Analysis of Low Level Opioids in Hair by UPLC/HRMS

1 Introduction

Opioids are a class of substances that include natural, semi-synthetic, and synthetic alkaloidal agents derived from opium or substances which have morphine-like activity. Naturally occurring opioids such as morphine and codeine are typically referred to as opiates. Heroin (diacetylmorphine) is a semi-synthetic opioid that is synthesized by the acetylation of morphine. In humans, heroin is rapidly metabolized to 6-monoacetylmorphine (6-AM) and morphine. Other common opioids include hydromorphone, hydrocodone, oxymorphone, oxycodone, methadone, meperidine, and tramadol. Buprenorphine and fentanyl are two potent synthetic opioids which are usually given at a much lower dose than other opioids. These compounds and their metabolites may be found in the hair of individuals who have been exposed to the drugs.

2 Scope

This procedure allows for the screening and confirmation of morphine, codeine, hydromorphone, hydrocodone, oxycodone, 6-AM, methadone, meperidine, tramadol, methadone, fentanyl, buprenorphine, and norburprenorphine at low levels in hair.

3 Principle

Hair samples are decontaminated with methylene chloride, water, and methanol washes before cryogrinding. The resulting hair powder is digested using a proteinase based solution. The resulting digest solution is cleaned up using solid phase extraction (SPE). Final extracts are analyzed by ultra performance liquid chromatography-high resolution mass spectrometry (UPLC/HRMS). Positive sample extracts are confirmed via UPLC/HRMS with tandem mass spectrometry.

4 Specimens

20 milligrams of hair is preferred for each analysis. Lower amounts of hair may be used with an increase in limit of detection

5 Equipment/Materials/Reagents

Specified items may be substituted with an equivalent material/product if necessary.

5.1 Hair Weighing, Decontamination, and Cryogrinding

Material / Equipment	Grade/Type	Supplier	Product/Part No.
Eppendorf vials, 2.0 mL, snap-cap	Polypropylene, curved/conical bottom	Fisher Scientific	05-408-138
Methylene chloride	HPLC/Optima	Varies	n/a
Water	Millipore/18mΩ	In-house	n/a
Methanol	HPLC/Optima	Varies	n/a
Laboratory balance	0.1 mg resolution	Varies	n/a
3.0 mm grinding balls	stainless steel	Retsch	22.445.0011
Cryogrinder	programmable	Retsch	MM200

5.2 Digestion

Material / Equipment	Grade/Type	Supplier	Product/Part No.
potassium phosphate, dibasic	ACS	Fisher	P-288-500
potassium phosphate, monobasic	ACS	Fisher	P-286-1
1.0 M potassium phosphate, dibasic	Weigh 87 g of potassium phosphate, dibasic to a 500 mL volumetric flask. Dilute to the mark with deionized water. Mix well and store refrigerated in glass. Stable for 3 months.		
1.0 M potassium phosphate, monobasic	Weigh 68 g of potassium phosphate, monobasic to a 500 mL volumetric flask. Dilute to the mark with deionized water. Mix well and store refrigerated in glass. Stable for 3 months.		
0.1 M potassium phosphate buffer, pH 8	Combine 94 mL of the 1.0 M potassium phosphate, dibasic solution and 6 mL of the 1.0 M potassium phosphate, monobasic solution. Dilute to 1 L with deionized water. Mix well and store refrigerated in glass. Stable for 3 months.		
Proteinase-K enzyme	Tritirachium album, ≥ 30 units/mg protein	Sigma-Aldrich	P-6556-100MG
Proteinase-K enzyme working solution, 40 mg/mL	Weigh 40 mg of proteinase-K into a tared Eppendorf snap-cap vial. Add 1mL of 0.1M potassium phosphate buffer, pH 8. Vortex and store refrigerated. Stable for 3 days. 1 mL of working solution is sufficient for 20 digests (0.050 mL per digest).		
Urea	Electrophoresis grade	Sigma-Aldrich	U-6504
Dithiothreitol (DTT)	≥ 98% (TLC)	Sigma-Aldrich	D0632
Calcium chloride, 1.0 M	Fluka/volumetric	Sigma-Aldrich	21114-1L

