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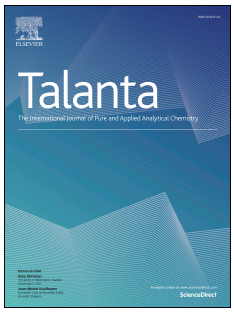

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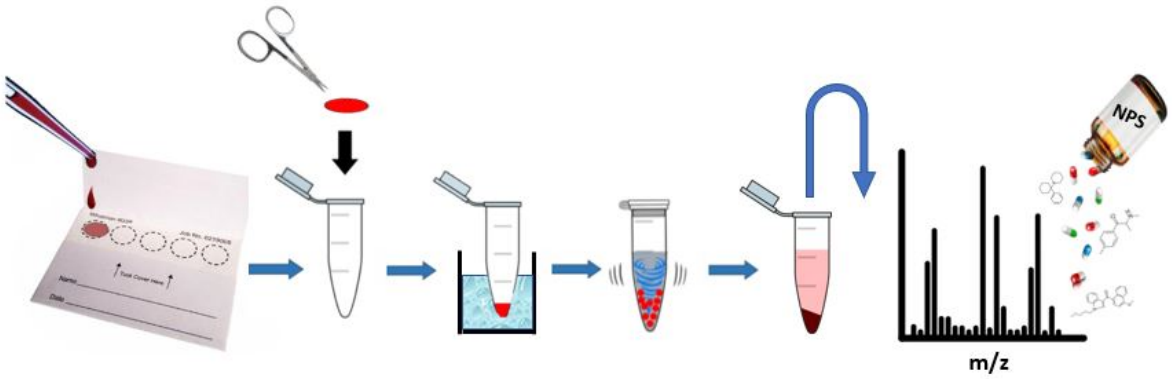

Development and validation of a UHPLC-HRMS-QTOF method for the detection of 132 New Psychoactive Substances and synthetic opioids, including fentanyl, in Dried Blood Spots

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Abstract

Dried Blood Spots (DBS) represents a promising micro-sampling technique in the field of forensic toxicology to carry out minimally invasive blood sample collection. In DBS, cheap, fast and easy sampling is combined with effortless store and transport. These properties aimed us to develop and validate a quick and easy procedure for the detection of a large and diverse range of emerging and alarming New Psychoactive Substances (NPS). A drop of whole blood sample was collected on a DBS card and dried for 3 hours, from which a total of 132 analytes (including NPS, synthetic opioids NSO and metabolites) plus 13 deuterated internal standards could be extracted using 500 µL of a methanol/acetonitrile mixture (3:1, v/v) and subsequently separated and identified by means of ultra-high-performance liquid-chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS). The extraction efficiency proved to be reproducible with yields ranging from 30% to 100% depending on the different classes of drugs. Trueness, repeatability, and intermediate precision fulfilled acceptance criteria for almost all synthetic opioids, cathinones and hallucinogens (bias and CV% below ±20%); in particular, the aggregate inter-day trueness data showed extremely limited deviation from the expected concentrations (–10% < bias% < +10%) for 114 target analytes out of 132. The calculated limits of detection ranged from 1.3 to 6.3 ng/mL, consistently exceeding the values experimentally tested. Moderate ion suppression was observed for most analytes, partly caused by blood fortification itself. Good stability of the target analytes at –20 °C, 4 °C, and 35 °C on DBS cards after drying was observed, even for long periods of time. Optimal storage condition appeared to be at 4 °C resulting in virtually no drugs degradation for up to 40 days. The novel analytical method based on DBS sampling, verified on venous whole blood real samples previously tested positive with our routine procedure, conveys remarkable potential in analytical toxicology, clinical analysis, and doping control.

Keywords: NPS, fentanyl, dried blood spots, validation, QTOF, HRMS, opioids

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Introduction

Drug abuse has certainly represented a dramatic social and health issue for a long time, but in recent years the diffusion of countless New Psychoactive Substances (NPS) in the illegal drug market introduced an unprecedented threat to the public health [1]. The adjective "new" does not necessarily indicate totally original compounds, but it may refer to substances initially synthesized and tested for their potential pharmaceutical properties and revalued years later as highly potent drugs of abuse [2,3]. NPS are often categorized into synthetic cannabinoids, synthetic opioids, cathinones and hallucinogen, but this simplistic classification does not express adequately the variety and complexity of their potency, combined effects, and risk profiles that intersect categories and often differentiate compounds belonging to the same category [4].

Nowadays, the NPS abuse in U.S and Europe is proliferating at unprecedented rate [5] and represents an increasing challenge to the established national and international drug policies. Difficulties associated with NPS monitoring include their high number, the speed with which they enter and exit the illegal drug market, the restricted and partial information about them, the incomplete knowledge about their composition and mixing, the variable and often unknown potency, and the difficulty for toxicological laboratories to find the analytical standards to provide confirmatory testing [6]. New analytical approaches and in-depth investigations of the different biological matrices, either conventional or alternative, are thus needed for effective monitoring, surveillance, drug control, and public health campaigns aimed to reduce this drug-related harm [7].

Dried blood spot (DBS) is a sampling technique based on the collection of a whole blood drop on a filter paper. It has been widely used in the diagnostic screening of neonatal metabolic disorders [8] and is now available for clinical diagnostics [9] as well as other applications, including forensic toxicology [10,11] and antidoping analyses [12]. With the development of modern mass spectrometers, several studies have been published on the use of DBS to monitor the blood concentration of a single or multiple target substances for neonatal diagnosis [13], drugs of abuse [14] and therapeutic drugs [15]. The collection of whole blood dried on a piece of filter paper provides several advantages including simplicity, speed, resistance to manipulations, and enhanced stability of the target analytes at room temperature. Compared to conventional venous blood sampling, the typical 30-50 µL collection of capillary blood, obtained by finger, ear, or heel prick, is minimally invasive. The minimum space occupied by the sampling paper as well as the reduced effects of environmental degradation result in a facilitated storage and shipment of DBS specimens [16]. The continuous sensitivity improvement enabled by mass spectrometric techniques encourages further a minimal blood volume handling, such as the one involved in DBS [17].

The present study aims to highlight the opportunities and potential benefits arising from the implementation of DBS as a complementary tool in forensic control programs to monitor the NPS

diffusion. To this purpose, the present approach combines DBS sampling with ultra-high-pressure liquid-chromatography (UHPLC) coupled to time-of-flight high resolution mass spectrometry (TOF-HRMS). The analytical method developed in this study achieved the simultaneous qualitative and quantitative analysis of 132 NPS/NSO analytes and metabolites (their full list is available in Table 1), with the goal of reaching adequately low LOQ levels using a simple and rapid extraction procedure.

Table 1. List of the 132 NPS/NSO substances under study (target analytes).

