APPLICATION NOTE | AN0022

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Determination of Drugs in Human Brain via Bidirectional Solid-Phase Extraction

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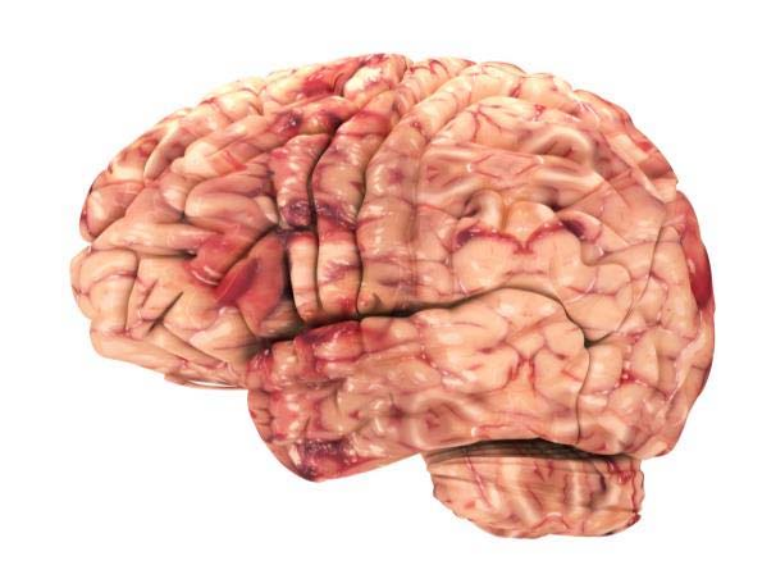
Content

1 Introduction

To clarify the role of drugs of abuse or other toxic substances in forensic investigations, the analysis of post-mortem tissue samples via Solid-Phase-Extraction (SPE) has successfully been applied. In the following application note a new automated approach is described that uses the so-called bidirectional SPE (BD-SPE); this specific approach is used when either matrices that are difficult to process, such as brain tissue with its high fat and protein content are used, or cross-contamination has to be excluded - due to the uniqueness of the extracted sample.

In brief, this approach uses standard 3 mL SPE cartridges which undergo standard conditioning, washing, drying, and elution steps, whereas the critical loading step, where the matrix enters the automation system, is performed in a reverse way. This means the homogenised and buffered sample, e. g. brain tissue, is not loaded from the 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 automation system, moreover it passes through the sorbent twice. Consequently it was called bidirectional SPE.



Human brain: A challenging matrix due to the high fat and protein content.

2 Method Development

2.1 Reagents and Materials

- 0.05 M Phosphate buffer pH 7.4 p.a.
- Water p.a.
- 0.1 N Acetic acid p.a.
- Ethyl acetate (Merck, Darmstadt, Germany)
- Isopropanol (Merck, Darmstadt, Germany)
- Triethylamine (Merck, Darmstadt, Germany)
- Ethyl acetate/isopropanol (3/1, v/v)
- Ethyl acetate/isopropanol/triethylamine (75/25/3, v/v/v)
- MSTFA (MACHEREY-NAGEL, Düren, Germany)
- Evolute CX-50 cartridges (50 mg, Biotage, Uppsala, Sweden)
- Standards in a composition recommended by the working group of extraction of the GTFCh (Society of Toxicological and Forensic Chemistry).
  All standards in methanol at a concentration of 1 mg/mL. For low controls 50 ng/g tissue for basic, and 500 ng/g for neutral/acidic substances were added to the samples prior to extraction; for high controls the levels were 10-times higher.
  - Benzoylecgonine (Lipomed, Arlesheim, CH)
  - Amphetamine (Lipomed, Arlesheim, CH)
  - Cocaine (Lipomed, Arlesheim, CH)
  - Codeine (Lipomed, Arlesheim, CH)
  - Diazepam (Lipomed, Arlesheim, CH)
  - Morphine (Lipomed, Arlesheim, CH)
  - Doxepin (Lipomed, Arlesheim, CH)
  - Metoprolol (Lipomed, Arlesheim, CH)
  - Methadone (Lipomed, Arlesheim, CH)
  - Phenobarbitone (Lipomed, Arlesheim, CH)
  - Ibuprofen (Sigma-Aldrich, St. Louis, MO, USA)

2.2 Sample Preparation

Thoroughly homogenise 2 g of brain, e. g in an IKA 15 mL tube (IKA, BMT-20-S tube with 6 steel balls) with an Ultra Turrax Tube Drive (IKA, Staufen, Germany). Take 0.5 g of homogenate sample and add 5.5 mL of phosphate buffer pH 7.4. Sonicate for 5 min and centrifuge at 600 x g for 20 min.
0.1 mL of the standard test mix in methanol was added to 0.5 mL phosphate buffer pH 7.4 in order to avoid any precipitation. This mixture was then added to the sample and mixed at highest speed for 1 min.

