Journal of Chromatography B, 879 (2011) 3367–3377

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Drugs of abuse in oral fluid collected by two different sample kits – Stability testing and validation using ultra performance tandem mass spectrometry analysis

H.M.E. Lund*, E.L. Øiestad, H. Gjerde, A.S. Christophersen

Norwegian Institute of Public Health, Division of Forensic Medicine and Drug Abuse, PO 4404, Nydalen, 0403 Oslo, Norway

ARTICLE INFO

Article history:
Received 29 June 2011
Accepted 1 September 2011
Available online 7 September 2011

Keywords:
Drugs of abuse
Oral fluid
Stability
Ultra-performance liquid chromatography–tandem mass spectrometry
Multi-component analysis

ABSTRACT

Oral fluid (OF) is an alternative matrix for monitoring drugs of abuse in workplace, clinical toxicology, criminal justice, and driving under the influence of drugs (DUID). OF is suitable for detection of drugs that have been taken recently. It is unproblematic to observe the collection and hence avoid the possibility of the samples being tampered. OF often contains compounds in low concentrations, and small volumes are often collected. It is therefore necessary to have a sensitive, multi component method for drug detection. In this study an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS–MS) method has been developed. The samples were prepared by liquid–liquid extraction (LLE) with ethyl acetate/heptane (4:1) and the separation was achieved by an Acquity HSS T3-column (2.1 mm × 100 mm, 1.8 μm particles). Mass detection was performed by positive ion mode electrospray MS–MS. 32 drugs of abuse were determined with a cycle time of 9 min. Stability of drugs in oral fluid before analysis is an important factor that must be evaluated for each sampling device. The collection devices Intercept® and StatSure Saliva Sampler™ were tested using pools of real samples containing various drugs. The testing showed that 6-MAM (6-acetylmorphine), cocaine and zopiclone were the least stable compounds. In the testing for short term stability, StatSure Saliva Sampler™ showed better results. The testing of 1 year of storage at −20 °C showed that most of the compounds were stable for both sampling devices, except for 6-MAM, cocaine and zopiclone. Samples of OF should be analysed as soon as possible after collection, and they should be kept frozen if immediate analysis is not possible.

1. Introduction

Oral fluid (OF) has gained focus as an alternative matrix for monitoring drugs of abuse in workplace testing, clinical toxicology, criminal justice, and driving under the influence of drugs programs (DUID) [1,2]. OF is suitable for detection of drugs that have been taken recently and is easily available for collection. Sample adulteration is more difficult compared to urine, as it is unproblematic to observe the collection process [3]. Both drugs and their metabolites can be detected in OF. The volume of the collected samples is often less than 1 mL, therefore multicomponent methods with low detection limits are needed [2,4]. Developments within analytical technology using liquid chromatography with tandem mass spectrometry (LC–MS/MS) have made it possible to simultaneously quantify several drugs at low concentrations in OF [5–9]. Ultra performance liquid chromatography (UPLC) has been introduced as a replacement for high performance liquid chromatography (HPLC) with potential of faster analysis, less solvent consumption, and improved resolution [10–14], and is considered as a promising technique for analysis of small volumes of OF [15–17].

We have previously used a method for detection of 32 drugs in OF with HPLC coupled with tandem mass spectrometry (MS/MS) [18]. The run time was 14 min and several components co-eluates. Switching to UPLC could give shorter analysis time and similar or better separation of the drugs. Badawi et al. has developed an UPLC-method for screening and quantification of 29 drugs in OF [15]. The run time was 20 min and satisfactory separations were achieved. We wanted to develop a UPLC-method for screening of drugs in OF with a shorter run time to facilitate analysis of larger volumes

Abbreviations: OF, oral fluid; DUID, driving under the influence of drugs programs; UPLC–MS–MS, ultra-performance liquid chromatography–tandem mass spectrometry; LLE, liquid–liquid extraction; 6-MAM, 6-acetylmorphine; LC–MS/MS, liquid chromatography with tandem mass spectrometry LC–MS/MS; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-ethylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; LSD, lysergic acid diethylamide; THC, Δ9-tetrahydrocannabinol; ES+, electro spray in the positive mode; R^2, the correlation coefficients; S/N, signal-to-noise ratio; LOD, limit of detection; LOQ, limit of quantitation; ME, matrix effects; RSD, relative standard deviation.

* Corresponding author. Tel.: +47 21 07 78 62.
E-mail address: hier@fhi.no (H.M.E. Lund).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.09.002

of samples. Two other UPLC–MS/MS methods with run times of approximately 8 min have been published [17,19]. These do however have smaller repertoire lacking e.g., benzodiazepines [19] and THC [17]. Sample preparation only by dilution as presented in these methods might in addition give long term instrumental problems as commercial sampling kits contain preservatives, surfactants and in some cases dyes, which might build up instrumental back-ground noise if not removed.

The suitability of 9 different commercial sampling kits for collecting samples for drug analysis have been evaluated by Langel et al. [20]. In this paper we have used two of these sampling kits, Intercept® and StatSure Saliva Sampler™, in the development of the analysis method. We also wanted to study the influence of different short term storage conditions and stability during storage past the 28 days tested in the paper by Langel et al. and in addition include more substances.

2. Experimental

2.1. Chemicals, reagents and materials

The reference compounds were obtained from several companies. 3,4-Methylenedioxyamphetamine (MDA) and 3,4-ethylenedioxyethylamphetamine (MDEA) were obtained from Alltech (Lexington, KY, USA); 3,4-methylenedioxymethamphetamine (MDMA), lysergic acid diethylamide (LSD), Δ9-tetrahydrocannabinol (THC), 3,4-methylenedioxymethamphetamine-d5, 7-aminoflunitrazepam-d7, amphetamine-d11, benzoylecgonine-d8, buprenorphine-d4, clonazepam-d4, cocaine-d3, methadone-d3, methamphetamine-d11 and nordiazepam-d5 from Cerriliant (Round Rock, TX, USA); 3-OH-diazepam, 6-acetylmorphine, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, flunitrazepam, methamphetamine, nordiazepam, oxazepam and zopiclone were purchased from Lipomed (Arlesheim, Switzerland). We obtained alprazolam, amphetamine, benzoylecgonine, bromazepam, diazepam, clonazepam, codeine, cocaine, meprobamate, methadone, morphine, nitrazepam and morphine-d3 from Sigma–Aldrich (St. Louis, MO, USA); buprenorphine and carisoprodol from RBI (Natick, MA, USA); fenazepam from Chiron AS (Trondheim, Norway); lorazepam from LGC (Middlesex, UK) and THC-d3 from High Standard Products Corp (Westminster, CA, USA). All the references were of ≥98% purity. Standard compounds were stored according to supplier recommendations (solid substances mainly at room temperature, ampules at 4 °C).

