Analysis of Plant Toxins

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1 Introduction

Plants contain a variety of chemicals and compounds, many of which can be toxic. Examples include alkaloids such as gelsemine (*Gelsemium*) and glycosides such as digoxin/digitoxin (*Digitalis*), oleandrin (*Nerium*), and cerberin (*Cerbera*).

2 Scope

Analyses	☒ Screening ☒ Confirmation ☐ Quantitation
Matrices	Whole blood
Analytes	Digoxin, digitoxin, cerberin, oleandrin, gelsemine
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

3 Principle

Specimens are diluted and adjusted to basic pH through a combination of aqueous buffers and organic solvent. The resulting solution is mixed and centrifuged. The supernatant is applied to a supported liquid extraction (SLE) column. Organic solvents are used to elute the analytes from the column. The eluent is concentrated, reconstituted and filtered. The prepared extract is analyzed by UPLC-HRMS/MS (ultra- performance liquid chromatography-high resolution tandem mass spectrometry). Three acquisition modes are utilized: full scan (FS; 35,000 resolution), selected ion monitoring (SIM; 35,000 resolution), and tandem mass spectrometry (MS^2; 17,500 resolution).

4 Specimen Criteria

Whole blood (0.2 mL per extraction)

5 Equipment

5.1 Equipment

Pipettors with disposable tips, various ranges
Vortexer
Centrifuge, 10,000 rpm capable
Positive Pressure Manifold
Evaporator/Concentrator
UPLC Column: Waters Acquity UPLC HSS C18 1.8 µm, 2.1 x 100 mm

5.2 Consumables

Item	Supplier*	Description	Part Number*
Eppendorf Tubes	Eppendorf	Safe-Lock Tubes 2.0mL (polypropylene)	0030 120.094
SLE Cartridge	Biotage	Isolute SLE+, 400 µL sample volume	820-0055-B-500
Glass Tube	Fisher	Disposable Culture Tube 12x75 mm	14-961-26
Centrifugal Filter	Corning	Costar Spin-X HPLC 0.2 µm with nylon filter	8169
ALS Vials	Waters	Screw Top Vial, 12x32 mm, PTFE/Silicone pre-slit cap (with 250 µL insert)	186000307C

*use of an equivalent product is allowable

5.3 Instruments

Thermo Fisher Q-Exactive with Waters Acquity I-Class UPLC System

5.4 Software

Component	Software / Version	Version
Operating System	Microsoft Windows	7 Pro SP 1
Mass Spectrometer	Foundation	3.1
	Xcalibur	3.1
	Q-Exactive Orbitrap MS	2.8 SP1
	Waters Acquity	3.0.0
Chromatography	Acquity Instrument Driver	1.51.3347
	Binary Solvent Manager	1.50.1521
	Column Manager	1.50.1678
	Sample Manager	1.50.2736

5.5 Chemicals/Reagents

5.5.1 Purchased

Item	Supplier*	Description	Part Number*
Water	Fisher	Optima, LC/MS grade (mobile phase and Reconstitution Solvent)	W6-4
Water	In-house	18 mΩ, distilled	n/a
Methanol	Thermo Scientific	UPLC-MS grade (mobile phase preparation)	A458

Methanol	Fisher	Optima LC-MS grade (sample preparation and solvents)	A454-4
Acetonitrile	Fisher	Optima LC-MS grade	A955-5
Isopropanol	Fisher	Optima grade	A451
Ammonium formate	Fisher	Optima LC-MS grade	A115
Dichloromethane	Fisher	Optima grade	D151-1
MTBE (Elution Solvent 2)	Sigma-Aldrich	Chromasolv, 99.9%	20257
Sodium phosphate, monobasic, monohydrate	Fisher	Certified ACS	S369
Sodium phosphate, dibasic, heptahydrate	Fisher	Certified ACS	S373
Ammonium hydroxide	Fisher	ACS Plus	A669S
*use of an equivalent product is allowable			

5.5.2 *Prepared*

Depending upon the batch size, the absolute amounts may be adjusted so long as the ratios of components are maintained.

