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Determination of antipsychotic drugs in oral fluid samples using dried saliva spots

Versão final após defesa

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Dedicatória

Esta dissertação é dedicada ao meu avô paterno, por toda a força e coragem que me forneceu.

“Progress is not achieved by luck or accident, but by working on yourself daily” – Epictetus

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Resumo Alargado

A esquizofrenia é considerada uma das doenças mentais que mais incapacidade causa em todo o mundo, afetando 1% da população. São múltiplas as causas que podem dar origem a esta doença, tais como fatores bioquímicos, hereditários, entre outros. Atualmente não se dispõe de uma cura mas a minimização dos seus sintomas é conseguida através da administração de medicamentos e/ou terapias psicossociais. Nos últimos anos, o tratamento desta doença mental teve um grande impacto na área da investigação, visto que o número de suicídios em relação a pacientes esquizofrénicos foi aumentando ligeiramente. O tratamento da esquizofrenia é realizado através de medicação antipsicótica que tem como objetivo principal atenuar os sintomas psicóticos. Consoante o diagnóstico do paciente, é importante que exista uma vigilância na administração de antipsicóticos de forma a evitar overdoses ou erros na medicação. Segundo o relatório da INFARMED, o consumo de antipsicóticos em Portugal entre 2000 e 2012 aumentou, tendo como principais causas a utilização prolongada destes fármacos e a facilidade com que se acede a estes. Estes tópicos levantam um importante problema de saúde pública, contribuindo para o desenvolvimento de investigações na área toxicológica e clínica.

Em relação ao desenvolvimento de técnicas analíticas para a deteção e quantificação dos antipsicóticos, a amostra de fluido oral tem vindo a ter um grande impacto devido à sua facilidade de recolha e por ser considerada uma amostra não invasiva. Ultimamente, como técnica de extração de amostra, os *dried saliva spots* (DSS) têm sido utilizados na deteção de diversos tipos de fármacos, visto ser um processo que requer um baixo volume de saliva e o seu processo ser bastante simples e rápido.

Tendo por base esta premissa o presente trabalho descreve o desenvolvimento, otimização e validação de um método analítico para a determinação de seis antipsicóticos (clorpromazina, levomepromazina, ciamemazina, clozapina, haloperidol e quetiapina) em saliva com recurso a DSS e a cromatografia gasosa acoplada à espectrometria de massas em tandem (GC-MS/MS). Os fármacos estudados foram selecionados com base na sua prescrição e associação a efeitos tóxicos. Os padrões internos utilizados foram a clorpromazina-d_3 e a promazina, visto que apresentam características químicas semelhantes às dos compostos em estudo. O método proposto foi totalmente validado quanto a seletividade, linearidade, limites de quantificação, precisão e exatidão, estabilidade e recuperação conforme os critérios internacionais sugerem. A otimização do processo de extração foi realizada de acordo com os seguintes parâmetros: escolha do solvente mais adequado (metanol acidificado), volume do solvente (2 mL), bem como o tempo de extração (5 minutos) e o tempo de secagem das amostras de fluido oral aplicadas nos DSS (1 hora). O método foi linear para todos os compostos numa gama de concentrações de 10-400 ng/mL, exceto para o haloperidol (5-100 ng/mL), apresentando coeficientes de determinação superiores a 0.99. A precisão e exatidão inter- e intra-dia foram realizadas de acordo com a validação bioanalítica do método, isto é, os coeficientes de

variação apresentam-se abaixo dos 15% e uma exatidão de ± 15% ou inferior, para todos os fármacos em estudo. As recuperações obtidas com esta técnica miniaturizada variaram entre 63% a 97%. De forma a verificar a fiabilidade do método, este foi aplicado a amostras de fluido oral procedentes de doentes em tratamento com estes fármacos. Salienta-se que o método que aqui se descreve é o primeiro trabalho que utiliza DSS como técnica de extração para a análise de antipsicóticos. Os resultados apresentados permitem afirmar que a técnica proposta apresenta uma excelente sensibilidade (5-10 ng/mL) para além de ser um procedimento que a nível prático mostra uma maior simplicidade e rapidez comparativamente aos outros métodos de extração. Por outro lado, o volume de amostra constitui uma grande vantagem, visto que se utiliza apenas 50 µL de fluido oral. Assim sendo, este método representa uma excelente alternativa às técnicas convencionais na área da toxicologia e da monitorização clínica, permitindo melhorar a assistência ao doente, diminuir o risco de toxicidade, reduzir reações adversas e/ou interações farmacológicas como também a falha na adesão à terapêutica.

Palavras-chave

Dried saliva spots, Antipsicóticos, Fluido oral, GC-MS/MS.

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 10-400 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 µL).

Keywords

Dried saliva spots, Antipsychotic drugs, Oral fluid, GC-MS/MS

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APDs	Antipsychotic drugs
CI	Chemical ionization
CLZ	Clozapine
CNS	Central nervous system
CPZ	Chlorpromazine
CYA	Cyamemazine
CYP	Cytochrome P450
DMS	Dried matrix spots
DSM	Diagnostic and statistical manual of mental disorders
DSS	Dried saliva spots
EI	Electron impact
EPS	Extrapyramidal symptoms
FID	Flame ionization detector
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography tandem mass spectrometry
HAL	Haloperidol
HPLC	High performance liquid chromatography
HPLC-UV	High performance liquid chromatography - ultraviolet detector
HPLC-DAD	High performance liquid chromatography - diode-array detector
ICD	International classification of diseases
IS	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LSD	Lysergic acid diethylamide
LVP	Levomepromazine
MEPS	Micro-extraction by packed sorbent
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NMDAR	N-methyl-D-aspartate receptor
PET	Positron emission tomography
PID	Photoionization detector
PRZ	Promazine
QTP	Quetiapine
SDAs	Serotonin and dopamine antagonists
SMD	Selective mass detectors

SPE	Solid phase extraction
SPECT	Single photon emission computed tomography
TCD	Thermal conductivity detector
TDM	Therapeutic drug monitoring
UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometer
UPLC-ESI-MS/MS	Ultra-performance liquid chromatography–electrospray ionization–tandem mass spectrometry
5-HT	5-hydroxytryptamine
5-HT2R	Serotonin type 2 receptors

Justification and Objectives

Antipsychotic drugs (APDs) are a specific type of drugs used in the treatment of schizophrenia, which affects several pathways in the Central Nervous System (CNS) and may cause adverse effects. These drugs have been object of several studies with the aim of reducing their side effects and ensuring the patient's comfort with the medication that is prescribed. Considering the clinical and toxicological analyses of APDs, the specimens preferentially used in studies for their determination and quantification are whole blood, serum and urine. However, oral fluid sample has achieved a great interest in the development of new analytical methods with possible application in the therapeutic drug monitoring (TDM). This alternative sample has advantages over others, namely a non-invasive collection, easy handing and representing the free fraction of the drug.

For these reasons, it is interesting to investigate the correlation between oral fluid and plasma concentrations and to develop analytical methods to quantify these drugs in order to apply them in TDM. The aim of these researches is to overcome the consequences that can result from the consumption of APDs.

On the other hand, a relevant topic to consider is the time and costs that several conventional methods consume. For this reason, the use of dried saliva spots (DSS) has gained more popularity in the determination of different classed of compounds.

The main goal of this work was to develop an analytical method using a simpler and more practical extraction technique for the determination of six APDs, chlorpromazine, levomepromazine, cyamemazine, clozapine, haloperidol and quetiapine in oral fluid samples. The proposed extraction technique was DSS followed by the analysis by gas chromatography-tandem mass spectrometry (GC-MS/MS). The method was validated according to international guidelines. Additionally, another important goal of this work was to evaluate the applicability of the proposed method in authentic samples.

The present work is divided into two chapters, in which chapter I introduces the subject based on the literature and chapter II corresponds to the accepted article.

Chapter I is devoted to the description of schizophrenia, antipsychotic therapy and consequently the APDs used for the study, the advantages of oral fluid sampling and the use of DSS as an extraction technique for the determination and quantification of different analytes.

Chapter II describes the entire experimental procedure performed to achieve the goal of this dissertation and corresponds to the article accepted entitled "Determination of antipsychotic drugs in oral fluid using dried saliva spots by gas chromatography-tandem mass spectrometry".

Chapter 1: Introduction

1 Schizophrenia: A Chronic Mental Illness

Mental illness can be defined as a medical condition involving changes in emotion, thinking or behaviour (or a combination of these). The diagnostic and statistical manual of mental disorders (DSM)-IV and the international classification of diseases (ICD)-10 are the major organizations of mental illnesses and have been extremely useful in clinical practice and in scientific research. Regarding scientific research, one of the main goals is the identification of subjects who constitute a high risk of developing a psychotic disease as well as recognise its pathogenesis and increase the capacity to predict other psychoses. Several reviews have shown that individuals with a serious mental illness, such as schizophrenia, have a higher mortality rate and the close monitoring of these individuals is important in order to minimize symptoms and possible disabilities [1, 2]. Schizophrenia is considered a psychiatric illness that affects about 1% of the population and is one of the top ten causes of disability worldwide [3, 4].

1.1 Diagnosis

The diagnosis of schizophrenia is made on a purely clinical basis of the patient. Considering the diagnostic criteria, the most commonly used methods require patients to have certain symptoms combinations for at least six months, continuously. However, a significant variability may occur among clinicians in the diagnosis when the diagnostic criteria is used in sets with standardized interviews [5]. Symptoms of schizophrenia are diverse and harmful. They frequently begin in late adolescence or early adulthood and continue throughout life. These manifestations can be divided into two types: positive and negative symptoms. The positive are characterized by auditory hallucinations (voices that talk with or about the patient), agitation, disorganised behaviour and delusions (paranoid beliefs). On the other hand, negative symptoms include inability to pay attention, loss of sensation of pleasure, reduced motivation, disorganization of thoughts and communication and social withdrawal. Both positive and negative symptoms differ in intensity over time; a patient may have predominantly one type of symptom at a specific time. Criminal behaviour is not concomitant with schizophrenia, though patients in response to hallucinations or certain delusions may commit violent acts [6]. Table 1 shows some of these symptoms caused by schizophrenia.
A swiss psychiatrist, Eugene Bleuler, was one of the first to introduce the term schizophrenia. For him, this illness was described as a “weakness of associative psychic acts” that caused a “loss of mental links between mental contents”. He recognized the three subtypes of schizophrenia initially presented by Emil Kraepelin (hebephrenic, catatonic and paranoid) as well as adding two new subtypes, the simple and latent. Bleuler’s main contribution was in

the recognition of the heterogeneity of schizophrenia [7]. The distinction between the types of paranoid, catatonic, hebephrenic and undifferentiated schizophrenia remained for many decades the primary approach to sub-schizophrenia. This difference has been extended to moderate classification systems, such as ICD-9 and the modified form DSM-III. In DSM-III and DSM-III-R, the hebephrenic type was identified as a "disorganized type" and this is classified by discord and disorganized behaviour and reduced or inadequate affect. Also, the type called catatonic is characterized by abnormal postures and/or actions, mutism or echolalia, while the paranoid type presents as a major symptomatology the hallucinations without the presence of disorganized speech and behaviours from the previous type. Another form that the DSM-IV criteria presents is denominated undifferentiated and is diagnosed when the patient does not accomplish the criteria for the previous subtypes but satisfies the general criteria for schizophrenia. In addition, two other categories, residual and schizoaffective disorder, were added in which the first represents individuals whose symptoms do not follow the six-month criteria and the second can be considered as a combination between schizophrenia and mood problems (depression or bipolar disorder) [8].

Table 1. Common symptoms of schizophrenia. Adapted from [6].

