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Fast and efficient analyses of the post-mortem human blood and bone marrow using DI-SPME/LC-TOFMS method for forensic medicine purposes

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ABSTRACT

For the first time the method DI-SPME/LC-TOFMS was used and developed in order to determine the large antidepressant drugs in real forensic cases. The aim of the study was to optimize the new DI-SPME/LC-TOFMS method for the quantification of the large group of psychotropic drugs such as benzodiazepines, selective serotonin reuptake inhibitors, selective serotonin and noradrenaline reuptake inhibitors, tricyclic antidepressants and sleeping pills "Z". The volume of the sample, adsorption time, post-adsorption purification and desorption time were precisely optimized. The validation parameters such as limit of detection and quantification, linearity, precision during and between days and the matrix effect were determined. All obtained values are within the acceptable range for toxicological analyses. The usefulness of the method was confirmed by analyzing the post-mortem samples. Drug concentrations were determined in real samples with high precision, which gives perspectives for the DI-SPME/LC-TOFMS routine application in toxicological and forensic analyses in the future.

1. Introduction

Due to complicated post-mortem processes that occur in the body after intake of drugs or poisons like redistribution, degradation, and contamination, it is necessary to provide a complex analysis of available body fluids like urine, blood or other alternative materials such as bone marrow. Material which can be helpful in post-mortem investigation is bone marrow aspirate (BMA), which is a semi-liquid form (it can be handled with a pipette) of bone marrow, placed inside the bones [1–3]. However, analysis of alternative materials often carried out with various limitations and compared to that analysis of BMA appears to have few practical advantages. It is a well-preserved post-mortem material, because of the physical barrier provided by cortical bone, which can be easily collected in large amounts and may be used as an alternative material to peripheral venous blood [4–6]. Due to high fat level and blood supply, it is possible to determine psychotropic drugs in bone marrow, which has been confirmed by Snamina et al. [7] with very satisfying results. On the other hand Cartiser et al. [2] provided a comprehensive summary on correlation studies between blood or plasma and bone marrow levels of different xenobiotics. The correlations varied from very low to almost absolute, depending on the drug.

The success and application of the method often depend on its capabilities, trueness and also time of whole analysis. Considering extraction of analytes as the most demanding, time-consuming and multi-step process it is regarded as a critical process to carry out in the whole analysis. The technique which may facilitate and simplify the extraction of analytes from complex samples like biological materials may be Direct Immersion Solid-Phase Micro Extraction (DI-SPME) [8–10]. It is an extraction technique based on the sorption of analytes on the stationary phase of fused silica, placed on the solid support. The DI-SPME procedure is based on the exposure of the fiber to the sample for sufficient time. This technique integrates extraction, concentration and analyte desorption into a single procedure [11,12]. As the technique is reducing sample handling, solvent use, time and cost it is prosperous for toxicological and forensic analysis. Due to the wide selection of fiber coatings and its sorption capabilities DI-SPME extraction is also used in food and environmental analyses, as well as studies for its *in vivo* application are carried out. The initial application of the DI-SPME technique in drug analysis was for psychotropic substances. Moreover, in recent years the increase of DI-SPME applications was observed with connection of liquid chromatography and capillary electrophoresis [13,14].

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The aim of the studies was to present the first time the application of newly developed DI-SPME/LC-TOFMS method for the detection and quantification of a large group of psychotropic drugs and its metabolites in the post-mortem biological matrix. The matter of analysis were very often misused antidepressants from groups like Benzodiazepines (BZDs), Selective Serotonin Reuptake Inhibitors (SSRI's), Serotonin and Norepinephrine Reuptake Inhibitors (SNRI's) and Tricyclic Antidepressants (TCA's). The post-mortem biological materials which were evaluated in this study were: human blood and bone marrow aspirate. In the article, the final protocol for the optimized extraction process was formulated. As the developed method does not require modification of the matrix, it is an additional advantage, which shortens and simplifies the whole process. Crucial parameters that were optimized were sample volume, time of adsorption, post-adsorption washing and time of desorption. Once optimized, the method was evaluated by determination of validation parameters and used for the analysis of forensic case samples.

2. Experimental

2.1. Chemicals and reagents

Drug standards of the analytes: alprazolam (ALP), amitriptyline (AMI), bromazepam (BRO), carbamazepine (CBZ), citalopram (CIT), clonazepam (CLO), clorazepate (CLOR), desipramine (DESI), diazepam (DIA), estazolam (EST), flunitrazepam (FLUN), fluoxetine (FLUOX), imipramine (IMI), lorazepam (LOR), lormetazepam (LORM), midazolam (MID), nitrazepam (NITR), nordazepam (NORD), nortriptyline (NORT), paroxetine (PAROX), prazepam (PRA), temazepam (TEM), tetrazepam (TETRA), venlafaxine (VEN), zolpidem (ZOL) were purchased from Lipomed AG (Arlesheim, Switzerland). The deuterated analogues of drugs: alprazolam-d5, bromazepam-d5, diazepam-d5, lorazepam-d4, lormetazepam-d3, midazolam-d4, nitrazepam-d5, temazepam-d5, venlafaxine-d6, zolpidem-d6 were also purchased from Lipomed AG (Arlesheim, Switzerland). The LC-MS grade chromatographic solvents acetonitrile, methanol, isopropyl alcohol, and sodium hydroxide were obtained from Fluka Analytical (Seelze, Germany). Analytical grade ammonium formate was purchased from Sigma-Aldrich (St. Louis, MA, USA), analytical grade formic acid was purchased from Merck (Darmstadt, Germany). Finally, the deionized ultrapure water (18.2 M Ω · cm, TOC < 5 ppb) was obtained and filtered through a Mili-Q Plus system (Milipore, Bedford, Ma, USA).