Compounds	Formula	Precursor theoretical Mass	Fragment theoretical Mass	Rt	ISTD
5-CHLORO-AB-PINACA	$C_{18}H_{25}ClN_4O_2$	365.17388	249.0776	5.07	JWH-081 D9
5-CHLORO-THJ018	$C_{23}H_{21}ClN_2O$	377.14152	249.0771	7.12	JWH-081 D9
5-F-AB-PINACA	$C_{18}H_{25}FN_4O_2$	349.20343	233.1066	4.61	JWH-081 D9
5-F-APINACA	$C_{23}H_{30}FN_3O$	384.24457	135.1168	7.18	JWH-081 D9
5-F-APP-PICA	$C_{23}H_{26}FN_3O_2$	396.20818	232.1111	4.93	JWH-081 D9
5-F-APP-PINACA	$C_{22}H_{25}FN_4O_2$	397.20343	233.1065	5.06	JWH-081 D9
5-F-CUMYL-PINACA	$C_{22}H_{26}FN_3O$	368.21327	233.1064	6.33	JWH-081 D9
5-F-NNEI MAPHTHYL isomer	$C_{24}H_{23}FN_2O$	375.18672	232.1112	6.42	JWH-081 D9
5-F-PB22	$C_{23}H_{21}FN_2O_2$	377.16598	232.1113	6.09	JWH-081 D9
AB-CHMINACA	$C_{20}H_{28}N_4O_2$	357.2285	241.1333	5.63	JWH-081 D9
AB-FUBINACA	$C_{20}H_{21}FN_4O_2$	369.17213	253.075	4.90	JWH-081 D9
AB-PINACA	$C_{18}H_{26}N_4O_2$	331.21285	215.1181	5.18	JWH-081 D9
ADBICA	$C_{20}H_{29}N_3O_2$	344.23325	214.121	5.41	JWH-081 D9
ADB-PINACA	$C_{19}H_{28}N_4O_2$	345.2285	215.1163	5.55	JWH-081 D9
AM-1220	$C_{26}H_{26}N_2O$	383.21179	98.0958	4.14	JWH-081 D9
AM-2201	$C_{24}H_{22}FNO$	360.17582	155.0488	6.49	JWH-081 D9
AM-2233	$C_{22}H_{23}IN_2O$	459.09279	98.0963	3.77	JWH-081 D9
AM-694	$C_{20}H_{19}FINO$	436.05682	230.9297	6.12	JWH-081 D9
APP-FUBINACA	$C_{24}H_{21}FN_4O_2$	417.17213	253.0755	5.23	JWH-081 D9
CB-13	$C_{26}H_{24}O_2$	369.18491	155.049	7.37	JWH-081 D9
CUMYL PEGACLONE	$C_{25}H_{28}N_2O$	373.22744	255.1475	6.66	JWH-081 D9
JWH-007	$C_{25}H_{25}NO$	356.20089	155.0483	7.30	JWH-081 D9
JWH-015	$C_{23}H_{21}NO$	328.16959	155.0493	6.48	JWH-081 D9
JWH-018	$C_{24}H_{23}NO$	342.18524	155.0493	6.84	JWH-081 D9
JWH-019	$C_{25}H_{25}NO$	356.20089	169.0651	7.46	JWH-081 D9

JWH-020	$C_{26}H_{27}NO$	370.21654	155.0483	7.75	JWH-081 D9
JWH-073	$C_{23}H_{21}NO$	328.16959	155.0487	6.73	JWH-081 D9
JWH-081	$C_{25}H_{25}NO_2$	372.19581	185.0593	7.15	JWH-081 D9
JWH-098	$C_{26}H_{27}NO_2$	386.21146	155.0481	7.32	JWH-081 D9
JWH-122	$C_{25}H_{25}NO$	356.20089	169.0644	7.46	JWH-081 D9
JWH-147	$C_{27}H_{27}NO$	382.21654	155.0481	7.79	JWH-081 D9
JWH-203	$C_{21}H_{22}ClNO$	340.14627	125.015	7.01	JWH-081 D9
JWH-210	$C_{26}H_{27}NO$	370.21654	183.0802	7.57	JWH-081 D9
JWH-250	$C_{22}H_{25}NO_2$	336.19581	121.0643	6.50	JWH-081 D9
JWH-251	$C_{22}H_{25}NO$	320.20089	214.1219	6.85	JWH-081 D9
JWH-302	$C_{22}H_{25}NO_2$	336.19581	214.1209	6.50	JWH-081 D9
JWH-307	$C_{26}H_{24}FNO$	386.19147	155.0486	7.29	JWH-081 D9
JWH-398	$C_{24}H_{22}ClNO$	376.14627	189.0102	7.68	JWH-081 D9
MAB-CHMINACA	$C_{21}H_{30}N_4O_2$	371.24415	241.1313	5.90	JWH-081 D9
MAM-2201	$C_{25}H_{24}FNO$	374.19147	169.0646	6.64	JWH-081 D9
MDMB-CHMINACA	$C_{22}H_{31}N_3O_3$	386.24382	241.1324	7.22	JWH-081 D9
MDMB-CHMICA	$C_{23}H_{32}N_2O_3$	385.24857	240.1382	6.75	JWH-081 D9
MMB-2201	$C_{20}H_{27}FN_2O_3$	363.20785	232.1116	5.61	JWH-081 D9
PB-22	$C_{23}H_{22}N_2O_2$	359.1754	214.1203	6.72	JWH-081 D9
RCS-4	$C_{21}H_{23}NO_2$	322.18016	135.0439	6.50	JWH-081 D9
RCS-8	$C_{25}H_{29}NO_2$	376.22711	121.0644	7.46	JWH-081 D9
STS-135	$C_{24}H_{31}FN_2O$	383.24932	135.1157	7.17	JWH-081 D9
UR-144	$C_{21}H_{29}NO$	312.23219	125.0954	7.43	JWH-081 D9
UR-144-5-OH	$C_{21}H_{29}NO_2$	328.22711	125.0957	5.88	JWH-081 D9
WIN-48	$C_{23}H_{26}N_2O_3$	379.20162	135.0429	3.75	JWH-081 D9
WIN-55	$C_{27}H_{26}N_2O_3$	427.20162	155.0482	5.56	JWH-081 D9
XLR-11	$C_{21}H_{28}FNO$	330.22277	125.0944	6.79	JWH-081 D9
25B-NBOMe	$C_{18}H_{22}BrNO_3$	380.08558	121.0651	3.94	MDPV D8
25C-NBOMe	$C_{18}H_{22}ClNO_3$	336.1361	121.0657	4.02	25I-NBOMe D3
25H-NBOMe	$C_{18}H_{23}NO_3$	302.17507	121.0654	3.59	25I-NBOMe D3
25I-NBOMe	$C_{18}H_{22}INO_3$	428.07172	121.0639	4.10	25I-NBOMe D3
2C-B	$C_{10}H_{14}BrNO_2$	260.02807	243.0025	2.80	MDPV D8
2C-P	$C_{13}H_{21}NO_2$	224.16451	207.1355	3.45	mCPP D8
3-4 DMMC	$C_{12}H_{17}NO$	192.13829	159.103	2.73	MDPV D8
4-F-METCAT	$C_{10}H_{12}FNO$	182.09757	149.0631	1.66	Mephedrone D3
4-MEC	$C_{12}H_{17}NO$	192.13829	174.1258	2.47	MDPV D8

