Journal of Pharmaceutical and Biomedical Analysis

Research Paper

Antidepressants detection and quantification in whole blood samples by GC-MS/MS, for forensic purposes

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Abstract

Depression is among the most prevalent psychiatric disorders of our society, leading to an increase in antidepressant drug consumption that needs to be accurately determined in whole blood samples in Forensic Toxicology Laboratories. For this purpose, this work presents a new gas chromatography tandem mass spectrometry (GC-MS/MS) method targeting the simultaneous and rapid determination of 15 common Antidepressants in whole blood: 13 Antidepressants (amitriptyline, citalopram, clomipramine, dothiepin, fluoxetine, imipramine, mianserin, mirtazapine, nortryptiline, paroxetine, sertraline, trimipramine and venlafaxine) and 1 Metabolite (N-desmethylclomipramine). Solid-phase extraction was used prior to chromatographic separation. Chromatographic and MS/MS parameters were selected to improve sensitivity, peak resolution and unequivocal identification of the eluted analyte. The detection was performed on a triple quadrupole tandem MS in selected ion monitoring (SIM) mode in tandem, using electronic impact ionization. Clomipramine-D_3 and trimipramine-D_3 were used as deutered internal standards.

The validation parameters included linearity, limits of detection, lower limit of quantification, selectivity/specificity, extraction efficiency, carry-over, precision and robustness, and followed internationally accepted guidelines. Limits of quantification and detection were lower than therapeutic and sub-therapeutic concentration ranges. Overall, the method offered good selectivity, robustness and quick response (< 16 minutes) for typical concentration ranges, both for therapeutic and lethal levels.

Keywords Antidepressants; GC-MS/MS; whole blood; Solid-phase extraction; Forensic Toxicology.

1. Introduction

Depression is one of the most prevalent psychiatric disorders in our society, characterized by poor concentration, reduced self-confidence, guilty thoughts, pessimism, disturbed sleep, ideas of self-harm or suicide [1]. It is one of the major causes of morbidity and is associated with increased mortality [2]. Its treatment usually follows pharmacotherapy, which has led to an exponential and alarming increased consumption of Antidepressants (ADs) over the past years.

There are numerous types of AD drugs available in the market. To choose the most suitable one depends on many variables, such as the particularity of depression, side effects, cost, drug-drug interactions, among others. [3]. AD drugs are typically grouped according to the neurotransmitter/receptor involved in the pharmacological action, leading to (i) first generation of ADs, including Heterocyclic ADs (tricyclic and tetracyclic antidepressants, TCAs) and Monoamine oxidase inhibitors (MAOIs); and (ii) second generation ADs, where selective serotonin re-uptake inhibitors (SSRIs) and selective serotonin and norepinephrine re-uptake inhibitors (SSNRIs) are included [3,4].

Although it may be founf in literature several analytical methods for the determination of ADs in serum, plasma, and urine, only few are applied to whole blood [5, 6, 7, 8]. Most of these methods are based on liquid chromatography (LC) approaches, using HPLC with MS [9-13] or tandem MS detection modes [5, 6, 9, 14-18], and U(H)PLC with tandem MS [19, 20]. Few of these employ UV/Diode array detection [21-24], with the inherent difficulties associated to drug identification. Some works also used gas-chromatography (GC) coupled to MS detection [7, 8, 25-28] or even nitrogen–phosphorus detection [29].

Considering that routine analytical applications must be less expensive, and since GC is, in fact, less expensive than LC coupled to mass spectrometry, GC methods seem more suitable within the forensic/clinical toxicology context [30]. Furthermore, considering the significant low blood concentration of drugs, and the legal implications behind critical results, the unequivocal identification of an AD drug becomes crucial. This is mainly achieved by the combination of the chromatographic system with a triple quadrupole detector (MS/MS), giving rise to highly selective methods, where target analytes are detected regardless of the sample matrix or co-eluting interferences. Thus, a new GC-MS/MS method would be an advantageous alternative for determining the most common AD drugs in whole blood, in a straight and simple way, achieving ultra-low detection limits and sensitive analyses in this type of samples.

The proposed method was developed and validated for 14 Antidepressants and was applied to real samples. The AD drugs were selected according to the most detected AD's at the Forensic Chemistry and Toxicology Service, National Institute of Legal Medicine and Forensic Sciences of Portugal. Thus, amitriptyline, citalopram, clomipramine, dothiepin, fluoxetine, imipramine, mianserin, mirtazapine, nortryptiline, paroxetine, sertraline, trimipramine and venlafaxine were included, along with one metabolite, *N*-desmethylclomipramine, integrating the TCA, SSRI and SSNRI groups (Fig. 1).

The concentration levels used in this study were selected according to therapeutic, toxic or lethal concentrations (Table 1) [31].

2. Experimental

2.1. Chemicals and solutions

High-purity water obtained by a Milli-Q purification system (Millipore, Bedford, MA) was used throughout the study. Amitryptiline, citalopram, clomipramine, N-desmethylclomipramine, dothiepin, fluoxetine, imipramine, mianserin, mirtazapine, nortryptiline, paroxetine, sertraline, trimipramine and venlafaxine (1 mg/mL) and deuterated internal standards (IS), clomipramine-D_3 and trimipramine-D_3 (100 µg/mL), were purchased from Cerilliant (Round Rock, Texas, USA). Methanol (MeOH, gradient grade) and ethyl acetate were supplied by Merck (Darmstadt, Germany).

Standards stock solutions of each drug, prepared in MeOH at 1 mg/mL, were stored at -20ºC. Standards solutions of the mixtures of all compounds at 5 mg/L were prepared by appropriate dilution of the stock solutions in MeOH. Single or multiple drug working solutions were prepared by accurate dilution of the previous solution in MeOH. Clomipramine-D_3 and trimipramine-D_3 were added before completing the volume with MeOH, leading to final concentrations of 5 mg/L.

2.2. Biological samples

Human whole blood was used throughout this study.

The selectivity study was conducted with *pools* of whole blood samples free from AD drugs, collected from autopsies performed at the National Institute of Legal Medicine and Forensic Sciences of Portugal, and stored at the Forensic Chemistry and Toxicology Service for analysis. These samples respected the legal deadlines for elimination and were previously analyzed by other analytical methods to confirm the absence of such drugs.

All the other validation parameters evaluation were performed using whole blood supplied from a local blood bank, Portuguese Blood Institute (IPS), and were handled according to the institute protocol and regulations concerning data privacy and sample handling.

All blood samples used in this study were stored at -20ºC, defrosted and homogenized with a vortex mixer before use.

2.3. Apparatus

Chromatographic analysis was conducted on a Bruker apparatus (gas chromatograph GC-450, coupled to a triple quadrupole mass spectrometer detector MS-300, a 1177 Split/Splitless injector port, an autosampler CP-8400 and a 5% phenyl-methyl capillary column (0.25mm I.D. × 30m length× 0.25µm film thickness - Factor Four VF 5ms). All this equipment was purchased from Bruker Daltonics. Helium (99.9995% from Air Liquide, Portugal) was maintained at 1.0mL/min in constant flow mode. The oven temperature started at 100ºC for 3 min., followed by an increase of 40ºC/min up to 200ºC and a subsequent increase of 7ºC/min to reach a final temperature of 295ºC, held for 10 min. The injector port was set at 250ºC and 10.4 psi, and the system was programmed to perform a 2.0 µL splitless injection. The total run time was 29.07 min.

