APPNOTE

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GERSTEL AppNote Nr. 229

# Automated Liquid-Liquid Extraction and Determination of NBOMes in Serum and Blotter Paper Samples using a Novel Robotic Autosampler and LC-MS/MS Platform

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## Keywords

Sample Preparation, LC-MS/MS, High Throughput Lab Automation, Forensics, NPS

## Abstract

The forensic community has experienced an increasing emergence of New Psychoactive Substances (NPSs) in recent years. The five main classes of NPSs include the synthetic cannabinoid compounds, stimulants such as the derivatives of cathinone, opioids such as fentanyl, psychedelics, and non-pharmaceutical benzodiazepines. The automated extraction and determination of other classes of NPS compounds using the GERSTEL MultiPurpose Sampler (MPS) robotic$^{PRO}$ have been previously developed and discussed [1,2,3]. In this work, we show that the same robotic sampler can be used to automate the extraction and determination of the psychedelic NPS compounds in serum samples and seized blotter paper samples.

The entire liquid-liquid extraction and subsequent analysis by LC-MS/MS is automated providing critically needed high throughput analysis for the psychedelic NPS compounds in either sample type. Using the GERSTEL MPS robotic$^{PRO}$ sampler, syringe transfer of all liquids involved in the liquid-liquid extraction as well as the controlled mixing, centrifugation, and evaporation of the samples for defined periods are performed. The resulting extracts are introduced into the Agilent Ultivo LC-MS/MS instrument for detection and quantification.

## Introduction

Psychedelic compounds, which produce marked alterations in perception, mood, and cognition, are widely used for recreational purposes. The stimulation of the 5-$HT_{2A}$ receptors is required for the psychedelic effects of compounds such as LSD, mescaline, and psilocybin [4]. The first N-(2-methoxybenzyl)phenethylamines (NBOMEs) were originally synthesized in a search for a pharmacological tool to study this 5-$HT_{2A}$ receptor. The first recreationally used NBOMe compound was 25I-NBOMe (2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine). Other NBOMe compounds were then found to be synthesized and introduced into the illicit drug market. For these compounds, the iodine was exchanged for other halogens such as bromine (25B-NBOMe) or chlorine (25C-NBOMe).

As a result of this study, we were able to show that automated liquid-liquid extraction performed by the GERSTEL MPS robotic$^{PRO}$ sampler could successfully be used for a variety of NBOMe compounds in either serum or blotter paper samples. NBOMe analytes isolated from the serum or blotter paper samples using the automated extraction procedures were introduced to an Agilent Technologies 1260 HPLC coupled with an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. The recovery of the NBOMe compounds when extracted from blotter paper samples was found to be 98.9 % for 25B-NBOMe, 93.6 % for 25C-NBOMe, and 97.3 % for 25I-NBOMe. Accuracy data averaged 101 % (range: 96.9 % - 105 %) and precision data averaged 2.22 % RSD (range: 0.790 % - 5.09 %) for all NBOMe compounds extracted from serum samples.

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## Experimental

*Materials*

All stock solutions for the analytes listed in Table 1 were purchased from Cerilliant. An intermediate analyte stock solution was prepared by combining the analyte stock solutions with (1:1) acetonitrile: water, resulting in appropriate concentrations of the drug compounds for method evaluation.

Deuterated analogues, $d_3$-25C-NBOMe and $d_3$-25I-NBOMe, were purchased from Cerilliant. An internal standard stock solution containing the deuterated internal standards was prepared in (1:1) acetonitrile: water at a concentration of 1000 ng/mL. A working internal standard solution was prepared in (1:1) acetonitrile: water at a concentration of 10 ng/mL. Table 1 shows which deuterated internal standards were used for the quantitation of the respective analytes.

Calibration standard and QC serum samples were prepared by making appropriate dilutions of the combined intermediate analyte stock solutions using analyte-free rat serum (Biochemed Services) to reach the concentrations listed in Table 1. Calibration standards were prepared using a dilution ratio strategy from the high concentration sample of 1:2:5:2:5:2:5:2:5. The high, middle, and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:10:10. Table 1 lists the concentrations for the highest calibration standard and the limit of quantitation found during this study.