Positive symptoms	**Negative symptoms**	**Other symptoms**
Hallucinations	Apathy	Cognitive symptoms
Delusions	Poverty of speech	Aggressive symptoms
Disorganised behaviour	Emotional withdrawal	Depression
Incoherence of speech	Anhedonia (lack of pleasure)	Anxiety
Agitation	Asociality	
Incongruity of emotions	Difficulty in abstract thinking	
Thought broadcast	Attention impairment	

1.2 Etiology

Over the past decade researchers have confirmed that schizophrenia is a brain disorder, not being potentiated only by psychosocial factors [9]. On other hand genetic findings affirm that schizophrenia is related to several factors and prenatal complications. Considering the obstetric complications, which frequently affects the development of fetal brain, it makes these individuals more susceptible to emerging schizophrenia [8].

The etiological process has generated some controversy but despite this, it is known that this pathology does not extend evenly, nor is focused on a specific region of the brain. The disorder is associated with variations in the cerebrocortical and limbic region. In addition, the ventricular enlargement and lower cortical volume, abnormalities on cytoarchitectural and abnormal septum pellucidum prevalence are neuropathological changes that characterize the pathology [9, 10].

1.2.1 Dopaminergic hypothesis

Over the years, it has been observed that schizophrenia would be associated with CNS abnormality, showing a dysregulation at dopaminergic receptors [9]. These receptors can be divided into two families: the D_1-family (D_1 and D_5) and the D_2-family (D_2, D_3 and D_4). From neuroanatomical studies it was possible to recognize that dopaminergic neurons perform different activities in four distinct pathways: mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular [11]. Regarding the mesolimbic pathway, one of the effects that schizophrenia causes is the excess of dopaminergic activity, whereas in the mesocortical pathway, it causes a reduction of the dopaminergic signal. The dopamine hypothesis is mostly related to D_2 receptors since studies with positron emission tomography (PET) and single photon emission computed tomography (SPECT) revealed that the occupation of D_2 receptors in vivo was an important step for the prognosis of adverse effects and antipsychotic therapy. These studies suggested that an occupancy for the D_2 receptors between 65% and 70% is required for the antipsychotic effects and an occupancy >80% shows a significant risk of extrapyramidal symptoms (EPS) [11–13]. Further studies with the arrival of the first typical antipsychotic, chlorpromazine, strengthened this hypothesis in which D_2 receptor antagonism leads to a reduction in dopaminergic activity and consequently to fewer positive psychotic symptoms (mesolimbic pathway). However, D_2 receptor antagonists exhibit disadvantages in which frequent development of EPS and an increase in prolactin levels are noted [13].

1.2.2 Serotoninergic hypothesis and other receptor relations

Historically, the serotonergic hypothesis proved to be important when it was found that lysergic acid diethylamide (LSD) had psychotic effects similar to schizophrenia. Although the 5-hydroxytryptamine (5-HT) receptor family divides into several subclasses, the serotonin type 2 receptors (5-HT2R) is one that influences the most on the nervous system leading to psychotic symptoms caused by the mental illness. These symptoms are caused by serotonergic agonists of type 2A receptors, such as LSD, so the antagonistic effect of antipsychotic drugs has emerged as an alternative to those previously used [9, 11]. It has also been suggested that dopamine and serotonin systems would be connected, since dopamine neurons may be innervated by serotonergic neurons in certain midbrain substructures, leading to the hypothesis that accounts for the mechanism of most atypical antipsychotics. This hypothesis is suggested as a combined modulation of the two major hypotheses, termed 5-HT2/D_2 receptor antagonism [11]. The evolution of scientific knowledge has led some studies to state that schizophrenia is a multifunctional disease in which other receptors such as adrenergic, histaminergic and muscarinic also have some influence on the symptomatology. In addition, N-methyl-D-aspartate receptor (NMDAR) shows to be abnormal in schizophrenia, and antagonists of these receptors can induce some negative symptoms and cognitive damages

[8]. The combined interactions of these receptor systems provides an improvement in antipsychotic therapy, due to the many side effects described for the latter [13].

1.3 Antipsychotic Therapy

The main form of treatment of schizophrenia is from neuroleptic drugs known as antipsychotic drugs (APDs). These act on the different receptors in order to minimize both positive and negative symptoms caused by this pathology. Since the 1950s, these drugs have been introduced and revolutionized many of strategies used in the past to treat many mental illnesses [14, 15]. In addition to drug therapy, some psychosocial therapies have been used, such as psychoeducation, assertive community treatment and cognitive behavioural therapy, in which all of them are complemented with pharmacotherapy [16].
APDs have been evaluated in several studies, since the discovery of chlorpromazine until the new recently available medications, which nowadays are possible to divide into two major classes: typical and atypical APDs. Table 2 resumes the different classes of APDs.

Table 2. Examples of APDs, their respective daily doses and blood therapeutic levels. Adapted from [6, 17–20].

	APDs	Daily oral dose (mg)	Therapeutic level (µg/L)
Typical Antipsychotics	Chlorpromazine	200-600	30-300
	Levomepromazine	25-50	15-60
	Cyamemazine	50-300	50-400
	Haloperidol	1-15	5-50
Atypical Antipsychotics	Clozapine	300-450	200-800
	Quetiapine	300-450	70-170
	Olanzapine	5-20	10-100
	Risperidone	2-6	10-100

1.3.1 Typical Antipsychotics

This group consists of the first APDs to be synthesized and therefore are often referred to as first generation or conventional. According to their chemical structure, typical antipsychotics are classified in phenothiazines, thioxanthenes, butyrophenones, perathiepines and diphenylpiperidines, presenting as common feature the high affinity for dopamine type-2 receptors. By blocking these receptors on the mesolimbic and mesocortical pathways, usually related to the mood and emotional behaviour, the positive and negative symptoms can be minimized. On the other hand, the striatum areas are also affected which can lead to thoughtful side effects due to their association with motor skills (Figure 1) [21, 22]. Concerning the hypotheses studied, and previously mentioned, a new group of APDs was formed with the aim of improving the therapeutic effects and minimizing the adverse effects caused by typical antipsychotics.

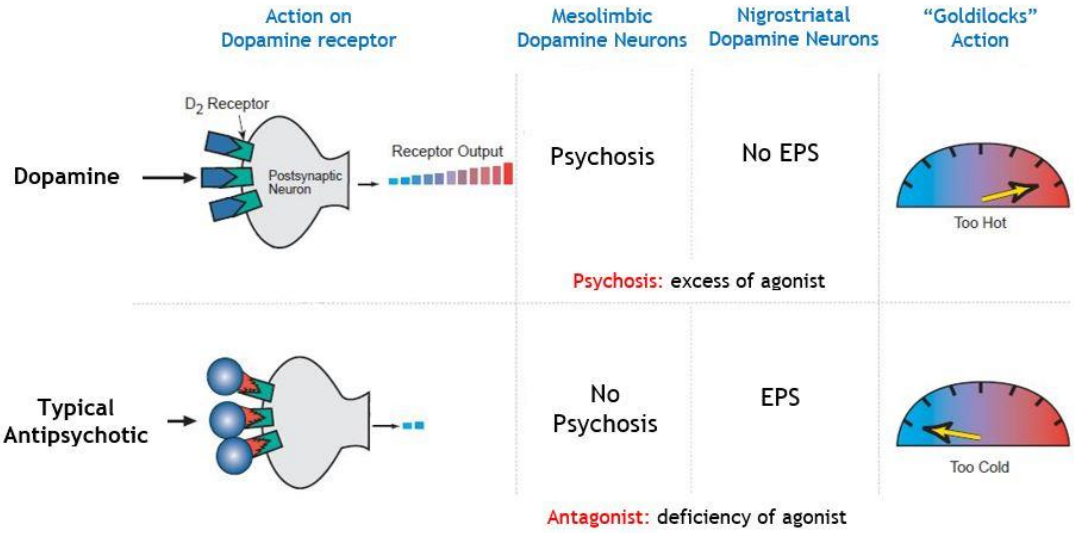


Figure 1. Action on dopamine receptors by dopamine and typical antipsychotic. Adapted from [23].

1.3.2 Atypical Antipsychotics

The new generation of antipsychotics, known as second generation or atypical antipsychotics, were introduced in the 1970s and constitute a very diverse group of drugs that include indoles (e.g., ziprasidone), benzamides (e.g., amisulpride), diazepines/oxazepines/thiazepines (e.g., clozapine, olanzapine , quetiapine), and others (e.g., risperidone, paliperidone) [24]. Although the affinity for dopaminergic receptors is the main target for which the therapeutic effect of the APDs is based, the serotonin and dopamine antagonists (SDAs) have allowed the atypical antipsychotics to be relevant as a therapy [24, 25]. The major difference between this group and typical antipsychotics is the high selectivity for 5-HT2R (5-HT2A, 5-HT2B, 5-HT2C) where many APDs, such as clozapine, employ their effect. In some cases, other receptors such as the 5-HT1A receptor and the adrenergic receptors (α1 and α2) also contribute for this combined modulation [26]. In relation to the 5-HT2A receptor, it was verified that the blockade of this with the D2 receptor blockade presents a differential activity in the nigrostriatal, mesolimbic and mesocortical projections. The diversified activities of this receptor in dopaminergic neurons leads to a decrease of EPS development, as well as the negative and positive symptoms and also cognitive symptoms caused by the pathology [21, 22]. All activities carried out in the different projections and subsequent effects are shown in Figure 2.

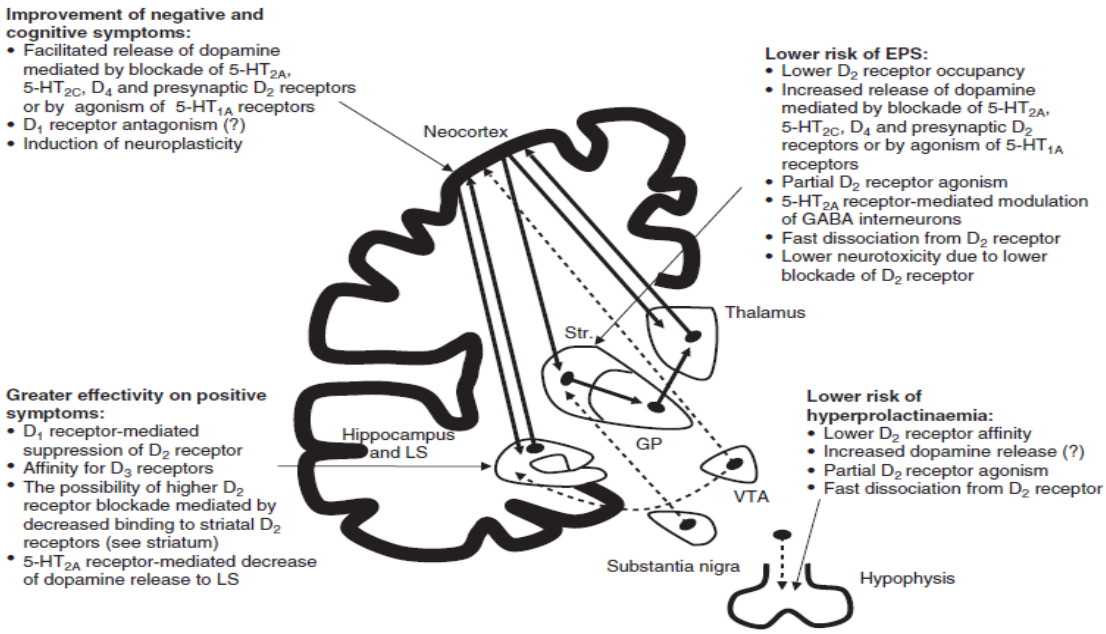


Figure 2. Illustration of the hypothetical mechanism of action of atypical antipsychotics. The pathways involve the limbic system (LS), the striatum (Str.), the globus pallidus (GP) and the ventral tegmental area (VTA). Adapted from [21].

