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Advancing drug testing in oral fluid: Comparison of microflow and analytical flow LC-Orbitrap analysis

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ABSTRACT

Drug abuse is still rising and being a serious issue for healthcare systems and workplace safety amongst others. Fast, sensitive, and reliable methods to confirm abuse with straightforward analytical techniques are thus required. Microflow liquid chromatography (LC)-high-resolution tandem mass spectrometry (HRMS/MS) offering sensitivity and sustainability merits further investigation. Thus, this study aimed to develop a qualitative microflow-based analytical strategy and to evaluate it in comparison to analytical flow LC-HRMS/MS for the detection of abused drugs in oral fluid (OF).

The presented procedure allowed the detection of 20 abused drugs as well as nine of their metabolites. Liquid-liquid extraction proved to be suitable for all analytes except for the cannabinoids, which required solid-phase extraction. Analytical flow LC required higher injection volume (five microliter) and an increased flow rate (500 μL/min) while microflow LC allowed reduced injection volume (one microliter), reduced solvent consumption, and reduced flow rate (100 μL/min). Chromatographic separation using two Hypersil Gold C_{18} columns with different inner column diameters followed by Orbitrap-based MS resulted in better peak shapes and considering the different injection volumes to an increased analyte sensitivity with limits of identification between 0.2–25 ng/mL.

Microflow LC was shown to be suitable and sustainable in analytical toxicology for small molecule analysis in OF. The current procedure allowed the detection of 29 compounds while providing sufficient analytical performance also in comparison to analytical flow LC making it a valuable strategy in the clinical and forensic setting.

1. Introduction

The abuse of drugs has risen from 240 million in 2011 to 296 million in 2021 in the global population aged between 15 and 64 [1]. Cannabis and cocaine are most consumed in Europe, followed by e.g., synthetic stimulants, heroin, or new psychoactive substances [2]. To test for such drugs in the clinical setting, different matrices e.g., urine, blood plasma, exhaled breath, hair, or oral fluid (OF) can be used [3–7]. Particularly OF gained increasing interest in the past and is now widely implemented in the field of clinical and forensic toxicology e.g., in driving under the influence of drugs programs, therapeutic drug monitoring, or doping control [8]. OF offers several benefits such as rapid, non-invasive, and easy sample collection particularly beneficial for children and people with poor venous access. Furthermore, the privacy of the patient is not interfered and infection risks are decreased compared to blood sampling [9]. Detection windows in OF rely on the drug dosage, dosing frequency, or the individual metabolism but are often shorter than in urine. Most likely due to the fact that OF drug levels show a similar pharmacokinetic profile to blood and drug concentrations are expected to be lower than in urine [10]. However, due to the process of ion trapping, it can be expected that basic substances such as amphetamines are present in OF in higher concentrations and for longer times, as they are ionized after diffusion from the blood into the acidic environment of the OF (pH = 6.2–7.4) and then retained in the matrix [10,11]. Despite these benefits, it should be considered that a sufficient volume (at least 1 mL) of OF is often necessary and that the matrix must be in appropriate conditions [10]. The contamination of the oral cavity with food/beverages or by smoking, inhalation, or insufflation of substances can influence the consistency of the OF and thus the analysis result. Furthermore, the volume of collected OF can also be affected by e.g., the 'dry-mouth'

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syndrome caused by cigarette smoking or the consumption of specific drugs such as cannabis, amphetamines, antipsychotics, antidepressants, or antihistamines, which lead to a reduced salivary flow [9,10]. However, these benefits and limitations associated with OF also lead to the fact that blood plasma is still the matrix of choice for drug quantification as concentrations correlate best with pharmacological or toxic effects [12]. Due to the pH value, which is more acidic compared to the relatively constant blood pH of 7.4, the lipid solubility, the protein binding of compounds, or the change in the salivary flow, OF is well suited for qualitative assessment of drugs, but the drug concentrations in blood plasma may not be fully reflected [13]. Methods to detect abused drugs in OF are mainly based on mass spectrometry (MS) as this technique provides sufficient sensitivity, selectivity, and specificity [14]. A crucial part before MS analysis is chromatographic separation. Most laboratories are equipped with LC pumps amenable to analytical columns with flow rates higher than 200 μL/min and column ID of 2.1 mm [15]. Nanoflow liquid chromatography (LC) characterized by a flow rate < 1 μL/min and inner column diameters (ID) between 50–100 μm is preferred for proteomics as well as metabolomics workflows. Although analysis based on nanoscale offers increased sensitivity, gradient elution typically takes more than one hour making this approach not suitable within the context of e.g., emergency toxicology [16]. Another option for miniaturization is using microflow LC. It provides the opportunity of reducing the injection volume, solvent consumption, column back pressure, matrix effects, but can also lead to higher sensitivity and lower operational costs [17–19]. Only a limited number of reports are available evaluating microflow LC pros and cons for drug analysis, particularly with a focus on abused drugs [20,15]. This study aimed to address this gap of knowledge by developing and validating a microflow LC-based high-resolution tandem MS (LC-HRMS/MS) method for the analysis of 29 compounds in OF with a further focus on its comparison to a method using analytical flow LC-HRMS/MS.

