Liquid chromatography tandem mass spectrometry determination of selected synthetic cathinones and two piperazines in oral fluid. Cross reactivity study with the Dräger DrugTest 5000.

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Abstract

Since the past few years, several synthetic cathinones and piperazines have been introduced into the drug market to substitute illegal stimulant drugs such as amphetamine and derivatives or cocaine due to their unregulated situation. These emerging drugs are not usually included in routine toxicological analysis. We develop and validated a LC-MS/MS method for the determination of methedrone, methylone, mephedrone, 3,4-methylenedioxypyrovalerone (MDPV), fluoromethcathinone, fluoromethamphetamine, 1-(3-chlorophenyl)piperazine (mCPP) and 3-trifluoromethylphenylpiperazine (TFMPP) in oral fluid. Sample extraction was performed using Strata X cartridges. Chromatographic separation was achieved in 10 min using an Atlantis® T3 column (100 x 2.1 mm, 3µm), and formic acid 0.1% and acetonitrile as mobile phase. The method was satisfactorily validated, including selectivity, linearity (0.2-0.5 to 200 ng/mL), limits of detection (0.025-0.1 ng/mL) and quantification (0.2-0.5 ng/mL), imprecision and accuracy in neat oral fluid (%CV= 0.0-12.7% and 84.8-103.6% of target concentration, respectively) and in oral fluid mixed with Quantisal™ buffer (%CV=7.2-10.3% and 80.2-106.5% of target concentration, respectively), matrix effect in neat oral fluid (-11.6 to 399.7%) and in oral fluid with Quantisal™ buffer (-69.9 to 131.2%), extraction recovery (87.9-134.3%) and recovery from the Quantisal™ (79.6-107.7%), dilution integrity (75-99% of target concentration) and stability at different conditions (-14.8 to 30.8% loss). In addition, cross reactivity produced by the studied synthetic cathinones in oral fluid using the Dräger DrugTest 5000 was assessed. All the analytes produced a methamphetamine positive result at high concentrations (100 or 10 µg/mL), and fluoromethamphetamine also at low concentration (0.075 µg/mL).

Keywords: synthetic cathinone, synthetic piperazine, LC-MS/MS, immunoassay cross reactivity, oral fluid

1. Introduction

In the last years, new non-regulated synthetic drugs have emerged into the market to avoid prosecution traditional illegal drugs consumption and sale [1]. Among these emerging drugs are the synthetic cathinones and piperazines, which users consume as an alternative to amphetamines or cocaine, as they mimic their stimulant effects. Synthetic cathinones are the main constituents of the so-called “salt baths” or “plant food” [2], and piperazine derivatives are the main ingredient of the “party pills” or “legal ecstasy” [1]. These products are sold on the Internet, head shops or gas stations without any legal control, as the presence of psychotropic compounds is never stated, and the label usually indicates that the product “is not intended for human consumption” or “not tested for hazards or toxicity” [1-3]. Among these drugs, mephedrone is a controlled substance in Europe since 2010; and several synthetic cathinones, including 3,4-methylenedioxypyrovalerone (MDPV), mephedrone and methylone, have been added to the Schedule I of the Control Substances Act of the USA in 2011 and 2014 [4, 5]. The regulation of these drugs motivates slight chemical variations to include new non-controlled substances, responsible for the great variability in the products composition, even within the same brand [2, 6]. This fact and the concentrations variability of the products increase the risk of acute toxicity. Up to date, few data about the toxicity in humans are available, and they are obtained mainly from reported intoxication cases or drug users forums [7-9]

An objective proof of the exposure to these drugs is not available in many cases, as these emerging drugs are not detected in traditional toxicological analysis [3]. Due to the structural similarity, some of these analytes cross-react with amphetamine and/or

methamphetamine in immunoassay screening devices [10, 11], which would not be confirmed using routine toxicological methods. To our knowledge, only one company commercialized specific immunoassay kits for the screening of MDPV, and for mephedrone and methcathinone [12]. With regard to confirmation methods, several GC-MS or LC-MS(MS) methods have been developed for the determination of one or more synthetic cathinones and/or piperazines, sometimes mixed with other groups of amphetamine type stimulants, in powder [13-15], blood, plasma or serum [14, 16-19], urine [14, 20] and oral fluid [21, 22].

The goal of the present work was to assess the possible cross-reactivity of the studied synthetic cathinones with the drug groups included in the Dräger DrugTest 5000, which is one of the on-site drug screening devices employed in mandatory drug testing performed by the Spanish traffic police. In addition, we developed and validated an LC-MS/MS method for the determination of 8 common synthetic cathinones and piperazines in oral fluid, which will be applied to authentic specimens collected on the road site, and sent to our laboratory for confirmation purposes.

2. Materials and Methods

2.1. Chemicals and Reagents

Methylone, methedrone, mephedrone, 1-(3-chlorophenyl)piperazine (mCPP), MDPV and 3-trifluoromethylphenylpiperazine (TFMPP) standards at 1 mg/mL, and methylone-d3, methamphetamine-d5, mephedrone-d3, mCPP-d8 and TFMPP-d4 at 0.1 mg/mL in methanol were purchased from Cerilliant™ (Round Rock, TX, USA). (±)-4-fluoromethamphetamine and 4-fluoromethcathinone (4-FMC) in solid form were

obtained as free bases from the National Measurement Institute, Australian Government (Lindfield, Sydney, Australia). Water was purified with a Milli-Q water system (Millipore, Le-Mont-sur-Lausanne, Switzerland). Reagent grade formic acid 98-100% was from Scharlau (Sentmenat, Spain). Chromasolv® gradient grade methanol, 2-propanol and reagent grade dichloromethane were from Sigma-Aldrich (Steinheim, Germany), and LC-MS grade acetonitrile from Panreac (Castellar del Vallès, Spain). Boric acid, potassium chloride and sodium hydroxide were from Merck (Darmstadt, Germany), and reagent grade ammonia solution 32% (v/v) was from Scharlau (Sentmenat, Spain). Strata X cartridges (3cc, 60 mg) were purchased from Phenomenex (Torrence, CA, USA). Salivette® and Quantisal™ oral fluid collection devices were from Sarstedt (Nümbrecht, Germany) and Immunalysis Corp. (Pomona, CA, USA), respectively. Dräger Drugtest 5000 (Dräger Safety AG & Co. KGaA, Lübeck, Germany) was kindly donated by Dräger.

