentrations or of which metabolites could be identified in the samples. More false positive results were observed due to an observed bias for prothipendyl. Determination of the exact concentration in a sample may only be required depending on the specific case circumstances. For this purpose, semi-quantification using each of the screening methods was investigated. Excellent results were observed using the tMRM method in combination with a small number of labelled internal standards (n = 12). Semi-quantification using the QTOF screening method was more laborious,

but limited results on selected compounds indicated equally good results. Overall, the developed semi-quantitative screening methods performed well and – following further validation on case samples – could be implemented for most compounds in routine toxicological analysis without the need for highly trained or specialised personnel.

Keywords

1 Forensic toxicological screening
2 Psychoactive pharmaceuticals
3 Liquid chromatography
4 High-resolution mass spectrometry
5 Triggered multiple reaction monitoring
6 Semi-quantification

Highlights

1 Different LC-MS screening methods for 128 psychoactive pharmaceuticals
2 Fit-for-purpose design for use in clinical and forensic toxicology
3 Validated against routine medico-legal case samples
4 Semi-quantification with a limited, well-thought set of internal standards

Abbreviations

Acetonitrile, ACN; antidepressant, AD; antipsychotic, AP; benzodiazepine & Z-drug, BZD; calibrator level #, CAL L#; compound of interest, cpd; data-dependent acquisition, DDA; data-independent acquisition, DIA; dynamic multiple reaction monitoring, dMRM; high-resolution mass spectrometry, HRMS; labelled internal standard, ISTD; liquid chromatography, LC; liquid-liquid extraction, LLE; lower limit of quantification, LLOQ; mass spectrometry, MS; methyl-tertiary-butyl-ether, MTBE; quadrupole – ion trap mass spectrometer, QTRAP; quadrupole time-of-flight mass spectrometry, QTOF; retention time, RT; solid-phase extraction, SPE; systematic toxicological analysis, STA; triggered multiple reaction monitoring, tMRM; triple quadrupole mass spectrometry, QQQ

1 Introduction

With an estimated 8000+ relevant substances, toxicological laboratories must rely heavily on screening methods in their investigations to accurately detect (and quantify) those compounds that may play a role in each individual case [1,2]. Systematic toxicological analysis (STA), sometimes also called general unknown screening, is the first step in a typical 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 [3–5]. Additionally, these methods should ideally be rapid, easy to perform, flexible, available around the clock and – most importantly – straightforward, comprehensive and specific in their results [6,7]. Liquid chromatography (LC) coupled to mass spectrometry (MS) has long been overlooked for STA applications given the inability to generate reproducible spectra between instruments and laboratories and the subsequent absence of large, commercially available reference libraries [3,8]. 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 [3,9–11].

Limited targeted LC-MS/MS screening assays have been developed and validated against case samples. 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 [12]. Viette et al. developed a LC method coupled to a hybrid triple quadrupole – ion trap mass spectrometer (QTRAP) for the detection of 300 substances in serum samples, using solid-phase extraction (SPE) as a sample preparation technique [13]. Similarly, Dresen et al. describe a multi-analyte method for the identification of 700 drugs and metabolites using LC-QTRAP. Their sample preparation was a dilute-and-shoot method using urine as a matrix [14]. For each of the above, the instruments were operated in positive ionisation mode and used targeted, multiple reaction monitoring mode for data acquisition. The potential for (semi-)quantitative data analysis was also investigated by Dresen et al., who concluded on the need for labelled analogues of each compound as internal standards for such purposes [14]. Lastly, Di Rago et al. and Orfanidis et al. published quantitative methods for the detection of 132 and 84 substances respectively, but self-reported either significant ion suppression and accuracy issues for multiple compounds (Di Rago et al.) or tested it against a limited number (n = 14) of case samples only (Orfanidis et al.) [15,16].

To increase sample throughput, targeted analyses could be operated in triggered multiple reaction monitoring (tMRM) mode. tMRM can be seen as a variation on the widely used dynamic multiple reaction monitoring (dMRM) methods, benefitting from faster cycle times, increased sensitivity and larger amounts of compounds of interest (cpds) that can be added in one analytical method. Similar to dMRM, the operator is required to program experimentally determined precursor-product ion transitions 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 is therefore continuously monitored. If the abundance of the primary transition exceeds a self-determined threshold, the instrument will start acquiring the secondary transitions. Up to ten secondary transitions may be defined per analyte. When and how to monitor each transition is an essential part of the method development.

A drawback of these targeted methods in STA is their limitation to a priori known compounds only, which can be overcome with untargeted, high-resolution mass spectrometric (HRMS) applications using quadrupole time-of-flight (QTOF) mass analysers [17–19]. 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 [20]. Table 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, which could potentially be explained by the complex composition of blood as a matrix, giving rise to a higher and more variable background signal, therefore potentially excluding relevant, low-concentrated analytes from fragmentation in DDA mode [3]. However, DIA suffers from complicated data interpretation as product ions from co-eluting analytes or endogenous compounds will make up the same spectrum [20].

Here we present two screening methods based upon either tMRM or QTOF analysis, and investigate their potential to determine semi-quantitative concentrations. Compared to other targeted methods, the tMRM method aimed to increased sample throughput by reducing the overall run time from around 20 min to 12 min. For the QTOF analysis, ease-of-use in a routine setting and the absence of a need for highly trained and specialised personnel were additional key criteria in the method development. Therefore, it was opted to investigate the suitability of a DDA method [21]. Of equal importance, the potential for semi-quantitative analysis of all compounds using a limited number of labelled internal standards (ISTDs) was investigated.

2 Materials and methods

2.1 Chemicals and stock solutions

Reference standards for 39 antidepressants (ADs), 37 antipsychotics (APs), 52 benzodiazepines and 2 Z-drugs (BZDs) were acquired from Cerilliant (Round Rock, Texas, US), Chiron AS (Trondheim, NO), Ciba-Geigy AG (Basel, CH), Duphar (Weesp, NL), H. Lundbeck A/S (Copenhagen, DK), Hoffmann-La Roche (Basel, CH), LGC Standards (Teddington, UK), Lipomed AG (Arlesheim, CH), Merck KGaA (Darmstadt, DE), Novartis (Basel, CH), Organon International (Oss, NL), Pfizer Inc. (New York City, New York, US), Pharmacia & Upjohn (Kalamazoo, Michigan, US), Sanico NV (Turnhout, BE), Sanofi (Paris, FR), Sigma Aldrich International GmbH (St. Gallen, CH), Specia (Paris, FR) and Toronto Research Chemicals (Toronto, CA). An overview of all included analytes of interest can be found in Table 2. Merck KGaA (Darmstadt, DE) also provided formic acid, methyl-tertiary-butyl-ether (MTBE), potassium bicarbonate and potassium hydroxide. Acetonitrile (ACN) was acquired from Biosolve (Valkenswaard, NL). Ultrapure water was produced in-house using an Elga Purelab water purification system from Veolia Water Technologies (Tienen, BE).

