WP2 - Qualitative and quantitative analysis of new psychoactive substances (NPS) in Europe, with focus on synthetic opioids and prescriptions opioids

Deliverable 7

Analytical procedure for quantitative analysis of NPS and prescription opioids

Different analytical methods were developed and validated to perform the quantitative analysis of (i) new psychoactive substances (NPS) in urban wastewater (WW) and pooled urine samples and (ii) prescription opioids in urban WW.

(i) NEW PSYCHOACTIVE SUBSTANCES (NPS)

Selection of NPS

A list of "priority NPS" was selected considering the NPS most frequently and recently recorded in the drug market, during seizures and overdose cases, according to the alert reports (2018-2020) from the Early Warning System of UNODC, EMCDDA and national EWS of Italy. This list has been updated every 6-months within the project (last update in September 2021). Among all "priority NPS", 63 NPS biomarkers were selected (Table 1) considering their presence in urban WW previously reported, their stability in this matrix, and the availability of analytical standards for identification and quantitation. Nineteen fentanyl analogues were included in the study, together with the prescription opioid fentanyl and its main metabolite norfentanyl. The other NPS were 20 synthetic cathinones, 8 phenethylamines, 6 synthetic cannabinoids, 3 tryptamines, 2

arylcyclohexylamine/ketamine analogue, 1 synthetic opioid, 1 aminorex derivative, and 1 piperidine/pyrrolidine.

In the case of fentanyl analogues, both parent substances and metabolites were selected as NPS biomarkers in WW. For the rest of NPS only parent drugs were considered because of their unknown metabolism and/or the limited availability of analytical standards.

Table 1. Biomarkers of new psychoactive substances (NPS) selected for investigation, divided by categories, and labeled internal standards used for quantitation. Metabolites are *in italic*.

Biomarkers to be monitored			**Internal Standards**
Categories	**Number of NPS**	**NPS (Abbreviation)**	
Synthetic opioids (fentanyl analogues)	21	**Fentanyl** ***Norfentanyl*** Acetylfentanyl *Acetylnorfentanyl* Alfentanyl Butyrylfentanyl *Butyrylfentanyl carboxy metabolite* *Butyrylnorfentanyl* Carfentanyl Cyclopropylfentanyl *despropionylfentanyl (4-ANPP)* *despropionyl-para-fluorofentanyl (4-F-ANPP)* Furanylnorfentanyl Beta-hydroxyfentanyl beta-hydroxythiofentanyl *Methoxyacetylnorfentanyl* *trans-3-methyl norfentanyl* *cis-3-methyl norfentanyl* Ocfentanil Phenylacetylfentanyl *Valerylfentanyl carboxy metabolite*	Fentanyl-D5 norfentanyl-D5 amphetamine-D6 (AMP-D6) methamphetamine-D9 (METHAMP-D9), 25-B-NBOMe-D3 25-C-NBOMe-D3 25-I-NBOMe-D3 ρ-methoxymethamphetamine-D3 (PMMA-D3) α-pyrrolidinovalerophenone (α-PVP-D8) mephedrone-D3 (MEPH-D3) methylone (METL-D3) buthylone (BUTL-D3) naphyrone (NAPH-D5) 3,4-Methylenedioxypyrovalerone-D8 (MDPV-D8) methoxetamine-D3 (MXE-D3)
Phenethylamines	8	25-B-NBOMe 25-C-NBOMe 25-I-NBOMe 25-iP-NBOMe Ephenidine (NEDPA) para-methoxyamphetamine (PMA)	

		para-methoxymethylamphetamine (PMMA) 6-(2-aminopropyl)benzofuran (6-APB)	
Synthetic cathinones	20	Buphedrone (BUPH) Butylone (BUTL) 4-Cl-α-pyrrolidinopropiophenone (4-Cl-α-PPP) Dimethylcathinone (DCAT) 3,4-dimethylmethcathinone (3,4-DMMC) Ethcathinone (ETHC) Ethylone (ETHL) 3,4-methylenedioxypyrovalerone (MDPV) 4-fluoromethcathinone (4-FMC) 4-methylethcathinone (4-MEC) Mephedrone, 4-methylmethcathinone (MEPH) Methcathinone (METC) Methedrone (METD) Methylone (METL) 3-methylmethcathinone (3-MMC) 1-Naphyrone (1-NAPH) Naphyrone (NAPH) Pentedrone (PENTD) Pentylone (PENTL) α-pyrrolidinovalerophenone (α-PVP)	
Synthetic cannabinoids	6	5-fluoropentyl-3-pyridinoylindole (5-Fpentyl-3-pyr) JWH-122 AB-CHMINACA ABD-FUBINACA CUMYL-PeGLACONE MDMB-CHMICA	
Synthetic opioid	1	Isotonitazene	
Arylcyclohexylamine	2	Methoxetamine (MXE) 2-Fluorodechloroketamine (2-FDCK)	
Aminorex derivative	1	4,4-dimethylaminorex (4,4-DMAR)	
Tryptamines	3	5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) N,N-dimethyltryptamine (N,N-DMT)	

		5-methoxy-N-isopropyl-N-methyltryptamine (5-MeO-MiPT)	
Piperidine and pyrrolidine	1	Ethylphenidate	

Sample preparation

Sample (pre)treatment protocols for urban WW and pooled urine have been adapted from previous works (Castiglioni et al, 2021; Gjerde et al, 2019) and applied both for qualitative (Deliverable 6) and quantitative analysis of NPS (present deliverable).

Urban wastewater. WW samples were vacuum-filtered and pooled for analyses. Pooled weekend and weekday samples (50 mL) were prepared mixing fixed aliquots from each day, as follows: Saturday, Sunday and Monday are pooled for "weekend" composite sample and Tuesday to Friday for "week" composite sample.