2. Materials and methods

2.1. Chemicals

Alprazolam, 7-aminoclonazepam, buprenorphine hydrochloride, clonazepam, cocaine hydrochloride, codeine-d_6, delta-9-tetrahydrocannabinol (THC), diazepam, diazepam-d_5, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), ketamine hydrochloride, lorazepam, lysergic acid diethylamide (LSD), methamphetamine hydrochloride, methadone, morphine hydrochloride, norbuprenorphine, nordazepam, nortilidine, *O*-desmethyltramadol, oxycodone hydrochloride, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol-d_3 (THC-COOH-d_3), and tilidine hydrochloride hemihydrate were purchased from Merck (Darmstadt, Germany). Bromazepam, codeine phosphate, and tramadol hydrochloride were obtained from VWR International GmbH (Darmstadt, Germany), fentanyl, 3,4-methylenedioxymethamphetamine hydrochloride (MDMA), and THC-COOH from LGC Standards (Wesel, Germany), 6-monoacetylmorphine (6-MAM) from Cerilliant Corporation (Texas, USA), amphetamine sulfate and benzoylecgonine (BZE) from Lipomed (Arlesheim, Swisse). Solid-phase extraction (SPE) cartridges (ISOLUTE C18 500 mg/3mL) were obtained from Biotage (Uppsala, Sweden), centrifuge glasses from neoLab Migge GmbH (Heidelberg, Germany), and Quantisal™ Oral Fluid Collection Devices from Abbott Rapid Diagnostics GmbH (Cologne, Germany). Isopropanol was purchased from Fisher Scientific (Schwerte, Germany). Amber colored glass vials and all other chemicals (LC-MS or analytical grade) were bought from VWR International GmbH (Darmstadt, Germany). Water was purified with a Millipore filtration unit (18.2 Ω × cm water resistance) from Merck.

2.2. Stock and working solutions

Stock solutions of each analyte (1 mg/mL) as well as the internal standards (IS, 1 mg/mL) were separately prepared in methanol except for 7-aminoclonazepam and 6-MAM which were dissolved in acetonitrile (ACN). Dilutions of the compounds and the quality control (QC) sample containing all abused drugs were prepared in purified water and freshly spiked in blank OF prior to the application of 1 mL onto the Quantisal swab. The swab was then placed into the Quantisal buffer solution followed by shaking for 2 h at 1000 rpm at room temperature (+22 °C). Aqueous dilutions of the IS were spiked in the Quantisal buffer prior to sample preparation. All solutions were handled in amber colored glass vials and stored at −20 °C. Final concentrations of the abused drugs and the IS in the QC sample are given in Table S1.

2.3. LC-HRMS/MS conditions

OF samples were analyzed using a Thermo Fisher (TF) Vanquish Duo ultra-high performance LC system consisting of a degasser, a binary pump, and a dual split sampler HT (TF Scientific, TF, Dreieich, Germany) coupled to a TF Orbitrap Exploris 120 system equipped with a heated electrospray ionization source. The instrument was calibrated prior to analysis according to the manufacturer's recommendations using external mass calibration. Chromatographic separation was performed on two Hypersil Gold C_{18} columns with different ID (analytical column, 100 mm × 2.1 mm, 1.9 μm; microflow column, 100 mm × 1 mm, 1.9 μm) using eluent A (2 mM aqueous ammonium formate plus formic acid (0.1 %, pH 3)) and eluent B ACN:methanol (1:1, *v:v*) plus water (1 %, *v/v*), and formic acid (0.1 %, *v/v*)). The gradients programmed on both columns and HRMS/MS conditions are described in the Supplementary Material.

2.4. Sample preparation – Liquid-liquid extraction

Abused drugs except for THC, THC-COOH, and THC-COOH-d_3 were extracted according to a published procedure with some modifications [21]. A volume of 1 mL Quantisal buffer was pipetted into a 15 mL centrifuge glass, spiked with 10 μL codeine-d_6 and 10 μL diazepam-d_5 (100 ng/mL final concentration each) followed by the addition of 1 mL isopropanol and vortexing (5 sec). The liquid–liquid extraction (LLE) was performed using 1 mL of cyclohexane:ethyl acetate (1:1, *v:v*), the mixture was then shaken (1000 rpm, 5 min), centrifuged (2000 rpm, 5 min), and the top layer decanted into a fresh 2 mL reaction tube. After the LLE was repeated, both top layers were combined and evaporated under nitrogen until complete dryness at 70 °C and reconstituted in a mixture of purified water:methanol (80:20, *v:v*) followed by the injection of 5 μL (analytical flow setup) or 1 μL (microflow setup) onto the LC-HRMS/MS system.

