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Chromatographic determination of antidepressants in plasma and saliva: Towards non-invasive therapeutic monitoring

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A R T I C L E I N F O

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A B S T R A C T

Drug monitoring of antidepressants in plasma and oral fluid represents a valuable tool in clinical practice, enabling the optimisation of treatment efficacy and the reduction of adverse effects. Given the significant interindividual variability in antidepressant response—driven by factors such as metabolism, drug-drug interactions, and adherence to therapy—drug monitoring facilitates dose adjustment based on measured drug concentrations, ensuring levels remain within the therapeutic window.

This study aimed at developing and validating a robust, rapid, and sensitive method for the simultaneous quantification of 21 selected antidepressants and their metabolites in only 100 μL of plasma and oral fluid. Sample preparation was performed using a simple protein precipitation protocol, followed by analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method was validated in accordance with internationally accepted bioanalytical guidelines, demonstrating linearity over the concentration range of 0.98–1000 ng/mL. Limits of quantification were established at 0.98 ng/mL for all analytes across both matrices.

The extraction procedure yielded high recovery rates, and the method showed excellent selectivity, sensitivity, precision, and accuracy, confirming its suitability for routine toxicological applications. The validated method was successfully applied to 142 paired authentic plasma and oral fluid specimens from patients undergoing antidepressant therapy. Antidepressant concentrations were determined in both matrices, and treatment adherence was considered high, being confirmed in 88.7 % of patients. Correlation analysis between plasma and

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oral fluid concentrations produced promising results for several of the compounds under investigation, reinforcing the potential utility of oral fluid as a non-invasive alternative matrix in drug monitoring.

1. Introduction

Depression is a severe and chronic mental health condition, predicted by the World Health Organization to become the second leading cause of global disease burden. Among the various therapeutic strategies, the use of antidepressants remains the most effective and widely adopted approach. In recent decades, their prescription has significantly increased—not only for depression but also for other psychiatric disorders [1–5]. However, the co-administration of these medications with other drugs introduces the potential for drug interactions, influenced by factors such as interindividual variability, uncertain dosing, and narrow therapeutic windows.

For these reasons, drug monitoring has become an essential tool in clinical practice. Drug monitoring ensures patient safety, supports treatment adherence, and facilitates dose individualisation. Monitoring antidepressant levels allows for tailored adjustments, helping to reduce costs and resource use, prevent non-adherence or treatment failure, and minimise adverse effects—ultimately improving therapeutic outcomes and patients' quality of life. Since individuals metabolise antidepressants differently, drug monitoring based on plasma or oral fluid concentrations ensures that drug levels remain within the therapeutic window [6–10].

Oral fluid is emerging as a promising alternative to plasma due to its non-invasive and straightforward collection, lower risk of adulteration, and shorter detection window—period of time after drug intake during which the drug (or its metabolites) can be reliably detected—which provides a better correlation with pharmacological effects. Although advantageous, oral fluid presents limitations, including limited sample volume, physicochemical factors affecting drug diffusion (e.g., pH, molecular weight, lipid solubility, pKa, and protein binding), and potential contamination leading to falsely elevated concentrations. However, its advantages makes it particularly suitable for frequent monitoring, especially in vulnerable populations or large-scale epidemiological studies. Nonetheless, analytical validation is crucial to establish reliable correlations between drug concentrations in oral fluid and plasma, as not all compounds exhibit comparable behaviour in these matrices [11–15]. Plasma, on the other hand, remains one of the most widely used biological specimens in drug monitoring and other clinical applications. Like oral fluid, it provides a relatively short detection window, enabling the correlation of drug levels with clinical symptoms or patient condition. Nevertheless, its invasiveness, requirement for trained personnel, and infection risk have driven the search for alternative matrices [8,14,16–18].

In addition to therapeutic monitoring, antidepressants are sometimes associated with misuse or overdose, whether accidental or intentional. This underscores the need for robust analytical methodologies capable of quantifying both parent drugs and metabolites in biological matrices for use in clinical and forensic toxicology [19–21]. When developing such methods, the isolation and concentration of analytes from biological matrices is a critical step. Commonly used extraction techniques include liquid-liquid extraction (LLE) [5,22–24], solid-phase extraction (SPE) [5,25–31], and protein precipitation [32–36]. The selection of an appropriate chromatographic method for detection is also vital, with various techniques reported, including gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) [37–41], or tandem mass spectrometry (MS/MS) [28,35,36,42–46]. More recently, time-of-flight (TOF) [47] and quadrupole time-of-flight (QTOF) [48] mass spectrometry have also been employed. Nevertheless, LC-MS and LC-MS/MS remain the preferred methods due to their high sensitivity, specificity, robustness, and ability to achieve low limits of quantification.

This study presents a validated LC-MS/MS methodology for the simultaneous identification and quantification of the most commonly prescribed antidepressants and metabolites—including amitriptyline, nortriptyline, bupropion, citalopram, desmethylcitalopram, clomipramine, desmethylclomipramine, duloxetine, fluoxetine, norfluoxetine, fluvoxamine, maprotiline, mirtazapine, *N*-demethylmirtazapine, paroxetine, sertraline, desmethylsertraline, trazodone, *meta*-chlorophenylpiperazine, venlafaxine, and *O*-desmethylvenlafaxine—using only 100 μL of plasma or oral fluid. The extraction procedure was based on protein precipitation, followed by LC-MS/MS analysis.

The method was applied to 142 paired samples (plasma and oral fluid) collected from patients undergoing antidepressant therapy at various healthcare institutions: Centro Hospitalar Universitário Cova da Beira, Unidade Local de Saúde da Guarda, and Casa de Saúde Bento Menni (Irmãs Hospitaleiras) da Guarda. The results demonstrate that this method is suitable for routine clinical and forensic toxicology, allowing for the reliable correlation of antidepressant and metabolite concentrations across matrices. Furthermore, the findings reinforce the potential of oral fluid as an alternative matrix for assessing treatment adherence and supporting personalised therapeutic monitoring.

2. Material and methods

2.1. Reagents and standards

Acetonitrile, methanol and water, LC–MS grade (≥99.9 %), were purchased from Honeywell Riedel-de-Haën™ (Seelze, Germany).

Certified analytical standards of amitriptyline (AMT), nortriptyline (NTP), bupropion (BUP), citalopram (CIT), desmethylcitalopram (DCIT), clomipramine (CMI), desmethylclomipramine (DCMI), duloxetine (DUL), fluoxetine (FLX), norfluoxetine (NFLX), fluvoxamine (FLV), maprotiline (MPT), mirtazapine (MTZ), *N*-demethylmirtazapine (DMTZ), paroxetine (PXT), sertraline (SRT), desmethylsertraline (DSRT), trazodone (TRZ), *meta*-chlorophenylpiperazine (m-CPP), venlafaxine (VLX) and *O*-desmethylvenlafaxine (DVLX) were acquired from Merck Portugal (Algés, Portugal), as well as the internal standard of clomipramine-d3 (CMI-d3). All analytical standards were purchased at a concentration of 1 mg/mL. The working solution was prepared by diluting the stock solutions with methanol:water (50:50, *v*/v) to a final concentration of 1000 ng/mL. A separate working solution of the internal standard was prepared at a concentration of 1 μg/mL, also using methanol:water (50:50, v/v). All solutions were stored in amber borosilicate glass vials, protected from light, at −20 °C.

2.2. Biological samples

Blank plasma and oral fluid samples used in all experiments for the present study were obtained from laboratory personnel. Authentic specimens of plasma and oral fluid were collected from patients undergoing treatment with the selected antidepressants at Centro Hospitalar Cova da Beira, Casa de Saúde Bento Menni - Irmãs Hospitaleiras (Guarda), and Unidade Local de Saúde da Guarda. For plasma separation, blood samples were collected in EDTA vacutainers and centrifuged for 10 min at 1500 ×*g*. Oral fluid samples were obtained by passive drooling (spitting) into disposable polypropylene tubes. All specimens were stored at −20 °C until analysis.

2.3. Liquid chromatographic and mass spectrometric conditions

For chromatographic analysis, an ExionLC™ AC liquid chromatography system (SCIEX, Darmstadt, Germany), coupled with a QTrap®

6500+ mass spectrometer (SCIEX, Darmstadt, Germany), was employed. Analyte separation was achieved using a Waters™ Acquity UPLC® HSS T3 column (1.8 μm, 2.1 mm × 100 mm; Waters, France).