WW samples (50 mL) were acidified to pH~2 with HCl (37 %), spiked with internal standards (2 ng of each compound), and extracted by SPE using Oasis® MCX cartridges (150 mg, 6 cc). Before the extraction, MCX cartridges were conditioned with 10 mL methanol (MeOH), 5 mL ultrapure water, and 5 mL of ultrapure water acidified to pH 2. Samples were manually loaded at a flow rate of about 5 mL min^{-1}. MCX cartridges were vacuum-dried for 10 min and eluted with 2 mL of MeOH and 2 mL of a 2% ammonia solution in MeOH. Eluates were dried under a gentle nitrogen stream, reconstituted in 80 µL of a mixture of ultrapure water: MeOH (90:10), centrifuged for 2 min at 2500 rpm, and transferred into glass vials for LC-MS/MS analysis.

Pooled urine. One mL of pooled urine sample was spiked with internal standard (2 ng of each compound) and hydrolysed with β-glucuronidase at 55ºC for 2 h (pH=4.5-5, buffer acetic acid/ammonium acetate). Then, urine extracts were acidified to pH~2 with HCl (9 %) and extracted

by SPE using Oasis® MCX cartridges (60 mg, 3 cc). Before the extraction, MCX cartridges were conditioned with 6 mL MeOH, 3 mL ultrapure water and 3 mL of ultrapure water acidified to pH 2. After sample loading, MCX cartridges were vacuum-dried for 5 min and eluted with 1 mL of MeOH and 1 mL of a 2% ammonia solution in MeOH. Eluates were dried under a gentle nitrogen stream, reconstituted in 200 µL of a mixture of ultrapure water: MeOH (90:10), centrifuged for 2 min at 2500 rpm, and transferred into glass vials for LC-MS/MS analysis.

LC-MS/MS analysis

WW samples and pooled urine samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), using the same instrumental methods. Chromatographic separation was done using an Agilent 1260 Series HPLC system with a binary high-pressure gradient pump and a refrigerated autosampler (4 °C). The chromatographic separation was carried out at 35ºC using an Atlantis® T3 (100 × 2.1 mm; 3 µm) column from Waters (Milford, MA) and a dual eluent system consisting of (A) 0.1% formic acid (FA) in Milli-Q water and (B) acetonitrile (can). The flow rate was 300 µL min^{-1} and the injection volume was 2 µL. Because of the high number of biomarkers, their different chemical properties and the lower levels of fentanyl analogues expected in WW, different instrumental methods were developed for the analysis of (i) fentanyl, norfentanyl and other fentanyl analogues; (ii) other NPS, including phenethyalmines, synthetic cathinones, tryptamines and other classes; (iii) the less polar NPS, including synthetic cannabinoids and some phenetylamines (NBOMe). A specific method for the analysis of isomers MEPH and 3-MMC was developed, as the co-elution of these NPS was observed using the methods previously described. A longer column Atlantis® T3 (150 × 2.1 mm; 3 µm) and a dual eluent system consisting of (A) 10 mM NH_4COOCH_3 in ultrapure water and (B) ACN were used for ensuring the separation of

both isomers. Multiple reaction monitoring (MRM) was chosen as acquisition mode, selecting the two/three most abundant fragmentation products of the protonated pseudo-molecular ions of each analyte and one fragmentation product of each deuterated compound.

More details about the chromatographic separation and MS conditions for each method, and MS/MS parameters, retention time (RT) and MRM ratio for each compound are reported below separately for each method:

Fentanyl analogues

LC gradient: The 26-min elution gradient was as follows: 0 min (2% ACN), 12 min (30% ACN), 16 min (60% ACN), 17 min (100% ACN, maintained for 2 min) and 19.5 min (2% ACN). The initial conditions were finally kept for 6.5 min (column equilibration).

ESI source parameters: curtain gas (CUR), 30; collision gas (CAD), 7; ion spray voltage (IS), 5500 V; source temperature, 500 °C; ion source gas 1 (GS1), 50 and gas 2 (GS2), 40.

MS/MS parameters: precursor and product ions, collision energy (CE) and cell exit potential (CXP)) and retention time (RT)

Analyte	RT (min)	Internal Standard	$[M+H]^+$ formula	Precursor ion [M-H]+ m/z	Product ions			DP
					m/z	CE	CXP	
Fentanyl	13.8	Fentanyl-d5	C14H21N2O	337.1	188.1*	30	12	60
					105.1	47	12	
					216.1	32	12	
NorFentanyl	9.1	Norfentanyl-d5	C14H21N2O	233.1	84.1*	23	12	50
					56.1	40	12	
					150.1	25	12	
Acetyl Fentanyl	12.3	Fentanyl-d5	C21H27N2O	323.1	188.1*	31	12	70
					105.1	47	12	
					202.1	30	12	
Acetyl Norfentanyl	7.1	Norfentanyl-d5	C13H19N2O	219.1	84.1*	22	12	55
					56.1	40	12	
					136.1	25	12	
Alfentanil	13.5	Fentanyl-d5	C21H33N6O3	417.1	268.1	24	14	60
					197.1	35	14	
					314.1	35	14	
Butyryl Fentanyl	15.1	Fentanyl-d5	C23H31N2O	351.1	188.1*	32	12	70

