Anal Bioanal Chem (2016) 408:3737–3749
DOI 10.1007/s00216-016-9458-3

RESEARCH PAPER

Ultra-rapid targeted analysis of 40 drugs of abuse in oral fluid by LC-MS/MS using carbon-13 isotopes of methamphetamine and MDMA to reduce detector saturation

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Received: 26 November 2015 / Revised: 14 February 2016 / Accepted: 1 March 2016 / Published online: 18 March 2016

Abstract The number of oral fluid samples collected by the road policing authority in Victoria, Australia, requiring confirmatory laboratory analysis for drugs proscribed under Victorian legislation (methamphetamine, MDMA and Δ9-tetrahydrocannabinol) has greatly increased in recent years, driving the need for improved analysis techniques to enable expedient results. The aim of this study was to develop an LC-MS/MS-based targeted oral fluid screening technique that covers a broad range of basic and neutral drugs of abuse that can satisfy increased caseload while monitoring other compounds of interest for epidemiological purposes. By combining small sample volume, simple extraction procedure, rapid LC-MS/MS analysis and automated data processing, 40 drugs of abuse including amphetamines, benzodiazepines, cocaine and major metabolites, opioids, cannabinoids and some designer stimulants were separated over 5 min (with an additional 0.5 min re-equilibration time). The analytes were detected using a Sciex® API 4500 Q-Trap LC-MS/MS system with positive ESI in MRM mode monitoring three transitions per analyte. The method was fully validated in accordance with international guidelines and also monitored carbon-13 isotopes of MDMA and MA to reduce detector saturation effects, allowing for confirmation of large concentrations of these compounds without the need for dilution or re-analysis. The described assay has been successfully used for analysis of oral fluid collected as part of law enforcement procedures at the roadside in Victoria, providing forensic results as well as epidemiological prevalence in the population tested. The fast and reliable detection of a broad range of drugs and subsequent automated data processing gives the opportunity for high throughput and fast turnaround times for forensic toxicology.

Keywords Oral fluid · LC-MS/MS · Drugs of abuse · Drugs and driving · Carbon-13

Introduction

Road safety is a strong focus in many jurisdictions, especially in Australia where random roadside drug and alcohol screening of drivers features as a common strategy to improve and deter the driving practices and behavior of individuals in the community [1–3].

Detectable blood levels of methylamphetamine (MA), 3,4-methylenedioxy-methylamphetamine (MDMA) and Δ9-tetrahydrocannabinol (THC) have an established link with dangerous driving practices [4–10]. Oral fluid (OF) offers an alternative matrix to blood due to ease of collection without the need for invasive sampling procedures or medically trained personnel [11, 12]. Furthermore, detectable levels of these drugs in OF indicate recent use and therefore likely impairment of a driver's reaction time and decision-making processes [10, 13–15]. Since 2004, police in the state of Victoria, Australia, have tested OF from drivers stopped

Parts of this paper were presented at the 2015 International Association of Forensic Toxicologists (TIAFT) meeting in Florence, Italy.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-016-9458-3) contains supplementary material, which is available to authorized users.

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randomly at temporary roadside stations for the presence of MA, MDMA and THC. These samples are then delivered to the laboratory for confirmation of presence of MA, MDMA and THC above cutoff concentrations specified in the Australian Standard AS4760-2006 [16]. The additional determination of other potentially impairing drugs allows for epidemiological analysis of drug use by drivers on Victorian roads.

As Victorian road safety schemes intensify, the number of samples screened by immunoassay and found positive on the roadside and subsequently requiring laboratory confirmation has increased significantly, from 200 per year in 2004 to over 8000 per year in 2015. Traditional multi-analyte GC-MS methods employing large sample volumes of up to 1 mL with intensive sample preparation techniques such as derivatisation [17–21] or LC-MS/MS chromatographic techniques using long separation times of up to 20 min [22–28] have proved challenging to maintain short turn-around times as bottlenecks in laboratory analysis inhibit prompt reporting of results. Utilising state-of-the-art ultra-high performance liquid chromatography (UHPLC) with solid-core chromatographic columns coupled with tandem mass spectrometry, we describe a rapid, precise, robust and efficient technique for analyzing a broad range of drugs of abuse in OF.

Experimental

Chemicals and reagents

A total of 40 analytes were targeted in this study. 6-Monoacetylmorphine, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, amphetamine, benzoylecgonine, buprenorphine, cocaethylene, cocaine, codeine, ecgonine methyl ester (EME), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), methylenedioxyamphetmaine (MDA), MDMA, MA, methylenedioxypyrovalerone (MDPV), morphine, norbuprenorphine, nordiazepam, oxycodone, pyrovalerone, THC, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) and zolpidem were purchased from PM Separations (Brisbane, Australia). Diazepam, fentanyl, hydromorphone, ketamine, lorazepam, ephedrine, temazepam and tramadol were obtained from the Forensic and Analytical Science Service (Sydney, Australia). Clonazepam, flunitrazepam and methadone were purchased from Australian Government Analytical Laboratories (Melbourne, Australia). Alprazolam, nitrazepam and oxazepam were received from Division of Analytical Laboratories, (Sydney, Australia). Phentermine was purchased from Sigma-Aldrich (Sydney, Australia), and mephedrone was purchased from National Measurement Institute (Sydney, Australia). Deuterated standards MDMA-d5 and THC-d3 were purchased from PM Separations (Brisbane, Australia). Acetonitrile (ACN), methanol (MeOH), hydrochloric acid (HCl), chloroform, isopropanol and formic acid were purchased from Merck (Darmstadt, Germany). Ammonium formate was purchased from Sigma-Aldrich (Sydney, Australia). Phosphate buffered saline (PBS) (0.1 M) was supplied by PathTech (Melbourne, Australia). All chemicals were of analytical grade or better and water was purified using a Merck Millipore Milli-Q Direct-8 Ultrapure Water System (Melbourne, Australia).

Specimens

Neat OF for validation purposes was obtained from drug-free volunteers. Samples were collected into 20 mL Sarstedt polypropylene tubes (Adelaide, Australia) and stored at −20 °C prior to analysis.

Apparatus

The analytical system consisted of a Sciex 4500 Q-Trap™ quadrupole mass spectrometer (Melbourne, Australia) operated in multiple reaction monitoring (MRM) mode and an electrospray ionization (ESI) TurboIonSpray™ source in positive mode. The liquid chromatograph system consisted of a Shimadzu Nexera X2 30 AD (Melbourne, Australia) which comprised of a degasser, a binary pump, autosampler and column oven.

