Toxtyper: Detection of opioids and prescription drugs at Miami Dade

Comprehensive detection and identification of prescription analgesics and illicit opioids in postmortem specimens using Toxtyper.

Abstract

Ongoing evolution of new designer drugs, also known as new psychoactive substances (NPS), requires continuous updating of screening methods when determining the suspected role of NPS in deaths. The open library concept of the Toxtyper system allows for rapid updates of methods linked to the appearance of new drugs. The following application note describes the development and validation of a sensitive screening method for the identification of the common prescription analgesics (e.g. oxycodone, methadone and buprenorphine) together with illicit substances such as fentanyl, beta-Hydroxythiofentanyl and other fentanyl analogs with detection limits in sub-ppb to low ppb range.

Keywords: Post-mortem specimens, Analgesics, Opiods, Drugs of Abuse Research, Toxicology Screening Research, Open library concept Toxtyper, amaZon speed, Ion Trap, DataAnalysis, LibraryEditor

Authors: Elisa N. Shoff · Miami-Dade Medical Examiner Department, Miami, FL, Zoltan Czentnar, Andrea Kiehne · BrukerDaltonik GmbH Bremen

Introduction

The Miami-Dade County Medical Examiner Department (MDME) Toxicology Laboratory observed an increase in the number of deaths involving heroin and fentanyl after new state laws regulating pain management clinics were enforced. At the same time, the use and abuse of oxycodone and related analgesics declined. Although heroin and fentanyl have been a constant presence in death investigation in South Florida, an increase in the number of cases was initially observed between 2010 and 2012 followed by a drastic increase between 2013 and 2016. Fentanyl, a pharmaceutical drug used in the medical community to treat acute pain, emerged in decedents illicitly either alone or in combination with heroin and other drugs.

Fentanyl is of great concern due to its high potency, typically 75-125 times more potent than morphine. Drug paraphernalia recovered at the terminal event and analyzed by the MDME laboratory indicated that fentanyl was mixed with heroin prior to use. This increased the lethality of the mixture substantially and deaths involving the combination became common. In many cases, the death was so sudden that the question arose as to whether the deceased knew they were ingesting fentanyl, as opposed to heroin.

The trend in heroin abuse elevated to a new high in 2015 with the introduction of fentanyl analogs. Miami-Dade County had already experienced the emergence of synthesized illegal drugs known as "bath salts" and "k2/spice". Many of these synthetic drugs appeared in cases in Miami-Dade County, and the lethality of these drugs were of less importance than their ability to affect behavior and judgment. The new trend in synthesized drugs expanded to include manufactured versions of fentanyl. These new fentanyl analogs began to appear in heroin cases in 2015 and a new trend evolved. Analysis of a number of cases in 2015 revealed two new fentanyl-like compounds, beta-Hydroxythiofentanyl (see figure 1) and 4-acetylfentanyl. In 2016, four new compounds emerged, para-fluorobutyryl fentanyl, para-fluoroisobutyryl fentanyl, furanyl fentanyl and carfentanil, rendering fentanyl itself almost obsolete.

Due to the increase in the number of cases involving heroin and fentanyl and the introduction of fentanyl analogs, the MDME developed a comprehensive and sensitive screening tool, using the Toxtyper LC-Ion Trap MS^n system, to identify common prescription analgesics (oxycodone, methadone and buprenorphine) and illicit substances (6-monoacetylmorphine, fentanyl and fentanyl analogs) in post-mortem specimens such as whole blood, urine, brain tissue and liver tissue.

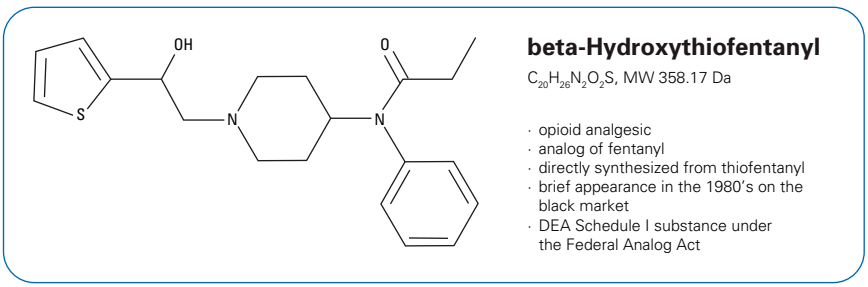

Fig. 1: Structure and general information about beta-Hydroxythiofentanyl.

