Journal of Chromatography Open 1 (2021) 100006

Contents lists available at ScienceDirect

Journal of Chromatography Open

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

Quantitative analysis of fentanyl, several analogues and metabolites in urine by parallel artificial liquid membrane extraction and liquid chromatography tandem mass spectrometry analysis

Flaminia Vincenti[a,b], Camilla Montesano[a,*], Simone Gobbi[a], Manuel Sergi[c], Roberta Curini[a], Dario Compagnone[c]

[a] Department of Chemistry, Sapienza University of Rome, Rome 00185 Italy
[b] Department of Public Health and Infectious Disease, Sapienza University of Rome, Rome 00185, Italy
[c] Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, 64100, Italy

ARTICLE INFO

Keywords:
Parallel artificial liquid membrane extraction (PALME)
Fentanyl
Synthetic opioids
Urine
LC-MS/MS

ABSTRACT

The rapid introduction of new psychoactive substances (NPS) has definitively changed the drug market. Among the several NPS that were identified in the last decades, fentanyl and its analogues deserve special attention. These are synthetic opioids with high potency and are associated with increasing number of deaths; for this reason, forensic toxicologists are paying close attention to these analytes and sensitive analytical methods for their detection in biological samples of drug users are needed.

The aim of this study was the development of a LC–MS/MS method for the determination of fentanyl, 23 analogues and metabolites in urine by exploiting parallel artificial liquid membrane extraction (PALME). This technique was shown to be particularly suitable for fentanyl extraction and allowed to obtain a high enrichment factor by using a few microliters of organic solvent (1-octanol) immobilized into a polyvinylidene fluoride (PVDF) membrane. The extraction was carried out on a 96 well plate providing high laboratory throughput.

The applied strategy allowed to measure concentrations ranging from 0.1 ng mL^{-1} for fentanyl and most analogues to 5 ng mL^{-1} for metabolites, by using an entry level mass spectrometer. Because of the different concentration levels generally found in real samples, linearity was studied in different ranges i.e. LOQ to 50 ng mL^{-1} for parent drugs and LOQ to 200 ng mL^{-1} for metabolites. All the validation parameters were found within the imposed limits, and notably matrix effect was not significant for all the analytes, showing the selectivity achieved by PALME extraction.

1. Introduction

In the last years illicit drug market is rapidly evolving, due to the introduction of new psychoactive substances (NPS) which are increasingly consumed among drug users [1]. Their chemical structures are constantly updated, trying to stay ahead of the national and international banning laws [2]. Amid the several NPS chemical classes, synthetic opioids and especially fentanyl and its analogues deserve special attention. In fact, fentalogs, as sometimes are referred these drugs, are associated with increasing number of deaths, especially in the USA; COVID-19 pandemic even worsened this trend both in North America [3] and Europe [4]. New fentanyl derivatives are continuously appearing in the market [5] so that forensic toxicologists are required to keep up with this ever changing market with effective analytical methods.

The analytical determination of fentalogs in biological matrices requires sensitive and accurate techniques, such as mass spectrometry (MS) coupled with separative techniques including gas (GC–MS) and liquid chromatography (LC–MS); on the other hand, sample pre-treatment may be of utmost importance in defining the detectable concentrations, the time required for analysis and the overall quality of the data in term of reproducibility and accuracy. In fact, sample preparation is crucial to remove interfering compounds from complex matrix such as biological specimens while maintaining high recoveries of analytes; several strategies consent at the same time to obtain high enrichment factors, making possible the detection of trace amounts of analytes even with poor sensitivity instruments. In the last years, different approaches were proposed for the determination of fentanyl and/or fentalogs and metabolites in urine, involving several sample preparation procedures such as liquid liquid extraction (LLE) [6–10], solid phase extraction (SPE) [11–14] or innovative miniaturized techniques such as single drop microextraction (SDME) [15], dispersive liquid liquid extraction (dLLME), hollow fiber liquid phase microextraction (HF-LPME) [16] and more recently micro extraction on packed sorbent (MEPS) [17]. All these procedures are gen-

* Corresponding author.
E-mail address: camilla.montesano@uniroma1.it (C. Montesano).

https://doi.org/10.1016/j.jcoa.2021.100006
Received 30 July 2021; Received in revised form 6 September 2021; Accepted 6 September 2021

erally limited to a few compounds and require an amount of organic solvent ranging from hundreds of μL to several mL. The most comprehensive methods [18,19] encompassed several fentanyls and metabolites; in these cases urine was 30 times diluted, making possible analyte detection only with extremely sensitive instruments.

Parallel artificial liquid membrane extraction (PALME) is a novel liquid phase micro extraction (LPME) based technique, characterized by a high-throughput and a few μL organic solvent consumption, perfectly adhering to the principles of green chemistry [20]. The proposed technique is based on a supported liquid membrane (SLM) in a 96-well plate configuration offering the possibility of processing several samples together. Extraction is based on a three phases system, exploiting a pH gradient to selectively transfer basic/acidic analytes from the original matrix to an acidic/basic aqueous extracting phase with an intermediate passage into an organic solvent immobilised within the pores of a membrane. The aqueous extract is directly compatible with LC–MS/MS and high enrichment factor are possible with no issues in term of matrix effect. PALME was firstly proposed in 2013 by Gjelstad et al. [21] for the extraction of pethidine, nortriptyline, methadone and haloperidol in plasma. More recent publications include other pharmaceuticals [22–26] and also NPS belonging to the piperazine, phenetylamine, cathinone [27] and benzodiazepine classes [28] in plasma, whole blood and dried blood spot [29]. To the best of our knowledge the use of PALME for urine pretreatment has never been reported to date, neither for fentalogs or in general synthetic opioids. The advantage of this matrix for toxicological applications are well-known in addition it can be easily collected, and high volumes are normally available, making possible a significant enrichment of analytes through PALME extraction. For most drugs, analyte concentration in urine is generally high; for fentalogs this is true for metabolites, while parent drugs concentration is normally in the low ng mL^{-1} or pg mL^{-1} range.

The aim of this study was the development of a new method for the determination of fentanyl, 23 analogues and metabolites in urine by exploiting PALME extraction and LC–MS/MS analysis. We showed that PALME is particularly suitable for fentanyl extraction, as a result of their basic nature, and can be easily adapted to new analogues and different matrices. The high enrichment factor obtained by means of the selected extraction strategy allowed to measure concentrations down to pg mL^{-1} for several fentalogs with an entry level mass spectrometer. The method was validated following SWGTOX guidelines.