Digestion Solution	To a 15 mL conical tube, add 308 mg of DTT, 900 mg of urea and 0.050 mL of 1.0M calcium chloride. Dilute to 10 mL with 0.1M potassium phosphate buffer, pH 8. Vortex and store in heating block at 55°C until use. Stable for one day. Makes 10 mL of solution, which is sufficient for 20 digests (0.5 mL per digest).		
Thermomixer	programmable, capable of 55°C and 1000 rpm	Eppendorf	Model 5355, R
5% potassium phosphate, monobasic	Weigh 25 g potassium phosphate (monobasic) and dilute to 500 mL with deionized water. Mix well. Store refrigerated, stable for 3 months.		

5.3 Extraction

Material / Equipment	Grade/Type	Supplier	Product/Part No.
Positive pressure manifold	multi-sample, capable of 25 psi	SPEWare	289-0004
Concentrator	multiple sample, capable of 40°C	SPEWare	279-0050
SPE Column, Polychrom Clin II	SCX strong cation exchange, 35 mg, 3 mL capacity	SPEWare/Cerex	650-353
Potassium bicarbonate	ACS	Fisher	P9144-500
Potassium carbonate	ACS	Fisher	P208-500
Potassium carbonate buffer, pH 9	Dissolve 20 g of potassium bicarbonate and 10 g of potassium carbonate in 500 mL of deionized water. Adjust the pH to 9 and then dilute to 1 L. Store at ambient temperature. Stable for at least 3 months.		
Hydrochloric acid	ACS	Fisher	A144-500
0.1 M hydrochloric acid	Aliquot 800 µL of hydrochloric acid and dilute to 96 mL with deionized water. Stable for at least 3 months. Store in glass at room temperature.		
Ethyl acetate	ACS	Fisher	E195-1
Ammonium hydroxide	ACS	Fisher	A6695-500
Elution Solvent (98% ethyl acetate with 2% ammonia)	To 40 mL of ethyl acetate, add 0.8 mL of ammonium hydroxide. Mix well. Prepare fresh, just prior to elution.		
0.2µ centrifugal filtration vial, Spin-X	0.2µ, nylon	Fisher	CLS8169
Reconstitution solvent	95:5 water:acetonitrile	Combine 5 mL acetonitrile with 95 mL water (both Optima grade) and 0.1 mL	

	with 0.1% formic acid	formic acid and mix well. Store in glass at room temperature. Stable for 3 months.

5.4 Liquid Chromatograpy/Mass Spectrometry

Material / Equipment	Grade/Type	Supplier	Product/Part No.
UPLC Binary Pump System	Acquity-I class	Waters	
Mass Spectrometer	Hybrid quadrupole-Orbitrap	Thermo Scientific	Q-Exactive
LC Column	BEH UPLC C18: 2.1x100 mm, 1.7u	Waters	186002352
Acetonitrile	HPLC/Optima	Fisher	A996-4
Water	HPLC/Optima	Fisher	W7-4
Formic Acid	reagent	Sigma/Fluka	94318
Mobile Phase A, Aqueous / Weak Wash	In a 500 mL graduated cylinder, add 250 mL of Optima grade water. Add 0.5 mL of formic acid. Dilute to 500 mL and mix well. Stable for 5 days. To prevent microbial growth discard after 5 days.		
Mobile Phase B, Organic / Strong Wash	In a 500 mL volumetric cylinder, add 250 mL of Optima grade acetonitrile. Add 0.5 mL of formic acid. Dilute to 500 mL and mix well. Stable for at least 3 months.		
Seal Wash, Solvent A2	Methanol:Water 1:1. Combine equal volumes of each solvent. Store at ambient temperature. Stable for at least 6 months.		
Solvent B2	Acetonitrile		

