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Simultaneous Screening and Quantification of 29 Drugs of Abuse in Oral Fluid by Solid-Phase Extraction and Ultraperformance LC-MS/MS

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BACKGROUND: The European DRUID (Driving under the Influence of Drugs, Alcohol And Medicines) project calls for analysis of oral fluid (OF) samples, collected randomly and anonymously at the roadside from drivers in Denmark throughout 2008–2009. To analyze these samples we developed an ultra performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method for detection of 29 drugs and illicit compounds in OF. The drugs detected were opioids, amphetamines, cocaine, benzodiazepines, and Δ-9-tetrahydrocannabinol.

METHOD: Solid-phase extraction was performed with a Gilson ASPEC XL4 system equipped with Bond Elut Certify sample cartridges. OF samples (200 mg) diluted with 5 mL of ammonium acetate/methanol (vol/vol 90:10) buffer were applied to the columns and eluted with 3 mL of acetonitrile with aqueous ammonium hydroxide. Target drugs were quantified by use of a Waters ACQUITY UPLC system coupled to a Waters Quattro Premier XE triple quadrupole (positive electrospray ionization mode, multiple reaction monitoring mode).

RESULTS: Extraction recoveries were 36%–114% for all analytes, including Δ-9-tetrahydrocannabinol and benzoylecgonine. The lower limit of quantification was 0.5 μg/kg for all analytes. Total imprecision (CV) was 5.9%–19.4%. With the use of deuterated internal standards for most compounds, the performance of the method was not influenced by matrix effects. A preliminary account of OF samples collected at the roadside showed the presence of amphetamine, cocaine, codeine, Δ-9-tetrahydrocannabinol, tramadol, and zopiclone.

CONCLUSIONS: The UPLC-MS/MS method makes it possible to detect all 29 analytes in 1 chromatographic run (15 min), including Δ-9-tetrahydrocannabinol and benzoylecgonine, which previously have been difficult to incorporate into multicomponent methods.

Recently, oral fluid (OF[3]; saliva) has been investigated as a sample for drug-of-abuse testing, especially for testing in the workplace and testing individuals suspected of driving under the influence of drugs *(1)*. Substances can be detected in OF for short periods of time, typically 12–24 h after consumption. OF is therefore suitable for detecting recent drug use, e.g., for roadside testing *(2)*. A major advantage of using OF instead of blood samples is the noninvasive nature of the collection procedure and the ability of nonmedical personnel to collect OF samples. Furthermore, OF can be collected under direct observation, which makes it difficult to substitute or adulterate samples.

OF is produced by a number of specialized glands and consists of about 98% water and trace amounts of proteins (normally present in plasma) in addition to electrolytes *(1)*. The pH of OF is typically 6.7 with a range of 5.6–7.9. OF pH affects the concentration of drugs. Several studies have investigated the detection of drugs in OF, as recently reviewed by Drummer *(3)*. Most of these studies focused on detection of amphetamines, cannabis, cocaine, and opiates.

Because only a limited amount of OF is available for drug analysis, it is crucial to have a multicomponent method with a low detection limit for sample analysis. Gunnar et al. reported a multicomponent method that uses GC-MS with fractionated solid-phase extraction

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[3] Nonstandard abbreviations: OF, oral fluid; SPE, solid phase extraction; LC-MS/MS, liquid chromatography–tandem mass spectrometry; THC, Δ-9-tetrahydrocannabinol; DRUID, Driving under the Influence of Drugs, Alcohol and Medicines project; UPLC-MS/MS, ultraperformance LC-MS/MS; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; MDEA, 3,4-methylenedioxy-N-ethylamphetamine; IS, internal standard; MRM, multiple-reactions monitoring; ME, matrix effect; LloQ, lower limit of quantification; UloQ, upper limit of quantification.

(SPE) and derivatization *(4)*. In recent years, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has often been used in forensic toxicology, allowing for easier sample preparation and a shorter time for sample analysis. Wood et al. and Mortier et al. have described multicomponent methods for detecting several drugs of abuse in OF, but neither Δ-9-tetrahydrocannabinol (THC) nor benzodiazepines were detected with these methods *(5, 6)*. Øiestad et al. reported a multicomponent method that detected both THC and several benzodiazepines by using liquid/liquid extraction *(7)*. However, the method had a very low recovery for benzoylecgonine (0.2%–0.3%). More recently, Concheiro et al. reported an SPE method that also detected both THC and benzodiazepines, but again had a relatively low recovery for benzoylecgonine (7.5%) *(8)*.

The European Commission recently initiated the DRUID (Driving under the Influence of Drugs, Alcohol and Medicines) project to assess the prevalence of psychoactive substance use by drivers in European countries *(9)*. As a partner in the DRUID consortium, we developed a multicomponent method for analysis of OF. Here, we describe the validated ultraperformance LC-MS/MS (UPLC-MS/MS) method that uses SPE extraction for screening and quantification of 29 drugs/illicit compounds included in the DRUID project. Twenty-two of the substances are commonly tested in all of the involved countries, and the rest are chosen only in Denmark.

Materials and Methods

CHEMICALS AND REAGENTS

The following compounds were purchased from Lipomed GmbH: morphine, amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), codeine, 6-acetylmorphine, methadone, cocaine, benzoylecgonine, 3,4-methylenedioxy-N-ethylamphetamine (MDEA), nordiazepam, 7-aminonitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, chlordiazepoxide, lorazepam, zopiclone, buprenorphine, 7-aminoflunitrazepam-d_3, and flunitrazepam-d_3. Bromazepam, flunitrazepam, and clonazepam were obtained from Roche A/S, and oxazepam and diazepam from Durascan Medical Products. We obtained the following substances from Cerilliant: THC, THC-d_3, amphetamine-d_5, methamphetamine-d_5, MDEA-d_5, MDMA-d_5, MDA-d_5, methadone-d_3, cocaine-d_3, benzoylecgonine-d_8, morphine-d_6, 6-acetylmorphine-d_6, codeine-d_6, tramadol-d_3, zolpidem-d_6, diazepam-d_5, demethyldiazepam-d_5, nitrazepam-d_5, oxazepam-d_5, alprazolam-d_5, clonazepam-d_4, 7-aminoclonazepam-d_4, and buprenorphine-d_4. Tramadol and nitrazepam were purchased from Nycomed Danmark A/S. Zolpidem was obtained from Tocris Bioscience, alprazolam from Pfizer ApS, and zopiclone-d_8 from Toronto Research Chemicals. All the reference substances were of ≥98% purity, except for buprenorphine-d_4 (97%).