For semi-quantitative purposes, a calibration curve of seven (ADs and APs) or six levels (BZDs) was prepared in blank plasma. The concentration ranges were kept similar to those of previously validated methods [22–24]. The ISTD solution (prepared in ACN) contained alprazolam-D_5 (200 ng/mL), bromazepam-D_4 (500 ng/mL), citalopram-D_6 (1500 ng/mL), diazepam-D_5 (1000 ng/mL), flupentixol-D_4 (100 ng/mL) melitracen-D_6 (150 ng/mL), mirtazapine-D_3 (600 ng/mL), olanzapine-D_3 (200 ng/mL), prazepam-D_5 (100 ng/mL), quetiapine-D_8 (2000 ng/mL), trazodone-D_6 (7500 ng/mL) and zopiclone-D_4 (200 ng/mL).

2.2 Sample preparation and chromatography

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 of the University of Antwerp in the framework of their medico-legal analyses, 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. The case samples were reanalysed using validated confirmation methods to generate an up-to-date list of detected analytes, as well as to determine reference concentrations for semi-quantitative purposes [22–24]. As the lower limit of quantification (LLOQ) was selected to represent sub-

therapeutic values (as determined *in vivo)*, any compounds with a concentration below the LLOQ were listed as “not detected”.

Additionally, the samples were re-extracted and analysed using the below described tMRM and QTOF methods. Blank blood samples for the preparation of the calibration levels were collected from healthy, drug-free volunteers in 9 mL Vacuette® K_2EDTA tubes (Greiner Bio One International GmBH, Kremsmünster, AT). These were centrifuged immediately after collection and the plasma was stored at -20 °C. The donation was approved by the ethical committee of the University Hospital Antwerp (EC/PC/avl/2018.039).

200 µL blood was spiked with 20 µL ACN and ISTD mix (or calibrator (CAL) and ISTD mix for calibration samples). Compounds of interest were subsequently extracted by liquid-liquid extraction using 65 µL carbonate buffer (pH 9.5) and 800 µL MTBE. Following mixing and centrifugation, the upper layer was evaporated to dryness and reconstituted in 25 µL ACN, after which 1 µL was injected into the chromatographic system (see further). More details on the sample preparation procedure, including data on the extraction efficiency and ion enhancement/suppression, can be found in previous publications [22–24].

The cpds and ISTDs were separated on a Zorbax Eclipse Plus C8 column (2.1 x 150 mm, 3.5 µm) using an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, California, US). The mobile phases consisted of A) water + 0.1% formic acid (V/V) and B) ACN:water (9:1) + 0.1% formic acid (V/V) at a flow rate of 0.5 mL/min. The gradient was increased from 5% to 95% mobile phase B in 9 min, leading to a total run time of 12 min from injection to injection.

2.3 Triggered multiple reaction monitoring screening

For tMRM analysis, the LC was coupled to an Agilent 6460 triple quadrupole mass spectrometer. The detector was operated using Jetstream electrospray ionisation in positive mode. Other MS parameters were gas (N_2) temperature: 300 °C, sheath gas flow: 11 L/min, nebuliser pressure: 30 psi, capillary voltage: 4000 V, and nozzle voltage: 500 V. Where possible, three transitions were monitored for cpds and two for ISTDs. The previously determined quantifier transitions were set as primary transitions [22–24]. Retention time windows were set to ± 0.25 min. Secondary transitions were acquired if the abundance of the primary ones exceeded 200 counts. Their tMRM-specific settings were trigger window 0.3 min, trigger entrance 0 cycles and trigger delay 2 cycles.

Data analysis was performed using the Agilent MassHunter Quantitative Analysis 10.0 (for QQQ) software (Agilent Technologies, Santa Clara, California, US). As secondary transitions are not monitored continuously and may only be triggered near to the apex of the peak (especially for cpds present at low concentrations in samples), relying on ion ratios for identification is generally not recommended. An ion spectrum library was therefore generated using a sufficiently high calibration level (CAL L5), against which each compound in the samples could be scored. A library match score ≥ 90 corresponded well with the true positive identification of a compound and was therefore set as the cut-off.

2.4 Quadrupole time-of-flight screening

Data 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, in line with commercially available Agilent databases. An abundance threshold of 2000 counts was set for precursor selection. In-run mass recalibration was ensured by continuous infusion of a calibrant solution containing purine (*m/z* 121.0508) and hexakis phosphazine (*m/z* 922.0098), their respective precursor ions were excluded from fragmentation throughout the run.

The acquired features were extracted using the Agilent MassHunter Profinder software 10.0 and compared to a subset, in-house database containing 144 entries, created through the injection of certified reference standards. Three fragmentation spectra and the retention time (RT) were entered into the database for each compound. A database match was returned if the *m/z*-value of the precursor ion differed by less than 20 ppm from that of the predicted one. Any features with a peak area < 10000 counts were excluded from further analysis. The resulting list was additionally filtered on match score (≥ 50; as determined by the software's algorithm), RT (≤ 0.2 min difference with the predetermined one) 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 with the Agilent MassHunter Qualitative Analysis 10.0 software. The automated workflow extracted all features selected by auto-MS/MS and compared their exact mass and fragmentation pattern with the previously mentioned database. The identified features were additionally filtered on match score (≥ 50; as determined by the software's algorithm), RT (≤ 0.2 min difference with the predicted one) 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 feature resulting in a match for at least one workflow was included in the final list of identified analytes.