5.5 Miscellaneous Laboratory Supplies

Material / Equipment	Grade/Type	Supplier	Product/Part No.
Pipettes	20µL to 1 mL range	varies	n/a
Heating block	n/a	Varies	n/a
Falcon 15mL conical tube	n/a	Fisher-Scientific	14-959-49D
Aluminum weighing dish	fluted sides, tab	Fisher-Scientific	08-732-100
Centrifuge	micro-vial size, 10,000 rpm capable	varies	n/a
Centrifuge	12x75 mm size, 1500 rpm capable	varies	n/a

Glass tubes, 12 x 75 mm	n/a	Fisher Scientific	14-961-26
Autosampler vials with inserts	9 mm with insert	Wheaton	09-1200-101
Autosampler caps	9 mm, Blue S/T	Wheaton	09-0034B

6 Standards and Controls

6.1 Internal Standards (Cerilliant, or other approved vendor. Stability determined by manufacturer)

Analyte	Concentration (mg/mL)	Solvent	Product No.	Aliquot for Stock (mL)
d_3-Morphine	0.1	methanol	M-003	0.250
d_6-Codeine	0.1	methanol	C-040	0.250
d_3-Oxymorphone	0.1	methanol	O-003	0.250
d_6-Oxycodone	0.1	methanol	O-005	0.250
d_3-Hydromorphone	0.1	methanol	H-006	0.250
d_3-Hydrocodone	0.1	methanol	H-005	0.250
d_3-Tramadol-13C	0.1	methanol	T-029	0.250
d_3-Methadone	0.1	methanol	M-008	0.250
d_4-Meperidine	0.1	methanol	M-036	0.250
d_4-Buprenorphine	0.1	methanol	B-901	0.025
d_3-Norbuprenorphine	0.1	methanol	N-920	0.025
d_5-Fentanyl	0.1	methanol	F-001	0.025
d_3-6-AM	0.1	acetonitrile	A-010	0.250

6.1.1 Stock Internal Standard Solution (methanol, 0.005/0.0005 mg/mL)

Aliquot the standards as indicated in section 6.1 (except d_3-6-AM) into a 5 mL volumetric flask. Dilute to the mark with Optima grade methanol. Store below 0°C in glass. Stable for at least one year.

6.1.2 Stock Internal Standard Solution, d_3-6-AM (acetonitrile, 0.005 mg/mL)

Aliquot the d_3-6-AM standard as indicated in section 6.1 into a 5 mL volumetric flask. Dilute to the mark with Optima Grade acetonitrile. Store below 0°C in glass. Stable for at least one year.

6.1.3 Working Internal Standard Solution (aqueous, 0.05/0.005 µg/mL)

Aliquot 0.050 mL of both of the stock internal standard solutions to a partially filled 5

mL volumetric flask. Bring to the mark with HPLC grade water. Store refrigerated in glass. Stable for approximately one week.

6.2 Controls (Cerilliant, Lipomed or other approved vendor. Stability determined by manufacturer)

Analyte	Concentration (mg/mL)	Solvent	Product No.	Aliquot for Stock (mL)
Morphine	1	methanol	M-030	0.250
Codeine	1	methanol	C-006	0.250
Oxymorphone	1	methanol	O-004	0.250
Oxycodone	1	methanol	O-002	0.250
Hydromorphone	1	methanol	H-004	0.250
Hydrocodone	1	methanol	H-003	0.250
Tramadol	1	methanol	T-027	0.250
Methadone	1	methanol	M-019	0.250
Meperidine	1	methanol	M-035	0.250
Buprenorphine	1	methanol	B-902	0.025
Norbuprenorphine	1	methanol	N-912	0.025
Fentanyl	1	methanol	F-002	0.025
6-AM	1	acetonitrile	A-009	0.250

6.2.1 Stock Control Solution (methanol, 0.01/0.001 mg/mL)

Aliquot the standards as indicated in section 6.2 (except 6-AM) into a 25 mL volumetric flask. Dilute to the mark with Optima grade methanol. Store below 0°C in glass. Stable for at least one year.