The simulated blotter paper sample replicates were prepared by pipetting 25 µL of a 200 ng/mL NBOMe intermediate stock solution onto a 1 cm x 1 cm blotter paper sample. Each simulated blotter paper sample was then placed in an open container at room temperature for 2 hours before being transferred into a separate empty vial prior to extraction.

All other reagents and solvents used were reagent grade.

**Table 1:** Mass Spectrometer Acquisition Parameters.

| Compound Name | Precursor Ion [m/z] | Product Ion [m/z] | | Fragmentation Voltage [V] | | CE [V] | | Ret Time [min] | High Std Conc. [ng/mL] | LOQ [ng/mL] |
|---|---|---|---|---|---|---|---|---|---|---|
| 25I-$D_3$-NBOMe | 431.1 | 124 | 92 | 110 | 110 | 20 | 60 | 4.76 | - | - |
| 25I-NBOMe[1] | 428.1 | 120.9 | 91 | 110 | 110 | 20 | 60 | 4.77 | 100 | 0.0100 |
| 25B-NBOMe[2] | 382.1 | 120.9 | 90.9 | 110 | 110 | 20 | 60 | 4.50 | 100 | 0.0100 |
| 25C-$D_3$-NBOMe | 339.1 | 124 | 92 | 110 | 110 | 20 | 50 | 4.37 | - | - |
| 25C-NBOMe[2] | 336.1 | 121 | 91 | 120 | 120 | 15 | 50 | 4.38 | 100 | 0.0100 |

*1 - Internal Standard 25I-$D_3$-NBOMe*
*2 - Internal Standard 25C-$D_3$-NBOMe*

## Instrumentation

All automated Prep Sequences were performed using a MPS robotic$^{PRO}$ sampler with GERSTEL quickMIX, Multi Sample Evaporation Station (mVAP), and CF200 Centrifuge Options as shown in Figure 1. All analyses were performed using an Agilent 1260 HPLC with a Phenomenex Kinetex PFP column, (3.0 x 100 mm, 2.6 µm) and an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. Sample injections were made using the GERSTEL LCMS Tool into a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

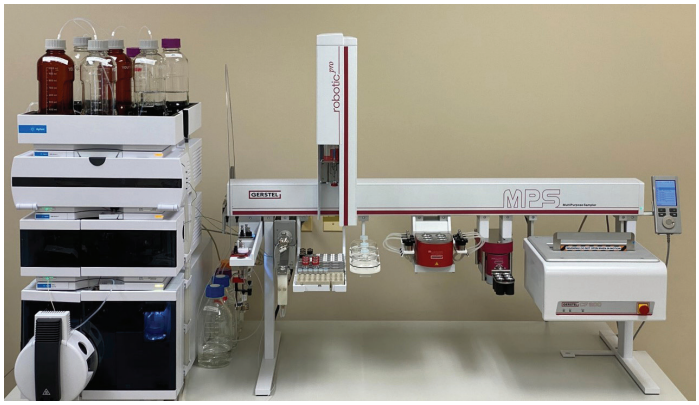


**Figure 1:** MPS robotic$^{PRO}$ sampler with GERSTEL quickMIX, mVAP, and CF-200 centrifuge options.

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*Serum Sample Pretreatment*

1. Pipette 500 µL of serum sample into a clean, round bottom 4 mL autosampler vial.
2. Pipette 10 µL of a 10 ng/mL working internal standard into each sample and cap with a magnetically transportable cap.
3. Automated MPS Prep Sequence for NBOMe Serum Samples
4. The MPS adds 0.2 mL of a 0.2 M NaOH solution to each vial.
5. The MPS mixes each vial for 30 seconds at 1500 rpm.
6. The MPS adds 3 mL of (9:1) hexane: ethyl acetate to each vial.
7. The MPS mixes each vial for 5 minutes at 1500 rpm.
8. The MPS centrifuges each vial for 10 minutes at 3000 rpm.
9. The MPS transfers 2.25 mL of the organic layer into a 10 mL high recovery vial.
10. The MPS adds 2.25 mL of (9:1) hexane: ethyl acetate to each vial.
11. The MPS mixes each vial for 5 minutes at 1500 rpm.
12. The MPS centrifuges each vial for 10 minutes at 3000 rpm.
13. The MPS transfers 2.25 mL of the organic layer into the 10 mL high recovery vial.
14. The MPS evaporates each 10 mL high recovery vial to dryness at 40 °C, 250 rpm, and 100 mbar.
15. The MPS reconstitutes each high recovery vial using 75 µL of (1:1) acetonitrile: water.
16. The MPS transfers the final extract into a 2 mL autosampler vial containing a high recovery conical insert.