3 Results and discussion

3.1 Qualitative screening

3.1.1 Triggered multiple reaction monitoring

Validated LC-QQQ confirmation methods yielded 438 hits with concentrations above their respective LLOQs in the medico-legal samples: 125 for ADs, 99 for APs and 214 for BZDs. Using a trigger threshold of 200 counts, 370 of these cpds had tMRM library match scores ≥ 90 (Figure 1). The most commonly missed AD was mCPP (n_{total} = 16, 75% false negatives), an active metabolite of trazodone, as well as a designer drug abused in its own right [25]. Its precursor trazodone was detected in all but one sample (range 35 ng/mL – 586 ng/mL), where the concentration upon confirmation analysis was found to be lower than the LLOQ. mCPP concentrations that were not detected in the tMRM screening ranged from the LLOQ (2 ng/mL) to 45 ng/mL. The APs norquetiapine and/or 7-OH-norquetiapine gave false negative results in 16 samples (80%, range 1 ng/mL – 107 ng/mL), in all of which quetiapine and its other metabolites could be detected (range 18 ng/mL – 726 ng/mL). Norcitalopram (11 ng/mL & 16 ng/mL), normirtazapine (1 ng/mL, 5 ng/mL, 12 ng/mL & 27 ng/mL) and nortriptyline (35 ng/mL & 42 ng/mL) could not be detected at concentrations significantly higher than their respective LLOQs (2 ng/mL, 0.5 ng/mL & 10 ng/mL). However, their metabolic precursors citalopram (15 ng/mL & 16 ng/mL), mirtazapine (2 ng/mL, 11 ng/mL, 11 ng/mL & 20 ng/mL) and amitriptyline (80 ng/mL & 69 ng/mL) were detected in these instances. Lastly, norolanzapine could not be identified via tMRM in any of six samples, with concentrations found upon confirmation ranging from at the LLOQ (1 ng/mL) to as high as 180 ng/mL, although olanzapine (range 2 ng/mL – 216 ng/mL) was identified in all. Of the remaining false negative samples, only five contained cpds present at a concentration significantly different from the LLOQ and had no related compounds detected: amisulpride (22 ng/mL and 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 returned by the software. As only one sample had tested positive for prothipendyl, no conclusions could be made as to whether this is a recurring trend or a one-off miss.

The majority of false positive results were observed for etizolam (12 out of 24 false positive hits). Interference by triazolam, with which etizolam shares its precursor and product ions and of which it

is separated by a 0.2 min RT difference only, was ruled out as neither compound could be detected via the confirmation analyses. Moreover, none of the defined ion transitions could be detected in blank matrix (neat and spiked with the ISTD mix) either. The exact nature of this interference remains to be determined (Figure S1). Mirtazapine was erroneously identified in one sample. Unlike in the true positive samples tested, 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 contained mirtazapine. A last sample was falsely positive for brotizolam. For the remaining ten cases with a false positive hit, confirmation analyses picked up precursor and product ions for most cpds. However, as their calculated concentrations were close to but below the LLOQ, these hits were excluded from the analyses. Additionally, pharmacokinetically related compounds were identified and confirmed in all of these samples (Table S1). As such, these could be considered true positive identifications.

Compared to other published methods, our sample preparation reduced the volume of sample needed (200 µL vs. 1 mL for Gergov et al. and Viette et al.) and overall turn-around times (vs. SPE by Viette et al. and SLE by Ong et al.) [12,13,26]. The use of QuEChERS as published by Orfanidis et al. was discarded based upon our own poor experiences with the extraction of BZDs [24,27]. Importantly, confidence in the findings was increased significantly by the acquisition of 3 MRM transitions, according to internationally accepted guidelines (vs. 2 by Di Rago et al. and 1 by both Dresen et al. and Gergov et al.) [12,14,15]. The simple sample preparation and LC parameters of our method allow for easy and fast incorporation of other drug classes, as illustrated by in-house experience with fentanyl analogues and antiarrhythmic drugs (unpublished results). The tMRM acquisition principle would avoid potential detector saturation due to the increased number of ion transitions to be monitored. Sensitivity and specificity might need to be re-evaluated if more targets are added.

3.1.2 Quadrupole time-of-flight

80% of the analytes detected using the validated LC-QQQ methods were correctly identified by the QTOF method (Figure 1). Of those that were missed, 65% had a concentration at or around the LLOQ (selected to reflect concentrations following sub-therapeutic dosing schemes if known). For the ADs, most problems were observed for mCPP, which would generally not be detected in concentrations below 40 ng/mL (50% false negative results). However, its precursor trazodone was picked up in these instances (range < 50 ng/mL – 586 ng/mL). Similarly, missed hits for norcitalopram (range 5 ng/mL – 16 ng/mL) showed the presence of citalopram (range 15 ng/mL – 219 ng/mL) in the same samples. The pairs amitriptyline – nortriptyline and mirtazapine – normirtazapine could not be accurately detected via the QTOF method. The concentrations obtained via the confirmation methods ranged from 10 ng/mL to 25 ng/mL and from 0.5 ng/mL to 27 ng/mL, respectively. Moclobemide was missed at a concentration > 5000 ng/mL, no other samples were positive for this compound. 7-OH-norquetiapine (range 1 ng/mL – 22 ng/mL) was the most missed of the APs, although commonly at low to near-LLOQ concentrations. Quetiapine and its other metabolites (range < 1 ng/mL – > 5120 ng/mL) would be detected in those cases. Remarkably, norquetiapine was falsely negative in four samples (19%), no related compounds were detected during screening or confirmation analysis. The missed concentrations were around 11 ng/mL, the LLOQ of norquetiapine was 3 ng/mL. Norolanzapine might not be detected at concentrations as high as 122 ng/mL, but its precursor olanzapine would (range 2 ng/mL – 155 ng/mL). Lastly, amisulpride was missed in all three positive samples (concentrations 20-50 ng/mL). As an estimated less than 5% of the bioavailable dose undergoes biotransformation, no metabolites were included in the methods that could be used to indicate its presence [28]. The BZDs, with the exception of α-OH-alprazolam (range 0.6 ng/mL – 5 ng/mL), would be accurately detected. α-OH-alprazolam is reported to be present in blood at a concentration of less than 10% of that of its precursor [28]. Alprazolam itself could be identified in all samples (range 7 ng/mL – 156 ng/mL). Nordazepam and oxazepam were both missed in the same sample at respective

concentrations of 617 ng/mL and 96 ng/mL. No diazepam or temazepam were present in the sample, nor were similar issues noted for these compounds in other samples, hinting at an instrumental issue for that particular sample. Other missed BZDs all had concentrations close to their LLOQs.