For the analyses following devices were utilized. The HPLC vials (1.5 mL) and inserts (200 µL) were purchased from VWR (Randor, PA, USA). The SPME-LC Probe 45 µm C18-Silica fibers (Supelco) were purchased from Merck (Darmstadt, Germany). Fibers condition were monitored with use of Scanning Electron Microscope (Phenom-World, Eindhoven, The Netherlands). Adjustable manual pipettes from Sartorius AG (Göttingen, Germany) were used. Digital Vortex Mixer and Thermal Shake Touch were purchased from VWR (Randor, PA, USA). Concentrator plus was from Eppendorf AG (Hamburg, Germany). The UltiMate 3000 RS liquid chromatography system (UHPLC; Dionex, Sunnyvale, CA, USA) equipped with a Hypersil Gold Phenyl column (50 mm × 2.1 mm I.D., particles 1.9 µm; Thermo Scientific, Bermen, Germany) was used for chromatographic analyses. Chromatograph was coupled to a mass spectrometer (MS) equipped with electrospray ionization source (ESI) and MicroTOF-Q II time of flight analyzer (TOF) (Bruker, Bermen, Germany). MS detection was performed based on signal intensity with positive ion $[M+H]^+$ mode, covering the range of target analytes values (ca. 237–337 m/z). Table 1. reports about adjusted MS parameters. The data acquisition and processing were performed using Chromeleon 6.8 (Dionex), HyStar 3.2, MicrTOFcontrol and Compass DataAnalysis software (Bruker), respectively. In order to obtain extracted ion chromatograms, expected masses of ions of all analytes, $[M+H]^+$, were calculated using IsotopePattern software (Bruker).

Table 1
Adjusted mass spectrometer parameters.

Equipment	Parameter [unit]	Value for MS coupled with LC-MS
ESI	ionization mode	positive
	capillary voltage [kV]	4.5
	nebulizer pressure [bar]	2.5
	dry gas flow [L/min]	5.5
	dry gas temperature [°C]	200
MS-TOF	mass range	50–800

2.2. The LC-MS method conditioning

The MS settings, gradient program, and mobile phase were chosen on previously done research for psychoactive substances [15,16]. The eluent A (0.1% formic acid in ultrapure water) and eluent B (acetonitrile) were pumped at a flow rate of 0.3 mL/min at 35 °C. The gradient program was performed as follows. First, mobile phase B increase from 15% (0.0 min) to 40% (4 min). Next, the content of 40% was used for 3 min (4.0–7.0 min) and then increase from 40% (7 min) to 70% (10 min). Subsequently, the mobile phase B content was decreased from 70% (10 min) to 15% (12.5 min) and held for 4.5 min (12.5–17.0 min) to equilibrate the column for the next injection. The injection volume was 5 µL.

The choice of those parameters allowed to obtain all analyzed substances in less than 10 min. Fig. 1. present extracted ion chromatograms of blank and spiked blood with 25 analyzed psychotropic substances (100 ng/mL).

2.3. Sample collection and preparation

Drug-free human blood samples as a surrogate matrix were provided by unknown examined donors from Blood Donation Center in Krakow, Poland. Case samples (bone marrow aspirates) were provided by courtesy of the Forensic Medicine Unit at the Department of Forensic Medicine of the Wroclaw Medical University (according to the Bioethical Commission Approval no 1072.6120.303.2018). All samples were stored frozen until use at the temperature of −20 °C. Blood and bone marrow aspirate were prepared 24 h prior to the analyses by spiked them with a known amount of internal standards solution and drugs solution in case of creation calibration curve using drug-free blood samples. Spiked samples were stored at the temperature of 4 °C until the extraction process. List of analytes with physical and chemical properties were presented in Table 2.

2.4. Optimization of DI-SPME

2.4.1. Sample volume

For toxicological analysis techniques requiring small sample volumes are favored but considering the SPME technique, some limitations are encountered. The first condition is to ensure complete coverage of coated sorbent, while the second is associated with the mechanism of extraction process. It is based on an equilibrium process, so the volume of the sample significantly effects this process. Using smaller sample volumes results in lower quantities of analytes and, in consequence, smaller quantities of the analytes can be extracted. Therefore, the process of adsorption must occur efficiently as well as the desorption process [17].

2.4.2. Adsorption time

To establish the time of reaching the equilibrium the analyses of amount extracted versus adsorption time have been made. The extractions were performed with the use of 200 µL of blood placed in the inserts, located internally in 1.5 mL HPLC vials with the agitation of 2200 rpm. To the desorption solution, IS solution was added to check the ratio of amount of analyte which was extracted to the initial amount

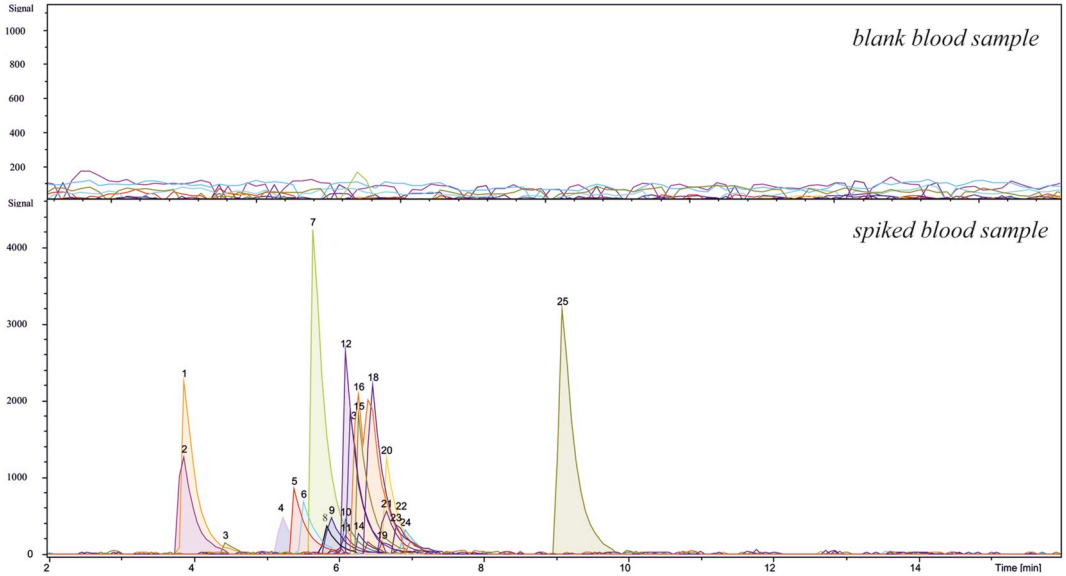

Fig. 1. Extracted ion chromatograms of blank and spiked blood sample. Peak assignment: (1) VEN; (2) ZOL; (3) BRO; (4) MID; (5) CBZ; (6) TETRA; (7) CIT; (8) NITRA; (9) NORD; (10) EST; (11) LOR; (12) DESI; (13) PAROX; (14) IMI; (15) CLO; (16) NORT; (17) ALP; (18) AMI; (19) TEM; (20) FLUN; (21) FLUOX; (22) CLOR; (23) LORM; (24) DIA; (25) PRA.

Table 2
List of analytes and their physical and chemical properties (active metabolites, therapeutic and toxic range concentration, pKa, logP, $[M+H]^+$, protein binding and retention time).