6.2.2 Stock Control Solution, 6-AM (acetonitrile, 0.01 mg/mL)

Aliquot the 6-AM standard as indicated in section 6.1 into a 5 mL volumetric flask. Dilute to the mark with Optima grade acetonitrile. Store below 0°C in glass. Stable for at least one year.

6.2.3 Working Control Solution (high, aqueous, 80/8 ng/mL)

Aliquot 0.080 mL of both of the stock standard solutions to a partially filled 10 mL volumetric flask. Dilute to the mark with HPLC grade water. Store refrigerated in glass. Stable for approximately one week.

6.2.4 Working Control Solution (low, aqueous, 4/0.4 ng/mL)

Aliquot 0.50 mL of the Working Standard Solution (High) to a partially filled 10 mL

volumetric flask. Bring to the mark with HPLC grade water. Store refrigerated in glass. Stable for approximately one week.

6.3 Column Performance Evaluation Mix (2/0.2 ng/mL):

Aliquot 0.05 mL of the Working Standard Solution (low, aqueous) to an autosampler vial. Aliquot 0.05 mL of reconstitution solvent and mix. Stable for approximately one week.

6.4 Negative Control Hair: Obtained from drug-free donors. Stored in paper or plastic at room temperature. Negative Control Hair does not expire.

6.5 Negative Control Hair Powder: Obtained by washing Negative Control hair with methylene chloride, water, and methanol; drying the hair, and pulverizing in a cryogrinder. Negative Control Hair Powder is stored in plastic at room temperature and does not expire.

6.6 Low Positive Control Hair Sample (5/0.5 pg/mg): Prepared by spiking 20 mg Negative Control Hair Powder with 0.025 mL of the Working Standard Control Solution (Low, Aqueous).

6.7 High Positive Control Hair Sample (100/10 pg/mg): Prepared by spiking 20 mg Negative Control Hair Powder with 0.025 mL of the Working Standard Control Solution (High, Aqueous).

7 Sampling

Not applicable.

8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

8.1 Hair Weighing, Decontamination, and Cryogrinding:

a. Visually inspect hair and record observations.

b. If segmental analysis is required, cut a portion of the hair sample into 2-cm segments.

c. Weigh approximately 20 mg of each hair sample (or segment) into a properly labeled 2.0 mL Eppendorf tube. Record weight to the nearest 0.1 mg.

d. Wash each hair sample with 1.0 mL methylene chloride by vortexing for approximately 1 minute. Discard this wash.

e. Wash each hair sample with 1.0 mL water (Millipore) by vortexing the sample for approximately 1 minute. Discard this wash.

f. Wash each hair sample with 1.0 mL HPLC Grade methanol by vortexing for approximately 1 minute. This wash may be capped and stored refrigerated for later analysis, if necessary.

g. Dry hair samples in a heating block or sample concentrator at approximately 40°C to evaporate any remaining solvent. Dry for 30 minutes or until samples are dry.

h. Cryogrind dry hair samples using the settings in Section 9.5 of this procedure. Tap tubes to loosen any cryoground hair from the cap before proceeding.

i. Similarly, prepare negative and positive control samples as directed in Section 6.

j. Open tubes, and add 0.5 mL Digestion Solution, 0.050 mL Proteinase-K Working Solution, and 0.020 mL Internal Standard Working Solution to each Eppendorf tube. Recap and vortex briefly.

k. Thermomix for 60 minutes at 55ºC and 750 rpm (may be extended if necessary).

l. Add 1 mL of 5% potassium phosphate monobasic, continue thermomixing at 55ºC and 750 rpm for five minutes.

m. Centrifuge samples at ~10,000 rpm for 5 minutes, decant supernatant to 12 x 75 mm glass tube.

n. Add an additional 1 mL of 5% potassium phosphate, monobasic.