Table 1: HPLC conditions used in the Toxtyper workflow.

LC settings	
LC system	UltiMate 3000 RSLC
Eluent A	H_2O, 0.1% formic acid, 2 mM ammonium formate, 1% acetonitrile
Eluent B	Acetonitrile, 0.1% formic acid, 2 mM ammonium formate, 1% H_2O
Column	Acclaim® RSLC 120 C18 2.2 µm, 120 A, 2.1 x 100 mm
Flow rate	500 µl/min
Gradient	0.0 to1.0 min: 1% B 1.0 to8.0 min: 1% B to 99% B, linear 8.0 to9.0 min: 99% B 9.0 to 9.1 min: 99% B to 1% B, linear 9.1 to 11 min: 1% B

Table 2: Toxtyper MS and MS^n parameters.

MS settings	
Source	Electrospray ionization (ESI)
Polarity	Zero Delay Alternating polarity (ZDA™)
MS Tuning	Smart Parameter Setting SPS™ using target mass 300 *m/z*
MS^n Acquisition	Data dependent using a Scheduled Precursor List with 42 compounds. Active Exclusion after 1 spectrum, reconsider if intensity increase by factor ≥ 5
ICC Target	200 000

Experimental

Sample Preparation

Whole blood samples were prepared using the following solid-phase extraction (SPE) method: United Chemical Technologies (UCT) Clean Screen® mixed mode extraction columns (200 mg, 10 mL) coupled with a UCT Positive Pressure Manifold were utilized for the SPE procedure. Aliquots of 1 mL whole blood were spiked with 50 µL of 1 mg/L D_5-fentanyl internal standard and diluted with 4 mL of 0.1 M phosphate buffer (pH 6). The samples were then vortexed and centrifuged for 10 minutes at 3500 rpm. The supernatant was submitted to the primed SPE columns for a dual-elution extraction procedure.

This extraction elutes acidic and neutral drugs first, in an elution solvent of hexane/ethyl acetate (50:50), then elutes basic drugs in an elution solvent of methylene chloride/isopropanol/ammonium hydroxide (78:20:2). All eluents are then evaporated at 40°C with N_2. The basic drug residue is reconstituted in 50 µL acetonitrile and the acidic/neutral drug residue is reconstituted in 50 µL Toxtyper eluent A/B (90:10). For the preparation of brain tissue, a homogenate is made by weighing out 5 g of brain and diluting the tissue with 5 g of water (1:1). The mixture is then homogenized in a blender and/or vortexed. 1 mL of this homogenate is aliquoted and submitted to the same extraction procedure described above.

For the preparation of liver tissue, a homogenate is made by weighing out 5 g of liver and diluting the tissue with 20 g of water (1:4). The mixture is then homogenized in a blender. 1 mL of this homogenate is aliquoted and submitted to the same extraction procedure described above.

LC-MS^n conditions

Five microliters of the sample were injected and separated using the LC conditions from table 1. All analytes eluted within a five minute window and were analyzed in alternating polarity mode using the Toxtyper system equipped with an electrospray ionization source. Full scan MS, MS^2 and MS^3 spectra were acquired from whole blood, brain and liver samples in data dependent MS/MS mode using a scheduled precursor list (SPL) including all compounds of interest. Additional MS method parameters are listed in table 2.

Data processing and reporting

All data was automatically processed using the embedded DataAnalysis (DA) software and customizable LibraryEditor. An automated method was utilized to extract MS and MS^n spectra, perform the library search and generate the final result report. For validation, all results (spectra, chromatographic peak intensity) were manually assessed in DA before being finalized.

Results

To create a more sensitive, streamlined screening method, versus the traditional comprehensive Toxtyper method of 900+ compounds, a specialized scheduled precursor list (SPL), including the retention times and *m/z* values of 42 compounds (cf. table 3) was developed. Out of these 42 compounds, 8 of them were added in-house to the Toxtyper custom library: beta-Hydroxythiofentanyl, acetyl-fentanyl, butyryl-fentanyl, para-fluorobutyryl fentanyl, para-fluoroisobutyryl fentanyl (FIBF), furanyl fentanyl, desproprionyl fentanyl (4-ANPP) and carfentanil. Using certified reference standards, 30 prescription analgesic and illicit opioids were fully validated in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) method validation guidelines. Additional non-validated compounds in the screening method were mainly metabolites and deuterated compounds.