The MS was operated in electron impact (EI) ionization mode, with the transfer line at 280ºC, the ionization source at 250ºC, and the manifold at 40ºC. During tandem mass spectrometric analysis, argon (99.9995% from Air Liquide, Portugal) was used as collision gas, and the pressure of collision was held at 2.0 mTorr. MS was performed in selected ion monitoring (SIM) mode, isolating parent ions from AD drugs, followed by new collision and analysis of the corresponding daughter ions. Data acquisition was performed using the software "MS workstation" version 6.9.2. Compound identification was obtained through an in-house library (NIST MS Search 2.0), which allowed a comparison of the obtained full scan mass spectra of each pure drug standard with the reference spectra.

2.4. Sample preparation

Whole blood (1mL) was diluted with water to a 5mL final volume before pre-treatment. 1 mL of blank whole blood samples were spiked with a suitable mixture of ADs solution and ISs, before dilution to 5mL. Afterwards, the samples were homogenized with a vortex mixer and centrifuged, with a Rotofix 32A centrifuge from Hettich (Buckinghamshire, England), at 4000 rpm, for 30 min.

Blood samples were pre-treated using a solid-phase extraction (SPE) procedure, for clean-up and pre-concentration, with Oasis HLB® cartridges 3cc (Waters Corp., Milford, MA, USA), connected to an automated extractor GX-271 ASPEC™ (Gilson, Inc., Middleton, WI) with a 406 Single Syringe Pump (Gilson, Inc., Middleton, WI). The cartridges were conditioned with 2 mL MeOH, followed by the addition of 2 mL deionised water. Then, the 5 mL diluted blood was passed through the cartridges at 1-2 mL/min. After isolation, cartridges were washed and rinsed with 2 mL MeOH in deionised water at 5%, and the analytes were eluted with 2 mL MeOH. Eluates were filtered with 0.45 µm syringe filters, GHP Acrodisc 13, Pall™ (Waters Corp., Milford, MA, USA), after drying in a rotary evaporator,

CentriVap Concentrator (Labconco Corp., Kansas City, Missouri), during 2 hours, at 45ºC, under vacuum. Before injection into the GC-MS/MS system, the extracts were recovered with 100 µL MeOH and then transferred into GC vials (32 × 12mm).

2.5. Method validation

For the specificity/selectivity and identification studies, 30mL of whole blood samples were used, corresponding to a *pool* of thirty blank real samples (blood samples of men/women, peripheral/cardiac blood samples, obtained *in vivo*/*post-mortem*). This *pool* was homogenized and two aliquots were prepared for analysis; the first one was analysed without addition of ADs and the second one was fortified with the group of ADs under study. The blood was fortified with analytical standards mixtures of ADs and IS's at 500 ng/mL.

The calibration curves were used to calculate LOD and LLOQ, and were performed with blank samples (Portuguese Blood Bank samples) fortified with ten different concentration levels of each AD. The concentrations ranged from 10–100 ng/mL for amitriptyline, citalopram, clomipramine, dothiepin, imipramine, mianserin, mirtazapine, trimipramine and sertraline; 20–110ng/mL for *N*-desmethylclomipramine; 30–120ng/mL for nortriptyline; 40–130ng/mL for venlafaxine; 80–170ng/mL for paroxetine; and 100–180ng/mL for fluoxetine.

The calibration curves used to assess linearity and the working range for each AD were similar to the previous ones, although using wider concentrations ranges. The upper limit was always approximately 4000 ng/mL, except for dothiepin (9910ng/mL), while the lower limit depended on the AD drug: 10ng/mL for trimipramine, mianserin, dothiepin and citalopram; 20ng/mL for imipramine and mirtazapine; 30ng/mL for fluoxetine, venlafaxine, amitriptyline, nortriptyline, clomipramine, *N*-desmethylclomipramine, and paroxetine; and 40ng/mL for sertraline.

For the extraction efficiency study, blank whole samples were spiked at different concentrations: 20 and 3000ng/mL for citalopram, imipramine, mianserin, mirtazapine and trimipramine, 20 and 7000ng/mL for dothiepin, and 40 and 3000ng/mL for amitryptiline, clomipramine, N-desmethylclomipramine, fluoxetine, nortryptiline, paroxetine, sertraline and venlafaxine. This validation parameter was designed in order to assess the extraction capability of the AD drugs whenever these are present in whole blood. Therefore, the extraction method was applied to two different batches. In the first batch, IS's and the analytes were added before the samples extraction (above mentioned pre-treatment). On the other hand, in the other batch, the ISs were added before the extraction process, while the analytes were added after this step. All these assays were performed considering two different concentrations, in triplicate for each compound. Results were obtained through a comparison between the peak area ratios of the first batch and the peak area ratios of the second batch.

Carry-over studies were accomplished during the linearity and working range evaluation, and during the extraction efficiency studies. In each of these validation parameters, blank whole samples were injected after the highest concentration standard of the analyte, namely 4000 ng/mL (approximately) and 9910 ng/mL in the study of the linearity and working range, and 3000 ng/mL and 7000 ng/mL in the extraction efficiency parameter.

The inter-day precision (intermediate precision) of 14 Antidepressants (fluoxetine, venlafaxine, amitriptyline, trimipramine, nortriptyline, mianserin, imipramine, mirtazapine, sertraline, dothiepin, citalopram, clomipramine, N-desmethylclomipramine and paroxetine) was determined at three concentration levels (30, 1000 and 1500 ng/mL) in fortified whole blood samples. Intermediate precision was calculated as relative standard deviation (RDS%), with a maximum acceptable value of 20%. Precision was further evaluated by analyzing in-house quality control (QC).

3. Results and discussion

3.1. GC-MS/MS method development

3.1.1. EI mass spectra

The EI represents an important role for mass spectrometry, since it allows the identification of a given organic compound. This identification is based on a fragmentation pattern of the compound, which works as a chemical fingerprint [32]. Thus, before any detector optimisation, the study began by an individual analysis of each standard solution, under SCAN mode. The final conditions obtained at this stage are depicted in Table 1.

For each spectrum, and to ensure higher sensitivity, the most representative ions obtained in SCAN mode were selected, in order to analyze them in SIM mode (MS_1). The choice of the selected ions for each analyte took into account some considerations, in order to allow an unambiguous identification of the compound and the inhibition of possible interferences. Ions as 207 and 281 were ignored, as they may also be the result of matrix interferences. Ions with high intensity, but low mass units (such as ion 44 and 58) were also not considered, since their fragmentation would originate still smaller fragments, which, subsequently, could be masked by the equipment analytical noise.

The precursor ion for each analyte was selected, and different collision voltages (between 2 and 50V) were applied, in order to obtain a proper voltage for a characteristic fragmentation of each precursor ion. The individual parameters used at the MS/MS detection are resumed in Table 2, while individual MS-MS spectra are shown in electronic supplementary material Figure S1.