*Blotter Paper Sample Pretreatment*

1. Transfer a spiked 1 cm x 1 cm blotter paper sample into a clean, round bottom 4 mL autosampler vial.
2. Pipette 10 µL of a 100 ng/mL working internal standard onto each sample and cap with a magnetically transportable cap.

*Automated MPS Prep Sequence for NBOMe Blotter Paper Samples*

1. The MPS adds 1 mL of (1:1) acetonitrile: methanol to each vial.
2. The MPS mixes each vial for 5 minutes at 1500 rpm.
3. The MPS centrifuges each vial for 10 minutes at 3000 rpm.
4. The MPS transfers 75 µL of water into a 2 mL vial containing a high recovery conical insert.
5. The MPS transfers 75 µL of the extract into the 2 mL vial.

*Automated MPS Sample Introduction*

1. Using the GERSTEL LCMS Tool, the MPS injects the extract into a 2 µL stainless steel sample loop (loop over-fill technique).

*LC Method Parameters*

| | | |
|---|---|---|
| Pump | Gradient (800 bar) | |
| | Flow rate = 0.5 mL | |
| Mobile Phase | A – 10 mM ammonium formate in water, with 0.1 % formic acid | |
| | B – acetonitrile with 0.1 % formic acid | |
| Gradient | Initial | 50 % B |
| | 4.0 min | 95 % B |
| | 5.0 min | 95 % B |
| | 5.1 min | 50 % B |
| | 10.0 min | 50 % B |
| Run time | 10 minutes | |
| Injection volume | 2.0 µL (loop over-fill technique) | |
| Column Temperature | 30 °C | |

*Mass Spectrometer Parameters*

| | |
|---|---|
| Operation | Electrospray positive mode |
| Gas Temperature | 350 °C |
| Gas Flow ($N_2$) | 5 L/min |
| Nebulizer pressure | 35 psi |
| Sheath Gas Flow ($N_2$) | 11 L/min |
| Sheath Gas Temperature | 400 °C |
| Capillary voltage | 4000 V |
| Nozzle voltage | 500 V |
| Delta EMV | 0 V |

The mass spectrometer acquisition parameters are shown in Table 1 with qualifier ions.

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## Results and Discussion

Figure 2 shows representative mass chromatograms for the NBOMe compounds and deuterated internal standards determined in an extracted low serum QC sample.

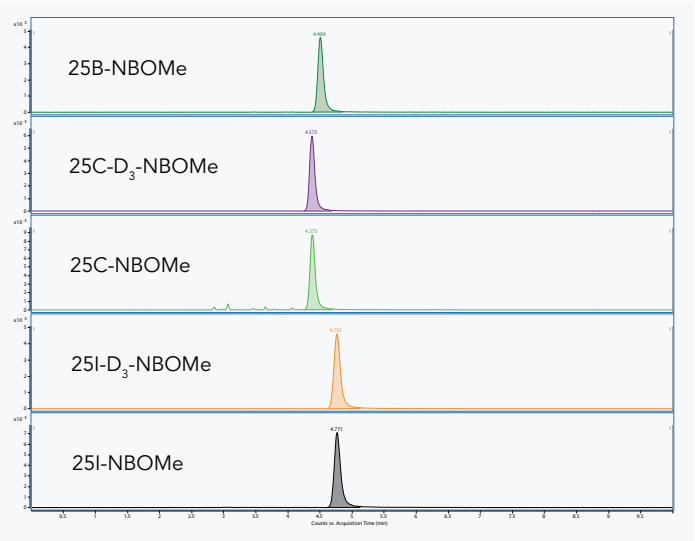


**Figure 2:** Representative mass chromatograms for extracted low serum QC sample.

The lower limits of quantitation for the drugs of abuse determined using this method were found to be 0.0100 ng/mL as can be seen in Table 1. Representative calibration curves are shown in Figures 3 A-C. Regression analysis resulted in $R^2$ values of 0.99 or greater.

