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ORIGINAL ARTICLE

# Development and validation of quantitative analytical method for 50 drugs of antidepressants, benzodiazepines and opioids in oral fluid samples by liquid chromatography–tandem mass spectrometry

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## Abstract

**Purpose** We developed and validated a method for quantitative analysis of 50 psychoactive substances and metabolites (antidepressants, benzodiazepines and opioids) in oral fluid samples using simple liquid–liquid extraction procedure followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

**Method** Oral fluid samples were collected using Quantisal™ device and extracted by liquid–liquid extraction with 1.0 mL of methyl *tert*-butyl ether and then analyzed using LC–MS/MS.

**Results** The method attended method validation criteria, with limits of quantification as low as 0.5 and 1.0 ng/mL, and linearity between 0.5–50.0 ng/mL for antidepressants, 0.5–25.0 ng/mL for benzodiazepines and 1.0–50.0 ng/mL to opioids. During method validation, bias and imprecision values were not greater than 16 and 20%, respectively. Ionization suppression/enhancement bias results were not greater than 25%. No evidence of carryover was observed. Sample stability studies showed that almost all analytes were stable at 25 °C for 3 days and at 4 °C for 7 days. Freeze–thaw cycles stability showed that most antidepressants and opioids were stable under these conditions. Autosampler stability study showed that all analytes were stable for 24 h, except for nitrazepam and 7-aminoclonazepam. Thirty-eight authentic oral fluid samples were analyzed; 36.8% of the samples were positive for 2 drugs. Citalopram was the most common drug found, followed by venlafaxine.

**Conclusions** The method was validated according to international recommendations for the 50 analytes, showing low limits of quantification, good imprecision and bias values, using simple liquid–liquid extraction, and was successfully applied to authentic oral fluid samples analysis.

**Keywords** Oral fluid · LC–MS/MS · Antidepressants · Benzodiazepines · Opioids · Quantitative mass analysis

## Introduction

Oral fluid is used as an alternative matrix for diagnostic in clinical and workplace applications; drug testing under driving, drug monitoring and criminal justice settings have been increasing over the last 20 years [1–6]. This matrix is constituted by saliva and other fluids, from minor and major salivary glands and gingiva; its presence in oral cavity allows easy collection and application for monitoring therapeutic or illicit use of drugs and for pharmacokinetic studies [7].

As compared to other matrices, oral fluid excelled due to rapid, noninvasive and observed collection, difficult adulteration and simpler analysis (when considering plasma with high contents of lipids and proteins, for example). Oral fluid is also considered as an alternative matrix to blood due to the good correlation of concentration found in both matrices for

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most analytes. This characteristic has been further investigated, making oral fluid a priority for on-site collection [1, 2, 8, 9].

Oral fluid has arisen as the primary option to access drug driving problems, considering the easy collection and possibility to access recent drug use without needing blood sampling. The Roadside Testing Assessment (ROSITA) study, the objective of which was assessing drug and alcohol driving problems in six countries in Europe and four American states, highly recommended the start of random drug testing for government officials [10]; oral fluid was considered the most relevant biological matrix applied for roadside testing situations [2]. This matrix is also useful to field sample collection, such as at parties and music festivals; one of the main objectives of this study is to establish solid patterns into drug consumption using oral fluid [11]. Mohr et al. [12] evaluated the use of synthetic stimulants and hallucinogens in an electronic dance music festival, and concluded that paired blood, urine and oral fluid sampling, was the best choice for monitoring these populations.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is an effective tool for detection compounds from different classes, with distinct chemical structures and physicochemical properties, even at low concentrations. The aim of this work was to develop and validate an analytical method for simultaneous and quantitative analysis of 50 psychoactive drugs of antidepressants, benzodiazepines and opioids, which are widely circulating in the world, in oral fluid samples, using simple liquid–liquid extraction and LC–MS/MS. After validation, the method was successfully applied to the analysis of 38 authentic oral fluid samples collected from volunteers attending parties and electronic music festivals from different cities in Brazil.

## Materials and methods

### Standards and chemicals

Certified reference materials of amitriptyline, bupropion, citalopram, desipramine, desmethylcitalopram, duloxetine, fluoxetine, imipramine, mirtazapine, nortriptyline, paroxetine, sertraline and trazodone were purchased from LGC Standards (Teddington, London, UK); certified reference materials of clomipramine, desmethylvenlafaxine, doxepin, hydroxybupropion, norfluoxetine, norsertraline, trimipramine, venlafaxine, 7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam, temazepam, zolpidem, 6-monoacetylmorphine, *N*-desmethyltramadol, buprenorphine, codeine, fentanyl, hydrocodone, hydromorphone, meperidine, methadone, morphine, naloxone, naltrexone, oxycodone, oxymorphone, tramadol, bupropion-$d_9$, citalopram-$d_6$, clonazepam-$d_4$, codeine-$d_3$, diazepam-$d_5$, duloxetine-$d_3$ and morphine-$d_3$ from Cerilliant (Round Rock, TX, USA); methanol, acetonitrile, ammonium formate and formic acid from Merck (Darmstadt, Germany); ultrapure deionized water was purified by Milli-Q from Millipore (Billerica, MA, USA). All solvents used in the extraction procedure were HPLC grade. Quantisal™ oral fluid collection devices and elution buffer were purchased from Immunalysis (Pomona, CA, USA).

### Calibrators, quality control, and internal standards

The stock solutions of the substances were prepared by dilution of the reference certified material in methanol. Dilutions of the stock solution in methanol were made to create calibrators at 2.5, 5, 25, 50, 100, 150, 250 ng/mL for amitriptyline, bupropion, hydroxybupropion, citalopram, desmethylcitalopram, desipramine, venlafaxine, desmethylvenlafaxine, doxepin, fluoxetine, imipramine, mirtazapine, nortriptyline, sertraline, trazodone, trimipramine, clomipramine, duloxetine, norfluoxetine, norsertraline and paroxetine (antidepressants); at 2.5, 5, 25, 50, 75 and 125 ng/mL for 7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam, lorazepam, midazolam, nitrazepam, nordiazepam, oxazepam, temazepam and zolpidem (benzodiazepines); and at 5, 25, 50, 100, 150 and 250 ng/mL for morphine, codeine, 6-monoacetylmorphine, buprenorphine, fentanyl, hydrocodone, hydromorphone, meperidine, methadone, naloxone, naltrexone, *N*-desmethyltramadol, oxycodone, oxymorphone and tramadol (opioids). In this work, zolpidem was reported in benzodiazepine's group, to embrace all method substances.

Quality control (QC) working solutions were prepared by another analyst (different from the individual preparing the calibrators). The low-quality control (LQC) solutions were prepared in methanol at concentrations of 15 ng/mL for opioids; 7.5 ng/mL for benzodiazepines; 7.5 ng/mL for amitriptyline, bupropion, hydroxybupropion, citalopram, desmethylcitalopram, desipramine, venlafaxine, desmethylvenlafaxine, doxepin, fluoxetine, imipramine, mirtazapine, nortriptyline, sertraline, trazodone and trimipramine and 15 ng/mL for clomipramine, norfluoxetine, paroxetine, duloxetine and norsertraline. Medium-quality controls (MQC) solutions were prepared in methanol at 125 ng/mL for antidepressants and for opioids and at 40 ng/mL for benzodiazepines. High-quality control (HQC) solutions were prepared in methanol at 200 ng/mL for antidepressants and for opioids and at 100 ng/mL for benzodiazepines. More information about QC working solutions are summarized in Table 1.

Internal standard (IS) solutions were made from dilutions of the stock solutions of certified reference materials, to produce a single IS mixture working solution at the

**Table 1** Linearity parameters, quality control concentrations and correlation coefficients ($r$) for all 50 substances of analytical method

| Analyte | Linearity (ng/mL) | LQC (ng/ml) | MQC (ng/ml) | HQC (ng/ml) | $r$ |
|---|---|---|---|---|---|
| Alprazolam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.994 |
| 7-Aminoclonazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.998 |
| 7-Aminoflunitrazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.996 |
| Amitriptyline | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.998 |
| Bromazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.999 |
| Buprenorphine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.998 |
| Bupropion | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.998 |
| Citalopram | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.997 |
| Clomipramine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.995 |
| Clonazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.999 |
| Codeine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.997 |
| Desipramine | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.999 |
| Desmethylcitalopram | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.997 |
| *N*-Desmethyltramadol | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.998 |
| Desmethylvenlafaxine | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.998 |
| Diazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.999 |
| Doxepin | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.997 |
| Duloxetine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.992 |
| Fentanyl | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.996 |
| Flunitrazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.996 |
| Fluoxetine | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.993 |
| Hydrocodone | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.996 |
| Hydromorphone | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.997 |
| Hydroxybupropion | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.999 |
| Imipramine | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.994 |
| Lorazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.998 |
| Meperidine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.997 |
| Methadone | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.993 |
| Midazolam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.999 |
| Mirtazapine | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.997 |
| 6-Monoacetylmorphine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.998 |
| Morphine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.998 |
| Naloxone | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.998 |
| Naltrexone | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.998 |
| Nitrazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.998 |
| Nordiazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.995 |
| Norfluoxetine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.993 |
| Norsertraline | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.995 |
| Nortriptyline | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.997 |
| Oxazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.997 |
| Oxycodone | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.986 |
| Oxymorphone | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.997 |
| Paroxetine | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.999 |
| Sertraline | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.992 |
| Temazepam | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.998 |
| Tramadol | 1.0–50.0 | 3.0 | 25.0 | 40.0 | 0.996 |
| Trazodone | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.996 |
| Trimipramine | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 0.999 |
| Venlafaxine | 0.5–50.0 | 1.5 | 25.0 | 40.0 | 1.000 |
| Zolpidem | 0.5–25.0 | 1.5 | 8.0 | 20.0 | 0.998 |

*LQC* low quality control, *MQC* medium quality control, *HQC* high quality control

concentration of 5 ng/mL for codeine-$d_3$, morphine-$d_3$, clonazepam-$d_4$ and diazepam-$d_5$, and 125 ng/mL for bupropion-$d_9$, citalopram-$d_6$ and duloxetin-$d_3$. All solutions were prepared in methanol and stored in amber glass vials at − 20 °C.