2.2. Oral fluid samples

Fresh oral fluid samples for the preparation of the calibrators and quality control (QC) samples were donated by the staff personnel, and collected with the Salivette® device. Authentic specimens from the roadside were collected using the Quantisal™ device. For the cross-reactivity study, a pool of blank oral fluid matrix was prepared by mixing oral fluid from 8 different people (10 mL each) collected by direct spitting. Donors were not allowed to eat or smoke 10 min before sample collection. After shaking and resting 60 min at room temperature, the supernatant of the pooled oral fluid was transferred to a clean tube.

2.3. Preparation of calibration and quality control (QC) solutions

A stock solution at 10 μg/mL in methanol was prepared from the individual standards at 1 mg/mL. Further dilutions in methanol were prepared at 1, 0.1, 0.01 and 0.002 μg/mL. An eight to nine-point calibration curve from 0.2 or 0.5 to 200 ng/mL was generated by addition of 25 to 100 μL of the appropriate working solution to 0.5 mL of blank oral fluid. Independent stock and working solutions at 10, 1, 0.1 and 0.01 μg/mL were prepared for the generation of low, medium and high QC samples (0.6, 20 and 150 ng/mL, respectively). An internal standard (IStd) mixture containing methylone-d3, mephedrone-d3, mCPP-d8, TFMPP-d4 and methamphetamine-d5 at 1 μg/mL was prepared by dilution of the individual IStd stock solution in methanol.

2.4. Sample preparation

The analytes of interest were extracted using the same solid phase extraction (SPE) procedure employed in our laboratory for the determination of opiates (morphine, codeine, 6-acetylmorphine), cocaine (cocaine and benzoylecgonine), amphetamines (amphetamine, methamphetamine, MDA, MDMA, MDEA), cannabis (THC), methadone, benzodiazepines (alprazolam, clonazepam, oxazepam, nordiazepam, lorazepam, flunitrazepam, diazepam), zolpidem, zopiclone, amitriptyline and diphenhydramine. The current protocol is a slight modification from that published elsewhere [23]. Briefly, 25 μL of IStd mixture and 2 mL of borate buffer pH 9 were added to 0.5 mL of oral fluid. The sample was loaded after conditioning the Strata X cartridge with 2 mL methanol and 2 mL water. After two washing steps with 2 mL water:methanol (95:5, v/v) and 2 mL water:methanol:NH4OH (70:29.5:0.5, v/v/v), analytes were eluted with 3 mL dichloromethane:2-propanol (75:25). After addition of 100 μL HCl 0.1%, the eluate was evaporated with nitrogen in a TurboVap LV

156 evaporator (Zymark, Hopkinton, MA, USA) at 35ºC, reconstituted in 75 µL formic acid
157 0.1%:ACN (90:10, v/v), and 20 µL were injected into the LC-MS/MS system.

158 2.5. LC-MS/MS

159 The HPLC system was an Alliance 2795 Separation Module with an Alliance series
160 column heater/cooler (Waters Corp., Mildford, MA, USA). Chromatographic separation
161 was performed with an Atlantis® T3 (2.1 mm x 50 mm, 3 µm) reversed-phase analytical
162 column (Waters, Mildford, MA, USA), maintained at 35ºC. Formic acid 0.1% (A) and
163 acetonitrile (B) were used as mobile phase at a flow rate of 0.3 mL/min. Gradient was
164 programmed as follows: 10% B from 0 to 0.5 min, linearly increased to 60% over 5.5
165 min, to return to initial conditions at min 6.1. Column was equilibrated until min 10. A
166 divert valve was set to direct the flow to the MS from 0.2 to 6 min, and to waste the
167 remaining time. The autosampler was maintained at 6ºC.

168 The mass spectrometer was a Quattro Micro ™ API ESCI triple quadrupole (Waters
169 Corp., Mildford, MA, USA). The instrument was operated in electrospray in the
170 positive mode (ESI+) to produce protonated molecules of the analytes with the
171 following optimized settings: capillary voltage 1.0 kV; source block and desolvation gas
172 (nitrogen) temperature 150 ºC and 450 ºC, respectively; desolvation and cone gas
173 (nitrogen) flow rate 550 L/h and 45 L/h, respectively. Data were recorded on multiple
174 reaction monitoring (MRM) mode. A post-column infusion of each individual analyte at
175 10 µL/mL connected with a “T” valve to the chromatographic effluent (formic acid
176 0.1%:ACN, 50:50, v/v) was employed to select multiple reaction monitoring (MRM)
177 transitions, cone voltages and collision energies for the target analytes and IStds. Data
178 acquisition was controlled with MassLynx 4.1 software and processed with QuanLynx

179 4.1 software (Waters Corp., Milford, USA).

180 2.6. Method validation

181 The following parameters were studied for method validation: selectivity, linearity,
182 limit of detection (LOD), limit of quantification (LOQ), imprecision, accuracy, matrix
183 effect, Quantisal™ and extraction recovery, dilution integrity and stability under
184 different conditions.

185 Selectivity was evaluated by assessment of endogenous and exogenous interferences.
186 Potential endogenous interferences were assessed by the analysis of blank oral fluid
187 samples collected with the Salivette® from 10 different sources fortified with the IStd
188 mixture. Exogenous interferences were assessed by the analysis of blank oral fluid
189 samples fortified with 43 common drugs of abuse and medicines at 500 ng/mL, which is
190 an intermediate concentration for most of the drugs within the usual concentrations
191 ranges observed in oral fluid specimens. The following drugs were tested: morphine,
192 codeine, 6-acetylmorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-
193 diphenylpyrrolidine (EDDP), amphetamine, methamphetamine, 3,4-
194 methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA),
195 3,4-methylendioxyethylamphetamine (MDEA), cocaine, benzoylecgonine, ecgonine
196 methyl ester, cocaethylene, THC, 11-nor-9-carboxy-THC, 11-hydroxi-THC, 8ß,11-
197 dihydroxy-THC, cannabinol, cannabidiol, lysergic acid diethylamide (LSD), ketamine,
198 norketamine, gamma-hydroxybutyric acid (GHB), nicotine, cotinine, fentanyl,
199 amitriptyline, diclofenac, naproxen, alprazolam, temazepam, lormetazepam, lorazepam,
200 flunitrazepam, 7-aminoflunitrazepam, clonazepam, diazepam, nordiazepam, oxazepam,
201 triazolam, nitrazepam and bromazepam at 500 ng/mL.

