



# Determination of antipsychotic drugs in oral fluid using dried saliva spots by gas chromatography-tandem mass spectrometry

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## Abstract

The present work describes the optimization and validation of an analytical method for the determination of six antipsychotic drugs (chlorpromazine, levomepromazine, cyamemazine, clozapine, haloperidol, and quetiapine) in oral fluid samples after solvent extraction from dried saliva spots, by gas chromatography coupled to tandem mass spectrometry. The method was fully validated, and the included parameters were selectivity, linearity, limits of quantification, precision and accuracy, stability, and recovery. The method was linear for all compounds from 10 to 400 ng/mL, except for haloperidol (5–100 ng/mL), presenting coefficients of determination higher than 0.99. Inter- and intra-day precision and accuracy were in conformity with the criteria usually seen in bioanalytical method validation; i.e., coefficients of variation were lower than 15% and an accuracy of 15% or better for all studied drugs. The recoveries obtained with this miniaturized technique ranged from 63 to 97%. The herein described method is the first to be reported using the dried saliva spots approach for the analysis of these antipsychotic drugs, proving great sensitivity apart from its simple and fast procedure. The method was considered a good alternative to the conventional techniques to be applied in clinical and toxicological analyses, even more taking into account the extremely low sample volume used (50  $\mu$ L).

**Keywords** Dried saliva spots · Oral fluid · GC-MS/MS · Antipsychotic drugs

## Introduction

Schizophrenia affects 1% of the population and is one of the ten leading causes of disability worldwide. It is usually characterized by negative and positive symptoms; the former are described by loss of movement and emotional blunting, while the latter involve symptoms such as delusions, hallucinations, and disorganized speech [1].

In recent decades, the increased number of suicides related to schizophrenia has raised some concern. Indeed, between 25 and 50% of patients suffering from an acute episode attempt suicide, and 10% of these attempts are successful [2]. The treatment of schizophrenia has been explored over the years, and it is based on the use of antipsychotic drugs (APDs). Antipsychotic medication is currently divided into two major classes: first-generation drugs, which include chlorpromazine, cyamemazine, levomepromazine, and haloperidol, and second-generation drugs including clozapine and quetiapine [3, 4]. The first generation, or conventional antipsychotics, are potent antagonists of dopamine D2 receptors, which

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are highly effective against psychotic symptoms. However, they have side effects like involuntary movement disorders due to the extrapyramidal system, many of which mimic the effects of Parkinson's disease. On the other hand, second-generation antipsychotics have lower affinity for dopaminergic receptors and high affinity for serotonin receptors (5-HT type 2A), leading to a decrease of extrapyramidal side effects and a reduced risk of tardive dyskinesia. Nevertheless, they have been associated to diabetes mellitus, hypotension, hypercholesterolemia, and moderate to severe weight gain [5].

Oral fluid is, nowadays, considered as an excellent alternative specimen in both clinical and forensic fields. The main advantages of this sample are the ease of collection, lower risk of adulteration, and a generally shorter window of drug detection, when compared to urine; hence, a better correlation is observed with the drug effects [6–8]. As a result, oral fluid has been used for quantification of antipsychotic drugs, and several extraction methods have been used, such as solid-phase extraction (SPE) [9], liquid-liquid extraction (LLE) [10–12], which is the most common technique with this type of sample, and microextraction by packed sorbent (MEPS) [13]. In order to overcome the disadvantages that these extraction methods present, techniques using less sample volume have been an increasingly sought subject.

The dried saliva spots (DSS) technique is a procedure for the analysis of oral fluid samples, involving the spotting of the liquid specimen onto a collection card and then letting it to dry. Oral fluid is a very attractive specimen and it is a useful alternative to blood and urine samples for investigations of drugs in biological fluids [7, 14, 15], and the DSS sampling technique is much simpler and faster compared to other techniques. DSS has been used mainly in clinical pharmacokinetics and therapeutic drug monitoring studies because of its advantages, including lower costs for storage and transport of samples as well as smaller sample volumes [16, 17]. The use of DSS in bioanalysis was first reported in a study to determine lidocaine by liquid chromatography-mass spectrometry [17]; however, DSS has been widely used in publications for the determination of several drugs, such as anticonvulsants [18], methadone and metabolites [19], and biological components [20]. Several analytical methods have been published for the detection and quantitation of this class of compounds in biological samples following the described extraction procedures, namely using gas chromatography coupled to mass spectrometry (GC/MS) [21] and tandem mass spectrometry (GC/MS/MS) [22], liquid chromatography (LC) coupled to MS/MS [11, 23–25], and time of flight detectors (TOF/MS) [26].

The present work describes the optimization and full validation of a sensitive method to determine several

antipsychotic drugs (chlorpromazine, levomepromazine, cyamemazine, clozapine, haloperidol, and quetiapine) in oral fluid using the DSS approach and GC-MS/MS. Only 50  $\mu$ L of sample is needed to accomplish the analysis, which is important for instance in those situations where sample amount is limited. In addition, the improved selectivity and sensitivity provided by GC-MS/MS further allowed low limits of quantitation to be obtained, making this method a good alternative when compared to existing procedures. To the best of our knowledge, this is the first application of DSS to determine these drugs in oral fluid samples, thus resulting in a great alternative to the classical techniques usually used in routine analysis.

## Experimental

### Reagents and standards

Standard solutions of haloperidol (HAL), clozapine (CLZ), and chlorpromazine (CPZ) as well as the trideuterated analogue of chlorpromazine (CPZ-d3) were purchased from LGC Promochem (Barcelona, Spain) at the concentration of 1 mg/mL. Promazine (PRZ), levomepromazine (LVP), and cyamemazine (CYA) were purchased from Sigma-Aldrich (Lisbon, Portugal). Quetiapine (QTP) was kindly donated by AstraZeneca PLC (London, UK). It is important to emphasize that PRZ (internal standard—IS) is not commercially available as therapeutic drug in Portugal, and therefore, its appearance in an authentic sample, impairing quantitative analysis, is highly unlikely to occur. *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) were acquired from Macherey–Nagel (Düren, Germany). Whatman™ 903 protein saver cards were acquired from Sigma-Aldrich (Sintra, Portugal).