2. Experimental

2.1. Chemicals

Standards of acetyl fentanyl (hydrochloride), acetyl norfentanyl (hydrochloride), acrylfentanyl (hydrochloride), alfentanyl (hydrochloride), butyryl fentanyl (hydrochloride), butyryl fentanyl carboxy metabolite, carfentanil, despropionyl para-Fluorofentanyl, fentanyl (hydrochloride), furanyl fentanyl (hydrochloride), methoxyacetyl norfentanyl (hydrochloride), norfentanyl, ocfentanil (hydrochloride), ortho/para-fluorofentanyl (hydrochloride), remifentanil (hydrochloride), sufentanyl (citrate), valeryl fentanyl carboxy metabolite, 4-ANPP, α-methyl fentanyl (hydrochloride), α-methyl thiofentanyl (hydrochloride), β-hydroxy fentanyl (hydrochloride), (±)-cis-3-methyl fentanyl (hydrochloride), (±)-cis-3-methyl thiofentanyl (hydrochloride) e (±)-trans-3-methyl norfentanyl (hydrochloride) and labeled internal standards fentanyl-d_5 (hydrochloride) e norfentanyl-d_5 were purchased from Cayman Chemical (Ann Arbor, MI, USA) in the form of methanolic solutions at 100 μg mL^{-1}.

Working solutions at 1 μg mL^{-1} were prepared for each analyte in methanol by diluting the original stock solution and were kept at -20 °C. A mixture of all the analytes was also prepared at the same concentration.

Solvents including, methanol, ethanol, 2-propanol and acetonitrile were of HPLC grade formic acid and were purchased from Sigma-Aldrich (Milwakee, WI, USA). All other solvents i.e. 1-octanol, dodecylacetate, trioctylamine (TOA) and dihexyl ether as well as sodium carbonate, sodium bicarbonate, sodium chloride, sodium hydroxide and ammonium formate were from the same purchaser.

Ultrapure water was produced by a Milli-Q Plus apparatus from Millipore (Bedford, MA, USA)

2.2. PALME extraction

Extraction was carried out by means of a 96-well MultiScreen-IP filter plate with polyvinylidene fluoride (PVDF) membranes with 0.45 mm pore size (Merck KGaA, Darmstadt, Germany) and a thickness of 100 μm; each well included the SLM in contact with the acceptor solution. The donor plate consisted of a 96-well polypropylene plate with 2.2 mL wells from Brand GMBH (Wertheim, Germany) and was clamped together with the acceptor plate for extraction.

PALME involves a few simple passages, briefly 950 μL of urine were mixed with 950 μL of carbonate buffer 0.5 M (pH 11) containing fentanyl-d5 at 10 ng mL^{-1} and norfentanyl-d5 at 20 ng mL^{-1} and inserted into the donor plate. 0.2 g of sodium chloride were added at this stage, homogenization was assured by vortex mixing. Afterwards, the acceptor plate, whose membrane was previously washed with ethanol and water as suggested by the purchaser, was placed upon the donor plate and 3 μL of 1-octanol containing 1% of trioctylamine was pipetted into the porous membrane to form the SLM. After a few minutes the acceptor solution constituted by 95 μL of formic acid 0.1% in H_2O:MeOH, 80:20 (v:v) was added. Finally, the acceptor plate was sealed; for extraction the whole assemblage was placed on an orbital shaker at 480 rpm for 120 min at room temperature. Three μL of the acceptor solution were finally injected into the HPLC–MS/MS system.

2.3. HPLC-MS/MS analysis

Liquid chromatography was performed on a HPLC Series 200 Micro-LC Pump and a Series 200 autosampler from Perkin Elmer (Norwalk, CT, USA). An API 2000 triple quadrupole from AB-Sciex (Toronto, ON, Canada) was used for mass spectrometry detection. Analytes were separated using a Kinetex F5 column (10 cm × 2.1 mm ID) from Phenomenex (Torrance, CA, USA) packed with core–shell particles of 2.6 μm. The column was kept at 30°C by means of an oven ThermaSphere™ TS-130 HPLC Column from Phenomenex® (Torrance, CA, USA). Acetonitrile:methanol, 75:25 (v:v) + 0.05% formic acid (A) and 5 mM ammonium formate + 0.1% formic acid in water (B), were used as mobile phases at flow rate of 0.2 mL min^{-1}.

Gradient scheme for the separation was as follows: phase A was kept at 0% for 0.5 min and then increased from 0 to 35% in 1.5 min, from 35% to 40% in 9 min; in the following 0.5 min phase A was brought to 100%, maintained in these conditions for 0.5 min and then re-equilibrated to the initial 0% in 3 min. The total run time was of 15.5 min.

All the analytes were detected in positive ionization, with a capillary voltage of 5000 V, nebulizer gas (air) at 35 psi, turbo gas (nitrogen) at 65 psi and curtain gas at 20 psi; the source temperature was 400 °C. For each analyte two multiple reaction monitoring (MRM) transitions were selected. MRM experiments were segmented in four windows in order to get enough data points across the peaks. All source and instrument parameters for the monitored analytes were tuned by injecting each single standard solution at a concentration of 50 ng mL^{-1} at 10 μL min^{-1} by a syringe pump. All the source parameters have been checked in flow injection analysis with the same chromatographic conditions. Peak areas for the selected ions were determined using AB-Sciex package Analyst 1.4 and quantitation was performed by the internal standard method. The selected transitions, together with the main LC–MS/MS parameters, are reported in Table 1.

Table 1
Liquid Chromatography- tandem mass spectrometry parameters for the selected analytes and internal standards (Rt: retention time; Q1: precursor ion mass; DP: declustering potential; EP: entrance potential; Q3: product ion mass; CE: collision energy; CXP: cell exit potential).