1.4 Adverse Effects

Side effects of APDs have been known since their introduction as the first line of treatment for schizophrenia. In a study by Leuchts, haloperidol was recognized as the drug to cause more EPS while olanzapine and clozapine were identified as the most likely to cause weight gain and metabolic dysfunction [22, 27]. In addition, the EPS can be divided in four categories: pseudoparkinsonism, akathisia, acute dystonia and tardive dyskinesia. The arising of these effects and others, such as high prolactin levels, sedation, hypotension and anticholinergic effects, are dependent on the dose provided to the patient. On the order hand, weight gain, which seem not to be dose dependent, is a sudden adverse effect that usually arises from the use of antipsychotic medication. Furthermore, abnormalities in the male reproductive system, for example, problems in ejaculation and erection were verified as a consequence of these drugs consumption [6, 28]. The knowledge of these effects and others that may appear with the use of both types of APDs (showed in Table 3) is very important so that certain dosages of medication can be avoided, and the well-being of the patient can be guaranteed.

Table 3. Main side effects of typical and atypical APDs. Adapted from [6].

Typical Antipsychotic	Atypical Antipsychotic
Movement disorders, such as dystonia, bradykinesia, tremor and akathisia	Moderate movement disorder
Anhedonia	Diabetes mellitus
Sedation	Sedation
Moderate weight gain	Moderate-to-severe weight gain
Temperature dysregulation	Myocarditis
Hyperprolactinemia	Seizures
Postural hypotension	Agranulocytosis
Prolonged QT internal	Hypercholesterolemia
	Anticholinergic effects

1.5 Antipsychotic drugs studied

In the follow lines a brief resume about the chemical properties of studied APDs is present (figures 3-8 and tables 4-9).

1.5.1 Chlorpromazine (CPZ)

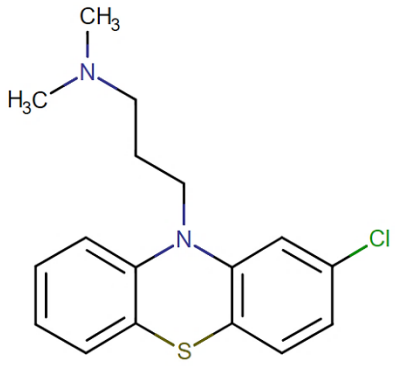


Figure 3 Chemical structure of chlorpromazine [27].

Table 4. Chemical properties of chlorpromazine. Adapted from [29].

Properties	**Chlorpromazine**
IUPAC name	[3-(2-chloro-10H-phenothiazin-10-yl)propyl]dimethylamine
Molecular formula	$C_{17}H_{19}ClN_2S$
Class / Subclass	Benzothiazine / Phenothiazines
Mechanism of action	Antagonist dopaminergic receptors essentially
Half time	Approximately 30 hours
pKa	9.3
Protein binding	> 90% to plasma proteins, primarily albumin

The CPZ is classified as a neuroleptic phenothiazine and was the first drug with therapeutic effects to be discovered. It belongs to the group of typical antipsychotics and presents a very low antagonistic effect on 5-HT2R but high antagonism at dopaminergic D_2 receptors [30]. The use of CPZ, as a therapeutic drug, marked an important step in the control of schizophrenia, which led to investigations aimed at explaining the mechanism of action of the pathology. Although the positive symptoms are minimized by this drug, it is known that this causes a series of adverse effects, mainly EPS and hyperprolactinaemia [31].

Pharmacokinetic

CPZ is metabolised in several metabolites during phases I and II of biotransformation, in which the main reactions are S-oxidation, mono-N-demethylation and di-N-demethylation as well as aromatic hydroxylation and N-oxidation [32–34]. The drug binds largely to the plasma protein albumin and after being consumed orally can reach a peak of concentration in 1-4 hours. Due to its lipophilicity and protein-binding, it is eliminated in 16-30 hours, which corresponds to its half-life. Considering its metabolism, which occurs in the liver, CYP2D6 is the enzyme that more frequently is responsible for this action. Finally, a large amount of conjugated and unconjugated metabolites is excreted in the urine and a small amount can still be excreted in the feces [35]. Some studies have shown that the major metabolites of CPZ are 7-hydroxychlorpromazine and mono-N-desmethylchlorpromazine, which are considered active metabolites and CPZ-5-sulfoxide, which appear to have no clinical effect [34].

1.5.2 Levomepromazine (LVP)

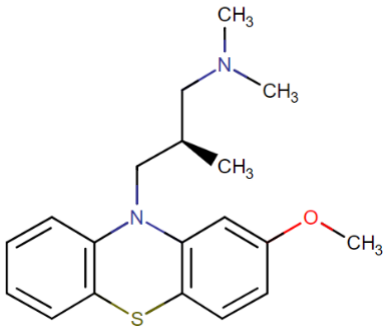


Figure 4. Chemical structure of levomepromazine [27].

Table 5. Chemical properties of levomepromazine [29].

Properties	**Levomepromazine**
IUPAC name	[(2R)-3-(2-methoxy-10H-phenothiazin-10-yl)-2-methylpropyl] dimethylamine
Molecular formula	$C_{19}H_{24}N_2OS$
Class / Subclass	Benzothiazine / Phenothiazines
Mechanism of action	Antagonist dopaminergic/serotonergic receptors
Half time	Approximately 20 hours
pKa	9.19

Another phenothiazine belonging to the first-generation antipsychotic group is the LVP and its clinical effects have made it widely used in psychiatric and non-psychiatric states. The LVP potency is due its effect mostly on the 5-HT2R being considered one of the phenothiazines that more antagonism presents for these receptors. Despite this, it is also a moderate antagonist of the adrenergic alfa-1, muscarinic and dopaminergic D_2 receptors [36].

Pharmacokinetic

The LVP, like the other APDs, is subjected to hepatic metabolism with O-demethylation, aromatic hydroxylation, S-oxidation and N-demethylation. The biotransformation of the drug results from the action of the enzyme CYP3A4, which is responsible for the S-oxidation and N-demethylation reaction. When administered orally, it reaches a peak concentration in 1-3 hours with a half-life between 15-30 hours. Some CYP isoenzymes, such as, CYP1A2, CYP3A2

and CYP2D6 are inhibited by this drug, contributing to problems regarding the metabolism of other concomitant drugs that compete for the same enzymatic system. In men, metabolised LVP produces two major metabolites called N-monodesmethyl LVP, which is pharmacologically active, and LVP sulphoxide, which is considered less active [36, 37].

1.5.3 Cyamemazine (CYA)

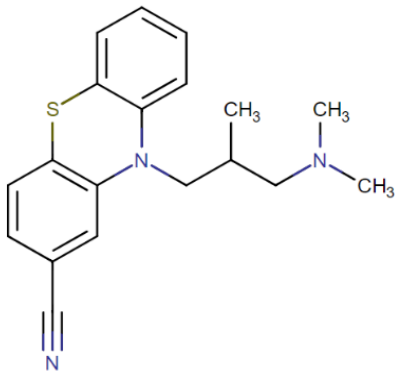


Figure 5. Chemical structure of cyamemazine [27]

Table 6. Chemical properties of cyamemazine [29].

Properties	**CYA**
IUPAC name	10-[3-(dimethylamino)-2-methylpropyl]-10H-phenothiazine-2-carbonitrile
Molecular formula	$C_{19}H_{21}N_3S$
Class / Subclass	Benzothiazine / Phenothiazines
Mechanism of action	Antagonist dopaminergic/serotonergic receptors
Half time	11 hours
pKa	9.42

CYA, also a phenothiazine, reveals great affinity to dopaminergic receptors, blocking specially D_2 type, as well as serotonergic receptors, 5-HT2C and with less extent 5-HT3. Initially, this drug was administered as an anxiolytic agent and later used as an APD in patients with suicidal and depressive tendencies [38].

Pharmacokinetic

As previously described, all phenothiazines are metabolized in the liver by plasma isoenzymes and excreted in the urine. Regarding this drug, its half-life is 11 hours when managed orally, which results in two main metabolites (CYA sulfoxide and monodesmethyl CYA), both excreted after 72 hours [39].

1.5.4 Haloperidol (HAL)

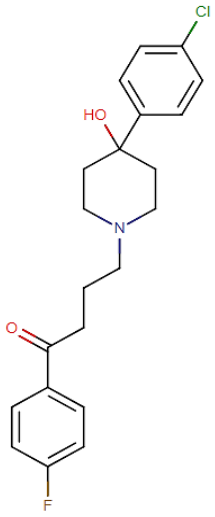


Figure 6. Chemical structure of haloperidol [27].

Table 7. Chemical properties of haloperidol [27].

Properties	**Haloperidol**
IUPAC name	4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one
Molecular formula	$C_{21}H_{23}ClFNO_2$
Class / Subclass	Organooxygen / Butyrophenones
Mechanism of action	Antagonist dopaminergic receptors
Half time	range 14-36 hours
pKa	8.66
Protein binding	90-94%

Unlike other typical antipsychotics, this drug is considered a butyrophenone which is well indicated when patients have severe behaviours as well as treatment for hyperactive children and those who show symptoms like impulsivity, aggressivity and mood lability. Its antipsychotic activity is essentially characterized by strong antagonism at dopaminergic D_2 receptors, such as CPZ, and α1-adrenergic receptors. The HAL can also be prescribed to people with Tourette's syndrome, to control tics and voice changes in children and adults [40, 41].

Pharmacokinetic

Biotransformation of HAL has been the subject of much research because of its complexity, since it is extensively metabolised in the liver and is part of several metabolic pathways such as glucuronidation, reduction/oxidation cycle of the benzylic carbonyl, oxidative N-dealkylation and oxidation of some piperidines. Considering the metabolic enzymes which are involved in its biotransformation, this drug is metabolized by CYP isoforms (CYP3A4), carbonyl reductase and uridine diphosphoglucose glucuronosyltransferase. Although HAL distributes freely in several tissues, it also binds strongly to plasma proteins and its half-life is between 14 to 36 hours (orally administered). The major metabolites are, in humans, p-fluorobenzoylpropionic acid, 4-(4-chlo- rophenyl)-4-hydroxypiperidine, reduced HAL, pyridinium metabolites and HAL glucuronide. It was observed that only 1% of the administered dose, is excreted through the urinary route considering that it undergoes extensive metabolization. It is also known that this drug co-administered with others may affect its pharmacokinetics and consequently modify its clinical effect on treatment [41, 42].

1.5.5 Clozapine (CLZ)

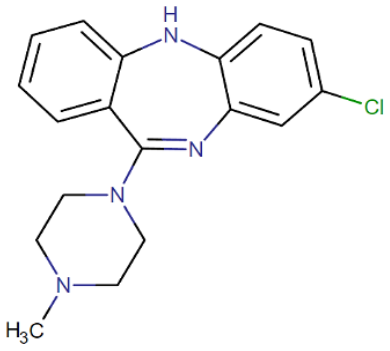


Figure 7. Chemical structure of clozapine [27].

Table 8. Chemical properties of clozapine [29].

Properties	**Clozapine**
IUPAC name	6-chloro-10-(4-methylpiperazin-1-yl)-2,9-diazatricyclo[9.4.0.0^{3},^{8}]pentadeca-1(15),3,5,7,9,11,13-heptaene
Molecular formula	$C_{18}H_{19}ClN_4$
Class / Subclass	Benzothiazine / Dibenzodiazepines
Mechanism of action	Antagonist dopaminergic/serotonergic receptors
Half time	range 6-30 hours
pKa	7.5
Protein Binding	97%

The first drug to be recognized as an atypical antipsychotic was CLZ and the knowledge of its actions on the brain receptors provided important evidence to discover the etiology of schizophrenia. CLZ, a tricyclic dibenzodiazepine, differs from typical antipsychotics because of its affinity for dopaminergic D_1 receptors and minor block at dopamine D_2 receptors [43]. In addition, the high affinity for 5-HT2AR was recognized as an important milestone in the hypothesis that exists a balance between the D_2/5-HT receptors, which constitutes one of the main characteristics of these antipsychotics [25]. Due to the fact that CLZ performs antagonism at 5-HT receptors, this suggests that there is a lower production of significant EPS, which makes it a drug of choice in the treatment of this particular psychotic disease [43, 44].

Pharmacokinetic

Plasma concentrations of CLZ act in a biphasic way, in which peaks may reach 1 to 4 hours when administered orally, followed by terminal elimination of 6 to 30 hours. CLZ is metabolised in the liver, by enzymes such as CYP1A2 and CYP3A4, in which the main reactions that occur are: N-oxidation, N-demethylation and dehalogenation. Its excretion is approximately 50% by urinary route and 35% by faecal route [45].