198 The chosen mass data allows an unequivocal distinction between all ADs, with well resolved peaks in less than
199 16 minutes, even when the drugs elute very closely within time. As seen in Table 2, some of these ADs, as the pair
200 citalopram and clomipramine and, also, mianserin, nortryptiline and trimipramine, demonstrated a slight overlap between
201 the individual peaks following their elution due to their retention times. From the identification point of view, this aspect
202 has no problem, since the detection provides specific information for each compound.
203
204 ### *3.2. Method validation*
205 The applicability of the developed method was tested following a set of international standards and guidelines,
206 such as those from EUROCHEM [33], ISO (*International Organization for Standardization*) [34], ICH (*International*
207 *Conference on Harmonization*) [35], WADA (*World Anti-doping Agency*) [36], TIAFT (*The International Association of*
208 *Forensic Toxicologists*) [31], SOFT (*Society of Forensic Toxicologists*) [37] and EMA (*European Medicine Agency*)
209 [38], in order to define the acceptance and validation criteria for procedures applied in Clinical and Forensic Toxicology.
210 These cover all the necessary steps to ensure a correct identification of the compounds and appropriate quantification.
211 This validation process involved the assessment of the following parameters: selectivity/specificity and identification
212 capability, limits of detection and quantification, linearity and working range, extraction efficiency, carry-over, inter-day
213 precision and robustness. Clomipramine-D_3 and trimipramine-D_3 were used as deuterated IS compounds, since they
214 exhibit a behaviour similar to the studied analytes during the extraction process, the chromatographic separation and the
215 ionization process. It mus be guaranteed that the mass ratio between the analyte and IS is not affected by any of these
216 operations [39].
217
218 #### *3.2.1. Specificity/Selectivity and Identification Capacity*
219 Specificity and selectivity may be defined as the ability of the method for identifying, unequivocally, all the
220 target-compounds, present in the ADs mixture or in the routine samples matrix, without suffering interference from other
221 compounds that may be present in the mixture or in the analysed samples [31,40].
222 The present study was conducted using two sample pools: one employed in a direct analysis, fortified only with
223 IS's at 500 ng/mL (without addition of ADs) and the other one fortified with the group of ADs and IS's under study, at a
224 concentration of 500 ng/mL.
225 The choice of the IS for each analyte was carried out through the evaluation of the areas ratio between each AD
226 standard and the two proposed IS's (ten replicates of each analyte). Thus, clomipramine-D_3 was used to study citalopram,

clomipramine, N-desmethylclomipramine, fluoxetine, mirtazapine, nortryptiline, paroxetine and sertraline, while trimipramine-D_3 was linked to amitryptiline, dothiepin, imipramine, mianserin, trimipramine and venlafaxine.

Following WADA, TIAFT and SOFT guidelines, evaluation criteria of these results for positive identification included: (1) the absolute retention time (tr) within 2% or ±0.1min of the retention time of the same analyte in the control sample; (2) the relative retention time (rrt) within <1% difference to a positive control; (3) the three diagnostic-ions unequivocally present in the sample spectrum; (4) the value of the signal / noise ratio for the less intense diagnostic-ion greater than 3; (5) acceptance criteria of relative intensities of the three selected diagnostic-ions as follows: if the relative ion intensity in the control sample was higher than 50%, it allowed an absolute tolerance of ±10%; if this value was between 25 and 50%, a relative tolerance of ±20% was accepted; if it was between 5 and 25%, an absolute tolerance of ±5% was allowed and, finally, for relative ion intensities of ±5% or less, a relative tolerance of ±50% was used [41]. The blank samples were negative in all these acceptance criteria [31,36,37].

Figure 2 shows a representative ion chromatogram obtained in SIM-SIM mode for a mixture of ADs and ISs. it was evinced that the method has the ability to identify, unequivocally, the target-compound present in the ADs mixture or in the routine samples matrix, without suffering interference from other compounds that are present in the mixture or in the analysed samples, based both on different retention times and specific target-ions.

According to positivity criteria, all the analytes were successfully and unequivocally identified in all the spiked samples, whereas in the blank samples no analyte was identified. Therefore, the method was considered specific, selective and with a suitable identification capacity for ADs determination in blood.

3.2.2. Limits of detection and quantification

These parameters were studied in compliance with therapeutic, toxic or lethal concentrations for ADs (Table 1) and according to the previous experience within the laboratory. Concentration levels varied from the sub-therapeutic concentrations to therapeutic concentrations, without sample dilutions, in order to have a representative concentration level of each studied analyte.

Following ICH guidelines, the limit of detection (LOD) was defined as the lowest tested concentration, which allowed to fulfil the qualitative acceptance criteria, and the lower limit of quantification (LLOQ) was defined as the lowest AD concentration that could be measured precisely [coefficient of variation (CV) of less than 20%] and accurately measured (concentration within ±20% of the nominal concentration). The identification criteria of limits included a correlation coefficient higher than 0.98, the smallest peak with a signal-to-noise ratio of at least 3 and the calibrators accuracy within ±20% [35,41,42].

The obtained LOD and LLOQ values are summarized in Table 3 and were calculated according to the equations $LOD = 3.3\ \sigma/s$ and $LLOQ = 10\ \sigma/s$, respectively, where σ represents the SD of γ-intercepts and s the slope from regression analysis. The LOD and LLOQ values for the 15 analytes varied from 3.1 ng/mL to 9.4 ng/mL, respectively, for trimipramine, which was the most sensitive compound, to 9.7 ng/mL and 29.3 ng/mL, respectively, for sertraline, which was the least sensitive. Overall, the obtained LOD and LLOQ values were lower than the therapeutic values described on literature (Table 1) and, consequently, the method allows the direct determination of these drugs levels in whole blood.

3.2.3. Linearity and working range

Similarly to the previous procedure, the linearity features of the proposed method were evaluated using calibration curves with ten levels of concentration of blank fortified samples. However, for this study, the working range started at a low reference level of therapeutic concentration and finished at a higher concentration, which covered almost all lethal reported cases. The linearity was checked by least-squares linear regression analysis, traduced by the analytical data presented in Table 3.

The linear regression data summarized in Table 4 shows that the correlation coefficients (r^2) were all > 0.99 and all the γ-intercepts included the zero value for a 95% of confidence degree (ISO 8466-1) [34,42]. Generally, the linear range with lower concentration was obtained for the following ADs: citalopram, dothiepin, mianserin and trimipramine (10 ng/mL), and the higher concentration for dothiepin (9910 ng/mL). These results indicated also that the working concentration range selected could be well applied to quantify the fifteen antidepressants in whole blood samples.

3.2.4. Extraction Efficiency

According to the above mentioned approach, the obtained values for efficiency ranged from 31 to 112% for all analytes, and are summarized in Table 3. The acceptance criteria for this parameter, implemented by the previous experience within the laboratory, considered an extraction percentage between 20 and 120% for a qualitative analysis of these drugs, also based on the low limits of detection achieved. All the obtained values are in compliance with these requirements.

3.2.5. Carry-over

Carry-over is a characteristic phenomenon of an analytical method which allows the interference assessment of higher analyte concentrations in the next sample result. Due to this and following EMA guidelines, carry-over studies were accomplished, simultaneously, with the linearity and the extraction efficiency studies. The study of carry-over phenomena in the linearity parameter was developed with the analysis of a blank whole blood sample, after the injection of the highest concentration level. In the extraction efficiency parameter, blank whole blood samples were, also, injected, interchangeably, with the replicates of the highest concentration level in the two procedures (mentioned in Section 2.5.) that allowed the accomplishment of the extraction efficiency analysis.

Both assessments shown that carry-over at high concentration levels did not occur. These results confirmed the inexistence of carry-over phenomena at the analytical stage (GC-MS/MS), as desired.

3.2.6. Inter-day precision

Intermediate precision was assessed by the low and high QC samples for antidepressants. RSD% was less than 20% at low and high QC levels for all compounds, as it can be observed in Table 3.

3.2.7. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small changes in method parameters ensuring that the analytical method is reliable during use [33]. This validation process was carried out by establishing different changes, some intentionally (automated and manual extraction procedure, different blank samples, different solvents, work solutions and different volumetric measuring equipment) and some unintentionally, as to the routine operation of equipment is concerned (different chromatographic columns, liner, septa, syringe, ionization source and ion volume state, among others). All these changes shall confirm the inexistence of relevant variations in the analytical results.