The working solutions were prepared by properly diluting the stock solutions with methanol to the final concentrations of 0.1, 1, and 10  $\mu$ g/mL for all APDs, with exception of HAL (0.02, 0.2, and 2  $\mu$ g/mL). The internal standards', CPZ-d3 and PRZ, working solutions were prepared in methanol at the concentrations of 0.1  $\mu$ g/mL and 0.5  $\mu$ g/mL respectively. All working and stock solutions were stored in the absence of light at 4 °C.

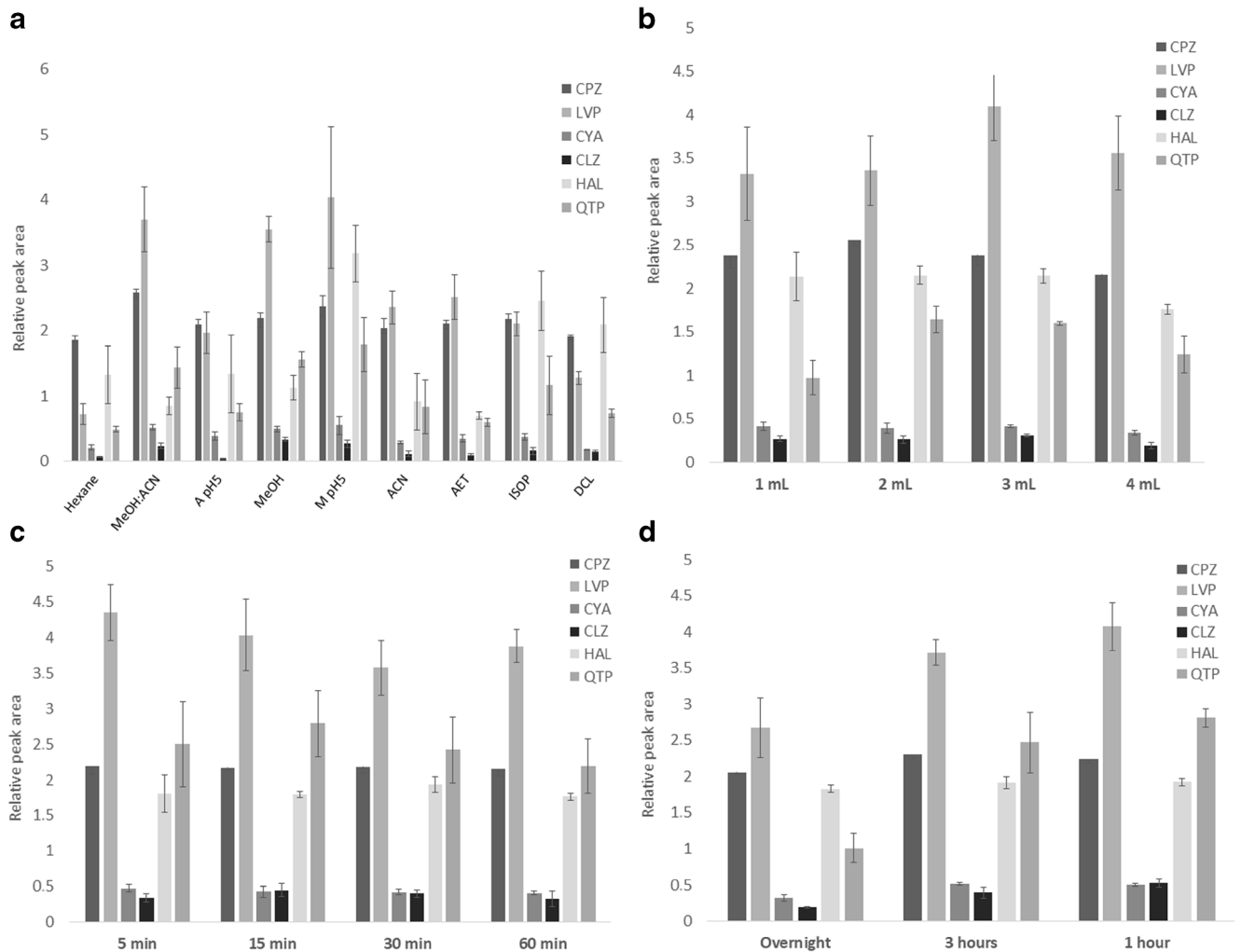
### Biological specimens

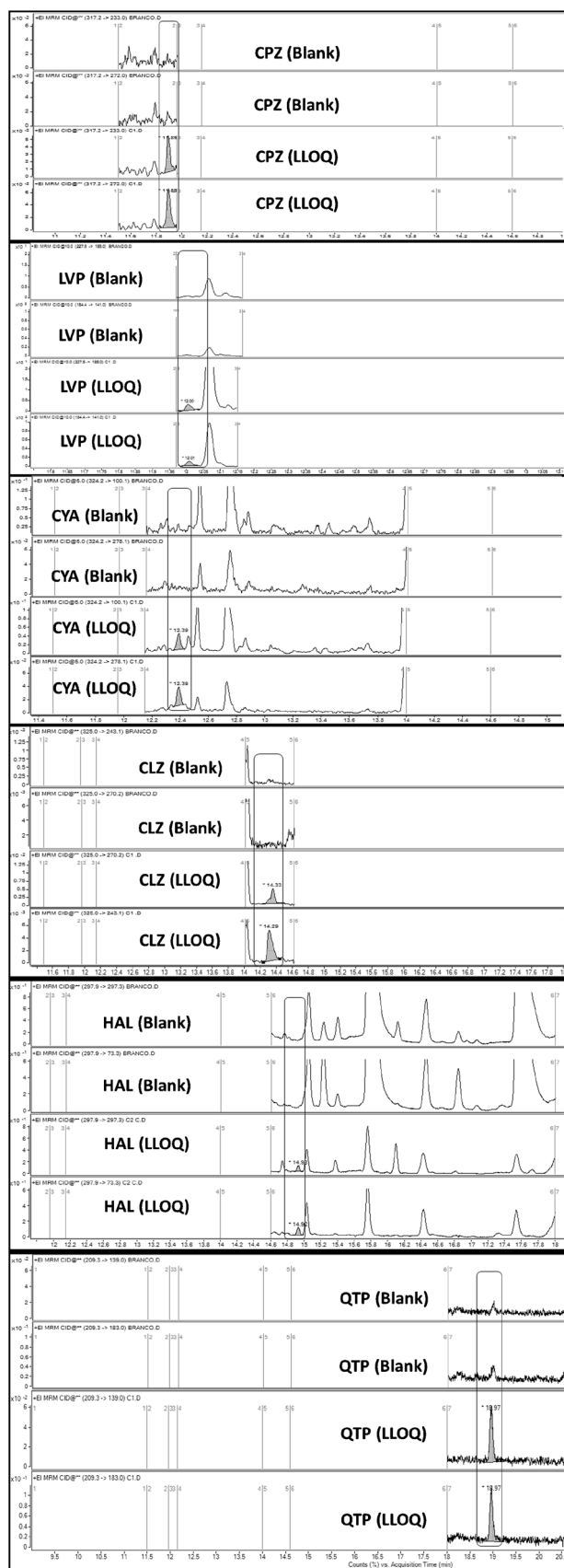
Blank oral fluid samples used in the present work were supplied by laboratory staff, and authentic samples were provided by the psychiatric center of the Hospital Cova da Beira, Covilhã, Portugal. All oral fluid samples were collected by spitting without the use of specific collection devices. These samples were stored frozen at –20 °C until analysis.