5-EAPB	$C_{13}H_{17}NO$	204.13829	131.0479	2.74	MDPV D8
6-APB	$C_{11}H_{13}NO$	176.10699	131.0492	2.38	MDPV D8
ALFA-PVP	$C_{15}H_{21}NO$	232.16959	91.0535	2.91	MDPV D8
Buphedrone	$C_{11}H_{15}NO$	178.12264	131.0700	1.83	MDPV D8
Butylone	$C_{12}H_{15}NO_3$	222.11247	174.0917	2.25	MDPV D8
Ethylone	$C_{12}H_{15}NO_3$	222.11247	174.0916	2.13	PCP D5
Ketamine	$C_{13}H_{16}ClNO$	238.09932	125.0149	2.42	MDPV D8
mCPP	$C_{10}H_{13}ClN_2$	197.084	154.0414	2.70	mCPP D8
MDPV	$C_{16}H_{21}NO_3$	276.15942	126.1278	2.90	MDPV D8
Mephedrone	$C_{11}H_{15}NO$	178.12264	144.0810	2.27	Mephedrone D3
Methedrone	$C_{11}H_{15}NO_2$	194.11756	146.0602	2.09	MDPV D8
Methylone	$C_{11}H_{13}NO_3$	208.09682	160.0763	1.78	MDPV D8
MXE	$C_{15}H_{21}NO_2$	248.16451	203.1058	2.64	MDPV D8
N-Ethylcathinone	$C_{11}H_{15}NO$	178.12264	132.0808	1.83	MDPV D8
N-ethylpentylone	$C_{14}H_{19}NO_3$	250.14377	202.1211	2.79	MDPV D8
PCP	$C_{17}H_{25}N$	244.20598	91.0534	3.27	PCP D5
PCP-4MEO	$C_{18}H_{27}NO$	274.21654	121.0638	3.41	PCP D5
Pentedrone	$C_{12}H_{17}NO$	192.13829	145.0883	2.50	Mephedrone D3
Pentylone	$C_{13}H_{17}NO_3$	236.12812	188.1065	2.70	MDPV D8
PMA	$C_{10}H_{15}NO$	166.12264	121.0641	2.10	MDPV D8
PMMA	$C_{11}H_{17}NO$	180.13829	121.0651	2.18	MDPV D8
Ritalinic acid	$C_{13}H_{17}NO_2$	220.13321	84.0806	2.30	MDPV D8
Trazodone	$C_{19}H_{22}ClN_5O$	372.15856	176.0804	3.30	MDPV D8
4-ANPP	$C_{19}H_{24}N_2$	281.20123	105.0689	3.44	Fentanyl D5
4-Fluorobutyrfentanyl	$C_{23}H_{29}FN_2O$	369.23367	188.1434	3.98	Fentanyl D5
4-methylfentanyl	$C_{23}H_{30}N_2O$	351.24309	202.1577	3.71	Fentanyl D5
3-methylnorfentanyl	$C_{15}H_{22}N_2O$	246.1732	69.6693	2.81	Fentanyl D5
Acetyl Fentanyl	$C_{21}H_{26}N_2O$	323.21179	188.1434	3.10	Fentanyl D5
Acrylfentanyl	$C_{22}H_{26}N_2O$	335.21179	188.1417	3.35	Fentanyl D5
AH-7921	$C_{16}H_{22}Cl_2N_2O$	329.1182	172.9545	3.50	Fentanyl D5
Alfentanil	$C_{21}H_{32}N_6O_3$	417.26087	268.1776	3.49	Fentanyl D5
Butyrfentanyl	$C_{23}H_{30}N_2O$	351.24309	188.1417	3.69	Fentanyl D5
Butyryl Fentanyl (carboxy metabolite)	$C_{23}H_{28}N_2O_3$	381.21727	188.1417	3.04	Fentanyl D5
Carfentanyl	$C_{24}H_{30}N_2O_3$	395.23292	113.059	3.65	Fentanyl D5
Cyclopropylfentanyl	$C_{23}H_{28}N_2O$	349.22744	188.1419	3.54	Fentanyl D5
Despropionyl p-Fluorofentanyl	$C_{19}H_{23}FN_2$	299.1918	105.0691	3.60	Fentanyl D5

Fentanyl	$C_{22}H_{28}N_2O$	337.22744	188.1434	3.55	Fentanyl D5
FuranilFentanil	$C_{24}H_{26}N_2O_2$	375.2067	188.1434	3.67	Fentanyl D5
Hydrocodone	$C_{18}H_{21}NO_3$	300.15942	199.0761	2.16	Fentanyl D5
MT-45	$C_{24}H_{32}N_2$	349.26383	181.0999	4.12	Fentanyl D5
OCFENTANYL	$C_{22}H_{27}FN_2O_2$	371.21293	188.1418	3.25	Fentanyl D5
Remifentanil	$C_{20}H_{28}N_2O_5$	377.2071	113.0603	2.92	Fentanyl D5
Sufentanil	$C_{22}H_{30}N_2O_2S$	387.21008	238.1268	3.82	Fentanyl D5
Tramadol	$C_{16}H_{25}NO_2$	264.19581	58.0648	2.71	Fentanyl D5
U-47700	$C_{16}H_{24}Cl_2N_2O$	331.13385	284.061	3.42	Fentanyl D5
Valerylfentanyl (carboxy metabolite)	$C_{24}H_{30}N_2O_3$	395.23292	113.0589	3.65	Fentanyl D5
4-ACETOXY DIPT	$C_{18}H_{26}N_2O_2$	303.2067	160.0747	3.10	MDPV D8
4-ACETOXY DMT	$C_{14}H_{18}N_2O_2$	247.1441	174.0899	2.58	MDPV D8
5-MeO-DALT	$C_{17}H_{22}N_2O$	271.18049	174.0912	3.10	MDPV D8
5-MeO-DIPT	$C_{17}H_{26}N_2O$	275.21179	174.091	3.31	MDPV D8
5-METOXY AMT	$C_{12}H_{16}N_2O$	205.13354	147.0672	2.25	PCP D5
DMT	$C_{12}H_{16}N_2$	189.13862		2.06	Mescaline D9
HARMINE	$C_{13}H_{12}N_2O$	213.10224	170.0826	2.88	MDPV D8
LSD	$C_{20}H_{25}N_3O$	324.20704	223.1227	3.13	MDPV D8
Mescaline	$C_{11}H_{17}NO_3$	212.12812	195.1019	1.98	PCP D5
Mitragynine	$C_{23}H_{30}N_2O_4$	399.22783	174.0914	3.69	MDPV D8
Psilocin	$C_{12}H_{16}N_2O$	205.13354	132.0808	1.78	MDPV D8
Norfentanyl	$C_{14}H_{20}N_2O$	233.16484	144.0808	2.46	Norfentayl D5
5-F-ADB	$C_{20}H_{28}FN_3O_3$	378.21875	233.1061	6.17	JWH-081 D9
ADB-FUBINACA	$C_{21}H_{23}FN_4O_2$	383.18778	253.0747	5.19	JWH-081 D9
β-Phenylfentanyl	$C_{28}H_{32}N_2O$	413.25874	188.1398	4.27	Fentanyl D5
Butyrilnorfentanyl	$C_{15}H_{22}N_2O$	247.18049	84.0800	3.00	Fentanyl D5
Ethylphenidate	$C_{15}H_{21}NO_2$	248.16451	84.0797	3.05	MDPV D8
Ethyltryptamine	$C_{12}H_{16}N_2$	189.13862	130.0643	2.65	MDPV D8
Furanylnorfentanyl	$C_{16}H_{18}N_2O_2$	271.1441	84.0798	2.55	Fentanyl D5
Hydroxy-fentanyl	$C_{22}H_{28}N_2O_2$	353.22235	204.135	3.11	Fentanyl D5
Hydroxy-thiofentanyl	$C_{20}H_{26}N_2O_2S$	359.17878	192.0829	2.94	Fentanyl D5
Fentanyl	$C_{26}H_{28}N_2O$	385.22744	188.1434	3.91	Fentanyl D5
5-MeO-DMT	$C_{13}H_{18}N_2O$	219.14919	58.0691	2.22	MDPV D8
Phenylacetylfentanyl	$C_{27}H_{30}N_2O$	399.24309	188.1408	3.05	Fentanyl D5
Eutylone	$C_{13}H_{17}NO_3$	236.12812	188.1060	2.64	MDPV D8
PCP-D5	$C_{17}H_{20}[2H]_5N$	249.23736	96.0854	3.24	