1.5.6 Quetiapine (QTP)

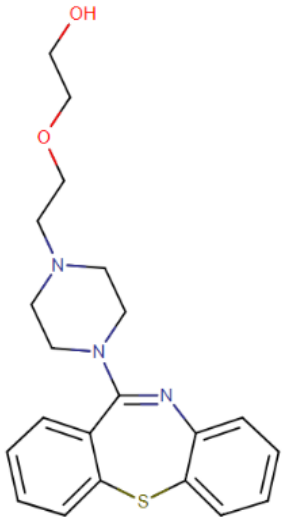


Figure 8. Chemical structure of quetiapine [27].

Table 9. Chemical properties of quetiapine [29].

Properties	**Quetiapine**
IUPAC name	2-[2-(4-{2-thia-9-azatricyclo[9.4.0.0^{3},^{8}]pentadeca-1(15),3,5,7,9,11,13-heptaen-10-yl}piperazin-1-yl)ethoxy]ethan-1-ol
Molecular formula	$C_{21}H_{25}N_3O_2S$
Class / Subclass	Benzothiazine / Phenothiazines
Mechanism of action	Antagonist Dopaminergic/serotonergic receptors
Half time	6 hours
pKa	7.06
Protein binding	83%

Considered an antipsychotic dibenzothiazepine, QTP is one of the most recent APDs in the treatment of schizophrenia as well as other psychotic disorders. Its activity is primarily on the 5-HT2R and D_2 receptors but also exhibits partial antagonism to the 5-HT1 and adrenergic α-2 receptors [6, 24, 46].

Pharmacokinetic

The metabolism of QTP is achieved through the hepatic enzymes, CYP3A4 and CYP2D6, and 83% of this drug binds to plasma proteins. After oral administration, it may reach a plasmatic peak concentration in 1-2 hours revealing a half-life of 6-7 hours. The major metabolites are 7-hydroxyquetiapine and 7-hydroxy-N-desalkylquetiapine which may accumulate in plasma but at non-relevant concentrations. The excretion of QTP is predominantly urinary as metabolites (73%) and unchanged (1%), after oral dosage [47, 48].

1.6 Consumption of Antipsychotics in Portugal

According to the report on psychiatric drugs from the National Authority of Medicines and Health Products, I.P. (INFARMED), the consumption of antipsychotics in Portugal has been increasing significantly during the years [49]. As it is shown in Table 10, there was an antipsychotic consumption in 2012 of 14 defined daily doses of a drug per 1000 habitants/day (DHD) which suggests a 171% increase over 2000.

Table 10. Evolution of Psychopharmaceutical Drugs by Therapeutic Subgroup between 2000 and 2012. Results expressed as DHD. Adapted from [49].

Year	**Anxiolytics / sedatives and hypnotics**	**Antipsychotics**	**Antidepressants**	**Lithium**
2000	91	5	26	0
2012	96	14	88	0,7
Rate (2000-2012)	6%	171%	240%	60%

Concerning the use of the different antipsychotics it was verified that in 2000 the typical ones predominated, mainly HAL and CYA and in general all of them remained unchanged throughout the study time. In relation to atypical antipsychotics, there has been an increase in the use of these drugs since the year 2000, mainly risperidone and olanzapine. A marked increase over time was observed for QTP and, although it was only reimbursed in 2002, it was the substance with the highest consumption. All these aspects can be observed in figure 9.

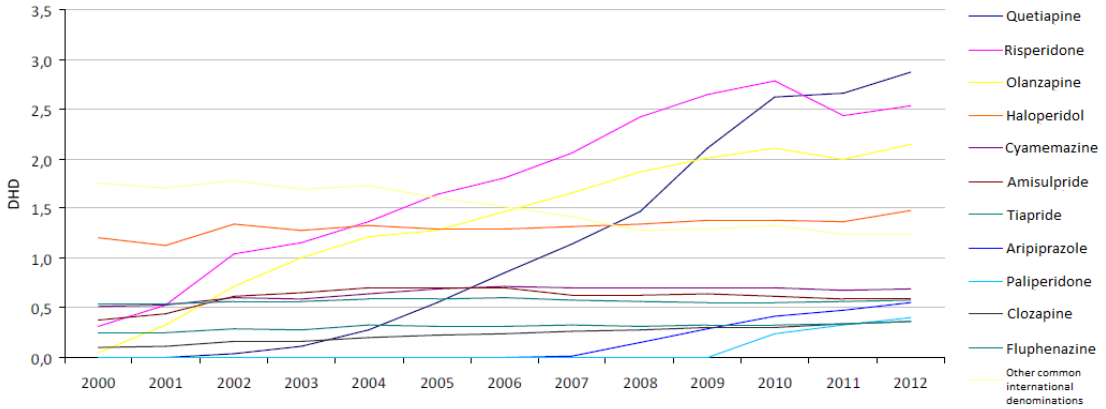


Figure 9. Evolution of the use of the main active substances (Antipsychotics) between 2000 and 2012. Adapted from [49].

According to the same source, the consumption levels in Portugal do not show significant differences in relation to the other countries that were subjected to this study (Denmark, Italy and Norway). In addition, the increase in the use of APDs may have the following justifications: increased duration of treatment, increased the prevalence of psychotic disturbances and off-label use.

Considering the National Program for Mental Health, which evaluate the state of mental illness in Portugal [50], it was verified that between 2013 and 2016 the consumption of psychotic drugs has been increasing over the last few years. As we can see in Table 11, the number of APDs box of tablets prescribed has risen steeply to double since 2013.

Table 11. Consumption of psychotic drugs (by packaging) between 2012-2016. Adapted from [50].

	Number of box of tablets required				
	2012	2013	2014	2015	2016
Psychotic drugs					
Anxiolytics, sedatives and hypnotics	-	7.345.279	12.011.514	12.868.760	12.971.342
Antipsychotics	-	2.146.672	3.871.060	4.457.535	4.863.952
Antidepressants	-	5.556.092	9.617.464	10.973.517	11.795.898

According to the same source and taking into account these values, some preventive measures were implemented for 2020 in order to minimize the consumption of APDs. These measures include: the monitoring of indicators in the field of mental health; monitoring the quality of treatment of psychiatric disorders; implementation of Integrated care in mental health; and also increase the registration of mental disorders in primary health care [50].

2 Determination of antipsychotics in oral fluid

2.1 The oral fluid as a sample

In recent decades, oral fluid has been used for many studies in the field of toxicology as well as therapeutic drug monitoring (TDM). This sample has been shown to be a good alternative to the most conventional samples such as blood and plasma [51]. The oral fluid originates from different salivary glands, three pairs of major glands and some minor glands, which has led many researchers to call this "mixed saliva" [52]. Saliva is a liquid component of the oral fluid, but it comes from a specific gland and lacks the other constituents that are blended in the mixed saliva [53]. In the herein described work, the two terms will be used with the same meaning since both are applied in these specific studies. Regarding its chemical characteristics, the oral fluid has a pH of 6.8 and consists of proteins, water and a few enzymes of digestion. The pH of this sample is a very relevant characteristic in the context that basic substances are influenced, presenting higher concentrations in the oral fluid compared to those in plasma [52]. In TDM, the sample collection technique is a process that can be divided into two types: stimulated saliva and unstimulated saliva. For collection of this specimen, both of these types, include diverse methods like spitting, draining, aspirating and by several types of absorbent materials (such as citric acid, rubber bands, paraffin wax and gum base) or devices (Quantisal™, Accu•SAL™, SalivaScreen® and OralLab®). Absorbent devices are used in many studies and considered more advantageous, for example in cases involving small children when the spitting method is not feasible [51, 52]. Regarding the storage of these samples, it is recommended freezing at -20 ºC after being collected. This storage is very important so that the analytes do not undergo degradation and prevent the development of microorganisms until the analysis [51]. Oral fluid has several advantages over conventional samples, such as the eased up and non-invasive collection, and difficulty of adulteration. In addition, the concentration determined in saliva represents the free fraction non-ionized, which is the pharmacologically active fraction of the drugs, present in blood (Table 12). Although this alternative sample presents some disadvantages, its use in the fields of toxicology, mainly in the analytical area, has proved to be very effective for the quantitative and qualitative analyses of several compounds [51, 54].

Table 12. Advantages and disadvantages of oral fluid. Adapted of [51].

Advantages	Disadvantages
Non-invasive	Vulnerability to alteration
The free-fraction (no protein binding)	Low sterility and samples might be subject to bacterial degradation over time (e.g. when collected by spitting)
Reflects efficiently the drug activity	Insufficient amount of sample (during collection)
Good stability at room temperature (when collected by certified devices)	Small detection window

Recently, oral fluid has been used in the detection of methamphetamines, amphetamines, barbiturates, benzodiazepines, cocaine, cannabinoids and other drugs [53]. Considering sample extraction, the most common methods are mainly liquid-liquid extraction (LLE), solid phase extraction (SPE) and microextraction by packed sorbent (MEPS) [51, 52]. A literature review was performed for the determination of several APDs. It is possible to observe the employment of these extraction techniques with different analytical methods (Table 13).

Table 13. Methods used to determine APDs from saliva samples.

APDs	Volume (µL)	Extraction Method	Analytical Method	LOD (ng/mL)	LOQ (ng/mL)	Recoveries (%)	Reference
RIS and metabolite	100	MEPS	HPLC-UV	0.7 and 1.0	2 and 3	> 89	[55]
CLZ, OLZ, QTP and RIS	200	LLE	LC-MS/MS	n.s.	5 for CLZ 1 for OLZ 2 for QTP 1 for RIS	95 for CLZ 77 for OLZ 105 for QTP 96 for RIS	[56]
HAL	300	LLE	HPLC-UV- PDA	1	3.1	90 - 93	[57]
RIS and metabolite	100	MEPS	LC with coulometric detection	0.17 and 0.17	0.5 and 0.5	>90	[58]
RIS and metabolite	25	LLE	LC-MS/MS	0.91	7.77	90–93 and 89–93	[59]
CLZ, HAL, OLZ, QTP and RIS	500	LLE	UHPLC-MS/MS	0.53 for CLZ 2.36 for HAL 0.16 for OLZ 0.30 for QTP 0.91 for RIS	15.57 for CLZ 14.05 for HAL 21.62 for OLZ 4.26 for QTP 7.77 for RIS	>80	[60]
CLZ, LVP, QTP and RIS	95	SPE	HPLC-DAD; LC-MS	5.48 for CLZ 5.63 for LVP	18.25 for CLZ 18.76 for LVP		[61]

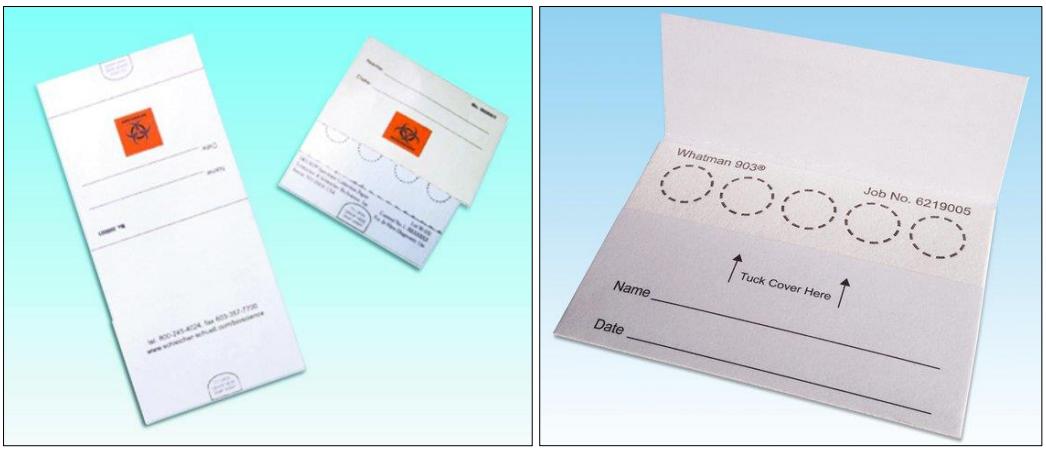
				2.77 for QTP 4 for RIS	9.24 for QTP 13.35 for RIS		
CPZ and HAL	1000	SPE	GC-MS	0.4 and 2.4	1 and 7.2	45 – 98	[62]
CLZ, QTP and RIS	500	LLE	LC-MS/MS	n.s.	10, 2 and 1		[63]
CPZ, OLZ and QTP	500	LLE	UHPLC-MS/MS	0.4 for CPZ 1.23 for OLZ 0.02 for QTP	1.34 for CPZ 4.11 for OLZ 0.07 for QTP	89 - 109	[64]
CPZ, LVP, CYA, CLZ, HAL, OLZ and QTP	200	SPE	GC-MS/MS	5 for CPZ, LVP, CYA, CLZ, QTP 1 for HAL and OLZ	10 for CPZ, LVP, CYA, CLZ, QTP 2 for HAL and OLZ	66 – 108	[65]