The robustness of the entire method was studied by changing several parameters of the procedure (the blank whole samples, solvents, work solutions and ISs) as well as the chromatographic components (the chromatographic column, liner, septum and syringe). In brief, neither a single parameter, nor a combination of the ones changed, showed a significant influence on the results of the method.

Overall, the method proved to be robust, prevented that equipment maintenance plans and an adequate traceability of reagents and standards solutions are ensured within the real laboratory environment.

3.2.8. Real blood samples

In forensic toxicology, whole blood is the main post-mortem matrix as it provides a direct link between the compound concentration and consequent effect [8]. Thus, the use of GC-MS/MS for a positive detection of antidepressants in whole blood may be a procedure applied routinely in a forensic toxicology laboratory.

A full breakdown of some real cases, where there was a definite cause of death found at autopsy, is shown in Table 7. For each drug in the forensic material (as whole blood in this case) the corresponding therapeutic serum concentration values were evaluated.

The majority of these samples were caused by suicide (57.6%), followed by road traffic accidents (18.2%) and natural causes (15.2%). Also, some of these samples represent undetermined causes (9.1%). The general age distribution revealed that the cases were observed mostly between 61 and 85 years (60.6%). For the ages between 43 and 59 years, the percentage of death cases was 24.2% and for the ages between 26 and 38 years 15.2% of the cases were recorded.

Around 52% of these cases were men and around 48% were women. Almost all the results revealed to be between the therapeutic range, with the exception of four cases, namely case number 7, 14, 20 and 22, which presented values above the therapeutic range and which correspond to causes of deaths in men. The predominant drug was sertraline (30.3%), followed by venlafaxine (21.2%) and mirtazapine (12.1%). The other drugs are present at percentages below 10%.

3.2.9. Perspective

A simple, sensitive and specific GC-MS/MS method for the determination of antidepressants in whole blood, after SPE treatment, was developed and validated according to international guidelines.

Overall, the validation parameters evaluation, both qualitative and quantitative, confirmed the method suitability to be applicable in routine toxicological analysis, since it allowed higher sensitivity when compared to other analytical techniques such as GC-MS and LC-MS. On the other hand, this method has shown to be dynamic, with further evolution perspectives and possibilities, such as adding other compounds, metabolites studies and characterization, or alternative internal standards addition.

3. Conclusion

The combination of GC with a triple quadrupole detector (GC-MS/MS) make it one of the most powerful analytical techniques for target compounds detection and quantitation in complex matrices. MS/MS is a highly selective

mass spectrometric technique, whereby target analytes are detected regardless of the sample matrix or co-eluting interferences and is a technique that permits to achieve ultra-low detection limits and sensitive analyses in this type of samples.

In this study, an analytical methodology was developed, in whole blood samples, to determine the retention times and their mass spectrum characterization, and study of acquisition parameters for 14 Antidepressants and 2 deuterated internal standards. It was possible to achieve a perfect identification and separation of all the studied compounds, by the retention time assessment, whose values were comprised between 8.48 and 15.86 minutes, and their mass spectrum acquired in SIM-SIM mode. The method was found to be specific and selective, with suitable LOD and LLOQ for routine within therapeutic, toxic and lethal levels, and accurate regarding the extraction efficiencies obtained with real samples.

Globally, the method can be applied in a forensic laboratory routine, because the studied parameters, qualitative and quantitative, confirmed the method suitability for this purpose, and when compared with other analytical techniques, such as GC/MS and HPLC/MS, used for ADs detection, it shown higher sensitive and acceptable results.

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Table 1. Reference values for therapeutic, toxic and lethal ranges for ADs in serum [31].

Compound	Reference range (mg/L)		
	Therapeutic	**Toxic**	**Lethal**
Fluoxetine	0.15 – 0.5	2	1.3 – 6.8
Venlafaxine	*sum* 0.25 – 0.75	*sum* 1 – 1.5	6.6 [b)]
Amitriptyline	0.05 – 0.3	—	—
Trimipramine	0.01 – 0.3	0.5	8.7 [b)]
Nortriptyline	0.05 [b)] / 0.075 – 0.25	> 0.25	1 - 3
Mianserin	0.015 – 0.07 / 0.14 [b)]	0.5 - 5	—
Imipramine	0.045 – 0.15	0.4 – 0.5	2
Mirtazapine	0.02 - 0.1 / -0.3 [b)]	—	—
Sertraline	0.05 – 0.25 / -0.5 [b)]	0.29 [b)]; 1.6 [b)]	—
Dothiepin	0.02 – 0.15 / 0.4 [b)]	0.8	1 [b)] / 5 - 19
Citalopram [a)]	0.02 – 0.2	—	0.5
Clomipramine	0.02 [b)]/ 0.09 – 0.25	—	—
N-Desmethylclomipramine	*sum* 0.15 – 0.55	*sum* 0.4	*sum* 1 - 2
Paroxetine	0.01-0.075/0.1 [b)] e 0.015 [c)]-0.15 [c)]/0.25 [b), c)]	0.35 – 0.4	—

Sum: included metabolite concentration; a) plasma concentration range; b) *Case Reports* values published in scientific papers about Forensic Toxicology; c) values for samples collected 1-2 hours following to substance administration.

Table 2. Acquisition parameters

Compound name	Retention time (min)	Ions selected in SIM mode (*m/z*)	MS_1 (*m/z*)	Collision Energy (V)	MS_2(*m/z*)		Retention time (min)
					Qualifier Ion 1	Qualifier Ion 2	
Fluoxetine	8.48	162, 183, 309	162	20	112	143	8.47
Venlafaxine	10.59	134, 135, 179	134	20	91	119	10.59
Amitriptyline	11.74	202, 203, 215	215	14	202	213	11.75
Trimipramine	11.92	193, 208, 294	294	10	84	99	11.92
Nortriptyline	11.94	202, 203, 220	202	25	176	200	11.95
Mianserin	11.96	193, 249, 264	193	20	165	178	11.98
Imipramine	12.04	193, 234, 280	234	20	117	218	12.04
Mirtazapine	12.41	195, 208, 265	195	30	151	167	12.42
Sertraline	13.60	159, 262, 274	262	15	116	227	13.61
Dothiepin	13.77	202, 203, 204	202	30	150	175	13.79
Citalopram	13.95	208, 221, 238	238	15	183	218	13.97
Clomipramine	14.02	268, 269, 314	314	10	58	85	14.05
N-Desmethylclomipramine	14.32	229, 268, 300	268	20	217	252	14.34
Paroxetine	15.86	138, 192, 329	192	15	70	135	15.87
IS: Trimipramine-d3	11.89	196, 211, 297	297	10	87	102	11.89
IS: Clomipramine-d3	14.01	271, 272, 317	317	10	61	88	14.00

Table 3. Method analytical validation data.