The guidelines for method validation include determination of detection limits, stability of the compounds over time and multiple injections, selectivity of targeted compounds when combined with non-targeted compounds,

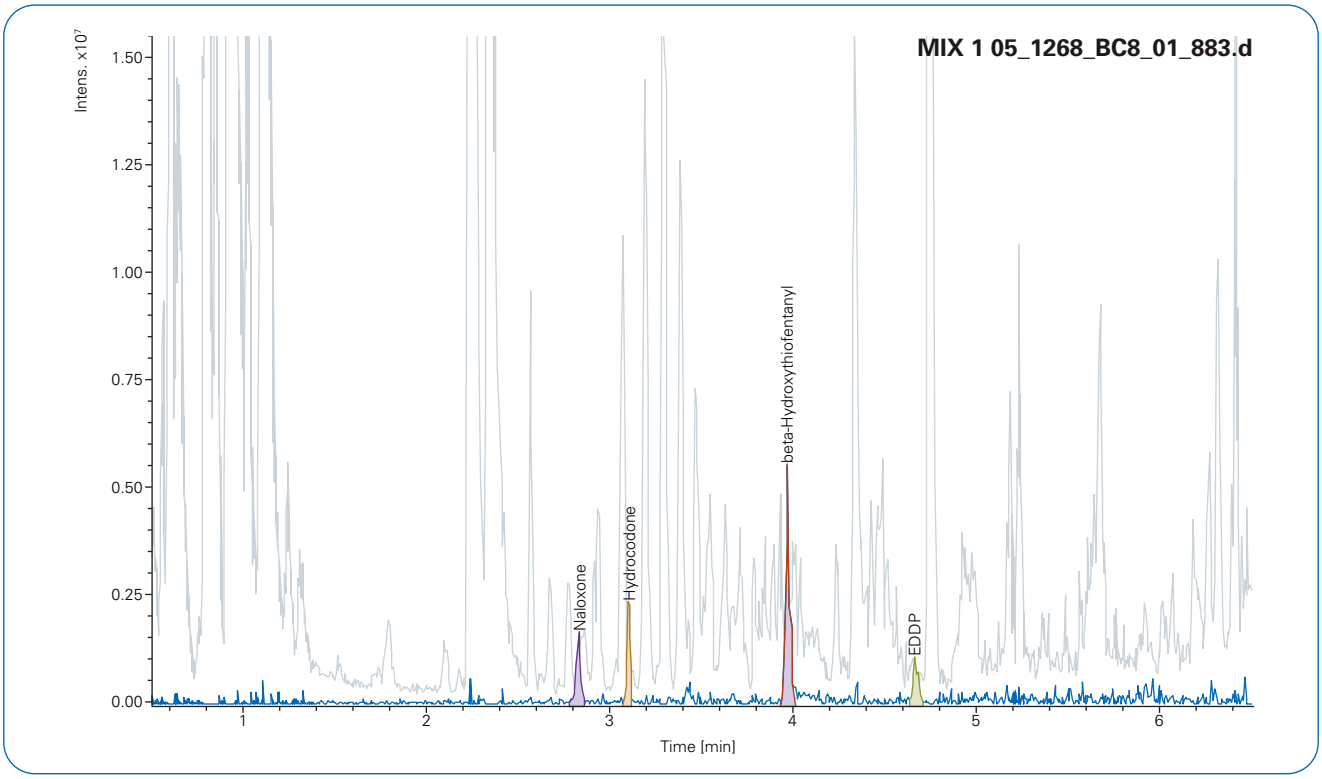

Fig. 2: Extracted ion chromatograms (EIC) of beta-Hydroxythiofentanyl, EDDP, hydrocodone and naloxone at their LOD of 0.5 ng/mL. MS Base Peak Chromatogram in grey.