2.5. Sample preparation – solid-phase extraction

THC, THC-COOH, and THC-COOH-d_3 were extracted using two modified SPE-based procedures [22,23]. First, a volume of 1 mL Quantisal buffer was spiked with 10 μL THC-COOH-d_3 (100 ng/mL final concentration) and diluted with 2 mL purified water followed by vortexing (5 sec). SPE cartridges were preconditioned with 1 mL methanol and 1 mL purified water followed by sample application. Three washing steps using 1 mL purified water, 1 mL sodium hydrogen carbonate solution (5 %), 1 mL purified water, and 200 μL acetone were performed followed by the application of full vacuum (1 min) prior to the two-step elution of the cannabinoids with 500 μL acetone each. Finally, full vacuum was applied again (30 sec). The extract was evaporated under nitrogen to complete dryness at 70 °C and reconstituted in an equal mixture of aqueous acetic acid (0.01 %, *v/v*) and methanol with acetic acid (0.01 %, *v/v*) followed by the injection of 5 μL (analytical flow setup) or 1 μL (microflow setup) onto the LC-HRMS/MS system.

2.6. Method validation

Method validation for the qualitative analysis of 29 abused drugs was performed using spiked OF samples (QC samples, analyte concentrations, see Table S1) according to international guidelines and recommendations including selectivity, carry-over, matrix effects (ME), recoveries (RE), and different stability data [24,25]. Recovery can be considered as the amount of analytes after sample workup compared to that of a solution containing the same initial amount of analytes [26]. Studies on co-eluting analytes, limits of identification (LOI), and detection (LOD) were additionally conducted [26–28]. Validation experiments, acceptance criteria, and software used for data handling are described in detail in the Supplementary Material.

2.7. Proof-of-concept

A total of 12 pooled OF samples were provided for reanalysis by Synlab MVZ Weiden (Germany). They were analyzed for the target analytes by comparing microflow and analytical flow LC-HRMS/MS.

3. Results

3.1. Method development and validation

Two procedures for the detection of 29 abused drugs including relevant metabolites extracted in OF based on microflow and analytical flow LC-HRMS/MS analysis were developed and validated with the further aim to compare the performance of both chromatographic settings. Two Hypersil Gold C_{18} columns differing in terms of the ID were chosen for chromatographic separation which was achieved within 16 min total runtime. Reconstructed ion chromatograms of the *m/z* of the abused drugs and IS extracted from OF and analyzed using microflow and analytical flow LC-HRMS/MS conditions are depicted in Figs. 1 and 2, respectively. Due to the adjustment of the injection volume and the flow rate, some LC parameters as well as the ion source settings of both LC setups had to be defined differently which are described in the Supplementary Material. Main analytical characteristics of the abused drugs are summarized in Table S2. Identification of the target compounds and IS was based on their protonated or deprotonated (*m/z* $[M+H]^+$ or $[M-H]^-$) precursor ion in full scan (FS) mode, the MS^2 spectrum, and the respective retention time. FS data of the compounds at the respective retention time were used to determine the LOD, while the criterion for defining the LOI was the presence of the MS^2 spectrum in addition to the FS peak. Determined LOD and LOI as well as cut-off concentrations reported are given in Table 1.

LLE proved to be suitable for all target analytes except for the cannabinoids THC and THC-COOH, where an SPE-based extraction procedure was required. Reproducibility of the LLE and SPE was given as all coefficients of variations (CV) were below ± 15 % except for THC where a CV of 21 % was calculated after analysis using the analytical column (see Table S3). Ion enhancement was detected for EDDP (40 %) in the presence of bromazepam on the analytical column and for methadone (48 %) in the presence of alprazolam and nordazepam on the microflow column.

Selectivity was given as no interferences were detected at the respective retention time. Carry-over was not observed after injection of an OF sample spiked with 500 ng/mL of the target compounds each. ME, RE as well as corresponding CVs for both LC setups are shown in Table 2. ME ranged between 27–156 % (analytical flow LC) and 16–123 % (microflow LC). Regarding reproducibility, CVs were ≤ 25 % except for methadone using microflow LC with a CV of 26 %. RE ranged between 1.3–17 % (analytical flow LC) and 1.2–16 % (microflow LC) with reproducible CVs ≤ 25 %. Aqueous stock and working solutions of the abused drugs indicated to be stable over eight weeks stored at −20 °C.