					105.1	49	12	
					230.1	31	12	
Butyryl Fentanyl carboxy met	12.4	Norfentanyl-d5	C23H29N2O3	381.1	188.1*	34	12	70
					105.1	48	12	
					260.1	33	12	
Butyryl Norfentanyl	10.8	Norfentanyl-d5	C15H23N2O	247.1	84.1*	24	14	50
					56.1	40	14	
					177.1	20	14	
Carfentanil	14.9	Fentanyl-d5	C24H31N2O3	395.1	335.1	23	14	70
					246.1	28	14	
					363.1	18	14	
Cyclopropyl Fentanyl	14.4	Fentanyl-d5	C23H29N2O	349.1	188.1*	32	14	60
					105.1	47	14	
					228.1	32	14	
4-ANPP	13.7	Fentanyl-d5	C19H25N2	281.1	105.1	40	13	60
					188.1	24	13	
					146.1	34	16	
4-FANPP	14.2	Fentanyl-d5	C19H24FN2	299.1	188.1*	23	14	60
					105.1	41	14	
					134.1	35	14	
Furanyl Norfentanyl	9.6	Norfentanyl-d5	C16H19N2O2	271.1	84.1*	20	14	50
					56.1	44	14	
					188.1	24	14	
Beta-Hydroxy Fentanyl	12.6	Fentanyl-d5	C22H29N2O2	353.1	204.1	29	12	70
					186.1	32	12	
					146.1	32	12	
Beta-hydroxythio fentanyl	11.9	Fentanyl-d5	C20H27N2O2S	359.1	192.1	31	12	60
					146.1	31	12	
					285.1	27	12	
Methoxy acetyl Norfentanyl	7.9	Norfentanyl-d5	C14H21N2O2	249.1	84.1*	19	14	50
					56.1	41	14	
					166.1	22	14	
3-methyl Norfentanyl (cis+trans)	10.4 (*trans*) 10.6(*cis*)	Norfentanyl-d5	C15H23N2O	247.1	98.1	22	14	55
					69.1	39	14	
					150.1	25	14	
Ocfentanil	12.3	Fentanyl-d5	C22H28FN2O2	371.1	188.1*	32	13	55
					105.1	48	13	
Phenylacetyl Fentanyl	16.0	Fentanyl-d5	C27H31N2O	399.1	188.1*	33	13	70
					105.1	55	13	
					278.1	35	13	
Valeryl Fentanyl carboxy met	12.4	Fentanyl-d5	C24H31N2O3	395.1	188.1*	34	13	60
					105.1	51	13	
					274.1	35	13	
Internal Standards								
Fentanyl-D5	13.8		C22H24D5N2O	342.1	188.1	32	14	65
Norfentanyl-D5	9.1		C14H16D5N2O	238.1	84.1	24	13	55

*MRM Transition used for Quantitation

Other NPS (method 1)

LC gradient: The 21-min elution gradient was as follows: 0 min (2% ACN), 10 min (50% ACN), 11 min (100% ACN, maintained for 3 min) and 14.5 min (2% ACN). The initial conditions were finally kept for 6.5 min (column equilibration).

ESI source parameters: curtain gas (CUR), 30; collision gas (CAD), 7; ion spray voltage (IS), 5500 V; source temperature, 400 °C; ion source gas 1 (GS1), 50 and gas 2 (GS2), 40.

MS/MS parameters: precursor and product ions, collision energy (CE) and cell exit potential (CXP)) and retention time (RT)

Analyte	RT (min)	Internal Standard	$[M+H]^+$ formula	Precursor ion [M-H]+ m/z	Product ions			DP
					m/z	CE	CXP	
NEDPA	8.6	α-PVP-D8	C16H20N	226.1	181.1*	18	14	40
					103.1	38	14	
PMA	5.9	METHAMP-D9	C10H15NO	166.1	121.1	23	12	40
					91.1*	39	12	
					149.1	12	12	
PMMA	6.3	PMMA-D3	C11H17NO	180.1	149.1*	15	12	40
					91.1	43	12	
6-APB	6.7	PMMA-D3	C11H14NO	176.1	131.1*	26	12	40
					159.1	12	12	
BUPH	5.5	METHAMP-D9	C11H16NO	178.1	131.1*	29	10	50
					130.1	43	11	
BUTL	6.2	BUTL-D3	C12H16NO3	222.2	174.1*	23	14	55
					131.1	46	11	
4-Cl-α-PPP	7.4	α -PVP-D8	C13H16ClNO	238.1	98.1	33	12	50
					139.1*	32	12	
DCAT	5.3	METHAMP-D9	C11H16NO	178.1	105.1*	27	12	60
					133.1	20	12	
3,4-DMMC	7.4	MEPH-D3	C12H18NO	192.1	159.1*	28	12	50
					158.1	43	10	
ETHC	5.5	METHAMP-D9	C11H16NO	178.1	130.1*	40	10	50
					132.1	23	10	
ETHL	5.8	METL-D3	C12H16NO3	222.2	174.1*	25	10	50
					146.1	36	12	
MDPV	7.7	MDPV-D8	C16H22NO3	276.2	126.1*	36	14	80
					135.1	36	15	
4-FMC	5.5	METHAMP-D9	C10H13FNO	182.1	149.1*	28	10	50
					148.1	43	14	
4-MEC	6.7	MEPH-D3	C12H18NO	192.1	145.1*	27	10	55
					146.1	24	10	
METC	5.1	METHAMP-D9	C10H14NO	164.1	131.1	26	12	65

					130.1*	40	12	
METD	5.9	MEPH-D3	C11H16NO2	194.1	161.1*	27	12	50
					146.1	37	12	
METL	5.4	METL-D3	C11H14NO3	208.2	160.1*	23	13	40
					132.1	36	14	
1-NAPH	9.3	NAPH-D5	C19H24NO	282.2	141.1*	36	14	70
					126.1	32	14	
NAPH	9.6	NAPH-D5	C19H24NO	282.2	141.1*	34	12	70
					211.1	26	14	
PENTD	6.5	METHAMP-D9	C12H18NO	192.1	132.1*	24	10	50
					91.1	32	10	
PENTL	7.2	BUTL-D3	C13H18NO3	236.2	188.1*	24	14	60
					175.1	29	13	
α-PVP	7.5	α-PVP-D8	C15H21NO	232.1	91.1*	32	12	60
					126.1	34	12	
MXE	7.1	METHOX-D3	C15H21NO2	248.1	121.1*	38	13	60
					175.1	26	13	
2-FDCK	6.1	α -PVP-D8	C13H16FNO	221.1	191.1*	18	14	50
					163.1	20	14	
					109.1	37	14	
4,4-DMAR	7.5	AMP-D6	C11H15N2O	191.1	148.1*	17	10	40
					131.1	27	10	
5-MeO-DMT	6.3	BUTL-D3	C13H19N2O	219.1	58.1*	16	12	50
					174.1	21	12	
N,N-DMT	6.1	BUTL-D3	C12H17N2	189.1	58.1*	14	12	40
					144.1	25	12	
5-MeO-MiPT	7.1	α-PVP-D8	C15H22N2O	247.1	86.1*	17	14	50
					174.1	26	14	
Ethylphenidate	8.0	α-PVP-D8	C15H22NO2	248.1	84.1*	36	13	70
					56.1	55	13	
					174.1	30	13	
Internal Standards								
α-PVP-D8	7.5		C15H13D8NO	240.1	91.1	32		60
AMP-D6	5.5		C9H7D6N	142.1	93.1	24		40
METHAMP-D9	5.9		C10H7D9N	159.1	93.1	28		40
METHOX-D3	7.1		C15H18D3NO2	251.1	124.1	38		40
MEPH-D3	6.2		C11H13D3NO	181.1	148.1	28		60
METL-D3	5.4		C11H10D3NO3	211.1	163.1	25		50
BUTL-D3	6.2		C12H12D3NO3	225.1	177.1	24		50
NAPH-D5	9.6		C19H18D5NO	287.1	141.1	36		65