Drug	Formula	Active metabolites	Therapeutic range [mg/L]	Toxic range [mg/L]	pKa	logP	$[M+H]^+$	Protein binding [%]	Retention time [min]
BZDs									
ALP	$C_{17}H_{13}N_4Cl$	α-hydroxyalprazolam, 4-hydroxyalprazolam	0.005–0.05 (s)	0.1–0.4 (s)	2.4	2.12	309.0901	70–80	6.35
BRO	$C_{14}H_{10}N_3OBr$	3-hydroxybromazepam	0.08–0.15 (p)	0.3–0.4 (s)	2.9; 11.0	2.05	316.0080	70	4.45
CLO	$C_{15}H_{10}N_3O_3Cl$	7-aminoclonazepam	0.02–0.07 (p)	> 0.1 (p)	1.5; 10.50	2.41	316.0483	86	6.29
CLOR	$C_{16}H_{11}N_2O_3Cl$	nordazepam, oxazepam	0.02–0.8 (s)	1.5–2 (b)	3.5; 12.50	2.05	337.0350	91	6.80
DIA	$C_{16}H_{13}N_2OCl$	nordazepam, oxazepam, temazepam	0.1–2.5 (p)	3–5 (b)	3.5	2.8	285.0789	89–99	6.93
EST	$C_{16}H_{11}N_4Cl$	4-hydroxyestazolam	0.055–0.2 (s)	no information	12.33	4.70	295.0745	93	6.10
FLUN	$C_{16}H_{12}N_3O_3F$	desmethylflunitrazepam	0.005–0.015 (s)	> 0.045 (b)	1.80	2.10	314.0935	77–80	6.61
LOR	$C_{15}H_{10}N_2O_2Cl_2$	-*	0.05–0.25 (p)	0.3–0.6 (p)	1.3; 11.50	2.40	321.0192	90	6.10
LORM	$C_{16}H_{12}N_2O_2Cl_2$	lorazepam	0.001–0.025 (s)	no information	11.60	2.2	335.0348	90	6.80
MID	$C_{18}H_{13}N_3FCl$	1-hydroxymethyl-midazolam	0.08–0.25 (s)	1–2.5 (b)	6.20	4.30	326.0855	95–98	5.08
NITRA	$C_{15}H_{11}N_3O_3$	-*	0.03–0.07 (s)	0.2–3 (b)	3.2; 10.80	2.25	282.0873	85–88	5.84
NORD	$C_{15}H_{11}N_2OCl$	oxazepam	0.02–0.8 (s)	1.5–2 (b)	3.5; 12.00	2.93	271.0633	97	5.84
PRA	$C_{19}H_{17}N_2OCl$	nordazepam, oxazepam	0.2–0.7 (s)	1–2 (b)	2.70	3.70	325.1102	97	9.09
TEM	$C_{16}H_{13}N_2O_2Cl$	oxazepam	0.03–0.9 (s)	> 1 (s)	1.60	2.20	301.0738	96	6.61
TETR	$C_{16}H_{17}N_2OCl$	diazepam, nordazepam	0.05–0.6 (p)	no information	4.30	3.20	289.1102	30–70	5.53
Sedatives/hypnotics									
ZOL	C19H21N_3O	-*	0.08–0.15 (s)	> 0.5 (s)	6.2	3.85	308.1757	92	3.87
Anticonvulsants									
CBZ	$C_{15}H_{12}N_2O$	10,11-epoxide	4–12 (s)	> 15 (p)	13.94	2.45	237.1102	75	5.40
Drug	Formula	Active metabolites	Therapeutic range [mg/L]	Toxic range [mg/L]	pKa	logP	$[M+H]^+$	Protein binding [%]	Retention time [min]
TCAs									
AMI	$C_{20}H_{23}N$	nortriptyline	0.1–0.2 (p)	> 0.3 (p)	9.40	4.94	278.1903	91–97	6.42
DESI	$C_{18}H_{22}N_2$	2-hydroxydesipramine	0.007–0.13 (p)	> 0.4 (p)	10.4	4.90	267.1856	70–90	6.10
IMI	$C_{19}H_{24}N_2$	desipramine, hydroxyimipramine, 2-hydroxydesipramine	0.1–0.3 (p)	> 0.50 (p)	9.53	4.47	281.2012	85–95	6.23
NORT	$C_{19}H_{21}N$	-*	0.05–0.15 (p)	> 0.25 (p)	10.1	4.51	264.1746	90–95	6.29
SNRI and SSRI									
VEN	$C_{17}H_{27}NO_2$	O-desmethylvenlafaxine, N-desmethylvenlafaxine,	0.2–0.75 (s)	1–1.5 (b)	10.10	2.9	278.2115	30	3.81
CIT	$C_{20}H_{21}N_2OF$	desmethylcitalopram	0.02–0.20 (p)	Lethal conc. 0.5	9.50	3.74	325.1710	50	5.65
FLUOX	$C_{17}H_{18}NOF_3$	norfluoxetine	0.15–0.5 (s)	1.3–6.8 (b)	4.05	4.05	310.1413	95	6.67
PAROX	$C_{19}H_{20}NO_3F$	-*	0.01–0.075 (s)	0.35–0.40 (s)	9.90	3.95	330.1499	95	6.16

(b)- blood.
(p)- plasma.
(s)- serum.
-* no data.