The mobile phases consisted of LC-MS grade water (A) and LC-MS grade methanol (B), with a flow rate of 0.4 mL/min. The gradient elution began at 90 % A and 10 % B, held for 50 s, followed by a linear shift to 5 % A and 95 % B over 8 min. This composition was maintained for 3 min before returning to the initial conditions at 11 min, followed by a re-equilibration phase lasting 3 min. The total run time was 14 min. The column temperature was maintained at 45 °C.

Samples were analysed in positive ionisation mode using multiple reaction monitoring (MRM), monitoring two transitions per analyte and one for the internal standard. Instrumental settings included an ion spray voltage of 4.5 kV, source temperature of 250 °C, ion source gases 1 and 2 set at 60, and curtain gas at 35. Nitrogen was used as the collision gas.

Data acquisition and processing were conducted using Analyst® Software version 1.7 and SCIEX OS version 2.1 (SCIEX, Darmstadt, Germany).

Table 1 presents the detection criteria for each antidepressant, including retention times, MRM transitions, and specific LC–MS/MS parameters.

2.4. Sample preparation

For the extraction of plasma and oral fluid specimens, 100 μL of sample was transferred into plastic Eppendorf vials and mixed with 100 μL of a methanol:water (50:50, *v*/v) solution. Subsequently, 10 μL of internal standard at a concentration of 1 μg/mL was added. Under agitation, 900 μL of ice-cold acetonitrile was added dropwise. The samples were then centrifuged at 21,100 ×*g* for 5 min at 5 °C. Following centrifugation, 50 μL of the supernatant was transferred into polypropylene autosampler vials. An aliquot of 3 μL of the final extract was injected into the LC-MS/MS system for analysis.

2.5. Statistical analysis

Plasma and oral fluid concentration data were processed and analysed using scatter plots in Microsoft Excel. To assess correlations, Pearson's correlation coefficient was applied for normally distributed data, while Spearman's rank correlation was used for data that did not follow a normal distribution. All statistical analyses were performed

Table 1
Retention times, selected MRM transitions, and optimised MS/MS parameters for the identification of the analytes.[a]

Analytes	Retention time (min)	Parent ion (*m/z*)	Product ions (*m/z*)	Decluster potential (V)	Entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)
AMT	6.49	278	233*	80	10	25	10
			91	80	10	35	10
NTP	6.50	264	233*	70	10	19	14
			91	70	10	29	10
BUP	4.90	240	184*	60	10	25	10
			131	60	10	45	10
CIT	5.50	325	109*	91	10	33	12
			262	91	10	27	10
DCIT	5.50	311	109*	71	10	29	12
			262	71	10	23	28
CMI	6.80	315	86*	96	10	23	10
			58	96	10	65	8
DCMI	6.80	301	72*	66	10	19	8
			44	66	10	65	20
CMI-d3 [a]	6.80	318	89*	96	10	23	10
DUL	6.40	298	154*	30	10	9	10
			44	30	10	67	6
FLX	6.50	310	44*	60	10	73	6
			148	60	10	11	12
NFLX	6.50	296	134*	30	10	9	16
			30	30	10	45	14
FLV	6.50	319	71*	50	10	19	8
			258	50	10	15	10
MPT	6.40	278	250*	100	10	25	10
			191	100	10	49	12
MTZ	4.40	266	195*	101	10	33	12
			72	101	10	23	8
DMTZ	4.40	252	195*	141	10	29	18
			209	141	10	33	16
PXT	6.18	330	192*	120	10	30	10
			70	120	10	49	10
SRT	6.79	306	275*	30	10	17	10
			158	30	10	33	10
DSRT	6.88	291	158*	10	10	29	18
			129	10	10	27	14
TRZ	4.90	372	176*	81	10	35	10
			148	81	10	48	10
m-CPP	4.20	197	118*	40	10	50	10
			119	40	10	34	10
VLX	5.30	278	260*	46	10	17	10
			58	46	10	57	8
DVLX	4.00	264	58*	46	10	51	26
			107	46	10	39	12

AMT: amitriptyline; BUP: bupropion; CIT: citalopram; CMI: clomipramine; CMI-d3: clomipramine-d3; DCIT: desmethylcitalopram; DCMI: desmethylclomipramine; DSRT: desmethylsertraline; DUL: duloxetine; FLX: fluoxetine; FLV: fluvoxamine; MPT: maprotiline; m-CPP: *meta*-chlorophenylpiperazine; MTZ: mirtazapine; DMTZ: *N*-demethylmirtazapine; NFLX: norfluoxetine; NTP: nortriptyline; DVLX: *O*-desmethylvenlafaxine; PXT: paroxetine; SRT: sertraline; TRZ: trazodone; VLX: venlafaxine.
[a] Internal standard; * Quantifier ion.

using IBM SPSS Statistics software, version 27.

3. Results and discussion

3.1. Method validation

The method was validated in accordance with the guidelines of the Food and Drug Administration (FDA) [49] and of the European Medicines Agency (EMA) [50] for all antidepressants under investigation, and for both plasma and oral fluid matrices. Validation followed a five-day protocol, with the parameters assessed including selectivity, linearity and limits, inter-day, intra-day, and intermediate precision and accuracy, carryover, extraction recovery, ion suppression/enhancement, and application to authentic samples.

The identification criteria for confirming the presence of analytes in real plasma and oral fluid specimens were based on the guidelines of the World Anti-Doping Agency (WADA) [51]. These included a signal-to-noise ratio of at least 3:1 and a relative retention time within ±1 % of that observed for the corresponding analyte in a spiked control sample (when a deuterated internal standard was not used), or within ±0.5 % when a deuterated analogue was employed as the internal standard.

To ensure a high level of confidence in analyte identification, two transitions per compound were monitored, along with their relative ion intensities. The maximum permitted tolerances for the relative ion intensities (expressed as a percentage of the base peak) were as follows: for intensities above 50 %, an absolute deviation of ±10 % was accepted; for intensities between 25 and 50 %, a relative tolerance of ±20 % was applied; for intensities between 5 and 25 %, an absolute deviation of ±5 % was accepted; and for intensities of 5 % or below, a relative tolerance of ±50 % was allowed.

3.1.1. Selectivity

The selectivity of the developed methodology was assessed by analysing pools of blank plasma and oral fluid samples obtained from different sources, in order to verify the presence of any potential interferences in the selected transitions and retention times for each antidepressant under investigation [49,50]. As all antidepressants were clearly identified in all spiked plasma and oral fluid samples, and no interferences were detected in the blank samples of either matrix, the method was deemed selective.

Supplementary Figs. S1 and S2 present representative chromatograms of blank plasma and oral fluid samples and plasma and oral fluid samples spiked at the lower limit of quantification (LLOQ), respectively. It is worth noting that the chromatographic signal of DSRT in oral fluid samples presented a slightly higher baseline noise compared to other analytes. While this did not compromise the quantification or identification criteria, it may reflect minor matrix-related interferences inherent to oral fluid. All other chromatograms exhibited stable baselines and well-defined peaks. This observation did not affect method validation parameters such as selectivity, precision, or accuracy for DSRT in oral fluid.

3.1.2. Calibration curves and limits

The linearity of the developed method was established using fortified plasma and oral fluid samples, processed and analysed according to the extraction procedure described above. The concentration range evaluated was 0.98–1000 ng/mL for all antidepressants in both biological matrices. The selected calibrator concentrations reflected those typically encountered in routine toxicological analyses. Linearity was assessed using eleven calibrators, each analysed in five replicates. Calibration curves were constructed by plotting the peak area ratio of each antidepressant and its respective metabolite to the internal standard against the nominal analyte concentration.

CMI-d3, a deuterated internal standard, was selected due to its structural similarity to the target compounds, which supports improved linearity, precision, and accuracy. Its use also prevents interference in authentic samples and reduces analyte loss during sample preparation.