LC-MS–grade methanol and acetonitrile were obtained from Fisher Scientific. Ammonium acetate and aqueous ammonia (25%) were obtained from Merck. Purified water was obtained with a Milli-Q system (Millipore). The ammonium acetate buffer used for the mobile phase and for reconstitution of sample extracts (2 mmol/L, pH 6.2) and sample pretreatment (0.1 mol/L, pH 4.1) was prepared monthly and stored at 4 °C. Both buffers were adjusted with hydrochloric acid to their final pH and filtered through a 0.22-μm Durapore membrane filter (Millipore) before use. The mobile-phase buffer used for the LC system was changed weekly. A pool of OF was prepared from samples from 10 drug-free employees, who volunteered and gave informed consent. The oral fluid was centrifuged, and the supernatant was stored at −20 °C until use.

SAMPLING OF ORAL FLUID

Unmodified oral fluid (200 mg determined by weighing) and OF collected by the Saliva-Sampler (StatSure Diagnostic Systems) device were collected for this study. In this sampler of saliva, a variable amount of absorbed OF (300–1500 mg) is diluted with a fixed buffer volume (1 mL). On the basis of measurements of 10 devices, we found that the mean buffer content amounted to 1080 mg (SD = 22 mg). By weighing the device with absorbed oral fluid, we determined the amount of oral fluid in each individual case (*x*or) by subtracting the average device weight. By use of the formula: *z*or = [(*x*or + 1080 mg)/*x*or] × 200 mg, we weighed (*z*or) OF-buffer mixture for extraction in an amount corresponding to 200 mg of pure OF. Correction was made for the exact weighed amount of OF + buffer. Thus, a quantitative determination of compound found in 200 mg oral fluid was achieved in each case. The minimum accepted *x*or amount was 600 mg, so that a duplicate determination could be carried out. Some experiments were carried out with synthetic OF (OraFlx negative, Dyna Tech Industries).

PREPARATION OF CALIBRATOR SOLUTIONS

All calibrator compounds and deuterated analogs were dissolved in methanol or acetonitrile as recommended by the manufacturer to concentrations of either 100 or 1000 mg/L and stored in ampoules at −20 °C before mixing. Two major working solutions in methanol containing all of the compounds at concentrations of 10 mg/L (except for THC and zopiclone, which were

diluted individually in methanol and acetonitrile, respectively, for stability reasons) were prepared monthly and stored at −20 °C in 125-μL ampoules. On the day of analysis, diluted calibrator solutions containing all 29 compounds for spiking of calibrators were prepared by further dilution of the 10 mg/L ampoules in methanol. Calibrators were prepared by spiking 200 mg of OF with 40 μL of calibrator solutions, yielding a final calibration range of: 0.5, 1.0, 10.0, and 100 μg/kg. Calibrators used for determination of drug concentrations in OF collected with the Saliva-Sampler device were mixed with 200 μL StatSure buffer solution before extraction.

Two primary internal standard (IS) stock solutions (A and B) 1 mg/L were prepared monthly in methanol. Solution A contained the following: deuterated IS: oxazepam, amphetamine, codeine, cocaine, benzoylecgonine, 6-acetylmorphine, methadone, methamphetamine, diazepam, MDA, zolpidem, MDMA, tramadol, and MDEA. Solution B contained: 7-aminoflunitrazepam, 7-aminonitrazepam, alprazolam, clonazepam, 7-aminoclonazepam, flunitrazepam, buprenorphine, nordiazepam, and nitrazepam. For practical reasons, the deuterated IS of morphine (1 g/L), THC (10 mg/L), and zopiclone (1 mg/L) were stored separately. Dilutions were freshly made in methanol to yield final concentrations of either 10 μg/L or 2.0 μg/L when spiked (20 μL) to 200 mg of OF, except zopiclone, which was diluted with acetonitrile. The IS concentration for each compound was chosen based on cutoff concentrations as decided by the partners in DRUID (Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue11). The IS solution was used for all validation experiments, calibrators, QCs, and samples.

QC samples containing all compounds were prepared in pooled OF and stored at −80 °C. The pooled OF was spiked with methanol or acetonitrile stock solutions of the compounds independently of the preparation of calibrator solutions. OF for calibration and QC was obtained from laboratory personnel on a voluntary basis after they gave informed consent.

CHROMATOGRAPHIC CONDITIONS

We performed the chromatography using an ACQUITY UPLC system (Waters Corporation). The column used was an Acquity UPLC HSS T_3 C_{18}, (100 mm × 2.1 mm, 1.8 μm) maintained at a temperature of 35 °C. A constant flow rate of 0.4 mL/min was used. The mobile phase was composed of solvents A (2 mmol/L ammonium acetate, pH 6.2) and B (100% methanol). The gradient program is shown in Table 1. The injection volume was 10 μL.

Table 1. UPLC gradient program (20 min total run time).