**Table 1** Retention time and GC-MS/MS parameters (quantitation transitions italicized)

Analyte	Retention time (minutes)	Transitions ( <i>m/z</i> )	Collision energy (eV)	Dwell time ( $\mu$ s)
PRZ*	11.12	283.1–238.2	15	50
CPZ-d3*	11.90	321.3–321.3	20	50
CPZ	11.91	<i>317.2–233.0</i>	10	50
		<i>317.2–272.0</i>	15	50
LVP	12.03	<i>227.5–185.0</i>	10	50
		<i>184.4–141.0</i>	10	50
CYA	12.41	<i>324.2–100.1</i>	5	50
		<i>324.2–278.1</i>	5	50
CLZ	14.31	<i>325.2–243.1</i>	5	50
		<i>325.0–278.2</i>	10	50
HAL	14.97	<i>297.9–297.3</i>	5	50
		<i>297.9–73.3</i>	20	50
QTP	19.10	<i>209.3–139.0</i>	20	50
		<i>209.3–183.0</i>	15	50

\*Internal standard

**Fig. 1** Effect of extraction solvent (a), effect of extraction solvent volume (b), effect of extraction time (c), and effect of drying time of APDs on the DSS (d)



**Fig. 2** Chromatogram of a blank sample and a spiked sample at the LLOQ for all compounds

## GC-MS/MS conditions

Chromatographic analysis was performed using an HP 7890A GC system equipped with a triple quadrupole mass spectrometer (model 7000B), both from Agilent Technologies (Waldbronn, Germany), a MPS2 autosampler, and a PTV injector from Gerstel (Mülheim an der Ruhr, Germany). Separation of the selected APDs was achieved with a capillary column (30 m × 0.25 mm ID, 0.25- $\mu$ m film thickness) with 5% phenylmethylsiloxane (HP-5MS), supplied by J & WScientific (Folsom, USA).

The initial oven temperature was held at 120 °C for 2 min, then raised to 300 °C at a 20 °C/min rate (maintained for 14 min), resulting in a total runtime of 25 min.

The derivatized extract (2  $\mu$ L) was injected in the splitless mode and the helium flow (carrier gas) was 0.8 mL/min at a constant rate. The mass spectrometer was set with a filament current of 35  $\mu$ A and electron energy 70 eV in the positive electron ionization mode, and nitrogen was used as collision gas at a flow rate of 2.5 mL/min. Data was acquired in the MRM mode using the MassHunter WorkStation Acquisition Software rev. B.02.01 (Agilent Technologies). Table 1 shows the MRM detection conditions for each APD including retention time, quantifier and qualifier transitions, collision energies, and dwell times.

## Sample preparation

The extraction procedure for APDs was as follows: after homogenization in the vortex-mixer for 10 s, 50  $\mu$ L of oral fluid was applied in the Whatman™ 903 protein saver card and dried for 1 h at 36 °C. Subsequently, the cards of each sample were cut with scissors, where upon 2 mL of methanol (pH = 5.0) with 25  $\mu$ L of IS solution (CPZ-d3 in 0.1  $\mu$ g/mL and PRZ in 0.5  $\mu$ g/mL) was added, and the extraction was performed with a roller mixer at 70 rpm for 5 min. The samples were then centrifuged for 15 min at 3500 rpm. The extract was evaporated to dryness under a gentle nitrogen stream and was afterwards derivatised with 50  $\mu$ L of MSTFA with 5% TMCS for 2 min in a microwave oven at 800 W. A 2- $\mu$ L aliquot of the derivatized extract was injected into the GC-MS/MS system.

## Validation procedure

The method was validated according to the guidelines of the Food and Drug Administration (FDA) [27], the International Conference on Harmonization (ICH) [28], the standard practices for method validation in forensic toxicology (SWGTOX) [29], and the European Bioanalysis Forum (EBF) [30]. The validation procedure was performed over a period of 5 days, and the studied parameters included selectivity, linearity and limits, intra- and inter-day accuracy and precision, recovery, stability, and dilution integrity.