Analytes	LOD and LLOQ data				Linearity data			Extraction Efficiency (%)	Inter-day precision (RSD%)
	Concentration Range (ng/mL)	R squared	LOD (ng/mL)	LLOQ (ng/mL)	Linear Range (ng/mL)	Intercept	R squared		
Fluoxetine	100-180	0.9944	7.1	21.6	30-3990		0.994	67	16.6
Venlafaxine	40-130	0.9935	9.3	28.3	30-3990	-44.943; 15.015	0.992	98	11.6
Amitriptyline	10-100	0.9982	4.0	12.1	30-3990	-0.018; 0.357	0.999	96	10.2
Trimipramine	10-100	0.9989	3.1	9.4	10-3970	-0.208; 0.256	0.999	81	10.0
Nortriptyline	30-120	0.9980	5.2	15.6	30-3990	-0.065; 0.893	0.998	42	14.4
Mianserin	10-100	0.9980	3.9	11.7	10-3970	-1.101; 4.042	0.997	82	10.2
Imipramine	10-100	0.9991	3.3	9.9	20-3980	-5.914; 2.005	0.995	87	8.1
Mirtazapine	10-100	0.9992	3.3	10.0	20-3980	-38.298; 6.090	0.997	84	12.4
Sertraline	10-100	0.9932	9.7	29.3	40-4000	-3.045; 0.617	0.997	49	12.1
Dothiepin	10-100	0.9964	5.2	15.9	10-9910	-0.062; 1.782	0.994	93	13.5
Citalopram	10-100	0.9977	5.0	15.2	10-3970	-0.102; 0.499	0.995	88	13.6
Clomipramine	10-100	0.9953	7.1	21.5	30-3990	-0.051; 0.570	0.998	79	8.9
N-Desmethylclomipramine	20-110	0.9939	6.8	20.7	30-3990	-0.062; 1.782	0.992	41	11.1
Paroxetine	80-170	0.9915	9.3	28.2	30-3990	-0.178; 7.322	0.994	53	15.3

y: peak area ratio of analyte injected amount versus analyte to IS; *x*: analyte concentration (ng/mL)

Table 4. Cases of Antidepressant concentrations in real blood samples measured in *postmortem* samples (n=33).

Case	Gender	Age	Cause of death	Drug	Concentration detected (ng/mL)
1	Male	26	Accident	Venlafaxine	655
2	Male	33	Suicide	Mirtazapine	179
3	Female	36	Undetermined	Clomipramine	189
4	Male	38	Suicide	Citalopram	157
5	Male	38	Suicide	Fluoxetine	372
6	Male	43	Natural	Venlafaxine	200
7	Male	44	Suicide	Trimipramine	1280
8	Female	45	Suicide	Venlafaxine	90
9	Male	47	Natural	Amitriptiline	163
10	Female	48	Suicide	Fluoxetine	368
11	Female	48	Suicide	Venlafaxine	127
12	Male	57	Suicide	Venlafaxine	80
13	Female	59	Suicide	Sertraline	104
14	Male	61	Suicide	Citalopram	580
15	Female	61	Accident	Venlafaxine	124
16	Male	64	Suicide	Fluoxetine	137
17	Female	64	Suicide	Sertraline	114
18	Female	65	Accident	Maprotiline	565
19	Female	68	Suicide	Mirtazapine	196
20	Male	70	Natural	Citalopram	770
21	Male	72	Suicide	Sertraline	106
22	Male	73	Accident	Sertraline	690
23	Female	73	Suicide	Sertraline	365
24	Male	74	Suicide	Sertraline	107
25	Female	74	Suicide	Amitriptiline	222
26	Female	77	Suicide	Mianserine	132
27	Male	78	Undetermined	Mirtazapine	60
28	Male	78	Accident	Sertraline	122
29	Female	78	Suicide	Sertraline	160
30	Female	78	Natural	Venlafaxine	478
31	Male	81	Accident	Sertraline	408
32	Female	81	Undetermined	Mirtazapine	172
33	Female	85	Natural	Sertraline	106