5.5.2.1 *Sample Buffer (0.1 M sodium phosphate buffer, pH 6.8)*

Step	Action	Amount	Component/Information
1	Acquire	1	Volumetric flask, 50 mL
2	Add	40 mL	Deionized water
3	Add	656 mg	sodium phosphate dibasic heptahydrate
4	Add	352 mg	sodium phosphate monobasic monohydrate
5	QS	50 mL	Deionized water
6	Mix		
7	Transfer		Amber glass
8	Storage		refrigerated
9	Stability		1 month
10	Prepares	50 mL	(500 samples)

5.5.2.2 *pH Modifier (2% ammonium hydroxide)*

Step	Action	Amount	Component/Information
1	Acquire	1	Eppendorf Tube, 2 mL polypropylene
2	Add	2.0 mL	Deionized water
3	Add	41 µL	Ammonium hydroxide
4	Mix		
5	Storage		In Tube
6	Stability		1 day
7	Prepares	2 mL	(40 samples)

5.5.2.3 *Elution Solvent 1 (95:5 dichloromethane:isopropanol)*

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 100 mL
2	Add	57 mL	dichloromethane
3	Add	3 mL	isopropanol
4	Mix		
5	Transfer		Amber glass
6	Storage		ambient
7	Stability		1 year
8	Prepares	60 mL	(40 samples)

5.5.2.4 *Reconstitution Solvent, Solvent A2 (50:50 methanol:water)*

Step	Action	Amount	Component/Information
1	Acquire	1	Graduated cylinder, glass, 25 mL
2	Add	12.5 mL	water (Optima LC-MS)
3	Add	12.5 mL	methanol (UPLC-MS grade)
4	Mix		
5	Transfer		Glass
6	Storage		ambient or refrigerated or frozen
7	Stability		6 months
8	Prepares	25 mL	(250 samples)

5.5.2.5 *Solvent A1 (5mM ammonium formate in water)*

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	250 mL	water (Optima LC-MS)
3	Add	79 mg	ammonium formate (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Storage		ambient or refrigerated
7	Stability		10 days
8	Prepares	250 mL	

5.5.2.6 *Weak Needle Wash (WNW) (10:90 methanol:water)*

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	225 mL	water (Optima LC-MS)
3	Add	25 mL	methanol (Optima LC-MS)
4	Mix		

5	Transfer		mobile phase bottle, glass
6	Storage		ambient
7	Stability		3 months
8	Prepares	250 mL	

5.5.2.7 Strong Needle Wash (SNW) (45:40:10:5 Methanol:Acetonitrile:Water:Isopropanol)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 500 mL
2	Add	225 mL	methanol (Optima LC-MS)
3	Add	200 mL	acetonitrile (Optima LC-MS)
4	Add	50 mL	water (Optima LC-MS)
5	Add	25 mL	isopropanol (Optima)
6	Mix		
7	Transfer		mobile phase bottle, glass
8	Storage		ambient
9	Stability		6 months
10	Prepares	500 mL	

5.5.2.8 Seal Wash (SW) (10:90 acetonitrile:water)

Step	Action	Amount	Component/Information
1	Acquire	1	graduated cylinder, glass, 250 mL
2	Add	225 mL	water (Optima LC-MS)
3	Add	25 mL	acetonitrile (Optima LC-MS)
4	Mix		
5	Transfer		mobile phase bottle, glass
6	Storage		ambient
7	Stability		3 months
8	Prepares	250 mL	

5.6 Standards/Controls

5.6.1 Purchased

Item	Supplier*	Description	Part Number*
Negative Control Matrix	Cliniqa	Blood	n/a
*use of an equivalent product is allowable			

5.6.2 Prepared

5.6.2.1 Primary Standards

Analyte	Supplier*	Description	Part Number*

Cerberin	Santa Cruz Biotechnology	1 mg powder	SC-480467
Digoxin	Cerilliant	1.0 mg/mL in methanol	D-029
Digitoxin	Cerilliant	1.0 mg/mL in methanol	D-067
Oleandrin	Phytolab	10 mg powder	89744
Gelsemine	Phytolab	10 mg powder	80457
Digoxin-d3	Cayman Chemicals	1 mg powder	10010657
*Use of an equivalent product is allowable. Store at about -20°C. Stability determined by manufacturer.			

5.6.2.2 Primary Standards in Methanol from Solid

For the standards in 5.6.2.1 that are in solid form, perform a dilution to yield a 1.0 mg/mL solution in methanol. For example, remove 1.0 mg of the oleandrin primary standard and add 1.0 mL of methanol. Store at about -20°C in amber glass.

