法務部法醫研究所
Institute of Forensic Medicine, Ministry of Justice

Evolving Methodologies Amenable to the Analysis of Postmortem Specimens

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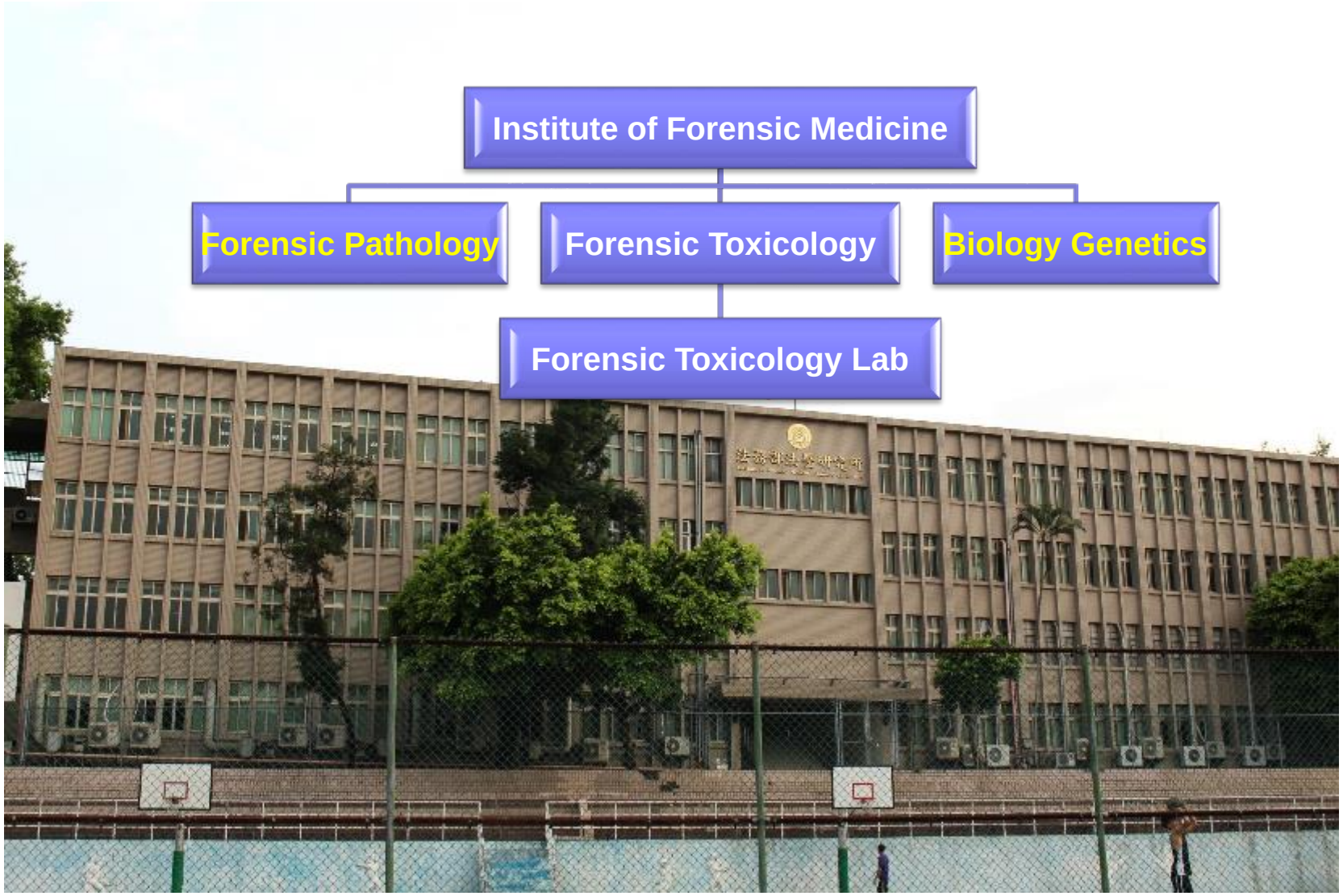
Presentation Outline