HPLC-grade methanol was obtained from Lab-Scan (Poch SA, Gliwice, Poland); ammonium formate from BDH (Bridgeport, NJ, USA) and concentrated formic acid was obtained from Merck (Darmstadt, Germany). Purified water was obtained with a Milli-Q system (Millipore, Billerica, MA, USA). The 10 mM ammonium formate buffer used for the mobile phase had a pH of 3.1. It was prepared from a 50 mmol/L stock solution of ammonium formate adjusted to pH 3.1 with acetic acid, by a 1:5 dilution with water. A 0.2 mol/L ammonium carbonate buffer, adjusted to pH 9.3 with ammonia, was used in the extraction method. Analytical grade *n*-heptane and HPLC-grade ethyl acetate were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of solutions

The stock solutions of zopiclone and zolpidem were made with acetonitrile, THC with ethanol, and other substances with methanol. Calibrator and QC solutions for zopiclone and THC were prepared in acetonitrile/water 30/70% (v/v), for other compounds in purified water. The stock solutions were stored at −20 °C before mixing, and the working solutions were stored at 4 °C.

The saliva collection devices contain salts and preservatives. Negative Calibrator Oral Fluid solution with the same contents as the Intercept® Oral Specimen Collection Device sample kits was purchased from Orasure Technologies Inc. (Bethlehem, PA, USA). The sample kits do in addition contain a blue dye, and Flag Blue Liquid Food Color was purchased from Chef's Classic (Minnetonka, MN, USA) and added to the Negative Calibrator Oral Fluid. For the Saliva Sampler™ Buffer 1000 Media was purchased from StatSure Diagnostic Systems Inc. (Framingham, MA, USA). 0.5 mL of these fluids were added the calibrator and QC samples. They are referred to as *zero calibrant solution regardless of manufacturer* and were making up the negative control.

The internal standards were dissolved in 50 mL water. The concentrations in preserved OF were 1.1–44.6 ng/mL.

2.3. Sampling of oral fluid

Two different collection devices were used to collect OF, the Intercept® Oral Specimen Collection Device and the Saliva Sampler™ from StatSure. They have been used for several projects at the Norwegian Institute of Public Health and they are widely used around the world. The collector pad of the Intercept® device has been treated with salts and citric acid to stimulate the saliva production. It is placed between gum and cheek and placed in the vial after a 2 min sampling time. The volume of OF that is collected has an expected mean value of 0.4 mL. The preservative–OF solution was transferred to 15 mL polypropylene tubes (Greiner Bio-One GMbH) after centrifugation at 1400 × *g* for 15 min. Aliquots of 0.5 mL preserved OF were transferred to separate 5 mL polypropylene tubes (Sarstedt AG & Co.) and stored at −20 °C until the time of analysis.

The Saliva Sampler™ device consists of an absorbing pad of cellulose with an indicator that turns blue when sufficient amount of OF is collected (1 mL). The indicator can turn blue even for smaller amounts of OF if sampling times are extended. The vial contains a colorless preservative solution of 1 mL, Buffer 1000. OF that is collected by this sampling device is unstimulated. Before analysis, the pad were removed from the plastic holder and placed in the bottom of the vial. A filter (Filter Sampler® Porex, Fairburn, GA, USA) was placed against the pad and pressure towards the filter reconstituted the preserved OF. 0.5 mL of OF solution were transferred to separate 5 mL polypropylene tubes (Sarstedt AG & Co., Rommelsdorf, Germany) and stored at −20 °C until the time of analysis.

2.4. Extraction procedure

The extraction procedure was the same for both sampling kits. 0.5 mL sample was extracted with liquid–liquid extraction, as described by Øiestad et al. [18]. The concentrations of the internal standards in the preserved fluid were 16.1 ng/mL MDMA-d5, 1.8 ng/mL 7-aminoflunitrazepam-d7, 44.6 ng/mL amphetamine-d11, 19.6 ng/mL benzoylecgonine-d8, 4.4 ng/mL buprenorphine-d4, 1.1 ng/mL clonazepam-d4, 1.8 ng/mL cocain-d3, 8.9 ng/mL methadone-d3, 13.0 ng/mL methamphetamine-d11, 14.7 ng/mL morphine-d3, 4.5 ng/mL nordiazepam-d5 and 4.0 ng/mL THC-d3.

2.5. LC–MS analysis

2.5.1. Equipment

LC was performed by using an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA). The column used was a HSS T3 C_{18} column (100 mm × 2.1 mm, 1.8 μm) obtained from Waters Corporation (Milford, MA, USA) maintained at a temperature of 65 °C. We used gradient elution with a mobile phase consisting of 10 mM ammonium formate buffer pH 3.1 (A) and methanol (B) with a flow rate of 0.5 mL/min. The gradient program is shown

Table 1
UPLC gradient program (9 min total run time).*

Time, min	A, %	B, %	Flow, mL/min
0	90	10	0.5
0.5	90	10	0.5
1.5	70	30	0.5
2.5	70	30	0.5
2.6	40	60	0.5
5.0	30	70	0.5
5.5	10	90	0.5
6.7	10	90	0.5
6.8	90	10	0.5

* A linear curve profile was used for the change in mobile phase composition.

in Table 1. The injection volume was 7.5 μL using partial loop injection with a needle overfill flush of 3 μL. Weak wash was performed with 600 μL methanol:water (10:90), and strong wash with 200 μL methanol:water (90:10), for each sample. The equipment and the gradient program was the same as described by Øiestad et al. [21].