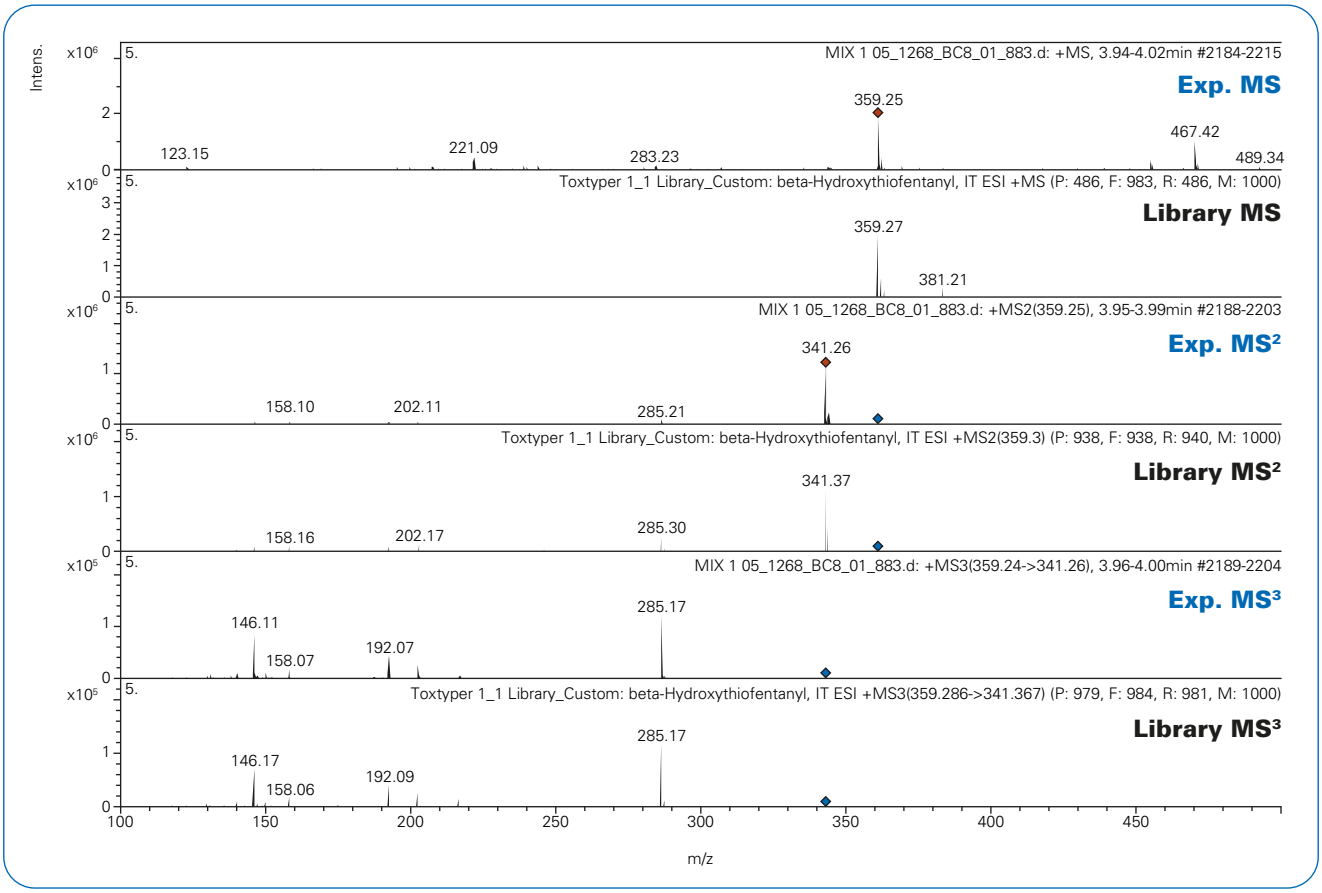

Fig. 3: Identification of beta-Hydroxythiofentanyl at a spiked concentration of 0.5 ng/mL. Comparison of experimental and library MS^n spectra.

Table 3: List of 42 compounds included in the screening method; the 30 validated compounds are in bold.