- Introduction — The laboratory
- Methodologies (adopted at various time periods)
  - Preliminary screen
  - Sample pretreatment
  - Confirmation/quantitation
- Highlights of selected methologies
  - Preliminary screen — LC-QTOF/MS
  - Confirmation/quantitation — LC-QQQ/MS
- What is next?
  - Sample pretreatment — QuEChERS

Introduction—The Institute



Introduction—The Lab

Forensic Toxicology Lab

Toxicologist	7
Research Assistant	11
Adm. Assistant	5
Total Personnel	**23**

Introduction—Major Intrumentation

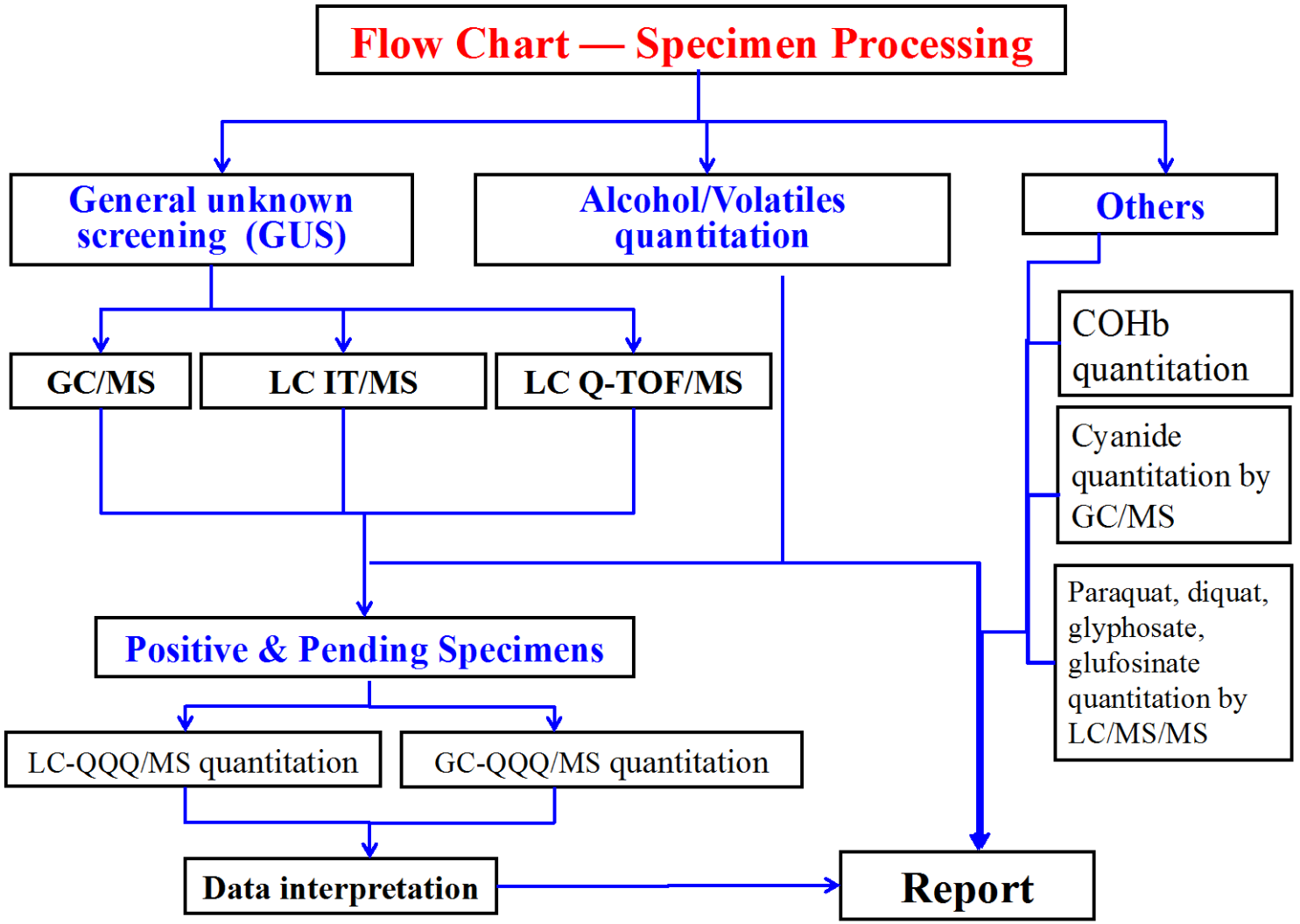
Instrument	#	Usage
FPIA (AxSYM)	1	Screen: AM, OPI, COC, THC, PCP, BAR, BEN …
GC/MS	7	GUS: 5-Library/in-house database (>450,000 cpds); Cyanide quant.
LC-IT/MS	4	GUS: In-house-database (>1500 cpds); Search by MS_2 spectra
LC-QTOF/MS	2	GUS: In-house-database (>1500 cpds); Search by formula, auto MS/MS, accurate mass
HS-GC	2	Quant.: EtOH/MeOH/acetone/isopropanol
HS-GC/MS	2	Conf.: (EtOH/MeOH/acetone/IPA/toluene/gasoline)
GC-QQQ/MS	2	Quant.: Tramadol, fluoxetine, mirtazapine, clothiapine, propofol, valproic acid …
LC-QQQ/MS	5	Quant.: Opiates, cocaine, THC, barbiturates, benzodiazepines, NPS, paraquat …)