## Samples

Blank oral fluid samples were mixed with Quantisal™ elution buffer according to the manufacturer's dilution (1:3, v/v), fortified with the working standard solutions and used for method development and validation.

To demonstrate that the analytical method was fit for purpose, oral fluid samples collected from volunteers participating in parties and electronic music festivals were analyzed ($n = 38$). The inclusion criteria were age greater than 18 years old and self-report use of the synthetic drug in the last 24 h. The sample collection was performed anonymously, and procedures performed in this study involving oral fluid samples from human volunteers were in accordance with the ethical standards of the University of Campinas committee (Comitê de Ética em Pesquisa da UNICAMP—CEP, CAAE 88770318.0.0000.5404), and with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

## Extraction procedure

To perform the liquid-liquid extraction (LLE), 500 µL of sample collected with Quantisal™ oral fluid device was transferred to a 5 mL polypropylene tube, followed by 25 µL of IS solution, 500 µL saturated solution of sodium tetraborate and 1 mL of methyl *tert*-butyl ether (MTBE). The mixture was vortexed using BenchMixer™ XL multi-tube vortexer (Benchmark Scientific, Sayreville, NJ, USA) for 2 min at 2500 rpm. After that, the samples were centrifuged at 987*g* for 5 min and the organic layer (700 µL) was transferred to a new 2 mL polypropylene tube and dried under nitrogen stream (10 psi/40 °C) using a TurboVap evaporation system (Biotage, Uppsala, Sweden). The samples were resuspended with 100 µL of a mixture solution (mixture of water and methanol 80:20, v/v, containing 0.1% formic acid and 2 mmol/L ammonium formate) and 1 µL was injected into LC–MS/MS system.

## Instrument parameters

The analysis was performed on a Nexera X2 ultra-high-performance liquid chromatography system coupled to an LCMS8060 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on a biphenyl column (Raptor, 100 × 2.1 mm, 2.7 µm; Restek, Bellefonte, PA, USA), maintained at 40 °C. The mobile phase consisted of ultrapure water containing formic acid (0.1%, v/v) and ammonium formate (2 mmol/L) (A) and acetonitrile (B). The flow rate was 0.4 mL/min, and the elution gradient initialized with 5% B maintained for 0.5 min, followed by a linear increase to 55% B in 5.5 min, and another linear increase to 100% B in 0.5 min, holding at 100% B for 1.5 min and returning to initial conditions over 0.2 min. The system was reequilibrated for 1.3 min before the next injection, with a total chromatographic run of 9.5 min.

The mass spectrometer was equipped with an electrospray ionization source operating in positive mode. The mass spectrometer conditions were: interface temperature at 400 °C, desolvation temperature at 350 °C, heat block temperature at 400 °C, drying gas ($N_2$) flow at 5 L/min, heating gas flow (air) at 15 L/min, nebulizing gas ($N_2$) flow at 3 L/min and collision-induced dissociation gas pressure (Ar) at 270 kPa. The analyses were performed in multiple reaction monitoring (MRM) mode. For each compound, two MRM transitions were selected, one as quantifier and one qualifier for confirmative identification, except for tramadol (only one transition was chosen). Individual chromatographic retention times and MRM information were presented in Table 2. Data were acquired and processed using LabSolutions 5.97 software (Shimadzu).

## Method validation

Method validation was performed based on the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines [13]. The parameters evaluated were limit of quantification (LOQ), linearity, interference studies, bias, imprecision, matrix effect, carryover, stability, dilution integrity and recovery.

## Identification criteria

Analytes identification criteria considered (1) a symmetrical chromatographic peak with retention time within ± 2% of the average calibrator retention time, (2) signal/noise ratio higher than 3 for both qualifier and quantifier ions and (3) the ratios of the two transitions within a maximum of ± 30% of those established by the calibrators, varying more for those with low intensity for the major transition [14].

## Limit of quantification

The LOQ was defined as the lowest concentration of the standard calibration curve that fulfilled identification criteria, with a signal-to-noise ratio of at least 10, acceptable bias and imprecision. The LOQ for all analytes was evaluated using three replicates per run, over 3 days with three different sources of the blank matrix.

**Table 2** Mass spectrometer parameters, retention times and internal standards for analyses of 50 analytes (antidepressants, benzodiazepines and opioids) in oral fluid samples using liquid chromatography–tandem mass spectrometry (LC–MS/MS)

| Analyte | Retention time (min) | MRM transitions (*m/z*) | Dwell time (ms) | Q1 pre bias (V) | CE (eV) | Q3 pre bias (V) | Internal standard |
|---|---|---|---|---|---|---|---|
| Alprazolam | 5.67 | 309.2 > 281.0 | 10 | −15 | −27 | −18 | Diazepam-$d_5$ |
| | | 309.2 > 205.1 | 10 | −23 | −45 | −20 | |
| 7-Aminoclonazepam | 3.79 | 286.0 > 222.0 | 10 | −20 | −26 | −23 | Clonazepam-$d_4$ |
| | | 286.0 > 250.0 | 10 | −21 | −21 | −16 | |
| 7-Aminoflunitrazepam | 4.23 | 284.0 > 135.0 | 10 | −20 | −27 | −13 | Clonazepam-$d_4$ |
| | | 284.0 > 227.0 | 10 | −11 | −26 | −25 | |
| Amitriptyline | 5.37 | 278.1 > 233.1 | 5 | −30 | −18 | −15 | Bupropion-$d_9$ |
| | | 278.1 > 191.0 | 5 | −14 | −27 | −12 | |
| Bromazepam | 4.64 | 318.0 > 182.1 | 10 | −12 | −33 | −18 | Diazepam-$d_5$ |
| | | 318.0 > 209.0 | 10 | −16 | −28 | −20 | |
| Buprenorphine | 4.80 | 468.3 > 55.0 | 30 | −14 | −54 | −20 | Codeine-$d_3$ |
| | | 468.3 > 396.0 | 30 | −14 | −40 | −26 | |
| Bupropion | 3.91 | 240.1 > 184.0 | 5 | −26 | −13 | −12 | Bupropion-$d_9$ |
| | | 240.1 > 166.0 | 5 | −28 | −19 | −29 | |
| Bupropion-$d_9$ | 3.90 | 249.1 > 185.1 | 5 | −26 | −13 | −19 | – |
| | | 249.1 > 131.1 | 5 | −12 | −28 | −12 | |
| Citalopram | 4.74 | 325.1 > 262.0 | 5 | −17 | −20 | −17 | Citalopram-$d_6$ |
| | | 325.1 > 109.0 | 5 | −17 | −28 | −21 | |
| Citalopram-$d_6$ | 4.73 | 331.1 > 190.0 | 5 | −16 | −27 | −19 | – |
| | | 331.1 > 262.0 | 5 | −16 | −21 | −17 | |
| Clomipramine | 6.00 | 315.1 > 270.1 | 5 | −10 | −19 | −30 | Duloxetine-$d_3$ |
| | | 315.1 > 242.0 | 5 | −16 | −27 | −15 | |
| Clonazepam | 5.60 | 316.0 > 270.0 | 10 | −24 | −26 | −12 | Clonazepam-$d_4$ |
| | | 316.0 > 214.1 | 10 | −23 | −40 | −22 | |
| Clonazepam-$d_4$ | 5.58 | 320.0 > 274.0 | 10 | −16 | −27 | −29 | – |
| | | 320.0 > 218.0 | 10 | −16 | −39 | −21 | |
| Codeine | 2.73 | 300.2 > 165.1 | 30 | −15 | −35 | −15 | Codeine-$d_3$ |
| | | 300.2 > 215.0 | 30 | −15 | −35 | −15 | |
| Codeine-$d_3$ | 2.72 | 303.0 > 165.0 | 30 | −12 | −40 | −17 | – |
| | | 303.0 > 199.0 | 30 | −12 | −30 | −20 | |
| Desipramine | 5.10 | 267.1 > 208.0 | 5 | −30 | −24 | −22 | Bupropion-$d_9$ |
| | | 267.1 > 72.1 | 5 | −29 | −18 | −12 | |
| Desmethylcitalopram | 4.64 | 311.1 > 262.0 | 5 | −16 | −18 | −17 | Citalopram-$d_6$ |
| | | 311.1 > 109.0 | 5 | −16 | −24 | −10 | |
| *N*-Desmethyltramadol | 3.55 | 250.2 > 44.0 | 5 | −29 | −13 | −16 | Codeine-$d_3$ |
| | | 250.2 > 232.1 | 5 | −28 | −9 | −15 | |
| Desmethylvenlafaxine | 3.17 | 264.1 > 58.0 | 5 | −29 | −22 | −22 | Bupropion-$d_9$ |
| | | 264.1 > 246.1 | 5 | −29 | −13 | −25 | |
| Diazepam | 6.32 | 285.0 > 193.1 | 10 | −11 | −27 | −15 | Diazepam-$d_5$ |
| | | 285.0 > 154.0 | 10 | −21 | −33 | −19 | |
| Diazepam-$d_5$ | 6.29 | 290.0 > 198.0 | 10 | −22 | −34 | −19 | – |
| | | 290.0 > 154.0 | 10 | −21 | −28 | −30 | |
| Doxepin | 4.86 | 280.1 > 107.0 | 5 | −30 | −22 | −10 | Citalopram-$d_6$ |
| | | 280.1 > 220.0 | 5 | −14 | −27 | −14 | |
| Duloxetine | 5.30 | 298.1 > 44.0 | 5 | −14 | −17 | −17 | Duloxetine-$d_3$ |
| | | 298.1 > 154.1 | 5 | −27 | −9 | −27 | |
| Duloxetine-$d_3$ | 5.29 | 301.1 > 157.0 | 5 | −25 | −8 | −25 | – |
| | | 301.1 > 47.0 | 5 | −15 | −15 | −17 | |