5.6.2.3 Intermediate Standards (10 µg/mL in methanol)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	2.5 mL	methanol (Optima LC-MS)
3	Add	50 µL	of each 1.0 mg/mL primary standard (excluding digoxin-d3)*
4	QS	5 mL	methanol (Optima LC-MS)
5	Mix		
6	Transfer		amber glass
7	Storage		about -20°C
8	Stability		2 years

Intermediate Internal Standard (10 µg/mL in methanol)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	2.5 mL	methanol (Optima LC-MS)
3	Add	1.0 g	of digoxin-d3
4	QS	5 mL	methanol (Optima LC-MS)
5	Mix		
6	Transfer		amber glass
7	Storage		about -20°C
8	Stability		2 years

5.6.2.4 Working Standard (0.25 µg/mL in methanol)

Step	Action	Amount	Component/Information
1	Acquire	1	volumetric flask, glass, 5 mL

2	Add	2.5 mL	methanol (Optima LC-MS)
3	Add	125 µL	of Intermediate Standard
4	QS	5 mL	methanol (Optima LC-MS)
5	Mix		
6	Transfer		Amber glass
7	Storage		about -20°C
8	Stability		2 years

5.6.2.5 Controls (0, 1 and 10 ng/mL in matrix)

Prepare controls according to the table below. After preparation of the 5 mL of solution, mix each control solution for 30 minutes prior to pipetting into Eppendorf centrifuge tubes (0.2 mL portions each). Store at about -20°C. Stable for two years.

Control	**Working Standard (5.6.2.5)**	**Addition Volume**	**Matrix Volume**	**Concentration**
Level	µg/mL	µL	mL	ng/mL
Negative	0.25	0	5	0
1 ng/mL	0.25	20	5	1
10 ng/mL	0.25	200	5	10

5.6.2.6 Internal Standard Solution (80 ng/mL in methanol)(ISS)

Aliquot 40 µL of the digoxin-d3 10 µg/mL solution to a 5 mL glass volumetric flask. QS with methanol (Optima LC-MS). Store at about -20°C in amber glass. Stable for two years.

5.6.2.7 System Suitability Sample (S^3)(10 ng/mL)

Prepare the S^3 portions according to the table below.

Step	**Action**	**Amount**	**Component/Information**
1	Acquire	1	volumetric flask, glass, 5 mL
2	Add	1.7 mL	methanol (Optima LC-MS)
3	Add	200 µL	of Working Standard
4	Add	625 µL	of ISS
5	QS	5 mL	water (Optima LC-MS)
6	Mix		
7	Transfer		Eppendorf vials in 0.2 mL portions
8	Storage		about -20°C along with controls
9	Stability		2 years

6 PROCEDURE

Step		Activity	Note	Reference/Lot
6.1	☐	Materials required per sample: 2 mL Eppendorf tube (1), SLE+ 400 µL cartridge (1), 12 x 75 mm glass tube (1), 0.2 µm centrifugal filter (1), ALS vial (1)		
6.2	☐	Thaw a control set (maintained at -20°C). (0, 1 and 10 ng/mL Controls, 200 µL each; System Suitability Sample (S^3), 10 ng/mL)	Control Lots, S^3	
6.3	☐	Aliquot 200 µL of each case specimen into a 2 mL Eppendorf tube.		
6.4	☐	Add 100 µL of Sample Buffer to each tube. (0.1M sodium phosphate, pH 6.8)	Sample Buffer	
6.5	☐	Add 50 µL of Internal Standard Solution (ISS)	ISS	
6.6	☐	Add 50 µL of pH Modifier. Cap vial. (scan NH_4OH)	pH Modifier	
6.7	☐	Vortex at 2000 rpm for 5 minutes at ambient temperature.		
6.8	☐	Centrifuge at 10,000 rpm for 5 minutes at ambient temperature.		
6.9	☐	Load Biotage SLE+ 400 µL cartridges onto positive pressure manifold. Place 12 x 75 mm tubes beneath.	Biotage SLE+ 400 µL	
6.10	☐	Apply 300 µL of supernatant to SLE+ cartridge		
6.11	☐	Apply a short pulse of maximum nitrogen pressure to load sample onto cartridge. Wait 5 minutes.		
6.12	☐	Apply 750 µL of Elution Solvent 1 to each cartridge (95:5 dichloromethane:isopropanol). Wait 5 minutes.	Elution Solvent 1	
6.13	☐	Apply 750 µL of Elution Solvent 1 to each cartridge. Wait 5 minutes. Apply low nitrogen flow for ~ 30 seconds to elute Elution Solvent 1.		
6.14	☐	Apply 750 µL of Elution Solvent 2 to each cartridge (MTBE). Wait 5 minutes.	Elution Solvent 2	
6.15	☐	Apply 750 µL of Elution Solvent 2 to each cartridge. Wait 5 minutes. Apply low nitrogen flow for ~ 30 seconds to elute Elution Solvent 2.		