Legend: CLZ (Clozapine); CPZ (Chlorpromazine); CYA (Cyamemazine); GC-MS (Gas chromatography – mass spectrometry); GC-MS/MS (Gas chromatography – tandem mass spectrometry); HAL (Haloperidol); HPLC-MS (High performance liquid chromatography – mass spectrometry); HPLC-DAD (High performance liquid chromatography with diode-array detector); HPLC-UV (High performance liquid chromatography - ultraviolet); HPLC-UV-PDA (High performance liquid chromatography ultraviolet-photodiode array detection); LC-MS (Liquid chromatography – mass spectrometry); LC-MS/MS (Liquid chromatography – tandem mass spectrometry); LLE (Liquid-liquid extraction); LOD (Limit of detection); LOQ (Limit of quantification); LVP (Levomepromazine); MEPS (Micro extraction by packed sorbent); n.s. (not specified); OLZ (Olanzapine); QTP (Quetiapine); SPE (Solid phase extraction); UHPLC-MS/MS (Ultra-high performance liquid chromatography - tandem mass spectrometer)

2.2 Dried Saliva Spots (DSS)

The dried saliva spots (DSS) are considered a subtype of the dried matrix spots (DMS), which have received some attention, in recent years, due to its advantages compared to other more conventional extraction techniques [66]. The advantages of DMS are lower costs for storage, practical and easy procedure, as well as small sample and organic solvents volumes required. The latter makes the DSS a useful tool in several TDM studies in oral fluid. The DSS extraction procedure is very similar to the one used for DMS, consisting on the addition of 50 or 100 µL of sample in the spot of DSS card, after which is allowed to dry. Subsequently the spots are transferred to a tube in which a certain amount of organic solvent and internal standard (IS) are added, followed by agitation and centrifugation for a certain time interval and speed. Finally, the supernatant is transferred to another clean tube for further analysis [67, 68]. Abdel Rehim et al. [69] were the first authors to report the use of DSS as an extraction technique to determine lidocaine in oral fluid samples by LC-MS/MS. The authors concluded that this technique demonstrates greater simplicity and speed in the saliva sample extraction process, which may outweigh the drawbacks of blood and plasma samples [69]. In addition, Masahiro Numako et al. [70] used the DSS method in the determination of biological components, enantiomers of lactic acid, in samples from diabetic, prediabetic and healthy patients using UPLC-ESI-MS/MS. They concluded that both the handling and transport, storage and/or pre-treatment of the samples becomes significantly easier [70]. Recently, DSS has been successfully applied for the determination of several drugs, such as anticonvulsants [71] and methadone and metabolites [72]. Figure 10 represents the paper cards commonly used in the DSS extraction technique. The herein described work is the first to report the use of this extraction technique in the determination of APDs.

Figure 10. Paper cards used in the DSS extraction technique. Adapted from [73].



2.3 Gas Chromatography coupled to Mass Spectrometry technique

The techniques of quantification and qualification of a wide range of compounds have evolved over time, so that it is more practical to carry out the analyses in the different fields: toxicology, clinical and forensic. Gas chromatography (GC) is one of the most widely used techniques and is considered to be the one with the most separation power used in the different types of analyses. This has been improved in terms of design, materials and methodology although its instrumentation has remained unchanged for the last 40 years. Considering its instrumentation, this technique consists of four key parts: a carrier gas, a column, a detector and a data system [74, 75]. The detectors are a very specific part of the equipment which may include a flame ionization detector (FID), thermal conductivity detector (TCD), photoionization detector (PID) and an atomic emission detector. On the other hand, selective mass detectors (SMD) and mass spectrometry (MS) had a great influence on the improvement of GC. The combination of these two devices results in a better sensitivity, specificity and an improved separation of the components to be analysed [75, 76]. The MS presents as main advantage, the more detailed information on the structure of the different compounds so that they are properly identified and quantified according to their mass-to-charge ratio (m/z). Usually, this equipment evolves a capillary column inserted directly into the ion source. The effluent leaving the GC is at a temperature around 300ºC, and, in the same way individual compounds will elute from the GC and enter the MS detector, commonly with electron ionization. At this time, the compounds are bombarded by a stream of electrons causing them to fragment. [77, 78].

For an analytical method to be used in the best way it is important to know all its process, as well as all its components that perform different functions and constitute some variables during its operation. Figure 11 shows all the components that constitute a GC-MS and these are characterized by:

- Carrier gas: It is referred to as the mobile phase and is usually constituted by an inert gas such as argon or helium, the latter being the most common. These gases are placed in pressurized compartments and use pressure regulators as well as flow meters in order to control the rate of gas flow.
- Injector: is the site where the sample is volatilized and where the gas is swept into the column.
- Column: It is where the stationary phase of the process is located, which may be a liquid into an inert solid support, usually inside a metal or glass tube. It is estimated that the column of a GC measures between 10-120 meters.
- Oven temperature: This oven can reach temperatures of 5 °C to 400 °C, and this temperature can be programmed. An important variable corresponds to the column temperature, which causes the oven to be equipped with a thermostat that controls the heat transfer.
- Ion source: The compounds before passing to the mass spectrometer are subjected to this source to which they give rise to ionized products. There are two types of

ionization: chemical ionization (CI) and electron impact (EI) in which the latter is the most commonly used since CI reveals a moderate ionization that produces a smaller fragmentation compared to EI.

- Vacuum system: For mass spectra to operate efficiently, it is necessary that they have high levels of vacuum.
- Detector: This component is one that generates the signals of the incident ions, which give rise to secondary electrons which are amplified or induce a current. The detector basically serves to detect the ion beam and turn them into a usable signal.
- Control Electronics: This panel serves to control the constituent parameters of the MS, which could also be controlled from a computer using specific software [77, 79].

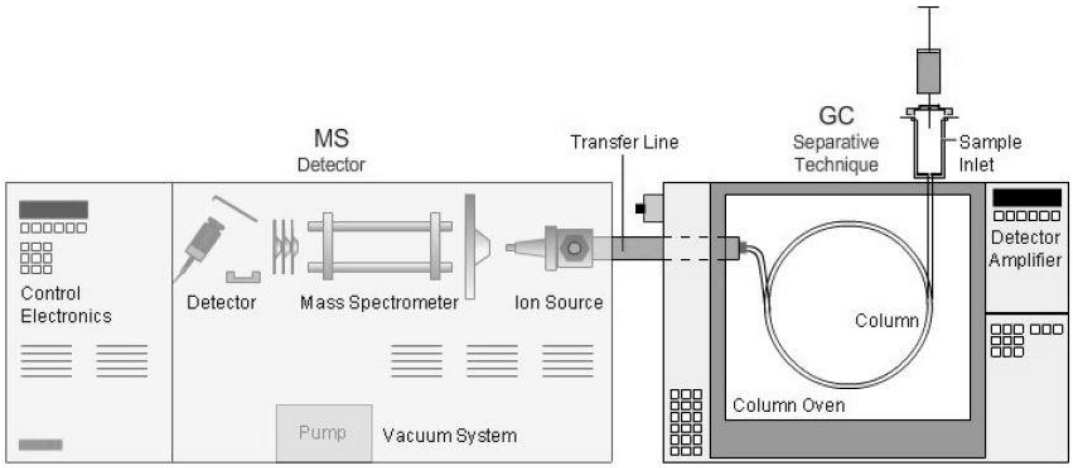


Figure 11. Schematic diagram of GC-MS [77].

This GC analytical method has as main requirement that all compounds are volatile and thermally stable and have demonstrated great utility in the detection and quantification of xenobiotics in several types of samples (e.g., saliva, blood, hair and urine) [76]. Its application is of great diversity, from doping control, toxicological analyses, to studies in the food analysis [74, 77]. Another field that has been using this method since the 70's is the metabolic profiling most properly organic acids and steroids in urine samples [79, 80].

Over time, these devices evolved due to the complexity of mixtures to be analysed, which led to the introduction of GC/MS analogue, constituting a new analytical method called gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). In this case, the components are ionized, then pass through the first stage of analysis, which by collision with a gas are, then, identified by the second stage of mass analysis. The tandem mass spectrometry (MS/MS) can be divided into different types of analysers of which the most common are the quadrupole, the magnetic sector and the electric sector. The characteristics of these types becomes relevant to the knowledge of the advantages and disadvantages that influence the appropriate choice of the analyser. The use of MS/MS has the ability to provide the sensitivity, selectivity, and speed that complex mixtures analysis requires [81].

In the present work GC-MS/MS was used, a triple quadrupole tandem mass spectrometer. This is one of the most used today and is composed of two quadrupole mass analysers and the second (which is in the center) is considered a collision quadrupole as shown in Figure 12 [81, 82]. Considering the characteristics of this equipment and the advantages it presents, as well as the information of its capacity to identify and quantify a great range of different substances with different chemical properties, has made this one the most suitable for the accomplishment of this work.

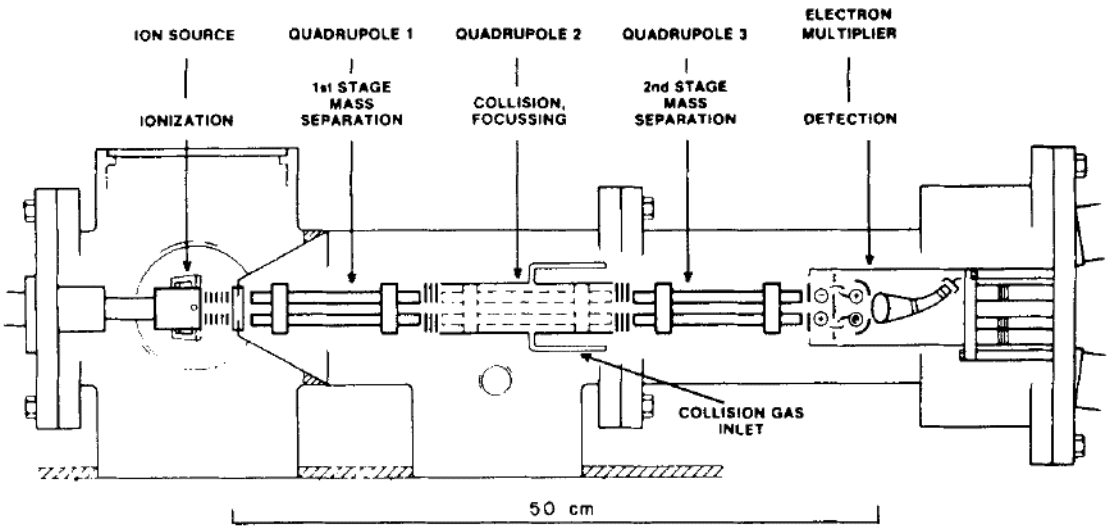


Figure 12. Scale drawing of triple quadrupole MS/MS instrument. Adapted from [81].