2.5.2. Mass spectrometry

MS detection was performed on a Quattro Premier XE triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). Ionisation was achieved by using electro spray in the positive mode (ES+) and multiple reaction monitoring (MRM) with one transition for each compound. The source block temperature was 120 °C and the capillary voltage 1.00 kV. The desolvation gas (nitrogen) was heated to 500 °C and the flow was set to 900 L/h. The cone gas (nitrogen) was delivered at a flow rate of 50 L/h and the collision gas (argon) pressure was maintained at 0.004 mbar in the collision cell. Data acquisition, peak integration and calculation were performed on a computer work station running MassLynx 4.1 software. Analytes were identified by comparing the retention times with the corresponding calibrators and QC samples. The internal standard chosen for each analyte, retention times, and MRM transitions are shown in Table 2.

2.6. Method validation

The validation was done according to guidelines given by Peters et al. [22]. It was primarily done with the Intercept® device, but evaluation of matrix effects and comparison with our previous HPLC method [18] were done with both sampling kits. The stability study was done with both devices as well.

2.6.1. Identification and quantitation

The components were identified by the retention time of the MRM transition for each component. Quantification was made by comparing the response (the ratio of the integrated peak height and the corresponding internal standard) of the analyte to the responses of the standard samples. 5-Point calibration curves were obtained with 3 replicates of each standard. Quadratic calibration curves were used with linearity weighing as $(1/x)$. Origo was included. The concentrations of the calibrator solutions shown in Table 3 corresponds to concentrations in the mixture of OF and Intercept® buffer.

2.6.2. Calibration curves

The correlation coefficients (R^2) for the calibration curves were determined by analysing 6 series with 3 parallels of each standard sample.

2.6.3. Limits of detection (LOD) and quantitation (LOQ)

LOD was determined by extracting standard samples of different diluted concentrations and defined as amount of drug giving a peak with signal-to-noise (S/N) ratio of 3. Authentic negative samples were analysed to evaluate background noise. LOD was set higher than the contribution from the internal standards. LOQ was set as the concentration where acceptable precision (RSD 20%) and bias (±20%) was achieved. S/N was measured for LOQ samples. A standard sample with a concentration near the LOQ sample was included in the calibration curve.

2.6.4. The retention stability of the internal standards

The retention stability of the internal standards was determined by evaluating the variability of the retention times of the internal standards within one series (inter-day) and within six series (intra-day).

2.6.5. Precision and accuracy

Within-day precision was estimated by analysis of separate preparations of QC samples at 2 concentrations in a single assay ($n = 10$). Between-day precision was determined by analysis of 3 replicates of 2 QC concentrations on 6 different days. For 6-MAM, amphetamine, benzoylecgonine, methamphetamine, and zolpidem between-day precision was determined on 5 days due to instrumental problems.

2.6.6. Specificity

To investigate the specificity of the method, we fortified zero calibrant solution with high concentrations of 86 selected prescription drug and extracted the samples as described earlier for the calibrators. The drugs tested were antidepressants, analgesics, antipsychotics, antiepileptic drugs, drugs that affect the cardio vascular system and other drugs that are commonly evaluated in forensic samples at our laboratory. A listing of these drugs and the concentrations tested are provided in Supplementary data Table 1. The found concentrations of peaks with retention times equal to components in the method were compared to the LOQ of the method. False positive results below LOQ were rejected as interfering peaks.

2.6.7. Carryover

To evaluate the carryover in the method, we fortified two calibrant solutions with components with concentrations 670 times higher than the lowest calibration standard for most drugs. For zopiclone and THC the concentrations were 130 times higher than the lowest calibration standard. Three blanks were analysed after each carryover test sample.

2.6.8. Matrix effects (ME)

ME were evaluated by the method proposed by Matuszewski et al. [23]. The analyte signals in the spiked mobile phase were compared with the analyte signals in the matrix fortified after extraction, and the ME was defined as ME% = (extracted matrix height/mobile phase height) × 100. A value below 100% indicates ion suppression, while a value above 100% indicates ion enhancement. Four replicates of mobile phase and six OF sample extracts from each sampling kit, obtained from six different drug-free persons, were analysed. The OF was collected by spitting, and diluted by the corresponding buffer solutions for each sample kit. For the Intercept® device, 800 μL buffer solution per 400 μL OF were added to six different samples. In addition, 3 samples were added 800 μL buffer solution per 200 μL OF. Samples collected with the StatSure Saliva Sampler™ contains about one part oral fluid per one part buffer, therefore 250 μL buffer solution was added to 250 μL oral fluid. The relative matrix effect was evaluated as the coefficient of

Table 2
MRM transitions and operating parameters for the analysed drugs.

Compound	MRM transitions, *m/z*	Cone voltage, V	Collision energy, eV	IS
Detection window 1				
Morphine	285.9>201	50	24	Morphine-d3
Morphine d3	288.9>201	50	24	
Window 2				
6-MAM	328>211	45	25	7-Aminoflunitrazepam-d7
7-Aminonitrazepam	251.9>121	40	25	7-Aminoflunitrazepam-d7
Amphetamine	135>91	35	20	Amphetamine-d11
Amphetamine d11	146.9>98	35	20	
Benzoylecgonine	289.9>168	30	20	Benzoylecgonine-d8
Benzoylecgonine-d8	297.9>171	30	20	
Codeine	299.9>214.9	45	25	7-Aminoflunitrazepam-d7
MDA	179.9>163	20	10	MDMA-d5
MDEA	208>163	20	15	MDMA-d5
MDMA	193.9>163	35	10	MDMA-d5
MDMA-d5	199>165	20	15	
Methamphetamine	149.9>91	35	20	Methamphetamine-d11
Methamphetamine-d11	160.9>97	35	20	
Window 3				
7-Aminoclonazepam	286>250	40	20	7-Aminoflunitrazepam-d7
7-Aminoflunitrazepam	283.9>135	40	25	7-Aminoflunitrazepam-d7
7-Aminoflunitrazepam-d7	291.1>138	50	23	
Cocaine	303.9>182	35	40	7-Aminoflunitrazepam-d7
Cocaine d3	306.9>185	35	40	
LSD	324>223.1	35	25	7-Aminoflunitrazepam-d7
Meprobamat	218.8>158	30	20	7-Aminoflunitrazepam-d7
Zolpidem	307.9>262.9	40	24	7-Aminoflunitrazepam-d7
Zopiclone	389.1>245	35	20	7-Aminoflunitrazepam-d7
Window 4				
3-OH-diazepam	300.9>255	30	20	Nordiazepam-d5
Alprazolam	309>280.9	45	25	Nordiazepam-d5
Bromazepam	315.9>182	40	30	7-Aminoflunitrazepam-d7
Buprenorphine	468.3>396.4	60	38	Buprenorphine-d4
Buprenorphine-d4	472.3>400.2	60	44	
Carisoprodol	260.9>176	30	20	7-Aminoflunitrazepam-d7
Clonazepam	315.9>269.9	40	25	Clonazepam-d4
Clonazepam-d4	319.9>273.9	40	25	
Flunitrazepam	313.9>268	40	25	Nordiazepam-d5
Lorazepam	320.9>275	30	20	7-Aminoflunitrazepam-d7
Methadone	310>265	40	18	Methadone-d9
Methadone-d9	319>268	40	18	
Nitrazepam	281.8>236	35	25	7-Aminoflunitrazepam-d7
Oxazepam	286.9>240.9	45	30	7-Aminoflunitrazepam-d7
Window 5				
Diazepam	284.9>193	40	30	7-Aminoflunitrazepam-d7
Fenazepam	350.9>206.1	40	37	7-Aminoflunitrazepam-d7
Nordiazepam	270.9>139.9	40	27	Nordiazepam-d5
Nordiazepam-d5	275.9>139.9	35	27	
Window 6				
THC	315.2>193	35	26	THC-d3
THC-d3	318.2>196	35	26	