Analyte	Time window	Rt (min)	Q1 (amu)	DP (V)	FP (V)	EP (V)	Q3 (amu)	CE (V)	CXP (V)
Methoxyacetyl norfentanyl	I	3.38	249.3	50	385	10	84.0	26	11
							105.8	34	17
Acetyl norfentanyl	I	3.46	219.3	52	385	9	84.0	27	10
							55.0	50	5
Norfentanyl	I	3.80	233.3	40	385	10	84.0	29	11
							56.0	40	6
(±)-trans-3-methyl Norfentanyl	I	4.07	247.3	51	385	10	97.9	27	14
							68.9	42	8
Remifentanyl	II	5.05	377.3	30	400	9	112.9	40	18
							228.1	27	13
Butyryl fentanyl carboxy metabolite	II	5.62	381.8	60	385	11	105.2	55	10
							188.3	35	9
Valeryl fentanyl carboxy metabolite	II	5.83	395.3	80	385	12	188.4	36	8
							105.2	64	11
β-hydroxy Fentanyl	II	6.01	353.3	53	400	10	204.2	31	10
							91.0	62	10
Acetyl fentanyl	II	6.23	323.4	70	385	10	105.2	50	15
							188.4	32	10
Ocfentanil	II	6.29	371.3	60	400	10	188.2	33	9
							105.2	60	14
Alfentanyl	II	6.70	417.4	43	385	10	268.4	25	13
							197.2	36	9
Acrylfentanyl	III	7.54	335.3	50	400	10	188.2	32	8
							105.2	51	14
α-methyl Thiofentanyl	III	7.67	357.3	41	400	12	124.9	40	19
							96.8	74	11
Fentanyl	III	7.70	337.4	52	385	12	105.2	51	15
							188.2	32	9
(±)-cis-3-methyl Thiofentanyl	III	8.10	357.3	45	400	11	208.2	34	12
							110.9	52	16
4-ANPP	III	8.18	281.3	45	385	10	105.2	43	14
							78.9	55	9
Furanyl fentanyl	IV	8.84	375.4	55	385	10	188.2	32	8
							105.2	55	15
p-fluorofentanyl	IV	8.96	355.3	52	400	9	105.2	58	12
							188.2	34	10
α-methyl Fentanyl	IV	8.97	351.3	50	400	10	91.0	75	12
							202.3	31	10
(±)-cis-3-methyl Fentanyl	IV	9.46	351.3	400	400	11	105.2	55	10
							202.2	34	8
Despropionyl para-Fluorofentanyl	IV	9.55	299.3	60	385	9	105.2	45	15
							188.3	26	9
Butyryl fentanyl	IV	9.94	351.4	77	385	12	188.2	33	10
							105.2	55	13
Carfentanyl	IV	10.03	395.3	66	385	11	335.4	25	16
							246.2	31	13
Sufentanyl	IV	10.94	387.3	31	400	9	238.2	29	12
							110.9	55	16
Fentanyl-d_5	III	7.70	347.2	50	400	12	105.2	51	15
Norfentanyl-d_5	I	3.80	238.3	31	400	10	84.0	30	12

2.4. Validation

The method was validated according to SWGTOX guidelines [30], by considering the following parameters: selectivity, linearity, recovery, precision, accuracy, matrix effect, limits of detection (LODs) and limits of quantification (LOQs). Both calibration standards and quality control samples (QC) were prepared in a pool of drug-free urine, using samples obtained from five different subjects.

2.4.1. Selectivity and carry-over

Selectivity was tested by analyzing five different samples of blank urine and verifying the absence of analyte signal. The possible interference deriving from ISs was assessed by adding the ISs mixture to five urine samples before extraction.

Carry-over was evaluated by injecting blank samples before and after the highest calibrator sample for five time and verifying the absence of target analyte signal in blank samples.

2.4.2. Identification, linearity and dilution integrity

For each compound, two product ions were selected; positive identification involved the detection of both fragments at a retention time within 2% from that of a standard and with quantitative to qualitative ion ratio within 20%. Linearity was evaluated from LOQ to 50 ng ml^{-1} for parent fentalogs and from LOQ to 200 ng mL^{-1} for metabolites; calibrator solutions were prepared at nine levels of concentration. Each point was analyzed five times. Analyte responses were normalized to internal standards and calibration was achieved by weighted linear least-squares regression (weighting factor 1/x). The squared determination coefficient (R^2) was used to roughly estimate linearity, while lack-of-fit test was used to verify the appropriateness of the linear model (95% significance level).

Dilution integrity was assessed by spiking urine samples with analyte concentrations exceeding the linear range. These samples were diluted with blank urine before analysis and processed as described above; three dilution factors, 5, 10 and 50

were tested and five determinations were performed for each level.

2.4.3. Limits of detection and quantification

To evaluate LODs and LOQs, drug-free urine samples from five different sources were spiked with a solution containing all the analytes at decreasing concentrations. All samples were processed and analyzed in duplicate over three separate runs. The background signal was recorded by analyzing a drug-free urine sample. LODs were established as the lowest concentration of a drug that provided a signal to noise ratio (S/N) equal or greater than 3 for the lower MRM transition and fulfiled the identification criteria. Limits of quantification (LOQs) were estimated likewise as the smallest concentration that gave a S/N ≥10. At LOQ, in addition to the verification of the identification criteria, RSD% and accuracy were required to be within ± 20% ($n = 5$).

2.4.4. Precision and accuracy

Precision and accuracy were calculated by using QCs samples at three concentration levels, i.e. LOQ value, 10 and 50 ng mL^{-1}. According to SWGTOX, precision was calculated as the relative standard deviation (RSD% = SD/mean × 100). Intra-day precision (repeatability) was estimated for each analyte at each concentration, from the areas of five independent QCs spiked before extraction; inter-day precision (reproducibility) was studied by analyzing five QCs samples at each concentration on five different days. Accuracy was calculated in terms of bias, as the relative deviation (%) of the mean concentration ($n = 5$), calculated by using a freshly prepared calibration curve, with respect to the corresponding spiked concentration. RSD% and accuracy were accepted at maximum values of 15% or 20% near LOQ.

2.4.5. Recovery and matrix effect

Extraction recovery (R%) was determined at two different concentrations, i.e. 5 and 50 ng mL^{-1}. Ten urine samples, obtained from different workers and family in the laboratory, were used for each concentration levels. Each matrix source was spiked with the appropriate amount of standard solutions and processed by PALME extraction, while an identical number of blank samples were processed and spiked with the same amount of standard solution after the extraction step. Recovery was calculated as the ratio of the mean peak area of the samples fortified before PALME (A) and the mean peak area of the samples fortified after extraction (B). R% = A/B × 100. To evaluate any potential interfering compounds included in the sample matrix, matrix effect (ME) was calculated for each analyte by comparing the mean peak areas in solvent (C) with the mean peak area in post extraction fortified samples. Accordingly, ME% = B/C × 100. Variability of matrix effect was evaluated by calculating at each concentration the RSD% of the calculated ME% values.