*MRM Transition used for Quantitation

Other NPS (method 2)

LC gradient: The 16-min elution gradient was as follows: 0 min (2% ACN), 7 min (100% ACN, maintained for 3 min) and 10.5 min (2% ACN). The initial conditions were finally kept for 5.5 min (column equilibration).

ESI source parameters: curtain gas (CUR), 30; collision gas (CAD), 7; ion spray voltage (IS), 5500 V; source temperature, 400 °C; ion source gas 1 (GS1), 50 and gas 2 (GS2), 40.

MS/MS parameters: precursor and product ions, collision energy (CE) and cell exit potential (CXP)) and retention time (RT)

Analyte	RT (min)	Internal Standard	$[M+H]^+$ formula	Precursor ion [M-H]+ m/z	Product ions			DP
					m/z	CE	CXP	
25-B-NBOMe	5.6	25-B-NBoMe-D3	C18H22BrNO3	380.1/382.1	91.1	62	14	60
					121.1*	63	11	
25-C-NBOMe	5.6	25-C-NBoMe-D3	C18H22ClNO3	336.1	91.1	57	11	50
					121.1*	23	11	
25-I-NBOMe	5.8	25-I-NBoMe-D3	C18H22INO3	428.1	91.1	66	13	50
					121.1*	27	13	
25-iP-NBOMe	6.0	25-I-NBoMe-D3	C21H30NO3	344.1	121.1	28	10	80
					91.1*	60	11	
5-Fpentyl-3-pyr	6.9	α-PVP-D8	C19H20FN2O	311.1	144.1*	50	14	80
					232.1	41	15	
Isotonitazene	5.5	25-C-NBoMe-D3	C23H31N4O3	411.1	100.1*	27	14	70
					72.1	52	14	
					107.1	59	14	
JWH-122	9.2	25-I-NBoMe-D3	C25H26NO	356.2	169.1*	35	12	60
					144.1	51	12	
					214.1	33	12	
AB-CHMINACA	7.6	25-I-NBoMe-D3	C20H29N4O2	357.1	241.1*	35	13	50
					312.1	20	12	
					340.1	12	13	
ADB-FUBINACA	7.2	25-I-NBoMe-D3	C21H24FN4O2	383.1	253.1*	33	14	45
					338.1	19	14	
					366.1	12	14	
CUMYLPegaclone	8.7	25-I-NBoMe-D3	C25H29N2O	373.1	255.1*	22	12	50
					119.1	33	12	
					174.1	26	14	
MDMB-CHMICA	8.8	25-I-NBoMe-D3	C23H33N2O3	385.1	240.1*	24	14	45
					144.1	50	14	
Internal Standards								
α-PVP-D8	7.5		C15H13D8NO	240.1	91.1	32		60
25-B-NBoMe-D3	5.5		C9H7D6N	142.1	93.1	24		50

25-C-NBoMe-D3	5.9		C10H7D9N	159.1	93.1	28		50
25-I-NBoMe-D3	7.1		C15H18D3NO2	251.1	124.1	38		55

*MRM Transition used for Quantitation

3-MMC and MEPH

LC gradient: The 40-min elution gradient was as follows: 0 min (2% ACN), 30 min (30% ACN), 30.5 min (100% ACN, maintained for 3 min) and 34 min (2% ACN). The initial conditions were finally kept for 6 min (column equilibration).

ESI source parameters: curtain gas (CUR), 30; collision gas (CAD), 7; ion spray voltage (IS), 5500 V; source temperature, 400 °C; ion source gas 1 (GS1), 50 and gas 2 (GS2), 40.

MS/MS parameters: precursor and product ions, collision energy (CE) and cell exit potential (CXP)) and retention time (RT)

Analyte	**RT (min)**	**Internal Standard**	**$[M+H]^+$ formula**	**Precursor ion [M-H]+ m/z**	**Product ions**			**DP**
					m/z	**CE**	**CXP**	
Mephedrone	23.7	MEPH-D3	C11H16NO	178.1	145.1*	28	15	60
					147.2	15	12	
					144.1	39	12	
3-MMC	24.0	MEPH-D3	C11H16NO	178.1	145.1*	28	15	60
					147.2	15	12	
					144.1	39	12	
Internal Standards								
MEPH-D3	23.4		C11H13D3NO	181.1	148.1	28		60

*MRM Transition used for Quantitation

Method validation

Quantitation was performed using deuterated compounds as surrogate standards (IS). The most abundant transition of each analyte was used for quantitation purposes, and the area was normalized with the corresponding IS. If the analogue deuterated is not available, another one with similar structure was selected considering their ability to compensate for matrix effect (recovery

values close to 100%). The identification and confirmation of positives in WW and pooled urine samples were based on the accomplishment of ion ratio abundances (MRM ratio) and RT, according to the European guidelines (European Commission, SANTE/11813/2019). MRM deviation between samples and analytical standards should be lower than 30%, and retention time error less than 0.1 min.