Acceptance criteria for the calibration curves included a coefficient of determination (R^2) of at least 0.99. In addition, the accuracy of each calibrator, expressed as mean relative error (RE or bias), was required to fall within ±15 % of the nominal concentration, except for the LLOQ, where a deviation of up to ±20 % was considered acceptable [49,50]. The calibration range was wide, and weighted least squares regressions of $1/x$ and $1/x^2$ were applied to correct for heteroscedasticity in both biological specimens. The method demonstrated linearity within the established calibration range for all analytes in both plasma and oral fluid samples.

The LLOQ was defined as the lowest concentration that could be measured with adequate precision and accuracy. Specifically, this was characterised by a coefficient of variation (CV, %) of less than 20 % and a mean relative error (RE, %) within ±20 % of the nominal concentration. [49,50].

The data from the calibration curves and limits for both plasma and oral fluid samples are presented in Tables 2 and 3, respectively.

Several published studies on this topic include the work by Wang et al. [36], who recently developed a method to determine some of the antidepressants featured in this study, using 100 μL of plasma. This method employed protein precipitation with methanol and analysis by LC-MS/MS. The authors obtained LLOQ values of 1.25 ng/mL for CIT and SRT, 2.5 ng/mL for AMT, BUP, DUL, MTZ and PXT, 3.75 ng/mL for NTP, 5 ng/mL for CMI, DCMI, NFLX, FLV, VLX, and DVLX, and 7.5 ng/mL for FLX; these values are significantly higher than those observed in our study. For LOD values, the only one lower than those in our study was 0.82 ng/mL for AMT and BUP.

Similarly, Phogole et al. [35], also using protein precipitation with acetonitrile as an extraction method, developed a methodology for determining SRT and DSRT in 200 μL of plasma, with the same chromatographic analysis. However, despite of using twice the biological sample volume, the authors achieved higher LLOQ values compared to Wang et al., namely 2.5 ng/mL for SRT and 10 ng/mL for DSRT.

Shin et al. [28] developed a method for quantifying a wide range of antidepressants and metabolites, including AMT, NTP, BUP, CIT, CMI, DUL, FLX, MTZ, PXT, SRT, TRZ, VLX, and DVLX, in oral fluid samples. Using 1 mL of biological sample, SPE, and LC-MS/MS analysis, the authors report an LLOQ value of 10 ng/mL for all compounds, which is ten times higher than the values obtained in the present study.

Additionally, de Castro et al. [25] developed a methodology using LC-MS/MS analysis and automated SPE for the extraction of 200 μL of oral fluid and plasma samples. The authors achieved LLOQ values of 2 ng/mL for NTP, CIT, DCMI, FLX, PXT, SRT, and VLX, 4 ng/mL for AMT and NFLX, and 10 ng/mL for CMI and FLV in plasma samples. For oral fluid, LLOQ values were 2 ng/mL for AMT, NTP, CIT, DCMI, FLX, NFLX, FLV, PXT, SRT, and VLX, and 10 ng/mL for CMI. Again, despite of using double the volume of biological samples, the authors obtained significantly higher LLOQ values.

3.1.3. Intra-day, inter-day, and intermediate precision and accuracy

In accordance with the FDA and EMA validation criteria [49,50], the precision of the method was expressed as the coefficient of variation (CV, %) between measured concentrations, with an accepted limit of ≤15 % for all concentrations, and ≤ 20 % for the LLOQ. Accuracy was evaluated in terms of the mean relative error (RE, %) between the measured concentrations, as determined using the calibration equation, and the nominal concentrations. A ± 15 % interval was established for all concentrations, except for the LLOQ, for which a range of ±20 % was deemed acceptable.

For intermediate precision and accuracy, the five concentration levels of quality controls (QCs) were analysed in triplicate over a 5-day period (n = 15). In plasma samples, the CVs obtained were typically lower than 13 %, with accuracy within the ±15 % interval. Similarly, for oral fluid samples, CV values below 13 % and mean RE values within the ±15 % interval were achieved. The results are presented in

Table 2
Linearity data ($n = 5$) in plasma.

Analytes	Weight	Linear range (ng/mL)	Linearity		R^{2a}	LLOQ (ng/mL)
			Slope[a]	Intercept[a]		
AMT	$1/x^2$	0.98-1000	0.0056 ± 0.0002	−0.0005 ± 0.0011	0.9918 ± 0.0010	0.98
NTP	$1/x^2$	0.98–1000	0.0066 ± 0.0003	0.0004 ± 0.0007	0.9932 ± 0.0025	0.98
BUP	$1/x^2$	0.98–1000	0.0033 ± 0.0002	−0.0004 ± 0.0007	0.9911 ± 0.0009	0.98
CIT	$1/x^2$	0.98–1000	0.0087 ± 0.0003	0.0059 ± 0.0077	0.9903 ± 0.0003	0.98
DCIT	$1/x^2$	0.98–1000	0.0062 ± 0.0001	0.0007 ± 0.0004	0.9912 ± 0.0014	0.98
CMI	$1/x^2$	0.98–1000	0.0072 ± 0.0003	−0.0010 ± 0.0006	0.9909 ± 0.0010	0.98
DCMI	$1/x$	0.98–1000	0.0045 ± 0.0002	−0.0023 ± 0.0008	0.9989 ± 0.0009	0.98
DUL	$1/x^2$	0.98–1000	0.0012 ± 0.0000	0.0000 ± 0.0002	0.9919 ± 0.0016	0.98
FLX	$1/x^2$	0.98–1000	0.0016 ± 0.0001	−0.0001 ± 0.0003	0.9909 ± 0.0005	0.98
NFLX	$1/x$	0.98–1000	0.0009 ± 0.0001	−0.0003 ± 0.0002	0.9980 ± 0.0011	0.98
FLV	$1/x^2$	0.98–1000	0.0020 ± 0.0001	0.0004 ± 0.0001	0.9914 ± 0.0019	0.98
MPT	$1/x^2$	0.98–1000	0.0093 ± 0.0001	0.0008 ± 0.0009	0.9928 ± 0.0022	0.98
MTZ	$1/x^2$	0.98–1000	0.0129 ± 0.0011	0.0003 ± 0.0019	0.9917 ± 0.0011	0.98
DMTZ	$1/x^2$	0.98–1000	0.0108 ± 0.0005	0.0014 ± 0.0010	0.9928 ± 0.0012	0.98
PXT	$1/x$	0.98–1000	0.0025 ± 0.0001	−0.0005 ± 0.0004	0.9987 ± 0.0003	0.98
SRT	$1/x$	0.98–1000	0.0035 ± 0.0002	−0.0009 ± 0.0009	0.9965 ± 0.0038	0.98
DSRT	$1/x$	0.98–1000	0.0003 ± 0.0000	−0.0001 ± 0.0001	0.9973 ± 0.0026	0.98
TRZ	$1/x^2$	0.98–1000	0.0098 ± 0.0004	−0.0005 ± 0.0014	0.9916 ± 0.0016	0.98
m-CPP	$1/x^2$	0.98–1000	0.0025 ± 0.0001	0.0000 ± 0.0007	0.9912 ± 0.0010	0.98
VLX	$1/x^2$	0.98–1000	0.0058 ± 0.0004	0.0003 ± 0.0013	0.9919 ± 0.0003	0.98
DVLX	$1/x$	0.98–1000	0.0034 ± 0.0006	−0.0025 ± 0.0006	0.9965 ± 0.0020	0.98

[a] : Mean values ± standard deviation.

Table 3
Linearity data ($n = 5$) in oral fluid.