**Table 2** (continued)

| Analyte | Retention time (min) | MRM transitions (*m/z*) | Dwell time (ms) | Q1 pre bias (V) | CE (eV) | Q3 pre bias (V) | Internal standard |
|---|---|---|---|---|---|---|---|
| Fentanyl | 4.66 | 337.2 > 105.0 | 30 | −17 | −39 | −18 | Codeine-$d_3$ |
| | | 337.2 > 188.0 | 30 | −18 | −24 | −18 | |
| Flunitrazepam | 6.00 | 314.2 > 268.1 | 10 | −12 | −26 | −17 | Diazepam-$d_5$ |
| | | 314.2 > 239.1 | 10 | −23 | −35 | −24 | |
| Fluoxetine | 5.25 | 310.0 > 148.1 | 5 | −15 | −9 | −15 | Bupropion-$d_9$ |
| | | 310.0 > 44.0 | 5 | −15 | −12 | −15 | |
| Hydrocodone | 3.07 | 300.1 > 199.0 | 30 | −16 | −31 | −20 | Codeine-$d_3$ |
| | | 300.1 > 171.0 | 30 | −15 | −39 | −17 | |
| Hydromorphone | 2.27 | 286.2 > 184.9 | 30 | −18 | −30 | −18 | Morphine-$d_3$ |
| | | 286.2 > 153.0 | 30 | −15 | −46 | −27 | |
| Hydroxybupropion | 3.42 | 256.0 > 167.0 | 5 | −13 | −22 | −16 | Bupropion-$d_9$ |
| | | 256.0 > 238.0 | 5 | −29 | −13 | −16 | |
| Imipramine | 5.23 | 281.1 > 208.0 | 5 | −30 | −26 | −13 | Duloxetine-$d_3$ |
| | | 281.1 > 193.0 | 5 | −14 | −41 | −12 | |
| Lorazepam | 5.31 | 321.0 > 275.0 | 10 | −23 | −23 | −12 | Diazepam-$d_5$ |
| | | 321.0 > 229.0 | 10 | −24 | −29 | −15 | |
| Meperidine | 3.83 | 248.2 > 220.1 | 30 | −12 | −22 | −22 | Codeine-$d_3$ |
| | | 248.2 > 174.1 | 30 | −10 | −20 | −17 | |
| Methadone | 5.40 | 311.2 > 266.0 | 30 | −25 | −15 | −17 | Codeine-$d_3$ |
| | | 311.2 > 105.0 | 30 | −23 | −28 | −18 | |
| Midazolam | 4.64 | 326.0 > 291.0 | 10 | −24 | −28 | −19 | Clonazepam-$d_4$ |
| | | 326.0 > 249.2 | 10 | −25 | −39 | −28 | |
| Mirtazapine | 3.74 | 266.1 > 195.1 | 5 | −13 | −26 | −12 | Bupropion-$d_9$ |
| | | 266.1 > 209.0 | 5 | −28 | −21 | −21 | |
| 6-Monoacetylmorphine | 2.89 | 328.1 > 165.2 | 30 | −24 | −39 | −16 | Codeine-$d_3$ |
| | | 328.1 > 211.2 | 30 | −16 | −27 | −13 | |
| Morphine | 1.95 | 286.2 > 152.1 | 30 | −18 | −55 | −30 | Morphine-$d_3$ |
| | | 286.2 > 201.0 | 30 | −10 | −27 | −20 | |
| Morphine-$d_3$ | 1.94 | 289.2 > 165.0 | 30 | −15 | −41 | −29 | – |
| | | 289.2 > 153.0 | 30 | −21 | −41 | −29 | |
| Naloxone | 2.66 | 328.1 > 310.0 | 30 | −10 | −20 | −20 | Codeine-$d_3$ |
| | | 328.1 > 268.0 | 30 | −17 | −27 | −30 | |
| Naltrexone | 2.94 | 342.1 > 324.0 | 30 | −10 | −22 | −22 | Codeine-$d_3$ |
| | | 342.1 > 282.0 | 30 | −17 | −28 | −29 | |
| Nitrazepam | 5.38 | 282.2 > 236.2 | 10 | −14 | −25 | −15 | Clonazepam-$d_4$ |
| | | 282.2 > 180.2 | 10 | −14 | −41 | −17 | |
| Nordiazepam | 5.58 | 271.2 > 140.2 | 30 | −14 | −29 | −13 | Diazepam-$d_5$ |
| | | 271.2 > 208.2 | 30 | −30 | −29 | −23 | |
| Norfluoxetine | 5.12 | 296.1 > 30.0 | 5 | −20 | −14 | −27 | Duloxetine-$d_3$ |
| | | 296.1 > 134.0 | 5 | −15 | −8 | −29 | |
| Norsertraline | 5.37 | 274.9 > 158.9 | 5 | −20 | −20 | −16 | Duloxetine-$d_3$ |
| | | 274.95 > 91.0 | 5 | −13 | −16 | −16 | |
| Nortriptyline | 5.24 | 264.1 > 233.1 | 5 | −13 | −15 | −10 | Citalopram-$d_6$ |
| | | 264.1 > 91.0 | 5 | −13 | −22 | −16 | |
| Oxazepam | 5.21 | 287.0 > 241.0 | 10 | −30 | −24 | −26 | Diazepam-$d_5$ |
| | | 287.0 > 104.1 | 10 | −29 | −36 | −20 | |
| Oxycodone | 2.96 | 316.1 > 298.0 | 30 | −16 | −20 | −20 | Codeine-$d_3$ |
| | | 316.1 > 241.0 | 30 | −16 | −29 | −24 | |

**Table 2** (continued)

| Analyte | Retention time (min) | MRM transitions (*m/z*) | Dwell time (ms) | Q1 pre bias (V) | CE (eV) | Q3 pre bias (V) | Internal standard |
|---|---|---|---|---|---|---|---|
| Oxymorphone | 2.10 | 302.1 > 284.0 | 30 | − 16 | − 20 | − 19 | Morphine-$d_3$ |
| | | 302.1 > 227.0 | 30 | − 15 | − 30 | − 22 | |
| Paroxetine | 5.09 | 330.0 > 192.1 | 5 | − 17 | − 21 | − 20 | Bupropion-$d_9$ |
| | | 330.0 > 70.1 | 5 | − 17 | − 29 | − 12 | |
| Sertraline | 5.53 | 306.2 > 275.0 | 5 | − 16 | − 13 | − 19 | Duloxetine-$d_3$ |
| | | 306.2 > 158.9 | 5 | − 15 | − 27 | − 15 | |
| Temazepam | 5.82 | 301.2 > 255.0 | 10 | − 15 | − 23 | − 11 | Diazepam-$d_5$ |
| | | 301.2 > 177.1 | 10 | − 15 | − 40 | − 18 | |
| Tramadol | 3.55 | 264.1 > 58.0 | 30 | − 13 | − 22 | − 22 | Codeine-$d_3$ |
| Trazodone | 4.40 | 372.1 > 176.1 | 5 | − 11 | − 25 | − 11 | Citalopram-$d_6$ |
| | | 372.1 > 148.0 | 5 | − 18 | − 34 | − 29 | |
| Trimipramine | 5.46 | 295.2 > 100.1 | 5 | − 15 | − 18 | − 19 | Citalopram-$d_6$ |
| | | 295.2 > 58.0 | 5 | − 15 | − 33 | − 10 | |
| Venlafaxine | 4.01 | 278.1 > 260.1 | 5 | − 14 | − 13 | − 12 | Bupropion-$d_9$ |
| | | 278.1 > 58.0 | 5 | − 30 | − 22 | − 25 | |
| Zolpidem | 4.14 | 308.1 > 235.0 | 10 | − 16 | − 35 | − 24 | Clonazepam-$d_4$ |
| | | 308.1 > 92.0 | 10 | − 17 | − 52 | − 16 | |

The quantifier multiple reaction monitoring (MRM) transitions are underlined

*CE* collision energy

## Linearity

Linearity was evaluated with calibration range from 0.5 to 50.0 ng/mL for antidepressants (except clomipramine, duloxetine, norfluoxetine, norsertraline, and paroxetine from 1.0 to 50.0 ng/mL), from 0.5 to 25.0 ng/mL for benzodiazepines and from 1.0 to 50.0 ng/mL for opioids. Linearity was evaluated with six-point calibration curves over 5 days, by linear least squares regression ($1/x^2$ weighting) for all analytes. Calibrators were required to quantify within ± 20% of each target concentration, with correlation coefficient ($r$) greater than 0.99.

## Interference studies

Oral fluid samples were fortified with common pharmaceuticals and drugs of abuse/metabolites at 200 ng/mL, extracted and injected into the LC−MS/MS. No peaks were visualized in each analyte's detection window that satisfied identification criteria. Supplementary Table 1 includes all pharmaceuticals evaluated as potential interferents (selectivity). Ten blank samples from different sources were extracted and analyzed to evaluate possible endogenous interferences. In addition, the potential contribution of native ions present in commercial deuterated ISs was evaluated comparing the blank oral fluid pool with and without IS additions. No interfering peaks should be visualized that satisfied identification criteria.

## Bias

Bias was evaluated in the triplicate analysis of fortified matrix samples, at three different concentrations (low, medium, and high) over 5 days. It was calculated considering the percentages of nominal deviation from the target concentration. The highest average acceptable bias from the target concentration was ± 20%. Results are presented in percentages.

## Imprecision

The imprecision was evaluated in the triplicate analysis of fortified matrix samples, at three different concentrations (low, medium, and high) over 5 days. Both within-run and between-run imprecisions were calculated using the one-way ANOVA ($p < 0.05$) approach with the varied factor (run number) as the grouping variable [13]. Using this approach, imprecision is considered as relative standard deviation percentage (%RSD) within the triplicate analysis in one day ($n = 3$) and for 5 days ($n = 15$) for each concentration. Imprecision values with %RSD less than 20% were considered acceptable.

## Matrix effect

Matrix effects were evaluated by comparison of target peak areas in six blank samples from different sources

fortified with analytes after extraction (at low and high QC levels) with the average target peak areas of a set of neat standards. Results were expressed as percentages considering a negative result indicative of matrix suppression, and a positive result of matrix enhancement.

### Carryover

Carryover was assessed analyzing blank samples immediately after the highest point of the calibration curve was analyzed. It was considered absent if all analyte's peak were below LOQ values.

### Stability

All the stability studies were conducted at low and high QC concentrations ($n = 6$) in triplicate. On day zero, they were aliquoted in 5 mL polypropylene tubes and stored at 25 °C (room temperature), 4 °C (refrigerator) and − 20 °C (freezer). After 3, 7, 15, 30 and 60 days, aliquots of each QC were fortified with IS and quantified using freshly prepared calibration curves. These drug concentrations were compared to those of the initial QC samples.