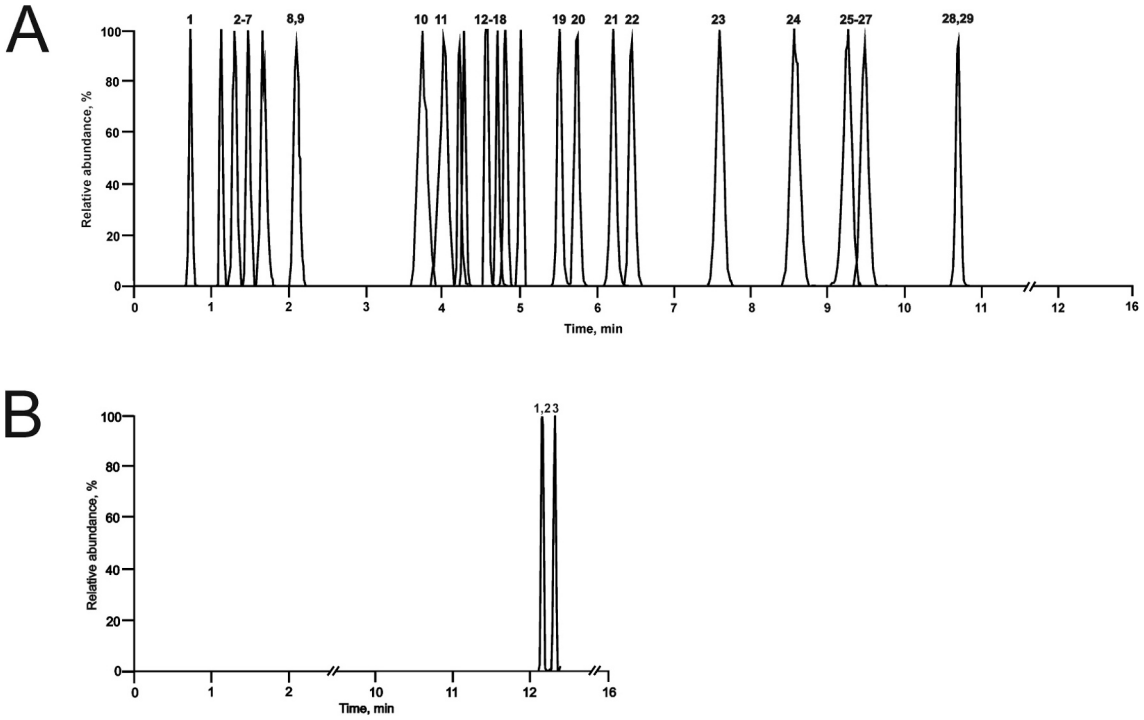

Fig. 1. Reconstructed ion chromatograms of the *m/z* (mass-to-charge ratio) of the abused drugs and internal standards (IS) spiked in blank OF followed by microflow LC-HRMS/MS analysis. All peaks at 100 % relative abundance. A) Extraction using liquid–liquid extraction (100 ng/mL each): Morphine (1), codeine (2), codeine-d_6 (3), oxycodone (4), 6-MAM (5), ODMT (6), amphetamine (7), MDMA (8), methamphetamine (9), 7-aminoclonazepam (10), BZE (11), ketamine (12), tramadol (13), cocaine (14), tilidine (15), nortilidine (16), LSD (17), norbuprenorphine (18), fentanyl (19), bromazepam (20), buprenorphine (21), EDDP (22), clonazepam (23), lorazepam (24), alprazolam (25), methadone (26), nordazepam (27), diazepam (28), diazepam-d_5 (29) B) Extraction using solid-phase extraction (50 ng/mL each): THC-COOH (1), THC-COOH-d_3 (2), THC (3).