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Chapter 2- Experimental procedure

1 Materials and Methods

1.1 Reagents and standards

Standard solutions of haloperidol (HAL), clozapine (CLZ), chlorpromazine (CPZ), as well as the trideuterated analogue of chlorpromazine (CPZ-d_3) 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 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 SigmaAldrich (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 µg/mL for all APDs, with exception of HAL (0.02, 0.2 and 2 µg/mL). The IS CPZ-d_3 and PRZ working solutions were prepared in methanol at the concentrations of 0.1 µg/mL and 0.5 µg/mL respectively. All working and stock solutions were stored in the absence of light at 4 ºC.

1.2 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. 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. The authors declare that they have no conflicts of interest. All oral fluid samples were collected by spitting without the use of specific collection devices. These samples were stored frozen at −20 ºC until analysis.

1.3 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 mx 0.25

mm ID, 0.25 µm film thickness) with 5% phenylmethylsiloxane (HP-5MS), supplied by J & W Scientific (Folsom, USA). The derivatized extract (2 µL) 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 settled with a filament current of 35 µA 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). The initial oven temperature was held at 120 ºC for 2 min, then raised to 300 ºC at a 20 ºC/min rate (maintained for 14 min), resulting in a total runtime of 25 min.

2 Sample Preparation

The extraction procedure for APDs was as follows: after homogenization in the vortex-mixer for 10 seconds, 50 µL of oral fluid were applied in the Whatman™ 903 protein saver card and dried for 1 hour at 36 ºC. Subsequently, the cards of each sample were cut with scissors, whereupon 2 mL of methanol (pH = 5.0) with 25 µL of IS solution (CPZ-d_3 at 0.1 µg/mL and PRZ at 0.5 µg/mL) was added, and the extraction was performed with a roller mixer at 70 rpm for 5 minutes. The samples were then centrifuged for 15 min at 3500 rpm. The extract was evaporated to dryness under a gentle nitrogen stream and then derivatised with 50 µL of MSTFA with 5% TMCS for 2 minutes in a microwave oven at 800W. A 2 µl aliquot was injected into the GC-MS/MS system.

3 Validation procedure

The method was validated according to the guidelines of the Food and Drug Administration (FDA) [1], the International Conference on Harmonization (ICH) [2], the Standard practices for method validation in forensic toxicology (SWGTOX) [3], and the European Bioanalysis Forum (EBF) [4]. 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.

4 Results and Discussion

4.1 Identification of the analytes under analysis

One of the first steps in the development of an analytical method is the identification of the selective compounds.

After the chromatographic conditions are defined, the different analyte standards are injected in scan mode in order to identify the ions that characterize the molecular structure of each compound. The proper identification of each APD is made by comparing the spectrum obtained in scan mode with the existing spectrum in the literature. In this work, all

compounds were individually analyzed at the concentration of 100 µg/mL, where the respective retention times were defined. Following the appropriate choice of ions from the mass spectrum, they were subjected to a fragmentation in product ion (PI) mode, under different collision energies (5, 10, 15 and 20 eV). The result of this fragmentation gives rise to the ion transitions that best represent the chromatographic signal, with the least possible interference and without the contribution of other analytes under study. Figure 1 and 2 show two examples of mass spectra of two different APDs and their respective spectra after PI mode analysis are shown in Figure 3 and 4.

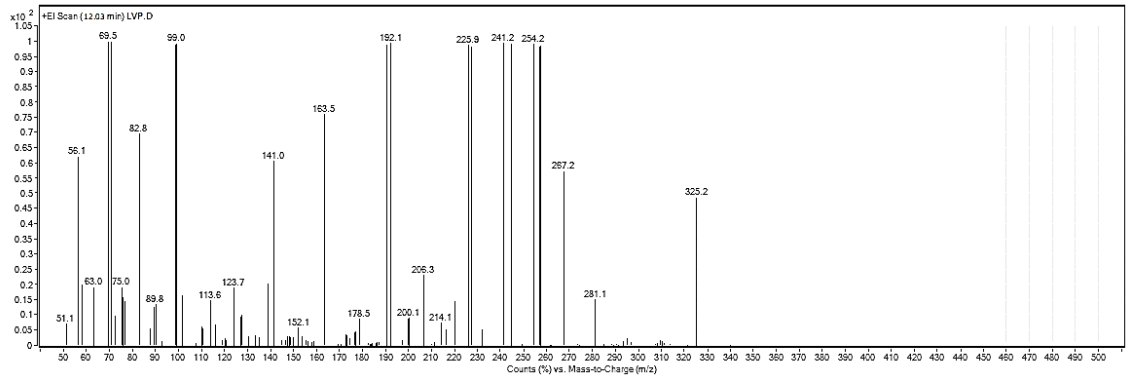

Figure 1. Mass spectrum of LVP in scan mode at the concentration of 100 µg/mL

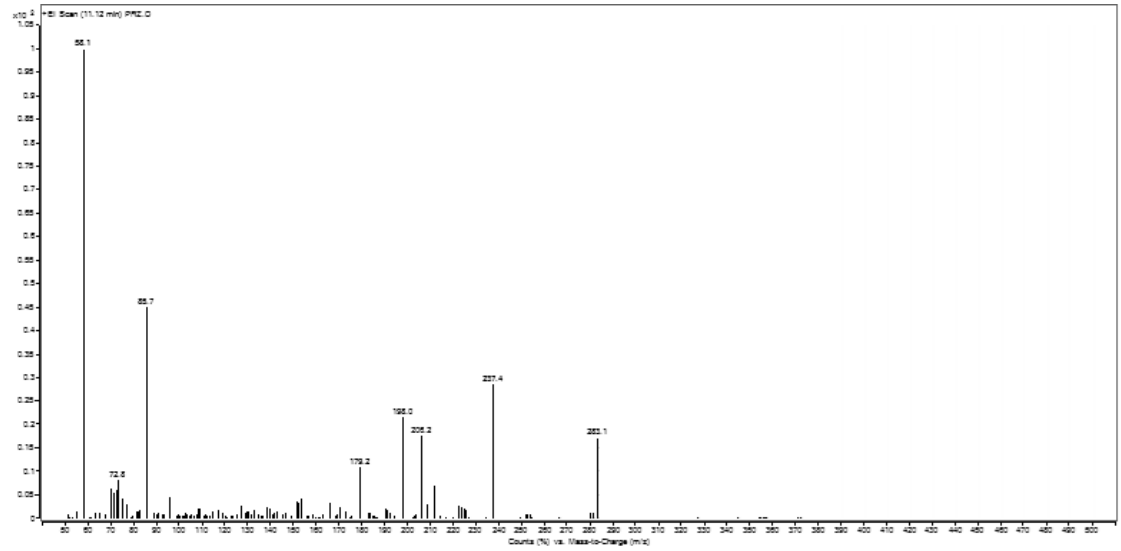

Figure 2. Mass spectrum of PRZ in scan mode at the concentration of 100 µg/mL

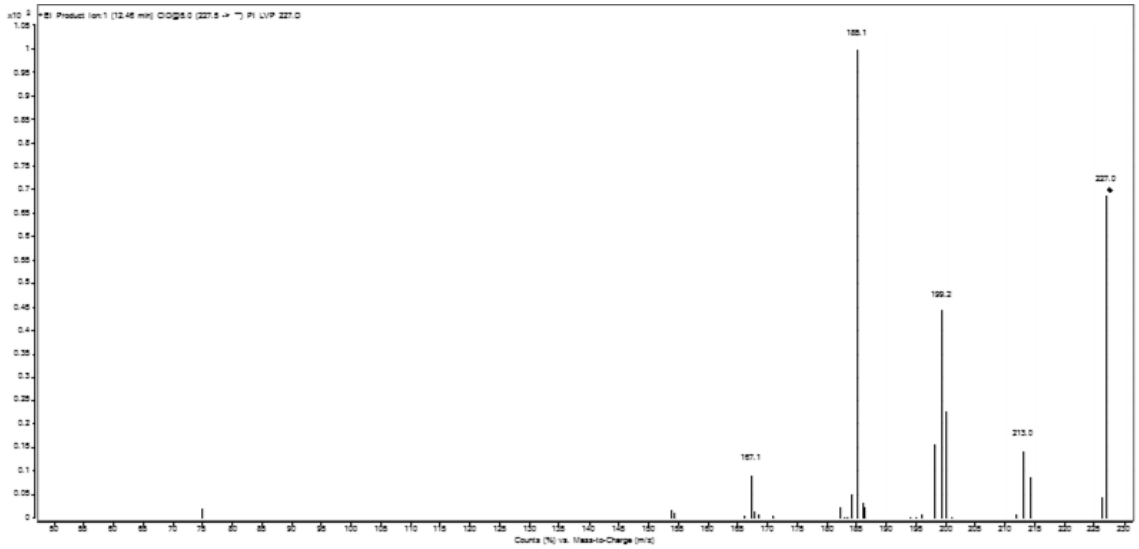

Figure 3. PI of ion 227.5 for LVP at the concentration of 100 µg/mL

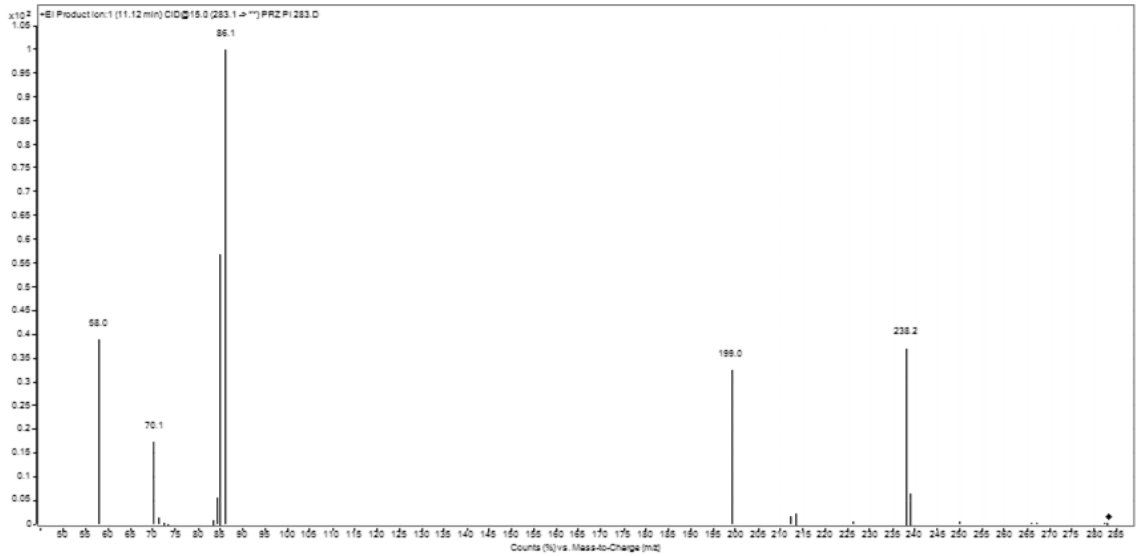

Figure 4. PI of ion 283.1 for PRZ at the concentration of 100 µg/mL

Usually, transitions are chosen based on the more intense ions and higher *m*/*z* ratio, which leads to the establishment of two different transitions: one to quantify and the other to qualify. After these transitions were selected, in order to increase the accuracy of the equipment for the analysis of the samples, the analysis segments were defined in multiple reaction monitoring (MRM) mode. Some parameters of the method in MRM have undergone an optimization to guarantee a greater sensitivity of the proposed method. The dwell time represents the time that the equipment spends in the acquisition of the data of each mass spectrum. Table 1 shows the MRM detection conditions for each APD including retention time, quantifier and qualifier transitions, collision energies and dwell time adopted.

Table 1. Retention time and GC-MS/MS parameters (quantitation transitions underlined).

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

*Internal standard

4.2 Optimization of the extraction process

According to the literature, the extraction procedure involving 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 [5–7]. In the present work, the type of solvent and volume, drying time of the DSS and homogenization time were evaluated and optimized.

4.2.1 Extraction solvent

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 minutes agitation time, overnight drying time and 15 minutes 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 (Figure 5).