Sample stability after three freeze–thaw cycles at − 20 °C was evaluated in triplicate on day zero and after quantifying each concentration, the other triplicates were stored at − 20 °C. After three freeze–thaw cycles (one cycle = 24 h), triplicates samples were quantified against a newly prepared calibration curve.

For evaluation of processed samples stability when storage in autosampler, low and high QCs and calibrator samples were extracted and analyzed immediately. These extracts were stored on the autosampler at 10 °C and reinjected after 12, 18 and 24 h. The peak areas of these stored QCs were compared to those obtained immediately.

In all stability studies, analytes were considered stable if the concentration was within ± 20% of the initial concentration.

### Dilution integrity

For dilution integrity studies, a triplicate of blank oral fluid samples was fortified with 500 ng/mL and diluted 20-fold in a blank oral fluid-Quantisal™ buffer mixture. If the measured concentration times the dilution factor is within ± 20% of the target concentration, the integrity of the dilution is established.

### Recovery

Recovery (extraction efficiency) was performed in two batches: the first using six replicates of blank samples fortified with analytes at the low and high concentrations, extracted with the proposed procedure and injected into the LC–MS/MS; the second, using six replicates of blank samples extracted by the proposed procedure and, the final extract was fortified with the analytes at low and high QC concentrations and injected into the LC–MS/MS. The average peak area of the samples fortified prior to extraction divided by the average peak area of the samples fortified after extraction is multiplied by 100 to give the percent extraction efficiency.

## Results

The solvent for the LLE was chosen by a mixture design of experiment [15]. Ethyl acetate, MTBE and hexane (contemplating the solvents to be most applied for these analytes by LLE) were evaluated individually as binary and ternary mixtures, using analytes' peak areas as the measure of response. The best results were achieved with MTBE as an extraction solvent. Methanol was the first option to reconstitute the dried extract but better chromatography peak symmetry was observed using water/methanol, both containing 0.1% formic acid and 2 mmol/L ammonium formate (80:20, v/v).

During chromatographic method optimization, methanol and acetonitrile were tested as organic mobile phase (B). Acetonitrile was chosen because it improved the chromatographic separation of specific analytes, such as morphine/hydromorphone, codeine/hydrocodone and desmethylvenlafaxine/tramadol, but it did not fully separate them when methanol was used. The use of acetonitrile also avoids interferences caused by similar isobaric interferences from the matrix in lorazepam MRM.

Meperidine and tramadol had adjusted mass spectrometry conditions differently from other analytes, due to their great sensibility at electrospray ionization. To prevent detector saturation and enlarge linearity, the third quadrupole resolution was set to "high" instead of "unit". The same was observed for methadone, although changing quadrupole resolution did not solve the problem. For this analyte, were adopted the less abundant ions (*m/z* 311.2 instead of 310.2), which allowed quantification and good linearity results.

The LOQ was defined as an administrative decision point concentration and established as 0.5 ng/mL for all benzodiazepines and for most part of antidepressants (amitriptyline, bupropion, hydroxybupropion, citalopram, desmethylcitalopram, desipramine, venlafaxine, desmethylvenlafaxine, doxepin, fluoxetine, imipramine, mirtazapine, nortriptyline, sertraline, trazodone and trimipramine) and as 1.0 ng/mL for

all opioids and some antidepressants (clomipramine, duloxetine, norfluoxetine, norsertraline, and paroxetine). Figure 1 is the combined MRM chromatogram of analytes at the LOQ levels.

Excellent performance and linearity were achieved with $r > 0.99$, fulfilling all identification parameters. No interference was observed among the ten different sources of blank oral fluid tested. The same was verified for evaluation of IS interferences and interferences from other commonly encountered pharmaceuticals and drugs of abuse. Calibration ranges, QC values and correlation coefficients are presented in Table 1.

The largest imprecision value in this validation was observed for norfluoxetine at low QC (3.0 ng/mL), with within-run imprecision of 20% and between-run imprecision of 19%. Bias was less than 16% for all analytes (Table 3).

The matrix effects biases were lower than 25% and no carryover was observed when analyzing blank samples immediately after the analysis of the highest point of the calibration curve. Recovery values were obtained comparing two different sets of samples. Most analytes had very similar values among low and high concentrations. Antidepressant extraction recovery values were not lower than 78%; opioids values ranged from 20 to 99%; and benzodiazepines values were not lower than 49%. The results for bias (accuracy) for each analyte are also shown in Table 3.

Stability results are presented on Tables 4, 5, 6 and showed that all antidepressants, benzodiazepines and opioids were stable in oral fluid collected with Quantisal™ device for at least 60 days at − 20 °C, except 7-aminoclonazepam (− 38 and − 41%, low and high QC, respectively), 7-aminoflunitrazepam (− 33 and − 38%), lorazepam (23 and 12%), nordiazepam (23 and 6%) naloxone (20 and 23%), naltrexone (25 and 12%) and norsertraline (21 and 14%). All studied analytes were considered stable at 4 °C for 7 days except nordiazepam (− 23 and − 1% at day 3) and methadone (21 and 11%) and at 25 °C for 3 days except sertraline (− 11 and − 23%) and flunitrazepam (− 21 and − 19%). Most antidepressants and opioids are stable after three freeze–thaw cycles, which tended not to be seen for benzodiazepines. Among 14 benzodiazepines, 6 presented great instability after freeze–thaw cycles, ranging from ± 21 to 35%. Autosampler stability study at 10 °C showed that all antidepressants and opioids were stable for 24 h, with results better than 11%, when peak areas of stored QCs were compared to those freshly prepared. Most of benzodiazepines remained stable in autosampler conditions after 24 h storage, except nitrazepam, 7-aminoflunitrazepam and 7-aminoclonazepam (relatively stable only for 18 h).

Dilution integrity studies were performed for all analytes which concentrations found in real samples were above the upper limit of the calibration range. The average diluted

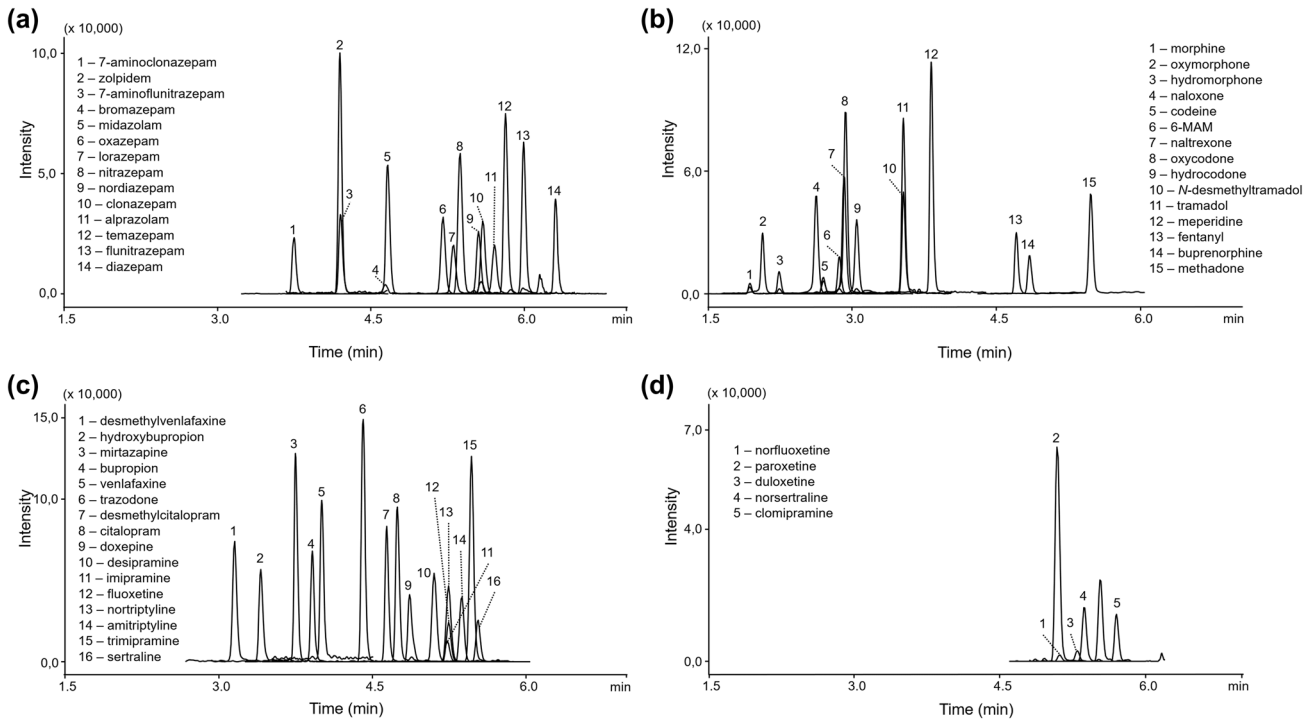


**Fig. 1** Combined multiple reaction monitoring (MRM) chromatograms of fortified oral fluid samples at the limit of quantification (LOQ). **a** Fourteen benzodiazepines analyzed at 0.5 ng/mL. **b** Fifteen opioids analyzed at 1.0 ng/mL. **c** Sixteen antidepressants analyzed at 0.5 ng/mL. **d** Five antidepressants analyzed at 1.0 ng/mL