The LOD was defined as the lowest concentration at which the two MRM transitions monitored for each analyte could be identified with a signal-to-noise >3 and appropriate ion ratio [24], and within ±0.2 min of the mean calibrators retention time. LOD was determined by the analysis of fortified blank oral fluid samples at decreasing concentrations. LOQ was defined as the lowest concentration that could be quantified with adequate precision (%CV <20%) and accuracy (% of target concentration ±20%). The LOQ was calculated by the analysis of 5 replicates at the lowest concentration of the calibration curve.

The best calibration model adjusted to our data was evaluated by the generation of 4 calibration curves from 0.2 or 0.5 ng/mL to 200 ng/mL, analyzed on 4 different days. Acceptance criteria included a coefficient of determination (r^2) ≥0.99, and residuals ≤15% at each concentration level, except at the LOQ, for which residuals ≤20% are accepted.

Imprecision and accuracy in neat oral fluid were evaluated at low, medium and high QC concentrations (0.6, 20 and 150 ng/mL) by the analysis of 5 replicates at each concentration on 4 different days (n= 20). Imprecision was evaluated by the calculation of the coefficient of variation (%CV) following Krouwer and Rabinowtz´ recommendations [25]. Accuracy was expressed as the percentage of the nominal concentration, and was required to be within 85-115% of the target concentration.

Intra-assay imprecision and accuracy in neat oral fluid mixed with Quantisal™ buffer were also assessed at medium QC concentration (20 ng/mL) by the analysis of 5 replicates analyzed in the same batch. These QC samples were quantified with a calibration curve prepared in neat oral fluid and analyzed in the same batch.

225 Quantisal™ recovery, extraction recovery and matrix effect in neat oral fluid and oral
226 fluid with Quantisal™ buffer were assessed at low and high QC concentrations. To
227 assessed recovery of the analytes from the Quantisal™ device, the collection pad was
228 inserted into the sample until 1 mL ± 10% oral fluid was collected (shown by the
229 colorant indicator which turns blue), and subsequently placed into the Quantisal™
230 buffer (n=5); average peak area in these samples was compared to average peak area
231 when 1 mL oral fluid was directly added into the Quantisal™ buffer (no collection pad
232 used) (n=5). All samples were refrigerated for 24 h before analysis to simulate real
233 conditions. Extraction recovery was calculated by comparing average peak area in oral
234 fluid samples fortified with the analytes before extraction (n=5) with average peak area
235 in blank oral fluid samples fortified after extraction (n=5). Matrix effect of neat oral
236 fluid was evaluated by comparing average peak area in blank oral fluid samples from 10
237 different sources fortified after extraction with average peak area when the analytes
238 were prepared in formic acid 0.1%:ACN (90:10, v/v) (n=10). Matrix effect originated
239 by the mixture of oral fluid and Quantisal buffer™ was assessed by comparing average
240 peak area in 2 mL blank oral fluid and buffer mixture (0.5 mL oral fluid + 1.5 mL
241 buffer) (oral fluid from 10 different sources) fortified after extraction with average peak
242 area when the analytes were prepared in formic acid 0.1%:ACN (90:10, v/v) (n=10).

243 Dilution integrity was assessed by the analysis of oral fluid samples fortified at 1
244 µg/mL, and diluted ten fold (n=3) with blank oral fluid.

245 Stability of the analytes in neat oral fluid was evaluated at low (n=5) and high QC
246 (n=5) concentrations under different conditions: stability in the autosampler for 72 h at
247 6ºC, 4ºC for 24 h, and after subjecting the sample to 3 freeze/thaw cycles. IStd was

248 added on the day of the analysis, and stability samples were compared to fresh QC
249 samples. Stability of the processed sample in the autosampler was assessed by re-
250 injection after 72 h storage. In addition, stability of the analytes in oral fluid mixed with
251 the Quantisal™ buffer after 3 freeze/thaw cycles was assessed at medium QC
252 concentrations (n=5). IStd was added on the day of the analysis, and stability samples
253 were compared to fresh QC samples.

254 2.7. Application to real specimens

255 As a proof of the method, 10 oral fluid specimens received in our laboratory during
256 2013 for confirmation of on-site positive results for opiates, amphetamine,
257 methamphetamine, cocaine and/or cannabis using the Dräger DrugTest 5000 were also
258 analyzed using the described LC-MS/MS method for the new synthetic drugs. Oral fluid
259 specimens were collected using the Quantisal™ device. Undiluted concentrations were
260 reported according to the proportion of oral fluid and buffer in the collection device.

261 2.8. Cross-reactivity study on the Dräger DrugTest 5000

262 Dräger DrugTest 5000 cross-reactivity was assessed for methylone, methedrone,
263 mephedrone, fluoromethamphetamine, fluoromethcathinone and MDPV. Independent
264 oral fluid solutions (1 mL) were prepared for each analyte at 100 μg/mL, and individual
265 measurements on the DrugTest 5000 were performed by addition of 280 μL of fortified
266 oral fluid over the DrugTest 5000 collector (n=2 for each analyte). Further oral fluid
267 dilutions were prepared (10, 1, 0.1, 0.01 μg/mL) and tested in the DrugTest 5000 until a
268 negative result was achieved. Dilutions at 0.075 and 0.05 μg/mL were also prepared for
269 fluoromethamphetamine.

3. Results

3.1. Method development and validation

Oral fluid samples were submitted to solid phase extraction using reversed-phase cartridges before injection into the LC-MS/MS system. Chromatographic elution of all the analytes was achieved in 5 min, and the total chromatographic run was 10 min. %CV for retention time over 50 injections was <2.1% for all the analytes. Quantification was based on the most prominent MRM transition. A second transition was monitored for qualitative purposes to fulfill the European Commission Decision 2002/657/EC identification criteria using mass spectrometric techniques [24]. Table 1 shows quantification and qualification transitions, cone voltages, collision energies, retention time and selected IStd for each analyte.