3. Results and discussion

3.1. LC–MS/MS method development

To obtain a satisfying separation of the analytes two columns with different retention mechanism were tested: Kinetex XB-C18 and Kinetex F5, both 100 × 2.1 mm (ID) and packed with 2.6 μm core-shell particles.

The second column clearly demonstrated a better resolution of the peaks and was selected for this reason. Different combinations of mobile phases were then tested to obtain a suitable separation of the analytes mainly focusing on the isobaric species (i.e. α-methyl fentanyl, cis-3-methyl fentanyl and butyryl fentanyl) for whom a baseline separation was obtained (Fig. 1). To achieve this result, the use of a binary organic phase (methanol/acetonitrile) was found as a key variable. The necessary peak resolution was obtained by using acetonitrile:methanol with a ratio 75:25 (v:v). On the other hand, as expected the addition of formic acid improved both peak shape and intensity by favoring positive ionization in ESI–MS.

For what concerns MS detection, two MRM transitions were optimized for each analyte by individual infusion. Due to the high number of analytes and taking into account the minimal settable dwell time for the employed mass spectrometer, it was necessary to separate the run in four acquisition windows; this expedient allowed to acquire at least 10 points per peak and was also beneficial in term of peak intensity.

3.2. Evaluation of PALME operational parameters

Several parameters may influence the recoveries in PALME: the pH of the acceptor and donor solutions, the eluent strength of the acceptor, the ionic strength of the donor, the solvent used in the SLM, the extraction time and agitation speed rate, among others. All these parameters were thoroughly evaluated in order to achieve good recoveries for the selected analytes. An univariate approach was used, for each tested condition urine samples ($n = 3$) were spiked at 5 ng mL^{-1} and absolute recovery was calculated by comparing peaks area obtained by the analysis of extracted urine with a reference sample at 50 ng mL^{-1} in 0.1% formic acid in water:methanol, 80:20 (v:v)

Given the basicity of fentanyl and fentalogs (pka ranging from 7.5 for remifentanil to 10.2 for trans-3-methyl Norfentanyl) a pH gradient from basic to acidic pH was required to transfer the analytes from urine to the donor solution above the SLM. Analytes in urine must be in their protonated form so that a pH above the pKa value is essential. As expected, the pH of the donor and acceptor solution were showed to be a central parameter for a successful extraction. Basic pH was achieved by diluting urine 1:1 with a carbonate buffer 0.5 M (pH 11); the use of sodium hydroxide 1 M was also attempted but remifentanil was not stable in these conditions. The addition of 0.2 g NaCl to the donor solution was useful in order to increase both the recovery and the reproducibility of the procedure, in fact ionic strength was shown to notably influence the partition between donor solution and organic solvent immobilized in the membrane as a consequence of salting out effect; given that urine may have a great variability due to the physiological nature of the sample, the addition of a constant amount of salt was found to have a leveling effect.

For what concern the acceptor, the acidic pH was achieved by adding formic acid at 0.1%, acceptor volume was set at 95 μL so that an enrichment factor of exactly 10 was obtained. Acceptor solution might be totally aqueous, however the addition of small amount of water-soluble organic solvents could be beneficial to improve analyte partitioning towards the acceptor phase. In this context, experiments with slight percentages of methanol (i.e. 10–20%) in the acceptor solution were carried out and a clear increase of the recoveries, especially for the last eluting analytes was obtained by increasing the methanol amount, so that 0.1% formic acid in water:methanol (80:20) was selected. Larger amounts of methanol were not tested in order to avoid partial solubility of the acceptor solution with the organic solvent serving as SLM.

Regarding the SLM, several solvents were tested. A suitable solvent must meet different requirements. First of all, it must have a good affinity with the analytes, it must have a low volatility and be water immiscible. In addition, it was previously reported by Vardal et al. [27] that the addition of trioctylamine 1% (TOA) in the selected organic solvent could be beneficial to avoid non-specific binding to the PVDF material. The effect of TOA was confirmed in our experiments (Fig. 2), however for most analytes it was not as notable as reported in the cited study.

As showed in Fig. 2, recoveries obtained with 1-octanol were excellent for all fentalogs and significantly lower for the metabolites. In order to try increasing the recoveries for these last compounds, other solvents were tested to impregnate the membrane, i.e. dodecylacetate and dihexyl eter. These solvents were selected as they were previously reported to be suitable for PALME and to be effective for the extraction of several drugs [23]. The results are shown in Fig. 3; only a slight increase of recoveries for parent compounds was obtained, while a significant worsening was found for metabolites, which are clearly more polar and have a lower partioning in the organic solvent, 1-octanol was