**Table 3** Method validation results for within- and between-run imprecisions, biases, matrix effects, and recoveries for analyses of 50 substances in oral fluid samples using LC–MS/MS

| Analyte | Within-run imprecision (%RSD) | | | Between-run imprecision (%RSD) | | | Bias (%) | | | Matrix effect (%) | | Recovery (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC | LQC | MQC | HQC | LQC | HQC | LQC | HQC |
| Alprazolam | 11 | 8 | 12 | 10 | 7 | 10 | 6 | 4 | 9 | −6 | −8 | 56 | 55 |
| 7-Aminoclonazepam | 9 | 8 | 11 | 9 | 7 | 10 | 9 | 10 | 9 | −4 | −25 | 72 | 49 |
| 7-Aminoflunitrazepam | 8 | 9 | 6 | 7 | 7 | 6 | 7 | 6 | 4 | −15 | −20 | 55 | 57 |
| Amitriptyline | 7 | 8 | 7 | 6 | 7 | 6 | 6 | −2 | 7 | −1 | −2 | 88 | 87 |
| Bromazepam | 9 | 11 | 14 | 8 | 9 | 11 | 6 | 6 | 9 | −1 | −1 | 74 | 67 |
| Buprenorphine | 8 | 3 | 8 | 7 | 3 | 7 | 1 | 0 | 2 | 3 | 10 | 99 | 88 |
| Bupropion | 8 | 3 | 3 | 6 | 2 | 3 | 7 | −11 | 0 | 24 | 25 | 105 | 130 |
| Citalopram | 5 | 3 | 6 | 4 | 2 | 5 | 6 | −10 | 4 | −3 | −3 | 92 | 84 |
| Clomipramine | 11 | 7 | 4 | 10 | 7 | 4 | −2 | −8 | 0 | 8 | 6 | 87 | 86 |
| Clonazepam | 5 | 8 | 4 | 5 | 7 | 4 | 3 | 4 | 2 | −3 | −3 | 72 | 77 |
| Codeine | 8 | 5 | 6 | 7 | 5 | 5 | 4 | 3 | 4 | 3 | 2 | 43 | 44 |
| Desipramine | 7 | 6 | 2 | 6 | 5 | 3 | 3 | −2 | 1 | −8 | −8 | 93 | 82 |
| Desmethylcitalopram | 4 | 3 | 5 | 4 | 3 | 4 | 3 | −9 | 2 | −5 | −4 | 85 | 78 |
| *N*-Desmethyltramadol | 11 | 7 | 6 | 10 | 6 | 5 | −1 | 0 | 2 | 6 | −2 | 65 | 63 |
| Desmethylvenlafaxine | 6 | 6 | 6 | 5 | 5 | 5 | 7 | −5 | 6 | −5 | −6 | 83 | 79 |
| Diazepam | 5 | 5 | 3 | 4 | 4 | 3 | 3 | 1 | 4 | −13 | −13 | 81 | 79 |
| Doxepin | 7 | 3 | 5 | 6 | 3 | 4 | 5 | −8 | 3 | 1 | 1 | 91 | 86 |
| Duloxetine | 8 | 6 | 2 | 8 | 6 | 3 | −5 | −5 | 1 | 0 | −7 | 87 | 87 |
| Fentanyl | 5 | 6 | 7 | 4 | 5 | 7 | −2 | 0 | 2 | 17 | 14 | 83 | 80 |
| Flunitrazepam | 5 | 5 | 4 | 5 | 4 | 3 | 0 | −1 | 2 | −6 | −7 | 80 | 73 |
| Fluoxetine | 9 | 10 | 8 | 8 | 8 | 7 | 7 | −4 | 6 | −7 | −9 | 92 | 84 |
| Hydrocodone | 7 | 13 | 11 | 6 | 11 | 10 | 2 | 2 | 5 | 5 | 5 | 48 | 48 |
| Hydromorphone | 8 | 5 | 11 | 7 | 5 | 9 | 8 | 4 | 4 | 7 | 7 | 23 | 23 |
| Hydroxybupropion | 7 | 7 | 8 | 6 | 6 | 6 | 8 | −4 | 7 | 0 | −1 | 86 | 85 |
| Imipramine | 4 | 6 | 7 | 5 | 6 | 6 | 4 | −4 | 3 | 3 | 1 | 92 | 86 |
| Lorazepam | 12 | 9 | 9 | 10 | 8 | 7 | 16 | 15 | 16 | −4 | −4 | 76 | 73 |
| Meperidine | 10 | 11 | 14 | 9 | 9 | 12 | −1 | 1 | 6 | 7 | −3 | 86 | 83 |
| Methadone | 17 | 11 | 6 | 14 | 9 | 6 | −1 | 2 | 1 | 11 | 1 | 86 | 83 |
| Midazolam | 7 | 6 | 11 | 7 | 5 | 9 | 13 | 11 | 10 | −7 | −7 | 80 | 76 |
| Mirtazapine | 6 | 7 | 4 | 5 | 6 | 4 | 8 | −1 | 3 | 10 | −4 | 85 | 86 |
| 6-Monoacetylmorphine | 8 | 6 | 4 | 7 | 6 | 4 | 6 | 2 | 0 | 22 | 15 | 70 | 70 |
| Morphine | 5 | 7 | 10 | 4 | 7 | 8 | 7 | 4 | 2 | 7 | 6 | 20 | 21 |
| Naloxone | 8 | 18 | 16 | 7 | 14 | 14 | 7 | 13 | 9 | −1 | −1 | 73 | 76 |
| Naltrexone | 10 | 13 | 13 | 9 | 11 | 11 | 9 | 7 | 5 | 15 | 15 | 79 | 81 |
| Nitrazepam | 10 | 12 | 2 | 8 | 10 | 3 | 3 | 1 | 0 | −1 | −1 | 78 | 74 |
| Nordiazepam | 6 | 4 | 4 | 6 | 4 | 4 | −3 | −6 | −3 | −7 | −8 | 79 | 73 |
| Norfluoxetine | 20 | 9 | 11 | 19 | 8 | 10 | −5 | −10 | −4 | 6 | −14 | 87 | 92 |
| Norsertraline | 13 | 11 | 10 | 11 | 10 | 9 | −3 | −13 | −4 | 17 | 4 | 88 | 91 |
| Nortriptyline | 5 | 1 | 3 | 5 | 2 | 3 | 8 | −5 | −1 | −6 | −5 | 92 | 86 |
| Oxazepam | 13 | 14 | 9 | 11 | 11 | 7 | 16 | 14 | 14 | −1 | −2 | 75 | 71 |
| Oxycodone | 8 | 11 | 9 | 7 | 9 | 8 | 7 | 7 | −1 | 6 | 5 | 59 | 59 |
| Oxymorphone | 11 | 13 | 16 | 9 | 11 | 13 | 13 | 10 | 10 | 8 | 6 | 32 | 35 |
| Paroxetine | 6 | 11 | 8 | 6 | 9 | 7 | −3 | −8 | 2 | 1 | −3 | 85 | 85 |
| Sertraline | 13 | 8 | 7 | 11 | 7 | 7 | 7 | −4 | 3 | −18 | −18 | 89 | 86 |
| Temazepam | 10 | 7 | 6 | 8 | 6 | 5 | 10 | 8 | 11 | −7 | −7 | 79 | 73 |
| Tramadol | 7 | 9 | 11 | 7 | 8 | 9 | 1 | 5 | 7 | 5 | −3 | 82 | 81 |
| Trazodone | 8 | 3 | 4 | 6 | 3 | 3 | 7 | −8 | 2 | −3 | −3 | 86 | 85 |

**Table 3** (continued)

| Analyte | Within-run imprecision (%RSD) | | | Between-run imprecision (%RSD) | | | Bias (%) | | | Matrix effect (%) | | Recovery (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LQC | MQC | HQC | LQC | MQC | HQC | LQC | MQC | HQC | LQC | HQC | LQC | HQC |
| Trimipramine | 5 | 3 | 1 | 5 | 3 | 1 | 4 | −8 | 0 | 0 | 0 | 87 | 84 |
| Venlafaxine | 18 | 12 | 6 | 15 | 10 | 5 | 7 | −3 | 7 | −1 | −2 | 86 | 83 |
| Zolpidem | 6 | 5 | 13 | 6 | 5 | 11 | 13 | 9 | 10 | −4 | −3 | 66 | 63 |

*RSD* relative standard deviation

concentrations were satisfactory within ± 10.5, 8.9, 2.4, 4.3, 1.9% of the target concentration for bupropion, citalopram, desmethylvenlafaxine, hydroxybupropion and venlafaxine, respectively.

Among all oral fluid real samples ($n = 38$) (Table 7), citalopram was the most common drug found, in 10 samples (26.3%), with concentrations varying between 1.5 to 150 ng/mL. Citalopram's main metabolite, desmethylcitalopram, was also detected in 7 of these samples, with concentrations within 0.6 to 5.5 ng/mL. Venlafaxine was the second most frequently found drug in 9 samples (23.7%) whereas in 5 of these samples were also possible to detect desmethylvenlafaxine (its main metabolite) in concentrations ranging between 0.6 to < 500 and 0.6 to 257 ng/mL, respectively. Bupropion was detected in 4 samples (10.5%), but its metabolite hydroxybupropion was more commonly found in 7 samples (18.4%), with concentrations within 1.2–334 and 0.6–189 ng/mL, respectively. All samples that had positive results to sertraline ($n = 2$) also were positive to norsertraline, at very low concentrations varying between 0.8 and 3.1 ng/mL. Clonazepam was found only in 2 samples (concentrations of 0.6 and 0.7 ng/mL) and among them only 1 was also positive to 7-aminoclonazepam (2.3 ng/mL). Clomipramine, duloxetine, mirtazapine, paroxetine and zolpidem had only 1 positive sample each and the concentrations found were between 0.5 and 14.1 ng/mL. Fourteen samples were positive for 2 of the investigated analytes, 10 samples had 1 analyte and only 1 sample showed positivity for 5 analytes (Fig. 2).

## Discussion

It is well known that psychoactive substances may affect brain functioning, altering attention, delaying reaction time, reducing alertness, which may lead to car injuries and fatalities [16, 17]. According to a meta-analysis of experimental studies carried out by European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), an usual dose of an antidepressant or anxiolytic can cause at least twice higher degree of impairment than $\Delta^9$-tetrahydrocannabinol smoking [18]. However, only a part of prescribed medicines and licit substances has been investigated in drivers across the world. In Driving Under the Influence of Drugs, Alcohol and Medicines (DRUID) project, which monitors 10,000 drivers per year in 18 countries in Europe, a list containing 25 substances without antidepressant drugs is embraced, containing a half of the 50 analytes in this method [18].

Several cutoff values for different substances are available for driving under the influence of legislations in oral fluid samples [10, 18–20]. For benzodiazepines and opioids, analytical cutoff values vary between 1–480 ng/mL [21]. To the best of our knowledge, no specific cutoff values are available for antidepressants in oral fluid.