Analytes	Weight	Linear range (ng/mL)	Linearity		R^{2a}	LOD/LLOQ (ng/mL)
			Slope[a]	Intercept[a]		
AMT	$1/x$	0.98-1000	0.0079 ± 0.0004	0.0003 ± 0.0016	0.9963 ± 0.0023	0.98
NTP	$1/x^2$	0.98–1000	0.0090 ± 0.0002	0.0031 ± 0.0002	0.9947 ± 0.0009	0.98
BUP	$1/x^2$	0.98–1000	0.0045 ± 0.0000	0.0009 ± 0.0004	0.9900 ± 0.0021	0.98
CIT	$1/x^2$	0.98–1000	0.0119 ± 0.0002	0.0111 ± 0.0004	0.9899 ± 0.0014	0.98
DCIT	$1/x$	0.98–1000	0.0081 ± 0.0004	0.0015 ± 0.0010	0.9977 ± 0.0014	0.98
CMI	$1/x^2$	0.98–1000	0.0097 ± 0.0000	0.0054 ± 0.0003	0.9918 ± 0.0019	0.98
DCMI	$1/x$	0.98–1000	0.0062 ± 0.0003	0.0017 ± 0.0009	0.9970 ± 0.0015	0.98
DUL	$1/x$	0.98–1000	0.0017 ± 0.0000	0.0002 ± 0.0002	0.9965 ± 0.0025	0.98
FLX	$1/x$	0.98–1000	0.0022 ± 0.0001	0.0006 ± 0.0002	0.9953 ± 0.0020	0.98
NFLX	$1/x^2$	0.98–1000	0.0010 ± 0.0001	0.0007 ± 0.0000	0.9948 ± 0.0016	0.98
FLV	$1/x^2$	0.98–1000	0.0028 ± 0.0001	0.0012 ± 0.0001	0.9939 ± 0.0025	0.98
MPT	$1/x^2$	0.98–1000	0.0120 ± 0.0005	0.0051 ± 0.0009	0.9955 ± 0.0010	0.98
MTZ	$1/x$	0.98–1000	0.0177 ± 0.0006	−0.0025 ± 0.0005	0.9962 ± 0.0011	0.98
DMTZ	$1/x^2$	0.98–1000	0.0139 ± 0.0007	0.0058 ± 0.0009	0.9943 ± 0.0007	0.98
PXT	$1/x^2$	0.98–1000	0.0032 ± 0.0003	0.0015 ± 0.0001	0.9949 ± 0.0012	0.98
SRT	$1/x^2$	0.98–1000	0.0043 ± 0.0004	0.0029 ± 0.0007	0.9966 ± 0.0002	0.98
DSRT	$1/x^2$	0.98–1000	0.0003 ± 0.0000	0.0005 ± 0.0001	0.9941 ± 0.0016	0.98
TRZ	$1/x^2$	0.98–1000	0.0136 ± 0.0003	0.0037 ± 0.0002	0.9924 ± 0.0023	0.98
m-CPP	$1/x^2$	0.98–1000	0.0034 ± 0.0001	0.0007 ± 0.0007	0.9930 ± 0.0011	0.98
VLX	$1/x^2$	0.98–1000	0.0074 ± 0.0001	0.0019 ± 0.0002	0.9912 ± 0.0010	0.98
DVLX	$1/x$	0.98–1000	0.0048 ± 0.0001	−0.0027 ± 0.0015	0.9972 ± 0.0020	0.98

[a] : Mean values ± standard deviation.

Supplementary Table S1.

Intra-day precision and accuracy were assessed at five concentration levels (QCs) by analysing six replicates on the same day ($n = 6$). The CV values obtained for plasma were below 10 % at the concentrations tested, with a RE value within the ±14 % range. For the oral fluid matrix, CV values were typically below 15 % for all concentrations, and the mean RE also fell within the ±15 % interval. The results are presented in Supplementary Table S2.

With regard to inter-day precision and accuracy, eleven concentration levels were evaluated over a 5-day period ($n = 5$), with CVs of less than 15 % and RE values within ±15 % for all concentrations, except for

the LLOQ, for which values were found within a ± 17 % interval for the plasma matrix. For oral fluid, CV values were generally below 12 %, and the mean RE values were within a ± 15 % range for all concentrations tested, except for the LLOQ, for which values within a ± 16 % interval were observed. The results are presented in Supplementary Table S3.

3.1.4. Carryover

Instrument carryover was assessed by analysing extracts from blank plasma and oral fluid samples immediately after the analysis of the highest calibrator on the calibration curve of the method. Carryover was deemed to be present if the peak area of the blank plasma or oral fluid sample exceeded 20 % of the peak area of the LLOQ for this method for each antidepressant and metabolite under study [49,50]. When peaks were present in the blank samples, their areas were smaller than said 20 % at all times, and as such the suitability of the method was not affected.

3.1.5. Extraction recovery

To evaluate the absolute recoveries, two sets of plasma and oral fluid samples ($n = 3$) were prepared at low (3.91 ng/mL), medium (125 ng/mL), and high (1000 ng/mL) concentrations for all the analytes under study and for both biological specimens. One of the groups, representing 100 % recovery, consisted of the extract of a blank sample only fortified with the compounds under study after extraction, while the other set involved spiking the blank samples with the analytes of interest before the extraction procedure. The recoveries were calculated by comparing the absolute peak areas of the antidepressants and metabolites from the second group with those from the first group [49,50]. The results are shown in Table 4.

The extraction efficiencies ranged from 75.9 % to 101.5 % for plasma, and from 66.4 % to 101.8 % for oral fluid. These results indicate that the method provides sufficient extraction efficiency for all the compounds under study in both specimens, considering that the extraction procedure is relatively simple. Furthermore, it is important to note that these recoveries represent the absolute extraction of the analytes of interest and did not impact the sensitivity of the methodology. Even using only 100 μL of biological sample, small amounts of the compounds were detected and quantified with adequate precision and accuracy, and with the intended LLOQs. Additionally, the method can be regarded as a powerful technique, resulting in a fast and efficient extraction of the target analytes with reduced consumption of organic solvents and biological sample.

3.1.6. Matrix effect (ion suppression/enhancement)

To assess ion suppression or enhancement phenomena, two groups of samples ($n = 3$) were prepared at low (3.91 ng/mL) and high (1000 ng/mL) concentrations for all the antidepressants and metabolites in biological specimens. The first set consisted of unextracted standards at the study concentrations, which were injected in triplicate. The second group was prepared using blank samples, fortified post extraction with the same concentrations as those of the first set. The extent of suppression or enhancement was evaluated by comparing the peak area ratios of the analytes of interest in both groups, with the values expected to fall within a range of 80 to 120 % [49,50]. As shown in Table 5, matrix effects ranged from 87.9 % to 154.2 % for plasma samples, and from 81.2 % to 132.4 % for oral fluid samples.

The results indicate that some of the analytes under study experience ionisation enhancement (values exceeding 120 %) at low concentrations, particularly in the plasma specimens. This increase may be attributed to various factors, such as the composition of the matrix, interferences present in the matrix, and the sensitivity of the analytical instrumentation, which can amplify the analyte signal. To ensure that this enhancement did not affect the LLOQ of the method, samples from different sources were used to evaluate this parameter; precision and accuracy at this concentration were deemed adequate, as described in subsection 3.1.3. As concluded, the matrix effect was not significant at high concentrations, did not compromise precision, and did not impact the assessment of positivity or the reliability of the results [52,53].

Table 4
Recoveries ($n = 3$).