**Table 2** Linearity data ( $n = 5$ )

Analyte	Linear range (ng/mL)	Linearity*		$R^2$ *	LLOQ (ng/mL)
		Slope	Intercept		
CPZ	10–400	$0.0012 \pm 0.0002$	$0.048 \pm 0.021$	$0.997 \pm 0.002$	10
LVP	10–400	$0.0032 \pm 0.0004$	$0.017 \pm 0.017$	$0.997 \pm 0.001$	10
CYA	10–400	$0.0009 \pm 0.0002$	$0.004 \pm 0.005$	$0.998 \pm 0.001$	10
CLZ	10–400	$0.0006 \pm 0.0002$	$0.005 \pm 0.004$	$0.996 \pm 0.001$	10
HAL	5–100	$0.0186 \pm 0.0070$	$0.069 \pm 0.040$	$0.996 \pm 0.004$	5
QTP	10–400	$0.0032 \pm 0.0008$	$0.019 \pm 0.020$	$0.998 \pm 0.001$	10

\*Mean values  $\pm$  standard deviation. The weighting factor was  $1/x$  for all analytes

## Results and discussion

### Extraction optimization

According to the literature, the extraction procedure involving dried matrix spots (DMS) is executed manually, which involves punching a disk from the center of the card and placing it in a clean tube. Then, a certain volume of organic solvent and an appropriate concentration of IS are added. Subsequently, homogenization in the roller stirrer and centrifugation are performed, after which the extracts are transferred into clean glass tubes for further analysis [16, 18, 31]. In the present work, the type of solvent and volume, drying time of the DSS, and homogenization time were evaluated and optimized. Concerning solvent type, nine different organic solvents were studied ( $n = 3$ ): hexane, methanol:acetonitrile (MeOH:ACN) (50:50, v/v), acidified acetonitrile (pH 5.0) (A pH 5.0), methanol, acidified methanol (pH 5.0) (M pH 5.0), acetonitrile (ACN), ethyl acetate (AET), isopropanol (ISOP), and dichloromethane (DCL). For this first assay, a volume of 3 mL was added to all samples; the remaining conditions were kept constant: 15-min agitation time, overnight drying time, and 15-min centrifugation at 3500 rpm were used. After analyzing the results and performed a statistical analysis, it was observed that the best solvent for the extraction of LVP, CYA, HAL, and QTP was M pH 5.0 without significant differences in relation to MeOH:ACN, with Friedmans statistic  $t = -0.149$  ( $p = 1.000$ ),  $t = 0.000$  ( $p = 1.000$ ),  $t = 3.280$  ( $p = 0.037$ ), and  $t = 0.447$  ( $p = 1.000$ ) respectively. Although CPZ and CLZ presented as best solvents MeOH:ACN and MeOH respectively, the statistical analysis did not show significant differences relatively to the remaining solvents with Friedmans statistic  $t = 0.447$  ( $p = 1.000$ ) and  $t = 0.596$  ( $p = 1.000$ ) correspondingly; therefore, M pH 5.0 was selected (Fig. 1a).

After the selection of the extraction solvent, four different volumes were tested ( $n = 3$ ): 1, 2, 3, and 4 mL. All other extraction conditions were kept constant. It was possible to observe that CPZ, HAL, and QTP showed better relative peak areas with a solvent volume of 2 mL, for which the Friedmans test values were  $t = 2.846$  ( $p = 0.027$ ), ( $p = 0.122$ ), and ( $p =$

0.086). For CPZ, the volume of 2 mL revealed significantly better results when compared to the other studied volumes. Although LVP, CYA, and CLZ showed better relative peak areas when a volume of 3 mL was applied, when compared with 2 mL of extraction solvent, no significant differences were observed (Friedman test CYA  $t = 0.206$  ( $p = 1.000$ ), LVP ( $p = 0.334$ ), and CLZ ( $p = 0.072$ )) (Fig. 1b). For this reason, the optimization continued using a volume of 2 mL, which is advantageous since the purpose is to adopt a miniaturized extraction procedure.

Regarding extraction time, four periods were studied ( $n = 3$ ): 5, 15, 30, and 60 min. The results obtained and analyzed with the Friedmans test exposed that a 5-min homogenization period in the roller mixer was better for CPZ, LVP, and CYA without significant differences ( $p = 0.801$ ,  $p = 0.241$ , and  $p = 0.532$ , respectively). For the remaining APDs, better results were obtained for 15 min; however, taking into account that there were no significant differences and the choice of every condition must be the same for all compounds, a time of 5 min was chosen (Fig. 1c).

Furthermore, an important step to take into consideration is the drying time of the DSS. The studied drying times were 1 h, 3 h, and an overnight period of time ( $n = 3$ ). The results showed that in 1 h of drying practically, all the compounds were extracted with a greater relative area peak, and by the Friedman test, no significant differences were detected (Fig. 1d). In all optimization experiments, the IS was added after extraction.

### Method validation

#### Selectivity

Selectivity is commonly considered as the ability of an analytical method to detect the target APDs while evaluating the presence of potential oral fluid endogenous interferences, such as mineral salts, mucins, and digestion enzymes. It is necessary to confirm that the quantitation of the analytes is not affected by the presence of interferences at their retention times [32]. In the present study, oral fluid samples from 10 different origins provided by laboratory staff were evaluated,