2.3 Instrumentation

2.3.1 The robotic system FREESTYLE

The FREESTYLE system is a fully automated sample preparation system and is used in nearly all areas that demand intensive sample preparation. This applies to food, environmental and pharmaceutical samples, but also to drug screening as well as to forensic and toxicological samples.
The system is based on the FREESTYLE BASIC platform that can be equipped with different modules suitable for the intended purpose – in this application with the FREESTYLE SPE module.

The racks for the samples and vials are hooked into the robotic system. The position of the racks is not predetermined but in the case of the BD-SPE the samples and the empty vials are placed in one rack.

All modules of the FREESTYLE system are managed through the same easy to use software with one user interface. Using the bidirectional SPE a software update is needed.

SPE module – robotic arm

Insertion of racks

2.3.2 Bidirectional SPE (BD-SPE) with FREESTYLE SPE

The following components are needed to process the BD-SPE automatically with a FREESTYLE SPE; see below. In brief the buffered, spiked, and homogenised samples are transferred to 10 mL vials. The system is equipped with 3 mL cartridges, empty 10 mL vials, and the vials for the eluate.

1.	FREESTYLE BASIC	P/N 12663-12
2.	FREESTYLE SPE	P/N 12668
3.	Rack for solvent supply	P/N 13156
4.	Rack for up to 18 SPE cartridges	P/N 13946-AD
5.	Clamping adapter (3 mL)	P/N 14892
6.	Reusable cap (3 mL)	P/N 14862
7.	Frame (100 mm)	P/N 11915 (2 needed)
8.	Rack, 30 positions, 75 x 12 mm vials, 5 mL	P/N 12117 (2 needed)
9.	Rack, 18 positions, 10 mL vials	P/N 14711 (2 needed)
10.	Vial, flat bottom, 10 mL	P/N V0010
11.	Software upgrade	P/N 14773

The 5 mL 75 x 12 mm reagent tubes have to be ordered from any local supplier.

After processing the eluates are evaporated to dryness with a vacuum concentrator (Christ Alpha RVC, Osterrode am Harz, Germany). The remaining residue is dissolved in 50 µL ethyl acetate and measured by GC-MS. After the first measurement the sample is evaporated again and derivatised with 50 µL MSTFA (20 min. at 80 °C) for the second GC-MS run.

2.3.3 Description of bidirectional SPE (BD-SPE)

The robotic arm of the FREESTYLE system takes the SPE cartridge and moves it to the sample vial. The SPE cartridge enters with the LUER tip into the sample. The sample is aspirated through the sorbent from bottom to top. The robotic arm moves the cartridge to an empty vial on the platform and releases the sample through the sorbent from top to bottom. Doing so, the sample is still available for further analysis.

This procedure can be repeated up to three times for a quantitative transfer.

BD-SPE: Sample is aspired through the sorbens from bottom to top and released in the reverse way.

Standard elution steps.

Robotic arm places used cartridge back into the rack.

2.3.4 Software Protocol

The following setting can be chosen to process the samples using BD-SPE on the FREESTYLE SPE system. The method is already defined and stored in the FREESTYLE software. It can also be changed and saved with a new name, if needed.

FREE-STYLE™
AUTOMATISIERTE PROBENVORBEREITUNG

LCTech FreeStyle - Report on Methods: SPE Date: 19.09.2016 Time: 14:41:45

Name: method_evolute_7.spe	SPE Column: LCTech_3ml.col
Extension cannula:	no
Processing speed selection:	Standard (organic solvents)
Rinsing intensity:	Standard rinsing cycle
Use pressure limitation function during loading and washing:	no