variation of the measured absolute matrix effects. Cocaine-d3 was added as internal standard for cocaine before this experiment.

2.6.9. Stability of the compounds in processed samples in case of delay in sample injection

The stability of the extracted samples was determined in case of delay in sample injection by reanalysing two assays of extracts. One assay was stored 4 days in the autosampler (10 °C) and one assay was frozen (−20 °C) for one week. Twelve actual OF samples were analysed in both assays.

2.7. Stability of the compounds during storage

To evaluate stability of various components in OF, we made pools of OF that was collected by both sampling devices. The samples were analysed before the study, and samples with the desired drugs were mixed and diluted with a mixture of blank oral fluid and zero calibrant solution to make batches with different concentrations. Three replicates of each sample were analysed to evaluate the stability of the drugs in collected OF samples. For the Intercept® device we collected samples from 70 persons that were attending an opiate maintenance treatment programme. Four different pools of samples were made with different dilutions. For the StatSure Saliva Sampler™ device we collected samples from 19 persons that were stopped by the police due to suspected driving under the influence of drugs. Two different pools were analysed.

Table 4 shows program for temperature conditions and time before sample analysis.

3. Results and discussion

3.1. Validation

3.1.1. Identification and quantitation

The retention times are shown with chromatograms in Fig. 1 and the MRM transitions are shown in Table 2. Several of the components co-eluates, but the separations were satisfactory because of the variation in *m/z* for the compounds. The 32 components and their internal standards were separated in less than 7 min.

Table 3
Validation results for oral fluid mixed with Intercept buffer.

Analyte	Calibration concentrations, ng/mL	Correlation coefficient ($n = 6$), R^2	LOQ, ng/mL	Theoretical concentratio, ng/mL	Within-day RSD, %	Between-day RSD, %	Bias, %
3-OH-diazepam	0.09 0.18 0.9 3.0 9.0	0.992	0.15	1.5 9.4	14 8	9 8	-1 -4
6-MAM	0.06 0.12 0.6 2.0 6.0	0.974	0.65	0.7 4.1	17 16	17 14	-4 11
7-Aminoclonazepam	0.05 0.10 0.5 1.7 5.0	0.985	0.06	0.6 3.6	11 10	15 10	3 9
7-Aminoflunitrazepam	0.008 0.02 0.08 0.3 0.8	0.990	0.02	0.1 0.9	13 5	10 7	11 9
7-Aminonitrazepam	0.04 0.08 0.4 1.3 4.0	0.960	0.10	0.5 3.1	8 10	12 10	-11 -9
Alprazolam	0.03 0.06 0.3 1.0 3	0.993	0.04	0.4 2.3	15 6	9 8	-1 2
Amphetamine	0.05 0.1 0.5 1.7 5	0.984	0.67	0.7 4.2	19 10	27 24	-7 -13
Benzoylecgonine	0.5 1.0 5.0 16.7 50.0	0.868	5.79	5.8 36.2	20 28	24 30	2 1
Bromazepam	0.5 1.0 5.0 18.0 54.0	0.981	6.22	6.2 38.9	10 13	19 14	4 12
Buprenorphine	0.08 0.16 0.8 2.7 8.0	0.994	0.19	0.9 5.8	17 8	13 7	30 23
Carisoprodol	1.7 3.4 17.0 58.0 174	0.972	2.62	26.2 163.9	21 31	20 19	8 15
Clonazepam	0.03 0.06 0.3 1.0 3.0	0.993	0.07	0.4 2.3	13 5	11 8	8 3
Cocaine	0.3 0.6 3.0 8.7 26.0	0.975	0.61	3.0 18.9	21 20	24 10	10 21

Table 3 (*Continued*)

Analyte	Calibration concentrations, ng/mL	Correlation coefficient ($n = 6$), R^2	LOQ, ng/mL	Theoretical concentratio, ng/mL	Within-day RSD, %	Between-day RSD, %	Bias, %
Codeine	0.1 0.2 1.0 3.3 10.0	0.979	0.15	1.5 9.3	18 19	16 13	-6 -10
Diazepam	0.05 0.1 0.5 1.7 5.0	0.982	0.06	0.6 3.6	13 11	18 11	-4 1
Fenazepam	0.06 0.12 0.6 2.0 6.0	0.979	0.14	0.7 4.4	10 10	17 10	-2 2
Flunitrazepam	0.02 0.04 0.2 0.7 2.0	0.993	0.05	0.3 1.6	13 8	9 10	4 3
Lorazepam	0.1 0.2 1.0 3.7 11.0	0.980	0.32	1.6 10.1	13 12	16 11	-3 0
LSD	0.01 0.02 0.1 0.33 1.0	0.974	0.01	0.2 0.9	22 13	25 10	14 16
MDA	1.2 2.4 12.0 39.3 118	0.983	1.79	17.9 111.9	21 6	12 11	23 18
MDEA	1.4 2.8 14.0 45.7 137	0.987	2.07	20.7 129.5	15 8	13 8	6 8
MDMA	1.3 2.6 13.0 43.3 130	0.995	1.94	19.4 121.2	13 4	11 9	22 20
Meprobamat	1.5 3.0 15.0 48.7 146	0.974	2.2	22.0 137.6	17 16	20 18	-2 3
Methamphetamine	0.1 0.2 1.0 3.3 10.0	0.990	0.12	1.3 7.8	9 6	19 10	1 4
Methadone	0.5 1.0 5.0 17.7 53	0.998	0.63	6.3 39.0	12 8	6 5	11 9
Morphine	0.1 0.2 1.0 4.7 14.0	0.997	0.32	1.6 10.2	10 3	8 8	-9 -11