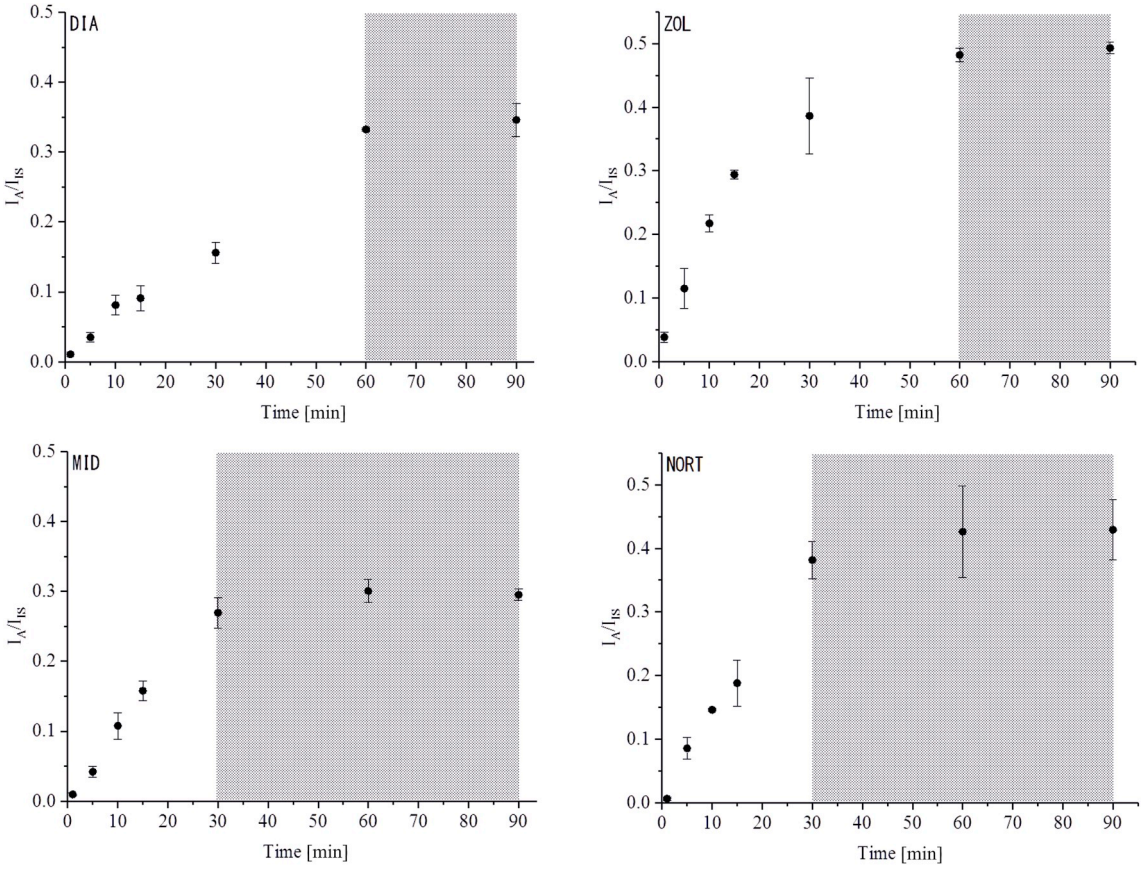

Fig. 2. Adsorption time plots for DIA, ZOL, NORT and MID.

of analyte. The chosen adsorption times were 1, 5, 10, 15, 20, 30, 60 and 90 min. For each time 3 repetitions have been made.

The determination of the concentration of analytes can be also performed by using pre-equilibrium times with very short sampling times, but this approach requires kinetic on-fibre standardization as a kinetic calibration method [18].

2.4.3. Post-adsorption washing

In the DI-SPME technique fiber is exposed directly to a sample. In case of biological samples like blood or bone marrow aspirate, residues of biological materials are deposited on the fiber coating. Omission of the rinsing step process which follows the adsorption results in clot formation, due to the composition of organic desorption solution. For this reason, it was decided to introduce an additional process such as washing in ultrapure water and mechanical purification.

2.4.4. Desorption time

Investigation of desorption time was performed for desorption times: 10, 30, 45 and 60 min. The desorption solution was adjusted to the type of the fiber coating and analytes [19]. The desorption process was carried out with the use of 200 µL of desorption solution of ACN:MeOH:0.1%HCOOH (2:2:1, v/v/v) placed in the inserts, located internally in 1.5 mL HPLC vials with the agitation of 2200 rpm.

2.5. Validation

Validation parameters were calculated according to standard practices for method validation by Scientific Working Group for Forensic Toxicology (SWGTOX) [20], Bioanalytical Method Validation Guideline for Industry (FDA) [21], strategy presented by Matuszewski [22] and previous analyses done by Wietecha-Posłuszny et al. [15]. For developed method parameters as linearity, limit of detection, limit of quantification, precision and matrix effect were evaluated. In validation process only blood samples were used as the best a surrogate matrix for BMA.

The first step of validation was evaluation of the linearity of the examined range. For the calibration process, the Interpolative Internal Standard Method (IISM) was applied [23]. The calibration curves were constructed by linear regression of area ratios of analytes peak to the IS peaks with the nominal concentrations (I_A/I_{IS}). The concentrations of analytes were tested within the therapeutic and toxic range of 30–300 ng/mL (30, 50, 100, 150, 200, 300 ng/mL). Slope and R^2 values were calculated and reported for three different runs. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated on the basis of standard deviation of the y-intercept (S_y) for the lowest calibrator and the average slope value (Avg_a) as Equations (1) and (2).

$$LOD = \frac{3.3 \cdot S_y}{Avg_a} \quad (1)$$

$$LOQ = \frac{10 \cdot S_y}{Avg_a} \quad (2)$$

Precision was calculated at three concentration levels (50, 150, 300 ng/mL) over the calibration range for four replicates for intraday precision per concentration (n = 4) and interday precision per twelve replicates (n = 12) in three separate analytical runs per day. The mean (Avg_c) and standard deviation (S) of the response were calculated using Equation (3) for each concentration to determine the precision as a coefficient of variation (CV). Results were evaluated according to the criteria that CV should not exceed 15%, with the exception of the LOQ, where CV should not exceed 20%.