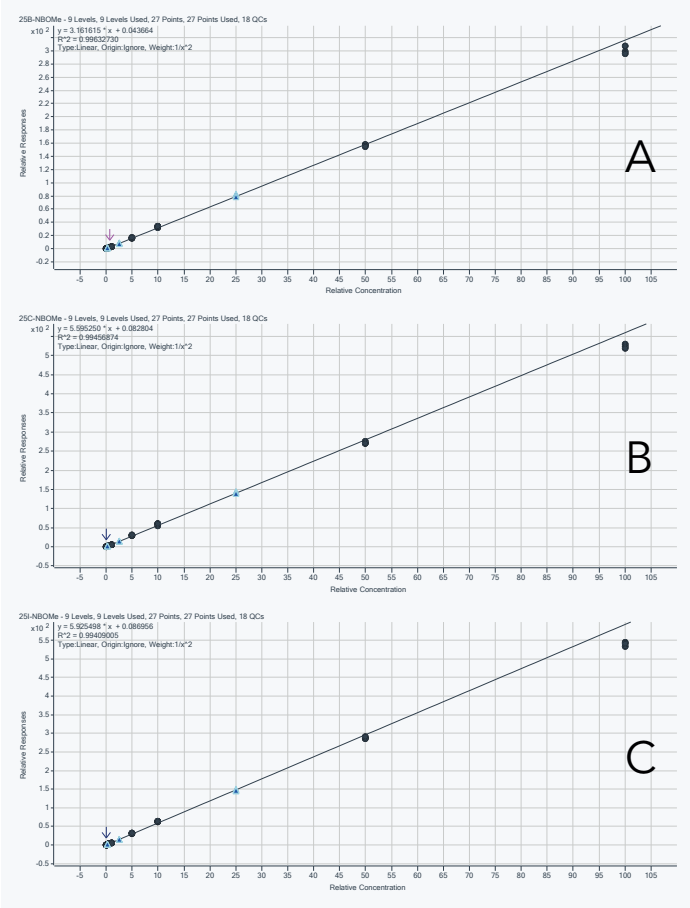


**Figure 3:** Calibration curves for 25B-NBOMe (A), 25C-NBOMe (B) and 25I-NBOMe (C).

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The accuracy and precision of the method were evaluated for all NBOMe compounds determined using QC samples at high, middle, and low concentrations. Table 2 shows the resulting accuracy and precision data for all drug compounds. Accuracy data averaged 101 % (range: 96.9 % - 105 %) and precision data averaged 2.22 % RSD (range: 0.790 % - 5.09 %) for all NBOMe compounds determined in serum samples.

**Table 2:** Serum QC sample % accuracy and % precision results.

| 25B NBOMe serum | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Name | Final Conc. | Accuracy | Name | Final Conc. | Accuracy | Name | Final Conc. | Accuracy |
| QC L 1 | 0.247 | 99.0 | QC M 1 | 2.56 | 102 | QC H 1 | 25.0 | 99.8 |
| QC L 2 | 0.252 | 101 | QC M 2 | 2.62 | 105 | QC H 2 | 25.8 | 103 |
| QC L 3 | 0.246 | 98.5 | QC M 3 | 2.48 | 99.1 | QC H 3 | 26.1 | 104 |
| QC L 4 | 0.218 | 87.2 | QC M 4 | 2.70 | 108 | QC H 4 | 26.3 | 105 |
| QC L 5 | 0.243 | 97.0 | QC M 5 | 2.50 | 100 | QC H 5 | 25.6 | 103 |
| QC L 6 | 0.247 | 98.9 | QC M 6 | 2.53 | 101 | QC H 6 | 25.5 | 102 |
| **mean** | 0.242 | 96.9 | **mean** | 2.56 | 103 | **mean** | 25.7 | 103 |
| **SD** | 0.0123 | 4.93 | **SD** | 0.0824 | 3.30 | **SD** | 0.472 | 1.89 |
| **% CV** | 5.09 | 5.09 | **% CV** | 3.21 | 3.21 | **% CV** | 1.84 | 1.84 |