Table 3 (*Continued*)

Analyte	Calibration concentrations, ng/mL	Correlation coefficient ($n = 6$), R^2	LOQ, ng/mL	Theoretical concentratio, ng/mL	Within-day RSD, %	Between-day RSD, %	Bias, %
Nitrazepam	0.03 0.06 0.3 1.0 3.0	0.981	0.33	0.3 2.1	11 8	15 10	-2 1
Nordiazepam	0.05 0.1 0.5 1.7 5.0	0.996	0.54	0.5 3.4	14 5	9 7	23 23
Oxazepam	0.05 0.1 0.5 1.7 5.0	0.980	0.11	0.6 3.6	11 7	19 12	2 4
THC	0.01 0.02 0.1 0.33 1.0	0.983	0.16	0.2 1.0	18 9	14 14	20 -1
Zolpidem	0.01 0.02 0.1 0.33 1.0	0.953	0.02	0.1 0.8	23 12	22 11	-11 1
Zopiclone	0.06 0.12 0.6 2.0 6.0	0.972	0.67	0.7 4.1	21 11	23 10	3 1

3.1.2. Calibration curves

The average values of R^2 are shown in Table 3. All of the components except for 6-MAM, codeine, benzoylecgonine, zopiclone, cocaine, 7-AN, meprobamat, LSD, zolpidem, carisoprodol and fenazepam had a R^2 of more than 0.98. The mentioned drugs had more curved calibration curves than the rest of the components. Of the above mentioned drugs, only benzoylecgonine, 7-AN and zolpidem had R^2 below 0.97. Benzoylecgonine should not be quantified at concentrations below the concentration level of Standard 3 because of sensitivity problems.

3.1.3. Limits of detection and quantitation

The results for LOD and LOQ are shown in Table 3. LOQ were considerably higher than LOD for some of the components. Lower concentrations than Standard 1 were not examined for practical reasons.

3.1.4. The retention stability of the internal standards

The mean RSD of the retention times of the internal standards were below 0.25% for all the internal standards. The internal standards had stable retention times.

Table 4
Time points and temperatures of storage for stability testing of different drugs in oral fluid.

Temperature	Point 0	1 Week	3 Months	1 Year
−20 °C	X		X	X
4 °C	X	X		
20 °C	X	X		

3.1.5. Precision and accuracy

The results for precision and accuracy can be seen in Table 3. For benzoylecgonine, buprenorphine, and carisoprodol the RSD value was up to 30%. Other compounds with RSD values above 20% were amphetamine (27%), cocaine (24%), LSD (25%), zolpidem (22%), and zopiclone (23%). For zopiclone it is known that it is unstable in methanolic solutions, and contact with MeOH at some point of the analysis might explain the variation in the results [24].

To achieve better precision, more internal standards could be used. The validation was done in a 3 month's period, and several working solutions of calibration samples and QC samples were made. The stock- and working solutions were from different batches for QC samples and calibrations samples, and variation in the manufacturing of them could have contributed to the large RSD values for the mentioned compounds. The within-day results were satisfactory for our purpose.

3.1.6. Specificity

Of the 86 substances phenytoin gave a false positive result above LOQ for flunitrazepam. In oral fluid analysis possible contamination with high concentrations from orally ingested compounds must in addition be kept in mind. If the results will be used to give negative sanctions for the sample donor, the sample should be confirmed positive with another method with other chromatographic conditions and two transitions before the results are sent to the client.

3.1.7. Carryover

No false positive results due to carryover were found from a sample fortified with a concentration 130 times the lowest standard for THC or zopiclone. The other compounds were tested with a concentration 670 times the lowest standard. Peaks with calculated concentrations of 1–3 times the lowest standard was found