$$CV = \frac{S}{Avg_c} \cdot 100\% \quad (3)$$

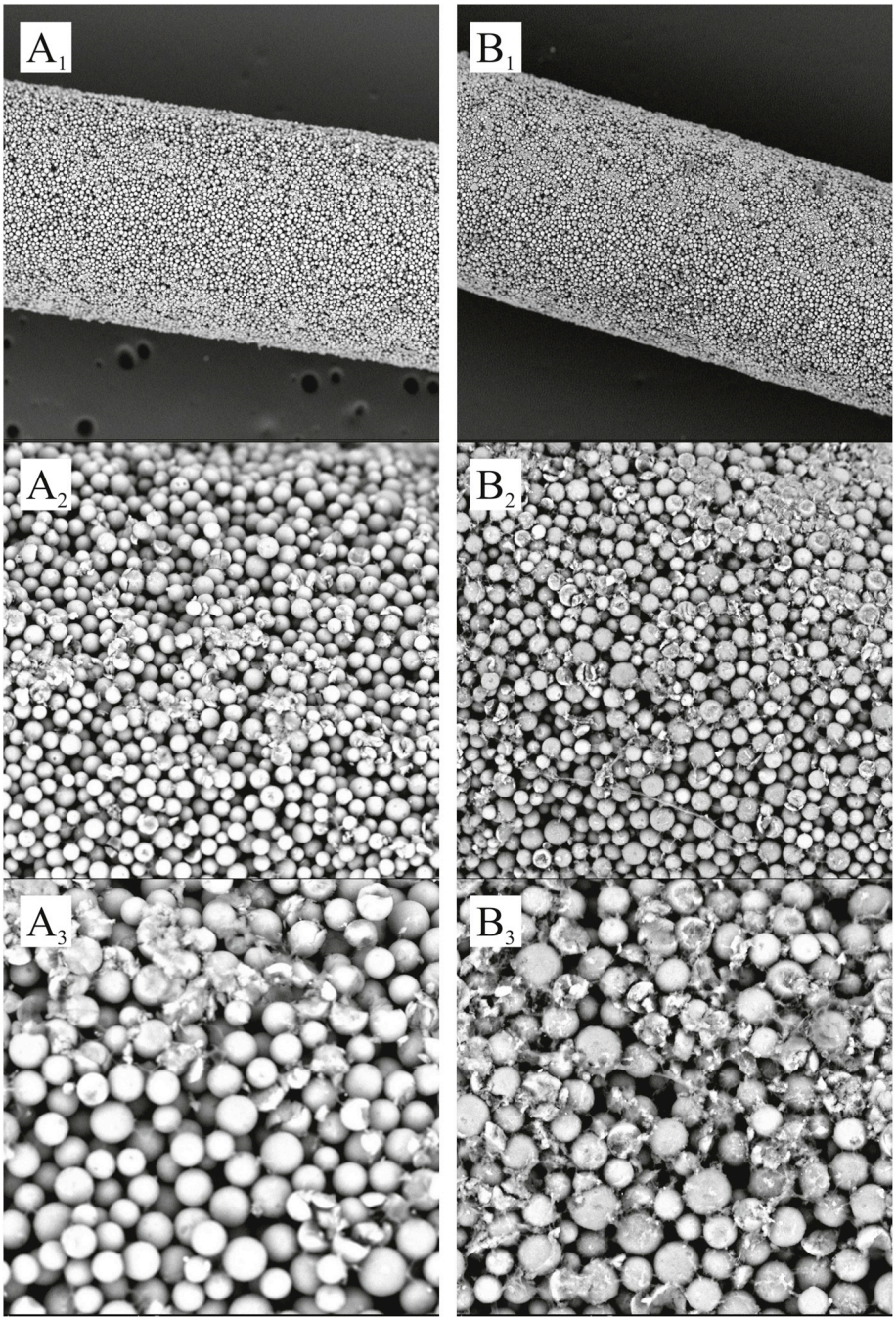

Fig. 3. SEM photographs of the fibers A (brand-new), B (after post-adsorption washing) in at magnification of 500 (1), 2000 (2) and 4000 (3) times.

The examination of 'absolute' ME was carried out by analysis of four different materials of various origins and in each case preparation of two measuring series. The first series (I) was prepared in accordance with the protocol for SPME, extraction was performed in biological material without analytes and later addition of known, appropriate amount of mix of analytes in the solution of the mobile phase. The second series (II) was prepared by addition of appropriate amount of mix of analytes to the mobile phase. The 'absolute' matrix effect was calculated using Equation (4), as a ratio of analytical signal obtained for series I and series II.

$$ME = \frac{I_I}{I_{II}} \cdot 100\% \quad (4)$$

2.6. Case samples

Bone marrow aspirates from 8 forensic cases (26/17, 95/17, 649/15, 161/17, 266/17, 396/17, 476/16, 412/16) were analyzed. The post-mortem samples were originated from the Forensic Medicine Unit at the Department of Forensic Medicine of the Wroclaw Medical University and were classified as samples that may contain psychotropic drugs.

3. Results and discussion

3.1. Optimization

3.1.1. Sample volume

Considering limited availability of sample, the adsorption was

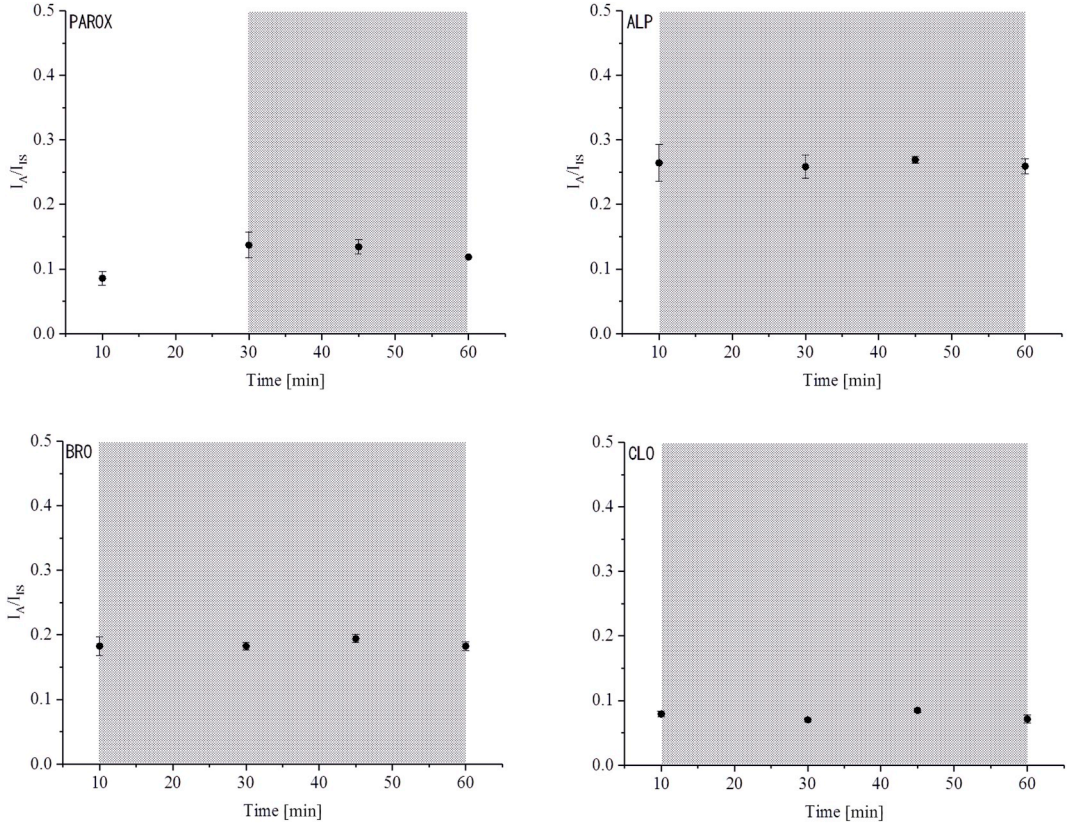

Fig. 4. Desorption time plots for PAROX, ALP, BRO and CLO.