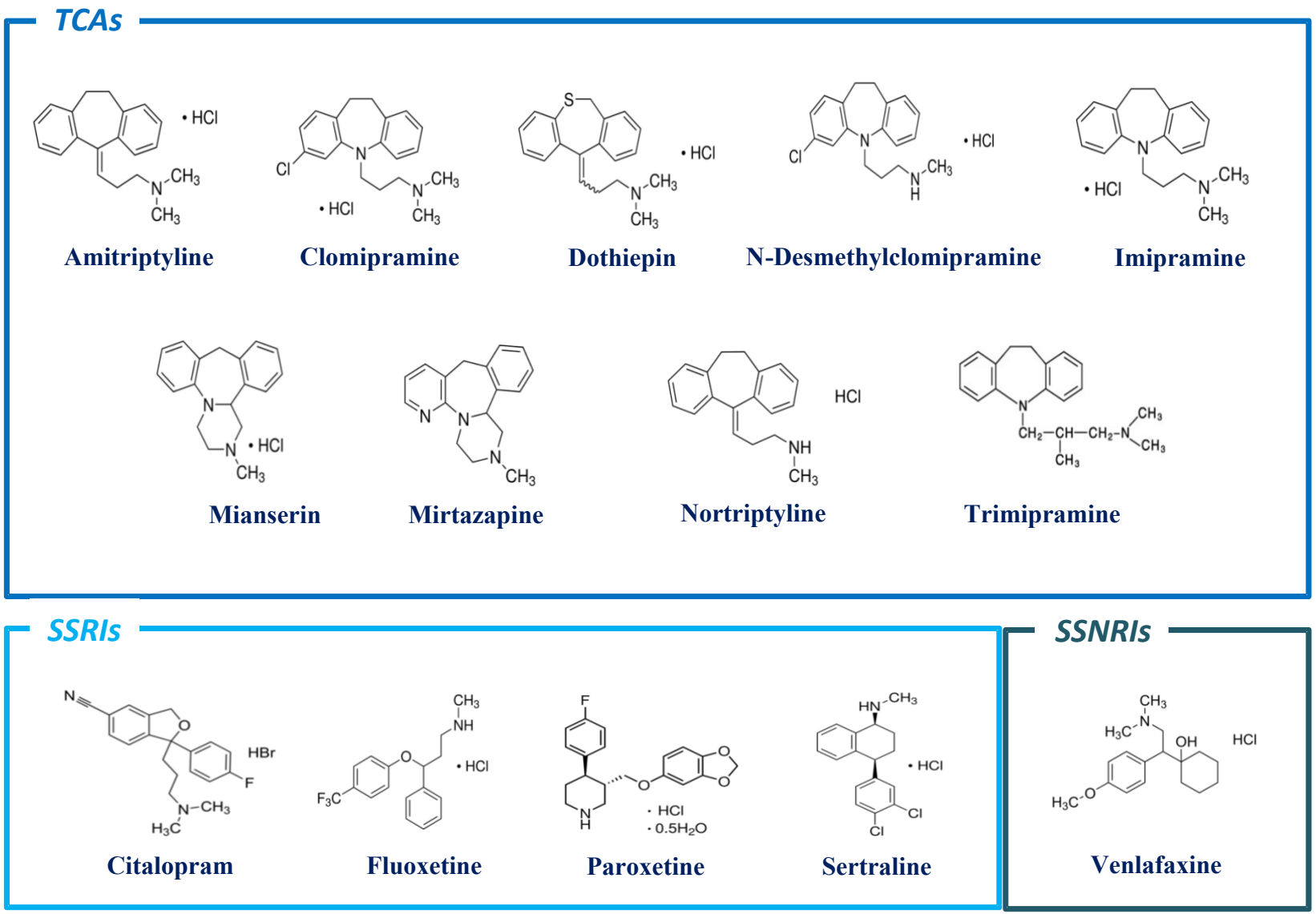


Fig. 1 - Chemical structures of the studied ADs

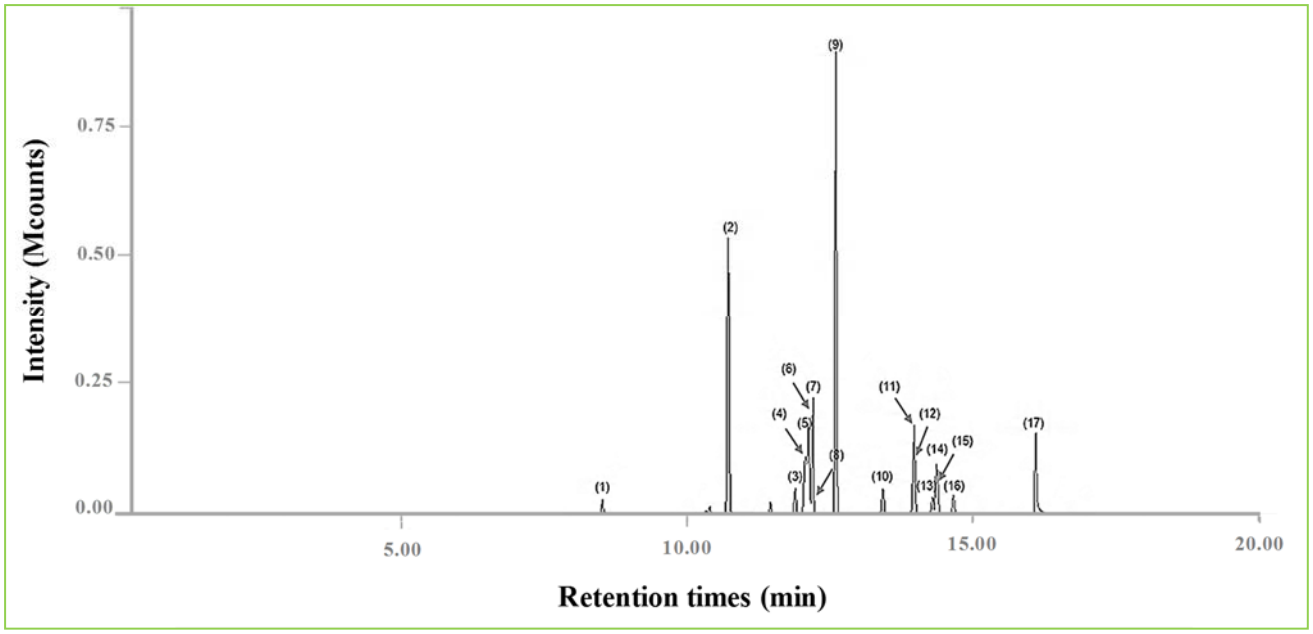


Fig. 2 GC-MS/MS extracted ion chromatogram, in SIM-SIM mode, for 15 ADs: (1) fluoxetine, (2) venlafaxine, (3) amitriptyline, (5) trimipramine, (6) nortriptyline, (7) mianserin, (8) imipramine, (9) mirtazapine, (10) maprotiline, (11) sertraline, (12) dothiepin, (13) citalopram, (15) clomipramine, (16) N-desmethylclomipramine, (17) paroxetine and 2 ISs: (4) trimipramine–*d3* and (14) clomipramine-*d3*.

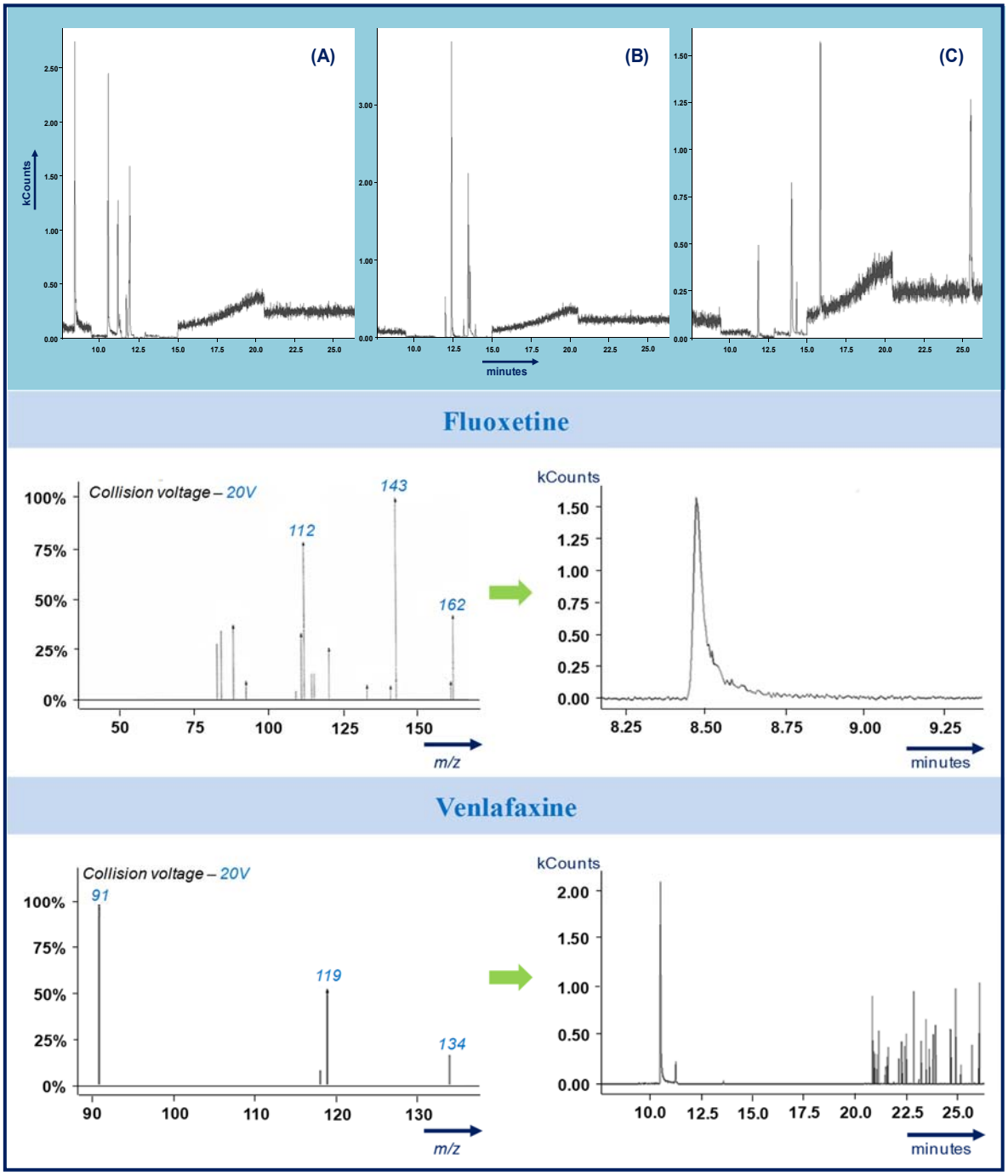


Fig. S1 Chromatograms of ADs mixtures. (**A**) mix with Amitriptyline, Fluoxetine, Imipramine, Venlafaxine, Mianserin, Nortriptyline and Trimipramine; (**B**) mix with Citalopram, Dothiepin, Maprotiline, Mirtazapine and Sertraline; (**C**) mix with Clomipramine, Clomipramine-*d3*, N-Desmethylclomipramine, Trimipramine-*d3* and Paroxetine, and the correspondents spectra of each analyte.