The method proved to be selective as no quantifiable interferences were detected in blank oral fluid specimens from 10 different individuals, or in blank samples fortified with common drugs of abuse and medicines. LOD and LOQ ranged from 0.025 to 0.1 ng/mL, and from 0.2 to 0.5 ng/mL, respectively (Table 2). Figures 1A and 1B shows MRM chromatograms of the quantifier transitions for all the analytes in a blank specimen, and in an oral fluid sample fortified at the LOQ, respectively.

Linearity of the compound-to-IStd ratio versus the theoretical concentration was verified using least-squared regression with 1/x weighting factor for all of the analytes, except for fluoromethamphetamine, for which data were better fitted assuming a quadratic model. Coefficients of determination were >0.99 for all the analytes, and

residuals within ±20% of the target concentration at the LOQ and ±15% at the remaining concentrations. Table 2 includes calibration parameters for all the analytes.

Results for imprecision and accuracy in neat oral fluid at low, medium and high QC concentrations are shown in Table 3. Intra-assay, inter-assay and total imprecision were <6.2%, <12.0% and <12.7%, respectively. Accuracy was satisfactory in all cases, ranging from 84.8% to 103.6% of the target concentration. Intra-assay imprecision of medium QCs prepared in neat oral fluid mixed with Quantisal™ buffer and quantified with a calibration curve prepared in neat oral fluid was <11% for all the analytes, and accuracy was between 80.2 to 106.5% of target concentration (Table 4).

Recovery from the Quantisal™ collection pad was >79.6%, and extraction recovery was >87.9% for all the analytes (Table 5). Matrix effect in neat oral fluid was observed for all the analytes except for mCPP and MDPV, with signal enhancement ranging from 31.9% to 399.7%. The presence of the Quantisal™ buffer produced some ion suppression compared to the results observed in neat oral fluid, resulting in no matrix effect for methylone, signal enhancement for methedrone, fluoromethamphetamine, fluoromethcathinone and mephedrone (ranging from 24.8% to 106.0%), and signal suppression for mCPP, MDPV and TFMPP (ranging from -25.1% to -69.9%) (Table 6). Similar results were observed for the respective IStds, thus compensating the possible imprecision and inaccuracy due to this effect.

Oral fluid samples fortified at 1 µg/mL and diluted 1:10 with blank oral fluid quantified within 75-99% of the target concentration.

All the analytes were stable in neat oral fluid in the autosampler at 6ºC for 72 h, except for methedrone and mephedrone at low QC, with 30.8% and 20.7% loss when compared to fresh QC, respectively. However, % loss only ranged from -13.8 to 11.8% for all the analytes when stored at 4ºC for 24 h and after 3 freeze/thaw cycles, proving the stability under these conditions. In addition, all the analytes were stable when stored in neat oral fluid mixed with Quantisal™ buffer and subjected to 3 freeze/thaw cycles, with % losses ranging from 3.8 to -14.8% (Table 7).

3.2. Application to real specimens

The present analytical method was applied to the analysis of 10 drivers' oral fluid specimens that tested positive to any of the drugs of abuse included in the DrugTest 5000 panel. Two specimens that were on-site positive to amphetamine and methamphetamine, and confirmed in the lab, also gave a positive result for MDPV (31.8 ng/mL) (figure 2A) and mCPP (89.8 ng/mL) (Figure 2B), respectively. The remaining specimens were negative for the studied analytes.

3.3. Cross-reactivity study

Table 8 summarizes results for the synthetic cathinones cross reactivity study using the Dräger DrugTest 5000. Oral fluid samples fortified at 100 µg/mL with any of the tested analytes gave a positive result for methamphetamine on the on-site device. As expected, a negative result was observed for the remaining compound groups included in the analyzer drug panel (amphetamine, opiates, cocaine, cannabis and benzodiazepines). To assess cross-reactivity cut-offs for methamphetamine, further dilutions at 10 µg/mL were prepared for each analyte with pooled oral fluid, obtaining a negative result for

fluoromethcathinone and MDPV. However, only oral fluid fortified with fluoromethamphetamine at 1 and 0.1 μg/mL were positive for methamphetamine. A negative result was observed at 0.01 μg/mL and, therefore, oral fluid solutions at intermediate concentrations (0.075 and 0.05 μg/mL) were also tested. Fluoromethamphetamine cross-reactivity cut-off for methamphetamine on the DrugTest 5000 was set at 0.075 μg/mL, as a negative result was observed at 0.05 μg/mL (n=2).

4. Discussion

The present manuscript describes the development and validation of a LC-MS/MS method for the determination of the synthetic cathinones methedrone, methylone, mephedrone, MDPV, fluoromethcathinone and fluoromethamphetamine and two synthetic piperazines, mCPP and TFMPP. In addition, cross reactivity caused by the above-mentioned synthetic cathinones using the Dräger DrugTest 5000 was evaluated.

For the mass spectrometric detection, a capillary voltage of 1 kV was selected as it allows the highest sensitivity (signal-to-noise) for most of the analytes. The extraction protocol was the same as the one employed in our laboratory for confirmation of positive oral fluid on-site results using a LC-MS/MS method that allows the determination of the classical drugs of abuse (cocaine, amphetamine and derivatives, opiates and cannabis), and some medicines. In addition, both methods share the same analytical column and mobile phase composition for the chromatographic separation [23]. Therefore, injecting twice the same extract, and selecting the appropriate chromatographic and MS method each time, we could perform the determination of all the analytes included in both analytical methods, saving time and money. Moreover, oral fluid volume available is usually scarce, and in some cases it would not be possible