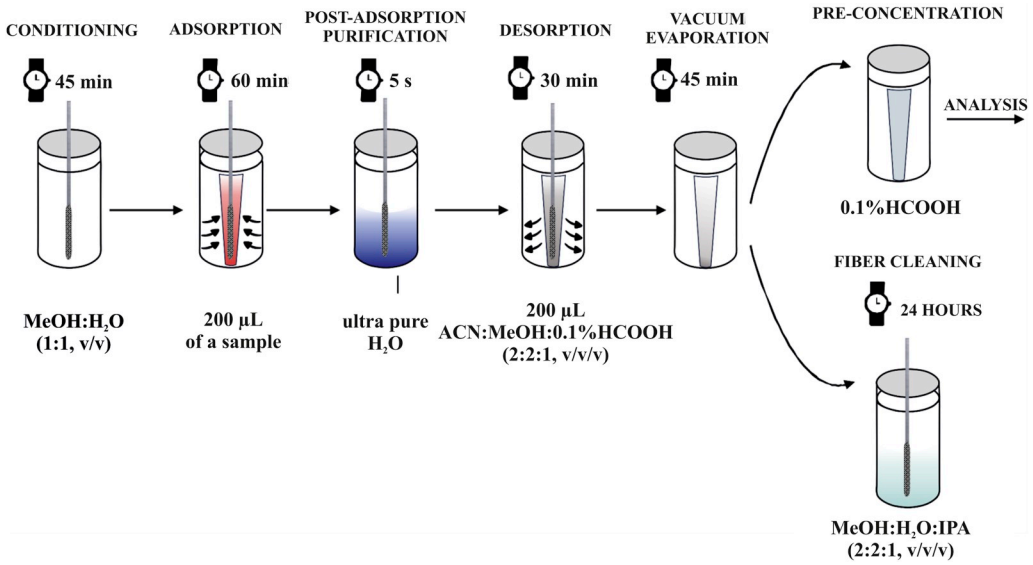

Fig. 5. Final procedure of DI-SPME method of sample preparation and fiber cleaning.

performed with the use of 200 µL. Sample solution was placed in 200 µL inserts, located internally in 1.5 mL HPLC vials. The optimized volume of the sample was chosen so as to cover up the 1.5 cm coating of SPME fiber.

3.1.2. Adsorption time

On the basis of the obtained signals for each time, adsorption curves were determined and the chosen plots were collected in Fig. 2. Equilibrium level on time was found to stabilize for different analytes in different times. The differences occur due to the diverse character of the analytes, a wide range of masses and various distribution constants (see Table 2). The equilibrium for some analytes of interest was found to reach equilibrium already after 30 min (NORT and MID) to even 60 min (DIA and ZOL), but choosing the shorter times would reduce the amount of extracted analyte and in consequence have an impact on the limit of detection. To ensure that equilibrium for all the analytes is reached the time of adsorption at room temperature was chosen for 60 min.

Table 3
Summary of the validation parameters for DI-SPME/LC-MS method.

Parameter		ALP	AMI	BRO	CBZ	CIT	CLO	CLOR	DESI	DIA	EST	FLU	FLUOX	IMI
Linearity [ng/mL]		LOQ – 300												
a		0.0107	0.0162	0.0225	0.0075	0.1030	0.0049	0.0081	0.0364	0.0095	0.0058	0.0249	0.0035	0.0118
b		0.0626	−0.1862	−0.0921	−0.0460	1.4159	0.0195	−0.0082	−0.1413	−0.0403	−0.1220	−0.6404	−0.0348	−0.2087
R^2		0.9970	0.9987	0.9975	0.9957	0.9991	0.9934	0.9957	0.9954	0.9978	0.9963	0.9968	0.9963	0.9975
LOD [ng/mL]		1.87	2.98	2.35	4.10	9.98	10.45	7.01	9.49	7.86	4.90	2.96	5.80	3.08
LOQ [ng/mL]		5.60	8.95	7.06	12.30	29.95	31.35	21.02	28.48	23.58	14.69	8.88	17.40	9.23
precision, 50 [ng/mL] CV [%]	interday	3.07	2.09	0.78	3.49	8.55	9.09	10.54	6.75	4.99	2.47	3.54	3.15	3.80
	intraday	10.72	8.82	7.95	7.80	11.4	9.27	7.09	10.78	10.09	6.39	6.51	8.01	11.00
precision, 150 [ng/mL] CV [%]	interday	5.08	9.68	5.85	4.32	4.29	12.59	2.38	1.84	2.45	3.57	2.85	1.47	9.40
	intraday	4.15	5.15	5.04	5.43	7.05	4.75	6.75	5.18	7.24	8.46	6.53	3.85	6.36
precision, 300 [ng/mL] CV [%]	interday	14.85	14.51	0.38	6.11	6.05	9.36	2.98	4.24	1.57	14.21	12.92	6.39	10.72
	intraday	8.98	7.14	2.58	3.72	3.64	2.27	0.98	5.62	4.66	6.64	3.22	2.29	5.80
'absolute' matrix effect, ME [%]	50	103.57	95.19	105.08	102.76	119.72	108.31	100.45	105.61	94.42	91.28	105.11	99.90	91.82
	150	91.29	100.10	107.61	102.08	107.18	109.24	110.96	101.63	95.88	100.19	92.51	106.01	107.64
	300	89.01	95.64	94.19	105.28	106.48	99.56	97.38	103.51	92.36	100.02	90.77	104.77	102.41