**Table 3** Intra-day, inter-day, and intermediate precision and accuracy

Compounds	Concentration	Inter-day ( <i>n</i> = 5)			Intra-day ( <i>n</i> = 6)			Intermediate ( <i>n</i> = 15)		
		Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)
CPZ	10	9.2 ± 1.3	14.3	-7.1	10.4 ± 0.9	8.9	4.3	20.2 ± 1.2	6.3	1.2
	25	26.5 ± 1.3	5.2	6.3	24.2 ± 2.2	9.4	-3.1			
	50	52.0 ± 2.3	4.5	4.1						
	100	93.5 ± 7.5	8.0	-4.2	105.2 ± 8.6	8.2	5.3	146.2 ± 9.0	6.1	-2.5
	200	207.5 ± 5.3	2.5	3.7						
	300	291.7 ± 5.6	1.9	-2.7				356.3 ± 23.3	6.5	1.8
	400	402.9 ± 9.9	2.4	8.0	374.4 ± 26.7	7.1	-6.3			
LVP	10	10.8 ± 1.0	9.5	8.8	10.0 ± 0.8	8.9	0.1	20.6 ± 1.3	6.6	3.4
	25	24.6 ± 2.3	9.4	-1.2	26.1 ± 2.8	10.8	4.4			
	50	46.5 ± 1.8	3.9	-6.8						
	100	95.9 ± 12.6	13.2	-4.0	89.4 ± 3.4	3.8	-10.5	148.8 ± 7.8	5.2	-0.7
	200	203.6 ± 4.6	2.2	1.8						
	300	301.2 ± 3.0	1.0	0.4				354.3 ± 24.1	6.8	1.2
	400	401.2 ± 7.2	1.8	0.3	418.7 ± 23.4	5.6	4.7			
CYA	10	10.6 ± 1.3	12.9	6.6	10.2 ± 0.7	7.2	2.2	20.7 ± 1.1	5.6	3.6
	25	25.3 ± 2.4	9.7	1.2	26.1 ± 1.6	6.2	4.4			
	50	46.3 ± 2.9	6.3	-7.3						
	100	96.1 ± 8.6	9.0	-3.8	91.9 ± 2.8	3.1	-8.0	145.3 ± 10.6	7.3	-3.0
	200	205.4 ± 2.8	1.3	2.7						
	300	300.4 ± 7.6	2.5	0.1				366.4 ± 37.0	10.1	4.6
	400	400.9 ± 4.5	1.1	0.2	445.0 ± 30.9	6.9	11.2			
CLZ	10	9.1 ± 1.2	13.3	-8.0	9.2 ± 0.6	7.2	-7.0	19.7 ± 1.6	8.1	-1.1
	25	23.1 ± 1.2	5.2	-7.2	25.3 ± 1.8	7.2	1.5			
	50	47.3 ± 2.8	6.0	-5.2						
	100	93.6 ± 5.3	5.7	-6.3	100.0 ± 5.9	5.9	0.0	146.4 ± 10.4	7.1	-2.3
	200	210.4 ± 8.1	3.8	5.2						
	300	293.3 ± 9.2	3.1	-2.2				329.5 ± 21.5	6.5	-5.8
	400	402.7 ± 10.7	2.6	0.6	423.6 ± 24.0	5.5	5.9			
HAL	5	4.7 ± 0.2	5.9	-4.7	4.7 ± 0.5	11.3	-4.0	5.2 ± 0.08	2.0	5.8
	10	10.2 ± 0.9	9.4	2.6						
	20	18.6 ± 2.6	14.3	-6.8	20.1 ± 2.6	12.9	0.6	29.3 ± 1.8	6.2	-2.2
	40	40.6 ± 4.0	9.9	1.6						
	60	59.5 ± 1.2	2.0	-0.7				69.6 ± 5.8	8.4	-0.4
	80	80.3 ± 2.0	2.5	0.4	86.3 ± 4.9	0.0	7.9			
	100	100.4 ± 1.1	1.1	0.4	103.4 ± 4.4	4.2	3.4			
QTP	10	10.1 ± 1.0	10.7	1.7	9.9 ± 1.2	12.3	-0.5	20.3 ± 1.1	5.6	1.9
	25	25.3 ± 2.4	9.5	1.4	27.6 ± 1.0	3.6	10.5			
	50	50.0 ± 2.3	4.7	0.1						
	100	93.2 ± 5.2	5.5	-6.7	103.1 ± 4.8	4.7	3.1	148.0 ± 13.0	8.8	-1.3
	200	203.6 ± 10.3	5.0	1.8						
	300	299.2 ± 11.3	3.7	-0.2				375.3 ± 20.7	5.5	7.2
	400	402.2 ± 4.6	1.1	0.5	377.0 ± 40.9	10.8	-5.6			

and considering the WADA criteria for acceptance [33], no interferences were observed at the retention times and selected transitions of the target analytes. In Fig. 2, we compare a blank sample for APDs and a sample fortified at the lowest limit of quantification (LLOQ). These samples were analyzed by the developed method.

### Calibration curves and limits

Spiked samples were processed and tested using the above described extraction procedure in the range of 10–400 ng/mL for all compounds, except for HAL (5–100 ng/mL). The linearity of the method was evaluated using seven calibrators

**Table 4** Absolute recovery ( $n = 3$ )

Analyte	Concentration (ng/mL)	Recovery* (%)
CPZ	10	87.0 ± 1.4
	100	92.8 ± 4.5
	400	96.6 ± 2.9
LVP	10	83.8 ± 5.0
	100	64.2 ± 4.9
	400	70.9 ± 5.1
CYA	10	88.4 ± 22.5
	100	62.5 ± 3.1
	400	77.3 ± 5.1
CLZ	10	91.9 ± 5.2
	100	82.7 ± 2.7
	400	94.4 ± 15.5
HAL	5	82.1 ± 1.3
	20	83.5 ± 11.9
	80	95.0 ± 15.5
QTP	10	88.6 ± 7.5
	100	74.4 ± 5.2
	400	91.9 ± 2.9

\*Mean values ± standard deviation

with five replicates. The calibration curves were obtained by plotting the peak area ratio between each analyte and the IS against concentration. In order to accept calibration curves, they had to comply with two parameters: (1) a determination coefficient ( $R^2$ ) of at least 0.99 and (2) the calibrators' accuracy within ± 15% (except in LLOQ, where ± 20% was considered acceptable). The adopted calibration ranges were wide, and as such weighted least squares regressions had to be adopted ( $1/x$  for all compounds). Calibration data is shown in Table 2. Comparing these results with those from other studies, we can assure that the herein described method offers greater advantages. A study performed by Pujadas et al. [34], who used samples of saliva collected by a device (Salivette®), presented LOQs of 7.2 ng/mL and 1.0 ng/mL for HAL and CPZ, respectively, using SPE and gas chromatography-mass spectrometry (GC-MS), however using 1000  $\mu$ L of sample. In the present work, we only use 50  $\mu$ L of sample, which is 20 times smaller, and we present LLOQs of 10 ng/mL for all the APDs except for HAL (5 ng/mL). On the other hand, Petruczynik et al. [9] also using a SPE method, however using high-performance liquid chromatography-mass spectrometry (HPLC-MS), obtained LLOQs for CLZ, QTP, and LVP of 18.25 ng/mL, 9.24 ng/mL, and 18.76 ng/mL, respectively, using 95  $\mu$ L of sample. Although the quantification limit for QTP is lower than ours, the other limits are above those herein presented. It is important to highlight once again that in this study, the authors used a sample volume higher than ours. Fisher et al. [10] published a method using 200  $\mu$ L of sample, which was collected by the spitting method, extracted by LLE

and analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS), obtaining LLOQs of 5 ng/mL and 2 ng/mL for CLZ and QTP respectively. Admitting that these results were better than ours, sample volume is the major parameter of comparison, and our method uses a miniaturized volume in relation to other studies. Furthermore, Rosado et al. [22] using the spitting method for sample collection, with a SPE technique and GC-MS/MS obtained the same LLOQs for all compounds (10 ng/mL), except for HAL which was 2 ng/mL. However, the volume of sample was 200  $\mu$ L.