Instrumental blanks were included in each analytical run to check for potential contamination. With the same aim, procedural blanks consisting of mineral water (50-100 mL) and urine samples (1 mL) were spiked with IS (2 ng) and processed together with every set of samples.

Urban wastewater. Method validation was performed in terms of linear range, accuracy, precision and sensitivity. In the case of fentanyl analogues, six-point matrix calibration curves were prepared freshly before each analytical run to compensate for matrix effect (range: 0.3-60 ng L^{-1}). Six-point calibration curves were built in the range 0.3-60 ng L^{-1} for the other NPS and between 0.3-200 ng L^{-1} for MEPH and 3-MMC. Recovery and repeatability of the analytical method were tested in raw WW (n=3) by spiking 50 mL aliquots with 50 ng L^{-1} of each analyte. An additional "blank aliquot" (i.e. the same raw WW without analyte spiking) was analyzed to correct recovery values for the background levels. Limits of detection (LOD) and quantitation (LOQ) of the whole method were directly estimated from raw WW samples as the values corresponding to signal-to-noise ratio (S/N) of 3 and 10, respectively. Figures of merit obtained for fentanyl analogues and NPS were satisfactory and are reported in Table 2 and Table 3, respectively.

Table 2. Relative recoveries (RR%), precision (relative standard deviation, RSD%), limits of detection (LOD) and limits of quantitation (LOQ) obtained for fentanyl, norfentanyl and 19 analogues in raw wastewater

Analyte	Linear range (ng L^{-1})	Concentration = 50 ng L^{-1}		LOD (ng L^{-1})	LOQ (ng L^{-1})
		RR (%)	RSD (%)		
Fentanyl	LOQ-60	**97**	1.2	0.04	**0.15**
NorFentanyl	LOQ-60	**104**	3.5	0.10	**0.37**
Acetyl Fentanyl	LOQ-60	**104**	1.0	0.10	**0.34**
Acetyl Norfentanyl	LOQ-60	**97**	2.4	0.15	**0.51**
Alfentanil	LOQ-60	**100**	2.4	0.17	**0.56**
Butyryl Fentanyl	LOQ-60	**99**	1.4	0.1	**0.33**
Butyryl Fentanyl carboxy met	LOQ-60	**102**	1.7	0.22	**0.74**
Butyryl Norfentanyl	LOQ-60	**94**	4.0	0.43	**1.4**
Carfentanil	LOQ-60	**102**	2.8	0.38	**1.3**
Cyclopropyl Fentanyl	LOQ-60	**97**	2.4	0.13	**0.43**
4-ANPP	LOQ-60	**49**	1.0	0.56	**1.9**
4-F-ANPP	LOQ-60	**49**	2.4	0.51	**1.7**
Furanyl Norfentanyl	LOQ-60	**99**	3.3	0.73	**2.4**
Beta-Hydroxy Fentanyl	LOQ-60	**98**	1.3	0.57	**1.9**
Beta-hydroxythio fentanyl	LOQ-60	**105**	0.7	0.48	**1.6**
Methoxy acetyl Norfentanyl	LOQ-60	**105**	4.4	1.1	**3.7**
trans-3-methyl Norfentanyl	LOQ-60	**80**	2.3	0.17	**0.55**
cis-3-methyl Norfentanyl	LOQ-60	**105**	3.8	0.62	**2.1**
Ocfentanil	LOQ-60	**69**	5.6	0.21	**0.70**
Phenylacetyl Fentanyl	LOQ-60	**108**	3.7	0.07	**0.22**
Valeryl Fentanyl carboxy met	LOQ-60	**105**	2.0	0.13	**0.44**

Table 3. Relative recoveries (RR%), precision (relative standard deviation, RSD%), limits of detection (LOD)and limits of quantitation (LOQ) obtained for 42 new psychoactive substances in raw wastewater

Analyte	Linear range (ng L^{-1})	Concentration = 50 ng L^{-1}		LOD (ng L^{-1})	LOQ (ng L^{-1})
		RR (%)	RSD (%)		
Method 1					
NEDPA	LOQ-60	**98**	0.80	0.07	**0.24**
PMA	LOQ-60	**84**	7.9	4.5	**7.8**
PMMA	LOQ-60	**79**	14	0.21	**0.71**
6-APB	LOQ-60	**100**	10.2	0.82	**2.8**
BUPH	LOQ-60	**75**	6.4	0.22	**0.72**
BUTL	LOQ-60	**94**	4.8	0.17	**0.58**
4-Cl-α-PPP	LOQ-60	**85**	6.5	0.18	**0.59**
DCAT	LOQ-60	**97**	11	0.26	**0.86**
3,4-DMMC	LOQ-60	**83**	8.9	0.10	**0.32**
ETHC	LOQ-60	**90**	7.1	0.26	**0.87**
ETHL	LOQ-60	**87**	13	0.18	**0.59**
MDPV	LOQ-60	**100**	3.3	0.11	**0.36**
4-FMC	LOQ-60	**82**	10	0.19	**0.63**
4-MEC	LOQ-60	**116**	6.3	0.19	**0.64**
METC	LOQ-60	**83**	9.4	0.20	**0.67**
METD	LOQ-60	**69**	14	0.19	**0.62**
METL	LOQ-60	**95**	9.1	0.14	**0.46**
1-NAPH	LOQ-60	**97**	3.2	0.08	**0.26**
NAPH	LOQ-60	**98**	4.9	0.03	**0.11**
PENTD	LOQ-60	**66**	10	0.47	**1.57**
PENTL	LOQ-60	**107**	12	0.16	**0.54**
α-PVP	LOQ-60	**101**	6.3	0.35	**1.17**
2-FDCK	LOQ-60	**90**	2.3	0.23	**0.77**
MXE	LOQ-60	**104**	6.3	0.12	**0.40**
4,4-DMAR	LOQ-60	**76**	4.7	0.06	**0.19**
4-AcO-DMT	LOQ-60	**104**	4.4	2.5	**7.5**
5-MeO-DMT	LOQ-60	**75**	5.6	0.12	**0.27**
N,N-DMT	LOQ-60	**99**	2.6	0.31	**0.81**