Time, min	%A	%B
0.0	98	2
4.0	75	25
5.8	62	38
7.3	55	45
8.6	45	55
9.6	35	65
11.0	32	68
11.1	15	85
16.1	15	85
16.2	98	2

MASS SPECTROMETRY

Mass spectrometry was performed using a Quattro Premier XE triple quadrupole (Waters). Positive electrospray ionization mode was used for all mass spectrometric analyses. The ionization parameters were a capillary voltage of 1 kV and source and desolvation temperatures of 120 and 400 °C, respectively. Cone and desolvation gas (N_2) flows were set at 1100 and 100 L/h, respectively. Argon was used as the collision gas at a pressure of 4.21×10^{-3} mBar, corresponding to a flow of 0.18 mL/min. The most suitable multiple-reaction monitoring (MRM) transitions, cone voltages, and collision energies were determined for all analytes and deuterated analogs by tuning on the analytes in calibrator solutions in the concentration of 1 mg/L dissolved in ammonium acetate buffer (pH 6.2) and methanol (vol/vol, 20:80). We injected the compounds into the mass spectrometer using the syringe pump coupled to the UPLC system with a tee fitting. The UPLC system delivered a constant flow of 0.4 mL/min.

MassLynx 4.1 (Waters Corporation) software with automated data processing (QuanLynx) was used with the MRM mode. The analytes were identified by the ratio of 2 characteristic MRM transitions and the retention time. The tolerance for the ratios was set to ±20%, except ±30% for ratios below 10%, and ±2% for the retention time. Quantification was performed by integration of the area under the curve from the specific MRM chromatograms of the analytes and their IS. The response (the ratio of the integrated area of the analyte and the corresponding IS) was compared to the calibration curve. The IS chosen for each analyte, retention times, and MRM transitions are shown in Table 2.

SAMPLE PREPARATION

A Gilson SPE robot (ASPEC XL4) (Gilson) equipped with Bond Elut Certify SPE (130 mg, 3 mL; Varian) columns

Table 2. Abbreviations, retention time, MRM transitions, and operating parameters for the analyzed drugs (MRM transitions are listed for each analyte with quantifier transition on top and qualifier transition below).

Compound	Abbreviation	Retention time, min	MRM transitions, *m/z*	Cone voltage, V	Collision energy, eV	IS
Detection window 1						
Morphine	MOF	4.63	286.16 201.09	43	24	Morphine-d_6
			286.16 165		38	
Amphetamine	AMF	5.92	135.9 118.81	16	9	Amphetamine-d_5
			135.9 90.70		16	
MDA	MDA	5.95	180.2 104.8	15	21	MDA-d_5
			180.2 162.93		11	
Benzoylecgonine	BZL	5.92	290.1 167.95	30	20	Benzoylecgonine-d_8
			290.1 104.75		29	
Detection window 2						
MDMA	MDMA	6.65	194.07 162.91	22	13	MDMA-d_5
			194.07 104.76		23	
Methamphetamine	MAMF	6.74	149.97 90.74	20	18	Methamphetamine-d_5
			149.97 118.84		10	
MDEA	MDEA	6.88	208.11 162.93	22	13	6-Acetylmorphine-d_6
			208.11 104.78		24	
6-Acetylmorphine	6-AM	6.53	328.09 164.93	43	38	6-Acetylmorphine-d_6
			328.09 211.03		25	
Codeine	COD	6.50	300.13 164.95	46	40	Codeine-d_6
			300.13 215.1		25	
7-Aminonitrazepam	7-AMN	6.59	252.11 120.85	40	26	7-Aminonitrazepam-d_5
			252.11 93.74		40	
7-Aminoclonazepam	7-AMC	6.66	286.04 120.8	40	30	7-Aminoclonazepam-d_4
			286.04 222.04		25	
Detection window 3						
7-Aminoflunitrazepam	7-AMF	7.36	284.08 134.92	45	27	7-Aminoflunitrazepam-d_3

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Table 2. Abbreviations, retention time, MRM transitions, and operating parameters for the analyzed drugs (MRM transitions are listed for each analyte with quantifier transition on top and qualifier transition below). (*Continued from page 2007*)

Compound	Abbreviation	Retention time, min	MRM transitions, *m/z*	Cone voltage, V	Collision energy, eV	IS
			284.08 227.11		22	
Tramadol	TRM	7.66	264.19 57.78	20	16	Tramadol-d_3
			264.19 246.15		11	
Detection window 4						
Cocaine	COC	8.51	304.11 182	32	20	Cocaine-d_3
			304.11 81.75		34	
Bromazepam	BRZ	9.44	315.90 181.94	37	32	Diazepam-d_5
			315.90 209		26	
Zopiclone	ZOP	9.38	388.90 244.96	18	18	Zopiclone-d_8
			388.90 216.96		36	
Detection window 5						
Clonazepam	CLZ	9.78	316.04 270.02	45	24	Clonazepam-d_4
			316.04 214		35	
Flunitrazepam	FLZ	9.89	314.06 268.13	40	25	Flunitrazepam-d_3
			314.06 239.15		32	
Nitrazepam	NTZ	9.75	282.12 236.1	45	25	Nitrazepam-d_5
			282.12 180.02		35	
Detection window 6						
Alprazolam	APZ	10.33	309.06 205.02	47	41	Alprazolam-d_5
			309.06 281.03		25	
Oxazepam	OXZ	10.28	286.99 240.98	32	22	Oxazepam-d_5
			286.99 268.98		15	
Chlordiazepoxide	CLDZ	10.79	300.03 227.03	25	25	Diazepam-d_5
			300.03 283.06		13	
Lorazepam	LRZ	10.28	321 275.03	30	20	Diazepam-d_5
			321 303		15	

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Table 2. Abbreviations, retention time, MRM transitions, and operating parameters for the analyzed drugs (MRM transitions are listed for each analyte with quantifier transition on top and qualifier transition below). (*Continued from page 2008*)