#### Intra-day, inter-day, and intermediate precision and accuracy

For these parameters, the precision acceptance criteria were coefficients of variation (CV)  $\leq 15\%$  for all concentrations, while accuracy was characterized in terms of mean relative error (RE) within ± 15% for all concentrations, except for LLOQ where ± 20% was accepted [27].

Intra-day precision and accuracy were evaluated by analyzing six replicates of three concentration levels, on the same day. The obtained CVs were typically lower than 13% at all studied concentrations for all APDs, with a RE within ± 11%.

The inter-day precision and accuracy were evaluated with seven calibrators within a 5-day period. The observed CVs were typically below 15% for all APDs, with an inaccuracy of ± 9%.

Regarding intermediate precision and accuracy, quality controls (QCs) with concentrations of 20, 150, and 350 ng/mL for CPZ, LVP, CYA, CLZ, and QTP and 5, 30, and 70 ng/mL for HAL were prepared and analyzed in triplicate over the 5-day validation protocol ( $n = 15$ ). The obtained CVs were below 11% with a RE within ± 8%. All data are showed in Table 3.

#### Recovery studies

The recovery relates the response of a spiked extracted sample and the response of a blank sample spiked with the target analytes after extraction [32]. Guidelines usually require that analyte recoveries are evaluated at low and high concentrations. In the present work, three different concentration levels were studied: 10, 100, and 400 ng/mL for all APDs, except for HAL (5, 20, and 80 ng/mL). The obtained recoveries are presented in Table 4, in which the lowest concentrations presented 82 to 92% of recovery, intermediate concentrations 63 to 93%, and the highest concentrations 71 to 97%. Di Corcia et al. [12], where the oral fluid samples were collected directly into a tube by the spitting method, obtained similar results in recoveries for both CPZ and QTP, 109% and 89% respectively, at 150 ng/mL. In the present study, with the same collection method, recoveries of 93% and 74% were obtained for CPZ and QTP respectively at 100 ng/mL using the DSS technique. Di Corcia et al. [12] used a common extraction method (SPE) being analyzed by ultra-high-performance liquid

**Table 5** Short-term, freeze/thaw, and long-term stability and accuracy ( $n = 3$ )

Analytes	Autosampler stability ( $n = 3$ )					Freeze/thaw ( $n = 3$ )					Long-term stability ( $n = 3$ )					
											8 days					
	Concentration	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured (ng/mL)	CV (%)	RE (%)
CPZ	20	20.5 ± 2.5	12.5	2.4	21.6 ± 0.1	0.6	8.3	19.5 ± 0.3	1.8	-2.4	21.9 ± 1.5	7.0	9.9	-7.3 ± 6.3	85.7	-136.9
	150	143.3 ± 6.0	4.2	-4.4	167.6 ± 5.5	3.3	11.7	163.8 ± 7.0	4.3	9.2	136.8 ± 4.7	3.4	-8.7	56.0 ± 15.9	28.4	-62.7
	350	371.8 ± 18.9	5.1	6.2	376.2 ± 11.9	3.1	7.4	375.6 ± 5.3	1.4	7.3	320.3 ± 26.6	8.3	-8.4	39.1 ± 15.4	39.4	-88.8
LVP	20	21.9 ± 0.1	0.5	9.5	20.9 ± 1.2	6.1	4.5	20.4 ± 2.1	10.7	2.0	18.6 ± 2.0	11.0	-6.9	0.3 ± 3.4	88.0	-98.0
	150	152.9 ± 20.1	13.1	1.9	148.1 ± 18.4	12.4	-1.2	135.1 ± 4.3	3.2	-9.9	133.2 ± 3.4	2.5	-11.1	45.3 ± 3.7	8.3	-69.7
	350	316.9 ± 27.1	8.5	-9.4	368.9 ± 29.0	7.8	5.4	361.5 ± 19.8	5.4	3.2	324.3 ± 40.4	12.4	-7.3	97.9 ± 23.6	24.1	-72.0
CYA	20	21.4 ± 1.5	7.4	7.3	20.5 ± 1.2	5.8	2.7	18.8 ± 1.8	9.7	-5.9	19.7 ± 2.0	10.5	-1.4	6.1 ± 3.6	60.5	-69.5
	150	159.8 ± 7.9	5.0	6.5	146.3 ± 11.0	7.5	-2.4	138.4 ± 3.0	2.1	-7.7	133.6 ± 3.7	2.8	-10.9	72.2 ± 2.1	2.9	-51.9
	350	362.6 ± 7.9	2.2	3.6	329.2 ± 7.2	2.2	-5.9	369.1 ± 3.3	0.9	5.4	333.1 ± 30.3	9.1	-4.8	130.9 ± 30.6	23.4	-62.6
CLZ	20	21.2 ± 1.6	7.8	6.2	19.4 ± 1.9	10.2	-2.9	20.1 ± 1.2	6.0	0.8	21.6 ± 1.3	6.3	8.3	7.8 ± 2.3	29.8	-60.6
	150	153.0 ± 19.3	12.6	2.0	160.1 ± 17.9	11.2	0.0	148.4 ± 9.9	6.7	-1.0	148.1 ± 18.6	12.5	-1.2	68.1 ± 5.3	7.9	-54.6
	350	378.6 ± 30.4	8.0	8.1	354.3 ± 37.5	10.6	0.0	384.2 ± 5.4	1.4	9.7	352.0 ± 41.3	11.7	0.6	138.7 ± 15.0	10.8	-60.4
HAL	5	4.6 ± 0.1	3.6	-7.8	5.0 ± 0.5	11.7	1.2	5.1 ± 0.4	8.5	3.6	5.2 ± 0.3	6.0	5.0	5.0 ± 0.1	2.1	1.3
	30	31.8 ± 4.1	13.0	6.1	31.4 ± 2.7	8.7	4.9	31.0 ± 2.3	7.4	3.5	29.3 ± 1.0	3.4	-2.2	31.8 ± 0.1	0.3	6.2
	70	69.0 ± 4.5	6.5	-1.4	67.4 ± 2.9	4.4	-3.7	76.4 ± 2.9	3.9	9.1	79.0 ± 0.2	0.3	12.9	64.4 ± 5.1	8.0	-7.9
QTP	20	20.6 ± 1.0	5.2	3.0	22.0 ± 0.4	1.8	10.1	20.1 ± 1.5	7.5	0.9	22.2 ± 0.7	3.4	11.1	21.0 ± 0.8	3.9	5.2
	150	168.0 ± 0.07	0.0	12.0	153.0 ± 13.6	8.8	2.0	139.8 ± 5.7	4.1	-6.7	159.2 ± 4.5	2.8	6.1	141.7 ± 3.0	2.1	-5.4
	350	338.2 ± 16.6	4.9	-3.3	340.5 ± 44.2	12.9	-2.6	356.2 ± 15.1	4.2	1.7	361.5 ± 30.9	8.5	3.2	305.4 ± 7.5	2.4	-12.7