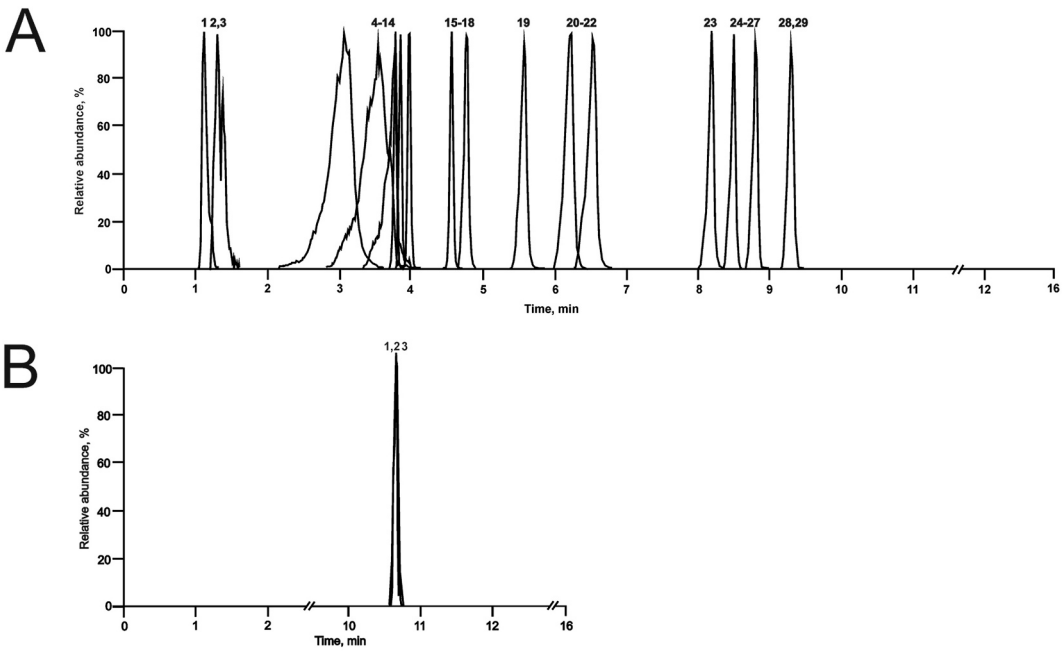

Fig. 2. Reconstructed ion chromatograms of the *m/z* (mass-to-charge ratio) of the abused drugs and internal standards (IS) spiked in blank OF followed by analytical flow LC-HRMS/MS analysis. All peaks at 100 % relative abundance. A) Extraction using liquid–liquid extraction (100 ng/mL each): Morphine (1), codeine (2), codeine-d_6 (3), oxycodone (4), 6-MAM (5), amphetamine (6), ODMT (7), MDMA (8), methamphetamine (9), ketamine (10), BZE (11), 7-aminoclonazepam (12), tramadol (13), cocaine (14), tilidine (15), nortilidine (16), LSD (17), norbuprenorphine (18), fentanyl (19), buprenorphine (20), bromazepam (21), EDDP (22), clonazepam (23), lorazepam (24), methadone (25), alprazolam (26), nordazepam (27), diazepam (28), diazepam-d_5 (29), B) Extraction using solid-phase extraction (50 ng/mL each): THC-COOH (1), THC-COOH-d_3 (2), THC (3).

Reanalysis of processed samples can be performed within 24 h stored in the autosampler (+20 °C, see Table S4) except for nordazepam which indicated a decrease > 15 %. In the Quantisal buffer, degradation > 15 % was observed for EDDP, methamphetamine, and tilidine if stored in the refrigerator (24 h, +4 °C, see Table S4), and for EDDP, THC, and tilidine if stored at room temperature (24 h, +22 °C, see Table S4). After one freeze and thaw cycle, all analytes remained stable (24 h, −20 °C, see Table S4). Storage in the Quantisal buffer solution over four weeks at −20 °C is not recommended for THC and THC-COOH as the concentration of both compounds decreased more than 15 % (see Table S4). Results of the stability validation experiments are summarized in Table S4.

3.2. Proof-of-concept

Table S5 summarizes the detection results obtained after analyzing 12 pooled OF samples using microflow and analytical flow LC-HRMS/MS. Due to the limited volume available, the SPE-based extraction was performed for sample ID 1–4 which revealed the consumption of THC. Sample ID 5–12 were extracted using LLE indicating consistent results in terms of analyte detection apart from norbuprenorphine which was not detectable using the analytical column (sample ID 6 and 7).

4. Discussion

Chromatographic separation was performed on Hypersil Gold C_{18} columns only differing in the ID (analytical column, ID 2.1 mm; microflow column, ID 1 mm). Compared to the analytical flow LC setup using flow rates between 250 to 500 μL/min, the micro-LC flow rate could be reduced to 100 μL/min, which decreased the solvent consumption. Additionally, reduced flow rates also led to reduced mobile phase entering the electrospray ionization (ESI) source and less contamination of the mass spectrometer equipment. He et al. focused on the detection and quantification of THC and THC-COOH in OF using microflow LC-Orbitrap analysis [29]. To achieve sufficient sensitivity with lower limits of quantification (LLOQ) down to 7.5 pg/mL, extracts had to be first subjected to an aQ trapping column for sample clean-up followed by chromatographic separation using an aQ LC column operated at a flow rate reduced to 20 μL/min. In the current study, the flow rate was set to 100 μL/min as the microflow-based method was used for analysis of all 29 compounds. Elution gradients applied on both columns vary particularly regarding the starting conditions to avoid pressure issues on the LC system, which also led to deviating retention times of some analytes as indicated in Figs. 1 and 2 and Table S2. Baseline separation could not be achieved for all compounds, but the impact of co-elution was tested resulting in ion enhancement for methadone on the microflow column and for EDDP on the analytical column. However, the analyte signals were not suppressed by their co-eluting compounds reducing the risk of not being identified. Importantly, the respective LOD and LOI for methadone and EDDP may be even lower if co-elution is avoided, which was not tested further. As clearly illustrated in Figs. 1 and 2, most of the analyte peaks detected on the microflow column elute with a smaller peak width than on the analytical column for several reasons. During method development, different analyte solvents (methanol, ACN, purified water) were tested regarding peak shapes. Purified water offered the best chromatographic performance on the microflow column and was therefore chosen together with a small amount of methanol as reconstitution solvent. Peak broadening also occurs e.g., due to higher injection volumes, higher ID, or slightly different flow rates. Chromatographic separation on the analytical column starts with 10 % of organic solvent, which might explain the broader peak shapes particularly for codeine, oxycodone, and 6-MAM. Additionally, the injection volume and the ID were higher compared to the microflow LC setup.