| 25C NBOMe serum | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Name | Final Conc. | Accuracy | Name | Final Conc. | Accuracy | Name | Final Conc. | Accuracy |
| QC L 1 | 0.249 | 99.7 | QC M 1 | 2.65 | 106 | QC H 1 | 24.7 | 99.0 |
| QC L 2 | 0.255 | 102 | QC M 2 | 2.70 | 108 | QC H 2 | 25.6 | 102 |
| QC L 3 | 0.248 | 99.1 | QC M 3 | 2.56 | 102 | QC H 3 | 25.7 | 103 |
| QC L 4 | 0.251 | 100 | QC M 4 | 2.64 | 106 | QC H 4 | 25.7 | 103 |
| QC L 5 | 0.246 | 98.3 | QC M 5 | 2.59 | 104 | QC H 5 | 25.5 | 102 |
| QC L 6 | 0.253 | 101 | QC M 6 | 2.58 | 103 | QC H 6 | 25.1 | 100 |
| **mean** | 0.250 | 100 | **mean** | 2.62 | 105 | **mean** | 25.4 | 102 |
| **SD** | 0.00334 | 1.34 | **SD** | 0.0528 | 2.11 | **SD** | 0.376 | 1.50 |
| **% CV** | 1.34 | 1.34 | **% CV** | 2.02 | 2.02 | **% CV** | 1.48 | 1.48 |

**Table 2 (cont.):** Serum QC sample % accuracy and % precision results.

| 25I NBOMe serum | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Name | Final Conc. | Accu-racy | Name | Final Conc. | Accu-racy | Name | Final Conc. | Accu-racy |
| QC L 1 | 0.247 | 98.7 | QC M 1 | 2.53 | 101 | QC H 1 | 24.6 | 98.6 |
| QC L 2 | 0.248 | 99.2 | QC M 2 | 2.66 | 106 | QC H 2 | 25.0 | 100 |
| QC L 3 | 0.245 | 98.2 | QC M 3 | 2.45 | 98.1 | QC H 3 | 25.2 | 101 |
| QC L 4 | 0.247 | 98.7 | QC M 4 | 2.63 | 105 | QC H 4 | 24.6 | 98.5 |
| QC L 5 | 0.242 | 97.0 | QC M 5 | 2.59 | 104 | QC H 5 | 25.2 | 101 |
| QC L 6 | 0.246 | 98.3 | QC M 6 | 2.61 | 104 | QC H 6 | 24.4 | 97.7 |
| **mean** | 0.246 | 98.4 | **mean** | 2.58 | 103 | **mean** | 24.9 | 99.4 |
| **SD** | 0.00194 | 0.777 | **SD** | 0.0750 | 3.00 | **SD** | 0.331 | 1.32 |
| **% CV** | 0.790 | 0.790 | **% CV** | 2.91 | 2.91 | **% CV** | 1.33 | 1.33 |

To assess the recovery of NBOMe compounds from extracted blotter paper samples, three spiked blotter paper samples were extracted using the automated liquid-liquid extraction procedure and the peak area results were compared to those from six replicate injections of a spiked recovery standard having concentrations equivalent to the extracted NBOMe compounds. Recovery of NBOMe compounds extracted from blotter paper samples was found to be 98.9 % for 25B-NBOMe, 93.6 % for 25C-NBOMe, and 97.3 % for 25I-NBOMe. Together with the data from the extracted serum samples, this data provides evidence that the MPS robotic$^{PRO}$ sampler can be used for the determination of NBOMe compounds in serum samples as well as in blotter paper samples.