The majority of false positive cases were for prothipendyl. Using the MassHunter Profinder software, no match was found with the database. However, when the fragmentation spectrum was also taken into account (MassHunter Qualitative Analysis software), prothipendyl was positively identified with a match score > 95 in a significant number of samples (n = 33). Further investigation into the nature of a potentially interfering analyte or confounding factor in the data algorithm is needed. A more strict RT criterion (the interference elutes 0.2 min earlier than the predicted RT) could also be applied. The ADs maprotiline and normaprotiline resulted in a combined six false positive findings, which could be attributed to respective interferences by amitriptyline and nortriptyline with which they share their exact mass and RT (Figure S2). Bupropion was incorrectly identified in one case that was also positive for OH-bupropion. Targeted confirmation analysis detected a signal for all of bupropion's ion transitions, but it was dismissed based upon the poor peak shape. 7-OH-quetiapine resulted in false positive hits in three samples, none of which contained quetiapine or any of its other metabolites. Two of those were also positive during confirmation analysis but the results were discarded as the calculated concentrations lay below that of the LLOQ. Similarly, six false positive results for oxazepam or temazepam could be attributed to compounds present below the LLOQ of the confirmation analysis. Lastly, diazepam, nordazepam and oxazepam all gave false positive hits in the same sample. Although likely correct identifications, confirmatory analysis did not support these findings; temazepam could not be detected via either method in that same sample. Other false positive results seemed to occur randomly (each in one or two samples only) and could not be attributed to interference by any known analyte or ISTD.

Compared to other published DDA methods, the major advantage of the presented method is in the sample preparation. Decaestecker et al. and Oberacher et al. both used more time-consuming SPE methods and required 1 mL and 2 mL of sample for their analyses, respectively [29,30]. Broecker et al. and Partridge et al. used the more simple LLE but still required 500 µL blood [31,32]. We managed to reduce the sample volume to 200 µL blood, whilst still achieving sufficient 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 post-mortem blood samples, which are frequently low in volume, analysis on multiple instruments without the need for re-extraction is highly advantageous. 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. The use of tailor-made R scripts could also speed up the data analysis and reduce analyst hands-on time [33]. 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. [34]. However, these usually require highly trained personnel – particularly when problem-solving or method modifications are needed – and therefore go beyond the scope of the current method.

3.2 Semi-quantification

To reduce time and money spent on often unnecessary accurate quantification of all compounds in any sample, screening methods ideally hold a semi-quantitative aspect. However, whereas validated, quantitative confirmation methods should display accuracies within ± 15% of the true concentration, such requirements are poorly defined for semi-quantitative purposes in international guidelines [35–37]. 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 accurate

concentration determination by confirmation methods) and the wider allowed range described by Paterson et al., we therefore deemed a deviation of ± 30% from the reference concentration (defined as the concentration determined by validated confirmation analyses) acceptable [35,38,39]. Results referred to in the sections below are expressed as average accuracy ± standard deviation. The calibration ranges were previously defined for the targeted confirmation methods and chosen to span the range from sub-therapeutic to potentially toxic *in vivo* concentrations. A tenfold dilution was successfully validated for higher concentrated samples [22–24].

3.2.1 Triggered multiple reaction monitoring

A total of 367 entries quantified by tMRM were included in the analysis, 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. The latter two groups were paired with both a structurally and RT-related ISTD for semi-quantitative purposes (Table S2).

Variable results were seen for cpds belonging to the ADs (Figure 2, Table S3). Aberrant concentrations were predominantly observed for mCPP (13% ± 112%), melitracen (55% ± 7%), norcitalopram (22% ± 13%) and sertraline (34% ± 64%). Venlafaxine displayed poor accuracies at higher concentrations (33% ± 33% for concentrations ≥ 1000 ng/mL). Its metabolite O-desmethyl-venlafaxine resulted in equally poor outcomes over the entire measured concentration range (45 ng/mL – 800 ng/mL, 31% ± 68%). For the APs, quetiapine and metabolites were the predominant cpds in the samples (Figure 3, Table S3). 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 reference value > 5120 ng/mL. Either value would be significantly higher than what is expected from normal therapeutic use and thus likely not change the overall case interpretation. 7-OH-quetiapine 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 confirmation ones (100% ± 17%), whereas the others showed larger discrepancies (116% ± 37%). Norquetiapine (34% ± 23%) and 7-OH-norquetiapine (18%) were less reliably detected in samples and coincidentally showed poor semi-quantification. Clotiapine concentrations were overestimated by 300% to 500%, regardless of the type of ISTD used. Semi-quantification of the BZDs was more reliable than for the other drug classes (Figure 4, Table S3). Only 7-amino-clonazepam resulted in significantly aberrant calculated concentrations (48% ± 21%).

Overall, the choice of ISTD strongly influenced the outcome of the results. Those cpds that did not have a labelled analogue present in the ISTD mix were generally more reliably semi-quantified using a RT-based ISTD. Of the different drug classes, the best results were observed for the BZDs. Potential differences in ionisation efficiencies might underlie these findings. The most notorious are matrix effects, originating from competition for a droplet's surface charge and from droplet precipitation due to the presence of non-volatile additives [38,40–42]. Previous publications have shown that for the current sample preparation and LC settings, no significant matrix effects arise [22–24]. 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 [38,43]. This may explain the better results for the BZDs, which are more spread out over the entire RT range and elute (on average) at higher mobile phase B percentages than the ADs and APs.

Maybe of greater significance than a purely mathematical approach, accuracy could also be evaluated from an interpretation point of view. Particularly for 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 administered in an equally high dose, and dosages are therefore tailored to a patient's needs. Result interpretation may therefore vary from low blood concentrations expected following normal therapeutic use to higher concentrations which could indicate either high dose chronic therapeutic use, moderately excessive use or excessive use, in line with the medical history amongst other things [44–48]. Provided the patient or deceased was prescribed the drugs, only around 10% of cases may have been inaccurately interpreted regardless of a deviation greater than ± 30% of the dMRM concentrations. Cases positive for venlafaxine and/or O-desmethylvenlafaxine and for sertraline should always be confirmed using fully validated confirmation methods. 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 [49,50]. Therefore, even though their semi-quantitative concentrations showed high accuracies, they should be interpreted with caution and never outside of the full scope of substances detected in a sample or of the case circumstances.

3.2.2 Quadrupole time-of-flight

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 [51–53]. For the untargeted data files as obtained with the here presented method, not enough data points were 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 and those samples with enough volume left were semi-quantitatively re-analysed. From a practical point of view, only linear trend lines were fitted to the data points, with excellent coefficients of determination ranging from 0.97 to 1.00 (Figure S3). The highest calibration levels for diazepam and trazodone significantly skewed the results and therefore were not included.