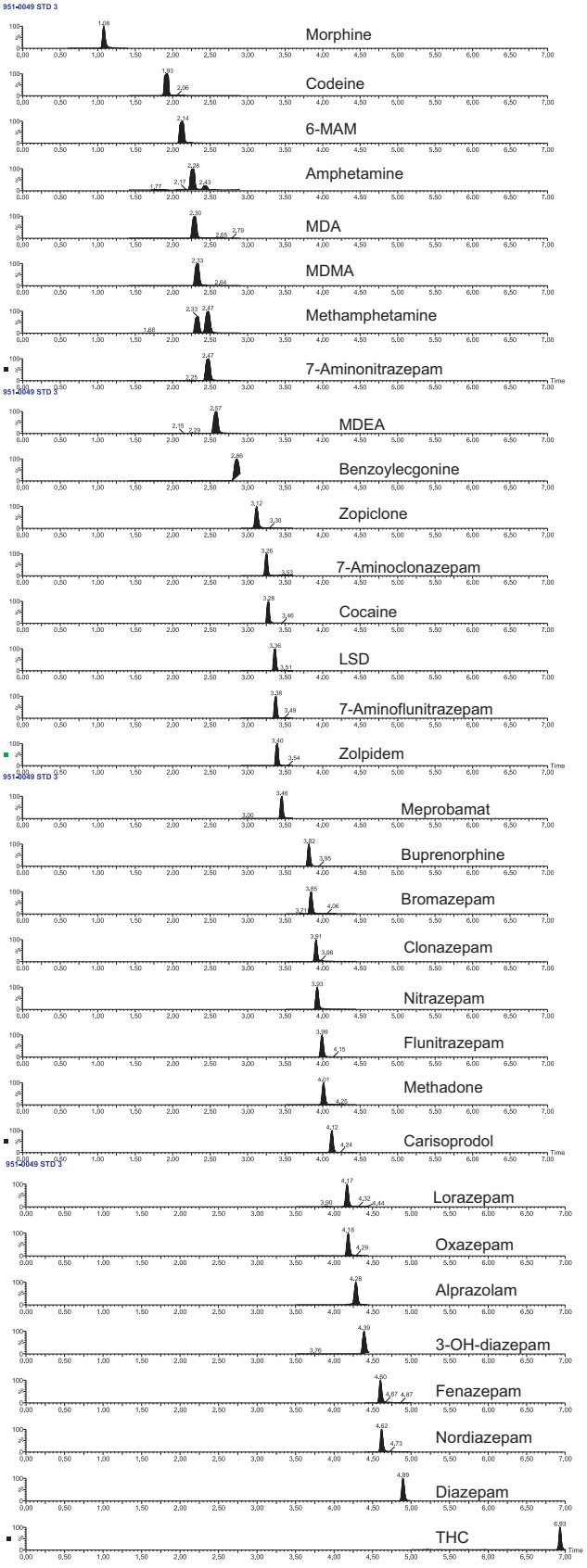

Fig. 1. Chromatograms of the highest calibration standard of one assay for all 32 compounds in the study.

for 7-aminoclonazepam, buprenorphine, diazepam, fenazepam, methadone and nordiazepam. For buprenorphine and LSD the second blank had a carry over of about 80% of the lowest standard, while the other compounds were blank. Evaluation of possible carry-over after high samples is therefore especially important for these compounds. THC and zopiclone were tested with a lower fortified sample than the other drugs. Concentration in real samples that are higher than that, might give false positive results.

3.1.8. Matrix effects

Results from ME-tests are given in Table 5. Both of the sampling kits were evaluated for ME. The buffer solution in the sampling kits is different, so ME could be different in samples collected with the two kits.

According to the manufacturers StatSure Saliva Sampler™ should collect OF with a ratio of 1:1 to the preservative solution, while the Intercept® device should have a ratio of 1:2. Consequently matrix effects were tested with these solutions. As have been demonstrated drug use is however associated with smaller oral fluid volumes [25], and for Intercept® matrix effects for an additional dilution of 1:4 ratio was tested (data not shown).

Matrix effects in OF-preservative solution from StatSure Saliva Sampler™ above 120% were seen for buprenorphine, clonazepam, cocaine, codeine, diazepam, fenazepam, lorazepam, LSD, MDEA, meprobamat, methamphetamine, methadone, morphine, nordiazepam, THC, zolpidem and zopiclone. When corrected with internal standards, cocaine and THC showed ion suppression, while buprenorphine, codeine, diazepam, fenazepam, lorazepam, LSD, meprobamat, zolpidem and zopiclone still showed ion enhancement.

Matrix effects in OF-preservative 1:2 mixture from the Intercept® device above 120% were seen for 6-MAM, buprenorphine, carisoprodol, clonazepam, cocaine, diazepam, fenazepam, LSD, methadone, morphine, nordiazepam, THC, zolpidem and zopiclone. When corrected with internal standard THC shows ion suppression, while 6-MAM, buprenorphine, carisoprodol, diazepam, fenazepam, lorazepam, LSD, zolpidem and zopiclone showed ion enhancement.

After correction with internal standard the same compounds showed ion enhancement or ion suppression for both sampling kits, except that cocaine showed some ion suppression and codeine and meprobamat ion enhancement only with the StatSure device, and 6-MAM, and carisoprodol showed ion enhancement only with the Intercept® device. More very high MEs was seen for StatSure Saliva Sampler™ compared to the Intercept® device. ME for Intercept® with OF:buffer solution 1:4 was fewer than for 1:2, but it does not appear to be a significant difference if the collected sample is with little OF in relation to the buffer solution. Under our daily operating conditions the Intercept® device is preferred because of the fewer very high MEs that were seen and because sample extraction from the device is easier.

More deuterated internal standard for the compounds that show great MEs could be included to reduce the matrix effects. Another possibility to correct for matrix effects is to change the internal standard for compounds that do not have their own internal standard to another compound than the one that were used in this study.

3.1.9. Stability of the compounds in processed samples in case of delay in sample injection

There were no significant deviations in the results when the samples were stored 4 days in the autosampler (10 °C) or in freezer (−20 °C) for 1 week and reanalysed.

Table 5
Evaluation of matrix effects for StatSure Saliva Sampler™ and the Intercept® collection device.

Compound	StatSure OF:preservative 1:1			Intercept® OF:preservative 1:2		
	ME (%)	Relative ME RSD (%)	ME corrected with internal standard (%)	ME (%)	Relative ME RSD (%)	ME corrected with internal standard (%)
3-OH-diazepam	118	4	79	116	4	85
6-MAM	119	11	114	263	44	408
7-Aminoclonazepam	94	1.5	97	89	5	143
7-Aminoflunitrazepam	104	27	106	92	8	149
7-Aminonitrazepam	65	10	67	51	25	82
Alprazolam	102	4	68	94	8	69
Amphetamine	73	40	69	70	24	41
Benzoylecgonine	95	4	138	89	6	96
Bromazepam	94	5	96	85	6	137
Buprenorphine	657	3	148	515	16	134
Carisoprodol	112	45	120	124	63	203
Clonazepam	134	18	90	123	11	92
Cocaine	188	30	79	254	32	98
Codeine	201	19	215	103	12	175
Diazepam	149	5	155	125	7	203
Fenazepam	211	12	221	173	6	284
Flunitrazepam	101	16	67	88	25	64
Lorazepam	133	14	135	111	12	177
LSD	304	38	330	152	30	270
MDA	99	5	84	85	20	85
MDEA	124	7	109	86	10	88
MDMA	86	39	76	106	13	109
Meprobamat	192	68	189	182	86	323
Methamphetamine	104	21	105	89	9	66
Methadone	285	16	97	257	34	91
Morphine	143	3	99	142	7	100
Nitrazepam	112	21	115	114	10	183
Nordiazepam	149	15	99	131	6	96
Oxazepam	105	21	110	116	27	193
THC	3358	6	63	1024	33	50
Zolpidem	324	19	339	190	19	316
Zopiclone	157	8	192	170	5	206

3.2. Stability of the compounds during storage

The evaluation of stability was performed for pools of real samples containing different substances. The possibility of formation of one substance from another must therefore be kept in mind. Especially for morphine and 6-MAM formation from heroin in the samples can be an important factor. Short-term stability relevant to transport of samples or storage before analysis was tested after one week at 4 °C or ambient temperature, while long-term storage was tested after storage at −20 °C for up to 1 year.