Parameter		LOR	LORM	MID	NITR	NORD	NORT	PAROX	PRA	TEM	TETR	VEN	ZOL
Linearity [ng/mL]		LOQ – 300											
a		0.0092	0.0087	0.0110	0.0150	0.0171	0.0088	0.0064	0.0113	0.0125	0.0074	0.0139	0.0115
b		−0.0091	0.1435	−0.0601	−0.0693	−0.0619	0.0550	0.0532	0.0819	−0.3801	0.0556	−0.1388	−0.1045
R^2		0.9961	0.9979	0.9968	0.9962	0.9996	0.9952	0.9960	0.9957	0.9996	0.9985	0.9976	0.9978
LOD [ng/mL]		10.14	10.21	7.1	3.85	3.14	4.93	5.34	5.74	14.27	4.18	5.46	1.98
LOQ [ng/mL]		30.41	30.64	21.31	11.56	9.41	14.80	16.02	17.22	42.80	12.55	16.39	5.93
precision, 50 [ng/mL] CV [%]	interday	8.60	5.11	2.48	4.12	1.07	4.93	3.32	3.55	3.66	4.52	1.63	0.48
	intraday	11.86	7.98	10.64	8.22	7.79	11.30	9.50	6.41	6.59	6.92	5.35	4.78
precision, 150 [ng/mL] CV [%]	interday	5.13	2.14	5.02	3.10	2.98	3.44	10.34	5.30	7.76	4.39	1.62	0.84
	intraday	5.93	5.66	5.71	6.29	6.75	5.53	6.08	5.30	7.11	6.91	6.35	4.39
precision, 300 [ng/mL] CV [%]	interday	12.49	11.67	6.14	5.65	9.67	3.83	8.63	7.53	4.15	4.31	4.36	3.73
	intraday	7.72	5.86	4.79	4.72	4.80	2.26	5.38	4.23	7.17	3.69	2.90	2.77
'absolute' matrix effect, ME [%]	50	98.27	109.25	110.48	147.01	112.52	90.28	113.76	113.26	112.68	109.00	111.07	114.27
	150	104.56	101.78	107.08	149.64	98.48	91.77	100.32	112.14	101.01	112.31	105.95	100.12
	300	94.48	97.23	97.07	145.09	103.00	93.77	98.64	105.93	97.62	97.64	100.95	105.71

Interday (n = 4), intraday n = 12.

Table 4
Drugs and metabolites found in analyzed case samples (BMA) using DI-SPME-LC-TOFMS method.

Case No.	Detected drugs	Concentration and SD [ng/mL]
26/17	IMI	38.78 ± 6.12
	BRO	37.18 ± 4.14
95/17	IMI	27.61 ± 3.70
649/15	ALP	415.86 ± 7.21
	IMI	44.31 ± 8.47
	BRO	147.51 ± 24.39
	ZOL	44.20 ± 1.35
	PAROX	26.33 ± 2.32
	VEN	20.10 ± 2.23
161/17	AMI	25.60 ± 1.29
	IMI	65.79 ± 4.81
	PAROX	19.81 ± 0.80
	NORT	23.50 ± 1.63
	VEN	16.63 ± 1.44
266/17	IMI	44.27 ± 0.88
396/17	IMI	35.76 ± 1.76
	BRO	51.57 ± 6.06
	DIA	< LOQ
476/16	IMI	36.77 ± 1.6
412/16	IMI	22.45 ± 2.48

3.1.3. Post-adsorption washing

Desorption process which follows the adsorption was carried out in an organic solvent what resulted in the formation of a clot, which was difficult to remove. Due to this post-adsorption coating purification was introduced between adsorption and desorption process to clear out interfering components from the coating of the fiber and avoid fouling of the coating. The post-adsorption purification was implemented after adsorption process and it consisted of two steps. The first step was to: wipe coating with dust-free materials, in case to remove formed clots, and then the second step was: the washing in the ultrapure water to providing to remove loosely bound residual of biological samples. The time of the washing step was selected on the basis of visual and microscopic assessment and last 5 s (with vortex agitation 5000 rpm).

The fibers were monitored with the use of Scanning Electron Microscope to control the usage of the fiber and process of cleaning. In Fig. 3 micrography's of brand-new fiber (A) and used fiber (B) which have been used for all carried out experiments are presented. On the used fibers, in minor parts, there are observed networks, which are probably the networks of the proteins, but the extent of the networks is not extensive and didn't affect the process of extraction, what proved the values of precision. It just indicates the need of fiber usage monitoring.

3.1.4. Desorption time

Equilibrium desorption time like in the adsorption process was obtained based on adsorption signal-times curves, several of which are presented in Fig. 4.

Desorption of analytes from the fiber is very rapid. As presented on the plots for most analytes, desorption maintained on the same level from 10 min to 60 min (ALP, BRO, and CLO). To provide complete desorption for most of the analytes the chosen desorption time in room temperature was 30 min.

3.1.5. The developed extraction procedure

This DI-SPME procedure is designed for the direct sampling of 25 psychotropic drugs from biological matrices like blood and BMA. The new procedure eliminates the need of protein precipitation, centrifugation or any other sample preparation. By reducing the number of steps, the time of the whole analysis is shortened, and the only limitation for a number of samples, which are analyzed, is a number of available fibers and number of places in the shaking plate. The method

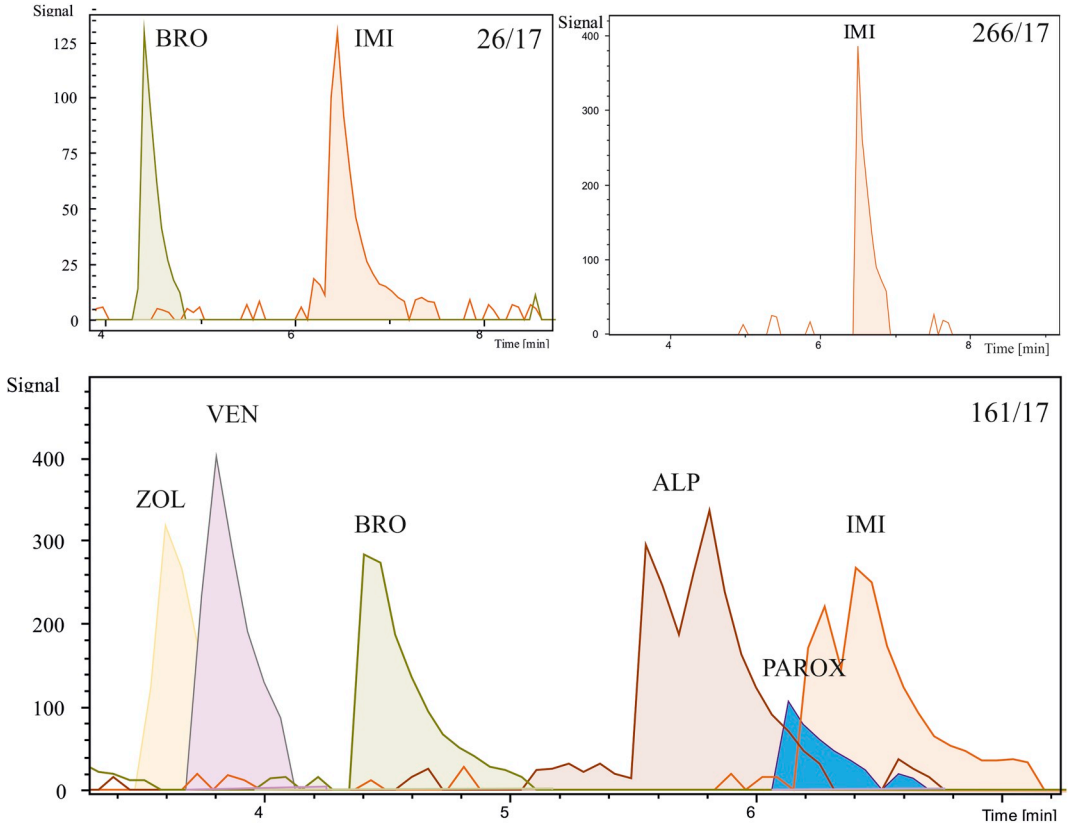

Fig. 6. Extracted ion chromatograms for 26/17, 161/17 and 266/17 case samples.

may in this way increase sample throughput because many fibers could be used simultaneously.