In contrast to blood plasma, where reference concentration ranges are known, cut-off concentrations are reported in OF or proposed by different agencies for drug-testing programs including driving under the influence of drugs or workplace testing [30]. These cut-off values, summarized in Table 1, were considered when evaluating the sensitivity of both LC setups [31–33]. LOD and LOI experiments were performed

Table 1
Limit of detection (LOD) and limit of identification (LOI) determined for each analyte ($n = 3$) as well as cut-off concentrations reported by the Substance Abuse and Mental Health Services Administration (SAMHSA), the European Workplace Drug Testing Society (EWDTS), and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). *m/z*, mass-to-charge ratio; n.d., not determined; –, not reported; BZE, benzoylecgonine; THC, delta-9-tetrahydrocannabinol; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; LSD, lysergic acid diethylamide; MDMA, 3,4-methylenedioxymethamphetamine; 6-MAM, 6-monoacetylmorphine; THC-COOH, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol; ODMT, *O*-desmethyltramadol.

Analyte	LOD, ng/mL		LOI, ng/mL		Cut-off concentration, ng/mL		
	Analytical column	Microflow column	Analytical column	Microflow column	SAMHSA	EWDTS	EMCDDA
Alprazolam	0.2	0.2	0.2	0.2	–	10	3.5
7-Aminoclonazepam	1.0	1.0	1.0	1.0	–	10	3.1
Amphetamine	10	25	10	25	25	30	360
Bromazepam	1.0	2.0	1.0	2.0	–	10	–
Buprenorphine	5.0	1.0	1.0	1.0	–	1.0	–
BZE	2.0	2.0	1.0	2.0	8.0	8.0	95
Clonazepam	1.0	1.0	1.0	1.0	–	10	1.7
Cocaine	0.2	1.0	0.2	1.0	8.0	8.0	170
Codeine	10	10	10	10	15	2.0	94
Codeine-d_6	n.d.	n.d.	n.d.	n.d.	–	–	–
THC	25	25	25	25	2.0	2.0	27
Diazepam	1.0	0.2	1.0	0.2	–	3.0	5.0
Diazepam-d_5	n.d.	n.d.	n.d.	n.d.	–	–	–
EDDP	1.0	0.2	1.0	1.0	–	20	–
Fentanyl	1.0	1.0	1.0	1.0	–	–	–
Ketamine	1.0	0.2	1.0	0.2	–	–	–
Lorazepam	1.0	2.0	1.0	2.0	–	3.0	1.1
LSD	1.0	1.0	1.0	2.0	–	–	–
MDMA	5.0	5.0	10	5.0	25	30	270
Methamphetamine	2.0	2.0	5.0	2.0	25	30	410
Methadone	1.0	1.0	1.0	1.0	–	20	22
6-MAM	5.0	5.0	5.0	5.0	2.0	2.0	16
Morphine	1.0	1.0	5.0	2.0	15	2.0	95
Norbuprenorphine	2.0	2.0	5.0	2.0	–	1.0	–
Nordazepam	1.0	2.0	1.0	2.0	–	3.0	1.1
Nortilidine	1.0	1.0	1.0	1.0	–	–	–
ODMT	1.0	2.0	1.0	2.0	–	–	–
Oxycodone	10	2.0	5.0	5.0	15	–	–
THC-COOH	5.0	5.0	5.0	5.0	–	–	–
THC-COOH-d_3	n.d.	n.d.	n.d.	n.d.	–	–	–
Tilidine	1.0	1.0	1.0	1.0	–	–	–
Tramadol	0.2	0.2	0.2	0.2	–	–	–

with fortified OF samples (0.2, 1, 2, 5, 10, 25 ng/mL). The extracts were injected onto the microflow column and afterwards onto the analytical column. When evaluating and interpreting the LOD and LOI, the different injection volumes (analytical flow LC, 5 μL; microflow LC, 1 μL) need to be considered, as the analyte concentration in the extract remains the same for both columns, but the absolute amount of substance applied on the analytical column is higher due to the fivefold higher injection volume. Thereby, microflow LC demonstrated to be more sensitive than analytical flow LC. Furthermore, microflow LC was used to achieve the cut-off concentrations for those compounds reported with some exceptions. THC e.g., could be detected down to 25 ng/mL on the microflow column. The LOD and LOI of THC did not meet the target values given by the Substance Abuse and Mental Health Services Administration and the European Workplace Drug Testing Society. This might be related to the presence of ion suppression or interferences e.g., by unknown compounds from the Quantisal buffer, not being removed during the SPE or to the low RE.