Variable outcomes were observed for the ADs (Figure S4, Table S4). Half of the citalopram concentrations were accurately determined, the remaining half were either over- or underestimated by 50%. Similar underestimations were also observed for melitracen and half of the trazodone-positive samples. For the APs, the investigated analytes were flupentixol, olanzapine and quetiapine (Figure S4, Table S4). In contrast to what had been observed for tMRM-based semi-quantification, 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 the reference ones. On the other hand, with the exception of three quetiapine concentrations classified as < 10 ng/mL (vs. reference concentrations 25 ng/mL, 49 ng/mL and 85 ng/mL, respectively), none of these findings are likely to have influenced the interpretation of the results in a forensic setting. Once more, semi-quantification of the BZDs was markedly better than for the other classes (Figure S4, Table S4). Although the highest calibrator had to be omitted for diazepam, none of the samples showed unacceptable deviations from the reference concentration. Based upon 25 samples, the average accuracy was 93% ± 15%.

Overall, the best results were obtained for the BZDs, where the order of magnitude corresponded well with that of the confirmation analysis for all four compounds (alprazolam, bromazepam, diazepam and prazepam) in all positive samples. 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 to avoid potential ion suppression (see higher) [38,43].

3.3 Considerations for routine implementation

Given the severity of a false identification in clinical or forensic analysis, it is recommended to screen any sample using a minimum of two or more methods based upon different physical or chemical principles. However, running samples on multiple methods often requires additional sample preparation – thus increasing turn-around times – and highly experienced personnel for result processing and interpretation. We sought to overcome these drawbacks by developing methods that benefit from a standardised sample preparation protocol, applicable to all investigated analytes, and minimal analyst experience, by relying on user-friendly vendor software and their standard settings.

The proposed LLE protocol was previously extensively tested against other sample preparation methods [24]. It has since been successfully implemented in routine analyses, including in the participation in external quality control and proficiency testing schemes [22–24]. It was originally created for the analysis of plasma samples, an often used matrix in therapeutic drug monitoring requiring less rigorous sample preparation and generally reflecting the unbound, biologically available fraction of the compounds [54,55]. However, the simple extraction principle was found to work well for multiple matrices, even providing sufficient clean-up for post-mortem blood samples. Inter-laboratory comparisons between samples have further proven its widespread applicability. The greatest influence on analyte concentration accuracy is expected to originate from pre-analytical issues such as inappropriate sample collection and storage rather than any steps in the analytical process.

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. The latter may be of particular 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 available, considerable time would need to be invested in their implementation (and validation) in to a targeted method [56,57]. 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 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 [57–59]. Additionally, 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 utilising an updated database can simply be re-run on the originally acquired data [57,58,60].

Semi-quantitative results are particularly useful in post-mortem casework, where amongst other factors bacterial metabolism, post-mortem redistribution and poorly established concentration-effect relationships necessitate cautious interpretation of findings in line with the case history and other available information [28,61–65]. A rough concentration estimation as obtained by the tMRM method is often equally valuable to one obtained by confirmation methods, which for the described method can be acquired after having processed the QTOF results without the need for additional sample preparation.

4 Conclusion

The qualitative performance of both screening methods (tMRM and QTOF) compared to that of the validated, confirmation methods was comparable, with 15% false negative results for the tMRM screening and 20% false negative results for the QTOF screening, generally for compounds present at low ng/mL concentrations. More false positive results were obtained for the QTOF (n = 83) versus the tMRM (n = 24) method. Exclusion of a known misidentification of prothipendyl following QTOF analysis significantly reduced these numbers. Overall, the untargeted acquisition of QTOF instruments is preferred for STA as it would allow for retrospective analysis and easier method expansion should information about new substances or more details about a given case become available.

The targeted tMRM method may provide complementary data to 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 forensic or post-mortem samples. The use of a limited number of ISTDs significantly reduces costs compared to validated confirmation methods, yet still providing highly similar concentrations. The sample preparation protocol has been preserved between both screening methods and the stability of the analytes after extraction was previously investigated for up to 72 h. Thus, positive findings following 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.

5 Conflict of Interest

The authors declare that they have no conflicts of interest.