**Table 3:** Results of automated liquid-liquid extraction of simulated blotter paper samples.

| 25B-NBOMe Results | | |
|---|---|---|
| Name | Analyte Resp. | Int. Std. Resp. |
| Blotter 1:1 extr 1 | 71270 | 24320 |
| Blotter 1:1 extr 2 | 71277 | 22699 |
| Blotter 1:1 extr 3 | 70998 | 22530 |
| **mean** | 71182 | 23183 |
| **SD** | 159 | 988 |
| **% CV** | 0.224 | 4.26 |
| Blotter recov std 1 | 78924 | 23807 |
| Blotter recov std 2 | 59508 | 18035 |
| Blotter recov std 3 | 68223 | 21604 |
| Blotter recov std 4 | 75150 | 22476 |
| Blotter recov std 5 | 75200 | 22807 |
| Blotter recov std 6 | 74626 | 22352 |
| **mean** | 71938 | 21847 |
| **SD** | 7004 | 2000 |
| **% CV** | 9.74 | 9.16 |
| Extraction using (1:1) acetonitrile: methanol | Recovery 98.9 | Recovery 106 |

**Table 3 (cont.):** Results of automated liquid-liquid extraction of simulated blotter paper samples.

| 25C-NBOMe Results | | |
|---|---|---|
| Name | Analyte Resp. | Int. Std. Resp. |
| Blotter 1:1 extr 1 | 127497 | 24320 |
| Blotter 1:1 extr 2 | 128767 | 22699 |
| Blotter 1:1 extr 3 | 125147 | 22530 |
| **mean** | 127137 | 23183 |
| **SD** | 1837 | 988 |
| **% CV** | 1.44 | 4.26 |
| Blotter recov std 1 | 146105 | 23807 |
| Blotter recov std 2 | 112336 | 18035 |
| Blotter recov std 3 | 135112 | 21604 |
| Blotter recov std 4 | 140879 | 22476 |
| Blotter recov std 5 | 141271 | 22807 |
| Blotter recov std 6 | 139504 | 22352 |
| **mean** | 135868 | 21847 |
| **SD** | 12055 | 2000 |
| **% CV** | 8.87 | 9.16 |
| Extraction using (1:1) acetonitrile:-methanol | Recovery 93.6 | Recovery 106 |

| 25I-NBOMe Results | | |
|---|---|---|
| Name | Analyte Resp. | Int. Std. Resp. |
| Blotter 1:1 extr 1 | 134612 | 24516 |
| Blotter 1:1 extr 2 | 133657 | 23614 |
| Blotter 1:1 extr 3 | 134405 | 23393 |
| **mean** | 134224 | 23841 |
| **SD** | 502 | 595 |
| **% CV** | 0.374 | 2.50 |
| Blotter recov std 1 | 150318 | 25374 |
| Blotter recov std 2 | 116831 | 19256 |
| Blotter recov std 3 | 133074 | 23267 |
| Blotter recov std 4 | 142998 | 24124 |
| Blotter recov std 5 | 143521 | 24441 |
| Blotter recov std 6 | 141211 | 23950 |
| **mean** | 137992 | 23402 |
| **SD** | 11747 | 2145 |
| **% CV** | 8.51 | 9.16 |
| Extraction using (1:1) acetonitrile:-methanol | Recovery 97.3 | Recovery 102 |

## Conclusions

As a result of this study, we were able to show:

- Psychedelic NPS compounds and internal standards in serum and blotter paper samples can be successfully extracted using automated liquid-liquid extraction procedures and determined using the Agilent Ultivo Triple Quadrupole Mass Spectrometer.
- These methods were readily automated using the GERSTEL MPS robotic$^{PRO}$ sampler.
- Linear calibration curves resulting in $R^2$ values 0.99 or greater were achieved for the NBOMe compounds determined in serum samples.
- The automated LLE-LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 101 % (range: 96.9 % - 105 %) and precision data averaged 2.22 % RSD (range: 0.790 % - 5.09 %) for all NBOMe compounds analyzed from serum samples.
- The recovery of NBOMe compounds extracted from blotter paper samples was found to be 98.9 % for 25B-NBOMe, 93.6 % for 25C-NBOMe, and 97.3 % for 25I-NBOMe.

## References

[1] GERSTEL Application Note No. 215, Automated Hydrolysis, Extraction and Analysis of Synthetic Cathinones in Urine using a Robotic Autosampler and LC-MS/MS Platform, **2020**.

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[4] J. Zawilska, M. Kacela, and P.Adamowicz, **2020** "NBOMes – Highly Potent and Toxic Alternatives of LSD", Front. Neurosci., 14:78.