All concentrations in ng/mL. Short-term and freeze/thaw stability were studied using neat oral fluid samples. Long-term stability was evaluated after spotting samples on DSS cards



**Table 6** Dilution integrity ( $n = 3$ )

Compounds	Concentration	Dilution factor								
		1:2			1:5			1:10		
		Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)	Measured	CV (%)	RE (%)
CPZ	750	810.1 ± 82.6	10.2	8.0	679.1 ± 37.3	5.4	-9.4	721.2 ± 77.12	10.6	-3.8
LVP	750	714.2 ± 14.8	2.0	-4.7	718.9 ± 32.6	4.5	-4.1	705.0 ± 91.1	12.9	-6.0
CYA	750	819.4 ± 52.4	6.4	9.2	745.5 ± 45.6	6.1	-0.6	742.7 ± 103.1	13.8	-0.9
CLZ	750	776.9 ± 49.8	6.4	3.5	735.1 ± 24.3	3.3	-1.9	762.2 ± 82.5	10.8	1.6
HAL	150	159.2 ± 19.1	12.0	6.1	142.6 ± 3.7	2.6	-4.8	159.2 ± 18.1	11.3	6.1
QTP	750	800.0 ± 4.4	0.5	6.6	812.7 ± 47.5	5.8	8.3	818.9 ± 43.4	5.3	9.1

All concentrations in ng/mL

chromatography-tandem mass spectrometry (UHPLC-MS/MS). Regarding the work by Rosado et al. [22], the recoveries obtained for the same compounds, in oral fluid samples using also the spitting method of collection, were 66 to 86% at low concentrations, and 90 to 108% at high concentrations. Additionally, Patteet et al. [11], using oral fluid samples collected by a specific device (Quantisal®), LLE as extraction technique and LC-MS/MS, obtained recoveries of 70%, 58%, and 65% for CLZ, HAL, and QTP, respectively, considerably lower than ours. In this work, the extraction procedure was optimized comprehensively, and this fact may have played an important role in what concerns recovery. The use of acidified methanol as extraction solvent may have also provided an enhanced capability of removing the analytes from DSS cards.

### Stability

Stability evaluation was divided into short-term, freeze/thaw, long-term, and autosampler stability [35]. Short-term stability was performed with neat oral fluid samples fortified at the concentrations of QCs and then left at room temperature for a 24-h period, time after which they were applied to the DSS. The results showed that the compounds were stable, presenting

CVs below 15% and RE within ± 12% when compared to freshly prepared samples. Concerning freeze/thaw stability, neat oral fluid QC samples were subjected to three cycles of freeze and thaw before being applied onto the card. All APDs demonstrated CV values typically lower than 11% and RE within ± 10%. Unlike short-term and freeze/thaw stability, long-term stability was assessed in DSS, and not in neat samples. Regarding long-term stability, fortified samples were applied to the card and were left on benchtop for specific time intervals (4 and 8 days). The results showed that all APDs were stable in the card for a 4-day period, and CVs typically lower than 13% and mean RE within ± 13% were obtained. However, for the 8-day period, only HAL and QTP remained stable, with CVs ≤ 9% and mean RE within ± 13%. Autosampler stability was also evaluated, in which all extracts (in the vials) were re-analyzed after 24 h at room temperature. The results revealed CVs below 13% and RE within ± 12%. Stability data is presented in Table 5.

### Dilution integrity

The integrity of the diluted samples is an important validation parameter, since authentic samples may be at a concentration

**Table 7** Analysis of authentic samples

Sample	Analyte (s)	Concentration (ng/mL)	Measured concentration (ng/mL) using the method [22]
1	QTP	13.0	11.9
2	HAL	6.2	5.5
3	HAL	17.4	18.8
4	QTP	20.5	22.0
5	QTP	7.7	7.5
6	HAL	8.9	9.6
7	CLZ	111.2	102.4
8	LVP	196.7	192.5
9	QTP	22.0	24.5
10	LVP/HAL	16.6/22.1	15.1/20.9
11	HAL	6.0	5.7