Analytes	Recoveries[a] (%)					
	3.91 ng/mL		125 ng/mL		1000 ng/mL	
	Plasma	Oral Fluid	Plasma	Oral Fluid	Plasma	Oral Fluid
AMT	77.0 ± 3.3	69.6 ± 3.3	86.9 ± 4.2	83.8 ± 3.5	83.9 ± 3.7	92.7 ± 6.1
NTP	84.4 ± 5.1	68.5 ± 0.1	88.5 ± 5.0	84.4 ± 4.2	86.9 ± 3.5	93.5 ± 6.7
BUP	85.8 ± 4.7	75.4 ± 2.3	93. ± 5.8	91.7 ± 3.5	88.5 ± 2.7	99.9 ± 6.3
CIT	98.5 ± 6.0	93.7 ± 3.4	89.4 ± 5.9	86.5 ± 4.9	85.3 ± 3.6	98.0 ± 8.9
DCIT	87.7 ± 4.6	74.5 ± 3.4	89.6 ± 4.1	85.6 ± 4.2	88.2 ± 3.6	100.0 ± 7.2
CMI	81.0 ± 1.8	69.7 ± 2.6	86.2 ± 6.9	84.5 ± 3.	83.5 ± 3.6	93.7 ± 3.7
DCMI	88.6 ± 7.6	69.8 ± 1.5	87.6 ± 3.7	85.0 ± 3.5	89.5 ± 3.0	97.7 ± 5.4
DUL	84.9 ± 4.5	75.0 ± 3.7	91.7 ± 6.3	90.5 ± 4.7	94.1 ± 2.6	100.3 ± 4.1
FLX	88.4 ± 3.6	68.9 ± 2.5	86.4 ± 5.8	87.0 ± 4.5	87.2 ± 3.4	94.6 ± 5.4
NFLX	88.1 ± 4.4	79.6 ± 0.2	93.3 ± 5.0	92.4 ± 3.0	87.3 ± 3.1	91.7 ± 3.7
FLV	85.4 ± 3.5	70.4 ± 3.6	90.2 ± 7.0	85.6 ± 4.6	86.1 ± 2.2	90.6 ± 4.4
MPT	84.4 ± 4.0	70.7 ± 2.7	86.4 ± 5.6	83.7 ± 3.8	84.7 ± 3.2	95.5 ± 6.1
MTZ	84.9 ± 9.6	68.4 ± 1.7	86.3 ± 4.3	85.9 ± 4.9	84.4 ± 3.2	94.7 ± 7.3
DMTZ	95.7 ± 11.3	77.7 ± 3.1	94.8 ± 5.4	92.7 ± 3.9	88.6 ± 2.9	99.2 ± 5.7
PXT	90.4 ± 7.3	70.3 ± 4.0	91.2 ± 5.5	86.9 ± 4.2	96.6 ± 3.1	100.8 ± 3.4
SRT	81.4 ± 3.5	66.4 ± 3.9	85.9 ± 4.8	83.1 ± 2.8	89.7 ± 1.8	92.5 ± 3.9
DSRT	97.5 ± 10.7	80.7 ± 11.5	101.5 ± 3.8	101.8 ± 5.0	87.1 ± 1.2	91.5 ± 5.8
TRZ	79.8 ± 4.9	69.9 ± 2.1	87.8 ± 5.5	85.9 ± 6.0	85.4 ± 3.0	98.1 ± 7.1
m-CPP	89.4 ± 6.2	72.5 ± 3.2	92.5 ± 4.6	88.1 ± 3.9	86.0 ± 2.9	96.5 ± 6.5
VLX	75.9 ± 3.5	70.1 ± 2.6	87.8 ± 6.4	85.5 ± 4.8	85.6 ± 4.9	94.1 ± 6.5
DVLX	78.2 ± 3.5	71.3 ± 0.9	87.3 ± 5.4	88.0 ± 4.4	85.6 ± 3.4	99.3 ± 7.8

[a] : Mean values ± standard deviation.

3.1.7. Stability

Stability of the analytes was determined under freeze/thaw cycles for both biological matrices at the concentrations of the QCs ($n = 3$), at 0.98, 3.91, 31.25, 250 and 1000 ng/mL. Plasma and oral fluid samples prepared for stability assessment were compared with freshly processed and analysed samples, using the same calibration curve for quantification. The comparison enabled calculation of mean RE values with respect to theoretical concentrations and the calculation of CV values. The compounds were considered stable when the criteria of CV values were below 20 % and RE values were within ±20 %.

For this evaluation, samples spiked at the specified concentrations were stored at −20 °C for 24 h, thawed at room temperature and refrozen under the same conditions for another 24 h. This cycle was repeated twice more, after which the samples were extracted and analysed. All analytes demonstrated stability for at least three freeze/thaw cycles in both matrices. Both for plasma and oral fluid samples, CV values remained below 14 % and mean RE values were within ±15 % across all concentrations. These findings confirm that these antidepressants remain stable for multiple freeze/thaw cycles and indicate that both biological matrices should preferably be stored under refrigerated

Table 5
Ion suppression/enhancement for selected antidepressants.

Analytes	Ion suppression/enhancement[a] (%)			
	3.91 ng/mL		1000 ng/mL	
	Plasma	Oral Fluid	Plasma	Oral Fluid
AMT	122.4 ± 2.7	98.8 ± 24.7	112.6 ± 7.6	104.2 ± 6.2
NTP	144.0 ± 1.7	128.2 ± 30.8	102.7 ± 6.2	101.2 ± 5.3
BUP	108.5 ± 6.2	92.8 ± 22.5	109.2 ± 8.0	104.2 ± 6.9
CIT	97.8 ± 2.7	81.2 ± 18.8	113.4 ± 8.4	107.1 ± 6.3
DCIT	151.3 ± 4.1	132.4 ± 30.4	102.9 ± 7.1	101.8 ± 5.6
CMI	116.6 ± 4.5	96.6 ± 23.4	112.3 ± 7.4	102.8 ± 8.0
DCMI	140.9 ± 2.6	130.0 ± 31.1	99.4 ± 5.7	96.7 ± 5.3
DUL	127.6 ± 5.0	108.8 ± 24.8	97.0 ± 7.2	94.0 ± 5.0
FLX	132.9 ± 5.9	116.8 ± 32.5	105.3 ± 6.6	101.6 ± 5.3
NFLX	122.0 ± 7.1	104.0 ± 29.2	108.1 ± 4.6	101.6 ± 5.7
FLV	118.6 ± 3.3	101.4 ± 23.4	112.4 ± 4.2	105.6 ± 4.1
MPT	154.2 ± 1.3	132.4 ± 32.1	101.8 ± 4.8	99.0 ± 5.4
MTZ	127.2 ± 2.7	108.1 ± 25.4	111.5 ± 7.0	107.4 ± 6.8
DMTZ	126.7 ± 4.0	111.8 ± 23.0	99.0 ± 5.3	94.0 ± 4.6
PXT	139.2 ± 5.9	129.4 ± 31.5	93.8 ± 4.5	92.0 ± 3.7
SRT	118.3 ± 4.5	102.3 ± 28.3	105.8 ± 3.9	102.1 ± 4.7
DSRT	87.9 ± 5.1	82.9 ± 23.7	109.5 ± 6.8	104.5 ± 5.5
TRZ	125.4 ± 1.0	110.1 ± 25.7	113.4 ± 8.2	108.1 ± 6.4
m-CPP	125.6 ± 2.8	113.9 ± 27.7	109.7 ± 7.1	105.2 ± 6.0
VLX	125.3 ± 3.2	102.7 ± 24.7	114.4 ± 7.6	110.5 ± 7.4
DVLX	140.5 ± 7.0	117.4 ± 36.7	123.6 ± 4.8	99.3 ± 7.9

[a] : Mean values ± standard deviation.

conditions, as analytes stability is not significantly compromised.

3.2. Method applicability

The method was successfully applied in the routine analysis of the target antidepressants in 142 paired authentic samples from different patients undergoing treatment at various health units. Each matched pair of oral fluid and plasma samples was obtained from a single individual, with only one such pair collected per patient throughout the study [51]. Plasma and oral fluid specimens were extracted and analysed on the same day and the concentrations of each analyte of interest in these samples are presented in Supplementary Table S4. Fig. 1 displays the chromatograms obtained for plasma and oral fluid sample 80.

For plasma samples, AMT concentrations varied between 4.4 and 20.0 ng/mL, NTP ranged from 2.6 to 33.8 ng/mL, BUP varied between 8.1 and 130.9 ng/mL, CIT concentrations ranged from 5.4 to 99.3 ng/mL, DCIT ranged between 3.5 and 23.2 ng/mL, DUL concentrations varied between 66.6 and 79.3 ng/mL, FLX ranged from 5.9 to 304.8 ng/mL, NFLX concentrations ranged from 48.7 to 316.4 ng/mL, FLV varied between 22.0 and 93.2 ng/mL, MTZ concentrations ranged from 3.6 to 96.1 ng/mL, DMTZ varied from 2.1 to 65.1 ng/mL, PXT ranged from 2.4 to 310.0 ng/mL, SRT concentrations ranged from 7.3 to 175.5 ng/mL, DSRT varied between 2.7 and 317.0 ng/mL, TRZ ranged from 5.5 to 1913.1 ng/mL, m-CPP concentrations ranged from 2.2 to 84.5 ng/mL, VLX varied between 18.3 and 366.7 ng/mL, and DVLX concentrations varied between 108.1 and 514.7 ng/mL. CMI and its metabolite DCMI were only detected in two samples, with concentrations of 126.0 and 278.4 ng/mL, and 250.5 and 430.2 ng/mL, respectively. MPT was detected in only one sample, with a plasma concentration of 78.5 ng/mL.