5-MeO-MiPT	LOQ-60	**53**	17	0.14	**0.45**
Ethylphenidate	LOQ-60	**95**	4.8	0.16	**0.46**
Method 2					
25-B-NBOMe	LOQ-60	**109**	3.8	0.06	**0.22**
25-C-NBOMe	LOQ-60	**99**	7.5	0.03	**0.10**
25-I-NBOMe	LOQ-60	**109**	7.0	0.04	**0.12**
25-iP-NBOMe	LOQ-60	**127**	2.4	0.07	**0.25**
5-Fpentyl-3-pyr	LOQ-60	**95**	16	0.02	**0.06**
JWH-122	LOQ-60	**90**	9.5	0.09	**0.32**
AB-CHMINACA	LOQ-60	**103**	4.9	0.3	**1.2**
ABD-FUBINACA	LOQ-60	**118**	4.1	0.21	**0.63**
CUMYL-PeGLACONE	LOQ-60	**100**	6.5	0.09	**0.3**
MDMB-CHMICA	LOQ-60	**120**	3.1	0.05	**0.17**
Isotonitazene	LOQ-60	**96**	15	1.8	**6.1**
Method 3					
Mephedrone	LOQ-200	**100**	3.4	0.17	**0.66**
3-MMC	LOQ-200	**99**	2.3	0.22	**0.78**

Pooled urine. Method validation was performed in terms of linear range, accuracy, precision and sensitivity, as in the case of WW samples. Six-point calibration curves were prepared freshly before each analytical run to in the range 0.03-3 ng mL^{-1} for all compounds. Recovery and repeatability of the analytical method were tested in pooled urine samples (n=3) by spiking 1 mL aliquots with 5 ng mL^{-1} of each analyte. An additional "blank aliquot" (i.e. the same pooled urine without analyte spiking) was analyzed to correct recovery values for the background levels. Limits of detection (LOD) and quantitation (LOQ) of the whole method were directly estimated from raw WW samples as the values corresponding to signal-to-noise ratio (S/N) of 3 and 10, respectively.

Figures of merit obtained for fentanyl analogues and NPS (Table 4 and 5) were satisfactory, except for 25iP-NBOMe and JHW-122 with recoveries close to 300%.

Table 4. Relative recoveries (RR%), precision (relative standard deviation, RSD%), limits of detection (LOD) and limits of quantitation (LOQ) obtained for fentanyl, norfentanyl and 19 analogues in pooled urine

Analyte	**Linear range (ng mL^{-1})**	**Concentration = 5 ng mL^{-1}**		**LOD (ng mL^{-1})**	**LOQ (ng mL^{-1})**
		RR (%)	**RSD (%)**		
Fentanyl	LOQ-3	**102**	4.4	0.05	**0.17**
NorFentanyl	LOQ-3	**105**	0.8	0.17	**0.57**
Acetyl Fentanyl	LOQ-3	**98**	3.5	0.05	**0.17**
Acetyl Norfentanyl	LOQ-3	**110**	2.0	0.07	**0.23**
Alfentanil	LOQ-3	**111**	5.7	0.03	**0.09**
Butyryl Fentanyl	LOQ-3	**113**	1.3	0.09	**0.3**
Butyryl Fentanyl carboxy met	LOQ-3	**92**	3.7	0.13	**0.44**
Butyryl Norfentanyl	LOQ-3	**98**	2.7	0.17	**0.57**
Carfentanil	LOQ-3	**114**	2.7	0.16	**0.52**
Cyclopropyl Fentanyl	LOQ-3	**110**	5.4	0.07	**0.24**
4-ANPP	LOQ-3	**89**	1.2	0.05	**0.17**
4-F-ANPP	LOQ-3	**107**	2.1	0.21	**0.70**
Furanyl Norfentanyl	LOQ-3	**108**	3.7	0.07	**0.22**
Beta-Hydroxy Fentanyl	LOQ-3	**104**	5.3	0.07	**0.24**
Beta-hydroxythio fentanyl	LOQ-3	**114**	6.8	0.08	**0.25**
Methoxy acetyl Norfentanyl	LOQ-3	**94**	3.5	0.14	**0.45**
trans-3-methyl Norfentanyl	LOQ-3	**100**	0.9	0.03	**0.09**
cis-3-methyl Norfentanyl	LOQ-3	**97**	3.8	0.13	**0.44**
Ocfentanil	LOQ-3	**113**	3.2	0.04	**0.12**
Phenylacetyl Fentanyl	LOQ-3	**130**	4.5	0.18	**0.6**
Valeryl Fentanyl carboxy met	LOQ-3	**104**	1.9	0.04	**0.13**

Table 5. Relative recoveries (RR%), precision (relative standard deviation, RSD%), limits of detection (LOD)and limits of quantitation (LOQ) obtained for 42 new psychoactive substances in raw wastewater