HPLC conditions

Gradient elution was performed on a Kinetex C18 (4.6 mm × 50 mm, 2.6 μm particle size) column from Phenomenex (Melbourne, Australia). The mobile phases consisted of 50 mM aqueous ammonium formate (3.15 g of ammonium formate dissolved in 1 L of deionized water) adjusted to pH 3.5 with formic acid (eluent A) and ACN with 0.1 % formic acid (eluent B) which were degassed by the integrated Shimadzu 30AD degasser. The autosampler was operated at 4 °C, and the autosampler needle was rinsed with 2 mL methanol before and after each injection. Gradient elution was programmed as follows: equilibration time −0.50–0.00 min 12 % eluent B; 0.00–0.25 min 12 % eluent B; 0.25–4.50 min, linear gradient to 100 % eluent B; 4.50–5.00 min, 100 % eluent B, resulting in a total run time of 5.5 min. The flow rate was 1.5 mL/min, and the column oven was maintained at 30 °C, resulting in a column backpressure of ~250 bar and a lifetime of over 7000 injections.

MS/MS conditions

For detection and quantitation, the following ESI inlet conditions were applied: gas 1, nitrogen (90 psi; 620.5 kPa); gas 2, nitrogen (90 psi; 620.5 kPa); ion-spray voltage, 5500 V in positive mode; ion-source temperature, 750 °C; curtain gas,

nitrogen (55 psi; 379.2 kPa). The dwell times were optimized using the scheduled MRM algorithm incorporated in Sciex Analyst® software version 1.6.2 (Melbourne, Australia). The MRM detection window was set at 6 s, and the target scan time was 0.12 s, resulting in at least 8–12 points across the peak above half height. All other settings were analyte-specific and were determined using Analyst® software in the automatic quantitative optimization mode. Three MRMs per analyte were monitored as per internationally accepted guidelines [29, 30], and the quantitation ion was selected based on evaluation of each transition's validation results (see Electronic Supplementary Material (ESM) Table S1, and Tables 1 and 2). Internal standard MDMA-d5 was paired with each analyte, except for THC and THC-COOH which were paired with THC-d3. All data processing was performed using Sciex MultiQuant® 2.1 (Melbourne, Australia) software.

Preparation of stock solutions, calibration standards and control samples

All stock solutions were prepared individually at 1 mg/mL in methanol and stored at −20 °C. Calibration standards were prepared from the above stock solutions by dilution in methanol. All solutions were prepared at concentrations 10-fold higher than the required calibration standards to allow for dilution into the required range with OF. The quality control (QC) samples were prepared using pooled OF and independently prepared solutions containing each compound at concentrations 10-fold higher than the corresponding QC samples and stored at −60 °C. Guidelines in the Australian Standard AS4760-2006 [16] concerning neat OF confirmatory testing concentrations of drugs proscribed under the Road Safety Act of Victoria (1986) [31] suggest cutoff values of 10 ng/mL for THC and 25 mg/mL for MA and MDMA [16]. These nominated cutoffs apply to neat OF only; however, many OF collection devices employ a buffer system to stabilize the collected OF and reduce drug instability that may occur during transport to the laboratory [32]. As buffer volume between manufacturers' OF collection devices may differ, dilution factors need be applied for accurate calculation of drug concentration in undiluted OF. In Victoria, the Securetec Drugwipe COMBO test kit (PathTech, Melbourne, Australia) is currently used, employing 2 mL of PBS to stabilize 1 mL of collected OF. This procedure results in a dilution factor of 3, and therefore, when applying AS4760-2006 guidelines to dilute OF collected via the device, actual drug cutoff concentrations of 3.33 ng/mL for THC and 8.33 ng/mL for MA and MDMA are required. For laboratory casework, control samples are spiked at these nominated cutoffs to confirm accuracy of reported confirmations. Finally, for validation purposes, in order to account for differences in cutoff concentrations for neat OF and OF diluted with PBS 1:2 collected at the roadside, two QC ranges (three levels each) were selected. The final OF concentrations of the calibration standards and QC samples are given in Table 3.

Extraction Procedure

The extraction procedure is that described by Beyer et al. [22]. In 2-mL polypropylene Sarstedt tubes (Adelaide, Australia), 200 μL of neat OF or OF diluted in PBS 1:2 was mixed with 10 μL of internal standard solution (final concentration of MDMA-d5 and THC-d3 at 20 and 100 ng/mL, respectively) and 20 μL of each calibration standard. After addition of 200 μL of 2 M ammonium carbonate buffer (pH 9.0), samples were vortexed and 1 mL of chloroform/isopropanol (9:1) was added. The samples were shaken for 5 min on a Talboys® Advanced Multi-tube Vortexer from Troemner (New Jersey, USA) at 1500 rpm. After centrifugation at 15,000 rpm for 2 min in an Eppendorf Microfuge (Melbourne, Australia), the upper aqueous layer was aspirated using a glass Pasteur pipette (ThermoFisher, Melbourne, Australia) and discarded. The solvent layer was transferred to an autosampler vial (Phenomenex, Melbourne, Australia) containing 10 μL 0.1 M HCl and dried under a gentle stream of nitrogen gas. The samples were then reconstituted in 50 μL of methanol, and 1 μL of the extract was injected into the LC-MS/MS system. Figure 1 shows an example chromatogram of an extracted OF:PBS LOW quality control sample (3.33 ng/mL).

Validation experiments

Each validation parameter was replicated for both neat OF and OF diluted 1:2 with PBS to account for any differences in matrix or dilution. This resulted in validation of three concentration levels in neat OF and the same levels in PBS-diluted OF at one third the concentration (see Table 3).

Selectivity

Selectivity experiments were carried out using OF collected from 20 drug-free volunteers. Samples were extracted as described previously without the addition of internal standard (IS). The samples were analysed to exclude any interference with endogenous peaks. Additionally, two zero samples (blank sample + IS) were analysed to check for absence of analyte ions in the respective peaks of the IS. Furthermore, two OF samples were fortified with a spike solution containing 234 common basic and neutral drugs and a spike solution containing 132 acidic and neutral drugs in order to exclude the interference on analytes included in the described method.

Matrix effects and extraction efficiency

Matrix effects (ME) and extraction efficiency (EE) were estimated in a post-extraction addition approach using

Table 1 Validation data for analytes in neat OF expressed as percentages. Values in bold-italics did not meet the acceptance criteria

