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# Determination of Drugs in Human Blood via Bidirectional Solid Phase Extraction

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# Content

## 1. Introduction

The analysis of forensic blood samples via Solid Phase Extraction (SPE), either post-mortem or of living persons, is a standard procedure in forensic labs for clarification of fatal cases or when drugs of abuse or toxic substances might have played a role in a criminal case. In the following application note an automated approach is described that uses the so-called bidirectional SPE (BD-SPE); this specific approach is applied when either difficult to process matrices are processed, or the likelihood of cross-contamination should be fully excluded from the very beginning. In particular the latter aspect is ultimately important in order to avoid any wrongful conviction.

In brief, the approach uses standard 3 mL SPE cartridges which are processed in a normal way in terms of conditioning, washing, drying, and elution steps, whereas the critical loading step, where the matrix enters the automation system, is loaded reversely. This means the diluted sample, e. g. blood, is not loaded on top of the cartridge, but aspirated via the Luer tip into the sorbent. As the aspirated sample is discarded into the waste in the "normal" direction afterwards, it never enters the system and passes the sorbent twice. Therefore, it was consequently called bidirectional SPE.

*Fig. 1: Blood sample*

# 2. Method Development

## 2.1 Reagents and Materials

- Ethyl acetate (p. a.)
- Methanol (LC/MS grade)
- Water (p. a.)
- Acetic acid (p. a.)
- 0.05M Ammonium acetate, pH 7.2 (LC/MS grade)
- Formic acid (LC/MS grade)
- Oasis HLB, 60 mg (Waters, USA)

## 2.2 Sample Preparation

The method can be applied to any blood samples typical in forensic investigations. Therefore, the blood originates either from living or deceased persons and thus represents all stages of biological degradation.

The sample preparation is straightforward and keeps the sample as unaltered as possible.

In this application note the samples are measured with LC-MS/MS. After a solvent exchange of the resulting eluates, and derivatisation with the corresponding derivatisation reagents, a measurement with GC-MS/MS is also possible.

- Add 2.5 mL water to 0.5 mL blood sample
- Vortex thoroughly
- Centrifuge at 5,000 rpm for 10 min.
- Pour the supernatant into a 10 mL sample vial
- Put the sample into the FREESTYLE SPE system

# 3. Instrumentation

## 3.1 Bidirectional SPE (BD-SPE) with FREESTYLE SPE

The following components are needed to process the BD-SPE automatically with a FREESTYLE SPE. In brief the blood sample is filled in 10 mL vials, the system equipped with 3 mL cartridges and the eluate vials.

After processing the combined eluates were evaporated to dryness in a nitrogen blow-down apparatus, filled up with the HPLC solvent and filled into HPLC vials for measurement with LC-MS/MS.

| | | | |
|---|---|---|---|
| 1. | FREESTYLE BASIC | P/N | 12663-12 |
| 2. | FREESTYLE SPE | P/N | 12668 |
| 3. | Rack for solvent delivery | P/N | 13156 |
| 4. | Rack for up to 18 SPE columns | P/N | 13946-AD (2 needed) |
| 5. | Column adapter for 3 mL SPE columns | P/N | 14612 (2 needed) |
| 6. | Cap for SPE-cartridges 3 mL | P/N | 14919 (2 needed) |
| 7. | Tray for 54 test tubes, 100 x 16 mm | P/N | 13948 |
| 8. | Sample rack, 18 x 10 mL-vials | P/N | 14711 (2 needed) |
| 9. | Vial, flat bottom; 10 mL | P/N | V0010 |
| 10. | SPE Software upgrade “Forensic” | P/N | 14773 |

14 mL 100 x 16 mm reagent tubes have to be ordered from any local supplier.

## 3.2 Software Protocol

The following setting is used to process the samples with the BD-SPE on the FREESTYLE SPE system.