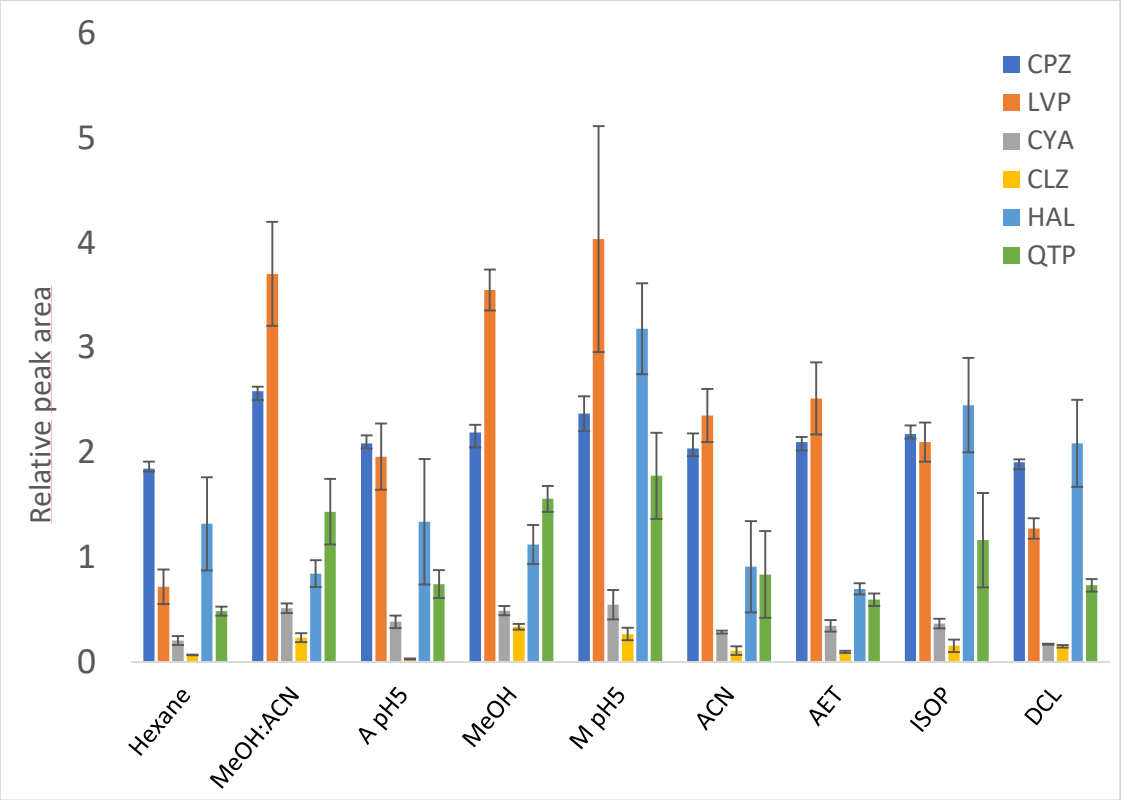

Figure 5. Evaluation of the extraction solvent.

4.2.2 Extraction solvent volume

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)) (Figure 6). 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.

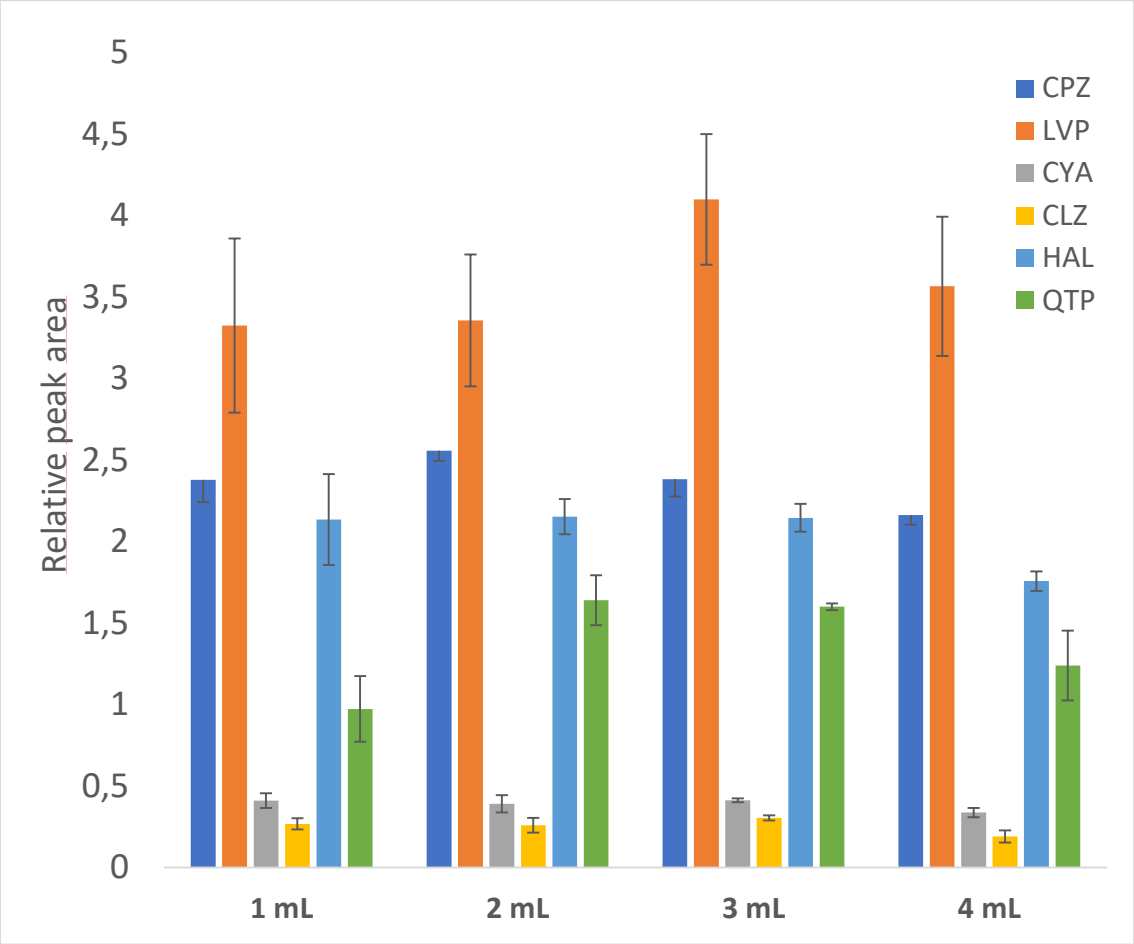

Figure 6. Evaluation of the extraction solvent volume.

4.2.3 Extraction time

Regarding extraction time, four periods were studied (n=3): 5, 15, 30 and 60 min. The results obtained and analysed with the Friedmans test exposed that a 5-minute homogenization period in the rollermixer 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 (Figure 7).

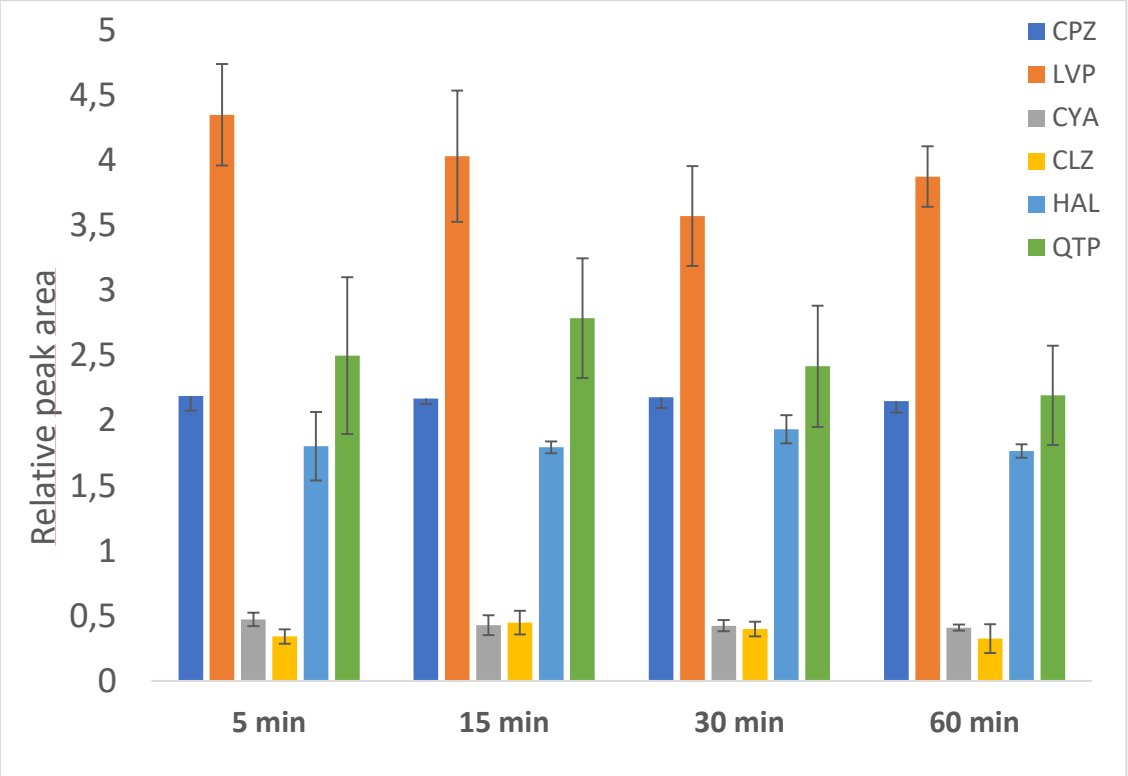

Figure 7. Evaluation of the extraction time.

4.2.4 Drying time of the DSS

Furthermore, an important step to take into consideration is the drying time of the DSS. The studied drying times were 1 hour, 3 hours and an overnight period of time (n=3). The results showed that in 1h of drying practically all the compounds were extracted with a greater relative area peak, and by the Friedmans test no significant differences were detected (Figure 8). In all optimization experiments the IS was added after extraction.

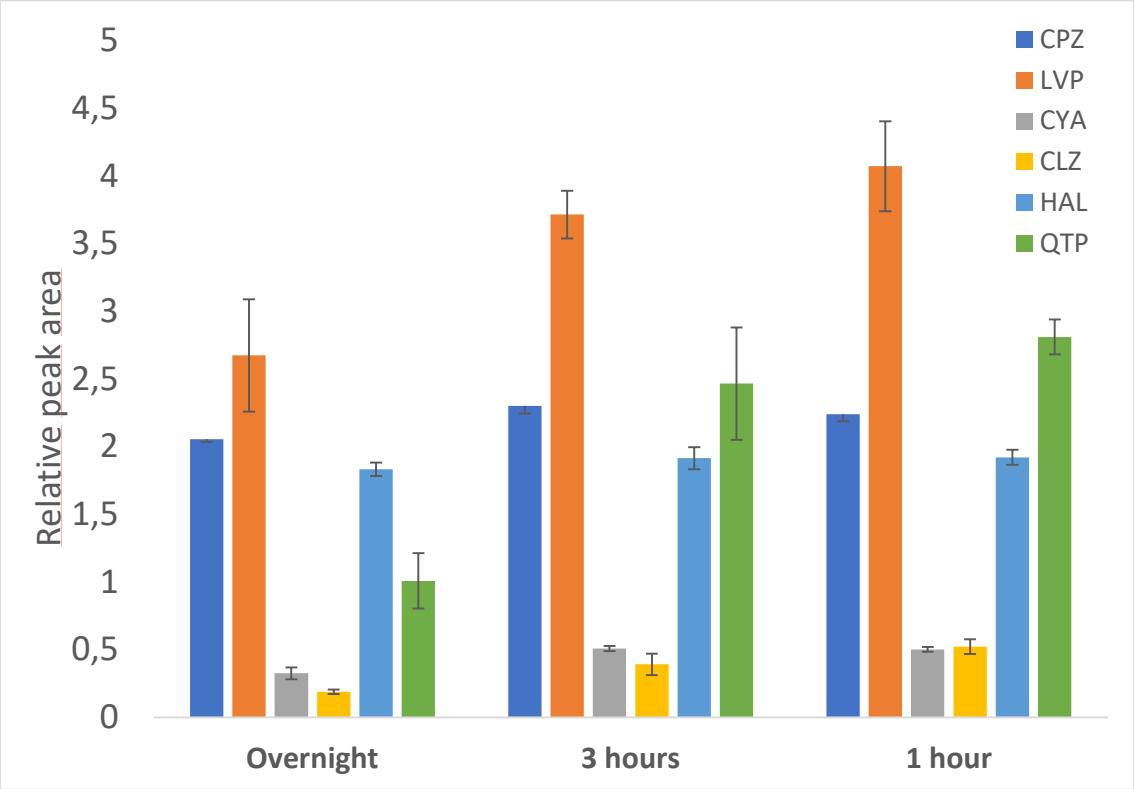

Figure 8. Evaluation of the drying time of the DSS.