8.2 Solid Phase Extraction:

a. Condition SPE extraction cartridges: 3 mL of methanol, followed by 1 mL of deionized water at 1-2 mL/minute.

b. Load sample onto cartridge at 1-2 mL/minute.

c. Wash cartridge with 1 mL of potassium carbonate buffer, 1 mL of water, 1 mL of 0.1 M hydrochloric acid, and 1 mL of methanol (each at 1-2 mL/minute).

d. Dry cartridge at 25 psi for 5 minutes.

e. Apply 2 mL of Elution Solvent at 1-2 mL/minute. Collect eluent in 12 x 75 mm test tubes.

f. Evaporate to dryness under nitrogen at 40ºC.

g. Reconstitute the dry residue in 75 µL of Reconstitution Solvent. Centrifuge the 12 x 75 mm tubes for 1 min at 1500 rpm to consolidate the solvent.

h. Transfer the solvent to a 0.2µ filtration vial. Centrifuge at 10,000 rpm for 5 minutes.

i. Transfer the eluent to an autosampler vial with insert.

j. Analyze 10 µL portions by UPLC/HRMS with the conditions given in section 9. Analysis may include full scan, SIM, or tandem mass spectrometry modes depending upon the case scenario. Positive case samples may be reinjected (with appropriate controls) by tandem UPLC/HRMS for confirmatory analysis.

8.3 Analysis of wash samples (optional):

a. For samples in which an opiate is identified above the LOD of the method, add 20 µL Internal Standard Solution to each wash.

b. Evaporate to dryness under a gentle stream of nitrogen at approximately 40ºC.

c. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.

d. Extract using the procedure in Section 8.2 above. Analyze 10 µL portions by LC/HRMS with the conditions given in section 9.

9 Instrumental Conditions

Appendix 2 contains an abbreviated version of the instrumental conditions in this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

9.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
A: 0.1% formic acid in water	total flow	0.40 mL/min		type	C18
	time (min)	% A	% B	length	100 mm
B: 0.1% formic acid in acetonitrile	0	98	2	internal diameter	2.1 mm
	0.5	98	2	particle size	1.7 µm
	5	50	50	temperature	50°C
	5.5	10	90	Autosampler Temp: 10°C Seal Wash: 5.00 min ACQ-SM: Weak Wash Vol: 600 µL Strong Wash Vol: 200 µL	
	7.5	10	90		
	7.75	98	2		
	9.75	98	2		
	total time	9.75 min			

9.2 Mass Spectrometer Parameters – Full Scan and SIM

Parameter	Full Scan	tSIM	Parameter	Value
Runtime	0 to 9.75 min	0 to 9.75 min	Mode	ESI
Polarity	positive	positive	Spray Voltage	+3.5 kV
In-Source CID	0.0 eV	0.0 eV	Capillary Temperature	375°C
Inclusion	-	on	Heater Temperature	350°C
Microscans	1	1	Sheath Gas	35
Resolution	35,000	35,000	Aux Gas	1
AGC Target	1e6	2e4	Sweep Gas	0
Maximum IT	50 ms	100 ms	S- Lens RF Level	70
MSX Count	-	1	For Inclusion List Values see Section 9.4	
Isolation Window	-	1.5 m/z		
Scan Ranges	1	-		
Scan Range	100-650 m/z	100-650 m/z		

9.3 Mass Spectrometer Parameters – Full Scan and Tandem MS

Parameter	Full Scan	tMS2	Parameter	Value
Runtime	0 to 9.75 min	0 to 9.75 min	Mode	ESI
Polarity	positive	positive	Spray Voltage	+3.5 kV
In-Source CID	0.0 eV	0.0 eV	Capillary Temperature	375°C
Inclusion	-	on	Heater Temperature	350°C
Microscans	1	1	Sheath Gas	35