Compounds		
3-Methyl fentanyl	Dihydrocodeine	**Norbuprenorphine**
6-Monoacetylmorphine	**EDDP**	Norcodeine
Acetaminophen (Paracetamol)	**Fentanyl**	**Norfentanyl**
Acetyl fentanyl	**Furanyl fentanyl**	Normorphine
Acetylsalicylic acid	**Heroin**	Noroxycodone
Alfentanil	**Hydrocodone**	Noscapine
beta-Hydroxythiofentanyl	**Hydromorphone**	O-Desmethyltramadol
Buprenorphine	**Ibuprofen**	**Oxycodone**
Butyryl fentanyl	**Meperidine**	**Oxymorphone**
Carfentanil	**Methadone**	**Para-fluorobutyryl fentanyl**
Codeine	**Morphine**	**Para-fluoroisobutyryl fentanyl**
D4-Acetaminophen (D4-Paracetamol)	**Naloxone**	**Salicylic acid**
D5-Fentanyl	**Naltrexone**	Sufentanil
Desproprionyl fentanyl	**Naproxen**	**Tramadol**

carryover and matrix effects caused by decomposed whole blood and tissue samples. The validation results are presented in the following sections.

Limit of Detection

Limit of detection was determined by spiking various concentrations of compounds into whole blood and extracting. The 26 basic compounds that were validated had a limit of detection between 0.5 ng/mL and 5 ng/mL (table 4). At 0.5 ng/mL extracted, the signal intensity for some of the fentanyl analogs exceeded expectation. However, examining these analytes at a lower concentration has not yet been carried out. Figure 2 exemplarily shows the MS Base Peak Chromatogram (MS BPC) of the blood sample overlaid by the extracted ion chromatograms (EIC) of naloxone, hydrocodone, beta-Hydroxythiofentanyl and EDDP obtained at their LOD of 0.5 ng/mL.

The remaining four acidic compounds, salicylic acid, ibuprofen, acetaminophen and naproxen, had validated limits of detection at 200 ng/mL, 50 ng/mL, 500 ng/mL and 50 ng/mL, respectively.

The MS^n spectra of all validated compounds at the LOD level are still in excellent agreement with the library spectra. Figure 3 shows the acquired MS^n spectra of beta-Hydroxythiofentanyl (0.5 ng/mL) together with matching library spectra giving purity scores > 900 for MS^2 and MS^3. The parameters used to assess the limit of detection included reproducibility both intra-day and inter-day for all compounds, consistency between ion ratios, and a calculated purity score of > 800.

Stability

Numerous case samples, positive for targeted compounds were re-injected 24 and 48 hours after extraction. All compounds appeared to be stable over this time period, presumably due to the refrigerated autosampler on the UltiMate 3000 RSLC system as well as the PTFE/Silicone septa composition of the autosampler vial caps from Phenomenex®. As example corresponding extracted ion chromatograms of beta-Hydroxythiofentanyl at time points 0, 24 and 48 hours after extraction are shown in Fig. 4.

Selectivity

All compounds were spiked at their respective limits of detection, in combination with a mixture of 80 untargeted drugs, spiked at a concentration of 500 ng/mL. All compounds of interest were properly detected at their respective detection limits, except for fentanyl which had an interference with quetiapine. However, no false positives were found.

Carryover

Carryover was assessed by running neat mixes of all compounds at a concentration of 1000 ng/mL, except for fentanyl and fentanyl analogs which were assessed at 100 ng/mL. Each

mix was then bracketed by blank solvents containing acetonitrile. None of the 24 targeted compounds were present in the subsequent blank runs, eliminating the possibility of carryover in the LC system.

Matrix Effects

Three compromised samples that previously screened negative (one decomposed blood, one decomposed brain homogenate and one decomposed liver homogenate) were extracted to assess the potential for false positive results due to endogenous interferences. Because of the state of post-mortem matrices, they tend to be more difficult to analyze. It is important to make sure that samples such as these do not produce false results. All three samples were extracted, with internal standard, and analyzed. No false positives were detected for any of the 42 compounds in the method.

Table 4: Limits of detection of basic compounds in whole blood samples.

Analyte	0.5 ng/mL	1 ng/mL	5 ng/mL
6-Monoacetylmorphine	X		
Acetyl-fentanyl	**X**		
beta-Hydroxythiofentanyl	**X**		
Buprenorphine	**X**		
Butyryl fentanyl	**X**		
Carfentanil	**X**		
Codeine		X	
Desproprionyl fentanyl	**X**		
EDDP	**X**		
Fentanyl	**X**		
Furanyl fentanyl	**X**		
Heroin	**X**		
Hydrocodone	**X**		
Hydromorphone	**X**		
Meperidine	**X**		
Methadone	**X**		
Morphine			**X**
Naloxone	**X**		
Naltrexone	**X**		
Norbuprenorphine		**X**	
Norfentanyl	**X**		
Oxycodone	**X**		
Oxymorphone	**X**		
Para-fluorobutyryl fentanyl	**X**		
Para-fluoroisobutyryl fentanyl	**X**		
Tramadol			**X**