357 to extract a different aliquot for each method. The method has been completely
358 validated in neat oral fluid, including the assessment of selectivity, linearity,
359 imprecision and accuracy, extraction recovery, matrix effect in neat oral fluid, dilution
360 integrity and stability under different conditions. All the studied parameters fulfilled the
361 accepted criteria. Although matrix effect (signal enhancement or suppression) was
362 observed for several analytes, %CV≤17% within the oral fluid samples from 10
363 different sources employed to assess this parameter proved its reproducibility. In
364 addition, the deuterated analogue was used as IStd when commercially available; and
365 for the remaining analytes, the deuterated compound that allows good calibration and
366 precision and accuracy results was selected. Authentic specimens were collected using
367 the Quantisal™, as it prevents from sample instability and irreversible adsorption of the
368 analytes to the pad. Therefore, recovery from the Quantisal™ device, matrix effect and
369 imprecision and accuracy in oral fluid mixed with Quantisal™ buffer were also
370 assessed during method validation to guarantee the quality of the results obtained in the
371 analyzed real specimens. Recovery from the Quantisal™ >80% proved almost no
372 retention of the analyte in the collection pad. As in neat oral fluid, matrix effect was
373 observed when the oral fluid was mixed with Quantisal™ buffer; however, this effect
374 was also reproducible within the 10 different oral fluid specimens employed to evaluate
375 this parameter (%CV≤16%). In addition, appropriate quantification of the real
376 specimens was guarantee by the assessment of the intra-assay imprecision and accuracy
377 results in oral fluid mixed with Quantisal™ buffer, which were within the accepted
378 ranges (%CV<15% and 85-115% of target concentration) for all the analytes, except for
379 MDPV, for which accuracy was slightly lower (80.2%). As a proof of the method, 10
380 real oral fluid specimens from drivers that tested positive to one or more drug classes

included in the DrugTest 5000 were analyzed with the present analytical method, observing a positive result for MDPV and mCPP in two specimens.

Only two authors reported the identification of synthetic cathinones in oral fluid. Strano-Rossi et al. [21] developed a ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) screening method for identification of several synthetic cannabinoids and new designer drugs, including some of the analytes quantified in the present method (methylone, mephedrone and MDPV). The DCD 5000 collector (Dräger Safety AG & Co. KGaA, Lübeck, Germany) was employed to collect blank and real oral fluid specimens. Oral fluid was recovered by centrifugation and analyzed after dilution, with a total chromatographic run of 14 min. LOD ranged from 1 to 5 ng/mL for most of the analytes, higher than our LODs (0.025 to 0.1 ng/mL); however, the volume of oral fluid was half from that used in the present method (0.25 and 0.5 mL, respectively). The method was adequately validated for identification purposes, and applied to oral fluid specimens collected during random traffic controls and samples from an external quality control. Amaratunga et al. [22] described a very fast UHPLC-MS/MS method (3 min total run time) for the quantification of 10 new designer drugs, including methedrone, mephedrone and MDVP. The method was developed and validated using 400 µL of oral fluid and Quantisal™ buffer mixture (100 µL of neat oral fluid). Oral fluid was extracted with MCX 96-well cartridges, achieving an LOQ of 1 ng/mL for all the compounds. In the LC-MS/MS method described in the present manuscript a higher volume of oral fluid is employed to achieve lower LOQs (0.2 ng/mL). This low LOQ could be necessary for some of the analytes, as few experimental data in oral fluid [21, 22] or plasma [14, 16, 18, 19] concentrations have been reported to date.

Synthetic cathinones are structurally related to amphetamines and derivatives, which could explain a false positive result for amphetamine and/or methamphetamine using the on-site screening device. For this reason, cross reactivity originated by the studied synthetic cathinones using the DrugTest 5000 also was assessed. All the tested analytes gave a positive methamphetamine on-site result when an oral fluid blank sample was fortified with either of the cathinones at 100 µg/mL, and most of them at 10 µg/mL. Therefore, the presence of these analytes in the specimens at high concentrations could justify a false positive for methamphetamine, which would not be confirmed with the traditional confirmation methods not including the analysis of the new synthetic drugs. However, only fluoromethamphetamine produced a false positive methamphetamine result below 1 µg/mL. Therefore, it will be the only analyte to produce cross reactivity on the DrugTest 5000 at low concentrations in oral fluid, with a cut-off of 0.075 µg/mL, just two-fold higher than the cut-off for methamphetamine. Amphetamine was negative in all cases; however, there is a wide range of new synthetic drugs on the market, and false positives due to other emerging drugs could not be discarded.

Nieddu et al. [11] evaluated the cross reactivity produced by 39 new synthetic amphetamine type stimulants different to those evaluated in the present study, using two oral fluid drug screening devices. Drugs of abuse included in the screening device panel were amphetamine, methamphetamine, cocaine, THC, phencyclidine and opiates. Oral fluid samples fortified with either of the analytes at different concentrations from 10 to 5000 ng/mL were evaluated. Only p-methoxyamphetamine (PMA) and p-methoxymethamphetamine (PMMA) cross react with amphetamine and methamphetamine, respectively, at concentrations ranging from 20-200 ng/mL, depending on the analyte and the screening device. The authors concluded that specific

immunoassay kits should be developed for the detection of these emerging drugs, as most of them could not be detected with the current screening devices.

In our opinion, users and toxicologists should be aware of the possibility of an apparently false amphetamine or methamphetamine result due to these new drugs of abuse. As a consequence, the laboratory should be able to detect as many emerging drugs as possible, and confirmation methods should allow the identification of at least emerging drugs of abuse that are currently regulated, in order to throw light on this field and to explain occurred presumptive false positive screening results.

5. Conclusion

The present manuscript describes the successful development and validation of a LC-MS/MS method for the determination of methylone, methedrone, fluoromethamphetamine, fluoromethcathinone, mephedrone, MDPV, mCPP and TFMPP in oral fluid. Two out of the 10 analyzed drivers' oral fluid specimens gave a positive result for mCPP and MDPV. Moreover, cross reactivity produced by the studied synthetic cathinones in the Dräger DrugTest5000 was assessed. All the analytes produced a positive result for methamphetamine at concentrations ranging from 0.075 to 100 µg/mL.

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Tables

Table 1. MRM transitions, cone voltage (CVolt), collision energy (CE), retention time (t_R) and internal standard (IStd) selected for each analyte.