Analyte	Linear range (ng mL^{-1})	Concentration = 5 ng mL^{-1}		LOD (ng mL^{-1})	LOQ (ng mL^{-1})
		RR (%)	RSD (%)		
Method 1					
NEDPA	LOQ-3	**64**	7.8	0.02	**0.07**
PMA	LOQ-3	**116**	2.5	0.16	**0.52**
PMMA	LOQ-3	**114**	2.7	0.01	**0.03**
6-APB	LOQ-3	**115**	7.6	0.02	**0.08**
BUPH	LOQ-3	**81**	5.6	0.01	**0.04**
BUTL	LOQ-3	**94**	1.5	0.07	**0.22**
4-Cl-α-PPP	LOQ-3	**96**	1.1	0.14	**0.45**
DCAT	LOQ-3	**97**	4.1	0.03	**0.11**
3,4-DMMC	LOQ-3	**92**	3.0	0.16	**0.53**
ETHC	LOQ-3	**82**	6.0	0.03	**0.10**
ETHL	LOQ-3	**110**	4.1	0.04	**0.12**
MDPV	LOQ-3	**113**	1.1	0.06	**0.20**
4-FMC	LOQ-3	**79**	8.1	0.19	**0.62**
4-MEC	LOQ-3	**85**	3.3	0.05	**0.15**
METC	LOQ-3	**80**	9.1	0.05	**0.17**
METD	LOQ-3	**125**	4.5	0.12	**0.41**
METL	LOQ-3	**114**	5.2	0.05	**0.18**
1-NAPH	LOQ-3	**110**	1.8	0.11	**0.5**
NAPH	LOQ-3	**107**	2.0	0.23	**0.78**
PENTD	LOQ-3	**92**	5.4	0.33	**1.1**
PENTL	LOQ-3	**104**	4.6	0.13	**0.42**
α-PVP	LOQ-3	**106**	1.5	0.05	**0.17**
2-FDCK	LOQ-3	**88**	3.8	0.01	**0.03**
MXE	LOQ-3	**117**	2.2	0.07	**0.22**
4,4-DMAR	LOQ-3	**76**	7.1	0.01	**0.03**
4-AcO-DMT	LOQ-3	**75**	9.0	0.72	**2.4**
5-MeO-DMT	LOQ-3	**111**	2.3	0.05	**0.17**
N,N-DMT	LOQ-3	**80**	3.0	0.03	**0.11**

5-MeO-MiPT	LOQ-3	**104**	7.6	0.03	**0.10**
Ethylphenidate	LOQ-3	**103**	3.1	0.12	**0.39**
Method 2					
25-B-NBOMe	LOQ-3	**110**	1.8	0.31	**1.03**
25-C-NBOMe	LOQ-3	**115**	4.1	0.20	**0.66**
25-I-NBOMe	LOQ-3	**111**	3.0	0.26	**0.85**
25-iP-NBOMe	LOQ-3	**335**	2.3	-	**-**
5-Fpentyl-3-pyr	LOQ-3	**118**	5.0	0.07	**0.22**
JWH-122	LOQ-3	**292**	2.3	-	**-**
AB-CHMINACA	LOQ-3	**85**	3.0	0.03	**0.11**
ABD-FUBINACA	LOQ-3	**84**	3.0	0.03	**0.11**
CUMYL-PeGLACONE	LOQ-3	**84**	2.5	0.04	**0.13**
MDMB-CHMICA	LOQ-3	**89**	2.5	0.01	**0.04**
Isotonitazene	LOQ-3	**94**	3.4	0.05	**0.17**
Method 3					
Mephedrone	LOQ-3	**113**	3.1	0.04	**0.12**
3-MMC	LOQ-3	**97**	3.6	0.08	**0.25**

(ii) PRESCRIPTION OPIOIDS

An analytical method for quantitative analysis of prescription opioids in urban wastewater has been developed and validated. The most relevant prescription opioids were selected considering their frequency of prescription in Europe and Italy (Deliverable 2). Then, a list of 24 biomarkers to be monitored in wastewater was created, including parent compounds and metabolites (Deliverable 5). All the analytical methods developed and validated were based on solid-phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A specific list of biomarkers was selected for analysis in Italy according to the most prescribed substances in our country (Deliverable 2) and availability of analytical standards.

The procedure developed is described in detail below.

The 16 biomarkers selected for analysis and the list of internal standards used for quantitation are summarized in Table 6. Fentanyl and norfentanyl were separately analyzed ad described above, because of the lower concentrations expected in wastewater.

Table 6. Biomarkers of prescription opioids monitored in urban wastewater (MN)

Biomarkers to be monitored in wastewater	***Internal Standards***
Fentanyl Norfentanyl	Fentanyl-d5 Norfentanyl-d5
Buprenorphine Norbuprenorphine Hydromorphone Naloxone Hydrocodone Oxycodone Noroxycodone Tapentadol Cis-tramadol O-desmethyl tramadol	Buprenorphine-d4 Hydromorphone-d3 Hydrocodone-d6 Oxycodone-d6 Tapentadol-d3 Cis-tramadol-13C, d3 Codeine-d6 Morphine-d3 Methadone-d3 EDDP-d3

Codeine Morphine Methadone EDDP	

Sample preparation

Sample (pre)treatment protocol has been adapted from previous works (Castiglioni et al., 2006) and validated for the analysis of prescription opioids. Briefly, aliquots of filtered WW samples (25 mL) were acidified to pH~2 with HCl (37 %), spiked with internal standards (2 ng of each compound) and extracted by SPE using mixed cation exchange cartridges and an automated system GX-274 ASPEC (Gilson, Middleton, WI, USA). Before the extraction, Oasis® MCX cartridges (60 mg, 3 cc) were conditioned with 6 mL methanol (MeOH), 3 mL ultrapure water, and 3 mL water acidified to pH 2. Samples were loaded at a flow rate of 5 mL min^{-1}, vacuum-dried for 10 min after percolation, and eluted with 2 mL of MeOH and 2 mL of a 2% ammonia solution in MeOH. Eluates are dried under a gentle nitrogen stream, reconstituted in 100 µL of a mixture of ultrapure water: MeOH (90:10), centrifuged for 2 min at 2500 rpm, and transferred into glass vials for LC-MS/MS analysis.

Instrumental analysis

LC-MS/MS analyses were done using an Agilent 1200 Series HPLC system with a binary high-pressure gradient pump and a refrigerated autosampler kept at +4 °C, coupled to a triple quadruple mass spectrometer TripleQuad 5500 ABSciex (Applied Biosystems, Concord, Ontario, Canada) equipped with a Turbo Ion Spray source. The chromatographic separation was carried out at room temperature using an Atlantis® T3 (100 × 2.1 mm; 3 µm) and a dual eluent system consisting of (A) 0.1% acetic acid in ultrapure water and (B) acetonitrile. The 24-min elution gradient was as

follows: 0 min (1% B), 12 min (30% B), 12.5 min (100% B), 16.5 min (100% B), 17 min (1% B), and 24 min (1% B). The flow rate was 200 μL min^{-1} and the injection volume was 2 μL.