In this work, we established a fully LLE and LC–MS/MS method for determining antidepressants, benzodiazepines, opioids and some of their main metabolites in oral fluid. The developed method was validated according to SWGTOX guidelines and performing stability studies for up to 60 days. The LOQ values were determined administratively, below available cutoff values established by EMCDDA, DRUID and European Workplace Drug Testing Society (EWDTS). Calibration range was chosen based on previous articles which reported similar concentrations in oral fluid samples for benzodiazepines [22–24] and for opioids [25, 26]. For antidepressants, calibration range proposed by prior publications were from 5 to 20,000 ng/mL [27–30], which is greater than applied in this method. Although a higher calibration range was firstly essayed, linearity could not be achieved due to the analytical sensibility implied by LC–MS/MS technique.

Solid-phase extraction (SPE) was presented as the extraction technique of the same analytes of this method in previously published papers [23–36]. Our results showed that LLE was a cheaper and preferrable alternative, providing great extraction efficiencies and adequate matrix effects, with low sample volume (500 µL) and low extraction solvent volume (1 mL) consumption. Quintela et al. [23] developed a method for 9 benzodiazepines using 500 µL of the sample, however, adopting 6 mL of extraction solvent and 15 µL injection volume. Kintz et al. [24] incorporated 17 analytes (including benzodiazepines and hypnotics) using the same sample volume and 3 mL of extraction solvent instead. For antidepressant drugs, Coulter et al. [29] developed a method

**Table 4** Antidepressant sample stability values expressed as percentage changes for autosampler (at 10 °C for 24 h), freeze/thaw cycles, room temperature (25 °C), refrigerator (4 °C) and freezer (− 20 °C) after 3, 7, 15, 30 and 60 days, respectively

| Analyte | Quality control concentration | Autosampler (10 °C) | R. Temp (25 °C) | | Refrigerator (4 °C) | | | Freezer (− 20 °C) | | | | | F/T cycles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 h | 3 days | 7 days | 3 days | 7 days | 15 days | 3 days | 7 days | 15 days | 30 days | 60 days | 3 cycles |
| Amitriptyline | LQC | 0 | −2 | 3 | −1 | 0 | −12 | −3 | 13 | −8 | 9 | −3 | 6 |
| | HQC | 0 | −9 | −7 | −2 | 15 | −4 | −3 | 15 | −1 | 10 | 9 | −3 |
| Bupropione | LQC | −7 | −7 | −14 | −5 | −6 | −2 | −6 | −3 | −8 | −4 | 0 | −8 |
| | HQC | −1 | −4 | −19 | −1 | 1 | −6 | −2 | −10 | −10 | 1 | −1 | −1 |
| Citalopram | LQC | −1 | −1 | −1 | 1 | −1 | −2 | −1 | −2 | −6 | −1 | −2 | −4 |
| | HQC | −1 | 1 | −9 | 0 | −1 | −7 | −2 | −10 | −10 | 0 | −2 | 0 |
| Clomipramine | LQC | −4 | −15 | −14 | 0 | −1 | −9 | −11 | −12 | −11 | 9 | −11 | −13 |
| | HQC | 7 | −14 | −13 | −6 | −4 | −9 | −14 | −14 | −3 | 12 | −6 | −18 |
| Desipramine | LQC | 0 | −7 | 7 | −8 | 2 | 2 | −10 | 10 | −4 | 7 | −3 | 6 |
| | HQC | 1 | −9 | 1 | −4 | 9 | 2 | −4 | 10 | −8 | 19 | 8 | −6 |
| Desmethylcitalopram | LQC | −2 | 2 | −2 | −2 | −2 | −3 | −5 | −3 | −3 | −4 | −4 | −2 |
| | HQC | −2 | 2 | −5 | 1 | 1 | 1 | −1 | −8 | −4 | 2 | 1 | 0 |
| Desmethylvenlafaxine | LQC | 1 | −1 | 15 | −1 | 4 | 1 | −2 | 10 | −2 | 10 | 8 | 15 |
| | HQC | 0 | −1 | 6 | −1 | 6 | 0 | −3 | 9 | −1 | 14 | 9 | −1 |
| Doxepine | LQC | −1 | −1 | −6 | −1 | −2 | −12 | −2 | −3 | −9 | −7 | −6 | −7 |
| | HQC | 0 | −1 | −11 | 0 | 1 | −12 | −1 | −8 | −14 | −2 | −6 | −4 |
| Duloxetine | LQC | 0 | 0 | −16 | 18 | 0 | −14 | −1 | −9 | −4 | −8 | 6 | −5 |
| | HQC | 0 | −7 | −7 | −3 | 3 | −15 | −5 | −10 | −19 | −4 | −6 | −8 |
| Fluoxetine | LQC | 3 | 18 | 24 | −3 | 4 | −19 | 0 | 4 | −9 | −3 | −16 | −5 |
| | HQC | 3 | −8 | −5 | 3 | 8 | −13 | −1 | 16 | −1 | 14 | 1 | −18 |
| Hydroxybupropione | LQC | 0 | 0 | 11 | 0 | 2 | −1 | −4 | 11 | −1 | 10 | 9 | 10 |
| | HQC | 0 | 2 | 7 | 3 | 7 | 2 | 1 | 9 | 2 | 16 | 13 | 3 |
| Imipramine | LQC | −3 | −9 | −1 | −7 | −10 | −10 | −6 | −19 | −4 | −12 | −8 | −10 |
| | HQC | 4 | 0 | −10 | −3 | −5 | −3 | −10 | −17 | −13 | −8 | −2 | −10 |
| Mirtazapine | LQC | 0 | 1 | 16 | 2 | 7 | 0 | −1 | 15 | −3 | 6 | 5 | 14 |
| | HQC | 0 | 0 | 9 | 1 | 12 | −3 | 0 | 12 | −3 | 13 | 6 | −1 |
| Norfluoxetine | LQC | 4 | −17 | −52 | 19 | 2 | 4 | −1 | −12 | 15 | 0 | −17 | 36 |
| | HQC | 2 | −15 | −29 | −8 | −13 | 0 | −13 | −10 | −17 | 3 | 0 | −17 |
| Norsertraline | LQC | 1 | −7 | −79 | 11 | −2 | 44 | 3 | −21 | 49 | 4 | 21 | 10 |
| | HQC | 1 | −5 | −38 | 6 | −8 | 15 | −1 | 11 | −2 | 12 | 14 | −7 |
| Nortriptyline | LQC | −1 | 2 | 0 | −2 | 5 | −1 | 2 | −2 | −5 | 4 | −5 | −8 |
| | HQC | −1 | −7 | −12 | −2 | 1 | −6 | −5 | −8 | −13 | 4 | 1 | −5 |
| Paroxetine | LQC | −3 | −3 | 4 | 5 | 4 | 3 | 1 | 1 | 13 | 16 | 8 | 34 |
| | HQC | 0 | −15 | −10 | −8 | 1 | −9 | −10 | 0 | −5 | 13 | 2 | −13 |

**Table 4** (continued)

| Analyte | Quality control concentration | Autosampler (10 °C) | R. Temp (25 °C) | | Refrigerator (4 °C) | | | Freezer (− 20 °C) | | | | | F/T cycles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 h | 3 days | 7 days | 3 days | 7 days | 15 days | 3 days | 7 days | 15 days | 30 days | 60 days | 3 cycles |
| Sertraline | LQC | 8 | − 11 | − 40 | 0 | − 5 | − 17 | − 2 | − 6 | − 15 | − 11 | − 11 | − 10 |
| | HQC | 9 | − 23 | − 49 | − 5 | − 10 | − 13 | − 11 | − 18 | − 11 | − 7 | − 7 | − 20 |
| Trazodone | LQC | − 1 | 1 | − 3 | 1 | 0 | 0 | 0 | − 1 | 1 | − 5 | 0 | − 7 |
| | HQC | − 1 | 1 | − 9 | 0 | 0 | − 4 | − 1 | − 8 | − 8 | 0 | 1 | − 10 |
| Trimipramine | LQC | 0 | − 5 | − 16 | 1 | 8 | − 10 | 0 | 9 | − 8 | − 7 | − 6 | − 13 |
| | HQC | 1 | − 9 | − 27 | − 1 | 6 | − 15 | − 4 | 0 | − 19 | − 6 | − 7 | − 5 |
| Venlafaxine | LQC | 2 | − 4 | 11 | − 3 | 2 | − 2 | − 5 | 8 | − 7 | 6 | 2 | 11 |
| | HQC | 0 | − 2 | 5 | − 2 | 6 | − 2 | − 4 | 6 | − 3 | 12 | 5 | − 2 |

*R. Temp* room temperature, *F/T cycles* freeze/thaw cycles. For LQC and HQC see Table 1

with 16 drugs, using SPE technique and applying 1.0 mL of oral fluid sample volume.

Coulter et al. [29] obtained an extraction recovery higher than 88% for antidepressants using Quantisal™ collection device. For benzodiazepines, Ngwa et al. [36] presented extraction recovery better than 54% in two different concentrations and SPE. For opioids, Truver and Swortwood [37] applied a SPE technique and Quantisal™ collector device; morphine, 6-monoacetylmorphine and buprenorphine had recovery results better than 85% using LOQ values of 5 ng/mL. In our method, morphine presented recovery results better than 20%; however, we achieved an LOQ 5 times lower (1 ng/mL).

In fact, low limits of detection/quantification are required in oral fluid analysis. Morphine and codeine are often found in concentrations ranging within 1–25 ng/mL in oral fluid samples [38, 39]. Fentanyl and oxycodone whatsoever appear to have lower and higher concentrations in oral fluid samples, respectively, justifying the necessity of more oral fluid disposition studies with controlled drug administration [38]. Jang et al. [40] reported very low concentrations for benzodiazepine drugs in oral fluid samples of chronic users. Alprazolam, clonazepam, diazepam and lorazepam concentrations ranged from 0.9 to 14.4 ng/mL supporting the need for more sensitive and selective techniques to analyze these drug classes. Even using LLE, our method had sensibility enough (or greater) than previously published methods.

In most recent articles, quantitative and qualitative analyses of novel synthetic opioids/clandestine opioids were published [37, 41], but such analytes were not dealt with this method. Additionally, a growing public need for opioid drug monitoring in the oral fluid has been arising, as an important clinical tool to evaluate the efficiency of opioid drug treatment in patients with cancer [38], during treatment for opioid addiction [42], and also for driving under the influence legislations [43, 44].

For benzodiazepines, bias and imprecision values of this method ranged from 0 to 16% and 3 to 14%, respectively, showing closer results to a previous method developed by Jang et al. [40] which had values within 0 to − 7.2% and 3.0 to 8.6%. Also similar values were obtained for antidepressants, with imprecision value average of 6% and bias average values of 5%, against 4.5 and 3.9%, respectively, in a previously published article by Shin et al. [45].