Introduction—# of Case & Publication

Cases\Year	2002	2009	2010	2011	2012	2013	2014	2015	2016	2017
Toxicology	884	2760	2832	3024	2927	3003	3513	3654	3557	3477
Case enquiry	110	265	355	443	499	618	650	764	759	752
Total	994	3025	3187	3467	3426	3621	4163	4418	4316	4229

Journal (2002–2017)	No.	Journal (2002–2017)	No.
J Anal Toxicol	16	*Anal Chem*	2
J Chromatrogr A	2	*J Forensic Sci*	1
Rapid Commun Mass Spectrom	1	*Anal Bioanal Chem*	1
Analyst	1	*Forensic Sci Rev*	3
Others	4	Total	31

Introduction—Flow Chart

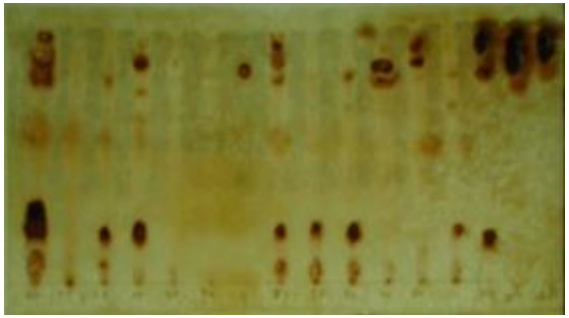


Methodology–Screen (1)

Immunoassay

EIA (EMIT®) (1980–1990)

TLC (1980–1990)



FPIA (TDx) (1990–2000)

FPIA (AxSYM) (2000–2013)

Methodology—Screen (2)

GC-MS

LC-IT/MS

Anal Chem **81**:9002–9011 (2009)
Rapid Commun Mass Spectrom **24**:75–84 (2010)

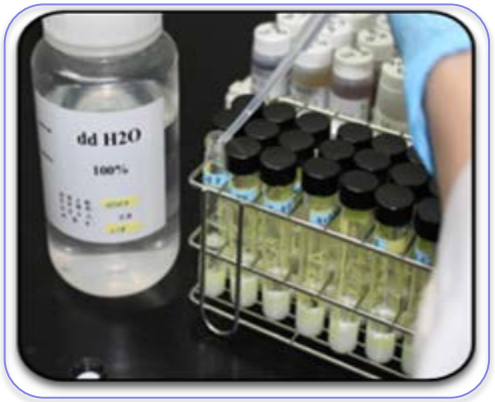
UHPLC-QTOF/MS

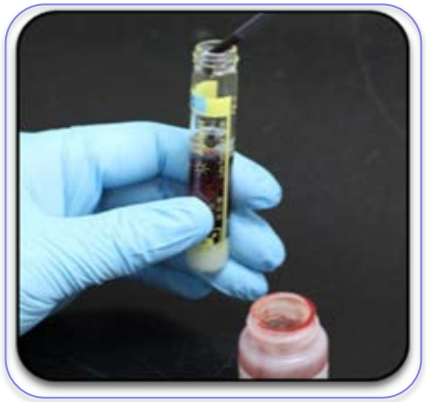
Automated library search
J Anal Toxicol **41**:421–430 (2017)