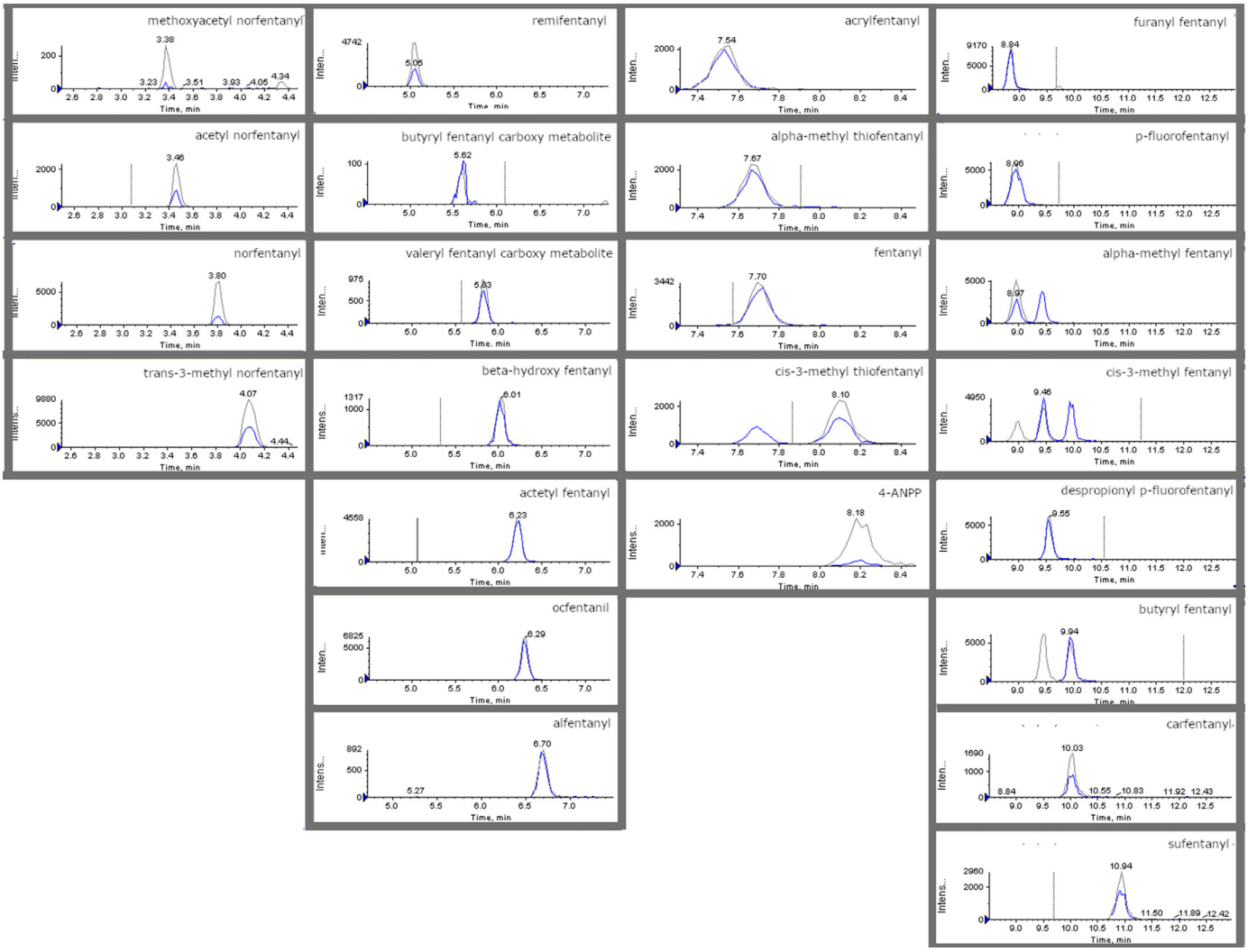

Fig.1. Extracted ion currents for the selected analytes obtained from the analysis of a spiked urine sample at 5 ng/mL-1.

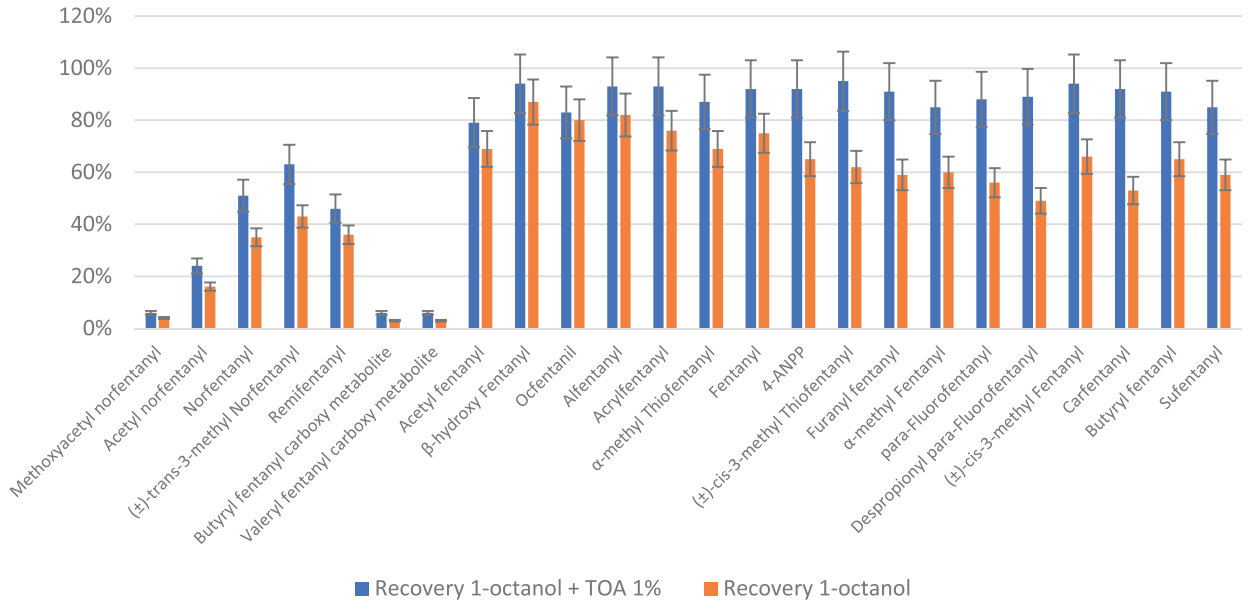

Fig.2. Effect of trioctylamine (TOA) 1% on extraction recovery.

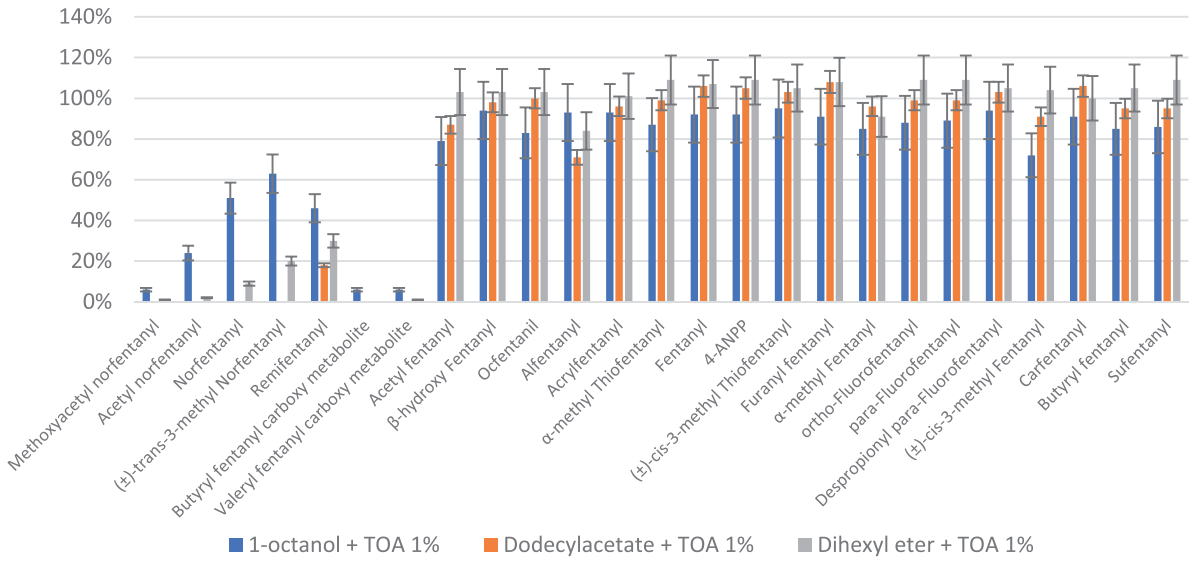

Fig.3. Recovery values obtained with different organic solvent into the porous membrane to form the SLM.