4.3 Method Validation

4.3.1 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 [8]. In the present study, oral fluid samples from 10 different origins provided by laboratory staff were evaluated considering the WADA criteria for acceptance [9]. No interferences were observed at the retention times and selected transitions of the target analytes. In Figure 9 we compare a blank sample for APDs, and a sample fortified at the lowest limit of quantification (LLOQ). These samples were analysed by the developed method.

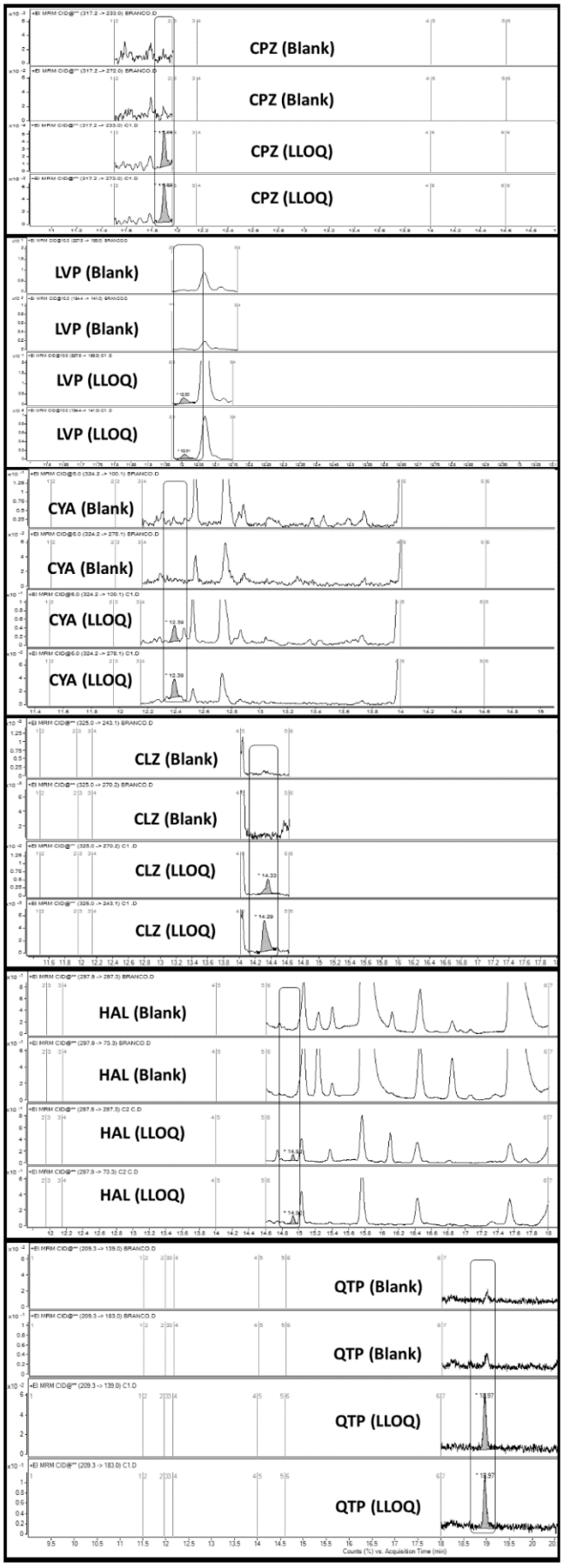

Figure 9. Chromatogram of a blank sample and a spiked sample at the LLOQ for all compounds.

4.3.2 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 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. [10], 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 µL of sample. In the present work, we only use 50 µL of sample, which is twenty 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. [11] 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 µL 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 greater than ours. Fisher et al. [12] published a method using 200 µL 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. [13] 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 µL.

Table 2. Linearity data (n=5).

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

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

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

For these parameters, the precision acceptance criteria were coefficients of variation (CV) ≤ 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 [1].

Intra-day precision and accuracy were evaluated by analyzing six replicates of four 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 five-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 five-day validation protocol (n=15). The obtained CVs were below 11% with a RE within ± 8%. All data are showed in Table 3.

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

		Inter-day (n=5)			Intra-day (n=6)			Intermediate (n=15)		
APD	**C**	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			

All concentrations in ng/mL; C – Nominal concentration; CV - coefficient of variation; RE - relative error.

4.3.4 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 [8]. 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. [14] 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. [14] used a common extraction method (SPE) being analysed by UHPLC–MS/MS. Regarding the work by Rosado et al. [13], 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. [15], 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.

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

4.3.5 Stability

Stability evaluation was divided into short-term, freeze/thaw, long-term and auto-sampler stability [16]. 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-hour 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 into the card. All APDs demonstrated CVs 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 hours at room temperature. The results revealed CVs below 13% and RE within ± 12%. Stability data is presented in Table 5.

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

Analyte	Concentration	Autosampler stability (n=3)			Short-term (n=3)			Freeze/thaw (n=3)			Long-term stability (n=3)					
											4 days			8 days		
		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.

4.3.6 Dilution integrity

The integrity of the diluted samples is an important validation parameter, since authentic samples may be at a concentration above the upper limit of quantification (ULOQ) of the adopted calibration curve [1]. 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 ±10% (Table 6).

Table 6. Dilution integrity (n=3).

Analyte	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

4.3.7 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, from 16.6 and 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 Figure 10 shows chromatograms of authentic samples positive for QTP at 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 [13], 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 analysed 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. [17] found concentrations of 40 ng/mL and 48 ng/mL for CLZ and QTP respectively, and these concentrations fall within the proposed calibration range. Patteet et al. [15] 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. [18] presented a multimethod that included CPZ and LVP with equal or greater LLOQs 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.

Table 7. Analysis of authentic samples.

Sample	**Analyte (s)**	**Concentration (ng/mL)**	**Measured concentration (ng/mL) using the method** [13]
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

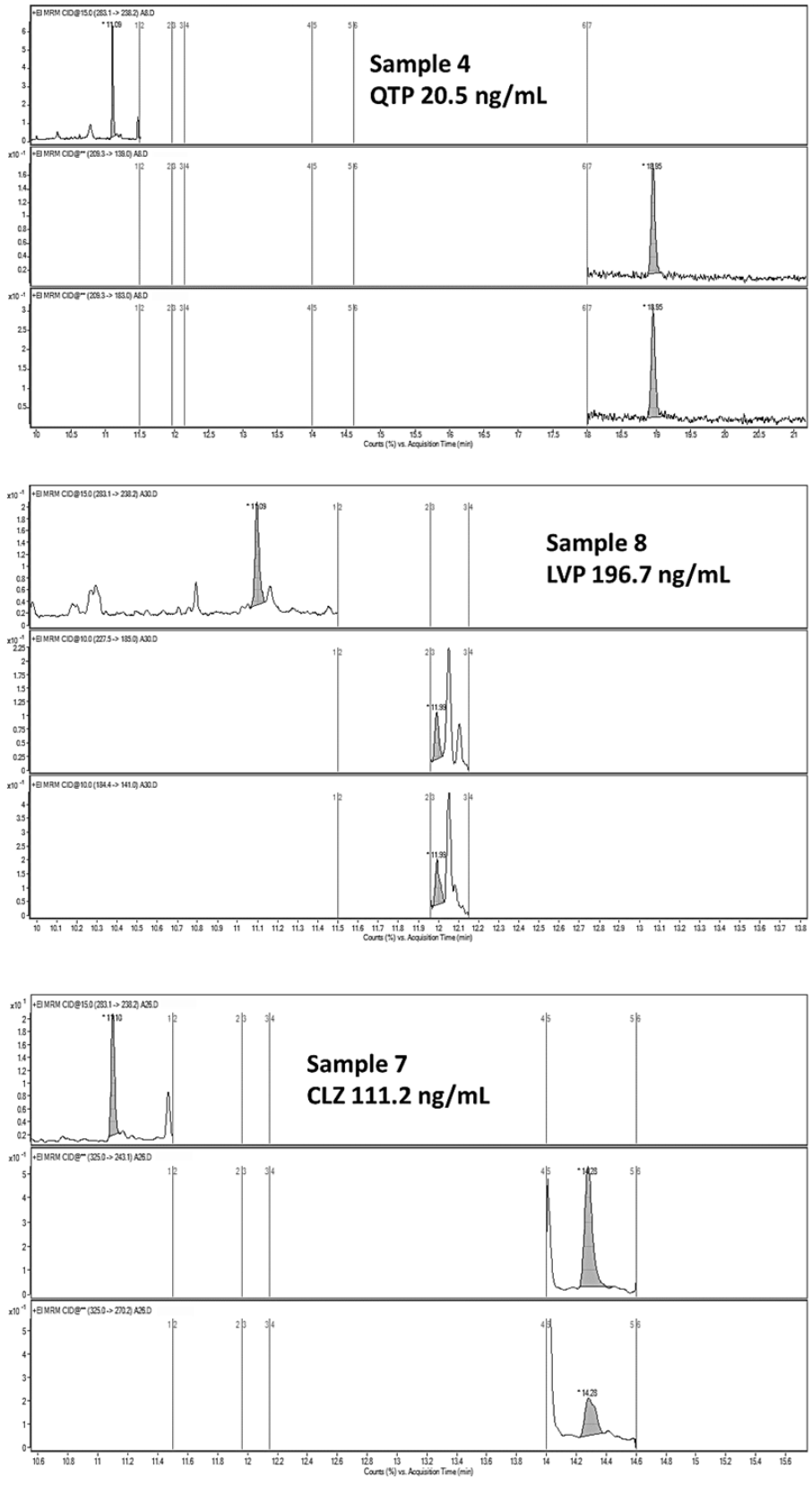

Figure 10. Chromatogram obtained by analysis of three positive authentic sample.

5 Conclusions

A fully validated method is described for the determination of chlorpromazine, 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 to 97%. This miniaturized procedure revealed to be simple, user friendly, associated to a rapid extraction procedure and requiring a small volume of sample (50 µL). This small sample volume, besides of being easily spotted into 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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Attachments

The present dissertation was disseminated in different events in the scientific area, as it was submitted to the publication.

Presentations in event:

DETERMINATION OF ANTIPSYCHOTIC DRUGS IN ORAL FLUID USING DRIED SALIVA SPOTS AND GAS CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY. (Poster communication)
Caramelo D., Rosado T., Gallardo E.
VII Encontro Nacional de Estudantes de Química (VII ENEQUI), FCT NOVA (Lisboa) March 2019

MONITORIZAÇÃO DE ANTIPSICÓTICOS EM AMOSTRAS DE FLUIDO ORAL COM RECURSO A DRIED SALIVA SPOTS: UMA FERRAMENTA ANALíTICA ÚTIL EM TOXICOLOGIA CLÍNICA. (Poster communication)
Caramelo D., Rosado T., Gallardo E.
Jornadas de Química e Bioquímica, UBI (Covilhã) April 2019

ANALYTICAL APPROACH TO DETERMINE ANTIPSYCHOTICS DRUGS IN ORAL FLUID BY DRIED SALIVA SPOTS (Poster communication accepted)
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