FREE-STYLE™
AUTOMATISIERTE PROBENVORBEREITUNG

LCTech FreeStyle - Report on Methods: SPE Date: 15.11.2016 Time: 10:37:47

| | |
|---|---|
| Name: FAST_5ML.spe | SPE Column: LCTech_3ml.col |
| Extension cannula: | no |
| Processing speed selection: | Speed up (aqueous solutions) |
| Rinsing intensity: | Standard rinsing cycle |
| Use pressure limitation function during loading and washing: | yes |
| Pressure limit for syringe pump: | 231 digits |
| Maximum count of triggered samples in series: | 3 Sample /s |

| Step: Conditioning | | Basic type: Conditioning | | Step: - ID: 688 |
|---|---|---|---|---|
| Volume: 8 ml | Suction Speed: 90 ml/min<br>Repetitions: 0<br>Waiting Time after Dosage: 0 sec. | Dispensing Speed: 10 ml/min<br>Waiting Time after Step: 0 sec. | Port: 7 Water<br>Dispense: into Waste | |

| Step: Drying | | Basic type: Drying - Drying by defined air volume | | Step: - ID: 689 |
|---|---|---|---|---|
| Air volume: 10 ml | Suction Speed: 100 ml/min | Dispensing Speed: 70 ml/min | Dispense: into Waste | |

| Step: Load | | Basic type: Load - Transfer Sample-Aliquot through tip of SPE column | | Step: - ID: 690 |
|---|---|---|---|---|
| Volume: 5 ml<br>Vial Type: Type1@10<br><br>without rinsing of vial | Suction Speed: 5 ml/min<br>Waiting Time after Dosage: 15 sec. | Dispensing Speed: 5 ml/min<br>Waiting Time after Step: 150 sec. | Dispense: into vials | Number of vials: 1<br>Vial Type: Type1@10 |

| Step: Washing | | Basic type: Washing | | Step: - ID: 691 |
|---|---|---|---|---|
| Volume: 10 ml | Suction Speed: 90 ml/min<br>Repetitions: 0<br>Waiting Time after Dosage: 0 sec.<br>Drying time: 50 min | Dispensing Speed: 5 ml/min<br>Waiting Time after Step: 0 sec.<br>Dispense: into Waste | Port: 7 Water | |

| Step: Drying | | Basic type: Drying - Nitrogen drying by defined time | | Step: - ID: 692 |
|---|---|---|---|---|
| Drying time with nitrogen 120 sec. | | | Dispense: into Waste | |

| Step: Eluting | | Basic type: Eluting | | Step: - ID: 693 |
|---|---|---|---|---|
| Volume: 1 ml | Suction Speed: 30 ml/min<br>Repetitions: 0<br>Waiting Time after Dosage: 0 sec.<br>Drying time: 0 min | Dispensing Speed: 1 ml/min<br>Waiting Time after Step: 0 sec. | Port: 1 MetOH<br>Dispense: into vials | Number of vials: 1<br>Vial Type: Type3@14 |

| Step: Eluting | | Basic type: Eluting | | Step: - ID: 694 |
|---|---|---|---|---|
| Volume: 1 ml | Suction Speed: 20 ml/min<br>Repetitions: 0<br>Waiting Time after Dosage: 0 sec.<br>Drying time: 0 min | Dispensing Speed: 1 ml/min<br>Waiting Time after Step: 0 sec. | Port: 8 Etil Asetat<br>Dispense: same vial as step ID: 693 | |

| Step: Drying | | Basic type: Drying - Drying by defined air volume | | Step: - ID: 695 |
|---|---|---|---|---|
| Air volume: 10 ml | Suction Speed: 100 ml/min | Dispensing Speed: 30 ml/min | Dispense: stay on actual position | |

*Fig. 2: Method report*

## 3.3 LC-MS/MS Measurement

Analytical instrumentation:

Agilent 1290 UPLC, Agilent 6460 Jetstream (AJS) Triple Quad LC/MS
Mobil Phase A: 2 mM Ammonium acetate, 0.1 % formic acid (in 5 % methanol)
Mobil Phase B: Methanol

Analytical Column: Poroshell 120 EC-C18
(4.6 x 150 mm; 2.7 micron; Agilent Technologies, USA)

Gradient Composition Method (Tab. 1)

| Time (min.) | A (%) | B (%) | Flow (mL/min) |
|---|---|---|---|
| 0 | 90 | 10 | 0.6 |
| 0.3 | 90 | 10 | 0.6 |
| 3 | 20 | 80 | 0.6 |
| 7 | 5 | 95 | 0.6 |
| 11.10 | 90 | 10 | 0.6 |