3.2.1. Stability testing with the Intercept® device

Table 6 shows the results from the stability study of compounds in OF collected with the Intercept® device. The average concentrations at time zero was set to 100%, and the other points are given as measured percentage of this concentration. The opiates seemed fairly stable for the first week except for 6-MAM. The increase in morphine when stored one week in room-temperature could be explained by formation from heroin, 6-MAM and codeine. Amphetamine, diazepam, THC and 7-aminoclonazepam showed a tendency towards decreasing, while the rest of the compounds seemed to be stable at both 4 °C and ambient temperature the first week. To maintain the information about zopiclone and heroin use, and it would however be best to deepfreeze samples as soon as possible after collection.

The morphine concentration seemed to increase over time at −20 °C, probably due to degradation from heroin, 6-MAM and/or codeine. 6-MAM decreases to approximately 30% of the initial concentration after one years storage. The methadone and buprenorphine concentrations were stable, as were the amphetamines. About 75% of the zopiclone concentration was left after 1 year in freezer. The benzodiazepines that were included in the study were stable, except for 7-aminoclonazepam that were reduced to about 30% after 1 year in freezer. The THC concentration was stable in this assay.

3.2.2. Stability testing with the StatSure Saliva Sampler™

Table 7 shows the results of the stability study of compounds in OF taken with the StatSure Saliva Sampler™. The other points are given as the average results measured as percentage of time zero. Both codeine and 6-MAM showed a tendency to decrease, while both morphine and methadone showed a slight increase for both storage conditions the first week. 6-MAM was however more stable than with the Intercept® device. The concentration of amphetamine was doubled the first week. Methamphetamine was present at a high concentration, and it might have been degraded to amphetamine and contributed to some of the increase in the amphetamine concentration. The stability of amphetamine after one week in the freezer or at ambient temperature is uncertain after this study. The doubling of the concentration could be caused by other factors than degradation from methamphetamine such as to low concentration of amphetamine in the calibration curve. Methamphetamine appeared to decrease in concentration, but the results could be caused by analytical variation in addition to a possible degradation to amphetamine. The cocaine concentration decreased the first week, in particular in ambient temperature. It might have been degraded into benzoylecgonine, where the concentration was doubled at ambient temperature. Zopiclone seemed to be stable in the samples that were stored at 4 °C, but the concentration was reduced with 44% at ambient temperature. Samples that might contain zopiclone should be frozen prompt after donation. All the included benzodiazepines, except from

Table 6
Results from the stability study of compounds in OF samples, Intercept® Specimen Collection Device.*

Compound	Time 0, ng/mL	1 week, fridge, change (%)	1 week, roomtemperature, change (%)	3 months, freezer, change (%)	11 months, freezer, change (%)
6-MAM sample 1	10.9	54%	47%	53%	29%
6-MAM sample 2	5.9	92%	86%	65%	34%
7-aminoclonazepam	1.1	67%	80%	44%	30%
Alprazolam	2.2	90%	85%	84%	89%
Amphetamine	12.1	65%	71%	95%	87%
Buprenorphine	42.4	85%	90%	71%	79%
Codeine sample 1	9.0	119%	123%	73%	105%
Codeine sample 2	3.9	117%	117%	69%	92%
Diazepam sample 1	3.8	90%	96%	89%	100%
Diazepam sample 2	2.6	84%	68%	72%	71%
Methadone sample 1	55.0	101%	99%	106%	110%
Methadone sample 2	51.4	100%	99%	108%	103%
Methamphetamine	22.5	87%	87%	82%	86%
Morphine sample 1	14.2	119%	135%	128%	150%
Morphine sample 2	4.7	127%	133%	119%	142%
Nordiazepam	3.2	95%	104%	99%	114%
Oxazepam sample 1	2.3	131%	105%	133%	135%
Oxazepam sample 2	9.3	100%	84%	71%	81%
THC sample 1	1.5	74%	95%	114%	120
THC sample 2	0.6	83%	76%	96%	113%
Zopiclone	14.3	31%	6%	83%	74%

* Sample 1/2 does not mean that the compounds were in the same solutions. Samples 1/2 is written to show that some compounds were measured in two samples.

Table 7
Results from the stability study of compounds in OF samples, StatSure Saliva Sampler™.

Compound	Time 0, ng/mL	1 week, fridge, change (%)	1 week, roomtemperature, change (%)	3 months, freezer, change (%)	11 months, freezer, change (%)
6-MAM	27.5	86%	78%	81%	49%
7-aminoclonazepam	3.4	109%	136%	76%	92%
Alprazolam	5.9	96%	104%	103%	100%
Amphetamine	15.9	192%	210%	128%	102%
Benzoylecgonine	6.0	101%	209%	67%	103%
Clonazepam	0.8	87%	98%	100%	86%
Cocaine	21.8	82%	45%	85%	83%
Codeine	17.1	75%	84%	72%	95%
Diazepam	1.9	106%	113%	117%	98%
Methadone	15.4	124%	127%	94%	95%
Methamphetamine	59.7	85%	88%	117%	87%
Morphine	58.6	109%	117%	124%	133%
Nordiazepam	1.7	100%	112%	97%	93%
Oxazepam	3.5	101%	114%	99%	105%
Zopiclone	2.5	112%	56%	111%	128%

7-aminoclonazepam, were stable the first week. 7-Aminoclonazepam increased at ambient temperature.

Long-term stability at −20 °C seem good for most compounds except for 6-MAM, cocaine and zopiclone. The change in concentration from 3 months to 1 year for 7-aminoclonazepam could be due to contribution from clonazepam.

3.2.3. Comparison of sample kits

For the compounds that were tested with both sample kits we found that the stability in stored samples was better with the StatSure Saliva Sampler™ than the Intercept® device for storage for one week at 4 °C or ambient temperature.