AH-7921 D6	$C_{16}H_1[2H]_6Cl_2N_2O$	335.15586	172.9546	3.64	
Norfentayl D5	$C_{14}H_{15}[2H]_5N_2O$	238.19622	84.0800	2.40	
Oxycodone D6	$C_{18}H_{15}[2H]_6NO_4$	322.192	304.1792	1.93	
Fentanyl D5	$C_{22}H_{23}[2H]_5N_2O$	342.25882	188.1416	3.50	
JWH-250 D5	$C_{22}H_{20}[2H]_5NO_2$	341.22719	121.0637	6.73	
JWH-081 D9	$C_{25}H_{16}[2H]_9NO_2$	381.2523	185.0577	7.24	
JWH-018 D9	$C_{24}H_{14}[2H]_9NO$	351.24173	155.0485	7.03	
Mephedrone D3	$C_{11}H_{12}[2H]_3NO$	181.14147	148.1064	2.35	
mCPP D8	$C_{10}H_5[2H]_8ClN_2$	205.13422	158.0661	2.65	
25I-nBOMe D3	$C_{18}H_{19}[2H]_3INO_3$	431.09055	124.0835	4.09	
Mescaline D9	$C_{11}H_8[2H]_9NO_3$	221.18461	171.0921	1.90	
MDPV D8	$C_{16}H_{13}[2H]_8NO_3$	284.20963	134.1778	2.97	

Reagents and Standards

All chemicals, including methanol, formic acid and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). All stock standard solutions were prepared in methanol at 1 mg/mL and stored at –20 °C until used. Working solution of 132 analytes (identified among the most common synthetic cannabinoids, synthetic opioids, synthetic cathinones and hallucinogens monitored nationally and internationally) and internal standard solution containing 13 deuterated NPSs, were prepared at the final concentration of 1 µg/mL by dilution with methanol. The analytical standards of the target analytes and deuterated internal standards were purchased from LGC Promochem (Milan, Italy) and Sigma-Aldrich (Milan, Italy) (purity > 99%, concentration between 0.1 mg/mL and 1 mg/mL), or kindly provided by the National Early Warning System (provided at a concentration of 0.02 mg/mL). DBS cards (FTA™ DMPK C) were purchased from Whatman™ GE Healthcare (UK).

Sample Preparation

For the preparation of spiked specimens used in the analytical method development and validation, a standard blood matrix was obtained by mixing different aliquots of blank whole blood obtained from volunteers (laboratory personnel) after having signed an informed consent. The absence of all the investigated analytes was verified by means of standard procedures routinely used in our lab for blood testing. The blank samples were then fortified at six concentration levels (5, 7.5, 10, 25, 50, 100 ng/mL) with an NPS working solution.

The blood samples were vigorously stirred prior to spotting a 30 µL aliquot on a DBS collection card using a calibrated pipette. The spots were allowed to dry for at least three hours at room temperature. The entire spots on the DBS card were punched out and transferred into an Eppendorf tube together with 500 µL methanol/acetonitrile (3:1, v/v) mixture. A 12.5 µL solution of the deuterated internal standards (ISTD) was added to the tubes to reach a final concentration of 25 ng/mL. After ultrasonication (215/860 W, 50/60 Hz) for 30 min at room temperature, the tubes were centrifuged for 5 min at 13000 g. The supernatant was transferred into a fresh tube prior to evaporating the solvent under a gentle nitrogen flow at room temperature. The dry residue was reconstituted with 50 µL methanol, centrifuged for 5 min at 13000 g, and 5 µL of the supernatant was injected into the UHPLC system.

Instrumentation

UHPLC separation was performed with a Phenomenex Kinetex C18 column (100 × 2.1 mm, 1.7 µm) maintained at 45 ºC on the SCIEX ExionLC™ AC system. The mobile phases consisted of water (A) and acetonitrile (B), both with formic acid 5 mM. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions: (A:B, v:v) isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration for 2.5 min to the initial condition. The total run time was 10 min.

All analyses were performed using a quadrupole/time-of-flight SCIEX X500R QTOF mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo VTM ion source operating in electrospray positive-ion mode (full MS and MS/MS parameters available in the Supplementary Table S1). Data acquisition involved a preliminary TOF-MS high-resolution full scan followed by a SWATH™ acquisition protocol which used a variable window setup (18 windows covering mass range from *m/z* 99.5 to 575.0 at 0.025 resolving power), resulting in a final cycle time of 0.933 sec. The qualitative identification of the 132 target analytes was based on the coincidence of their retention times, precursor ion and characteristic fragment ion *m/z* values (mass error accepted < 5 ng/mL) (Table S1) as identification parameters. Data were acquired using the SCIEX OS 1.5 Software.

Method validation

The validation strategy was based on a protocol recently published [18]. Each standard of blank whole blood fortified at six concentration levels (5, 7.5, 10, 25, 50, 100 ng/mL) was analysed nine times in three working sessions (i.e., 3×3) along ten days. This dataset of 54 analysis formed the groundwork on which the statistical evaluation of several validation parameters was founded, including calibration, intra- and inter-day trueness, repeatability and intermediate precision (at 6 concentration levels), limit of detection (LOD), limit of quantification (LOQ). Recovery, matrix effect and stability

parameters were evaluated with further independent experiments. Other independent analyses on purposely spiked samples were performed later to verify the actual detection of the target analytes at the calculated LODs (see the following paragraphs). An *ad hoc* Excel® sheet was built in-house to adapt the routine developed by Desharnais et al. [19]. All the equations employed to compute the validation parameters have been omitted from this text and can be found elsewhere [20].