Compound	Abbreviation	Retention time, min	MRM transitions, *m/z*	Cone voltage, V	Collision energy, eV	IS
Zolpidem	ZOL	10.45	308.13 235.12	45	35	Zolpidem-d_6
			308.13 91.74		49	
Detection window 7						
Nordiazepam	NDZM	10.95	271.05 139.87	45	30	Nordiazepam-d_5
			271.05 164.89		29	
Diazepam	DZM	11.27	285.1 153.9	43	26	Diazepam-d_5
			285.1 193.05		31	
Methadone	MDN	11.39	310.21 265.15	25	15	Methadone-d_3
			310.21 104.81		27	
Detection window 8						
THC	THC	14.44	315.12 193.05	35	20	THC-d_3
			315.12 259.15		23	
Buprenorphine	BUP	14.54	468.1 54.85	55	50	Buprenorphine-d_4
			468.1 100.8		48	

was used for SPE. The columns were conditioned with 2 mL of methanol and 2 mL of purified water. OF samples (200 mg) spiked with 20 μL of IS solution were diluted with 5 mL of ammonium acetate (0.1 mol/L, pH 4.1)/methanol (vol/vol, 90:10) buffer and introduced into the SPE columns at a constant flow rate of 1 mL/min. The columns were washed with 2 mL of purified water followed by 2 mL of purified water/methanol (vol/vol, 95:5). For elution, we prepared a mixture of 98 mL acetonitrile and 2 mL aqueous ammonium hydroxide solution (25 g ammonium hydroxide in 100 g aqueous solution). The elution was carried out in 2 steps by eluting twice with 1.5 mL into 1 collection tube without intermediate drying of the columns. Eluates were evaporated at room temperature under a stream of nitrogen and redissolved in 200 μL of mobile phase [2 mmol/L ammonium acetate buffer, pH 6.2/methanol (vol/vol, 20:80)].

MATERIALS AND METHOD VALIDATION

Calibration. To determine whether a linear or a quadratic calibration curve should be used, we performed an experiment with 9 calibration points (in 3 replicates). The concentration points used were 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0, and 100 μg/kg. The samples were prepared in 200 mg of OF spiked with a maximum of 60 μL of stock solution diluted in Milli-Q water to appropriate concentrations (THC was diluted in methanol) and 20 μL of IS solution.

Imprecision and recovery. To evaluate imprecision and recovery, we analyzed 4 replicates at 4 concentrations on 2 different days. The 4 concentrations analyzed were: 0.5, 1.0, 10.0, and 100 μg/kg. A calibrator series was freshly prepared for every run, based on 200 mg of pooled OF spiked with all analytes, yielding the concentration points 0.5, 1.0, 10.0, and 100 μg/kg. Before analysis, 4 different stock samples (3 mL each) representing the 4 concentrations were prepared by spiking pooled OF with all of the analytes. On day 1 of analysis, 4 samples (200 mg of OF) were taken from each of the 4 stock samples. All 16 samples (4 replicates for each concentration) and the calibrators were spiked with 20 μL of IS, as described above for the preparation of cal-

ibrator solutions, and extracted by SPE. The procedure was repeated on day 2 of analysis. The percentage recovery was estimated as: (measured concentration/added concentration) × 100. The imprecision was determined as the pooled intraday imprecision. The long-term, total imprecision was determined from the control measurements (1 low and 1 high control included in each run) over a period of 2 months.

Matrix effect (ME), extraction recovery, carryover, and ion suppression. We evaluated the ME of OF on the peak area responses *(10, 11)*. Two sets of 6 OF samples, obtained from 5 different drug-free persons, and 1 sample from the pooled OF were extracted according to the SPE procedure. Set 1 was spiked with all analytes after extraction (B), and set 2 was spiked before extraction (C). All oral fluid samples had a final concentration of 10.0 μg/kg. Three replicates of 10.0 μg/L reference solutions in mobile phase (A) were analyzed directly with the UPLC-MS/MS system. We calculated the ME for each analyte by comparison of the absolute peak areas. The ME results obtained in this study were calculated as follows:

$$ME = [1 - (B/A)] \times 100\%,$$

where A equals the peak area of standards in mobile phase and B is the peak area obtained for blank OF samples spiked with analytes after extraction. An ME value >0 indicates ionization suppression and a value <0 indicates ionization enhancement. Extraction recoveries were calculated as the mean absolute peak areas of all 6 samples spiked before SPE (C) and compared with absolute peak areas from samples spiked after SPE (B). Carryover was evaluated by running a blank sample after the highest calibrator.

We tested the impact of ion suppression and enhancement from ionization of components for all analytes and IS. The analytes and IS were injected continuously into the mass spectrometer in mixtures of a maximum of 5 compounds, selected so that all of the compounds in the mixture had similar responses, and so that none of the compounds had a $\Delta[M+H]^+$ smaller than 3 *m/z*. Furthermore, all of the compounds had baseline resolution chromatography. To produce a constant increased response in both MRM channels for each analyte, the compounds were injected postcolumn [10.0 μg/L in a mixture of 2 mmol/L ammonium acetate (pH 6.2) and methanol (vol/vol 80:20) at a constant flow rate of 2 μL/min] by use of the syringe pump and tee-fitting connected to the UPLC system (delivering a constant flow of 0.4 mL/min). The slightly increased baseline responses were monitored following injection (10 μL) from the autosampler with extracted blank OF samples from 5 different drug-free volunteers. Furthermore, all analytes in mobile phase (100 μg/L) were injected individually from the autosampler simultaneously with the flow of analytes from the syringe pump. We performed individual injection of the analytes to evaluate possible enhancement or depression from coeluting analytes. We then compared the acquired postinjection baseline responses to the baseline response after injection of a blank mobile phase.