Analyte	Matrix effect						Extraction efficiency						Accuracy (A), precision (P), repeatability (R)								
	LOW		MED		HIGH		LOW		MED		HIGH		LOW			MED			HIGH		
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	A	P	R	A	P	R	A	P	R
6-Monoacetylmorphine	71	[67–76]	71	[67–74]	62	[60–65]	82	[79–86]	86	[79–90]	81	[77–88]	2.8	5.9	4.7	1.5	3.3	3.6	2.0	5.6	4.8
7-Aminoclonazepam	76	[74–77]	76	[73–78]	71	[67–75]	***72***	***[43–84]***	***61***	***[44–89]***	66	[53–78]	0.1	11.6	7.8	−4.3	10.2	10.5	1.7	8.6	3.8
7-Aminoflunitrazepam	87	[83–91]	84	[80–87]	80	[78–82]	***75***	***[47–84]***	62	[47–80]	71	[55–82]	−1.0	10.5	8.6	−3.1	9.7	11.4	2.4	8.6	6.4
7-Aminonitrazepam	83	[78–87]	82	[79–86]	77	[73–80]	***70***	***[35–81]***	56	[39–76]	69	[50–83]	1.5	12.3	11.3	−1.6	9.6	9.6	2.9	5.8	5.1
Alprazolam	82	[80–84]	84	[81–89]	77	[75–78]	***81***	***[60–89]***	89	[85–91]	78	[67–89]	1.7	***22.1***	19.0	1.5	9.4	8.2	5.1	11.1	8.7
Amphetamine	99	[96–102]	97	[93–100]	88	[85–89]	83	[79–85]	83	[79–86]	81	[75–86]	0.9	4.5	3.4	1.3	2.7	1.5	2.5	4.6	4.0
Benzoylecgonine	91	[84–97]	88	[82–93]	82	[76–84]	34	[32–35]	37	[33–39]	35	[33–39]	0.7	5.9	3.2	1.8	3.7	3.2	0.7	5.0	4.7
Buprenorphine	88	[84–91]	87	[84–91]	78	[77–81]	80	[78–82]	83	[78–86]	82	[76–88]	4.6	4.8	5.4	1.3	6.3	6.2	1.5	6.5	4.8
Clonazepam	94	[91–99]	90	[86–94]	80	[76–83]	88	[87–90]	89	[83–93]	83	[75–92]	3.7	9.2	7.8	0.1	5.1	4.3	0.3	10.7	8.9
Cocaethylene	95	[89–99]	92	[89–95]	84	[82–89]	86	[81–94]	88	[84–92]	87	[82–95]	3.1	6.9	3.8	−0.4	7.0	1.7	***24.2***	***24.6***	***25.4***
Cocaine	92	[89–94]	86	[82–90]	87	[74–100]	87	[83–89]	88	[81–95]	***91***	***[74–114]***	1.1	6.5	3.7	10.4	14.4	7.0	5.2	***17.7***	***34.3***
Codeine	88	[82–90]	86	[82–91]	77	[74–79]	87	[83–89]	87	[80–91]	83	[75–89]	0.7	4.9	3.2	1.0	2.6	2.3	1.9	5.6	4.7
Diazepam	88	[86–90]	84	[80–90]	79	[76–81]	86	[84–91]	88	[82–93]	84	[77–89]	1.4	6.0	3.9	1.5	2.0	1.7	1.7	5.2	4.5
EME	85	[80–88]	88	[81–99]	91	[85–95]	82	[80–85]	82	[71–87]	70	[62–84]	5.9	7.1	4.4	−2.4	5.1	1.6	6.9	***18.3***	13.4
EDDP	105	[101–107]	98	[90–107]	93	[88–100]	58	[54–61]	71	[65–78]	65	[51–75]	12.5	8.8	8.7	5.5	9.6	8.1	−0.7	***17.7***	9.5
Ephedrine	95	[90–97]	91	[86–96]	85	[83–88]	73	[70–77]	79	[74–84]	72	[68–79]	1.0	7.1	5.8	1.3	4.1	3.5	−4.1	11.5	11.2
Fentanyl	90	[86–92]	82	[72–93]	78	[73–82]	82	[67–87]	94	[83–107]	81	[70–94]	5.4	12.0	11.2	−3.7	14.1	12.7	−7.4	***26.4***	13.2
Flunitrazepam	88	[85–89]	86	[83–91]	79	[76–81]	85	[83–87]	88	[81–93]	82	[74–90]	1.3	5.7	5.0	0.2	3.8	3.6	0.6	6.6	5.6
Hydromorphone	91	[85–102]	89	[84–93]	76	[74–80]	83	[72–87]	83	[75–91]	80	[69–85]	3.5	6.0	5.0	2.1	4.6	3.4	2.7	6.2	6.2
Ketamine	93	[86–97]	89	[85–91]	82	[80–83]	86	[80–92]	89	[84–93]	88	[81–93]	0.1	4.7	2.7	1.4	2.4	1.4	2.6	5.5	5.0
Lorazepam	112	[100–116]	108	[103–116]	90	[85–98]	81	[75–91]	85	[77–95]	85	[74–94]	1.0	7.0	6.0	−0.7	2.8	3.5	1.1	6.7	3.9
MDA	109	[106–112]	101	[98–105]	87	[83–89]	84	[80–88]	87	[80–94]	81	[73–89]	3.4	6.5	5.2	1.1	2.2	2.3	3.3	6.0	6.0
MDMA	99	[95–102]	98	[95–102]	86	[75–95]	83	[79–88]	87	[85–90]	80	[70–91]	1.6	5.3	4.5	−0.1	4.5	5.1	2.7	6.4	6.3
C^{13}-MDMA	98	[94–101]	97	[92–100]	85	[83–87]	80	[78–83]	84	[78–88]	80	[73–86]	0.2	6.9	6.1	0.1	5.7	6.0	1.6	7.0	3.4
MDPV	89	[86–90]	92	[88–95]	82	[78–89]	89	[83–92]	88	[83–92]	86	[80–94]	1.7	4.6	2.7	0.6	4.0	2.5	1.0	5.9	3.9
Mephedrone	99	[97–103]	97	[93–100]	88	[85–90]	83	[79–87]	85	[79–89]	82	[74–90]	4.6	4.6	3.9	−0.2	2.4	1.9	2.5	10.9	6.9
Methadone	94	[88–98]	91	[88–94]	79	[75–85]	85	[81–87]	89	[85–94]	81	[77–85]	0.5	4.8	3.6	0.8	2.6	2.6	1.3	4.5	4.1
Methylamphetamine	96	[92–100]	95	[92–98]	75	[72–78]	80	[76–85]	84	[79–88]	83	[76–90]	1.8	6.6	5.1	1.4	3.0	3.0	1.8	4.5	4.1
C^{13}-Methylamphetamine	97	[92–101]	93	[89–98]	83	[82–84]	80	[77–83]	83	[78–86]	79	[74–84]	1.1	5.8	5.0	0.8	4.7	4.7	−0.4	7.1	5.7
Morphine	95	[89–99]	93	[90–97]	86	[82–91]	75	[71–79]	78	[74–80]	75	[69–84]	1.8	5.6	3.8	2.3	2.8	2.1	2.5	4.8	4.4
Nitrazepam	70	[63–74]	70	[65–73]	64	[61–66]	85	[78–96]	87	[77–93]	83	[77–91]	−2.1	6.0	6.5	−1.2	3.7	4.8	−1.3	5.4	6.4
Norbuprenorphine	88	[82–96]	81	[78–87]	78	[73–81]	80	[72–85]	88	[78–95]	80	[74–86]	4.7	11.1	10.6	0.1	5.0	5.1	0.3	8.2	5.1
Nordiazepam	87	[85–90]	84	[80–88]	79	[73–82]	87	[83–90]	91	[85–101]	83	[74–90]	1.4	7.7	8.9	0.4	7.0	6.3	−0.8	7.9	5.2
Oxazepam	92	[88–94]	88	[85–92]	79	[77–80]	86	[78–93]	90	[84–93]	85	[76–95]	1.5	8.9	6.8	0.2	5.7	3.7	2.1	5.2	4.5
Oxycodone	85	[80–86]	84	[81–89]	75	[73–77]	88	[85–90]	87	[84–89]	84	[73–91]	0.5	5.1	4.8	1.9	3.7	3.3	3.9	8.4	6.6