Calibration

Calibration curves were generated from the peak-area ratio between each analyte and the ISTD quantifier transitions; the ratio was then plotted on the y-axis against the nominal analyte concentration to generate and estimate the curve that best fits and predicts the data distribution, with the support of statistical tests [21]. In the first step of the calibration process, homoscedastic vs. heteroscedastic distribution of the data points was evaluated by analyzing the residues distribution (9 data-points at 6 concentration levels) and comparing the variances at low, medium and high concentration levels. If the variance increased with concentration, the system proved heteroscedastic ($p < 0.05$) and a weighted model was adopted, using either a 1/x weighting factor (when the variance increased proportionally to the concentration) or a $1/x^2$ weighting (when a quadratic increase of the variance was observed). Then, the order of the calibration model (linear vs. quadratic) was selected on the basis of Mandel and lack-of-fit tests, using a significance level of 95%.

LOD and LOQ

The Hubaux-Vos method [22] was used for the calculation of the LOD values. The original method is based on the hypothesis that the data distribution is homoscedastic. Since this condition is usually not met, the weighting factors were included in the Hubaux-Vos calculation of the LOD, as described in the Currie's method [23]. LOQs were attributed to the lowest concentration within the respective calibration range yielding trueness values within the accepted limit (typically, bias% < ±20%).
The calculated LOD values were then experimentally tested by spiking the blank matrix with the target analytes at the approximate LOD concentrations and verifying that the signal-to-noise ratio (S/N) was higher than 3.

Trueness and Precision

The procedure used to calculate the intra-day trueness (expressed as bias %) considers separately the three calibration curves of the same day: two of these are cyclically used for the construction of a calibration model, which is used to perform a back-calculation of the points of the third curve. The concentration values calculated by repeating the procedure for the three days are used to determine

the bias %. The calculation of the inter-day trueness is similar, but the calibration constructed with the six data-points at each level collected in two days is cyclically used to back-calculate the concentration relative to the data-points of the third day. The results are then averaged.

Repeatability was independently assessed for the three days of analysis. For each day of validation, the calibration model was used to calculate the concentrations of the three experimental replicates; then, the coefficient of variation (CV%) was determined for each concentration level by averaging the precision obtained for the three days. The intermediate precision followed a similar procedure, that make use of the nine replications collected during all three days. In practice, the protocol used for calculating trueness and precision is based on the data collected for the 9 calibration curves, obtained in the three days [24].

Matrix effect and extraction recovery

The matrix effect was estimated at the concentration level of 10 ng/mL by comparing the experimental results obtained from three blank whole blood samples and MeOH solutions, equally spiked after the extraction step [25]. The ionization suppression/enhancement for each target analyte was expressed as the mean percentage ratio between the two measured signals. Owing to coelution, several spiked analytes are potentially present within a single ESI droplet, resulting in the possibility that the matrix effect may be partially due to their compresence, which is unlikely in a real blood sample. For this reason, the matrix effect for the compounds whose ion suppression exceeded 50% was tested again by spiking the blank blood with the single substance.

The extraction recovery was determined by comparing the experimental results obtained from three whole blood samples spiked at the concentration level of 100 ng/mL, before and after the extraction step. It was expressed as the mean percentage ratio between the two signals with its uncertainty expressed as extraction repeatability (CV%) from the three replications. Also in this case, the recovery was measured again for the analytes whose value was lower than 50% in order to evaluate a potential correlation between recovery and the compresence of analytes commonly present/absent in the same real sample.

Stability

Stability was evaluated at low (10 ng/mL) and high (100 ng/mL) concentrations levels. Following the deposition of the drop of blood and after three hours of drying, the samples were placed at the temperature conditions of −20 °C, 4 °C and 35 °C and stored before analysis for, respectively, 1, 3, 7 and 40 days. The conditions were chosen to simulate the possible storage scenarios, from the mildest

to the most critical conditions, so as to evaluate i) the transfer of the cards from the sampling site (*e.g.*, workplace) to the testing laboratory, ii) a short-term storage of the cards before analysis, and iii) a long-term storage of the cards, in the event that a counter-analysis is requested after a long time interval, for example when a positive result is challenged by the sample donor. The stability conditions were observed through trend lines that describe the variation of the concentration calculated at the different storage temperatures and after different storage intervals. Since a 15% deviation from the nominal value is compatible with the experimental uncertainty, the presence of degradation effects was positively detected above this 15% limit [26].

Application on real samples

The applicability of the developed procedure on real samples was verified on venous whole blood samples previously analyzed and tested positive by means of a previously published UHPLC-MS/MS method, which is periodically reviewed and updated according to new compounds entering the market [27]. At the time of analysis, the blood real specimens were obtained by venous sampling and then stored until analysis under controlled conditions, at the temperature of 4 °C. A drop of blood was spotted on the card and then treated as previously described. Whenever the effective drug concentration exceeded the calibration range, the samples were diluted to fit the quantitation range considered in the curve. The results obtained from the present method based on DBS and our standard procedure on venous blood were then compared to evaluate the performance of the new procedure in identifying and quantifying some of the analytes presented in Table 1.

Results and discussion

The present method proved adequate for the individual detection of all 132 target analytes and 13 internal standards at concentrations equal or lower than 10 ng/mL. In this work, a C18 column was used to obtain the best separation of the investigated compounds. The chromatographic run was completed in 10 min, including the final re-equilibration time, in agreement with the efficiency requirement needed for routine application. As shown in Table 1, 90 out of 132 compounds elute in the first 4.5 min, while 38 of the remaining analytes (mostly synthetic cannabinoids) elute between 6.5 and 7.75 min. Even when co-elution of chromatographic peaks was observed, high resolution mass spectrometry guaranteed separate quantifications of the coeluting substances by means of their differences in precursor ion and characteristic fragment ion *m/z* values (mass error accepted < 5 ng/mL). A typical example of extracted ion chromatogram is presented in Figure 1, in which the group of 24 synthetic opioids - spiked in the whole blood at 10 ng/mL concentration - is represented.