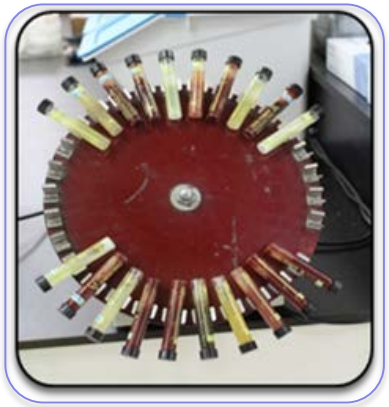
Liquid-Liquid Extraction

Test tube

Toxi-Tube®



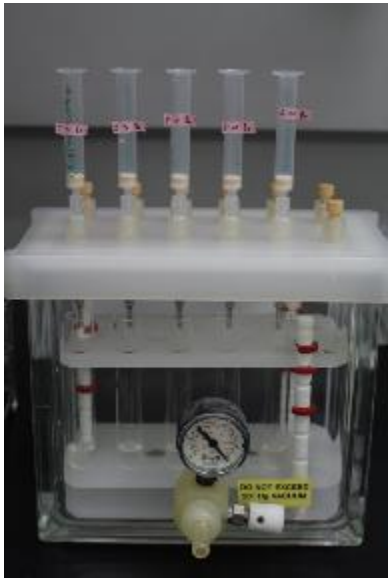




Methodology—Sample Prep (2)

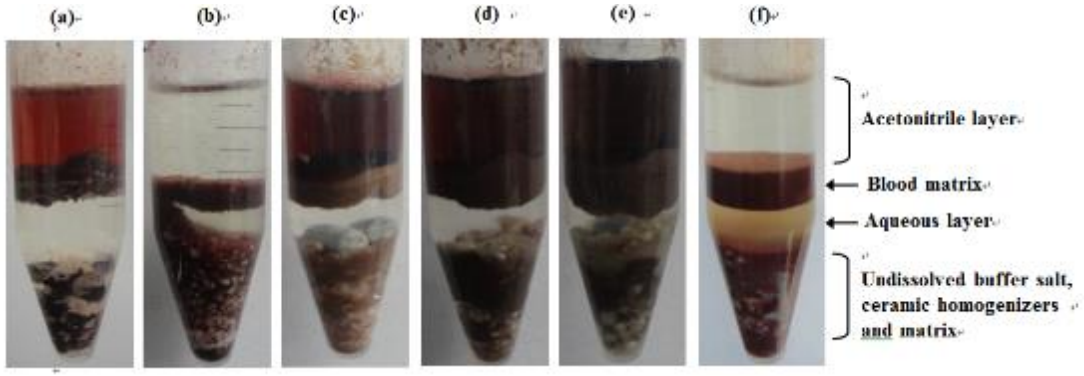
Solid-Phase Extraction (SPE)

SPE — Manual



SPE — Automated

Dispersive SPE QuEChERS (Quick, Easy, Cheap, Effective, Rugged, & Safe)



Methodology–Conf./Quant.