Analyte	MRM transition	CVolt (V)	CE (eV)	t_R (min)	IStd
Methylone	<u>208.4>160.2</u> 208.4>190.3	22	17 13	1.69	Methylone-d3
Methylone-d3	211.3>163.2	22	19	1.67	
Methamphetamine-d5	155.1>120.9	22	11	2.18	
Methedrone	<u>194.4>176.3</u> 194.4>161.2	20	13 19	2.21	Metamphetamine-d5
Fluorometamphetamine	<u>168.3>109.0</u> 168.3>137.2	22	21 11	2.85	Mephedrone-d3
Fluoromethcathinone	<u>182.3>164.2</u> 182.3>149.2	22	13 19	3.01	Methylone-d3
Mephedrone	<u>178.3>160.2</u> 178.3>145.1	20	12 18	3.03	Mephedrone-d3
Mephedrone-d3	181.4>163.3	22	13	3.01	
mCPP	<u>197.3>154.2</u> 197.3>118.9	32	19 25	4.27	mCPP-d8
mCPP-d8	205.3>158.2	36	21	4.25	
MDPV	<u>276.3>126.2</u> 276.3>175.2	28	27 23	4.57	mCPP-d8
TFMPP	<u>231.3>188.2</u> 231.3>118.9	36	23 31	4.92	TFMPP-d4
TFMPP-d4	235.3>190.3	36	21	4.92	

Underlined transitions were used for quantification. FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 1-(3-trifluoromethylphenyl)piperazine

592 Table 2. Calibration parameters, limit of detection (LOD) and quantification (LOQ) for
593 all the analytes.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Linearity (ng/mL)	Intercept ± SD (n=4)	Slope (x) ± SD (n=4)	Slope (x^2) ± SD (n=4)	r^2 ± SD (n=4)
Methylone	0.1	0.2	0.2-200	0.0010 ± 0.0074	0.3757 ± 0.2177	-	0.9996 ± 0.0002
Methedrone	0.05	0.2	0.2-200	0.0363 ± 0.0163	0.3811 ± 0.1820	-	0.9987 ± 0.0003
FMAMP	0.1	0.2	0.2-200	0.0101 ± 0.0095	0.3315 ± 0.0740	0.00005 ± 0.0004	0.9996 ± 0.0002
FMCAT	0.1	0.2	0.2-200	-0.0144 ± 0.0412	0.3471 ± 0.1584	-	0.9990 ± 0.0012
Mephedrone	0.1	0.2	0.2-200	0.0154 ±0.0188	0.3603 ± 0.1363	-	0.9996 ± 0.0002
mCPP	0.05	0.2	0.2-200	0.0464 ± 0.0207	0.4487 ± 0.2200	-	0.9996 ± 0.0001
MDPV	0.025	0.5	0.5-200	0.1916 ± 0.0712	1.1422 ± 0.7611	-	0.9977 ± 0.0016
TFMPP	0.05	0.2	0.2-200	0.0133 ± 0.0161	0.4802 ± 0.22313	-	0.9997 ± 0.0001

FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 1-(trifluoromethylphenyl)piperazine

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599 Table 3. Imprecision and accuracy at low (0.6 ng/mL), medium (20 ng/mL) and high
600 (150 ng/mL) QC concentracions.

Analyte	Intra-assay imprecision (n=20; %CV)			Inter-assay imprecision (n=20; %CV)			Total imprecision (n=20; %CV)			Accuracy (n=20; %CV)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
Methylone	2.7	2.9	2.4	1.3	2.8	4.3	3.0	4.0	4.9	89.8	90.7	91.5
Methedrone	5.7	3.0	3.7	0.0	7.6	3.2	5.7	8.2	4.9	92.8	102.5	95.3
FMAMP	5.2	3.2	2.4	8.1	1.7	2.7	9.6	3.6	3.7	92.5	94.5	96.9
FMCAT	4.1	3.3	3.5	8.7	5.1	1.0	9.6	6.1	3.6	92.8	89.0	89.8
Mephedrone	3.3	2.6	3.1	2.8	3.8	4.4	4.3	4.7	5.4	91.2	93.4	93.7
mCPP	3.6	4.3	2.4	1.9	0.0	2.9	4.1	4.3	3.8	91.8	93.3	93.5
MDPV	5.2	5.1	4.3	5.7	5.7	12.0	7.7	7.7	12.7	84.8	103.6	92.4
TFMPP	5.3	2.4	3.1	4.3	3.1	4.0	6.8	4.0	5.0	97.0	98.2	97.9

FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 1-(3-trifluoromethylphenyl)piperazine

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606 Table 4. Intra-assay imprecision and accuracy results in QC samples (20 ng/mL)
607 prepared in neat oral fluid mixed with Quantisal™ buffer.

Analyte	Imprecision, n=5 (%CV)	Accuracy, n=5 (%target concentration)
Methylone	7.5	96.4
Methedrone	8.6	104.7
FMAMP	8.8	92.4
FMCAT	10.2	106.5
Mephedrone	10.3	100.6
mCPP	8.0	99.1
MDPV	7.2	80.2
TFMPP	8.3	98.3
FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 1-(3-trifluoromethylphenyl)piperazine		

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615 Table 5. Extraction recovery and recovery from the Quantisal™ oral fluid collector at
616 low (0.6 ng/mL) and high QC (150 ng/mL) concentrations.

Analyte	Extraction recovery, n=5 (%CV)		Recovery from the Quantisal™, n=5 (%CV)	
	Low QC	High QC	Low QC	High QC
Methylone	103.5 (8.3)	99.8 (4.4)	103.7 (4.0)	88.1 (2.9)
Methylone-d3	97.2 (7.7)	95.4 (5.2)	107.8 (4.1)	85.5 (2.6)
Metamphetamine-d5	83.2 (6.6)	88.3 (5.3)	104.1 (5.0)	85.7 (1.4)
Methedrone	101.7 (5.6)	97.2 (6.8)	98.6 (3.9)	93.4 (5.7)
FMAMP	98.7 (7.6)	92.9 (5.1)	107.7 (4.7)	88.9 (2.2)
FMCAT	100.5 (8.3)	96.7 (5.6)	102.5 (5.3)	86.7 (2.5)
Mephedrone	87.9 (7.9)	92.1 (6.2)	106.5 (4.9)	87.3 (2.3)
Mephedrone-d3	87.5 (6.3)	90.2 (6.8)	109.4 (4.4)	84.7 (2.6)
mCPP	118.2 (6.4)	109.0 (5.4)	98.0 (5.7)	84.1 (1.6)
mCPP-d8	102.5 (4.6)	105.7 (6.1)	99.6 (3.7)	81.1 (1.9)
MDPV	134.3 (2.4)	118.2 (4.0)	93.1 (8.3)	82.8 (2.4)
TFMPP	115.9 (8.3)	103.4 (3.1)	88.8 (5.8)	79.6 (1.7)
TFMPP-d4	94.3 (5.9)	95.8 (3.4)	91.0 (5.6)	76.5 (2.2)
FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 1-(3-trifluoromethylphenyl)piperazine				

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Table 6. Neat oral fluid matrix effect and oral fluid mixed with Quantisal™ buffer matrix effect at low (0.6 ng/mL) and high QC (150 ng/mL) concentrations.