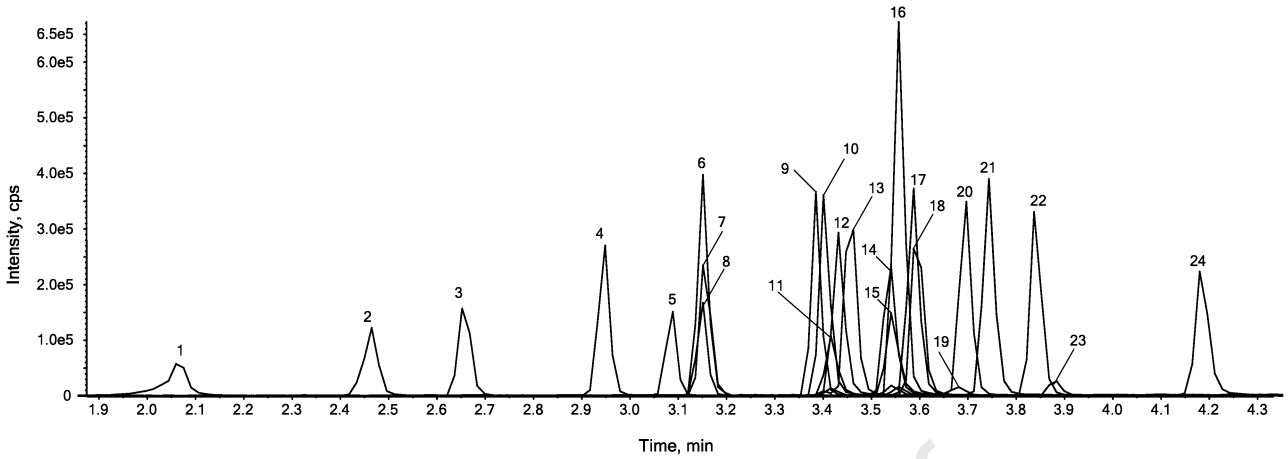

Figure 1. Chromatographic profile of 24 synthetic opioids included in the panel within a 1.9 - 4.3 min retention time interval. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition method, obtained from the 10 ng/mL neat standard mixture. Method was built using the Scheduled Algorithm Pro in SCIEX OS Software. In the figure: 1) Hydrocodone, 2) Norfentanyl, 3) Butyryl norfentanyl, 4) Remifentanil, 5) Butyryl fentanyl carboxy metabolite, 6) Acetyl Fentanyl, 7) Ocfentanyl, 8) Carfentanyl, 9) Alfentanil, 10) Acrylfentanyl, 11) U-47700, 12) 4-ANPP, 13) Fentanyl, 14) Despropionyl p-fluorofentanyl, 15) AH-7921, 16) FuranilFentanil, 17) Butyrylfentanyl, 18) Cyclopropylfentanyl, 19) Valeryl fentanyl carboxy metabolite 20) 4-F-Butyrylfentanyl, 21) 4-Methyl fentanyl, 22) Tramadol, 23) Sufentanil, 24) MT-45.

The complete results of the validation experiments for DBS samples fortified with 132 analytes at six concentrations levels (5, 7.5, 10, 25, 50 and 100 ng/mL) and 13 internal standards are reported in the Supplementary Tables S2-S7. Table S2 reports the outcome of the calibration process. Analysis of residues and variances of calibration data points at low, medium and high concentration levels showed that heteroscedastic distributions were present for all the target analytes, making the introduction of weighting factors in the calibration (either $1/x$ of $1/x^2$) beneficial. Moreover, the quadratic term of the calibration model proved statistically significant for all but one (JWH-015) tested substances. Consequently, a quadratic calibration model was chosen for all analytes.

The LOD values, calculated using the corrected Hubaux-Vos algorithm, ranged from 1.3 ng/mL for ethylphenidate and 1.8 ng/mL for 4-F-butyrylfentanyl up to 6.3 ng/mL for UR-144. The LOD was then verified by spiking blank blood samples with decreasing concentrations (7.5, 5, 2 ng/mL) until a response equivalent to three times the background noise was observed. This verification process proved that for 74 analytes out of 132 (56%), a 2 ng/mL LOD was experimentally verified, namely a lower LOD value than the calculated ones (Table S3). This evidence suggests that the Hubaux-Vos algorithm corrected with Currie's method provide reliable yet conservative LOD values.

The experimental results obtained for LOD values confirmed that the overall method sensitivity is adequate for the detection of NPS extracted from DBS in routine applications. In fact, the 2 ng/mL

limit consistently assessed in the present study represents the actual lower LOD measured in several other studies [28–30] and an acceptable requirement for NPS detection in blood, based on information available from case reports [31–33]. On the other hand, specific recommendations for DBS detection in blood have not been established yet, as long as little clinical information is available at the moment.

The extraction recovery was found to depend on the different classes of drugs considered (Figure S4), being higher for the class of synthetic cannabinoids (range 50%-100%) particularly for the JWH-series, and lower for synthetic cathinones and hallucinogens (range 30%-60%) and fentanyl analogues and synthetic opioids (range 30-50%). Relatively low extraction recoveries are expected from DBS cards [34], also taking into account that a large number of analytes have to be extracted simultaneously from the same dried droplet. However, the extraction repeatability measured from three replications proved to be satisfactory for the large majority of substances (CV% lower than 15% for 113 out of 132) [26], with the peculiar exception of the JWH-series that combines high extraction yield with relatively lower repeatability (10 out of 17 showed CV% in the range 15%-28%).

Further tests were performed in order to evaluate a possible correlation between the recovery yield of fentanyl, norfentanyl and ketamine and their compresence, as they are frequently present in the same real sample. The results showed (Table S5) that the extraction recovery did not vary significantly, no matter if only these three substances were spiked in the blank blood or all 132 analytes were simultaneously present in the sample.

Intermediate precision and repeatability (expressed as percent variation coefficient, CV%) and trueness (expressed as bias %) for all 132 target analytes are reported in Table S3. Notably, the validation procedure adopted in the present study allows precise and accurate calculation at all concentrations involved in the calibration process (6 calibration levels), not only at low, intermediate and high concentrations, as most recommended validation protocols entail. An example is reported in Figure 2, showing precision and trueness trends for AH-7921. The repeatability and intermediate precision and trueness trend for all the classes of compounds studied is reported in the Supplementary Figure S6.

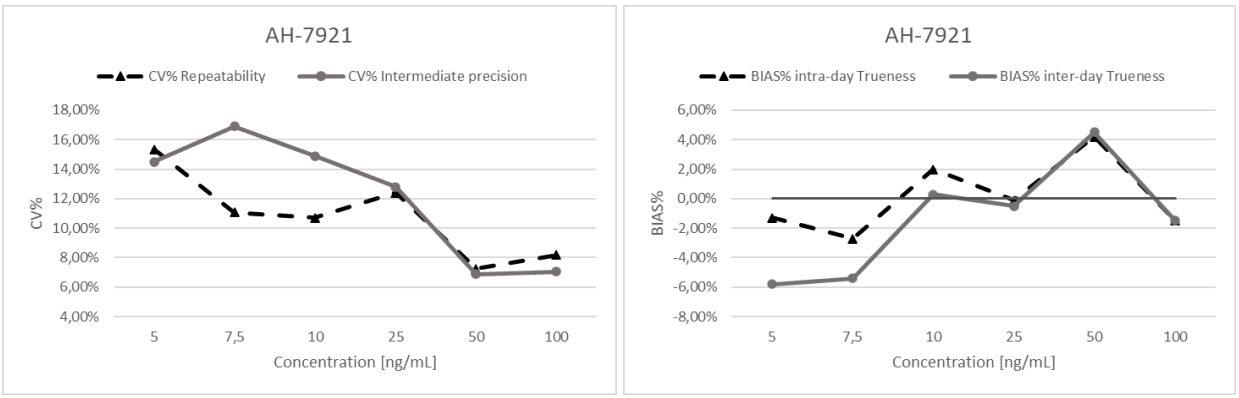

Figure 2. Intermediate precision and repeatability (CV%) and trueness (bias%) trends for the synthetic opioid AH-7921 at the different calibration levels.

In particular, the aggregate inter-day trueness data are reported in Figure 3, showing extremely limited deviation from the expected concentrations (-10% < bias% < +10%) for 114 target analytes out of 132. The inter-day trueness is an especially important performance parameter because it provides a reasonable estimation of the quantitative measurement reliability under routine conditions and – indirectly – the quality of calibration. This positive outcome partly relies on its averaging the single results of repeated determinations, forasmuch as the random sources of variability that influence the precision results (extraction yield, matrix effect, *etc.*) may find equalization. Therefore, it is highly recommended to collect blood droplets on three separate DBS cards and average their results whenever accurate quantitative determinations are needed.