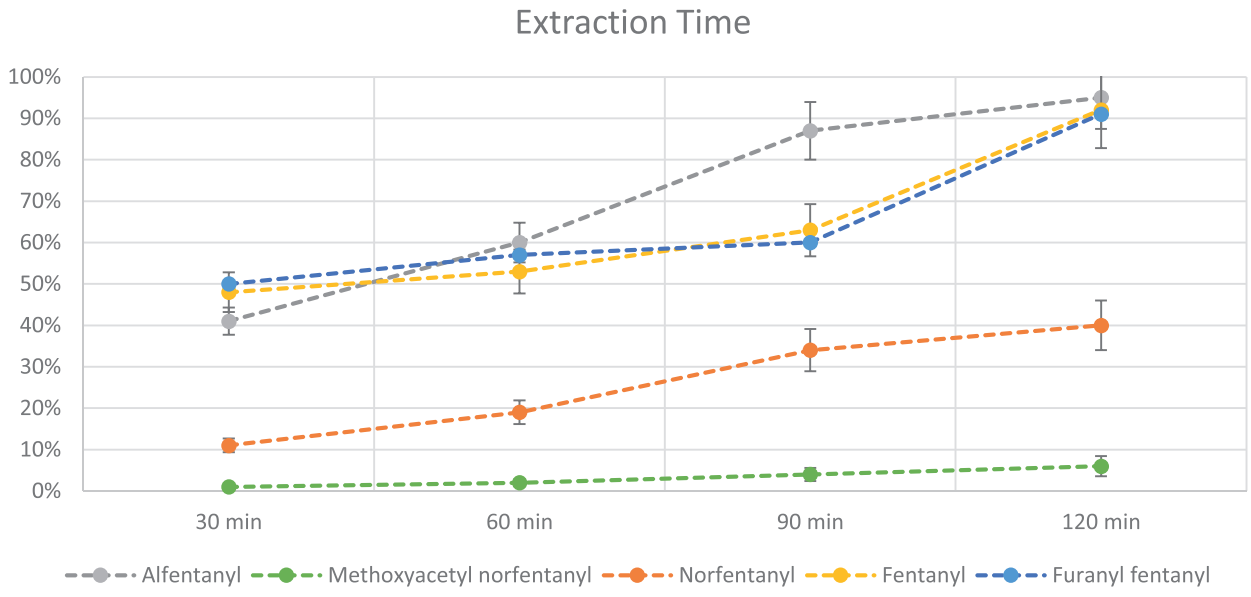

Fig. 4. Recovery as a function of extraction time for five representative analytes.

finally selected for SLM formation. It must be observed that metabolites are generally more concentrated than parent compounds in real samples, so the lower recoveries were not deemed as a great issue and were considered acceptable.

Extraction time was finally investigated in fortified urine samples. Extraction recoveries were evaluated at four time points, i.e. 30 min, 60 min, 90 min and 120 min (n = 3). The results obtained are reported in Fig. 4 for some representative analytes. In general, it appeared that most analytes had slow kinetics with no equilibrium established even after 120 min. Just a few analytes (i.e. alfentanil, remifentanil and the metabolites) reached equilibrium after 90 min of extraction. Vardal et al. [27] observed that kinetics was mainly related to log P, with polar molecules having a slower kinetic, however a clear relationship between extraction kinetics and molecular properties was not established in that study. In our experiments we observed an opposite trend with the metabolites and the fentalogs with lower log P reaching an equilibrium at 90 min. An extraction time of 120 min was finally selected, longer extraction times were not tested to avoid further increase of analysis time, considering that recoveries for most analytes were deemed satisfactory.

3.3. Validation

The described method for the detection and quantification of 23 fentalogs and metabolites was validated according to SWGTOX guidelines and all the measured parameters were found within the imposed limits.

No significant signals of the selected analytes resulted from the analysis of blank urine proving a good selectivity of the method; also, the ISs did not show relevant signals for the corresponding non-deuterated analytes.

LOQ ranged from 0.1 ng mL^{-1} for most parent compounds to 5 ng mL^{-1} for methoxyacetyl norfentanyl (Table 2). These values are generally in agreement with those reported in the literature, Busardò et al. reported considerably lower limits with a top level mass spectrometer. According to the concentrations normally reported in real cases metabolites concentrations are tens to hundreds of times higher than parent compounds, so that higher LOQ for metabolites are not an issue. On the other hand the sensitivity obtained for fentanyl and fentalogs is generally satisfactory to detect real sample concentrations that were shown to span from less than 1 ng mL^{-1} for example for fentanyl to several

hundreds of ng mL^{-1} for metabolites [18]. Lower concentrations were reported for carfentanyl in a few cases [31]. The achievement of low limits of detection, such as those obtained in this study, is than particularly important for these analytes, because of their potency and consequently low active concentrations.

Because of the generally different concentration levels between parent and metabolites investigated in this study, linearity was studied in different ranges i.e. LOQ to 50 ng mL^{-1} and LOQ to 200 ng mL^{-1} for metabolites. Heteroscedasticity was evaluated by using an *F*-test on the lowest and highest calibration levels (95% significance level) and a weighting factor equal to (1/x) was adopted for all analytes. Linearity of the curves was confirmed using lack-of-fit and Mendel tests, satisfying SWGTOX guidelines criteria.

In the presence of samples exceeding the upper limit of quantification (ULOQ), which is represented by the highest calibrator value, dilution would be required. Dilution integrity was assessed by preparing urine samples spiked at 1000, 2000 and 10,000 ng mL^{-1} (5 samples for each concentration) and diluted 1:5, 1:10 and 1:50, respectively; accuracy and precision was within 15% for all the analytes in the fifteen samples.