The first step of the procedure was conditioning of the fibers. For analysis of psychotropic drugs SPME LC Probe 45 µm C18-Silica was chosen. The composition of conditioning solution was recommended by the producer in the data sheet enclosed to the product. Conditioning of the coatings took place in the 1.5 mL of a solution of MeOH:H_2O (1:1, v/v) with continuous shaking of 2200 rpm. Conditioning of the fibers lasted 45 min. The next step was adsorption. It was performed with the use of 200 µL of sample placed in the inserts, located internally in 1.5 mL HPLC vials with the agitation of 2200 rpm. After adsorption purification was followed. The fiber was wiped with a dust-free tissue than washed in the ultrapure water for 5 s with the vortex agitation 5000 rpm. Desorption was carried out with the use of 200 µL of desorption solution of ACN:MeOH:0.1%HCOOH (2:2:1, v/v/v) placed in the inserts, located internally in 1.5 mL HPLC vials with the agitation of 2200 rpm. Desorption time was fixed for 30 min. After desorption fibers were placed in the cleaning solution (MeOH:H_2O:isopropanol, 2:2:1,v/v/v). The desorption solutions were evaporated with use of a vacuum evaporator at a temperature of 45 °C. Afterwards, 50 µL of the mobile phase (0.1% HCOOH) was added and vortexed in the speed of 2500 rpm. Samples prepared in accordance with this procedure (Fig. 5) were analyzed with the use of LC-TOF MS system in order to method validation and next application in case samples measurements.

3.2. Validation

Developed method DI-SPME/LC-TOFMS was validated. Parameters like linearity, limit of detection LOD, limit of quantification LOQ, precision and matrix effect were evaluated for all analytes of interest. The obtained parameters were summarized in Table 3.

Prepared blood samples that were analyzed were spiked with analytes 24 h before analyses on 3 levels of concentrations: low 50 ng/mL, medium 150 ng/mL and high 300 ng/mL. As internal standards adequate deuterated analytes were used at a concentration of 100 ng/mL.

For analysis of linearity for each analyte six-point calibration curves were prepared. Calibration curves were prepared with the use of blood, which were spiked with a known amount of mix of analytes and their deuterated analogues obtaining concentrations: 30, 50, 100, 150, 200, and 300 ng/mL. Linearity was determined in the range from LOQ for each analyte to the highest concentration of calibrator, equal to 300 ng/mL. The parameter of the coefficient of determination show agreement with acceptance criteria of $R^2 > 0.995$, and only for one analyte, CLO, coefficient of variation is below this criterion. For 24 analytes linear model was obtained. The calculated LOQs enable determination of 16 analytes in the range of both therapeutic and toxic concentrations (see Table 2), which creates opportunities for clinical exploitation and application [24]. For drugs CIT, CLO, CLOR, DESI, FLU, LORM PAROX, PRA, TEM and TETR determination of low therapeutic concentrations is not possible, in this case only the middle therapeutic level is possible to determine.

The obtained LOD and LOQ for analytes of interest fall respectively within the scope of 1.87–14.27 ng/mL and 5.60–42.80 ng/mL. These values for all drugs enable analysis of chosen drugs at a toxic level and also allow the method to be extended to analysis many drugs of interest at the therapeutic level.

Calculated values of precision (CV) for intraday analyses do not exceed 14.51% (AMI), while the variabilities of analyses carried out interday do not exceed 11.86% (LOR). For all analytes, obtained precision meets the established criteria, what is a very satisfactory result. On this basis, it was concluded that the method is characterized by good repeatability and reproducibility.

Obtained 'absolute' ME results for 24 analytes meets the established criteria. The one which exceeds the set range is NITR, where strong ion enhancement is observed. It can be seen that the influence of the matrix for the lowest concentration is the highest for almost all analytes. The

experience carried out shows that for most analytes there is no significant influence of ME observed. The accuracy of the method wasn't evaluated due to the lack of adequate reference material.

3.3. Post-mortem case samples

The DI-SPME/LC-TOFMS method can be used successfully for blood samples (see Paragraph 3.2 Validation) and bone marrow aspirates samples 26/17, 95/17, 649/15, 161/17, 266/17, 396/17, 476/16, 412/16 presented in this section. For analyzed cases, from one to six drugs from all analyzed drug groups were determined. The obtained results were presented in Table 4. The standard deviations of concentrations were determined for each sample and it was usually less than 15%. In Fig. 6 extracted ion chromatograms for selected case samples are shown. In analyses which were carried out, 9 different drugs were determined: BRO, IMI, ALP, ZOL, PAROX, VEN, AMI, NORT and DIA. In most of the samples, IMI was determined, which may mean that although it was withdrawn from treatment, drug poisoning is still used, and unfortunately very popular. The metabolite of IMI is DESI which was also the subject of this research, but it was not detected together with IMI – probably because of low content in the samples. In the case of 161/17 sample, AMI was detected with its metabolite NORT. One drug, DIA, were detected only because its determined value was below the LOQ parameter.

Due to the fact that BMA is an alternative material, it is difficult to classify obtained concentrations as therapeutic or toxic.

4. Conclusions

The present study showed that DI-SPME/LC-TOFMS method is rapid and allows to simplify the labor-intensive and usually complicated process of isolating analytes from the complex biological matrix. Moreover, this method is suitable for quantitative analyses and SPME fibers may be used repeatedly, as no fiber damage or 'fouling' is observed, with the applied cleaning and conditioning.

The DI-SPME/LC-TOFMS method was developed and validated on spiked blood samples and used in determination of 25 psychotropic drugs from post-mortem BMA samples.

In the future, researches could be focused on the enlargement of the number of analytes and also on prospecting if there is any correlation between concentrations of analytes in biological materials, in this case between blood and BMA.

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