For oral fluid specimens, AMT concentrations varied from 3.1 to 36.2 ng/mL, NTP ranged from 2.5 to 134.9 ng/mL, BUP varied from 36.7 to 361.6 ng/mL, CIT concentrations ranged from 30.0 to 3546.4 ng/mL, DCIT ranged from 3.3 to 111.7 ng/mL, DUL concentrations ranged from 3.1 to 18.4 ng/mL, FLX ranged from 8.2 to 324.0 ng/mL, NFLX concentrations ranged from 3.5 to 236.0 ng/mL, FLV varied from 87.4 to 216.7 ng/mL, MTZ concentrations ranged from 4.0 to 1548.0 ng/mL, DMTZ varied from 1.1 to 970.0 ng/mL, PXT ranged from 2.6 to 611.6 ng/mL, SRT concentrations ranged from 1.0 to 776.1 ng/mL, DSRT ranged from 4.1 to 752.0 ng/mL, TRZ varied from 1.0 to 1454.0 ng/mL, m-CPP concentrations ranged from 1.3 to 624.0 ng/mL, VLX varied from 64.4 to 1276.7 ng/mL, and DVLX concentrations ranged from 146.9 to 873.9 ng/mL. The two samples of CMI and its metabolite DCMI were detected, with concentrations of 120.0 and 216.7 ng/mL, and 404.0 and 597.1 ng/mL, respectively. The only sample of MPT was detected in oral fluid, with a concentration of 95.1 ng/mL.

The minimum and maximum calculated ratios between parent compounds and their respective metabolites varied across analytes and biological matrices. In plasma, AMT / NTP ratios ranged from 0.6 to 2.4, while in oral fluid varied between 0.3 and 2.0. For CIT / DCIT, ratios were higher, ranging from 1.4 to 6.8 in plasma and 4.2 to 44.6 in oral fluid. The CMI / DCMI ratio values were comprehended between 0.5 and 0.6 in plasma and 0.3 to 0.4 in oral fluid. FLX / NFLX showed a broader range in plasma (0.1–2.7) and slightly wider values in oral fluid (0.1–3.9). MTZ / DMTZ ratios spanned from 0.5 to 3.0 in plasma and 0.5 to 5.9 in oral fluid. The SRT / DSRT ratio values varied from 0.3 to 2.3 in plasma and 0.3 to 11.7 in oral fluid. TRZ / m-CPP displayed the widest range in plasma (2.7–178.5) and a considerable range in oral fluid as well (0.3–41.1). Finally, VLX / DVLX ratios ranged from 0.1 to 1.4 in plasma and from 0.4 to 2.0 in oral fluid.

Plasma and oral fluid concentrations of antidepressants and metabolites were determined for 126 of the 142 real samples, revealing treatment adherence in 88.7 % of the patients. Therefore, the applicability of the method was demonstrated, and it can be successfully implemented in routine analysis, allowing the identification and quantification of these antidepressants and their main metabolites whenever present.

3.3. Oral fluid/plasma correlation and concordance assessment

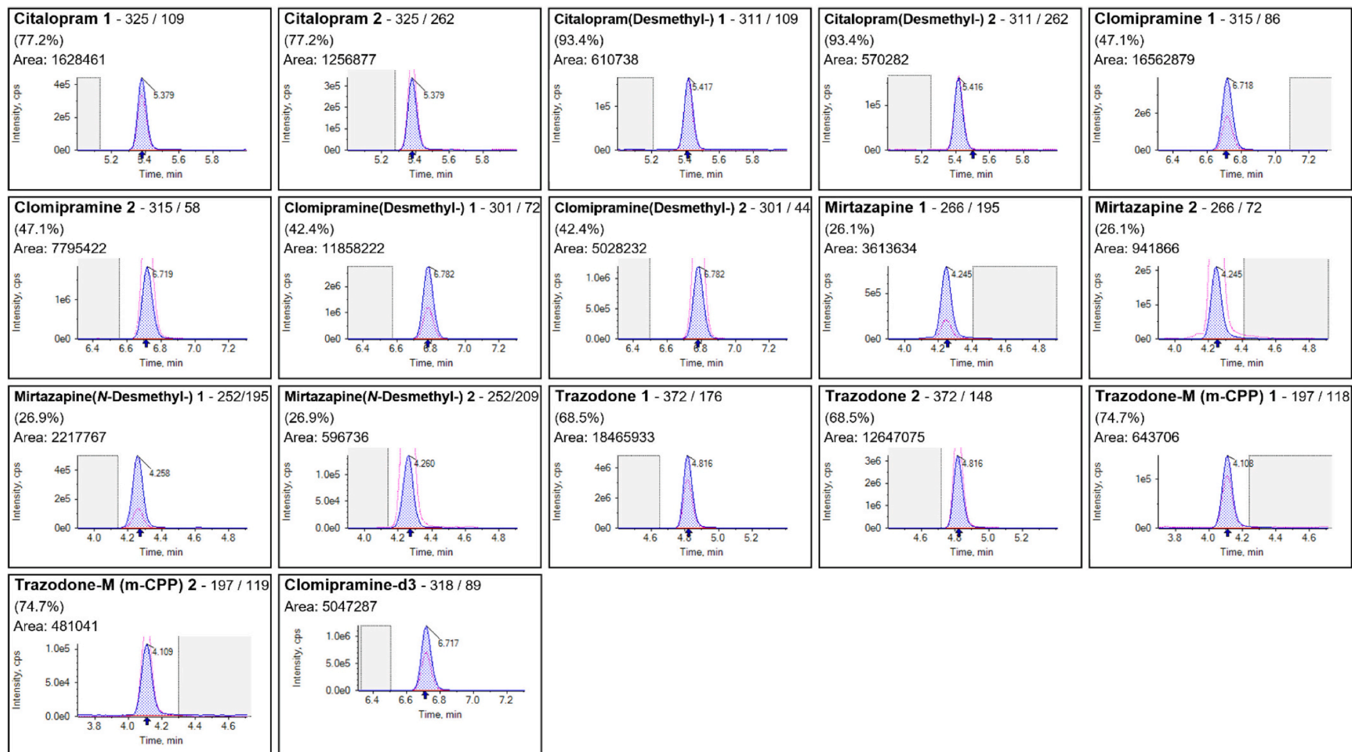
A correlation between oral fluid and plasma concentrations can be observed for some drugs. To investigate this, a correlation study was carried out by plotting the dispersion of each antidepressant and metabolite concentration in plasma (X-axis) against oral fluid (Y-axis) in scatter plots.

For **group A** (Supplementary Fig. S3), given the lower number of samples ($n = 3$) for each drug, it is only possible to predict, according to the R^2 values, a behavioural trend. AMT, its metabolite (NTP), and FLV showed a very good rate of explanation and a positive correlation, indicating that oral fluid concentration can be a good predictor of plasma concentration, with R^2 values of 0.9396, 1.0000, and 0.9889, respectively. On the other hand, DUL displayed a high data dispersion, resulting in a very low R^2 value of 0.0591, making it impossible to draw conclusions.

Regarding **group B** (Supplementary Fig. S3), a linear distribution tendency was identified through the dispersion of the points, and Pearson's correlation study was conducted. Based on this, the correlation results obtained were all positive with the following statistically significant and strong values: $r = 0.958$ ($p = 0.010$) for BUP, $r = 0.829$ ($p = 0.001$) for PXT, $r = 0.888$ ($p = 0.008$) for VLX, and $r = 0.769$ ($p = 0.043$) for DVLX.