Langel et al. [46] reported good stability at − 18 °C for morphine, codeine, diazepam and alprazolam for 28 days, using Quantisal™ as collector device. Grabenauer et al. [47] similarly noted that for 6-monoacetylmorphine, codeine, hydromorphone, oxymorphone and morphine were stable in the neat oral fluid at both refrigerator (8 °C) and freezer (− 20 °C) temperatures for up to 4 weeks. Hydrocodone was reported by Grabenauer et al. [47] to be unstable under refrigerator conditions for over 7 days, but our results

**Table 5** Benzodiazepines sample stability values expressed as percentage change for autosampler (at 10 °C for 24 h), freeze/thaw cycles, room temperature (25 °C), refrigerator (4 °C) and freezer (−20 °C) for 3, 7, 15, 30 and 60 days, respectively

| Analyte | Quality control concentration | Autosampler (10 °C) | | | R. Temp (25 °C) | | Refrigerator (4 °C) | | | Freezer (−20 °C) | | | | | F/T cycles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 12 h | 18 h | 24 h | 3 days | 7 days | 3 days | 7 days | 15 days | 3 days | 7 days | 15 days | 30 days | 60 days | 3 cycles |
| Alprazolam | LQC | −3 | −4 | 0 | −11 | −23 | 4 | −10 | −4 | 5 | −18 | −5 | −5 | −7 | 6 |
| | HQC | −5 | −2 | 2 | −9 | −23 | 2 | −16 | −7 | −1 | −19 | −15 | −17 | −7 | −1 |
| 7-Aminoclonazepam | LQC | −19 | −19 | −33 | 12 | −2 | 11 | −12 | −2 | −5 | −37 | −40 | −43 | −38 | 18 |
| | HQC | −18 | −18 | −26 | 11 | −8 | 5 | −16 | −4 | −6 | −36 | −40 | −43 | −41 | 8 |
| 7-Aminoflunitrazepam | LQC | −18 | −20 | −23 | 11 | −5 | 13 | −14 | −11 | −4 | −32 | −40 | −40 | −33 | 12 |
| | HQC | −19 | −17 | −23 | 13 | −11 | 4 | −18 | −12 | −3 | −33 | −40 | −40 | −38 | 6 |
| Bromazepam | LQC | −8 | −3 | −14 | −5 | −15 | −6 | 0 | −17 | −16 | 0 | −5 | −8 | 18 | −22 |
| | HQC | −5 | −1 | −15 | 0 | −6 | −5 | −1 | −9 | −9 | −3 | −8 | −14 | 9 | −20 |
| Clonazepam | LQC | 4 | 0 | −2 | −10 | −24 | 3 | 1 | 3 | 1 | 4 | 6 | 10 | 13 | 1 |
| | HQC | 1 | −1 | −8 | −17 | −32 | −8 | −10 | −3 | −8 | −10 | −9 | −6 | −3 | −10 |
| Diazepam | LQC | −4 | −5 | −7 | −7 | −15 | −4 | 0 | 0 | −6 | −5 | 2 | 3 | −1 | −2 |
| | HQC | −10 | −6 | −14 | −5 | −8 | −4 | −5 | −1 | −8 | −7 | −1 | −4 | −4 | −6 |
| Flunitrazepam | LQC | 1 | 2 | 4 | −21 | −27 | −14 | −5 | −2 | −12 | −10 | −3 | 0 | 1 | −27 |
| | HQC | −1 | 1 | −2 | −19 | −17 | −12 | −5 | 1 | −13 | −5 | −2 | −9 | −5 | −17 |
| Lorazepam | LQC | 7 | 6 | 8 | −11 | −27 | −1 | −11 | 14 | 0 | −9 | 0 | 2 | 23 | −9 |
| | HQC | 2 | 0 | 1 | −3 | −15 | 0 | −7 | 9 | −4 | −11 | 1 | −5 | 12 | −7 |
| Midazolam | LQC | −1 | −2 | −1 | 17 | −16 | 10 | −11 | −9 | 12 | −20 | −19 | −13 | −5 | 24 |
| | HQC | −2 | −3 | −1 | 11 | −12 | 10 | −13 | −3 | 11 | −16 | −13 | −11 | −4 | 21 |
| Nitrazepam | LQC | −11 | −11 | −31 | −5 | −10 | 0 | 1 | 3 | −10 | 8 | 15 | 10 | 10 | −19 |
| | HQC | −15 | −16 | −32 | −10 | −12 | −11 | 0 | −2 | −18 | 3 | −1 | −3 | −2 | −27 |
| Nordiazepam | LQC | 8 | 5 | 8 | −4 | −23 | −1 | 0 | 12 | 4 | −3 | 15 | 9 | 23 | −35 |
| | HQC | 7 | 3 | 5 | 2 | −11 | −23 | 3 | −6 | −13 | −6 | 0 | −4 | 6 | −34 |
| Oxazepam | LQC | 4 | 5 | 5 | −15 | −22 | −6 | −6 | −1 | −9 | −12 | −4 | 1 | 17 | −5 |
| | HQC | 1 | 1 | 0 | 0 | −13 | −1 | −3 | −1 | −5 | −14 | −1 | −7 | 4 | −9 |
| Temazepam | LQC | −2 | −1 | −1 | −2 | −9 | −3 | −2 | 9 | −5 | −15 | −1 | 0 | 5 | −4 |
| | HQC | −2 | 0 | −3 | 0 | −2 | 0 | −5 | 6 | −5 | −11 | 1 | −4 | 0 | −6 |
| Zolpidem | LQC | −1 | −4 | 0 | 13 | −7 | 15 | −9 | 0 | 16 | −19 | −11 | −13 | 0 | 33 |
| | HQC | 1 | −2 | 1 | 10 | −13 | 6 | −17 | −2 | 9 | −19 | −15 | −16 | 0 | 23 |

**Table 6** Opioids sample stability values expressed as percentage changes for autosampler (at 10 °C for 24 h), freeze/thaw cycles, room temperature (25 °C), refrigerator (4 °C) and freezer (− 20 °C) for 3, 7, 15, 30 and 60 days, respectively

| Analyte | Quality control concentration | Autosampler (10 °C) | R. Temp (25 °C) | | Refrigerator (4 °C) | | | Freezer (− 20 °C) | | | | | F/T cycles |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 h | 3 days | 7 days | 3 days | 7 days | 15 days | 3 days | 7 days | 15 days | 30 days | 60 days | 3 cycles |
| Buprenorphine | LQC | 2 | 5 | 9 | 7 | 16 | 10 | − 2 | 14 | 2 | − 5 | 15 | − 1 |
| | HQC | 7 | − 6 | 4 | − 7 | 8 | 0 | − 11 | − 2 | − 1 | − 13 | 6 | − 2 |
| Codeine | LQC | 4 | 8 | 11 | 11 | 13 | 6 | 8 | 12 | 8 | 10 | 10 | − 5 |
| | HQC | 2 | 1 | 6 | − 1 | 3 | 7 | − 1 | 0 | 7 | 3 | 3 | 1 |
| *N*-Desmethyltramadol | LQC | 4 | 2 | 5 | 6 | 11 | 9 | 0 | 12 | 9 | 7 | 6 | − 2 |
| | HQC | 11 | − 5 | 13 | − 5 | 13 | 10 | − 3 | 12 | 9 | − 6 | − 3 | 0 |
| Fentanyl | LQC | 1 | 0 | 0 | 6 | 10 | 7 | − 2 | 13 | 9 | 3 | 1 | − 8 |
| | HQC | 2 | − 6 | 12 | − 4 | 14 | 11 | − 7 | 4 | 13 | 0 | 1 | − 1 |
| Hydrocodone | LQC | − 1 | 2 | − 1 | 4 | 5 | 7 | − 1 | 8 | 8 | 9 | − 2 | − 2 |
| | HQC | 0 | − 3 | 10 | − 3 | 13 | 14 | − 2 | 0 | 15 | 2 | − 5 | 1 |
| Hydromorphone | LQC | − 1 | 2 | 4 | 10 | 4 | 14 | 9 | 5 | 17 | 19 | − 5 | 0 |
| | HQC | 4 | 0 | 5 | 3 | 6 | 12 | 0 | 5 | 12 | 13 | − 2 | 4 |
| Meperidine | LQC | 3 | − 10 | 3 | − 8 | 12 | − 6 | − 20 | 16 | − 4 | − 8 | − 13 | − 20 |
| | HQC | 2 | − 19 | 18 | − 17 | 17 | 0 | − 14 | 8 | 0 | − 8 | − 13 | − 18 |
| Methadone | LQC | 1 | − 1 | 5 | 8 | 21 | 15 | 5 | 17 | 8 | 8 | − 1 | − 4 |
| | HQC | 2 | − 12 | 6 | − 5 | 11 | 10 | − 5 | 1 | 9 | − 7 | − 4 | 0 |
| 6-Monoacetylmorphine | LQC | 5 | − 1 | − 8 | 2 | 7 | 4 | − 2 | 6 | 5 | 4 | 4 | − 6 |
| | HQC | 9 | − 11 | − 8 | − 8 | 0 | 0 | − 7 | 0 | 3 | − 5 | 3 | − 3 |
| Morphine | LQC | 2 | 2 | 9 | 4 | 6 | 8 | 2 | 2 | 6 | 6 | − 3 | − 6 |
| | HQC | 1 | 0 | 16 | 1 | 8 | 11 | 1 | 7 | 8 | 8 | − 1 | 2 |
| Naloxone | LQC | − 1 | 2 | 11 | − 5 | 0 | 0 | − 11 | − 4 | 8 | − 9 | 20 | − 12 |
| | HQC | 3 | 2 | 2 | − 4 | − 1 | 11 | − 6 | − 4 | 17 | 4 | 23 | − 5 |
| Naltrexone | LQC | 0 | 7 | 23 | 8 | 19 | 11 | 0 | 13 | 13 | 10 | 25 | − 1 |
| | HQC | − 1 | − 6 | 0 | − 10 | 0 | 5 | − 8 | − 4 | 6 | − 4 | 12 | − 3 |
| Oxycodone | LQC | 1 | − 1 | − 1 | 2 | 5 | 5 | − 2 | 8 | 6 | 4 | − 1 | − 5 |
| | HQC | − 1 | − 3 | 15 | − 7 | 15 | 12 | 0 | 7 | 15 | 7 | 5 | 0 |
| Oxymorphone | LQC | 0 | 0 | − 1 | 9 | 18 | 16 | 6 | 13 | 17 | 15 | 4 | − 3 |
| | HQC | 0 | − 1 | − 3 | 3 | 15 | 15 | 3 | 13 | 17 | 17 | 6 | 0 |
| Tramadol | LQC | 1 | 0 | − 2 | 5 | 3 | 6 | − 7 | 4 | 8 | 3 | 1 | − 6 |
| | HQC | 0 | − 6 | 11 | − 4 | 10 | 16 | − 5 | − 1 | 18 | 0 | − 1 | 2 |