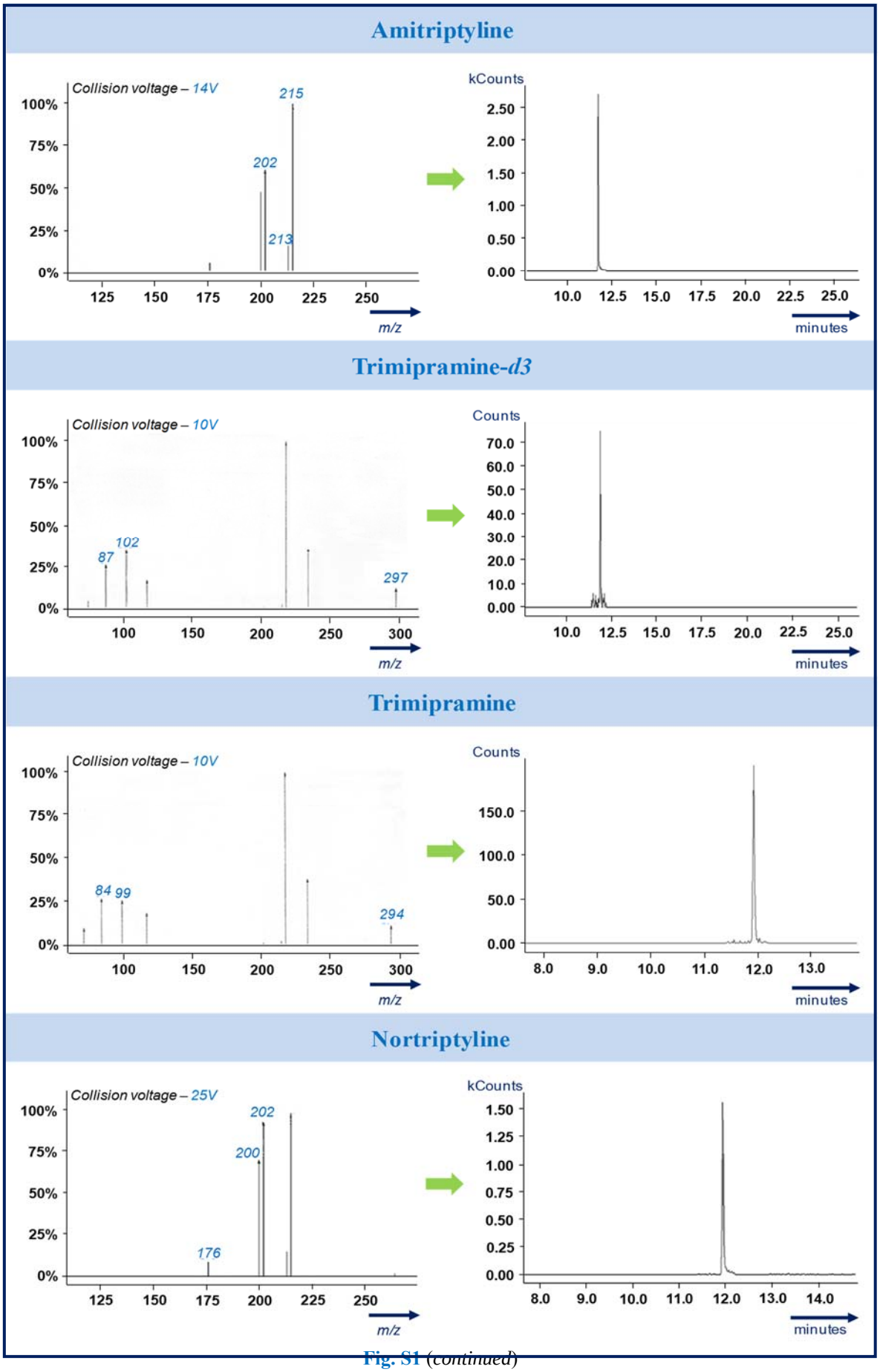
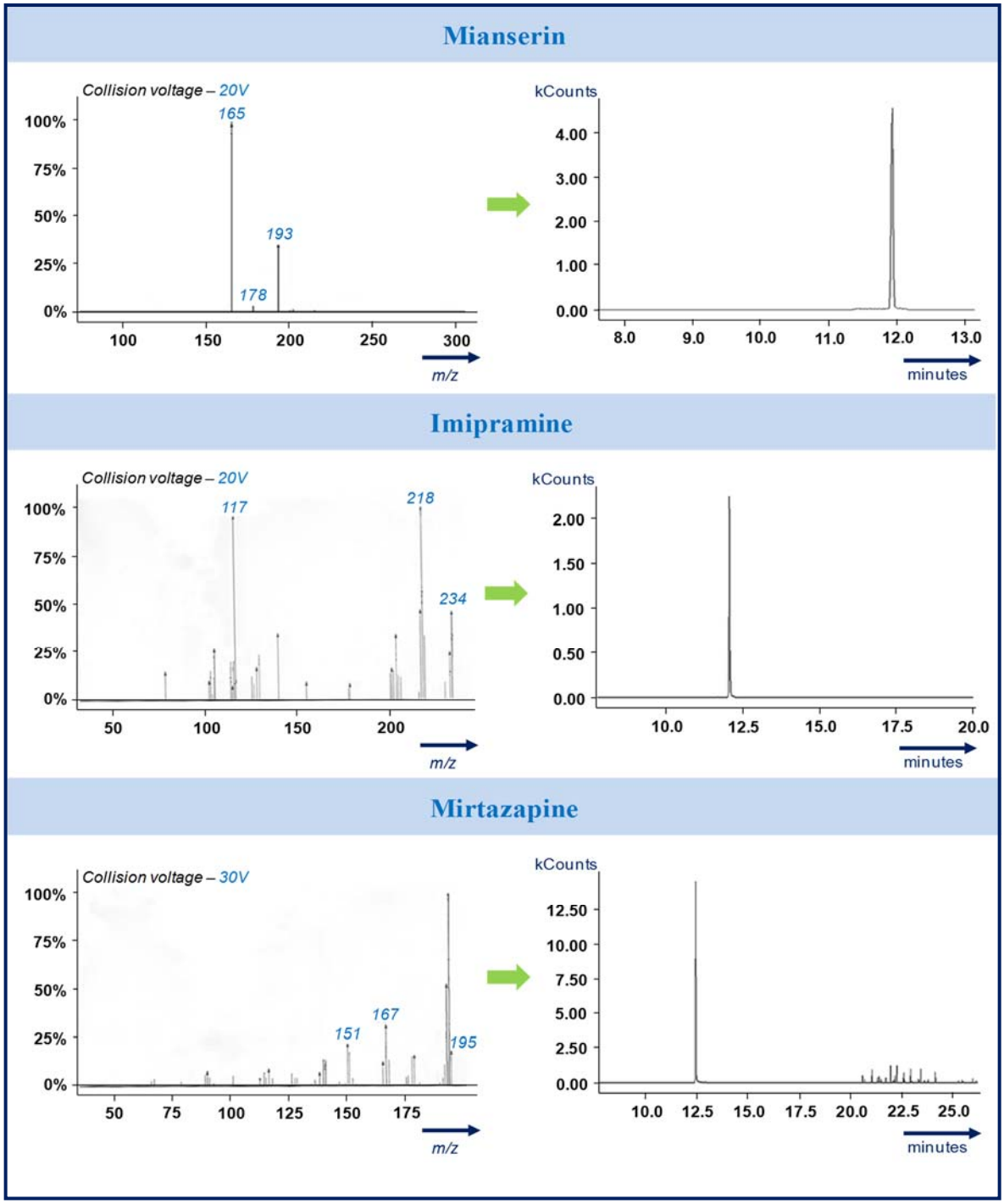


Fig. S1 (*continued*)