Table 1 (continued)

Analyte	Matrix effect						Extraction efficiency						Accuracy (A), precision (P), repeatability (R)											
	LOW		MED		HIGH		LOW		MED		HIGH		LOW			MED			HIGH					
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	A	P	R	A	P	R	A	P	R			
Phentermine	89	[85–93]	89	[84–96]	77	[69–82]	84	[83–90]	85	[80–89]	85	[74–95]	2.2	5.5	4.3	2.1	3.9	4.5	2.8	7.7	6.2			
Pyrovalerone	91	[87–95]	91	[87–96]	84	[82–87]	85	[83–88]	89	[83–94]	85	[82–89]	3.6	6.3	5.6	4.3	5.8	5.2	4.2	5.7	5.2			
Temazepam	96	[94–98]	92	[86–95]	82	[81–84]	84	[73–88]	89	[82–94]	82	[74–89]	1.4	6.4	4.7	1.2	2.9	3.5	1.2	6.3	5.8			
THC	89	[88–92]	86	[81–91]	78	[75–80]	75	[70–82]	75	[67–87]	70	[67–77]	1.2	3.5	3.1	1.6	2.5	2.2	1.3	2.7	1.6			
THC-COOH	97	[90–102]	92	[90–94]	83	[81–84]	76	[74–80]	73	[65–78]	67	[62–76]	−2.5	9.2	6.4	0.9	7.3	6.1	−1.4	7.7	7.3			
Tramadol	95	[91–99]	93	[89–96]	85	[79–92]	87	[85–91]	89	[85–91]	84	[72–94]	1.9	5.1	3.4	0.9	2.6	1.9	2.0	4.6	3.7			
Zolpidem	99	[94–102]	93	[91–95]	80	[71–99]	88	[86–90]	88	[82–93]	***88***	***[67–98]***	4.7	6.5	3.0	−1.2	4.2	1.8	10.1	14.3	***15.6***			
MDMA-d5	92	[81–98]	96	[93–102]	89	[85–92]	87	[80–101]	81	[76–87]	75	[69–79]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a			
THC-d3	91	[81–97]	96	[89–100]	89	[83–94]	76	[64–84]	70	[63–75]	64	[57–72]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a			

three concentrations [33]. Three sets of samples were prepared. Sample set 1 consisted of neat standard containing the analytes at designated 'LOW', 'MED' and 'HIGH' concentrations (see Table 3) in methanol. For preparation of sample set 2, blank OF samples from 10 different sources were first extracted as described previously. The samples were then reconstituted with methanol containing the analytes at a concentration of the same level as set 1. For preparation of sample set 3, blank OF samples from the same sources as those in set 2 were spiked with 20 μL of each standard at the same concentrations as set 1 and were extracted as described previously. Extraction efficiencies were estimated by comparison of peak areas from sample set 3 with those from the corresponding sample set 2 and reported as percentages. Matrix effects were estimated by comparison of peak areas from sample set 2 with those of corresponding sample set 1 and reported as percentages. Values below 75 % indicate ion suppression, whereas values above 125 % indicate ion enhancement. Acceptance criteria was a difference of less than 20 % from the mean response for each group.

Linearity

Aliquots of OF were spiked at concentrations shown in Table 3 and extracted as described previously to obtain calibration standards. Replicates ($n = 6$) at each of the eight concentration levels were analysed. The acceptance criteria was r^2 values of greater than 0.99.

Accuracy and precision

Quality control samples 'LOW', 'MED and 'HIGH' were prepared at concentrations described in Table 3. Two samples of each QC concentration were measured over a period of eight consecutive assays. Daily calibration curves were used to calculate the concentration of the QCs. Accuracy was calculated for each analyte as bias determined by calculating the percentage deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Precision data (given as relative standard deviations [RSDs]) for within-day (repeatability) and time-different intermediate precision (combination of intra- and inter-day effects) of the method were calculated using with the grouping-variable 'day'. The acceptance ranges for within-day and intermediate precision were ≤15 % RSD (≤20 % RSD at 'QC LOW') and ±15 % for bias (±20 % at 'QC LOW') of the nominal values [29, 30]. Accuracy and precision calculations were repeated for the three MRMs for each analyte in order to ascertain the most suitable transition for quantitation.

Table 2 Validation data for analytes in OF in PBS, expressed as percentages. Values in bold-italics did not meet the acceptance criteria