*Tab. 1: Chromatographic conditions*

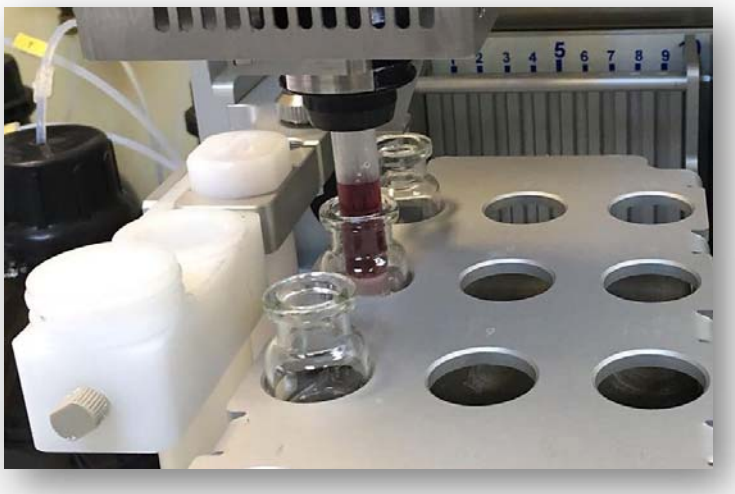

*Fig. 3: BD-SPE loading step of a blood sample at the Istanbul Forensic Lab*

## 4. Results

All analytes shown below were quantified against external calibrations measured with the corresponding analytical standards in typical concentration ranges (1 - 100 ng/mL) expected for human blood. The data represent a long term validation process over several months.

Tab. 2 and 3 show the recoveries of 162 analytes. Tab. 1 represents standard drugs and pharmaceuticals, respectively, whereas Tab. 3 shows 64 new synthetic drugs. All recoveries were obtained at a concentration of 100 ng/mL.

| | Compound | Recovery [%] |
|---|---|---|
| 1 | 6-Acetylmorphine | 47.7 |
| 2 | 7-Aminoclonazepam | 74.0 |
| 3 | Alprazolam | 71.4 |
| 4 | Amisulpride | 52.5 |
| 5 | Amitriptyline | 27.6 |
| 6 | Amlodipin | 62.2 |
| 7 | Amphetamine | 43.0 |
| 8 | Atenolol | 30.7 |
| 9 | Atropin | 59.4 |
| 10 | Benzoylecgonine | 45.7 |
| 11 | Biperiden | 58.2 |
| 12 | Bromazepam | 45.0 |
| 13 | Buprenorphine | 19.6 |
| 14 | Carbamazepine | 70.1 |
| 15 | Chlordiazepoxide | 5.4 |
| 16 | Chlorpheniramine | 51.2 |
| 17 | Chlorpromazine | 26.2 |
| 18 | Citalopram | 52.6 |
| 19 | Clobazam | 91.3 |
| 20 | Clomipramine | 56.9 |
| 21 | Clonazepam | 58.3 |

| | Compound | Recovery [%] |
|---|---|---|
| 22 | Clozapine | 32.0 |
| 23 | Cocaine | 43.2 |
| 24 | Codeine | 30.8 |
| 25 | Desipramine | 36.4 |
| 26 | Dextromethorphan | 30.9 |
| 27 | Diazepam | 96.9 |
| 28 | Diclofenac | 53.2 |
| 29 | Diltiazem | 60.4 |
| 30 | Diphenhydramine | 47.8 |
| 31 | Doxepin | 27.6 |
| 32 | Doxylamine | 72.0 |
| 33 | Etodolac | 70.1 |
| 34 | Famotidine | 19.8 |
| 35 | Fentanyl | 77.3 |
| 36 | Fluconazole | 23.1 |
| 37 | Flunitrazepam | 16.6 |
| 38 | Fluoxetine | 34.2 |
| 39 | Flurazepam | 40.6 |
| 40 | Fluvoxamine | 39.7 |
| 41 | Haloperidol | 37.6 |
| 42 | Hydroxyzine | 58.9 |
| 43 | Imipramine | 114.0 |
| 44 | Ketamine | 48.4 |
| 45 | Lansoprazole | 20.6 |
| 46 | Lidocaine | 63.7 |
| 47 | Loperamide | 56.1 |
| 48 | Lorazepam | 93.8 |
| 49 | MDA 3,4-Methylenedioxyamphetamine | 92.0 |
| 50 | MDEA 3,4-Methylenedioxyethamphetamine | 72.2 |