Analyte	Neat oral fluid matrix effect, n=10 (%CV)		Oral fluid + Quantisal™ buffer matrix effect, n=10 (%CV)	
	Low QC	High QC	Low QC	High QC
Methylone	131.4 (10.5)	102.8 (8.6)	17.4 (8.6)	-0.3 (7.3)
Methylone-d3	129.5 (6.8)	106.3 (7.9)	23.5 (8.6)	6.2 (5.4)
Metamphetamine-d5	196.5 (10.8)	272.4 (10.5)	110.9 (6.8)	84.1 (3.9)
Methedrone	113.3 (8.5)	110.6 (10.0)	29.3 (6.4)	24.8 (15.0)
FMAMP	266.6 (17.1)	315.4 (11.1)	89.9 (7.5)	62.2 (3.7)
FMCAT	306.3 (11.6)	399.7 (9.1)	83.0 (9.2)	81.0 (3.3)
Mephedrone	240.6 (13.0)	270.3 (10.0)	106.0 (8.0)	75.3 (3.4)
Mephedrone-d3	256.4 (8.2)	284.8 (9.2)	131.2 (7.4)	84.0 (4.0)
mCPP	-6.1 (10.8)	-11.6 (5.8)	-25.1 (14.1)	-26.6 (6.7)
mCPP-d8	-6.8 (8.7)	-9.2 (5.2)	-18.4 (6.9)	-21.3 (6.1)
MDPV	-7.7 (15.7)	-1.1 (11.4)	-65.7 (9.2)	-54.6 (10.4)
TFMPP	31.9 (12.8)	13.9 (12.5)	-65.2 (16.2)	-69.9 (10.9)
TFMPP-d4	20.4 (10.7)	6.7 (11.6)	-61.4 (7.3)	-68.7 (11.5)

FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 1-(3-trifluoromethylphenyl)piperazine

Tabla 7. Stability in neat oral fluid at low (0.6 ng/mL) and high (150 ng/mL) concentrations under different conditions: processed sample in the autosampler for 72 h, 4ºC for 24 h, and 3 freeze/thaw cycles; and stability in mixed oral fluid + Quantisal buffer after 3 freeze/thaw cycles at medium concentration (30 ng/mL). Data expressed as % loss compared to fresh controls.

		Neat oral fluid			Oral fluid+Quantisal™ buffer
Analyte	QC	Autosampler 72h	4ºC for 24 h	Three freeze/thaw cycles	Three freeze/thaw cycles
Methylone	Low	14.8	-7.4	-7.4	-
	Medium	-	-	-	3.1
	High	8.7	-5.9	-3.2	-
Methedrone	Low	30.8	-11.5	7.7	-
	Medium	-	-	-	-10.8
	High	11.4	-6.7	-4.5	-
FMAMP	Low	15.4	11.5	-3.8	-
	Medium	-	-	-	-4.6
	High	9.9	0.2	-4.4	-
FMCAT	Low	10.3	10.3	6.9	-
	Medium	-	-	-	-14.8
	High	7.2	-1.9	-5.8	-
Mephedrone	Low	20.7	-13.8	-3.4	-
	Medium	-	-	-	3.8
	High	15.3	-4.0	-1.8	-
mCPP	Low	0.0	-7.1	3.6	-
	Medium	-	-	-	-3.0
	High	-6.1	-4.1	-1.8	-
MDPV	Low	17.6	-5.9	11.8	-
	Medium	-	-	-	0.5
	High	0.1	-4.2	-0.6	-
TFMPP	Low	-11.8	-8.8	5.9	-
	Medium	-	-	-	1.4
	High	-4.3	-1.7	-1.2	-

FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 1-(3-trifluoromethylphenyl)piperazine

Table 8. Selected synthetic cathinone cross reactivity for amphetamine (AMP) and methamphetamine (MAMP) using the Dräger DrugTest 5000.

		Methedrone	Methylone	MDPV	Mephedrone	FMCAT	FMAMP
100 µg/mL	Test 1	+ MAMP	+ MAMP	+ MAMP	+ MAMP	+ MAMP	+ MAMP
	Test 2	+ MAMP	+ MAMP	+ MAMP	+ MAMP	+ MAMP	+ MAMP
10 µg/mL	Test 1	+ MAMP	+ MAMP	NEG	+ MAMP	NEG	+ MAMP
	Test 2	+ MAMP	+ MAMP	NEG	+ MAMP	NEG	+ MAMP
1 µg/mL	Test 1	NEG	NEG	NT	NEG	NT	+ MAMP
	Test 2	NEG	NEG	NT	NEG	NT	+ MAMP
0.1 µg/mL	Test 1	NT	NT	NT	NT	NT	+ MAMP
	Test 2	NT	NT	NT	NT	NT	+ MAMP
0.075 µg/mL	Test 1	NT	NT	NT	NT	NT	+ MAMP
	Test 2	NT	NT	NT	NT	NT	+ MAMP
0.05 µg/mL	Test 1	NT	NT	NT	NT	NT	NEG
	Test 2	NT	NT	NT	NT	NT	NEG
0.01 µg/mL	Test 1	NT	NT	NT	NT	NT	NEG
	Test 2	NT	NT	NT	NT	NT	NEG

FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; MDPV: 3,4-methylenedioxypyrovalerone; NEG: negative; NT: not tested