The used extraction strategy was based on described OF-based sample preparation procedures for the extraction of abused drugs [21,34–36]. The double LLE performed by Coulter et al. proved to be suitable to extract all analytes sufficiently except for THC and THC-COOH. To minimize health risks, hexane was replaced by cyclohexane in the current study for LLE and both cannabinoids had to be extracted via SPE [22,23]. Despite the performance of the SPE, RE for the cannabinoids remained low as indicated in Table 2. Another approach for the quantification of THC and THC-COOH was established by Concheiro et al. [37]. Quantisal buffer and OF (4:1) were mixed followed by SPE extraction and LC-HRMS/MS analysis. They achieved LLOQ of 0.5 ng/mL (THC) and 0.015 ng/mL (THC-COOH) and RE of 94 % (THC) and 72 % (THC-COOH). However, these different sample pretreatments have clearly to be considered when evaluating LOD and RE. In addition to the SPE applied in this study, the method developed by Scheidweiler et al. for the detection of the cannabinoids was also tested. In contrast to this study, sample extracts were analyzed using LC-triple quadrupole/linear ion trap MS which may be a reason for different LOD and LOI. Consequently, due to the usage of less extraction solvents, we only considered their reconstitution mixture because of enhanced peak shapes [23]. Moreover, SPE-based extraction, which would cover all compounds, was not further investigated in order to keep this approach more feasible e.g., in emergency situations, as performing LLE is faster than SPE.

As reported, LC-HRMS/MS with ESI is very prone to ME. Although sample clean-up can reduce matrix components which might interfere, ME are also dependent on the bio-fluid being analyzed. Proteins, amino acids, immunoglobulins, or mucins are present in OF leading to ion suppression or enhancement [38]. However, ME observed during method validation were reproducible with CVs $\leq$ 25 %. The ion enhancement of methadone on the microflow column might explain the CV of 26 %. Microflow LC is also associated with the reduction of ME [17]. However, it must be considered that the presence of ME depends on factors such as co-eluting matrix components and the compound itself.

Cocaine indicated stability issues if stored in the Quantisal buffer at room temperature (+22 °C). OF can accelerate the hydrolytic degradation of cocaine to BZE, especially at elevated temperatures. As no corresponding increase in the peak area of BZE was observed, when analyzing patient samples, the results of cocaine and BZE should be both considered [39]. Method validation revealed that storage at −20 °C was considered most suitable to avoid degradation for EDDP,

Table 2
Matrix effects (ME), recoveries (RE), and CVs of the 29 abused drugs in OF ($n = 6$) after microflow LC-HRMS/MS and analytical flow LC-HRMS/MS analysis. LC-HRMS/MS, liquid chromatography-high-resolution mass spectrometry; CV, coefficient of variation¸ BZE, benzoylecgonine; THC, delta-9-tetrahydrocannabinol; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; LSD, lysergic acid diethylamide; MDMA, 3,4-methylenedioxymethamphetamine; 6-MAM, 6-monoacetylmorphine; THC-COOH, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol; ODMT, *O*-desmethyltramadol.

Analyte	Matrix effect, %, (CV, %)		Recovery, %, (CV, %)	
	Analytical column	Microflow column	Analytical column	Microflow column
Alprazolam	114 (14)	81 (25)	15 (11)	13 (15)
7-Aminoclonazepam	56 (10)	50 (20)	10 (12)	7.4 (15)
Amphetamine	125 (10)	109 (22)	1.3 (13)	1.2 (9.0)
Bromazepam	156 (15)	85 (15)	13 (14)	13 (12)
Buprenorphine	100 (11)	95 (24)	10 (10)	10 (12)
BZE	35 (12)	46 (23)	5.2 (19)	3.9 (12)
Clonazepam	111 (12)	93 (23)	12 (11)	10 (15)
Cocaine	93 (10)	61 (21)	15 (13)	13 (15)
Codeine	27 (9.1)	16 (24)	14 (20)	14 (15)
THC	58 (15)	67 (18)	4.8 (13)	2.6 (14)
Diazepam	98 (11)	63 (22)	9.9 (11)	9.4 (15)
EDDP	112 (11)	79 (22)	7.6 (19)	7.7 (14)
Fentanyl	66 (13)	70 (20)	11 (13)	9.6 (15)
Ketamine	53 (10)	61 (25)	17 (14)	16 (15)
Lorazepam	137 (15)	82 (24)	12 (15)	10 (12)
LSD	92 (6.4)	28 (22)	14 (9.2)	13 (5.0)
MDMA	77 (6.9)	80 (19)	2.3 (8.8)	2.0 (12)
Methamphetamine	81 (7.0)	80 (19)	2.0 (7.3)	1.9 (14)
Methadone	109 (14)	88 (26)	11 (13)	10 (14)
6-MAM	67 (15)	50 (20)	15 (15)	13 (14)
Morphine	65 (14)	39 (13)	3.8 (13)	2.5 (4.1)
Norbuprenorphine	78 (9.7)	123 (23)	10 (7.4)	8.5 (15)
Nordazepam	120 (13)	78 (23)	7.7 (12)	6.8 (15)
Nortilidine	91 (11)	52 (22)	14 (13)	12 (15)
ODMT	74 (9.1)	58 (22)	12 (13)	11 (13)
Oxycodone	79 (13)	56 (18)	12 (9.2)	8.5 (14)
THC-COOH	77 (12)	84 (24)	10 (11)	10 (12)
Tilidine	88 (14)	55 (22)	14 (20)	12 (15)
Tramadol	73 (7.8)	64 (22)	15 (11)	14 (13)