GC-MS

- Buprenorphine & its metabolites [urine]: *Chromatrogr A* **1217**:1688–1694 (2010)
- Methadone in blood, plasma & oral fluid: *Anal Bioanal Chem* 405:3921–3928 (2013)

LC-QQQ/MS

- Amphetamines & opiates in hair: *J Anal Toxicol* **39**:183–191 (2015)
- Opiates, methamphetamine, buprenorphine, methadone & metabolites in oral fluid: *J Anal Toxicol* **39**:472–480 (2015)
- Methamphetamine, ketamine, opiate & metabolites in urine; *J Anal Toxicol* **41**:679–687 (2017)

Highlight—LC-QTOF for GUS Screen (1)

J Anal Toxicol **41**:421–430 (2017)

Instrumentation & Key Parameters

- Agilent 1290 UHPLC
  - Column: Agilent Zorbax SB-Aq
- Agilent 6540 QTOF/MS
  - Electrospray/positive mode
  - Quadrupole: isolating precursor ions
  - Linear hexapole collision cell: 10, 20, 40 eV
  - TOF-MS: MS, 100–1000 m/z; MS/MS, 50–800 m/z
  - Data Collection: MassHunter Acquisition
  - Personal Compound Database & library software
    - Molecular Feature: mass (main isotope), isotope pattern; mass (M+1 & M+2)
    - Formula
    - Auto MS/MS

J Anal Toxicol **41**:421–430 (2017)

LC Parameters & Sample Preparation

- Mobile phase & elution
  - Mobile phase: 0.1% formic acid in water (A); methanol (B)
  - Initial solvent (A:B, 88:12) 4 min; decrease to 0%A in 8 min; held 1 min for rinsing; increase to 88% in 1 min
- Standard
  - 1–5 µg/mL in initial gradient solvent
- Specimen (urine/blood)
  - Toxi-Tubes® A
  - Buffer: sodium carbonate/bicarbonate, pH 9.0
  - Solvent: dichloromethane/dichloroethane/*n*-heptane/ethyl acetate (1/1/1/1, v)
  - Extract dried; dissolved in initial gradient solvent

Highlight—LC-QTOF for GUS Screen (3)

J Anal Toxicol **41**:421–430 (2017)

ESI QTOF-MS Library—I

- Ions
  - Precursor ion: protonated ions
  - MS/MS spectra: 10, 20, 40 eV
- In-house library with 1,200 relevant compounds
  - Compound: name, chemical formula, chemical structure, monoisotopic mass (5 decimal places; automatically calculated)
  - Retention time
  - CID MS/MS spectra: 3 spectra (10, 20, 40 eV), each average over the width of eluting peak

Highlight—LC-QTOF for GUS Screen (4)

J Anal Toxicol **41**:421–430 (2017)

ESI QTOF-MS Library—II

LC–QTOF-MS in-house database—data for 21 opioids

Drugs	Retention time (min)	Formula	Monoisotopic mass	$[M+H]^+$ mass	Observed mass	Mass error	LOD (ng/mL)
Normorphine	1.331	---	271.12084	---	271.12067	---	5.0
Dihydromorphine	1.333	---	287.15214	---	287.15510	---	1.0
Morphine	1.351	---	285.13649	---	285.13647	---	2.5
Noroxymorphone	1.405	---	287.11576	---	287.11575	---	5.0
Oxymorphone	1.494	---	301.13141	---	301.13152	---	5.0
Hydromorphone	1.678	---	285.13649	---	285.13666	---	5.0

6-Acetylmorphine	3.934	---	327.14706	---	327.14710	---	10
6β-Naltrexol	4.026	---	343.17836	---	343.17860	---	2.5
6-Acetylcodeine	7.154	---	341.16271	---	341.16286	---	2.5
Heroin	7.248	---	369.15762	---	369.15777	---	5.0
Norbuprenorphine	8.258	---	413.25661	---	413.25659	---	5.0
Buprenorphine	8.921	---	467.30356	---	467.30349	---	2.5

Highlight—LC-QTOF for GUS Screen (5)