Resolution	17,500	17,500	Aux Gas	1
AGC Target	1e6	2e4	Sweep Gas	0
Maximum IT	50 ms	100 ms	S- Lens RF Level	70
MSX Count	-	1	For Inclusion List Values see Section 9.4	
Isolation Window	-	1.5 m/z		
Scan Ranges	1	-		
Scan Range	100-650 m/z	100-650 m/z		

9.4 Mass Spectrometer Parameters – Inclusion List for tSIM and tMS2

Mass (m/z)	Polarity	Start (min)	End (min)	NCE (%)	CS (z)	Name
286.14445	Positive	1.40	1.62	55	1	morphine
302.13935	Positive	1.60	1.74	50	1	oxymorphone
286.14445	Positive	1.70	1.88	55	1	hydromorphone
300.16007	Positive	2.11	2.28	55	1	codeine
316.15500	Positive	2.28	2.45	42	1	oxycodone
328.15500	Positive	2.30	2.50	55	1	6-AM
300.16007	Positive	2.40	2.59	55	1	hydrocodone
264.19648	Positive	3.05	3.24	10	1	tramadol
248.16517	Positive	3.30	3.45	45	1	meperidine
414.26456	Positive	3.50	3.65	62	1	norbuprenorphine
337.22809	Positive	3.88	4.02	35	1	fentanyl
256.17025	Positive	4.04	4.12	70	1	diphenhydramine*
468.31150	Positive	4.16	4.30	62	1	buprenorphine
310.21720	Positive	4.68	4.78	30	1	methadone

**inclusion optional*

9.5 Cryomill Parameters

Cycles	1
Precool	Auto
Run time	6.5 min
Rate	25 hz

10 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control.

10.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

10.1.1 Retention Time

The retention time of the peak should be within ± 2% of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard or Positive Control.

10.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

10.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of a reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

11 Calculations

Not applicable.

12 Measurement Uncertainty

Not applicable.

13 Limitations

a. Limit of Detection (LOD):

The LOD is administratively set at 10 pg/mg for all drugs except fentanyl, buprenorphine, and norbuprenorphine, which each have an LOD of 1 pg/mg. Lower amounts may be

reported if the decision criteria in Section 10 are met.

b. Processed Sample Stability:

All analytes are stable in processed sample extracts for at least four days except for buprenorphine and norbuprenorphine. After three days norbuprenorphine partially converts to buprenorphine.

c. Selectivity:

A low level interferent for tramadol is observed in some samples which coelutes and has a similar parent mass (264.195). In order to establish tramadol as detected in a sample, there must also be a) tandem mass spectrometry that passes Tox 104 criteria, b) have a detectable isotope trace (265.199) with a 17 ± 2 % ratio, and c) have an estimated concentration of greater than 1 pg/mg. No other interferences were observed.

d. Diphenhydramine and other commonly detected drugs:

Diphenhydramine and other common over-the-counter/prescription drugs may be detected by this method. In order to report such a drug as detected in a case sample, that drug must not be detected in the Negative Control Hair used for the batch.

14 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

15 References

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

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(1993), pp. 121-135.

Dioumaeva, I., “LC/MS/MS of Buprenorphine and Norbuprenorphine in Whole Blood Using Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 Column”, Application Note, 2013.

FBI Laboratory Safety Manual.

Instrument Subunit SOP Manual; FBI Laboratory Chemistry Unit.

Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Rev. #	Issue Date	History
0	12/19/14	New document.

Approval

Redacted - Signatures on File

Appendix 1: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS Materials Preparation and Procedure for bench use (page 1)

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Appendix 1: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS Materials Preparation and Procedure for bench use, continued (page 2)

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Appendix 1: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS Materials Preparation and Procedure for bench use, continued (page 3)

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Appendix 2: Abbreviated version of the Low Level Opioids in Hair by UPLC/HRMS Instrumental Parameters

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