**Table 7** Results for 38 authentic oral fluid samples collected using Quantisal™ and analyzed using the developed method

| Sample number | Number of detected analytes in the sample | Analytes found | Concentration (ng/mL) |
|---|---|---|---|
| 1 | 0 | ND | < LOQ |
| 2 | 0 | ND | < LOQ |
| 3 | 0 | ND | < LOQ |
| 4 | 0 | ND | < LOQ |
| 5 | 0 | ND | < LOQ |
| 6 | 0 | ND | < LOQ |
| 7 | 0 | ND | < LOQ |
| 8 | 0 | ND | < LOQ |
| 9 | 1 | Mirtazapine | 14.1 |
| 10 | 1 | Paroxetine | 5.8 |
| 11 | 1 | Venlafaxine | 0.9 |
| 12 | 1 | Venlafaxine | 0.6 |
| 13 | 1 | Venlafaxine | 0.7 |
| 14 | 1 | Clomipramine | 1.2 |
| 15 | 1 | Duloxetine | 1.5 |
| 16 | 1 | Citalopram | 15.6 |
| 17 | 1 | Nitrazepam | 2.5 |
| 18 | 1 | Codeine | 4.6 |
| 19 | 2 | Venlafaxine | 81.0 |
| | | Desmethylvenlafaxine | 257 |
| 20 | 2 | Desmethylcitalopram | 0.6 |
| | | Citalopram | 15.8 |
| 21 | 2 | Hydroxybupropion | 4.1 |
| | | Citalopram | 1.5 |
| 22 | 2 | Norsertraline | 1.5 |
| | | Sertraline | 0.8 |
| 23 | 2 | Desmethylvenlafaxine | 0.6 |
| | | Venlafaxine | 3.6 |
| 24 | 2 | Norsertraline | 3.1 |
| | | Sertraline | 2.2 |
| 25 | 2 | Desmethylvenlafaxine | 27.0 |
| | | Venlafaxine | 17.0 |
| 26 | 2 | Desmethylcitalopram | 4.4 |
| | | Citalopram | 150 |
| 27 | 2 | Citalopram | 2.3 |
| | | Nitrazepam | 1.8 |
| 28 | 2 | Desmethylcitalopram | 4.8 |
| | | Citalopram | 75.1 |
| 29 | 2 | Desmethylcitalopram | 3.0 |
| | | Citalopram | 33.8 |
| 30 | 2 | Hydroxybupropion | 5.0 |
| | | Bupropion | 1.2 |
| 31 | 2 | Hydroxybupropion | 71.8 |
| | | Bupropion | 24.0 |
| 32 | 2 | Desmethylvenlafaxine | 231 |
| | | Venlafaxine | > 500 |
| 33 | 3 | Desmethylvenlafaxine | 474 |
| | | Hydroxybupropion | 189 |
| | | Bupropion | 334 |
| 34 | 3 | Desmethylvenlafaxine | 1.0 |
| | | Venlafaxine | 2.3 |
| | | Codeine | 12.0 |
| 35 | 3 | 7-Aminoclonazepam | 2.3 |
| | | Nitrazepam | 1.1 |
| | | Clonazepam | 0.6 |
| 36 | 4 | Hydroxybupropion | 0.9 |
| | | Venlafaxine | 2.1 |
| | | Citalopram | 17.3 |
| | | Desmethylcitalopram | 1.5 |
| 37 | 4 | Desmethylcitalopram | 2.3 |
| | | Hydroxybupropion | 36.9 |
| | | Bupropion | 11.1 |
| | | Citalopram | 16.0 |
| 38 | 5 | Citalopram | 38.2 |
| | | Clonazepam | 0.7 |
| | | Zolpidem | 0.5 |
| | | Desmethylcitalopram | 5.5 |
| | | Hydroxybupropion | 0.6 |

*ND* not detected, *LOQ* limit of quantification

ensured its stability for up to 15 days (Table 6), which assured Quantisal™ buffer efficiency in compound stability. In our results, 7-aminoclonazepam showed poor stability at − 20 °C for 7 days (Table 5), in accordance with the literature [48, 49]. Alprazolam, clonazepam, diazepam, nordiazepam and oxazepam, which were stable for 60 days in freezer conditions (Table 5), agreeing with the results by Lurd et al. [49].

Our results showed that all antidepressants investigated in this method were stable under freezer conditions (− 20 °C) for 60 days, at room temperature (25 °C) for 3 days and at refrigerator temperature (4 °C) for 7 days. Stability performed in autosampler (10 °C) demonstrated that all antidepressants remained stable for 24 h (Table 4), which is in accordance with the results of Coulter et al. [29] and Shin et al. [45].

Among method limitations, the lack of stability under − 20 °C of 7-amino metabolites (as 7-aminoclonazepam and 7-aminoflunitrazepam) appeared as a problem which Quantisal™ was unable to solve. Vindenes et al. [50] reported that 7-amino metabolites are more commonly found in the oral fluid than the parent drug, which implies that collection

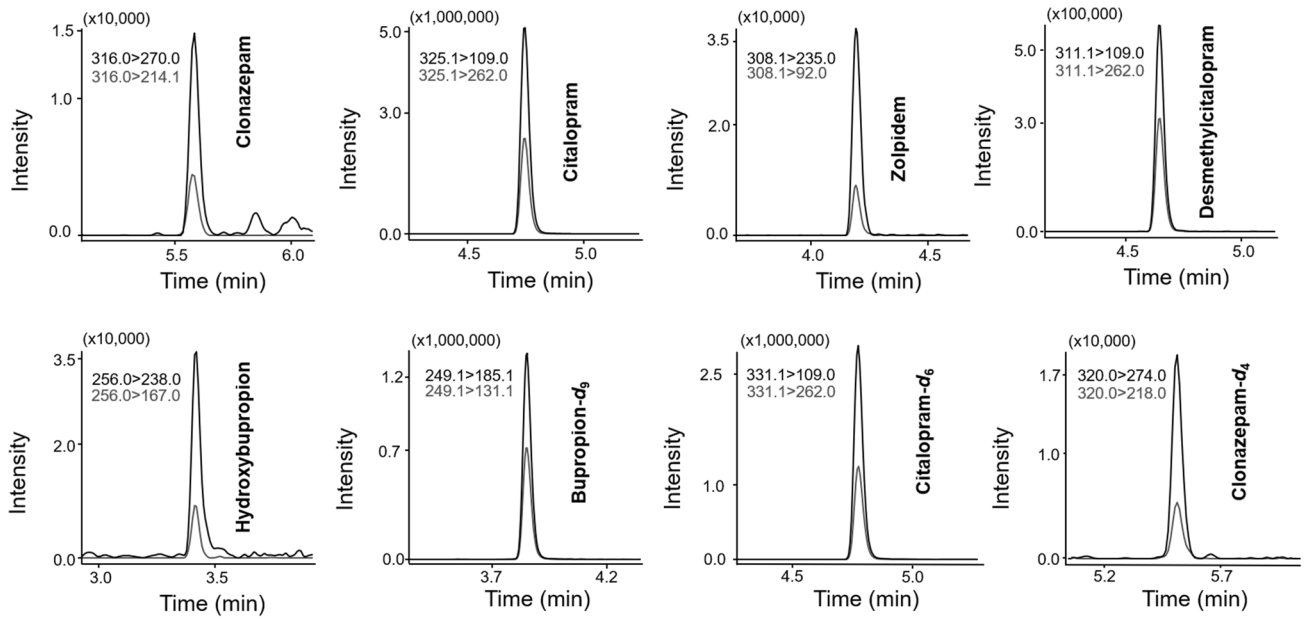


**Fig. 2** MRM chromatograms of an authentic oral fluid sample (#38) with positive results for clonazepam (0.7 ng/mL), citalopram (38.2 ng/mL), zolpidem (0.5 ng/mL), desmethylcitalopram (5.5 ng/mL) and hydroxybupropion (0.6 ng/mL) with their respective internal standards

and process sample should be done as soon as possible. The similar phenomenon was observed for sertraline and norsertraline at room temperature (25 °C) (Table 4), which appears to be a problem for on-site collection and storage during more than 3 days. Another limitation of our method was the impossibility of distinguishing citalopram and escitalopram (optical isomers).

To prove that the developed method fit the purpose, 38 oral fluid samples were analyzed. All samples were collected from volunteers above 18 years old in parties and electronic music festivals. The prevalence of the present drugs, which are circulating psychoactive drugs, is much higher than that of so-called new psychoactive substances (NPS) in current human society [51]. This is the reason why we have presented the details of a simple and sensitive analytical method for the 50 psychotropic drugs (largely prescription drugs) in this article.

## Conclusions

A sensitive method based on LLE and LC–MS/MS was developed to quantify 50 psychoactive drugs in oral fluid samples, with very low limits of quantification, adequate bias and imprecision. Besides the 50 MS/MS analyses incorporated in the method, a fast chromatographic run was developed, allowing analysis below 10 min and using a very simple liquid–liquid extraction procedure.

Finally, very low concentrations of the studied analytes are found in many of authentic samples, in most cases below 5 ng/mL, which justifies the need of a sensitive and specific method for monitoring drugs in oral fluid.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest associated with this manuscript.

**Ethical approval** All procedures performed in this study involving urine samples from human volunteers were in accordance with the ethical standards of the University of Campinas committee (Comitê de Ética em Pesquisa da UNICAMP—CEP, CAAE 58187816.6.0000.5404), and with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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