The MS analysis was performed in positive mode under the following conditions: ion spray voltage (IS), 5000 V; curtain gas (CUR), 30; collision gas (CAD), 7; source temperature, 500 °C; ion source gas 1 (GS1), 50 and gas 2 (GS2), 40. Multiple reaction monitoring (MRM) was chosen as acquisition mode, selecting the two or three most abundant fragmentation products of the protonated pseudo-molecular ions of each analyte and one fragmentation product of each deuterated compound. Retention times (RT) and individual MRM parameters for all compounds are reported in Table 7. All data were acquired and processed using Analyst® 1.6.1 and MultiQuant™ 2.1 software (AB Sciex).

Method validation

For method validation, linear range, accuracy, precision and sensitivity were evaluated and showed in Table 8. Seven-point calibration curves were prepared freshly before each analytical run in the range 1.2-280 ng L^{-1}. Linearity ($r > 0.9985$) was demonstrated for all compounds in the studied range. Recovery and repeatability of the analytical method were tested in raw wastewater (n=3) by spiking 25 mL aliquots with 200 ng L^{-1} and/or 1000 ng L^{-1} of each analyte, depending on the concentration levels expected in wastewater. An additional "blank aliquot" (i.e. the same raw wastewater without analyte spiking) was analyzed to correct recoveries for the background levels. Satisfactory recoveries and repeatability were obtained for all compounds, varying between 95 and 114 (RSD<4.4%). Limits of quantitation (LOQ) were directly estimated from raw wastewater samples as the values corresponding to signal-to-noise ratio (S/N) of 10. LOQ ranged from 0.9 ng L^{-1} (hydrocodone) to 6.4 ng L^{-1} (cis-tramadol).

The analysis of morphine, methadone and EDDP was performed using a previous method already established in our lab for illicit drugs analysis. MRM parameters and method validation can be found in Castiglioni et al., 2006.

Table 7. Retention time (RT) and MRM parameters (i.e. precursor and product ions, collision energy (CE) and cell exit potential (CXP)) for the prescription opioids and their deuterated analogues

Analyte	RT (min)	Internal Standard	$[M+H]^+$ formula	Precursor ion [M-H]+ m/z	Product ions			DP
					m/z	CE	CXP	
Codeine	5.8	Codeine-d6	C18H22NO3	300.1	215.1	33	18	50
					165.1	53	18	50
Hydrocodone	6.3	Hydrocodone-d6	C18H22NO3	300.1	*199.1*	38	17	50
					128.1	68	14	50
Oxycodone	6.1	Oxycodone-d6	C18H22NO4	316.1	*241.1*	38	16	60
					256.1	34	18	60
Noroxycodone	6.1	Hydromorphone-d3	C17H20NO4	302.1	187.1	31	13	70
					227.1	37	13	70
					229.1	30	13	70
Buprenorphine	8.9	Buprenorphine-d4	C29H42NO4	468.1	*396.1*	54	13	100
					414.1	43	13	100
					55.1	82	13	100
Norbuprenorphine	7.9	Buprenorphine-d4	C25H36NO4	414.1	*187.1*	48	14	60
					211.1	50	14	60
					223.1	54	14	60
Hydromorphone	5.3	Hydromorphone-d3	C17H20NO3	288.1	*185.1*	40	12	60
					157.1	51	12	60
					227.1	35	12	60
Tapentadol	7.4	Tapentadol-d3	C14H24NO	222.1	107.1	32	13	60
					121.1	28	13	60
					135.1	24	13	60
Naloxone	5.8	Oxycodone-d6	C19H22NO4	328.1	*212.1*	50	13	50
					253.1	36	13	50
					268.1	35	13	50
Cis-tramadol	7.2	Cis-tramadol 13C,D3	C16H26NO2	264.1	*58.1*	11	12	50
					246.1	16	12	50
O-desmethyltramadol	6.2	Cis-tramadol 13C,D3	C15H24NO2	250.1	*58.1*	20	12	45
					232.1	15	12	45

Internal Standards								
Codeine-d6	5.8	-	C18H16NO3D6	306.1	165.1	55	18	50
Hydrocodone-d6	6.3	-	C18H16NO3D6	306.1	202.2	41	14	50
Oxycodone-d6	6.1	-	C18H16NO4D6	322.1	247.1	38	16	60
Hydromorphone-d3	5.3	-	C17H17NO3D3	289.1	185.1	40	14	90
Buprenorphine-d4	8.9	-	C29H38NO4D4	472.1	400.1	53	14	90
Tapentadol-d3	7.4	-	C14H21NOD3	225.1	107.1	35	12	50
Cis-tramadol13C,D3	7.2	-	^{13}CC15H23NO2D3	268.1	58.1	12	12	50
MRM transition used for quantitation								

Table 8. Recoveries, repeatability and limits of quantitation (LOQ) obtained for each prescription opioid in urban wastewater

Analyte	**Conc = 200 ng L^{-1}**		**Conc = 1000 ng L^{-1}**		**LOQ (ng L^{-1})**
	Relative recovery (RR%)	**Relative Standard Deviation (RSD%)**	**Relative recovery (RR%)**	**Relative Standard Deviation (RSD%)**	
Codeine	-	-	103	3.9	4.4
Hydrocodone	-	-	105	1.8	0.9
Oxycodone	-	-	107	4.1	2.6
Noroxycodone	100	1.8	-	-	1.6
Buprenorphine	98	0.8	-	-	5.8
Norbuprenorphine	95	1.0	-	-	2.5
Hydromorphone	101	0.9	-	-	1.6
Tapentadol	116	3.3	114	3.8	3.5
Naloxone	96	3.2	-	-	2.1
Cis-tramadol	113	0.8	97	3.8	6.4
O-desmethyltramadol	112	4.4	104	4.4	2.4

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