Precision and accuracy values calculated at LOQ, medium and high concentrations are listed in Table 2, and always fall within the established limits (15%). Recoveries and matrix effects, calculated at two concentration values are shown in the same table. Recoveries are among 6 and 70%, even if they are quite low for metabolites, they are reproducible. Matrix effect is not significant for all the tested analytes; even if urine is a relatively simple matrix it must take into account that the sample was enriched up to 10 times, so the results nicely show that PALME is a selective extraction technique. This is mainly due to the three-phase system which includes several extraction stages, i.e. liquid liquid partitioning between the sample and the organic solvent, a physical barrier regulated by the pore size of the membrane and the pH gradient which only consent to extract basic analytes.

Autosampler stability of processed samples was confirmed by analyzing the extracts at $t = 0$, 2, 6, 24 h and 48 h. Specific studies of short and long term stability were not carried out since fentanyls were previously shown to be stable in urine at -20 °C for at least 2 months, only remifentanil was found to be unstable at higher temperatures [12]. For this reason it is recommended to maintain urine samples at -20 °C if not immediately analyzed .

4. Conclusions

In this paper we described the development of a new method for the quantitative analysis of fentanyl, 23 analogues and metabolites in urine by PALME extraction and LC–MS/MS analysis. A particular focus was dedicated to the extraction procedure, which was showed to be suitable for the pre-treatment of urine before instrumental analysis, especially when the target analytes may be in low concentration. Urine is a matrix normally available in considerable volume, so a high enrichment may be achieved, as shown in this study. Very often, sample preparation is neglected for urine samples and a simple dilution may be involved, however such strategy is not adequate when sensitivity may be an issue. Fentanyl and its analogues are analytes that are gaining close attention in the forensic laboratories and, due to their potency, sensitive analytical methods are required for their detection in biological samples, being their circulating concentration generally low. The use of the latest mass spectrometers may be an option for low concentrations detection, however high sensitivity may be achieved even with entry level instruments as long as sample enrichment is obtained during pretreatment. The achieved limits of quantification make the presented method suitable for application in forensic laboratories. A limitation of our study was that no samples from drug users were analyzed, however taking into account the literature data about the expected concentrations in human urine, we expect that the developed method would be suitable for fentanyls detection and quantification in real samples

In our opinion, PALME has a high potential in forensic toxicology, being a green, low cost and high-throughput technique, several drugs of abuse show basic properties and are then prone to be extracted by this technique. The workflow involved is very convenient on a laboratory scale considering that 96 samples can be processed simultaneously in a couple of hours with reduced manual processes; following the extraction, the acceptor plate can be rapidly transferred to a suitable plate and directly loaded into the autosampler.

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.

CRediT authorship contribution statement

Flaminia Vincenti: Investigation, Data curation. **Camilla Montesano:** Writing – original draft, Methodology, Conceptualization. **Simone Gobbi:** Visualization, Investigation. **Manuel Sergi:** Conceptualization, Writing – review & editing. **Roberta Curini:** Supervision, Writing – review & editing. **Dario Compagnone:** Writing – review & editing.

References

[1] Peacock A, Bruno R, Gisev N, Degenhardt L, Hall W, Sedefov R, White J, Thomas KV, Farrell M, Griffiths P. New psychoactive substances: challenges for drug surveillance, control, and public health responses. Lancet 2019. doi:10.1016/S0140-6736(19)32231-7.

[2] Zaami S. New psychoactive substances: concerted efforts and common legislative answers for stemming a growing health hazard. Eur Rev Med Pharmacol Sci 2019;23:9681–90. doi:10.26355/eurrev_201911_19529.

[3] Manchikanti L, Vanaparthy R, Atluri S, Sachdeva H, Kaye AD, Hirsch JA. COVID-19 and the opioid epidemic: two public health emergencies that intersect with chronic pain. Pain Ther 2021;10:269–86. doi:10.1007/s40122-021-00243-2.

[4] di Gaudio F, Mortali C, Tini A. Opioid epidemic spread from Northern and Eastern Europe to Mediterranean Area. Clin Ther 2021;172:209–10. doi:10.7417/CT.2021.2315.

[5] Vincenti F, Pagano F, Montesano C, Sciubba F, Di Cocco ME, Gregori A, Rosa FD, Lombardi L, Sergi M, Curini R. Multi-analytical characterization of 4-fluoro-furanyl fentanyl in a drug seizure. Forensic Chem 2020;21. doi:10.1016/j.forc.2020.100283.

[6] Huynh NH, Tyrefors N, Ekman L, Johansson M. Determination of fentanyl in human plasma and fentanyl and norfentanyl in human urine using LC-MS/MS. J Pharm Biomed Anal 2005;37:1095–100. doi:10.1016/j.jpba.2004.09.024.

[7] M. Gergov, P. Nokua, E. Vuori, I. Ojanpera, Simultaneous screening and quantification of 25 opioid drugs in post-mortem blood and urine by liquid chromatography – tandem mass spectrometry, 186 (2009) 36–43. 10.1016/j.forsciint.2009.01.013.

[8] Cooreman S, Deprez C, Martens F, Van Bocxlaer J, Croes K. A comprehensive LC-MS-based quantitative analysis of fentanyl-like drugs in plasma and urine. J Sep Sci 2010;33:2654–62. doi:10.1002/jssc.201000330.

[9] Rojkiewicz M, Majchrzak M, Celiński R, Kuś P, Sajewicz M. Identification and physicochemical characterization of 4-fluorobutyrfentanyl (1-((4-fluorophenyl)(1-phenethylpiperidin-4-yl)amino)butan-1-one, 4-FBF) in seized materials and post-mortem biological samples. Drug Test Anal 2017;9:405–14. doi:10.1002/dta.2135.

[10] Coopman V, Cordonnier J, De Leeuw M, Cirimele V. Ocfentanil overdose fatality in the recreational drug scene. Forensic Sci Int 2016;266:469–73. doi:10.1016/j.forsciint.2016.07.005.

[11] Thevis M, Geyer H, Bahr D, Schänzer W. Identification of fentanyl, alfentanil, sufentanil, remifentanil and their major metabolites in human urine by liquid chromatography/tandem mass spectrometry for doping control purposes. Eur J Mass Spectrom 2005;11:419–27. doi:10.1255/ejms.761.