| | Compound | Recovery [%] |
|---|---|---|
| 51 | MDMA 3,4- Methylenedioxymethamphetamine | 55.4 |
| 52 | Metformin | 15.0 |
| 53 | Methadone | 67.0 |
| 54 | Methamphetamine | 45.7 |
| 55 | Methylecgonine | 7.4 |
| 56 | Metoclopramide | 65.8 |
| 57 | Metoprolol | 112.2 |
| 58 | Metronidazole | 4.2 |
| 59 | Mianserin | 32.6 |
| 60 | Midazolam | 90.0 |
| 61 | Mirtazepine | 55.2 |
| 62 | Moclobemide | 79.7 |
| 63 | Morphine | 60.0 |
| 64 | Naproxen | 32.3 |
| 65 | Nifedipine | 76.8 |
| 66 | Nordiazepam | 78.1 |
| 67 | Nortriptyline | 29.1 |
| 68 | Opipramol | 59.3 |
| 69 | Ornidazole | 17.4 |
| 70 | Oxazepam | 99.2 |
| 71 | Oxcarbazepine | 35.3 |
| 72 | Pantoprazole | 86.8 |
| 73 | Paracetamol | 5.9 |
| 74 | Paroxetine | 25.5 |
| 75 | Pentobarbital | 17.8 |
| 76 | Pentoxifylline | 22.2 |
| 77 | Pethidine | 42.7 |
| 78 | Pheniramine | 74.1 |
| 79 | Phenobarbital | 16.8 |
| 80 | Phenytoin | 91.6 |

| | Compound | Recovery [%] |
|---|---|---|
| 81 | Prilocaine | 52.2 |
| 82 | Propafenone | 80.2 |
| 83 | Propranolol | 80.2 |
| 84 | Propyphenazone | 39.2 |
| 85 | Pseudoephedrine | 26.5 |
| 86 | Quetiapine | 50.0 |
| 87 | Risperidone | 75.0 |
| 88 | Sertraline | 39.9 |
| 89 | Sildenafil | 91.3 |
| 90 | Tadalafil | 69.9 |
| 91 | THC Δ9-Tetrahydrocannabinol | 122.0 |
| 92 | THC-COOH | 60.0 |
| 93 | Thiopental | 40.5 |
| 94 | Thioridazine | 24.9 |
| 95 | Tramadol | 77.7 |
| 96 | Vardenafil | 83.4 |
| 97 | Venlafaxine | 46.2 |
| 98 | Verapamil | 69.4 |

*Tab. 2: Standard drugs and pharmaceuticals*

| | Compound | Recovery [%] |
|---|---|---|
| 1 | (+-) CP 47,497 C8 | 10.0 |
| 2 | (+-) CP 47,497 | 5.8 |
| 3 | (+-) WIN 55,212-2 | 82.4 |
| 4 | (+-) CP 55,940 | 34.1 |
| 5 | 5-F-AKB-48-4-hydroxypentyl | 118.6 |
| 6 | 5-F-PB-22-3-carboxy-indole | 60.8 |
| 7 | 5-F-AB-Pinaca | 97.2 |
| 8 | 5-F-AKB-48 | 40.6 |
| 9 | 5-F-ADB | 69.9 |
| 10 | AB-Chminaca | 79.7 |
| 11 | AB-Fubinaca-M2 | 94.4 |
| 12 | AB-Pinaca-5-hydroxylpentyl | 100.4 |
| 13 | AB-Pinaca-pentanoic acid | 91.8 |
| 14 | AB-Chminaca-M1 | 92.5 |
| 15 | AB-Chminaca-M2 | 104.3 |
| 16 | ADB-Pinaca-pentanoic acid | 92.5 |
| 17 | ADB-Pinaca | 118.3 |
| 18 | AKB-48-N-5-hydroxypentyl | 84.8 |
| 19 | AKB-48-N-pentanoic acid | 105.1 |
| 20 | AM 2201 | 85.8 |
| 21 | AM 2201-6-hydroxyindole | 36.0 |
| 22 | AM 2201-N-4-hydroxypentyl | 36.1 |
| 23 | HU 210 | 6.7 |
| 24 | JWH 0814-hydroxynaphthyl | 40.0 |
| 25 | JHW 081 N-5-hydroxypentyl | 50.5 |
| 26 | JWH 122 N-4-hydroxypentyl | 82.7 |
| 27 | JWH 122 N-5-hydroxypentyl | 82.7 |
| 28 | JWH 203 N-pentanoic acid | 12.4 |
| 29 | JWH 203 | 51.3 |