Analyte	Matrix effect						Extraction efficiency						Accuracy (A), precision (P), repeatability (R)								
	LOW		MED		HIGH		LOW		MED		HIGH		LOW			MED			HIGH		
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	A	P	R	A	P	R	A	P	R
6-Monoacetylmorphine	69	[63–74]	71	[68–74]	63	[62–66]	79	[77–82]	84	[77–93]	88	[82–97]	8.4	7.3	3.4	5.3	6.8	2.6	−7.5	3.4	3.5
7-Aminoclonazepam	46	[42–52]	46	[42–51]	44	[42–46]	73	[61–86]	79	[68–88]	76	[60–92]	7.2	9.4	6.6	4.0	5.8	5.2	−6.8	5.5	3.7
7-Aminoflunitrazepam	50	[48–50]	51	[50–52]	49	[47–52]	78	[71–85]	80	[76–89]	***76***	***[54–90]***	4.4	5.6	2.6	1.7	6.1	5.5	−10.3	4.5	3.6
7-Aminonitrazepam	85	[78–89]	82	[81–85]	75	[70–80]	71	[58–86]	73	[59–87]	***73***	***[56–91]***	9.7	4.4	4.5	6.3	8.0	4.5	−8.1	6.5	3.8
Alprazolam	83	[75–85]	83	[81–84]	81	[79–83]	***72***	***[42–90]***	***79***	***[54–89]***	***76***	***[26–100]***	14	9.8	6.1	0.8	***22.2***	***23.5***	−13.7	***19.6***	***18.4***
Amphetamine	99	[97–102]	97	[95–99]	93	[90–96]	82	[80–83]	82	[77–86]	83	[79–89]	0.03	5.5	1.8	−1.7	5.9	5.3	−13.2	5.5	2.2
Benzoylecgonine	92	[84–100]	90	[87–92]	85	[82–88]	34	[32–36]	34	[31–37]	37	[34–42]	11	4.4	3.5	4.8	5.6	5.8	−7.8	7.6	3.9
Buprenorphine	89	[80–94]	88	[87–89]	85	[82–86]	80	[78–82]	81	[79–84]	84	[79–96]	8.7	4	3.1	5.3	7.0	4.0	−9.1	5.6	2.1
Clonazepam	95	[90–99]	96	[94–97]	93	[89–101]	85	[76–92]	87	[84–91]	85	[79–95]	2.7	12.1	8.4	3.3	12.3	7.8	−8.5	6.3	3.2
Cocaethylene	91	[90–92]	90	[87–93]	87	[84–91]	86	[84–89]	87	[79–92]	90	[85–100]	7.7	5.7	2.8	2.6	5.4	5.9	−12.0	5.4	2.9
Cocaine	36	[35–37]	36	[35–37]	38	[37–40]	87	[84–90]	85	[79–90]	86	[81–93]	3.6	6	3.5	0.9	5.7	5.7	−12.1	6.7	2.9
Codeine	83	[77–85]	82	[80–83]	79	[77–81]	85	[81–87]	83	[78–89]	86	[80–94]	7.7	5.9	3.5	2.5	5.8	3.7	−8.2	4.6	3.4
Diazepam	88	[82–92]	89	[86–90]	86	[83–89]	86	[85–87]	87	[85–90]	87	[81–96]	7.1	2.5	1.7	4.1	6.1	4.7	−8.5	5.4	2.1
EME	90	[87–95]	90	[88–92]	87	[84–89]	80	[77–82]	80	[77–85]	84	[79–92]	−5	8.1	4.7	9.5	4.0	4.8	−5.4	5.3	2.5
EDDP	128	[119–134]	112	[111–114]	105	[103–109]	39	[34–42]	58	[49–66]	64	[59–74]	−1.8	13.6	2.9	7.9	10.9	6.0	−3.8	10.4	7.0
Ephedrine	89	[81–94]	92	[90–94]	87	[81–93]	72	[68–77]	70	[64–76]	72	[67–80]	4.9	7.1	4.5	0.7	7.9	5.3	−10.5	6.9	4.4
Fentanyl	75	[66–91]	86	[73–93]	79	[66–89]	***92***	***[70–123]***	89	[77–105]	85	[65–102]	7.8	13.8	7.4	6.0	14.8	11.0	−8.9	12.6	11.7
Flunitrazepam	88	[84–90]	90	[88–92]	85	[83–88]	86	[84–88]	87	[80–93]	86	[82–95]	6.9	4.4	1.8	3.9	7.1	5.0	−9.9	5.7	2.4
Hydromorphone	53	[50–57]	53	[52–53]	51	[48–52]	80	[76–85]	81	[76–85]	84	[80–93]	2.4	5.2	5.3	−0.9	5.7	3.6	−11.8	5.6	5.9
Ketamine	92	[89–96]	91	[90–93]	89	[86–92]	87	[85–88]	85	[80–90]	89	[84–95]	3.1	5.9	3.6	1.0	7.2	6.6	−11.5	6.1	3.9
Lorazepam	127	[119–137]	125	[119–130]	125	[120–128]	91	[84–97]	88	[81–93]	86	[78–91]	3.2	5.0	3.6	3.6	8.1	6.4	−8.3	6.7	3.6
MDA	113	[108–120]	111	[110–113]	108	[105–113]	84	[79–86]	83	[77–87]	85	[77–94]	2.1	6.0	5.4	0.3	6.2	5.9	−11.0	5.1	3.4
MDMA	100	[92–104]	98	[96–101]	99	[94–102]	84	[81–87]	80	[76–91]	82	[75–86]	4.9	3.3	3.1	3.2	7.7	8.4	−9.1	7.7	3.0
C^{13}-MDMA	102	[96–106]	109	[106–116]	99	[96–104]	78	[69–86]	78	[70–86]	82	[77–92]	6.1	6.7	5.8	1.5	9.7	9.8	−11.3	8.1	5.0
MDPV	33	[32–35]	33	[32–35]	33	[32–36]	85	[81–89]	84	[79–90]	87	[81–93]	4.1	6.2	2.8	1.5	4.6	4.1	−10.0	4.9	3.4
Mephedrone	105	[99–110]	104	[103–105]	101	[98–106]	81	[79–84]	79	[77–84]	83	[77–89]	3.0	6.3	3.5	2.3	5.6	4.4	−10.7	6.3	3.4
Methadone	97	[93–101]	98	[94–102]	97	[93–101]	85	[83–88]	83	[79–91]	86	[78–94]	2.6	6.3	3.1	−2.3	5.2	5.4	−13.2	5.3	4.6
Methylamphetamine	102	[99–107]	101	[97–104]	97	[92–104]	79	[73–84]	78	[73–82]	82	[75–92]	3.8	5.4	3.1	0.3	7.0	4.3	−11.0	4.7	3.8
C^{13}-Methylamphetamine	99	[94–103]	99	[93–103]	98	[91–105]	82	[76–85]	79	[74–84]	80	[74–89]	−0.1	6.4	6.4	−0.6	5.5	4.0	−11.8	5.1	3.3
Morphine	80	[75–83]	80	[77–84]	77	[75–80]	73	[70–75]	72	[69–80]	77	[74–84]	7.9	5.2	4.0	5.3	6.5	3.9	−8.2	4.9	5.1
Nitrazepam	53	[49–56]	54	[52–56]	52	[47–55]	86	[76–92]	86	[82–98]	89	[81–101]	6.8	7.1	7.6	1.4	8.4	5.7	−12.4	10.2	3.7
Norbuprenorphine	43	[37–53]	39	[30–46]	42	[35–49]	81	[70–94]	***90***	***[72–116]***	91	[77–109]	1.3	***25.3***	18.8	1.4	***15.2***	***16.4***	−14.3	8.9	6.8
Nordiazepam	87	[78–96]	89	[83–97]	87	[84–89]	85	[77–93]	87	[82–94]	84	[77–89]	9.8	7.0	4.4	6.5	10.5	5.1	−9.6	8.8	5.5
Oxazepam	95	[88–102]	91	[84–96]	86	[70–92]	85	[74–91]	87	[82–91]	***91***	***[67–124]***	7.4	4.4	3.8	7.0	8.1	5.7	−6.9	3.8	3.2
Oxycodone	72	[66–75]	69	[68–71]	70	[67–73]	88	[85–91]	88	[82–94]	88	[85–92]	5.6	3.7	3.6	2.7	5.0	4.6	−10.0	5.3	3.2

Table 2 (continued)