For **group C** (Supplementary Fig. S3), Spearman's correlation was employed due to the considerable dispersion of the data and the fact that they do not follow a strictly linear pattern. This correlation method aims to understand the monotonic relationship (positive or negative) between the variables under study, even in the absence of proportional variation. Being a more robust method, Spearman's correlation is less sensitive to outliers and provides a more reliable measure of association. The Spearman's correlation coefficients (r_s) were 0.465 ($p = 0.045$) for FLX, 0.389 ($p = 0.090$) for NFLX, 0.355 ($p = 0.089$) for MTZ, and 0.459 ($p = 0.027$) for DMTZ, showing weak to moderate correlations. The higher dispersion in the scatter plot indicates that it is not possible to establish a reference value in oral fluid capable of predicting therapeutic levels in plasma. For SRT, $r_s = 0.599$ ($p = 0.001$), for DSRT, $r_s = 0.594$ (p = 0.001), for TRZ, $r_s = 0.515$ ($p < 0.000$), and for m-CPP, $r_s = 0.620$ (p < 0.000), were obtained, leading to moderate correlations between

A



B

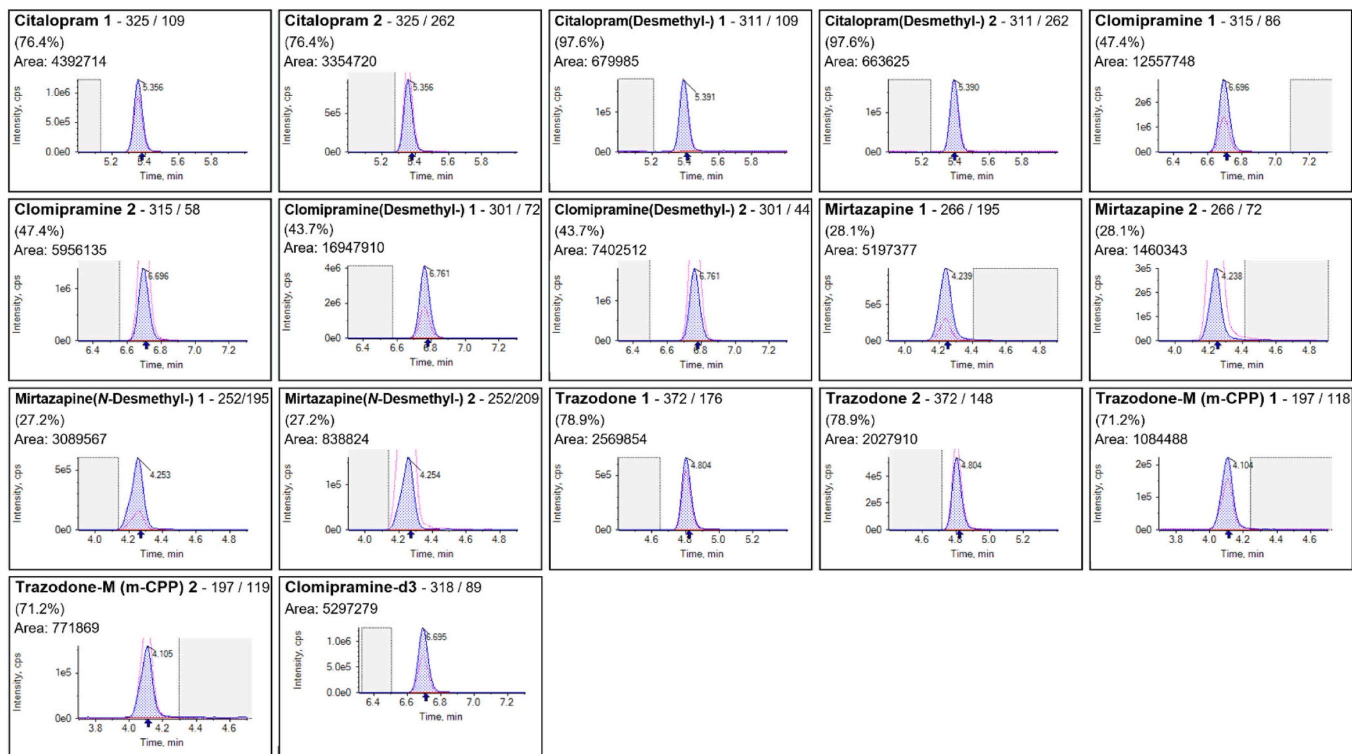


Fig. 1. Chromatograms obtained after analysis of an authentic specimen positive for antidepressants: (A) plasma and (B) oral fluid sample, belonging to a patient undergoing treatment with CIT, CMI, MTZ, and TRZ.

variables and suggesting a general tendency. An $r_s = 0.727$ ($p = 0.007$) was achieved for CIT, and an $r_s = 0.770$ ($p = 0.009$) for DCIT, showing better results with a considered strong correlation.

Additionally, the remaining compounds, CMI, DCMI, and MPT, were not considered due to the insufficient number of samples in which they were detected ($n = 2$ for CMI and DCMI, and $n = 1$ for MPT).

Overall, the results obtained can be considered promising, particularly for BUP, PXT, VLX, DVLX, CIT, and DCIT, as these show higher correlation values, some of which are close to 1.

Table 6 presents the ratios between oral fluid and plasma concentrations, along with the minimum and maximum values of these ratios per analyte. As an example, through this quotient, it is possible to infer that, on average, the oral fluid concentration of PXT was 79 % of the plasma concentration.

To further analyse the interchangeability of oral fluid and plasma concentrations, a study of Bland-Altman plots was conducted to assess the observed differences and determine the magnitude of variation between the pairs of concentrations. For each sample pair, the concentration difference (concentration difference between oral fluid and plasma samples (Y-axis)) is plotted versus the respective average concentration (X-axis). The 21 analytes present in this study, were categorized into three distinct groups based on the availability of data, the number of matched sample pairs, and the percentage of values falling within the limits of agreement (±1.96 Standard Deviations (SD) from the mean difference).

Analytes CMI, DCMI, and MPT lacked data for both the number of points (less than 3 sample pairs) and consequently, the percentage of agreement, precluding any statistical evaluation of concordance for these compounds. These analytes cannot be evaluated using the current dataset and require further investigation before any conclusions can be drawn regarding matrix comparability.

For **group A** (Supplementary Fig. S4), the majority of compounds (CIT, DCIT, FLX, MTZ, DMTZ, PXT, SRT, DSRT, TRZ, and m-CPP) demonstrated high but not perfect agreement, with concordance percentages ranging from 90 % to 96 %. These antidepressants and metabolites generally presented with adequate to large numbers of matched samples, supporting the robustness of the Bland-Altman evaluation. Notably, analytes TRZ and m-CPP were assessed with 50 and 45 paired observations, respectively, and yielded concordance values of 92 % and 93 %. Such results support their potential for reliable substitution between oral fluid and plasma matrices. DSRT also performed well, with 96 % agreement across 28 paired samples. For FLX and SRT, high concordance levels were observed, with 95 % and 93 % of pairs, respectively, within the limits of agreement. These analytes also had a relatively large number of matched samples ($n = 19$ and $n = 29$, respectively), supporting the reliability of the estimates. Similarly, CIT, PXT, and MTZ showed 92 % of pairs within the limits of agreement, based on 12, 12, and 24 paired observations, respectively. DMTZ exhibited 91 % concordance across 23 paired measurements. On the lower end of this group, the metabolite DCIT showed 90 % agreement based on 10 samples; while still acceptable, this result may reflect a degree of variability that warrants further scrutiny.

For **group B** (Supplementary Fig. S4), eight analytes (AMT, NTP, BUP, DUL, NFLX, FLV, VLX, and DVLX) exhibited perfect agreement, with 100 % of paired values lying within the defined limits of agreement. While this level of concordance is indicative of strong alignment between oral fluid and plasma concentrations, caution must be exercised in interpreting results for analytes with a low number of sample pairs. In particular, AMT, NTP, BUP, DUL, and FLV had 5 or fewer data points, which undermines the statistical reliability of the observed concordance. Conversely, compounds NFLX, VLX, and DVLX had between 7 and 20 data points, offering a more credible basis for interpreting the observed agreement. Among these, NFLX, with 20 paired observations and 100 % concordance, stands out as a particularly robust candidate for inter-matrix comparability.

Therefore, these findings support the suitability of several analytes for cross-matrix comparison, particularly those demonstrating both high concordance and sufficient sample sizes. However, analytes lacking data or supported by small datasets should be interpreted cautiously, and further validation is recommended to confirm the reliability of these preliminary observations.