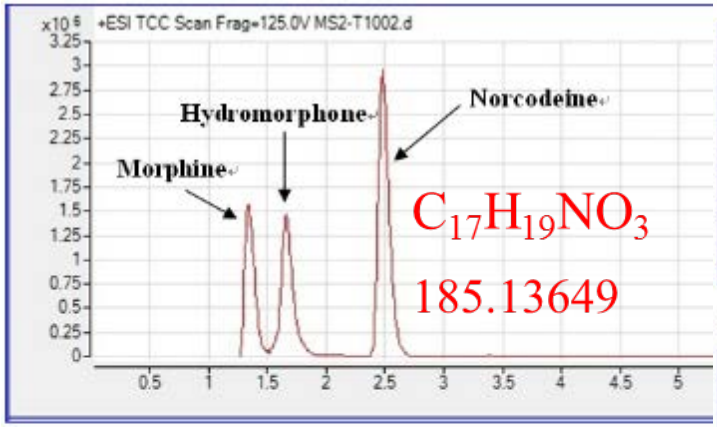
J Anal Toxicol **41**:421–430 (2017)

Analyte Identification—3 Search Algorithms

(A) Molecular feature: match score morphine (96.68)

(B) Formula: morphine (99.45), hydromorphone (99.62), norcodeine (99.95)

(C) Auto MS/MS: morphine (98.08), hydromorphone (95.94), norcodeine (98.12)



(A)

Compound Table

Compound Label	RT	Mass	Name	Formula	Tgt Mass	Diff (ppm)
Cpd 1: Morphine	1.346	285.1359	Morphine	C17H19NO3	285.1365	-1.94

Compound Label	Name	m/z	RT	Algorithm	Mass
Cpd 1: Morphine	Morphine	286.1431	1.346	Find by Molecular Feature	285.1359

MFE

(B)

Compound Table

Compound Label	RT	Mass	Abund	Name	Formula	Tgt Mass	Diff (ppm)	MFG Formula	DB Formula	DB Diff (ppm)
Cpd 8: Morphine	1.343	285.136	20696	Morphine	C17H19NO3	285.1365	-1.63	C17H19NO3	C17H19NO3	1.63
Cpd 7: Hydromorphone	1.66	285.136	64229	Hydromorphone	C17H19NO3	285.1365	-1.72	C17H19NO3	C17H19NO3	1.72
Cpd 6: Norcodeine	2.477	285.1361	145734	Norcodeine	C17H19NO3	285.1365	-1.49	C17H19NO3	C17H19NO3	1.49

Compound Label	Name	m/z	RT	Algorithm	Mass
Cpd 8: Morphine	Morphine	308.1253	1.343	Find By Formula	285.136
Cpd 7: Hydromorphone	Hydromorphone	308.1254	1.66	Find By Formula	285.136
Cpd 6: Norcodeine	Norcodeine	308.1256	2.477	Find By Formula	285.1361

FBF

(C)

Compound Table

Compound Label	RT	Mass	Name	Formula	MFG Formula	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 13: Morphine	1.344	285.1359	Morphine	C17H19NO3	C17H19NO3	C17H19NO3	2.13	3
Cpd 19: Hydromorphone	1.716	285.1357	Hydromorphone	C17H19NO3	C17H19NO3	C17H19NO3	2.9	3
Cpd 23: Norcodeine	2.48	285.136	Norcodeine	C17H19NO3	C17H19NO3	C17H19NO3	1.86	3

Compound Label	Name	m/z	RT	Algorithm	Mass
Cpd 13: Morphine	Morphine	286.1431	1.344	Auto MS/MS	285.1359
Cpd 19: Hydromorphone	Hydromorphone	286.1429	1.716	Auto MS/MS	285.1357
Cpd 23: Norcodeine	Norcodeine	286.1432	2.48	Auto MS/MS	285.136

AMM

Highlight—LC-QTOF for GUS Screen (6)

J Anal Toxicol **41**:421–430 (2017)