[12] Wang L, Bernert JT. Analysis of 13 fentanils, including sufentanil and carfentanil, in human urine by liquid chromatography-atmospheric-pressure ionization-tandem mass spectrometry. J Anal Toxicol 2006;30:335–41. doi:10.1093/jat/30.5.335.

[13] Poklis J, Poklis A, Wolf C, Mainland M, Hair L, Devers K, Chrostowski L, Arbefeville E, Merves M, Pearson J. Postmortem tissue distribution of acetyl fentanyl, fentanyl and their respective nor-metabolites analyzed by ultrahigh performance liquid chromatography with tandem mass spectrometry. Forensic Sci Int 2015;257:435–41. doi:10.1016/j.forsciint.2015.10.021.

[14] Shoff EN, Zaney ME, Kahl JH, Hime GW, Boland DM. Qualitative identification of fentanyl analogs and other opioids in postmortem cases by UHPLC-ion trap-MSn. J Anal Toxicol 2017. doi:10.1093/jat/bkx041.

[15] H. Ebrahimzadeh, Y. Yamini, A. Gholizade, A. Sedighi, S. Kasraee, Determination of fentanyl in biological and water samples using single-drop liquid – liquid – liquid microextraction coupled with high-performance liquid chromatography, 6 (2008) 193–199. 10.1016/j.aca.2008.07.047.

[16] Saraji M, Khalili Boroujeni M, Hajialiakbari Bidgoli AA. Comparison of dispersive liquid-liquid microextraction and hollow fiber liquid-liquid-liquid microextraction for the determination of fentanyl, alfentanil, and sufentanil in water and bi-

ological fluids by high-performance liquid chromatography. Anal Bioanal Chem 2011;400:2149–58. doi:10.1007/s00216-011-4874-x.
[17] da Cunha KF, Rodrigues LC, Huestis MA, Costa JL. Miniaturized extraction method for analysis of synthetic opioids in urine by microextraction with packed sorbent and liquid chromatography—tandem mass spectrometry. J Chromatogr A 2020;1624:461241. doi:10.1016/j.chroma.2020.461241.
[18] Busardò FP, Carlier J, Giorgetti R, Tagliabracci A, Pacifici R, Gottardi M, Pichini S. Ultra-high-performance liquid chromatography-tandem mass spectrometry assay for quantifying fentanyl and 22 analogs and metabolites in whole blood, urine, and hair. Front Chem 2019;7:184. doi:10.3389/fchem.2019.00184.
[19] Mannocchi G, Pirani F, Gottardi M, Sirignano A, Busardò FP, Ricci G. Determination of nine new fentanyl analogues and metabolites in consumers' urine by ultra-high-performance liquid chromatography-tandem mass spectrometry. Eur Rev Med Pharmacol Sci 2021;25:4394–9. doi:10.26355/eurrev_202106_26149.
[20] . In: Ahluwalia VK, Kidwai M, editors. Basic principles of green chemistry BT - new trends in green chemistry. the Netherlands, Dordrecht: Springer; 2004. p. 5–14. doi:10.1007/978-1-4020-3175-5_3.
[21] Gjelstad A, Rasmussen KE, Parmer MP, Pedersen-Bjergaard S. Parallel artificial liquid membrane extraction: micro-scale liquid-liquid-liquid extraction in the 96-well format. Bioanalysis 2013;5:1377–85. doi:10.4155/bio.13.59.
[22] Roldán-Pijuán M, Pedersen-Bjergaard S, Gjelstad A. Parallel artificial liquid membrane extraction of acidic drugs from human plasma. Anal Bioanal Chem 2015;407:2811–19. doi:10.1007/s00216-015-8505-9.
[23] Olsen KN, Ask KS, Pedersen-Bjergaard S, Gjelstad A. Parallel artificial liquid membrane extraction of psychoactive analytes: a novel approach in therapeutic drug monitoring. Bioanalysis 2018;10:385–95. doi:10.4155/bio-2017-0250.
[24] Ask KS, Lid M, Øiestad EL, Pedersen-Bjergaard S, Gjelstad A. Liquid-phase microextraction in 96-well plates - calibration and accurate quantification of pharmaceuticals in human plasma samples. J Chromatogr A 2019;1602:117–23. doi:10.1016/j.chroma.2019.06.013.
[25] Pilařová V, Sultani M, Ask KS, Nováková L, Pedersen-Bjergaard S, Gjelstad A. One-step extraction of polar drugs from plasma by parallel artificial liquid membrane extraction. J Chromatogr B Anal Technol Biomed Life Sci 2017;1043:25–32. doi:10.1016/j.jchromb.2016.09.019.
[26] Ask KS, Bardakci T, Parmer MP, Halvorsen TG, Øiestad EL, Pedersen-Bjergaard S, Gjelstad A. Parallel artificial liquid membrane extraction as an efficient tool for removal of phospholipids from human plasma. J Pharm Biomed Anal 2016;129:229–36. doi:10.1016/j.jpba.2016.07.011.
[27] Vårdal L, Askildsen HM, Gjelstad A, Øiestad EL, Edvardsen HME, Pedersen-Bjergaard S. Parallel artificial liquid membrane extraction of new psychoactive substances in plasma and whole blood. J Chromatogr B Anal Technol Biomed Life Sci 2017;1048:77–84. doi:10.1016/j.jchromb.2017.02.010.
[28] Vårdal L, Wong G, Øiestad ÅML, Pedersen-Bjergaard S, Gjelstad A, Øiestad EL. Rapid determination of designer benzodiazepines, benzodiazepines, and Z-hypnotics in whole blood using parallel artificial liquid membrane extraction and UHPLC-MS/MS. Anal Bioanal Chem 2018;410:4967–78. doi:10.1007/s00216-018-1147-y.
[29] Ask KS, Øiestad EL, Pedersen-Bjergaard S, Gjelstad A. Dried blood spots and parallel artificial liquid membrane extraction–a simple combination of microsampling and microextraction. Anal Chim Acta 2018;1009:56–64. doi:10.1016/j.aca.2018.01.024.
[30] Scientific working group for forensic toxicology. SWGTOX Stand Pract Method Valid Forensic Toxicol 2013. doi:10.1093/jat/bkt054.
[31] Brunetti P, Pirani F, Carlier J, Giorgetti R, Busardò FP, Lo Faro AF, 2017-2019 A. Update on acute intoxications and fatalities from illicit fentanyl and analogs. J Anal Toxicol 2021;45:537–54. doi:10.1093/jat/bkaa115.