Although the Bland-Altman plots primarily serve to evaluate agreement, they also offer crucial insights into the direction and magnitude of differences between oral fluid and plasma concentrations. The analysis of the mean differences reveals that, for several analytes, the bias was relatively small (e.g., analytes DCIT: 8.29; PXT: –6.76; SRT: 2.49), suggesting minimal systematic error between matrices. However, other compounds showed more pronounced mean differences, either positive or negative. For instance, TRZ (−527.44), CIT (498.78), and VLX (453.92) exhibited substantial absolute differences, indicating a marked discrepancy between oral fluid and plasma levels that may affect their interchangeability, even in the presence of high percentage agreement. Some analytes with perfect or near-perfect concordance still demonstrated large mean differences (e.g., FLV: 94.23; DVLX: 238.31), underscoring the importance of considering both the statistical and clinical significance of the observed differences. In contrast, analytes such as FLX (−58.52) and DSRT (−54.93) exhibited high agreement (≥95 %) but systematic underestimation in oral fluid, reflecting a consistent directional bias. These findings demonstrate that, for analytes with large sample sizes and high concordance (e.g., analytes TRZ and m-CPP), the limits of agreement were generally narrow (±1.96SD), indicating a consistent relationship between oral fluid and plasma concentrations. This consistency supports the potential of oral fluid as a reliable alternative matrix.

Although some degree of bias was observed in certain compounds under study, its identification provides valuable insight into the nature of the differences between matrices. Overall, the results reinforce that high concordance is a promising indicator of comparability, and that evaluating both the direction and magnitude of the bias can further inform the clinical relevance of using oral fluid in place of plasma.

These findings suggest that oral fluid analysis is suitable for detecting the aforementioned analytes. However, given that the response to antidepressants varies significantly between individuals and differs between the various compounds, it is essential to apply this methodology to a larger number of patients for all analytes and their respective metabolites under study. This will help ensure its practical applicability in

Table 6
Ratio oral fluid/plasma concentration study.

Analytes	Average Ratio[a] (Oral Fluid / Plasma)	Precision (CV (%))	Min.	Max.
AMT	1.57 ± 0.64	40.37	0.70	2.21
NTP	2.54 ± 1.24	48.90	0.96	3.99
BUP	4.97 ± 2.60	52.22	2.27	8.41
CIT	10.48 ± 18.01	171.81	1.42	69.00
DCIT	1.34 ± 1.36	101.87	0.32	5.12
CMI	0.87 ± 0.09	10.05	0.78	0.95
DCMI	1.50 ± 0.11	7.49	1.39	1.61
DUL	0.12 ± 0.10	83.15	0.04	0.26
FLX	0.83 ± 0.72	87.13	0.07	2.37
NFLX	0.54 ± 0.53	98.95	0.05	2.09
FLV	3.18 ± 0.67	21.19	2.33	3.97
MPT[b]	1.21 ± 0.00	–	–	–
MTZ	8.99 ± 17.88	198.81	0.21	69.86
DMTZ	8.33 ± 18.21	218.68	0.10	83.36
PXT	0.79 ± 0.78	98.29	0.09	2.13
SRT	0.81 ± 1.57	194.33	0.03	7.32
DSRT	0.67 ± 1.17	176.27	0.04	5.17
TRZ	0.21 ± 0.21	99.44	0.01	1.00
m-CPP	1.42 ± 1.02	71.90	0.18	3.14
VLX	5.86 ± 4.83	82.35	2.97	17.48
DVLX	1.99 ± 1.00	50.51	0.93	4.10

[a] : Mean values ± standard deviation; CV - coefficient of variation; [b]: MPT was only detected in one sample (n = 1).

monitoring treatment adherence and therapeutic levels of antidepressants.

It is also important to note that there are few publications focused on studying the correlation of antidepressants between plasma (serum or whole blood) and oral fluid, and those that do often study only a small number of analytes [25,54–58]. To the best of our knowledge, this is the first study to be applied to a substantial number of patients and compounds within this class of medication.

4. Conclusions

This study describes the validation of an analytical method that has been shown to be sensitive, selective, precise, and accurate for the identification and quantification of 21 antidepressants and their metabolites (AMT, NTP, BUP, CIT, DCIT, CMI, DCMI, DUL, FLX, NFLX, FLV, MPT, MTZ, DMTZ, PXT, SRT, DSRT, TRZ, m-CPP, VLX and DVLX) in plasma and oral fluid samples using protein precipitation and LC-MS/MS.

The combination of this extraction technique with tandem mass spectrometry resulted in a simple and rapid procedure. The method was linear within the range of 0.98–1000 ng/mL for all drugs in both biological specimens, using only 100 μL of either plasma or oral fluid. The sensitivity achieved, combined with the reduced sample volume required, offers a clear advantage, particularly when sample availability is limited, allowing multiple tests to be performed on the same specimen.

Adequate recovery values were obtained for both specimens, ranging from 75.87 to 101.52 % for plasma and from 66.39 to 101.78 % for oral fluid samples, with the intended LLOQ achieved for all analytes. The method was applied to 142 real samples (both plasma and oral fluid) from individuals undergoing treatment with the antidepressants under study. After determining the respective concentrations, treatment adherence was calculated at 88.7 %.

The correlation between the levels of these antidepressants and metabolites was also studied, with very promising results for BUP, PXT, VLX, and DVLX, showing Pearson's correlation coefficients of $r = 0.958$, $r = 0.829$, $r = 0.888$, and $r = 0.769$, respectively. For CIT and DCIT, Spearman's correlation yielded $r_s = 0.727$ and $r_s = 0.770$, respectively.

Furthermore, these results support the routine use of this methodology in the determination of these compounds in clinical and forensic toxicology analyses. Additionally, this application demonstrates that oral fluid has the potential to be used in the study of treatment adherence and in drug monitoring as a replacement for plasma.

CRediT authorship contribution statement

Sofia Soares: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Luana Rosendo:** Writing – review & editing, Validation, Investigation, Formal analysis. **Suzana Fonseca:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis. **Nuno Gonçalves:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis. **João M. Franco:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Tiago Rosado:** Writing – review & editing, Validation, Supervision, Conceptualization. **Mário Barroso:** Writing – review & editing, Validation, Supervision, Conceptualization. **Vítor Hugo Santos:** Writing – review & editing, Resources, Investigation. **Cristina Rei:** Writing – review & editing, Resources, Investigation. **Patricia Amantegui:** Writing – review & editing, Resources, Investigation. **António Pissarra da Costa:** Writing – review & editing, Resources. **Telma Chaves:** Writing – review & editing, Resources. **Rita Valente:** Writing – review & editing, Resources. **Fábio Duarte:** Writing – review & editing, Resources. **Susana Pacheco:** Writing – review & editing, Resources. **Marco Martins:** Writing – review & editing, Resources. **Kátia Dias:** Writing – review & editing, Resources. **Patricia Costa:** Writing – review & editing, Resources. **Rui Costa:** Writing – review & editing, Resources. **Sílvia Castro:** Writing – review & editing, Resources. **Diana Sousa:** Writing – review & editing, Resources. **Diana Figueiredo:** Writing – review & editing, Resources. **Isabel Soares:** Writing – review & editing, Resources. **Salomé Mouta:** Writing – review & editing, Resources. **Bianca Jesus:** Writing – review & editing, Resources. **Ana Pires:** Writing – review & editing, Resources. **Cândida Ribeiro:** Writing – review & editing, Resources. **Sónia Lobo:** Writing – review & editing, Resources. **Leonor Correia:** Writing – review & editing, Resources. **Sofia Malés:** Writing – review & editing, Resources. **Fátima Vale:** Writing – review & editing, Resources. **Carina Moita:** Writing – review & editing, Resources. **Carolina Moura:** Writing – review & editing, Resources. **Joana Sousa:** Writing – review & editing, Resources. **Luís Rafael Afonso:** Writing – review & editing, Resources. **Rita Santinho Costa:** Writing – review & editing, Resources. **Eugenia Gallardo:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Informed consent statement

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the Universidade da Beira Interior (code number CE-UBI-Pj-2023-007), by the Ethics Committee of the Centro Hospitalar Cova da Beira (study number 07/2023), by the Ethics Committee of the Casa de Saúde Bento Menni - Irmãs Hospitaleiras Guarda (available in the meeting report number 3/2022) and by the Ethics Committee of the Unidade Local de Saúde da Guarda (available in the meeting report number 41/2023). Informed consent was obtained from all subjects involved in the study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2025.124782.

Data availability

Data will be made available on request.

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