6-MAM, cocaine and zopiclone were the least stable compounds in the study, and for these compounds freezing immediately after collection and storage at −20 °C will give the best result. The concentrations of the compounds in this study were either in the middle or upper area of the calibration curves. Lower concentrations might be more vulnerable to loss of compound.

Langel et al. [20] have tested stability of compounds in OF from StatSure Saliva Sampler™ and the Intercept® device for alprazolam, amphetamine, cocaine, codeine, diazepam, MDMA, morphine and THC in a 28 days period. The samples consisted of spiked OF, not real samples. The samples were stored at −18 °C for 14 and 28 days. 6 replicates were analysed. Alprazolam and amphetamine were the least stable compounds, and the concentrations dropped when the samples were prepared with the buffer from StatSure Saliva Sampler™ and stored for 28 days. The other compounds were more stable. When the samples were prepared with buffer solution from the Intercept® device, amphetamine and cocaine were the least stable compounds. The concentrations decreased with 25% and 28%, respectively for amphetamine and cocaine after 28 days. These results are in accordance with our results. Alprazolam was more stable in our study than in Langel's study.

Giovanni and Fucci [26] have described stability for some compounds in OF. Morphine was stable for 90 days and 6-MAM for 1 week at different temperatures. They also described another study that showed that flunitrazepam and 7-aminoflunitrazepam were unstable when the samples were stored in refrigerator for 48 h [27]. Amphetamines were studied in OF at 3 different temperatures for 10 weeks, and there was a time dependent degradation of the components. THC has also been described as an unstable compound. Giovanni and Fucci did not describe the temperatures or concentrations in the different studies, and neither if the samples were spiked samples or real, nor which collection devices that were used.

This study shows that many compounds in OF are stable, however 6-MAM, cocaine, and zopiclone were less stable. Transport by e.g., ordinary mail might be possible, although for some compounds and sampling kits this will lead to loss of compound. Analysis after up to a year for e.g., large epidemiological studies where thousands of samples are collected within a short period of time will be possible for most of the compounds tested, although loss of 6-MAM must be expected.

4. Conclusions

The developed screening method is intended for determination of drugs of abuse in OF. 32 compounds were analysed in 7 min (cycle time 9 min), a significant reduction from our previous method. The stability testing showed that 6-MAM, cocaine, and zopiclone were unstable the first week of the testing. The results were in accordance with earlier studies, and there was no significant difference in the long term stability in the two sampling kits we tested. In the testing for stability at short term, StatSure Saliva Sampler™ gave better results. The testing of 1 year of storage at −20 °C showed that most of the compounds were stable in both sampling kits. Samples of OF should be analysed as soon as possible after collection, and they should be kept frozen if immediate analysis is not possible.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.09.002.

References

[1] W. Bosker, M. Huestis, Clin. Chem. 55 (2009) 1910.
[2] O. Drummer, Clin. Biochem. Rev. 27 (2006) 147.
[3] Y. Caplan, B. Goldberger, J. Anal. Toxicol. 25 (2001) 396.
[4] N. Samyn, M. Laloup, G. De Boeck, Anal. Bioanal. Chem. 388 (2007) 1437.
[5] S. Simões, A. Ajenjo, J. Franco, D. Vieira, M. Dias, Rapid Commun. Mass Spectrom. 23 (2009) 1451.
[6] M. Sergi, E. Bafile, D. Compagnone, R. Curini, G. D'Ascenzo, F. Romolo, Anal. Bioanal. Chem. 393 (2009) 709.
[7] M. Concheiro, A. de Castro, Ó. Quintela, A. Cruz, M. López-Rivadulla, Anal. Bioanal. Chem. 391 (2008) 2329.
[8] M. Wood, M. Laloup, M. Fernandez, K. Jenkins, M. Young, J. Ramaekers, G. de Boeck, N. Samyn, Forensic Sci. Int. 150 (2005) 227.
[9] R. Dams, C. Murphy, R. Choo, W. Lambert, A. De Leenheer, M. Huestis, Anal. Chem. 75 (2003) 798.
[10] S. Chesnut, J. Salisbury, J. Sep. Sci. 30 (2007) 1183.
[11] M. Churchwell, N. Twaddle, L. Meeker, D. Doerge, J. Chromatogr. B 825 (2005) 134.
[12] D. Nguyen, D. Guillarme, S. Rudaz, J. Veuthey, J. Sep. Sci. 29 (2006) 1836.
[13] M. Swartz, J. Liquid Chromatogr. Relat. Technol. 28 (2005) 1253.
[14] N. Wu, A. Clausen, J. Sep. Sci. 30 (2007) 1167.
[15] N. Badawi, K. Simonsen, A. Steentoft, I. Bernhoft, K. Linnet, Clin. Chem. 55 (2009) 2004.
[16] M. Huestis, Clin. Chem. 55 (2008) 2079.
[17] I.-T. Wang, Y.-T. Feng, C.-Y. Chen, J. Chromatogr. B 878 (2010) 3095.
[18] E. Øiestad, U. Johansen, A. Christophersen, Clin. Chem. 53 (2007) 300.
[19] S. Strano-Rossi, L. Anzillotti, E. Castrignanò, M. Felli, G. Serpelloni, R. Mollica, M. Chiarotti, Anal. Bioanal. Chem. 401 (2011) 609.
[20] K. Langel, C. Engblom, A. Pehrsson, T. Gunnar, K. Ariniemi, P. Lillsunde, J. Anal. Toxicol. 32 (2008) 393.
[21] E. Øiestad, U. Johansen, Å. Øiestad, A. Christophersen, J. Anal. Toxicol. 35 (2011) 280.
[22] F.T. Peters, O.H. Drummer, F. Musshoff, Forensic Sci. Int. 165 (2007) 216.
[23] B. Matuszewski, M. Constanzer, C. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[24] K. Langel, T. Gunnar, K. Ariniemi, O. Rajamäki, P. Lillsunde, J. Chromatogr. B 879 (2011) 859.
[25] H. Gjerde, J. Mordal, A. Christophersen, J. Bramness, J. Mørland, J. Anal. Toxicol. 34 (2010) 204.
[26] N. De Giovanni, N. Fucci, Curr. Pharm. Anal. 4 (2008) 258.
[27] N. Samyn, G. De Boeck, V. Cirimele, A. Verstraete, P. Kintz, J. Anal. Toxicol. 26 (2002) 211.