6.16	☐	Evaporate eluent to dryness at 45°C. Let cool for 5 min.		
6.17	☐	Reconstitute with 100 µL of Reconstitution Solvent to the bottom of the 12 x 75 mm tube. Vortex well.	Reconstitution Solvent	
6.18	☐	Transfer 100 µL extract to 0.2 µm centrifugal filter. Centrifuge at 10,000 rpm for 5 minutes.	Costar 0.2 µ filter	
6.19	☐	Transfer extract to Waters ALS vial with 250 µL insert. Cap with Waters pre-slit 12 x 32 mm vial cap.		
6.20	☐	Analyze 20 µL of extract		

LC MATERIALS

Component	Description	Reference/Lot
Solvent A1	5mM ammonium formate in water	
Solvent B1	Methanol	
Solvent A2	Methanol:Water 50:50	
Solvent B2	Acetonitrile	
Weak Needle Wash (WNW)	Methanol:Water 10:90	
Strong Needle Wash (SNW)	Methanol:Acetonitrile:Water:Isopropanol 45:40:10:5	
Seal Wash (SW)	Acetonitrile:Water 10:90	
UPLC Column	Waters Acquity UPLC HSS C18 1.8 µm, 2.1 x 100 mm	

7 ANALYTICAL PARAMETERS

7.1 Mass Spectrometry

7.1.1 Heated Electrospray Ionization, Global Settings and Tune File

HESI source

Sheath gas flow rate	50
Aux gas flow rate	10
Sweep gas flow rate	0
Spray voltage (|kV|)	3.50
Spray current (µA)	
Capillary temp. (°C)	275
S-lens RF level	70.0
Aux gas heater temp (°C)	300

Global Settings	
User Role	Advanced
Use lock masses	off
Lock mass injection	—
Chrom. peak width (FWHM)	6 s
Time	
Method duration	5.00 min
Customized Tolerances (+/-)	
Lock Masses	—
Inclusion	—
Exclusion	—
Neutral Loss	—
Mass Tags	—
Dynamic Exclusion	—

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0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

7.1.2 Inclusion List

Method editor — Inclusion List

File Edit Help Done

	Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	(N)CE	MSX ID	Comment
1	323.17540	C20H22N2O2	+ H	1	Positive	1.40	2.00	60		gelsemine
2	798.46343	C41H64O14	+ NH4	1	Positive	2.90	3.10	10		digoxin
3	577.33711	C32H48O9	+ H	1	Positive	3.32	3.62	10		cerberin and oleandrin
4	782.46852	C41H64O13	+ NH4	1	Positive	3.58	3.90	10		digitoxin
* 5										

The start/stop times listed are nominal. Due to normal column aging and variation in mobile phase preparation, small adjustments to the start and stop times may be required based upon the system suitability sample results.

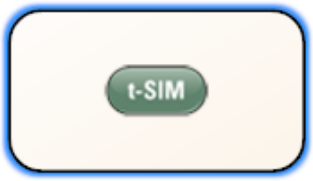
7.1.3 Scan Events

Properties of Full MS — SIM

General	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Full MS — SIM	
Microscans	1
Resolution	35,000
AGC target	5e5
Maximum IT	50 ms
Number of scan ranges	1
Scan range	300 to 840 m/z
Spectrum data type	Profile

Properties of Targeted-SIM

General	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Inclusion	on
SIM	
Microscans	1
Resolution	35,000
AGC target	2e5
Maximum IT	200 ms
MSX count	1
Isolation window	1.0 m/z
Isolation offset	0.0 m/z
Spectrum data type	Profile