Results

CALIBRATION CURVE

We investigated the analyte/IS peak area response ratio in pure OF (online Supplemental Table 1) and OF mixed 1:1 with StatSure buffer (Table 3). The calibration curve was fitted to either a linear or quadratic regression curve. Three measurements at 9 concentrations were performed. The criteria were set to a correlation coefficient $R > 0.990$ ($R^2 > 0.980$) and deviations from the fitted curve <15% [at the lower limit of quantification (LloQ) of <20%]. For calibrators prepared in pure OF, a weighted $(1/x)$ linear regression fit was achieved with $R^2 > 0.980$ for all analytes except THC, buprenorphine, 7-aminoclonazepam, clonazepam, lorazepam, 6-acetylmorphine, and codeine, for which $R^2 > 0.980$ was obtained by fitting to a weighted $(1/x)$ second-order regression (Masslynx 4.1 software) (online Supplemental Fig. 1). The calibration range obtained for all analytes in pure OF was 0.5–100 μg/kg, except for cocaine, bromazepam, and nitrazepam, which had a range of 1.0–100 μg/kg (online Supplemental Table 1). For calibrators prepared in OF mixed with StatSure buffer, linearity was achieved for all compounds in the range 0.5–100 μg/kg (Table 3). The highest calibrator (100 μg/kg) defined the upper limit of quantification (UloQ) for all of the analytes. All of the samples with concentrations higher than the UloQ were diluted with purified water (1 + 9). Online Supplemental Fig. 2 shows a chromatogram of a pure OF sample supplemented with 7.0 μg/kg of the compounds. Examples of chromatograms of positive OF samples obtained by the Saliva-Sampler in the DRUID project are displayed in Fig. 1.

LIMIT OF QUANTIFICATION, IMPRECISION, AND RECOVERY

The LloQ was determined as the lowest concentration yielding imprecision (CV) of ≤20% and bias of ±20% with fulfillment of retention time and ion ratio tolerances *(12, 13)*. For pure OF, the LloQ was determined to be 0.5 μg/kg for all analytes, except for cocaine, bromazepam, and nitrazepam (1.0 μg/kg) (online Supplemental Table 1). For OF mixed with StatSure buffer, the LloQ was 0.5 μg/kg for all analytes (Table 3). Some analytes could have yielded lower LloQs, but based on the DRUID cutoff limits, the set LloQs were adequate. The CV and recovery were determined at 4

Table 3. Validation results for OF mixed with StatSure buffer.

Analyte	DRUID cutoff, μg/L	Calibration range, μg/kg	Correlation coefficient, R^2	LloQ, μg/kg	Theoretical concentration, μg/kg	Measured concentration, μg/kg (n = 8)	Recovery, %	Imprecision CV, %	Extraction recovery (SD), % (n = 6)	ME, %
THC	1	0.5–100	0.9960	0.5	0.5	0.6	112	12.2	33 (19)	2.1
					1	1.0	98.2	4.5		
					10	9.5	95.0	6.2		
					100	95.0	94.7	4.5		
Buprenorphine	1	0.5–100	0.9940	0.5	0.5	0.5	92.3	7.2	91 (13)	−9.2
					1	0.9	91.7	2.5		
					10	9.7	96.7	3.7		
					100	104.8	105	3.7		
7-Aminonitrazepam	1	0.5–100	0.9953	0.5	0.5	0.5	96.5	6.8	84 (7.1)	19
					1	1.0	100	5.2		
					10	10.3	103	5.9		
					100	100	101	3.0		
7-Aminoclonazepam	1	0.5–100	0.9944	0.5	0.5	0.5	105	10.9	88 (10)	36
					1	1.0	95.0	11.9		
					10	9.1	91.1	5.9		
					100	103	103	2.4		
7-Aminoflunitrazepam	1	0.5–100	0.9916	0.5	0.5	0.5	105	5.0	87 (13)	36.1
					1	1.0	95.1	8.9		
					10	9.6	95.9	6.7		
					100	99	99.3	4.1		
Bromazepam	5	0.5–100	0.9920	0.5	0.5	0.5	103	10.5	92 (23)	6.1
					1	0.9	94.9	3.4		
					10	9.3	93.2	2.7		
					100	96	96.1	6.6		
Zopiclone	10	0.5–100	0.9985	0.5	0.5	0.5	92.8	15.0	106 (7.5)	8.0
					1	1.1	110	7.2		
					10	10.1	101	7.0		
					100	107	107	2.3		

Continued on page 2012

Table 3. Validation results for OF mixed with StatSure buffer. (*Continued from page 2011*)

Analyte	DRUID cutoff, μg/L	Calibration range, μg/kg	Correlation coefficient, R^2	LloQ, μg/kg	Theoretical concentration, μg/kg	Measured concentration, μg/kg (n = 8)	Recovery, %	Imprecision CV, %	Extraction recovery (SD), % (n = 6)	ME, %
Nitrazepam	1	0.5–100	0.9986	0.5	0.5	0.5	107	5.4	88 (21)	−0.3
					1	0.9	94.0	12.5		
					10	98	97.6	6.5		
					100	103	103	6.1		
Oxazepam	5	0.5–100	0.9912	0.5	0.5	0.5	94.8	9.9	87 (29)	−3.5
					1	1.0	101	3.1		
					10	10.6	106	1.8		
					100	102	102	2.7		
Clonazepam	1	0.5–100	0.9894	0.5	0.5	0.5	98.5	9.5	99 (25)	16.8
					1	1.1	106	8.7		
					10	11.0	107	6.0		
					100	102	102	3.4		
Lorazepam	1	0.5–100	0.9874	0.5	0.5	0.5	97.7	10.4	85 (20)	−2.1
					1	1.0	103	5.8		
					10	11.2	112	5.1		
					100	91.4	91.4	1.4		
Chlordiazepoxide	5	0.5–100	0.9953	0.5	0.5	0.5	99.8	8.1	91 (4.5)	−22.7
					1	0.9	93.8	6.8		
					10	8.8	87.7	7.7		
					100	102	102	3.1		
Alprazolam	1	0.5–100	0.9991	0.5	0.5	0.5	99.0	8.5	91 (9.7)	9.5
					1	1.0	99.3	3.4		
					10	10.0	100	3.9		
					100	98	98.1	3.9		
Flunitrazepam	1	0.5–100	0.9947	0.5	0.5	0.5	90.5	11.7	100 (26)	19.6
					1	1.1	110	6.4		
					10	11.8	118	6.1		
					100	96	97.6	5.4		