| | Compound | Recovery [%] |
|---|---|---|
| 30 | JWH 210 5-hydroxyindole | 64.5 |
| 31 | JWH 210 N-4-hydroxypentyl | 66.0 |
| 32 | JWH 210 N-5-hydroxypentyl | 66.0 |
| 33 | JWH 210 N-pentanoic acid | 85.4 |
| 34 | JWH 210 | 22.6 |
| 35 | JWH 250 N-4-hydroxypentyl | 60.7 |
| 36 | JWH 250 N-pentanoic acid | 39.4 |
| 37 | JWH 398 N-5-hydroxypentyl | 74.9 |
| 38 | JWH 398 N-pentanoic acid | 77.6 |
| 39 | JWH 018 N-pentanoic acid | 53.0 |
| 40 | JWH 018 | 49.3 |
| 41 | JWH 018 N-5-hydroxypentyl | 5.4 |
| 42 | JWH 019 | 32.0 |
| 43 | JWH 073 | 61.6 |
| 44 | JWH 073 4-hydroxybutyl | 25.1 |
| 45 | JWH 073 N-butanoic acid | 16.4 |
| 46 | JWH 081 | 40.4 |
| 47 | JWH 200 | 42.1 |
| 48 | JWH 201 | 71.3 |
| 49 | JWH 250 N-5-hydroxypentyl | 61.4 |
| 50 | JWH 250 | 26.8 |
| 51 | MAM 2201 4-hydroxypentyl | 44.5 |
| 52 | MAM 2201 N-pentanoic acid | 69.0 |
| 53 | MAM 2201 | 71.8 |
| 54 | PB-22-3-carboxyindole | 50.9 |
| 55 | RCS-4 | 76.0 |
| 56 | RCS-4-N-5-carboxypentyl | 65.8 |
| 57 | RCS-4-N-5-hydroxypentyl | 67.2 |
| 58 | RCS-8 | 31.6 |
| 59 | UR-144 N-5-hydroxypentyl | 84.0 |

| | Compound | Recovery [%] |
|---|---|---|
| 60 | UR-144 N-pentanoic acid | 81.3 |
| 61 | UR-144 | 35.6 |
| 62 | XLR-11 6-hydroxyindole | 47.6 |
| 63 | XLR-11 N-4-hydroxypentyl | 47.1 |
| 64 | XLR-11 | 57.0 |

*Tab. 3: Synthetic drugs*

**Recovery Values:** 4.2 - 122 %

**Repeatability within a day, n = 6:** 0.8 -10 % RSD

**Reproducibility for 5 days work:** 2.3 - 18 % RSD

From the data it can be seen that the performance criteria obtained with this approach meet the requirements for a screening tool in forensic analysis of blood samples even at advanced degradation stages.

Cross-contamination measurements conducted with samples spiked at a level of 10 ppm showed no measurable signals in blanks processed afterwards. Thus any wrongful conviction can definitely be excluded.

## 5. Conclusions

The BD-SPE approach is fit-for-purpose in the field of forensic investigations in routinely conducted blood sample analysis, where the blood samples may originate from living as well as deceased persons.

The sample preparation is reduced to a minimum thus avoiding any alteration of the sample and its chemical nature, respectively.

The methodology may be applied to acidic, neutral, and basic chemicals that are relevant in typical routine forensic examinations, such as illicit drugs, pharmaceuticals or toxic substances.

Due to the unique approach any cross-contamination is explicitly excluded as the sample never enters the automation system.

The recoveries are sufficiently high for a routine screening method, show an excellent repeatability as well as reproducibility, and thus can be considered as very robust.

## 6. Acknowledgements

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*Fig. 4: Complete Set-up of the robotic system FREESTYLE at the Istanbul Forensic Lab*

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