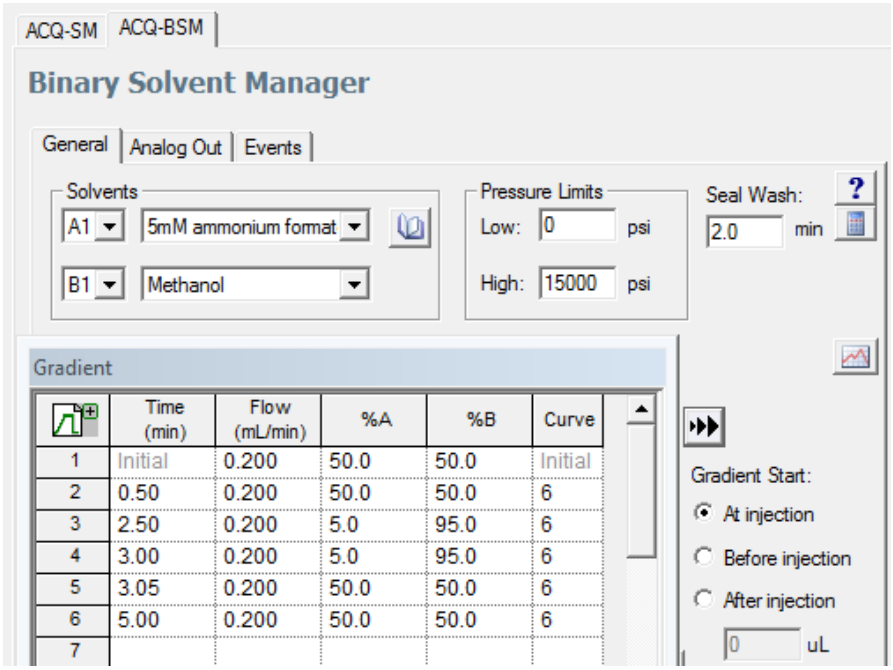


Properties of PRM

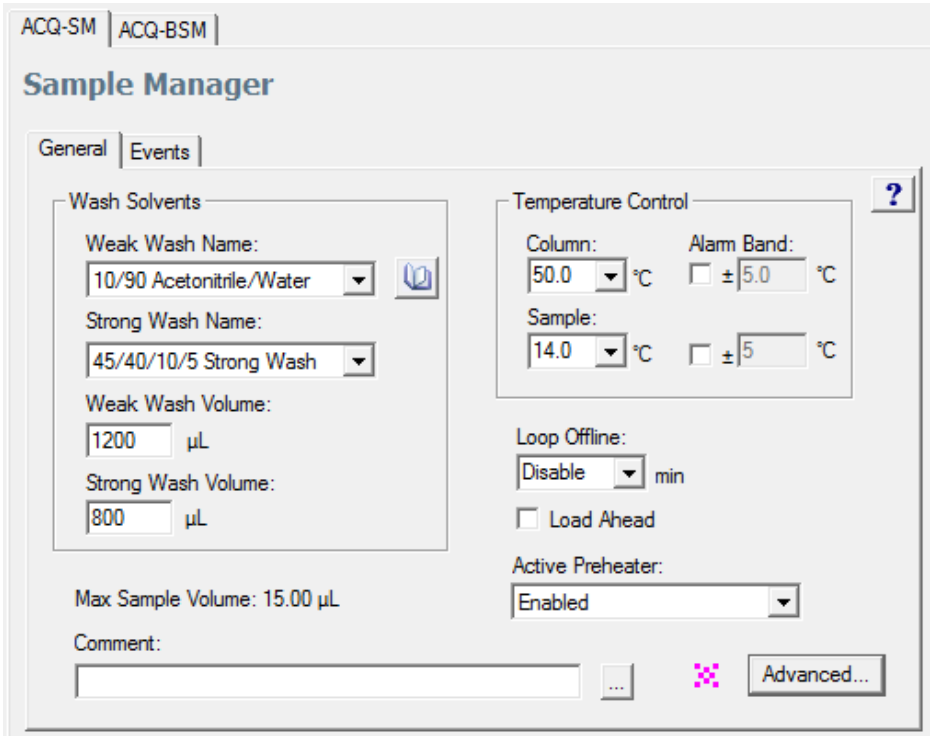
General	
Runtime	0 to 5 min
Polarity	positive
In-source CID	0.0 eV
Default charge state	1
Inclusion	on
MS²	
Microscans	1
Resolution	17,500
AGC target	2e5
Maximum IT	100 ms
Loop count	1
MSX count	1
MSX isochronous ITs	on
Isolation window	1.0 m/z
Isolation offset	0.0 m/z
Fixed first mass	—
(N)CE / stepped (N)CE	nce: 35
Spectrum data type	Profile

7.2 Liquid Chromatograph (LC) Parameters

7.2.1 Solvent Manager



7.2.2 Sample Manager



8 DATA ANALYSIS

8.1 Decision Criteria

In order for a chromatographic peak to be used for identification, the following criteria must be met:

Retention Time	Mass Accuracy	Signal To Noise	Preceding Negative Sample Response
± 5 % of concurrent standard or extracted control	± 5 mmu	≥ 3	≤ 10

8.1.1 Analyte Specific Decision Criteria

Analyte	Scan Mode	Retention Time†	Adduct / Fragment	m/z
Digoxin	SIM	2.99	$M+NH_4$	798.463
	MS^2	2.99	Fragment	651.373
			Fragment	97.065
			Fragment	391.247
	MS^2 spectra are concentration dependent. Refer to TOX-104.			
	Full Scan*	2.99	$M+NH_4$	798.463
			M+H	781.436
	**The inclusion of full scan data is optional. Digoxin undergoes in-source fragmentation, as well as forms multiple adducts.*			
Digitoxin	SIM	3.65	$M+NH_4$	782.469
	MS^2	3.65	Fragment	635.380
			Fragment	97.065
			Fragment	375.253
	MS^2 spectra are concentration dependent. Refer to TOX-104.			

	Full Scan*	3.65	$M+NH_4$	782.469
	**The inclusion of full scan data is optional. Digitoxin forms primarily the ammonium adduct.*			
Cerberin	SIM	3.53	M+H	577.337
	MS^2	3.53	Fragment	203.091
			Fragment	171.065
	MS^2 spectra are concentration dependent. Refer to TOX-104.			
	Full Scan*	3.53	M+H	577.337
			$M+NH_4$	594.364
	**The inclusion of full scan data is optional. Cerberin forms primarily the protonated adduct as well as an ammonium adduct at a lower abundance.*			
Oleandrin	SIM	3.41	M+H	577.337
	MS^2	3.41	Fragment	373.237
			Fragment	433.258
			Fragment	113.060
	MS^2 spectra are concentration dependent. Refer to TOX-104.			
	Full Scan*	3.41	M+H	577.337
			$M+NH_4$	594.364
	**The inclusion of full scan data is optional. Oleandrin forms primarily the protonated adduct as well as an ammonium adduct at a lower abundance.*			
Gelsemine	SIM	1.63	M+H	323.175
	MS^2	1.63	Fragment	70.065
			Fragment	236.106
			Fragment	195.067
	MS^2 spectra are concentration dependent. Refer to TOX-104.			
	Full Scan*	1.63	M+H	577.337

	**The inclusion of full scan data is optional. Gelsemine does not form additional adducts.*			
Digoxin-d3	Full Scan	2.98	$M+NH_4$	801.482

† The retention times listed are nominal. Due to normal column aging and variation in mobile phase preparation, small adjustments to the start and stop times may be required based upon the system suitability sample results.

8.1.2 Batch Acceptance

8.1.2.1 Control Criteria

Target analytes shall not be detected in the Negative Control. The S^3, 1 and 10 ng/mL Positive Control shall have all target analytes identified. (Either a positive control or an unextracted standard may be used for mass spec/ion ratios comparisons as needed). For an individual case, the target analytes required may vary.

8.1.2.2 Internal Standard

The internal standard shall be recovered via full scan for each control and unknown sample.

9 REPORTING

Refer to TOX-100 and TOX-101 for guidance.

10 CORRECTIVE MEASURES

Refer to TOX-101 for potential responses to QC failure(s).

11 PERFORMANCE CHARACTERISTICS

11.1 LOD

Analyte	Matrix	LOD (ng/mL)
Digoxin	Blood	0.5
Digitoxin	Blood	1
Cerberin	Blood	0.1
Oleandrin	Blood	0.1
Gelsemine	Blood	0.1

11.2 Carryover

Carryover was not detected.

12 Limitations

12.1 Interferences

No interferences identified.

12.2 Isomers

Cerberin and oleandrin are isotopomeric isomers. Baseline or near baseline resolution of these two analytes is required to differentiate on the basis of the protonated ion alone. However, the analytes do have different tandem mass spectra.

12.3 Source of Analytes

While digoxin (and digitoxin, to a lesser extent) are available as highly purified preparations for medical use, other plant toxins are often present in unprocessed or less purified forms. Potential poisonings from these types of scenarios may generate multiple analytes and metabolites that may be similar in structure and mass spectra to validated analytes. A combination of full scan, SIM, and MS^2 analyses may be used to investigate potential additional analytes of interest.

13 Safety

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

14 Revision History

Revision	Issued	Changes
01	02/11/2022	Document reformat. 5.6.2.4 - Expanded internal standard intermediate preparation to a table format.