Continued on page 2013

Table 3. Validation results for OF mixed with StatSure buffer. (*Continued from page 2012*)

Analyte	DRUID cutoff, μg/L	Calibration range, μg/kg	Correlation coefficient, R^2	LloQ, μg/kg	Theoretical concentration, μg/kg	Measured concentration, μg/kg (n = 8)	Recovery, %	Imprecision CV, %	Extraction recovery (SD), % (n = 6)	ME, %
Nordiazepam	1	0.5–100	0.9969	0.5	0.5	0.5	103	5.8	96 (22)	15.3
					1	1.1	107	4.0		
					10	11.0	110	4.1		
					100	108	108	5.5		
Diazepam	5	0.5–100	0.9963	0.5	0.5	0.5	102	5.6	95 (9.8)	13.2
					1	1.0	98.9	4.1		
					10	10.0	100	4.1		
					100	103	103	2.0		
Zolpidem	10	0.5–100	0.9936	0.5	0.5	0.5	104	4.4	91 (4.6)	5.4
					1	1.0	98.5	5.9		
					10	9.8	98.4	3.2		
					100	109	109	1.5		
Methadone	20	0.5–100	0.9983	0.5	0.5	0.5	91.3	6.0	88 (11)	−24.6
					1	1.0	95.9	1.5		
					10	10.1	101	3.9		
					100	99.7	99.7	1.3		
Tramadol	20	0.5–100	0.9981	0.5	0.5	0.5	105	8.2	93 (6.5)	−3.4
					1	1.1	105	3.4		
					10	10.2	102	3.4		
					100	108	108	4.1		
6-Acetylmorphine	5	0.5–100	0.9956	0.5	0.5	0.5	108	11.1	87 (16)	−1.7
					1	1.1	108	7.0		
					10	10.5	105	4.5		
					100	101	101	3.6		
Benzoylecgonine	10	0.5–100	0.9939	0.5	0.5	0.5	101	11.9	47 (9.1)	12.6
					1	1.0	103	5.1		
					10	9.6	96.3	5.4		
					100	97	97.0	2.2		

Continued on page 2014

Table 3. Validation results for OF mixed with StatSure buffer. (*Continued from page 2013*)

Analyte	DRUID cutoff, μg/L	Calibration range, μg/kg	Correlation coefficient, R^2	LloQ, μg/kg	Theoretical concentration, μg/kg	Measured concentration, μg/kg (n = 8)	Recovery, %	Imprecision CV, %	Extraction recovery (SD), % (n = 6)	ME, %
Codeine	20	0.5–100	0.9958	0.5	0.5	0.5	102	10.3	113 (13)	4.6
					1	1.0	95.8	6.4		
					10	10.0	100	5.9		
					100	100	100	6.4		
Cocaine	10	0.5–100	0.9917	0.5	0.5	0.5	102	8.0	103 (20)	14.9
					1	1.1	106	6.6		
					10	9.8	98.1	2.9		
					100	104	104	1.6		
Morphine	20	0.5–100	0.9886	0.5	0.5	0.6	119	16.7	48 (21)	19.8
					1	1.0	103	10.6		
					10	10.5	105	4.0		
					100	104	104	3.8		
Amphetamine	25	0.5–100	0.9921	0.5	0.5	0.5	100	16.4	69 (10)	−39
					1	1.0	98.8	5.1		
					10	10.0	100	3.4		
					100	103	103	2.6		
Methamphetamine	25	0.5–100	0.9992	0.5	0.5	0.5	108	13.2	76 (18)	8.7
					1	0.9	94.0	4.5		
					10	10.1	101	5.8		
					100	98	97.8	4.1		
MDA	25	0.5–100	0.9984	0.5	0.5	0.5	99.3	7.1	78 (12)	−14.7
					1	1.0	99.0	4.2		
					10	10.2	102	3.6		
					100	105	105	2.9		
MDMA	25	0.5–100	0.9944	0.5	0.5	0.5	96.8	9.6	72 (9.5)	−9.8
					1	1.0	100	5.1		
					10	10.4	104	4.1		
					100	104	104	3.1		
MDEA	25	0.5–100	0.9960	0.5	0.5	0.5	99.3	6.0	92 (9.9)	−1.5
					1	1.0	98.3	3.1		
					10	10.0	99.7	1.7		
					100	101	101	4.5		