Figures

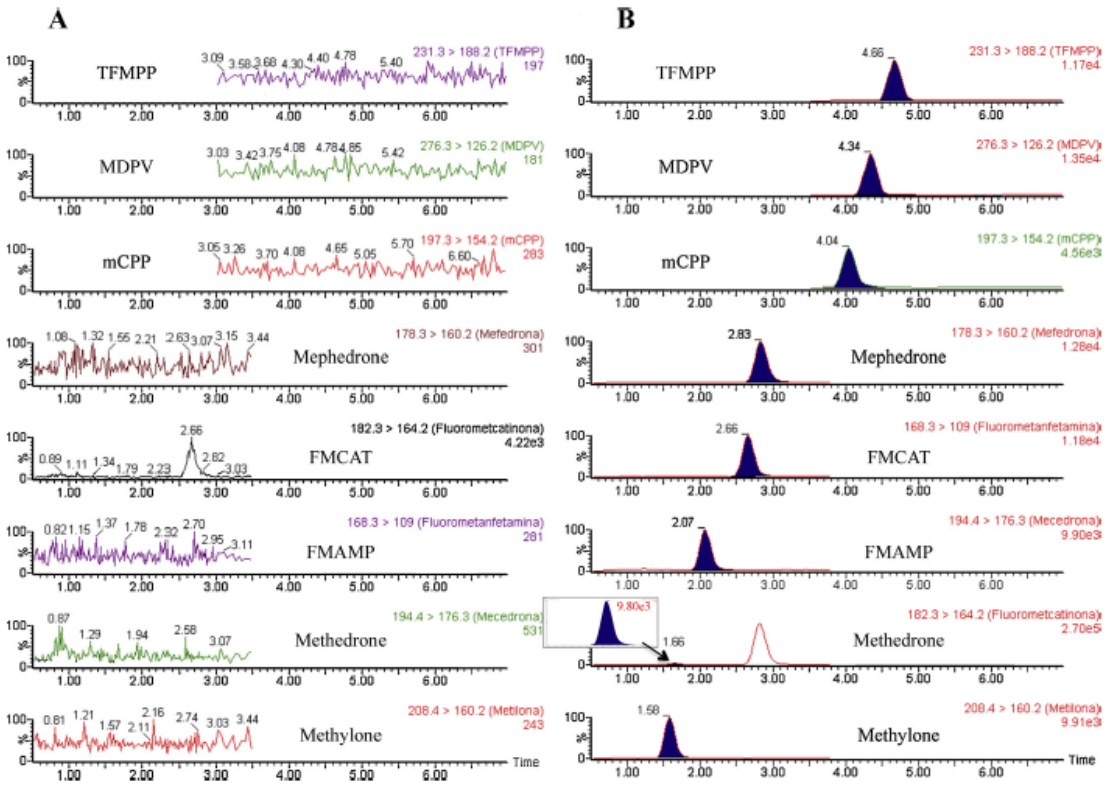

Figure 1. MRM chromatograms of the quantifier transition of the analytes in a blank oral fluid sample (1A) and a blank oral fluid fortified at the LOQ (1B). In Fig. 1B, methedrone peak was amplified to better distinguish it from the background noise. FMAMP: Fluoromethamphetamine; FMCAT: Fluoromethcathinone; mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone; TFMPP: 3-trifluoromethylphenyl)piperazine.

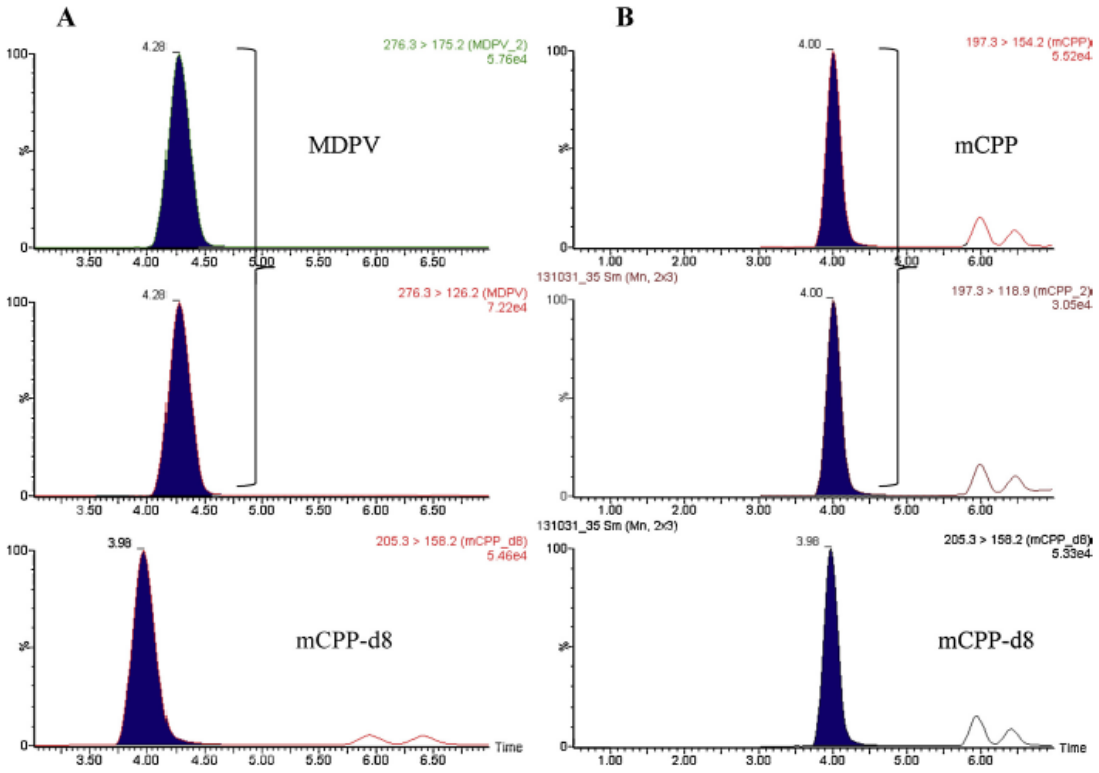

Figure 2. Chromatograms of the two precursor-to-product MRM transitions for MDPV (2A) and mCPP (2B) and their respective internal standards in two oral fluid specimens containing 31.8 ng/mL and 89.8 ng/mL of MDPV and mCPP, respectively. mCPP: 1-(3-chlorophenyl)piperazine; MDPV: 3,4-methylenedioxypyrovalerone.