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Figures

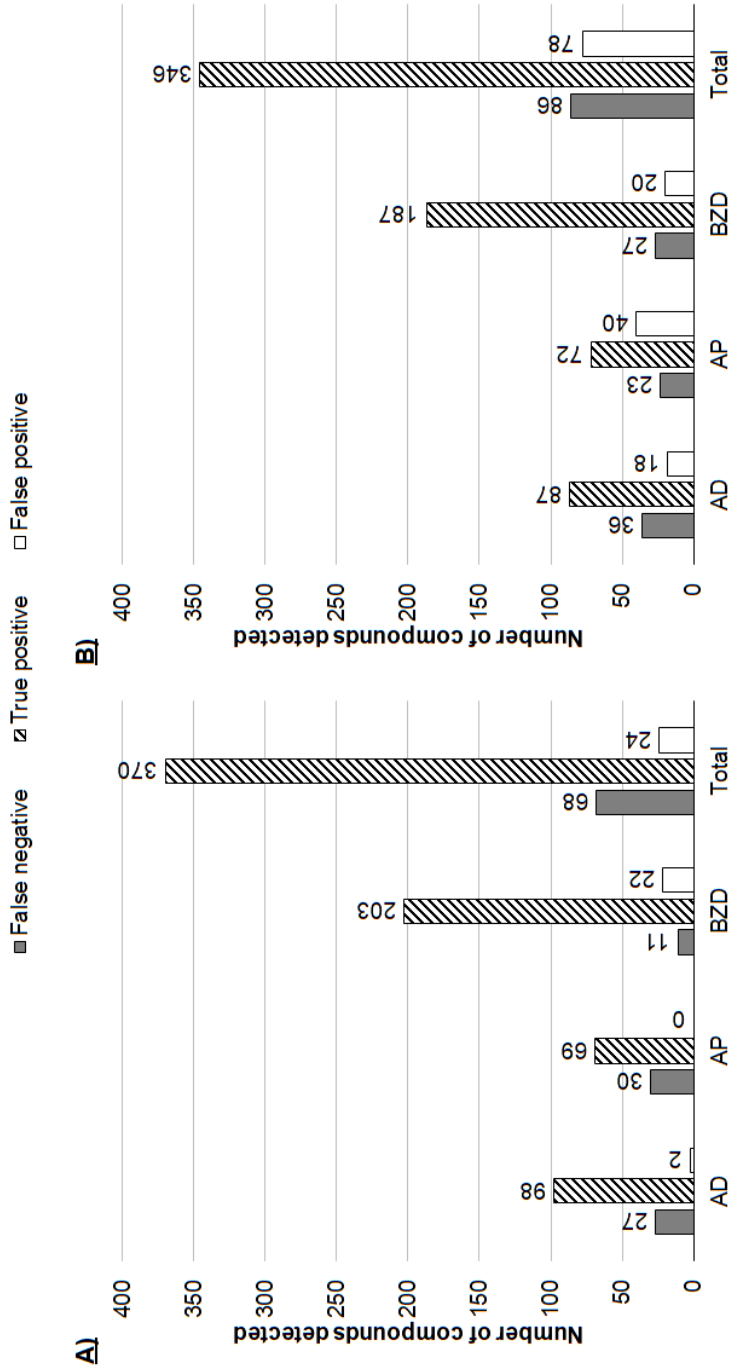

Figure 1. Screening of medico-legal samples by A) tMRM and B) QTOF analysis. Results were compared to those obtained with validated confirmation methods. The majority of compounds were correctly identified. False negative results were predominantly observed for mCPP and quetiapine metabolites – although their precursors could be detected in the same samples – or for compounds with concentrations near their respective lower limits of quantification. The higher number of false positive results for the QTOF analysis could be significantly reduced when taking into account a systematic bias for prothipendyl. Antidepressant, AD; antipsychotic, AP; benzodiazepine & Z-drug, BZD; quadrupole time-of-flight mass spectrometry, QTOF; triggered multiple reaction monitoring, tMRM.

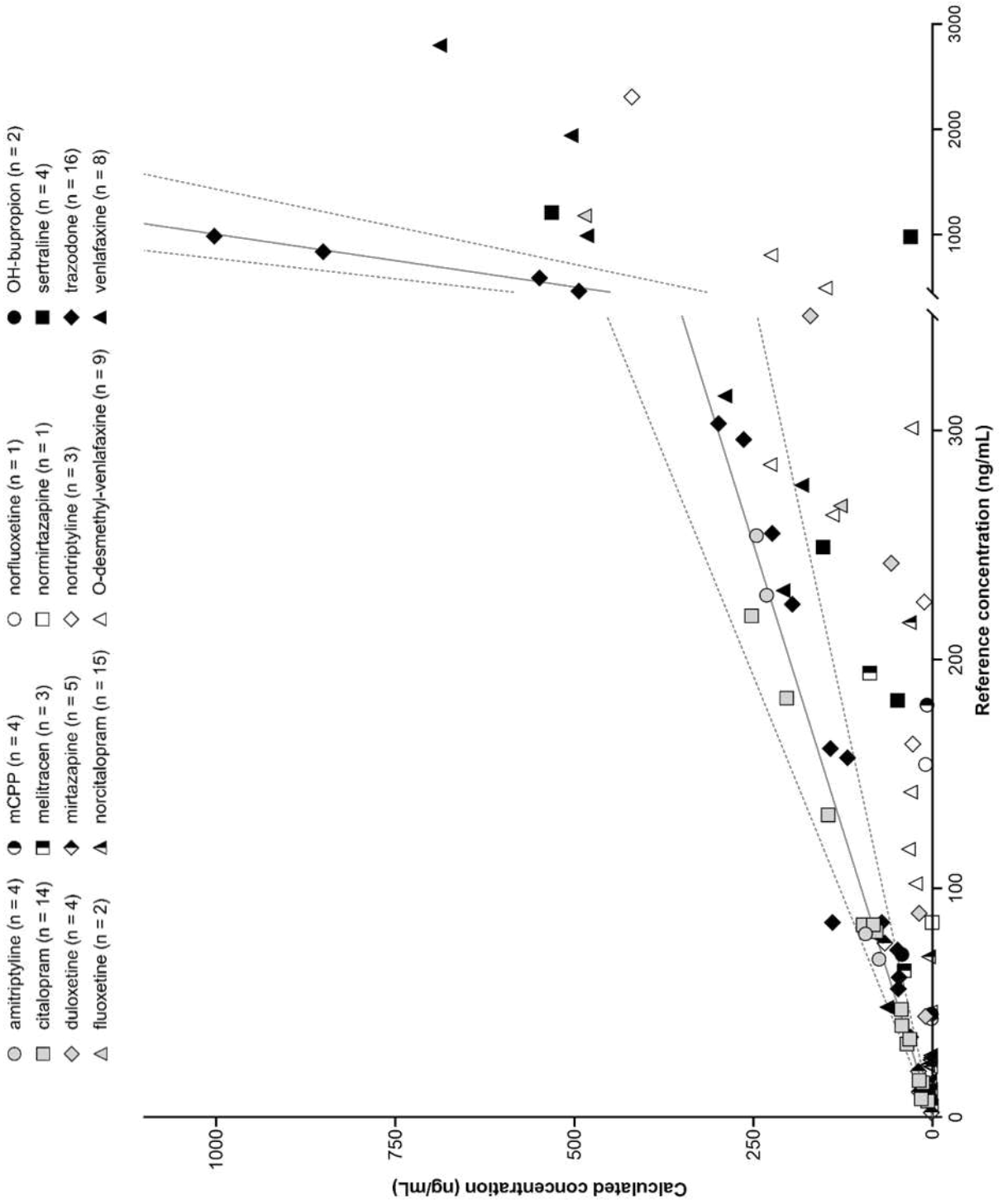

Figure 2. Semi-quantitative concentrations of the antidepressants as determined by tMRM screening. The reference concentration was obtained from validated confirmation methods. The dotted lines represent a spread of ± 30% of the accuracy compared to the reference concentration (full line). The concentrations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