methamphetamine, and tilidine. Results of the stability studies for the cannabinoids THC and THC-COOH were consistent with published findings, as +4 °C and −20 °C were found to be ideal storage temperatures and higher temperatures increase degradation [23,39]. However, Djilali et al. also described surface adsorption of the cannabinoids on polypropylene plastic containers which might be responsible for a loss during long-term storage at −20 °C [40].

The comparison of microflow with analytical flow LC-HRMS/MS using pooled OF samples demonstrated that microflow LC-HRMS/MS allowed a higher detection rate than analytical flow LC-HRMS/MS. Summarized in Table S5, norbuprenorphine was only detectable after microflow LC (sample ID 6 and 7) possibly due to an increased sensitivity. Reconstructed ion chromatograms exemplified for pooled sample ID 6 obtained after microflow LC-HRMS/MS analysis is depicted in Fig. S1. The oral mucosa can be contaminated with THC after cannabis smoking or vaporization leading to higher concentrations in OF compared to its inactive metabolite THC-COOH. This metabolite is reported to be more detectable in plasma as it enters the oral fluid in small quantities via the mucous membranes [41]. This might be related to the detection of THC only (sample ID 1–4), but not THC-COOH. In addition, OF samples had to be pooled to achieve the required volume for sample extraction, which may affect sensitivity and thus detection results due to sample dilution. Nevertheless, the abuse of cannabis could be proved apart from the LC configuration applied. OF also served as a suitable matrix to determine heroin consumption (sample ID 11). In urine, misinterpretations might arise when analyzing morphine-related compounds. The detection of morphine (or its glucuronide) can also reflect the intake of heroin, codeine, ethylmorphine, or morphine itself [42]. However, heroin is metabolized to 6-MAM as an indicative marker, which is more frequently detectable in OF than in urine due to its short half-life [10].

Limitations have to be considered particularly for THC and THC-COOH. An additional SPE must be performed and due to the low RE, reported cut-off concentrations may not be achieved. Moreover, LC pumps must be tested for changing pressure profiles (analytical flow LC, 420 bar; microflow LC, 330 bar) and the required LC equipment (e.g., columns) must be available. Nevertheless, microflow LC strikes the balance between sufficient sensitivity and greater sustainability, and this approach can be extended to other matrices, making it worth exploring and useful in analytical laboratories.

5. Conclusions

We report first data comparing microflow LC-HRMS/MS with analytical flow LC-HRMS/MS for the analysis of 29 abused drugs in OF as alternative sample matrix. The natural cannabinoids THC and THC-COOH had to be enriched with SPE, while all other compounds only required LLE extraction. Twelve pooled OF samples were analyzed to successfully prove the applicability of the developed procedures but also indicated that microflow LC reached a higher overall detection rate than analytical flow LC. Results, with exception of THC and THCCOOH, indicate that the LC miniaturization towards microflow LC is promising to overcome challenges of analytical flow LC. It provides more sustainability by offering enhanced peak shapes, lower solvent consumption, reduced injection volumes, and higher sensitivity for most compounds. The current study demonstrated that microflow-based LC-HRMS/MS analysis shows suitability for clinical or forensic applications and should be considered as alternative to analytical flow LC-HRMS/MS.

Ethical approval

Not applicable.

CRediT authorship contribution statement

Aline C. Vollmer: Writing – review & editing, Writing – original

draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Lea Wagmann:** Writing – review & editing, Conceptualization. **Armin A. Weber:** Writing – review & editing, Methodology. **Markus R. Meyer:** Writing – review & editing, Supervision, Resources, Investigation, Data curation, Conceptualization.

Informed consent

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.113508.

Data availability

Data will be made available on request.

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