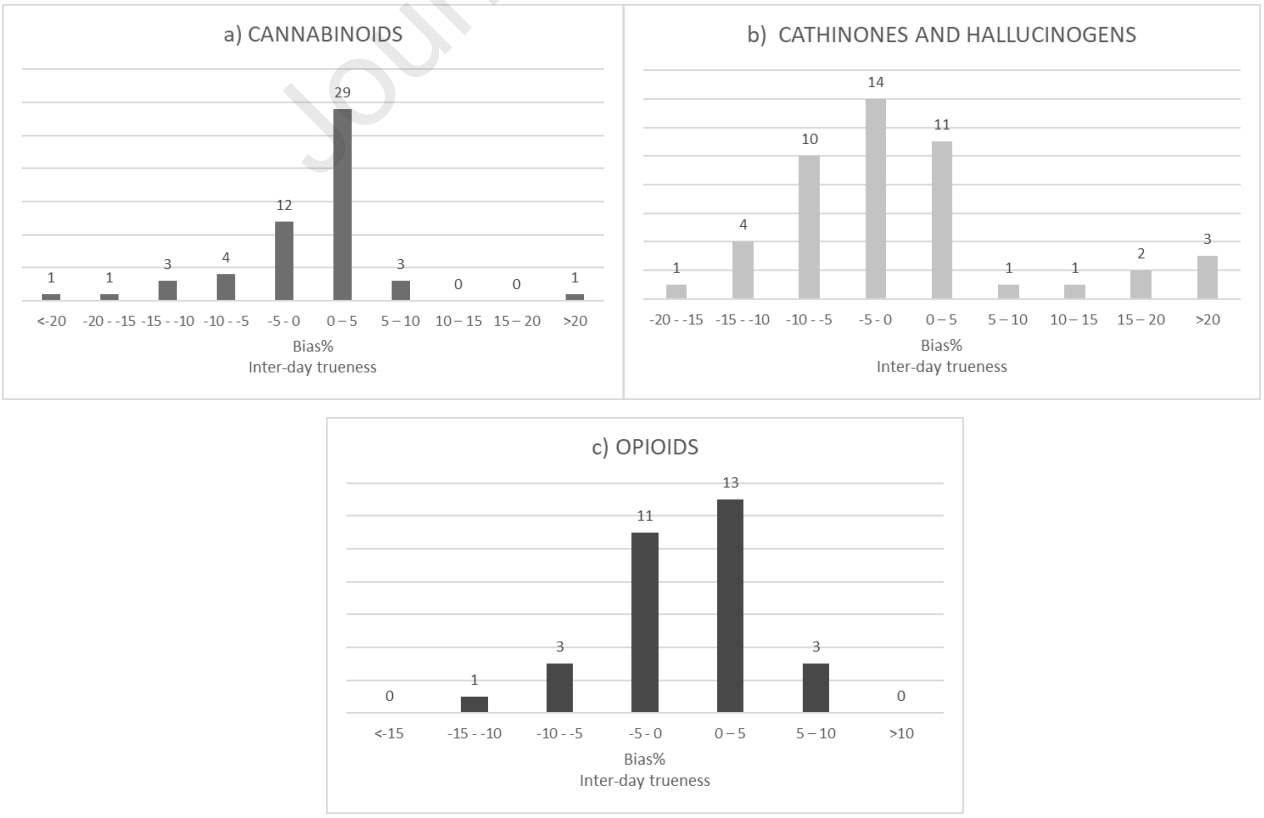

Figure 3. Inter-day trueness (bias%) trends for the class of a) synthetic cannabinoids, b) cathinones and hallucinogens and c) synthetic opioids.

Precision data, described by CV% from repeated independent analyses, show rather homogeneous results within each class and between different classes of substances under study (Supplementary Table S3 and Figure S6). Therefore, the observed data variability can realistically be attributed to the analytical method itself rather than to specific chemical properties of the investigated substances or their interaction with the DBS card. Quite interestingly, the average intermediate precision result (CV% = 15.5%, N = 132) is only slightly higher than the intra-day variability (CV% = 11.4%, N = 132), as is expected for a substantially stable method and calibration.

Electrospray ionization produced moderate ion suppression, resulting in matrix effect values ranging between negligible to –50% for synthetic cannabinoids (average –29%), between negligible to –40% for synthetic cathinones and hallucinogens (average –24%) and fentanyl analogues and synthetic opioids (average –27%) (Table S7). Due to the presence of many spiked analytes inside the single blood drop and the crowding of peaks around certain retention times (for example, in the interval 3.4-4.0 min), we considered the possibility that part of the matrix effect could be attributed to their coelution and interaction. To test this hypothesis, we measured again the matrix effect using blood droplets spiked with only the five substances whose ion suppression value exceeded –50% in the first set of experiments. Indeed, ion suppression decreased for all target analytes, on average from –62% to –29%. In particular, the effect reduction for 5-chloro-TH-J018 was from –69% to –9%, for AM-2201 from –57% to –43%, for JWH-007 from –66% to –7%, for MMB-2201 from –58% to –38%, and for WIN-48 from –59% to –49%. These results suggest that the recorded ion suppression initially attributed to the blood matrix is likely to be influenced by the coelution of several spiked analytes. In real samples, the simultaneous presence of more than five drugs in a single blood sample is quite implausible to occur, even in the worst cases. In conclusion, the ion suppression data reported in Table S6 should be considered as upper limits observed under stressed conditions rather than expected values in real toxicological contexts. Again, whenever accurate quantitative determination of a single substance is needed, specific experiments should be planned to complete the validation for that specific substance and an on-purpose calibration model may be prudentially built, so as to avoid overestimation of the matrix effect.

The largest part of the target analytes showed good stability on DBS cards after drying, even for long periods of time. At both concentration levels tested, deviations from the nominal value fell within 15% for most of them. Quite obviously, the NPS stability proved to be slightly more affected when a storage temperature of 35 °C was maintained, in combination with a high storage period and low

analyte concentration. Figure 4 reports an example of this trend. In particular, synthetic cannabinoids – and especially the JWH group – showed lower stability than the other classes of NPS. An example of particularly poor stability is evident in Figure 5 for JWH-015: on one hand, acceptable conservation (> 90%) is guaranteed during the initial 7 days at any storage temperature, on the other hand it is evident that significant degradation occurred after 40 storage days even if the DBS cards were maintained at –20 °C. In summary, the stability data suggest that storage of the DBS cards at –20 °C is not essential, the most convenient storage condition apparently being at 4 °C. As a matter of fact, extremely limited degradation is observed at 4 °C for periods fully compatible with routine analytical processing, while an acceptable level of preservation is maintained up to 40 days for most NPS. Specific caution should be exercised in counter-analyses involving the confirmation of JWH-series positive testing. Further studies will be needed to evaluate the compounds stability in real samples, similarly to what has been done on post-mortem samples for psychoactive substances [11]. Indeed, spiked samples may not always display the same stability profile as real samples.