Step: Conditioning		Basic type: Conditioning		Step: - ID: 646
Volume: 4 ml	Suction Speed: 15 ml/min Repetitions: 2 Waiting Time after Dosage: 10 sec.	Dispensing Speed: 5 ml/min Waiting Time after Step: 5 sec.	Port: 8 Phosphate Buffer Dispense: into Waste	
Step: Drying		Basic type: Drying - Drying by defined air volume		Step: - ID: 647
Air volume: 20 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: 648
Volume: 5 ml Vial Type: Type1@10 without rinsing of vial	Suction Speed: 5 ml/min Waiting Time after Dosage: 60 sec.	Dispensing Speed: 1 ml/min Waiting Time after Step: 150 sec.	Dispense: into vials Number of vials: 1 Vial Type: Type1@10	
Step: Washing		Basic type: Washing		Step: - ID: 649
Volume: 9.5 ml	Suction Speed: 20 ml/min Repetitions: 0 Waiting Time after Dosage: 0 sec.	Dispensing Speed: 5 ml/min Waiting Time after Step: 0 sec. Dispense: into Waste	Port: 1 Water	
Step: Drying		Basic type: Drying - Drying by defined air volume		Step: - ID: 650
Air volume: 10 ml	Suction Speed: 100 ml/min	Dispensing Speed: 50 ml/min	Dispense: into Waste	
Step: Washing		Basic type: Washing		Step: - ID: 652
Volume: 3.5 ml	Suction Speed: 15 ml/min Repetitions: 0 Waiting Time after Dosage: 0 sec.	Dispensing Speed: 5 ml/min Waiting Time after Step: 0 sec. Dispense: into Waste	Port: 10 1 M Acetic Acid	
Step: Drying		Basic type: Drying - Nitrogen drying by defined time		Step: - ID: 653
Drying time with nitrogen 120 sec.			Dispense: into vials Number of vials: 1 Vial Type: Type1@8	
Step: Eluting		Basic type: Eluting		Step: - ID: 654
Volume: 3 ml	Suction Speed: 10 ml/min Repetitions: 0 Waiting Time after Dosage: 40 sec.	Dispensing Speed: 0.5 ml/min Waiting Time after Step: 0 sec.	Port: 7 EA/IP 3:1 Dispense: into vials Number of vials: 1 Vial Type: Type1@8	
Step: Drying		Basic type: Drying - Drying by defined air volume		Step: - ID: 655
Air volume: 20 ml	Suction Speed: 100 ml/min	Dispensing Speed: 50 ml/min	Dispense: stay on actual position	
Step: Eluting		Basic type: Eluting		Step: - ID: 656
Volume: 3 ml	Suction Speed: 10 ml/min Repetitions: 0 Waiting Time after Dosage: 0 sec.	Dispensing Speed: 0.5 ml/min Waiting Time after Step: 0 sec.	Port: 9 EA/IP/TEA Dispense: into vials Number of vials: 1 Vial Type: Type1@8	
Step: Drying		Basic type: Drying - Drying by defined air volume		Step: - ID: 657
Air volume: 20 ml	Suction Speed: 100 ml/min	Dispensing Speed: 50 ml/min	Dispense: stay on actual position	

2.3.5 GC-MS Measurement

The samples were measured on an Agilent 6890 with a 5973net mass selective detector (Agilent, Santa Clara, CA, USA).
An autosampler ALS 7683 and a Zebron ZB-5MSi capillary column (15 m x 0.25 mm ID, 0.25 µm; Phenomenex, Torrance, CA, USA) was used.

Injector temperature	280 °C
Injection mode	1 µL, splitless
Carrier gas	Helium at 1.6 mL/min
Column oven	Initial temperature 100 °C; hold for 2 min. Heat with 25 °C/min to 200 °C Heat with 20 °C/min to 300 °C; hold for 7 min.
Transfer line temperature	300 °C
MS analysis	EI scan mode

3 Results

With this procedure acceptable recoveries for the wide spectrum of analytes (mean between 28-75%; n=6) could be obtained. Furthermore, the repeatability (1.4-7.6 % RSD; n=8) and reproducibility (3.6-18.3 % RSD; n=8) are very good in the complex matrix of brain and thus reliable results for this critical application are assured.

4 Conclusions

The presented BD-SPE approach proofed fit-for-purpose in the field of forensic investigations - even with very difficult matrices such as brain samples.
The methodology may be applied to acidic, neutral, and basic substances that are relevant in routine forensic investigations.
Due to the unique approach of BD-SPE any cross-contamination is explicitly excluded as the sample only enters the extraction cartridge and never the automation system.
The recoveries are sufficiently high for a routine screening method and show an excellent repeatability as well as reproducibility, and thus the procedure can be considered as very robust.
As the sample is not discarded into the waste but into a second vial, it can be used for further investigations.

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