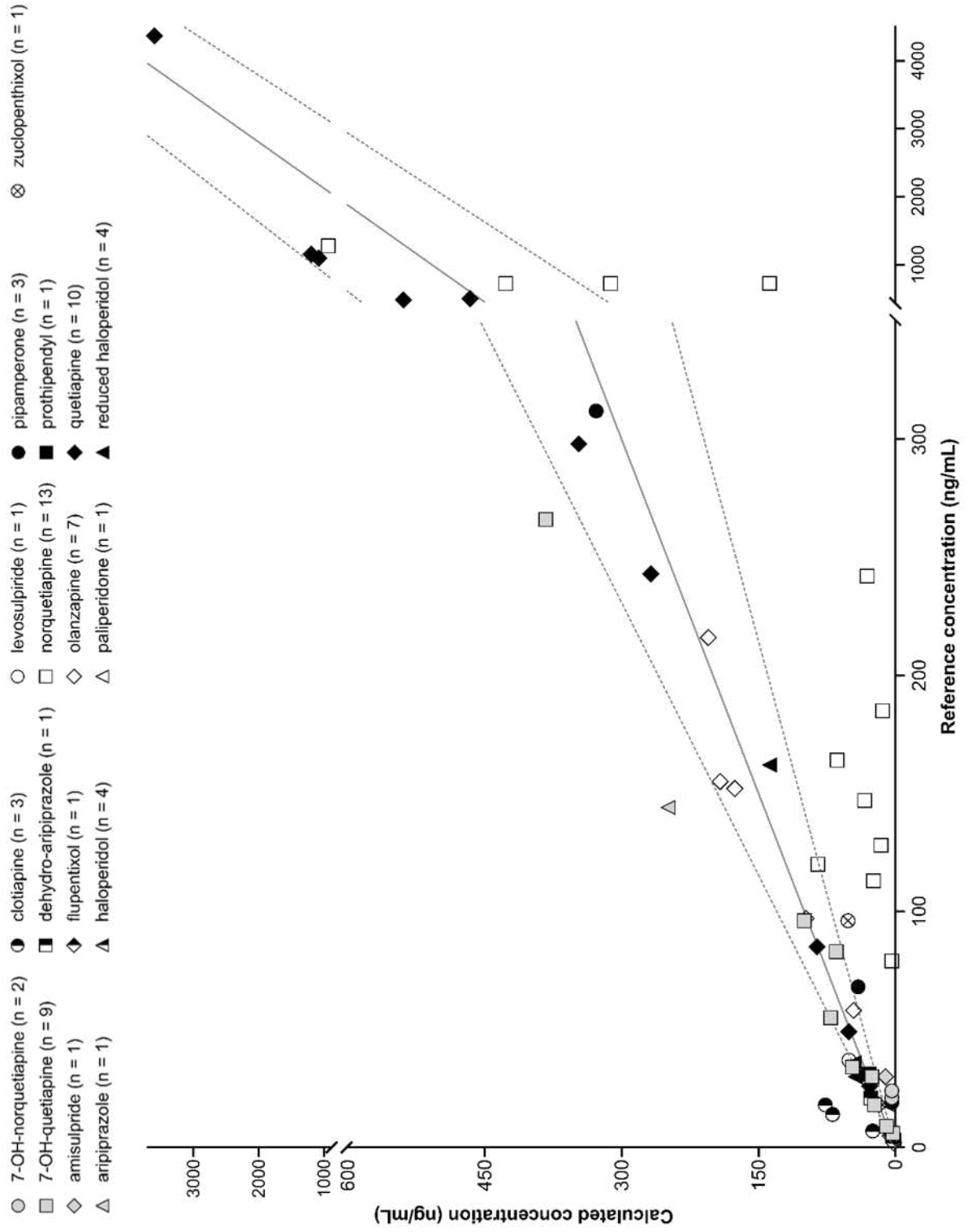

Figure 3. Semi-quantitative concentrations of the antipsychotics as determined by tMRM screening. The reference concentration was obtained from validated confirmation methods. The dotted lines represent a spread of ± 30% of the accuracy compared to the reference concentration (full line). The concentrations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