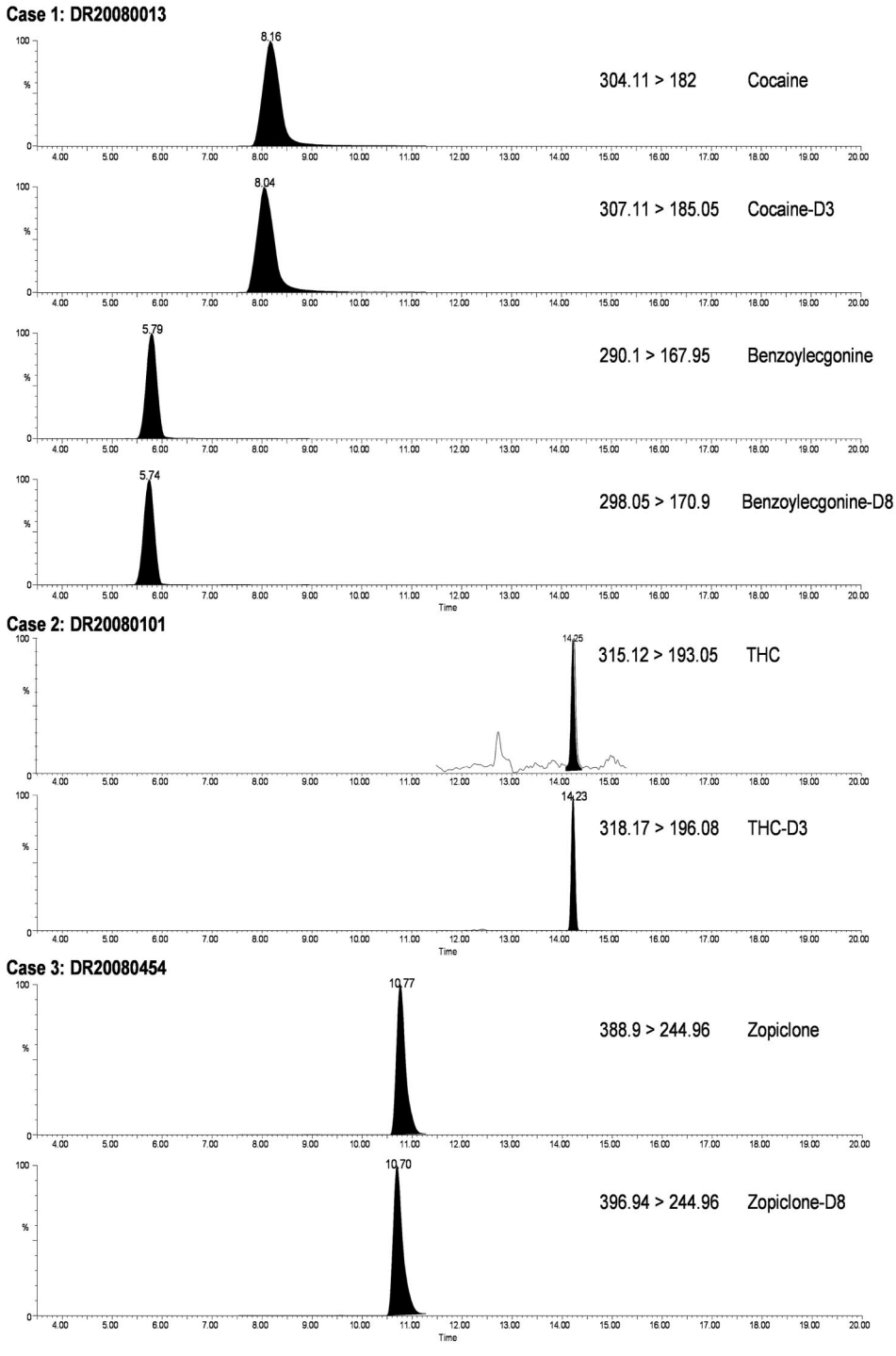

Fig. 1. Three positive OF cases: 1: cocaine (51 μg/kg) and benzoylecgonine (9.0 μg/kg); 2: THC (1.6 μg/kg); 3: zopiclone (130 μg/kg).

The *x* axis shows the time in minutes and the *y*-axis the relative response (0%–100%).

concentrations, including the LloQ and UloQ and 2 intermediate concentrations. The CV and bias were generally accepted at a maximum of 15% (LloQ 20%) *(13)*. For pure OF, all analytes fulfilled the precision criteria at all concentrations, except codeine, which had a CV estimate slightly exceeding the limit (16.8%) at 1.0 μg/kg (online Supplemental Table 1). The recoveries were satisfactory and not significantly different from the limits for all tested concentrations, except that clonazepam and lorazepam had biases slightly above 15% at concentrations above the LloQ (17.9% and −16.7%, respectively), and amphetamine, methamphetamine, and MDMA had values >20% at LloQ (21.5%–25.5%). For OF mixed with StatSure buffer, all compounds fulfilled the precision and recovery criteria, except flunitrazepam, which had a bias of 17.5% at the 10.0 μg/kg level.

MATRIX EFFECTS, ION SUPPRESSION, EXTRACTION RECOVERIES, AND CARRYOVER

The matrix effects from OF and OF mixed with StatSure buffer provided as percentages for all analytes, are listed in online Supplemental Table 1 and Table 3. The analytes had MEs within ±35% for pure OF and within ±39% for OF mixed with buffer, i.e., minor to moderate MEs. Online Supplemental Table 2 shows the MEs for the IS, which were within the same range as for the compounds.

The extraction recoveries (RE) were determined in 6 different sources of OF, and were calculated as mean peak areas. The RE was calculated from 6 different sources spiked before extraction (C) and compared with the 6 different sources of blank OF spiked with all of the analytes after extraction (B); i.e., RE (%) = (C/B) × 100%. Extraction recoveries were all above 50%, except those for THC, which were 33%–36%. (Table 3 and online Supplemental Table 1). The extraction recoveries for the IS were of the same order of magnitude as the corresponding compounds, and only THC-d_3 had a recovery below 50% (32%) (online Supplemental Table 2). Carryover was <1% for all compounds, except for bromazepam, which was <2% (n = 5 determinations).

Ion suppression from OF was also tested by infusion experiments for all analytes using OF from 5 different drug-free volunteers. The experiments showed that there were no major ion suppression or enhancement in OF from any of the 5 volunteers. Ion suppression in synthetic OF and the buffer solution of the Saliva-Sampler was also tested (data not shown). No critical ion suppression was observed in the buffer solution, but the synthetic OF caused ion suppression of up to almost 100% for 9 of the analytes (chlordiazepoxide, diazepam, nordiazepam, flunitrazepam, oxazepam, alprazolam, zolpidem, and MDON).

Table 4. Cases of 15 roadside tests of drivers with compound concentrations exceeding the DRUID cutoff limits.

Case no.	Compound	Concentration, μg/kg
DR 13	Cocaine	51
	Benzoylecgonine	9.0
DR 39	THC	8.0
DR 78	Zopiclone	13
DR 101	THC	1.6
DR 259	Codeine	140
DR 268	Codeine	32
DR 278	Tramadol	430
DR 316	Codeine	21
DR 318	THC	15
DR 320	Codeine	160
DR 325	Tramadol	6400
DR 371	Tramadol	24
DR 454	Zopiclone	130
DR 517	Amphetamine	26
	Cocaine	390
	Benzoylecgonine	85
DR 610	Codeine	48

CVs of the retention times and ion ratios are shown in online Supplemental Table 3. It was observed that the CVs for the retention times were <1% for all compounds. For the ion ratios, the CVs were <10%, with the exception of tramadol (10.8%), which had a very low ratio (1.35%).