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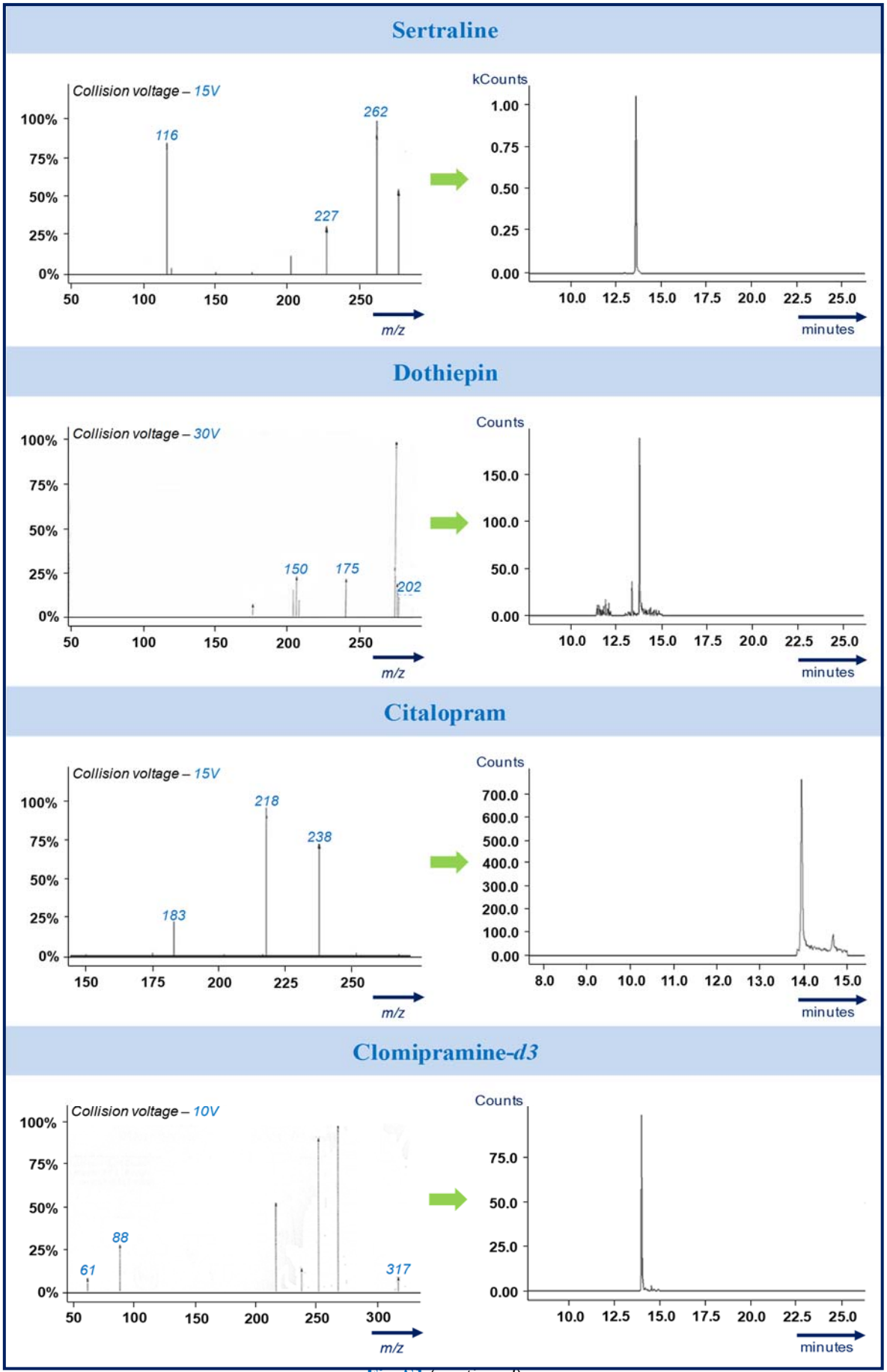

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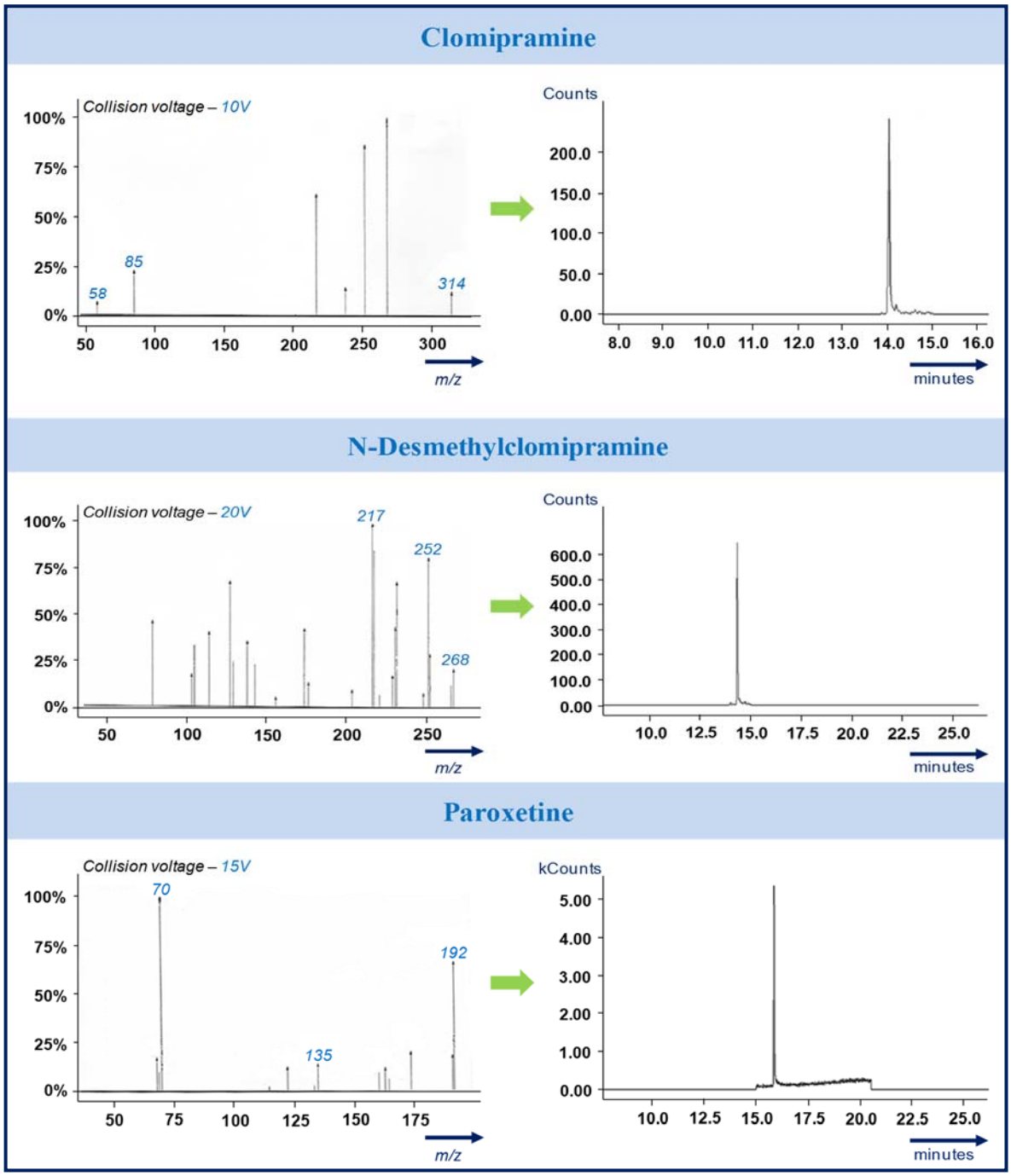

Fig. S1 (*continued*)