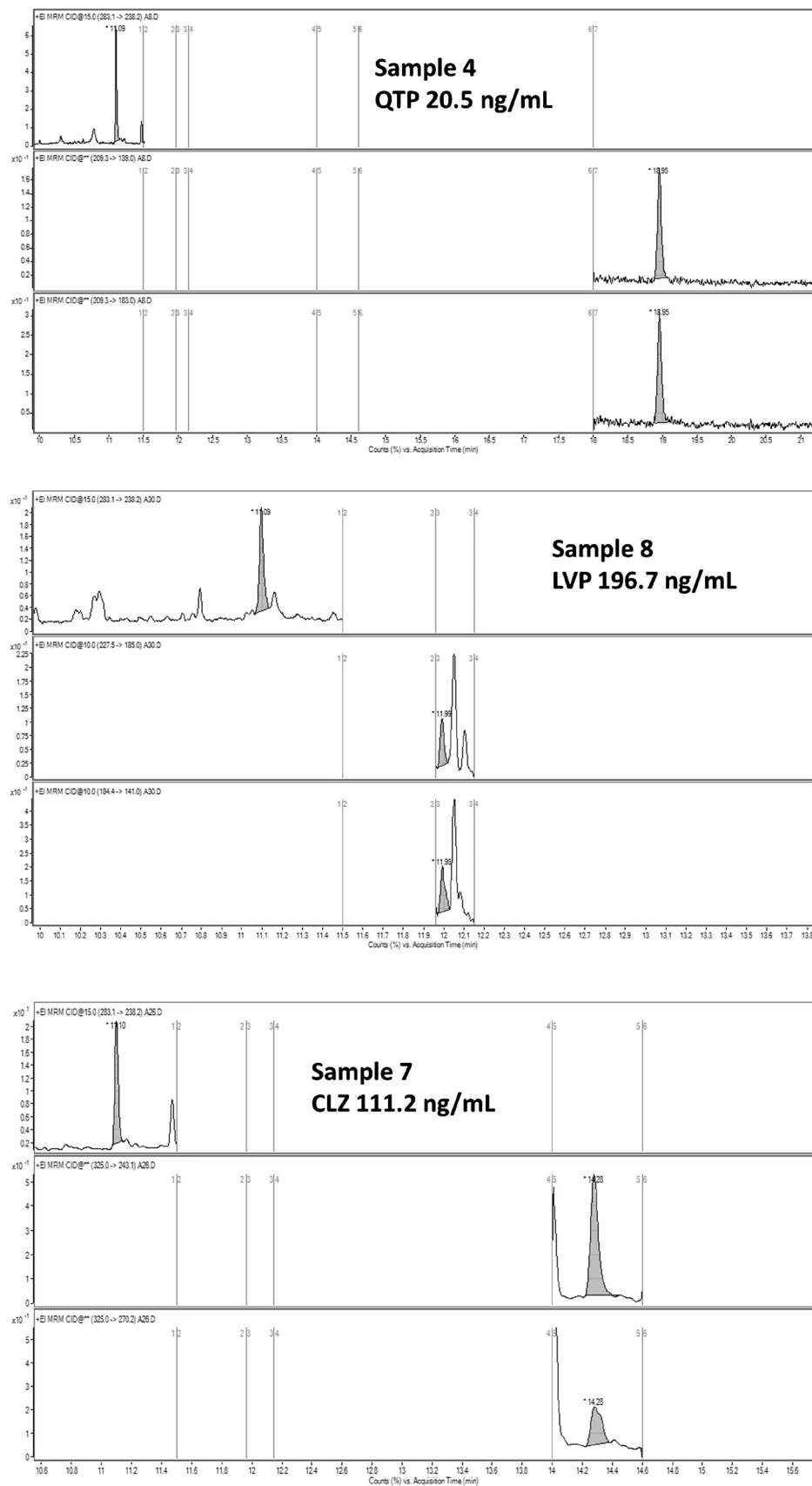


Fig. 3 Chromatogram obtained by analysis of three positive authentic sample

above the upper limit of quantification (ULOQ) of the adopted calibration curve [27]. Thus, three dilution factors (1:2, 1:5, and 1:10) were tested, using a concentration of 750 ng/mL for all APDs, except for HAL (150 ng/mL). This dilution was made with blank oral fluid, and the samples were applied on the DSS after dilution, and the results showed CVs below 15% for all APDs with a RE within  $\pm 10\%$  (Table 6).

### Method applicability

The described method was applied in routine analysis of the target APDs in authentic oral fluid samples belonging to patients under treatment in the psychiatric center of the Hospital Cova da Beira, Covilhã, Portugal. The concentrations ranged from 6.3 to 22.1 ng/mL for HAL, 7.7 to 22.0 ng/mL for QTP, and 16.6 to 196.7 ng/mL for LVP. CLZ was detected in only one sample, at 111.2 ng/mL. The concentrations found in authentic oral fluid samples are shown in Table 7, and Fig. 3 shows chromatograms of authentic samples positive for QTP (20.5 ng/mL, LVP at 196.7 ng/mL and CLZ at 111.2 ng/mL). The present method was also compared to a previous published manuscript [22], where SPE was used for sample preparation. Few studies report concentrations of the studied compounds in oral fluid, and these depend obviously on the administered dose; in addition, our method was only applied to 11 samples so far, meaning that more samples need to be analyzed in order to allow proper conclusions. However, there are a few studies where the found concentrations are close to ours. For instance, Fisher et al. [36] found concentrations of 40 ng/mL and 48 ng/mL for CLZ and QTP, respectively, and these concentrations fall within the proposed calibration range. In addition, Patteet et al. [11] reported an excellent comparison between oral fluid and serum samples concentrations and described the ratios found. The LOQs obtained by those authors are very similar to the ones achieved in the present work. Furthermore, Langel et al. [37] presented a multimethod that included CPZ and LVP with equal or greater LOQs than ours, which also corroborates the linear range herein adopted. Concerning CYA, this was the only APD for whom it was not possible to demonstrate the ability of the method, since its concentrations in biological fluids are not well described.

### Conclusions

A fully validated method is described for the determination of clorpromazine, levomepromazine, cyamemazine, clozapine, haloperidol, and quetiapine in oral fluid samples using the dried saliva spots approach. Analyte extraction from the spot was fully optimized, being successfully applied to authentic samples, and allowing recoveries between 63 and 97%. This miniaturized procedure revealed to be simple, user friendly,

associated to a rapid extraction procedure, and requiring a small volume of sample (50  $\mu$ L). This small sample volume, besides of being easily spotted onto the DSS card and of drying quickly, allows further tests and exams to be performed on the remaining of the sample. This is important, since there are situations where oral fluid sampling is not easily performed, and sample amount is an issue. The limits of quantification with this small sample volume were 5 ng/mL for haloperidol and 10 ng/mL for the remaining target antipsychotic drugs. The analytes were found stable on the dried saliva spot at room temperature for 4 days, and this period could even be extended to 8 days for quetiapine and haloperidol. This is the first developed method for the determination of antipsychotic drugs in oral fluid using this approach and gas chromatography-tandem mass spectrometry, and was proven as a great alternative for a routine clinical and toxicological analysis.

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### Compliance with ethical standards

The present study was approved by the ethics committee from Centro Hospitalar Cova da Beira and has been conducted according to ethical standards. The analyzed authentic samples belonged to individuals who provided an informed consent for their use, and all analyses were carried out according to the ethical standards of the institution.

**Conflict of interest** The authors declare that they have no conflicts of interest.

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