Total imprecision values for the compounds for a period of 2 months are shown in online Supplemental Table 4. At the low control concentration, the CVs were 5.9%–19.4%. At the high concentration, the CVs were 6.8%–15.8%.

PRELIMINARY RESULTS OF ROADSIDE TESTING

In the Danish part of the DRUID study, the Department of Transport, Technical University of Denmark organizes sampling of OF at the roadside in cooperation with the police. Locations are chosen in a random way so that representative roads (small, medium, and larger roads) are selected. Participation is voluntary. Up to now some hundred oral fluid samples have been subjected to analysis. Eleven were unfit for analysis and were discarded. For 15 cases samples had 1 or more compounds present in concentrations exceeding the DRUID cutoff limit (Table 4). Four samples contained codeine, 4 THC, 3 tramadol, and the rest amphetamine, cocaine, and zopiclone. A precise evaluation of

the frequency of positive results awaits testing of a larger number of samples.

Discussion

The method described in this report meets the requirements for the cutoffs decided on for the DRUID project (online Supplemental Table 1 and Table 3), and for some compounds the LloQ is lower than required. The automated SPE method is simple and has few steps, allowing for effective processing of large numbers of samples. An LC-MS/MS method with automated SPE that uses a Gilson robot for the extraction was recently reported *(8)*. The authors used different SPE columns and solvent for elution than we did, and had a low recovery for benzoylecgonine (7.5%), whereas our method showed 66% recovery for benzoylecgonine. The authors of that report also used more than twice the volume of sample (500 μL), which is less optimal for OF because the amount of sample is limited. We chose to weigh the samples because this approach is commonly used in our laboratory. Pipetting volumes could be easier, however, and would be adequate. When we prepared calibrators by spiking pooled OF, up to 40 μL drug solution was added. This high volume may be seen as a limitation of our method, and it would have been better to add a smaller amount of a more concentrated solution, e.g., 10 μL. However, because the following step is a dilution with a large volume of buffer, this point is probably of minor importance.

The UPLC-MS/MS equipment used in this method allows determinations with an adequate LloQ, and good chromatographic separations were obtained. Twenty-nine compounds were analyzed in 15 min, indicating that high throughput of samples could be possible. A low volume of extract (10 μL) was injected into the UPLC-MS/MS system, which provided a good resolution of the compounds and resulted in only low MEs for most of the drugs (online Supplemental Table 1 and Table 3). The selected brand of methanol has been shown to exhibit a low degree of ion suppression *(14)*. The instrument also benefited from the low amount of loaded matrix, and periods of approximately 1 month between cleaning the cone were achieved. The ease of sample preparation without a derivatization step makes the LC-MS technique superior to GC-MS.

When using MS/MS detection, it is important to investigate for the presence of MEs *(10, 11)*. Coeluting components from the matrix could interfere with the analytes in the MS interface, resulting in a reduced or enhanced MS response. The composition of endogenous compounds in OF could vary from 1 person to another. It is therefore important to investigate results obtained with samples from several different individuals when studying MEs in a method validation *(10, 11)*. We investigated MEs from OF sampled from 5 different people and a pool of OF prepared with samples from 10 people. Only moderate MEs were observed, and there was no difference in MEs between individual sample donors. The CVs of the ion ratios allow a 95%-tolerance region that is sufficiently narrow for the compounds to meet commonly used regulatory guidelines *(15)*.

The use of deuterated IS can normalize the effect of the matrix and should be included whenever possible *(12, 13)*. We were able to use deuterated IS for almost all of the analytes for this method, except for bromazepam and chlordiazepoxide, for which we could not purchase the substances, and lorazepam, for which reliable results could not be obtained using the deuterated IS. Instead, diazepam-d_5 was used as an IS.

The method was fully validated for detecting all 29 of the compounds in OF mixed with buffer from the StatSure Saliva-Sampler used in the DRUID project (Table 3). A recent evaluation of 9 sample devices concluded that the StatSure Saliva-Sampler was most suited for yielding a high recovery of THC and other frequently used drugs of abuse *(16)*. It was further shown for 9 tested compounds that more than 69% were recovered after 28 days of frozen storage (–18 °C).

We detected major ion suppression for some compounds in our method in the synthetic OF manufactured by Dyna Tech. This ion suppression may give rise to problems when this type of synthetic OF is used in proficiency tests.

The ease of sample collection and the detection of recent drug use make OF a promising choice for roadside testing *(17)*. A review showed that amphetamines, cannabis, cocaine, and opiates are readily detected in OF and exhibit a pharmacokinetic pattern similar to that found in plasma *(3)*. Amphetamines, cocaine, and opiates tend to concentrate, owing to their basic nature in the acidic OF, whereas THC (because of high lipophilicity) and benzodiazepines (because of high protein binding) are more difficult to detect, because of low excretion from blood to OF *(18, 19)*. On the other hand, THC is deposited in the oral cavity when smoked; thus, detection of high THC concentrations in OF may indicate very recent drug use *(20)*. The aim of the DRUID project is to assess the feasibility of using OF instead of blood for detection of drugs in drivers. When the project is finished, epidemiological data concerning drug abuse in a traffic context will be evaluated, as well as practical aspects related to sampling of OF and analysis in forensic laboratories.

The relatively low cutoffs selected for the DRUID project (Table 3) demand analytical methods with low limits of detection. To fulfill this demand, it is important to have good recoveries for all of the compounds

measured, which may be difficult to attain *(7, 8)*. In the initial development phase of our method, we focused on attaining adequate recoveries for THC, buprenorphine, and morphine, and succeeded in achieving recoveries exceeding 50% for all of the compounds but THC (32%–36%, which was found to be acceptable).

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