Analyte	Matrix effect						Extraction efficiency						Accuracy (A), precision (P), repeatability (R)											
	LOW		MED		HIGH		LOW		MED		HIGH		LOW			MED			HIGH					
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	A	P	R	A	P	R	A	P	R			
Phentermine	93	[90–99]	91	[86–97]	88	[83–93]	90	[85–95]	84	[74–96]	89	[82–94]	5.2	7.8	7.4	1.0	5.8	5.1	−12.3	5.1	3.2			
Pyrovalerone	93	[88–97]	93	[93–94]	90	[88–94]	83	[81–85]	82	[78–87]	85	[78–93]	4.6	5.6	2.3	3.1	7.4	5.8	−9.5	5.5	2.8			
Temazepam	96	[88–103]	94	[83–99]	87	[67–95]	86	[77–96]	89	[84–101]	***93***	***[63–135]***	8.6	5.0	4.4	3.9	7.5	6.0	−8.5	6.6	4.8			
THC	89	[85–93]	90	[86–95]	89	[86–92]	87	[78–93]	84	[79–88]	***79***	***[52–91]***	6.3	4.1	2.4	3.1	4.4	3.1	−7.4	3.9	3.2			
THC-COOH	96	[87–103]	99	[96–101]	93	[91–95]	89	[80–107]	80	[72–88]	81	[66–90]	9.6	7.6	5.8	5.4	4.4	3.3	−5.5	5.3	5.0			
Tramadol	82	[80–86]	80	[78–81]	79	[75–82]	85	[81–90]	86	[82–90]	86	[82–95]	4.8	4.5	1.7	1.4	5.0	3.9	−10.6	4.8	3.0			
Zolpidem	45	[43–46]	46	[45–47]	46	[45–48]	88	[85–90]	85	[80–90]	87	[81–96]	3.4	6.2	4.3	1.0	5.6	4.3	−11.3	5.9	3.4			
MDMA-d5	94	[89–97]	100	[96–104]	98	[93–102]	79	[77–80]	80	[74–84]	77	[75–80]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a			
THC-d3	89	[86–92]	98	[97–100]	97	[93–100]	81	[76–86]	81	[75–90]	***73***	***[49–83]***	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a			

Processed sample stability

For estimation of analyte stability in the processed samples under the conditions of LC-MS/MS analysis, 'LOW', 'MED' and 'HIGH' samples ($n = 3$ of each) were extracted as previously described. The resulting extracts at each concentration level were pooled. Aliquots of these pooled extracts at each concentration level were transferred to a single autosampler vial and injected into the LC-MS/MS system and analysed using the described method over a period of 24 h. The time intervals between repeat injections of the QC samples was 40 min. Stability of each analyte was expressed as a percentage of the peak area of the analyte at injection time zero ($t = 0$). The acceptance criteria was a drug stability greater than 80 %.

Freeze/thaw and bench top stability

Combined freeze/thaw and bench-top stability were evaluated by analyses of QC samples ($n = 6$ at each concentration) prior to (control samples) and after eight freeze/thaw cycles (stability samples). For each cycle, the samples were kept at −60 °C for at least 22.5 h. The thawed samples were kept at room temperature for 1 h prior to the next freeze cycle to incorporate bench-top stability. The experiments were carried out together with the accuracy and precision experiments, and the concentrations of the control and stability samples were calculated via daily calibration curves. Stability was tested against acceptance criteria of greater than 80 % for the ratio of the means (stability samples vs. control samples).

Long-term stability

Experimental design for the study of long-term stability was similar to the freeze/thaw stability. Analyte stability for long-term storage was evaluated by analysis of QC samples ($n = 6$ at each concentration) before (control samples) and after storage for 12 weeks at −60, −20, and 4 °C and room temperature (stability samples). Stability was measured against acceptance criteria of greater than 80 % for the ratio of the means (stability samples vs. control samples).

Lower limits of quantification and limit of detection

The lower limit of quantification (LLOQ) in MRM mode was defined as the lowest point of the calibration curve and had to fulfill the requirement of a signal-to-noise ratio of greater than 10:1 [29, 30]. The limit of detection (LOD) was not systematically investigated.

Application to authentic samples

Applicability experiments were carried out on authentic OF casework collected via Securetec Drugwipe COMBO test kit

Table 3 Calibration ranges of standards and concentrations of QCs for analysis of neat and diluted OF

Matrix	Calibration standards (ng/mL)								Quality controls (ng/mL)		
	1	2	3	4	5	6	7	8	LOW	MED	HIGH
Neat OF	1	2.5	5	10	15	25	35	50	10	25	45
OF:PBS (1:2)	1	2.5	5	10	15	25	35	50	3.33	8.33	15

at the roadside and sent to the authors' laboratory for toxicological analysis. Four thousand four hundred ninety-seven authentic case samples have been analysed using the described method.

Results and discussion

Carbon-13 isotope application

Basic, hydrophilic compounds are more likely to be found in OF. While acidic, neutral or hydrophobic compounds will also partition into OF, they are usually found at lower concentrations, and target confirmation concentrations will usually be lower. MA and MDMA are typically readily found in OF, while THC is transferred in a much smaller proportion, with its presence usually attributed to recent smoking or eating of consumables containing THC or leeching from surrounding tissue [15, 21, 34]. This, in combination with the described chromatographic system optimised for detection of basic analytes, results in detector signals proportionally much larger for methylamphetamines. Figure 2a shows typical detector saturation in a case of large MA concentrations in OF, with detector 'dropout' due to the excessive signal. Although a detector response of such magnitude is encountered at concentration levels far exceeding recommended confirmation cutoffs, guidelines require MRMs to match those set by a known standard [30]. Due to the degree of detector saturation observed for these compounds in this method, the natural isotope carbon-13 is used to satisfy identification requirements. Carbon-13 is a stable isotope and exists as 1.1 % of all carbon atoms. Its low relative abundance in small organic molecules can be utilised to satisfy detection confirmation criteria via mass spectrometry through selection of parent ion masses one Dalton greater than the most abundant isotope. Figure 2b shows the carbon-13 signal for the same transition