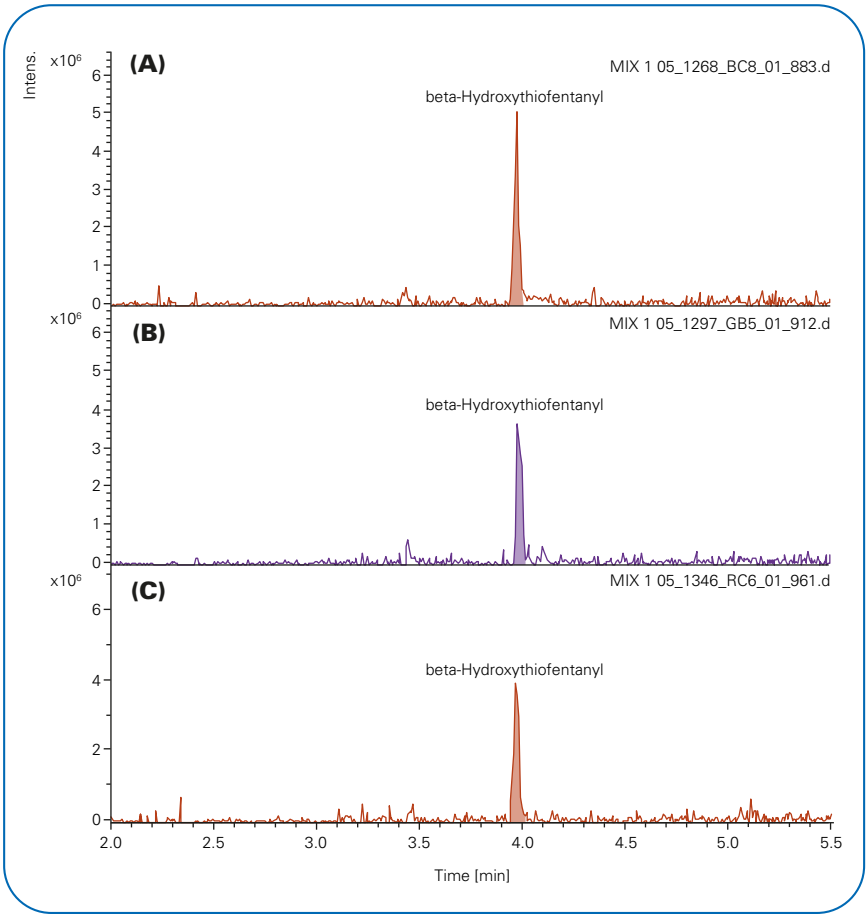

Fig. 4: 24 and 48 hour stability shown for beta-Hydroxythiofentanyl. Extracted ion chromatograms (EIC) directly after injection (A), after 24 hours (B) and after 48 hours (C).

Conclusion

In summation, a comprehensive, sensitive and customizable screening method for prescription analgesics and illicit opioids is presented. Due to the increase in opioid abuse in the United States, specifically South Florida, a method with robust spectral data (MS^2/MS^3), challenging detection limits, and the ability to scan for a wide range of compounds in limited time was needed.

By utilizing a targeted scheduled precursor list, focusing on one specific classification of drugs, proper sensitivity for real-life post-mortem case samples can be achieved. Twenty-two out of the thirty compounds validated for this method had a limit of detection of ≤ 0.5 ng/mL. For some of these compounds, this is well below concentration range of interest (oxycodone, hydromorphone) but for other compounds (buprenorphine, fentanyl and fentanyl analogs), 0.5 ng/mL concentration could be a contributing factor in the cause of death, depending on the circumstances of the case and the decedent in question. Therefore, it is suggested that this customized scheduled precursor list dependent MS^n method should be utilized for the detection of abused opioids and prescription analgesics, when analyzing whole blood, brain or liver tissue samples.

Learn More

You are looking for further Information? Check out the Link or scan the QR Code.

www.bruker.com/cannabinoids

For research use only. Not for use in diagnostic procedures.

Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com - www.bruker.com