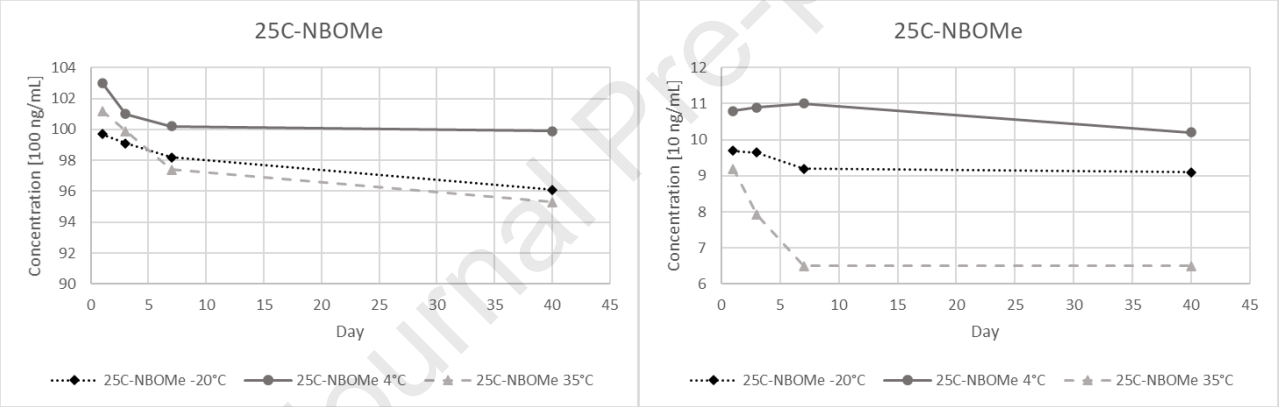

Figure 4. Stability of 25C-NBoMe at different temperature conditions (–20, 4, 35 °C) during 1, 3, 7, 40 days.

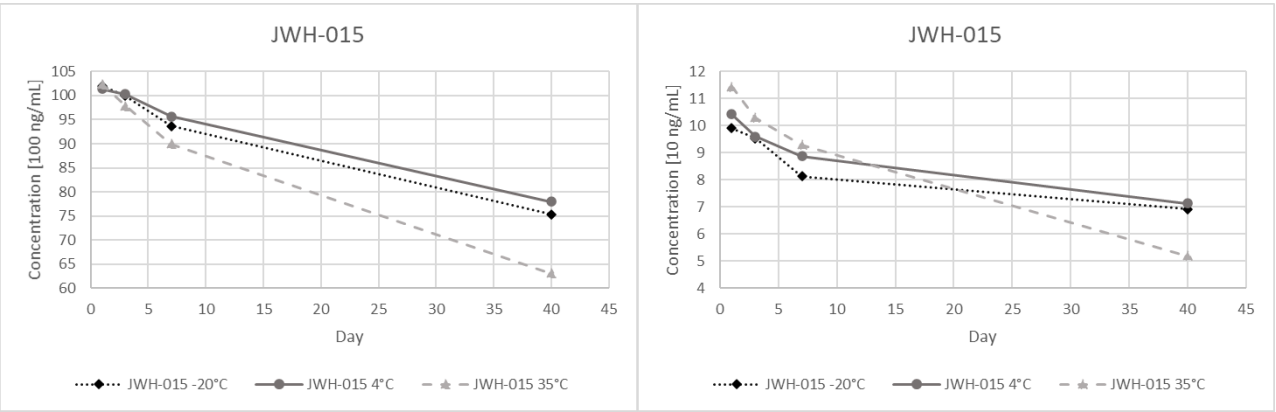

Figure 5. Stability of JWH-015 at different temperature conditions (–20, 4, 35 °C) during 1, 3, 7, 40 days.

The proven stability of NPS on DBS cards, combined with ease of sampling and minimal storage volume, represents a key asset of the DBS technique confirming its use as an alternative and innovative sampling method in troublesome conditions, for example when the sampling site is far from the laboratory and/or few days are required after sampling until the analysis is made possible.

Application to real samples

After completing the validation, the present method was applied to seven venous whole blood samples which previously tested positive to ketamine and fentanyl in our laboratory. The results summarized in Table 2 show that the new procedure based on DBS sample collection allowed confirmation of the ketamine and fentanyl positive testing, whenever their concentrations was higher than the corresponding LOQ of the method. Also, the quantitative results proved consistent with those measured with the routine procedure based on large blood volume sampling. These results demonstrate the ability of the new technique to detect and correctly quantify the substances present in the sample.

Table 2. Ketamine and fentanyl concentrations determined on venous whole blood (ng/mL) from real toxicology casework and measured with both the routine laboratory procedure and the present DBS method. Values between parentheses are rough estimations below LOQ; n.d. = not detected.

ID Sample	**Routine method (UHPLC–ESI-MS/MS)**		**DBS method (UHPLC-QTOF-HRMS)**	
	Ketamine (ng/mL)	Fentanyl (ng/mL)	Ketamine (ng/mL)	Fentanyl (ng/mL)
S1	n.d.	0.3	n.d.	< LOQ
S2	37.5	n.d.	42.0	n.d.
S3	493	n.d.	597	n.d.
S4	447	0.2	600	n.d.
S5	74.0	2.0	88.0	< LOQ (~2.3)
S6	405	n.d.	700	n.d.
S7	275	2.1	140	< LOQ (~2.5)

Conclusions

The need to test biological samples for drugs of abuse keeps increasing in several social contexts, especially in workplace and road controls, so as to guarantee safer conditions for workers, drivers, and third parties, but the progressive introduction of NPS in the illegal market significantly complicated these tests and expanded the number of targeted analytes. It is nonetheless essential to discriminate the subject who are under the effect of drugs from those who may have consumed drugs days earlier, i.e. out of the control context. The matrix of choice for this discrimination is blood, but blood sampling is prevented in almost all circumstances by ethical and practical reasons.

The present method overcomes both problems by combining the use of UHPLC-QTOF-HRMS instrumentation with a simple and minimally invasive DBS sample collection for detecting as many as 132 traditional drugs and NPS selected from EMCDDA reports [35] and articles [36,37], together with their metabolites, on only 30 μL whole blood. The analytical method's validation confirmed its reliability for the extraction and accurate analysis of this wide array of structurally different NPS within an adequate concentration interval (typically, 5-100 ng/mL). The initial comparison on real toxicology samples between our traditional routine procedure based on high volume blood sampling and the new DBS procedure provides a preliminary confirmation of the potential applicability of the latter technique on a vast scale in several fields, including workplace drug testing, road controls, and drug monitoring in clinical and forensic contexts.

Prospectively, the new method will be applied on real capillary whole blood samples in comparison with venous blood. Further investigation will be devoted to the hematocrit effect (HT) and its influence on the volumetric blood sampling method, either a single drop deposited on the card (requiring a corrective factor for the HT effect) or other micro-sampling systems (VAMS).

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Highlights

- Dried Blood Spot allows fast & easy sampling, store, transport for drug detection
- Method validation for New Psychoactive Substances detection in 30 µL whole blood
- Simultaneous detection of 132 synthetic opioids, cathinones, hallucinogens & fentanyl
- High-Resolution Mass Spectrometry allows targeted and untargeted analysis of unknown
- Limits of detection in the 1.3-6.3 ng/mL range are achieved

Declaration of competing interests

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.