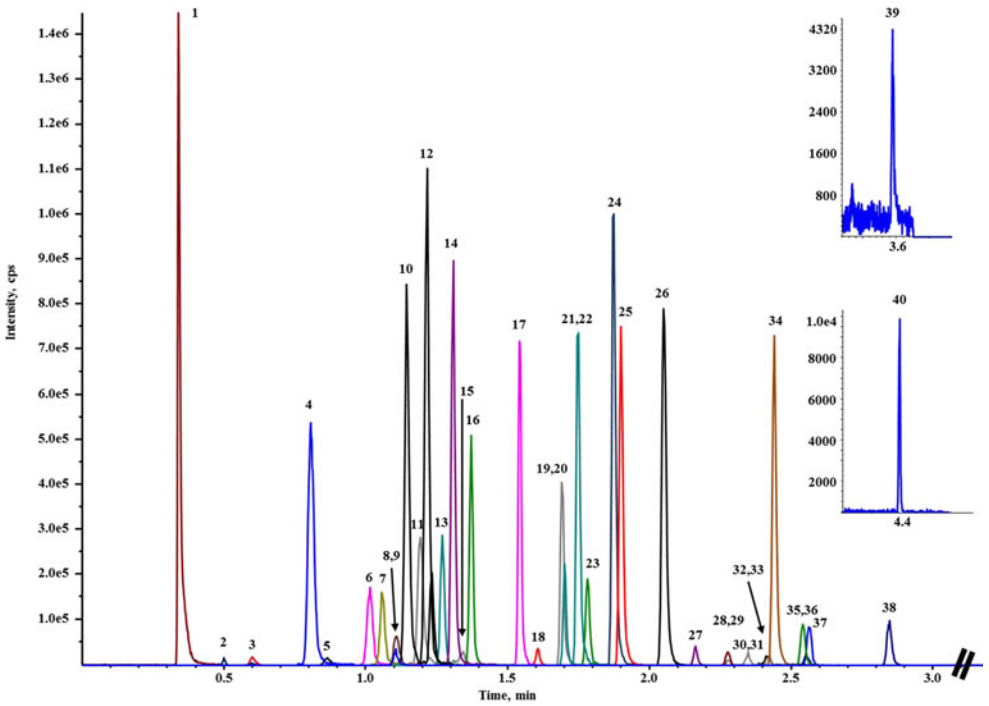

Fig. 1 Example chromatogram of the LOW quality control (OF:PBS, 3.33 ng/mL extracted). *1* EME, *2* morphine, *3* hydromorphone, *4* ephedrine, *5* codeine, *6* amphetamine, *7* oxycodone, *8* 6-monoacetylmorphine, *9* MDA, *10* MA, *11* 7-aminonitrazepam, *12* MDMA, *13* phentermine, *14* benzoylecgonine, *15* mephedrone, *16* ketamine, *17* tramadol, *18* 7 aminoclonazepam, *19* cocaine, *20* MDPV, *21* zolpidem, *22* norbuprenorphine, *23* 7-aminoflunitrazepam, *24* cocaethylene, *25* pyrovalerone, *26* fentanyl, *27* buprenorphine, *28* oxazepam, *29* EDDP, *30* lorazepam, *31* nitrazepam, *32* alprazolam, *33* clonazepam, *34* methadone, *35* temazepam, *36* flunitrazepam, *37* nordiazepam, *38* diazepam, *39* THC COOH, *40* THC

of that in Fig. 2a. Using this technique, reference ratios of carbon-12 transitions set within the calibration range of the method (1–50 ng/mL) can be used to match those calculated using carbon-13 isotope transitions to confirm the presence of MA and MDMA in authentic casework when carbon-12 transition ratios fall outside the acceptable limits due to detector saturation. This technique allowed for reliable confirmation of MRM ratios for MA and MDMA across a greater dynamic range. While this method only utilised carbon-13 isotopes of MA and MDMA (the only reportable compounds from Victorian legislation that exhibited significant saturation effects), the technique may be applied to additional small molecules of interest.

Validation Data

No interferences were observed among the selectivity experiments for both neat OF ($n = 20$) and OF diluted in PBS ($n = 20$).

Matrix effects (ME) were acceptable for all compounds in both neat OF and OF diluted in PBS (see Tables 1 and 2). Neat OF exhibited less ion suppression than OF in PBS, with all analytes displaying greater than 75 % mean response across LOW, MED and HIGH concentrations, with the exception of 6-monoacetylmorphine and nitrazepam. However, these analytes showed acceptable variation in response (within 20 % of the mean).

In OF diluted with PBS, analytes most affected by ion suppression (<50 % mean response) at the investigated concentrations were 7-aminoclonzepam, 7-aminofluintrazepam, cocaine, MDPV, norbuprenorphine and zolpidem; however, their variation was acceptable. Also yielding less than 75 % mean response (ion suppression) in OF diluted in PBS across all concentrations were 6-monoacetylmorphine, hydromorphone, nitrazepam and oxycodone. Although these compounds showed marked ion suppression, their variation in response was acceptable and all were detectable at the LOQ (1 ng/mL). Ion enhancement (>125 % mean response) was observed for EDDP and lorazepam; however, again, these ranges were within 20 % of the mean.

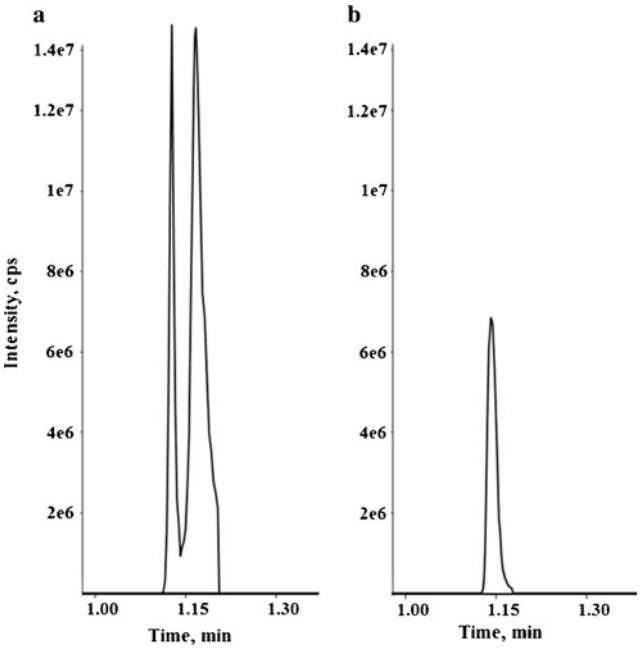

Fig. 2 Case example of detector signal for **a** methylamphetamine (transition 150.039 → 119.1 m/z) and **b** methylamphetamine-carbon-13 isotope (transition 150.993 → 120.1 m/z), demonstrating detector saturation and acceptable peak detection, respectively

Extraction efficiencies (EE) were above 75 % for most analytes in both neat OF and OF in PBS. In neat OF and OF diluted in PBS, the 7-amino benzodiazepine metabolites and alprazolam showed an unacceptable variation in EE. It is recommended that oral fluid concentrations of these analytes derived via this method be treated with caution due to the variation in extraction efficiency. Cocaine and zolpidem also showed large variation in neat oral fluid, however only at HIGH concentration. In OF diluted in PBS, fentanyl, norbuprenorphine, oxazepam and temazepam also showed high variation, but only at a single concentration levels. THC and THC-COOH also had high variation in EE only at HIGH concentration; however, these analytes' paired IS, THC-d3, also showed similar variation in the paired matrix reducing these effects during precision and accuracy experiments. Of the compounds investigated, only alprazolam returned poor results across all concentration levels in OF in PBS.

Assessment of linearity was based on comparisons of linear and quadratic regression analysis each with no weighting, $1/x$ and $1/x^2$. Residual plots were also generated to examine suitability of fit. A quadratic weighting of $1/x$ was selected for neat OF while OF in PBS yielded the best regression values with a quadratic fit and no-weighting. For the regression types and weighting selected, r^2 values were greater than 0.99 for all analytes except for alprazolam (0.90) and THC-COOH (0.97) in OF in PBS and 7-aminonitrazepam (0.98), alprazolam (0.97) and THC-COOH (0.98) in neat OF (data not shown).