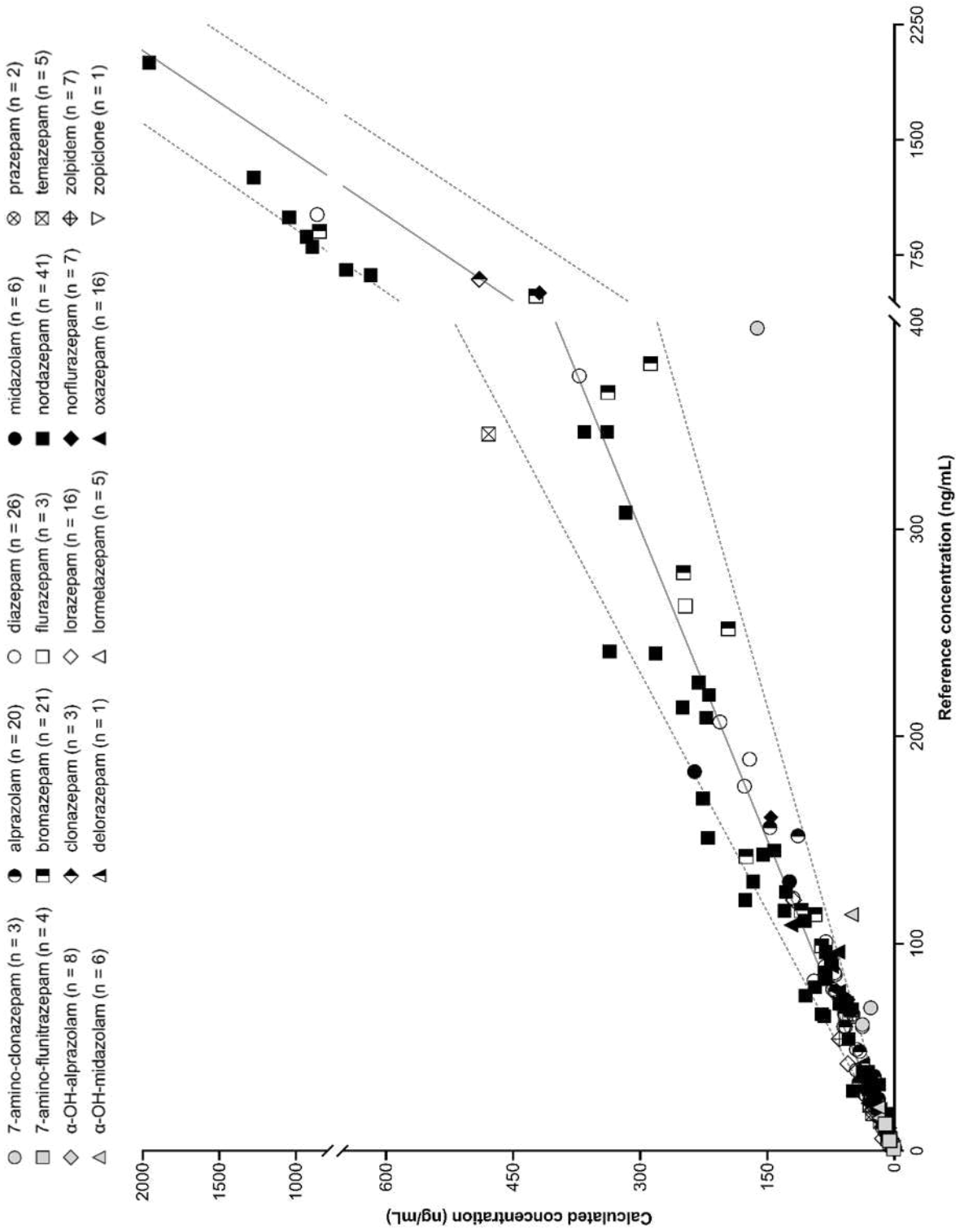

Figure 4. Semi-quantitative concentrations of the benzodiazepines and Z-drugs as determined by tMRM screening. The reference concentration was obtained from validated confirmation methods. The dotted lines represent a spread of ± 30% of the accuracy compared to the reference concentration (full line). The concentrations of compounds above the upper limit of quantification could not be accurately determined and therefore are not plotted. Triggered multiple reaction monitoring, tMRM.

Tables

Table 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. [66]. Data-dependent acquisition, DDA; data-independent acquisition, DIA; liquid chromatography, LC; mass spectrometry, MS; not applicable, N/A; (quadrupole) time-of-flight, (Q)TOF.

Acquisition MS	DDA/DIA	Data analysis n° analytes	Confidence	(Semi-)quantification	Article
TOF	N/A[a]	175	L3	no	Dalsgaard et al., 2012 [67]
TOF	N/A[a]	227	L3	no	Sundström et al., 2013 [68]
TOF	N/A[a]	50500	L4	no	Polettini et al., 2008 [69]
QTOF	DDA	> 300	L1	yes	Decaestecker et al., 2004 [29]
QTOF	DDA	320	L1	yes[d]	Partridge et al., 2018 [32]
QTOF	DDA	1208	L2a	no	Oberacher et al., 2013 [30]
QTOF	DDA	> 7500	L1 - L2a[c]	no	Broecker et al., 2010 [31]
QTOF	DIA	30	L1	no	Dalsgaard et al., 2013 [70]
QTOF	DIA	37	L1	yes	Pasin et al., 2015 [71]
QTOF	DIA	39	L1	yes	Roemmelt et al., 2015 [72]
TOF	DIA	151	L1	no	Teng et al., 2015 [73]
QTOF	DIA	> 185	L2a	yes[d]	Bidny et al., 2017 [74]
QTOF	DIA	> 250	L1	no	Krotulski et al., 2020 [75]
QTOF	DIA	256	L1	no	Pedersen et al., 2013 [70]
QTOF	DIA	> 950	L1	yes[d]	Rosano et al., 2014 [76]
QTOF	DIA	1353	L1	no	Grapp et al., 2018 [17]
QTOF	DIA	1500	L1 - L2a[c]	no	Kinyua et al., 2015 [21]
TOF	DIA	> 2000	L2a	no	Lung et al., 2016 [77]
QTOF	Both[b]	82	L1	no	Marin et al., 2015 [78]
QTOF	Both[b]	1208	L2a	no	Arnhard et al., 2015 [4]
QTOF	Both[b]	1326	L2a	no	Roemmelt et al., 2014 [79]
QTOF	**DDA**	**144**	**L1**	**yes[d]**	**Current method**

[a] Fragmentation ions were not acquired. [b] Both DIA and DDA acquisition 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.

Table 2. Psychoactive substances included in the semi-quantitative screening methods. Labelled internal standard, ISTD.

Antidepressants	Antipsychotics	Benzodiazepines & Z-drugs	ISTDs
agomelatine	7-OH-norquetiapine	3-OH-flubromazepam	alprazolam-D_5
amitriptyline	7-OH-quetiapine	4-OH-midazolam	bromazepam-D_4
atomoxetine	amisulpride	7-amino-clonazepam	citalopram-D_6
bupropion	aripiprazole	7-amino-flunitrazepam	diazepam-D_5
citalopram	asenapine	7-amino-nitrazepam	flupentixol-D_4
clomipramine	bromperidol	α-OH-alprazolam	melitracen-D_6
desipramine	chlorpromazine	α-OH-midazolam	mirtazapine-D_3
dosulepin	clotiapine	α-OH-triazolam	olanzapine-D_3
doxepin	clozapine	adinazolam	prazepam-D_5
duloxetine	dehydro-aripiprazole	alprazolam	quetiapine-D_8
fluoxetine	droperidol	bentazepam	trazodone-D_6
fluvoxamine	flupentixol	bromazepam	zopiclone-D_4
imipramine	fluphenazine	brotizolam	
maprotiline	fluspirilene	chlordiazepoxide	
mCPP	haloperidol	clobazam	
melitracen	iloperidone	clonazepam	
mianserin	levomepromazine	clonazolam	
mirtazapine	levosulpiride	cloniprazepam	
moclobemide	loxapine	clotiazepam	
norcitalopram	lurasidone	cloxazolam	
norclomipramine	norasenapine	delorazepam	
nordosulepin	norclozapine	deschloro-etizolam	
nordoxepin	norolanzapine	diazepam	
norfluoxetine	norquetiapine	diclazepam	
normaprotiline	OH-iloperidone	ethyl loflazepate	
normianserin	olanzapine	etizolam	
normirtazapine	paliperidone	flubromazepam	
nortrimipramine	perphenazine	flubromazolam	
nortriptyline	pimozide	flunitrazepam	
O-desmethyl-venlafaxine	pipamperone	flurazepam	
OH-bupropion	prothipendyl	halazepam	
opipramol	quetiapine	loprazolam	
paroxetine	reduced haloperidol	lorazepam	
reboxetine	risperidone	lormetazepam	
sertraline	sertindole	meclonazepam	
tianeptine	tiapride	medazepam	
trazodone	zuclopenthixol	metizolam	
trimipramine		midazolam	
venlafaxine		nifoxipam	
		nitrazepam	
		norclobazam	
		nordazepam	
		norflunitrazepam	
		norflurazepam	
		oxazepam	
		phenazepam	
		prazepam	
		pyrazolam	
		temazepam	
		tetrazepam	
		triazolam	
		zolpidem	
		zopiclone	