Precision, accuracy and repeatability calculations were performed for all 3 studied MRMs of each analyte at three concentration levels (see Tables 1 and 2), with the best performing transition selected for quantitation. In neat OF, alprazolam did not meet the acceptance criteria at LOW concentration, with a precision error value of 22.1 %. At HIGH concentration, cocaethylene had unacceptable accuracy, precision and repeatability (24.20, 24.6 and 25.4 %, respectively). Precision values at HIGH concentration were out of range for EME (18.3 %), EDDP (17.7 %) and fentanyl (26.4 %), while cocaine also showed poor precision (17.7 %) and repeatability (34.3 %). Finally, zolpidem did not have acceptable repeatability at HIGH concentration, with 15.6 % error. For OF in

Table 4 Number of positive detections in 4497 oral fluid cases collected under the Road Safety Act of Victoria (1986)

Drug	Number of detections	Percentage of cases analysed
Methylamphetamine	3304	73.5 %
Amphetamine	3195	71.0 %
THC	2422	53.9 %
Ephedrine	1185	26.4 %
MDMA	462	10.3 %
MDA	371	8.2 %
Codeine	347	7.7 %
Morphine	263	5.8 %
Nordiazepam	260	5.8 %
Cocaine	219	4.9 %
Diazepam	203	4.5 %
6-Monoacetylmorphine	192	4.3 %
Methadone	154	3.4 %
Benzoylecgonine	150	3.3 %
EDDP	125	2.8 %
Tramadol	118	2.6 %
Oxycodone	92	2.0 %
EME	90	2.0 %
Buprenorphine	71	1.6 %
Ketamine	68	1.5 %
Oxazepam	63	1.4 %
Alprazolam	59	1.3 %
Norbuprenorphine	43	1.0 %
THC-COOH	38	0.8 %
Hydromorphone	21	0.5 %
Temazepam	21	0.5 %
Cocaethylene	13	0.3 %
Phentermine	12	0.3 %
Fentanyl	10	0.2 %
Clonazepam	10	0.2 %
7-Aminoclonazepam	9	0.2 %
Nitrazepam	6	0.1 %
Mephedrone	6	0.1 %
7-Aminonitrazepam	4	0.1 %
Lorazepam	2	0.04 %
Zolpidem	1	0.02 %
Pyrovalerone	nil	nil
MDPV	nil	nil
Flunitrazepam	nil	nil
7-Aminoflunitrazepam	nil	nil

PBS, alprazolam did not meet the acceptance criteria for both precision and repeatability (22.2 and 23.5 % at LOW and 19.6 and 18.4 % at HIGH) while nitrazepam at LOW concentration had unacceptable precision (25.3 %) and at MED concentration precision (15.2 %) and repeatability (16.4 %) were outside the acceptable range. Of all compounds quantitated via this method, only alprazolam yielded consistently unacceptable results, likely due to the variation observed in extraction efficiency study.

Despite some analytes not meeting the acceptance criteria in one or more validation experiments (most notably the 7-aminobenzodiazepines and alprazolam), these compounds were included in the final method and are monitored for epidemiology purposes only.

All drugs were stable on the autosampler, with responses greater than 80 % of initial injection after 24 h in both matrices (data not shown). Freeze/thaw stability studies also showed that most of the studied analytes were stable for up to at least eight freeze/thaw cycles, with the exception of the 7-amino benzodiazepine metabolites in PBS-diluted OF, which had degraded to 31–70 % of initial response across all concentration levels. In neat OF, THC was found to be unstable decreasing to 35–49 % of original concentration after eight cycles. This was also shown in previous studies of THC in oral fluid [32].

Analytes spiked in PBS-diluted OF exhibited greater long-term stability than those spiked in neat OF (see ESM Table S2). In neat OF fluid, most analytes were stable at −60 and −20 °C, with the exception of THC which yielded 66 and 50 % of initial response across after 12 weeks storage, respectively. In neat OF, benzodiazepines and the 7-amino metabolites were least stable with large reductions in response at storage temperatures above −20 °C. Most significantly, clonazepam, flunitrazepam and nitrazepam were undetectable after 2 weeks storage at room temperature. 6-Monoacetylmorphine and mephedrone also demonstrated instability when stored at room temperature. Cocaine and its metabolites' EME and cocaethylene were undetectable after 8 weeks storage. Conversely, benzoylecgonine concentrations increased significantly (129–173 %), indicating possible conversion from cocaine through chemical, bacterial or enzymatic action [32, 35].

The addition of PBS buffer to OF appeared to stabilize spiked drug concentrations, with most analytes stable for up to 12 weeks at the temperatures investigated, with the exception of the 7-aminobenzodiazepine metabolites, MDPV, mephedrone, oxazepam, THC and THC-COOH, which showed instability after 12 weeks when stored above 4 °C. Again, large reductions in responses were observed for cocaine at temperatures 4 °C and above, with significant increases for benzoylecgonine. THC showed improved stability with the addition of PBS buffer when stored at less than 4 °C. MA and MDMA showed stability at all temperatures in both matrices. These data highlight the importance of appropriate OF buffering systems and storage temperatures if delays in laboratory analysis are expected.

Applicability

The laboratory has, at the time of writing, used the described method to analytes 4497 cases collected under the Victorian Road Safety Act (1986) [31]. Total drug detections in these cases above the method's LOQ (1 ng/mL) are listed in Table 4. The most commonly detected drugs are those proscribed under the Road Safety Act, along with their major metabolites, MA (73.5 %) and amphetamine (71.0 %), THC (53.9 %) and MDMA (10.3 %) and MDA (8.2 %) The high prevalence of these analytes in OF can be explained by the fact that the purpose of the samples delivered to the laboratory was for confirmation of these analytes, after being found positive by immunoassay on the roadside. Ephedrine (26.4 %) was also commonly detected; however, it is likely an impurity incorporated as a commonly used precursor in the clandestine synthesis of MA. Cocaine and its major metabolites were found in around 5 % of cases. Of concern, the heroin metabolite 6-monoacetylmorphine was detected in 192 cases (4.3 %). Ketamine is an anesthetic used in hospitals during medical treatment; however, it can be abused due to its ability to produce hallucinogenic effects in large doses and was found in 1.5 % of cases. The only analytes not detected in this group were flunitrazepam, MDPV and pyrovalerone. These data are in line with previous studies involving OF collected in Victoria under the Road Safety Act (1986) [36]. While detection of the listed analytes does not necessarily indicate driver impairment at the time of collection, the prevalence of other drugs in these cases is of interest for road policing strategies.

Conclusion

In summary, the method described is a suitably rapid, accurate and precise technique for the simultaneous detection of 40 basic and neutral drugs of abuse in both neat OF and OF diluted in PBS. This technique has proven to be extremely reliable, allowing the laboratory to continue to meet increased sample load and stakeholder demands while maintaining stringent and defensible, forensic drug-confirmation requirements whilst significantly reducing costs through reduced analysis time.

Acknowledgments The authors would like to acknowledge the analytical advice of Dr. Jochen Beyer from the Department of Forensic Medicine, St. Gallen, Switzerland, Dr. Bradley Patterson from Sciex, Australia and Dr. Frank Peters from the Institute of Forensic Medicine, Jena, Germany. We would also like to acknowledge Victoria Police for their support of this research.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was provided by all individuals involved with the study.

Ethics approval Ethics approval for method validation studies is not required from the Research Advisory Committee at the Victorian Institute of Forensic Medicine. Permission for use of oral fluid specimens

collected via random roadside drug screening procedures for epidemiology studies was provided by Victoria Police.

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