CAP PT Samples—T-A, T-B, T-C 2014 Toxicology

Specimen	Drug	LC-QTOF/MS	LC-IT/MS	GC/MS
T-01 Serum	Oxycodone	Yes	Yes	Yes
	Oxymorphone	Yes	Yes	—
	Buprenorphine	Yes	Yes	—
	Norbuprenorphine	Yes	—	—
T-02 Urine	Oxycodone	Yes	Yes	Yes
	Oxymorphone	Yes	Yes	—
	Buprenorphine	Yes	Yes	—
	Norbuprenorphine	Yes	—	—
T-06 Serum	Fentanyl	Yes	Yes	—
	Norfentanyl	Yes	—	—
	Methanol	Yes	Yes	—
	EDDP	Yes	—	—
T-07 Urine	Fentanyl	Yes	Yes	—
	Norfentanyl	Yes	Yes	—
	Methanol	Yes	Yes	Yes
	EDDP	Yes	Yes	Yes

Highlight—LC-QTOF for GUS Screen (7)

J Anal Toxicol **41**:421–430 (2017)

No. of Compounds Identified

—In 10 opiate postmortem cases (blood & urine)—

# Drug identified	Method		
	LC-QTOF/MS	LC-IT/MS	GC/MS
Total	146	97	31
6-Acetylmorphine	11	10	6
6-Acetylcodeine	4	5	3
Heroin	2	2	0

Highlight—LC-MS/MS for Conf./Quant. (1)

J Anal Toxicol **41**:679–687 (2017)

Instrumentation & Key Parameters

- Agilent 1200 Infinity LC system
  - Column: Agilent Zorbax SB-Aq
- Agilent 6410 QQQ/MS
  - Electrospray, positive mode
  - Precursor ion isolation width: 4 amu
  - Acquisition: dynamic multiple reaction monitoring
  - Collision energy: optimal for each precursor ion
  - Two transition ions for each analyte

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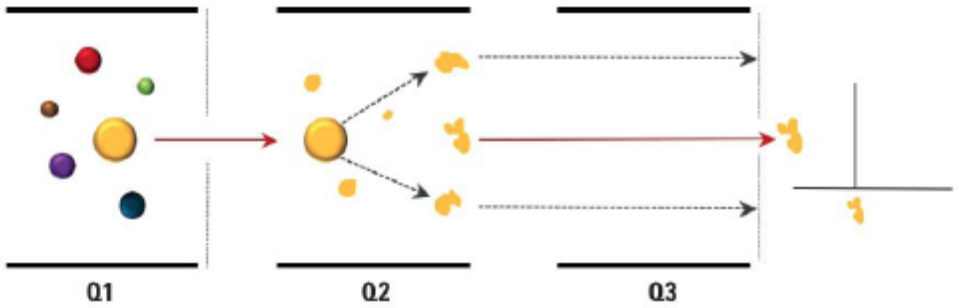
Dynamic Multiple Reaction Monitoring

J Anal Toxicol **41**:679–687 (2017)

Source: Agilent Technologies 5990-3595EN



- "… (Q1) selects and transmits a precursor ion with a specific *m/z*. This ion is then fragmented in … (Q2 collision cell), and a specific product ion with a defined *m/z* is selected and transmitted in … (Q3)"
- "Ion transitions and a retention time window for each analyte are stored in a method. MRM transition lists are then built dynamically throughout an LC/MS run, based on the retention time window for each analyte. … analytes are only monitored while they are eluting from the LC"

J Anal Toxicol **41**:679–687 (2017)

LC Parameters & Sample Preparation

- Mobile phase & elution
  - Mobile phase: 0.1% (v/v) formic acid in water (A); methanol (B)
  - Initial solvent (A:B, 90:10) 1.5 min; decrease to 0%A in 5.5 min; held 1 min for rinsing; increase to 90% in 1 min; held 1 min
- Standards
  - 50, 125, 250, 500, 1000 ng/mL in water
- Specimens (urine/blood)
  - Buffer: 0.1 M phosphate
  - SPE: ACCUBOND EVIDEX (Agilent), Biotage/Caliper/Zymark RapidTrace SPE Workstation
  - Elution: ethyl acetate/methanol/NH_4OH (75:25:2, v/v/v)
  - Extract dried; dissolved in initial gradient solvent; centrifuged

Highlight—LC-MS/MS for Conf./Quant. (4)

Transition and MS/MS Parameters

—Ten analytes & their internal standards—

J Anal Toxicol **41**:679–687 (2017)

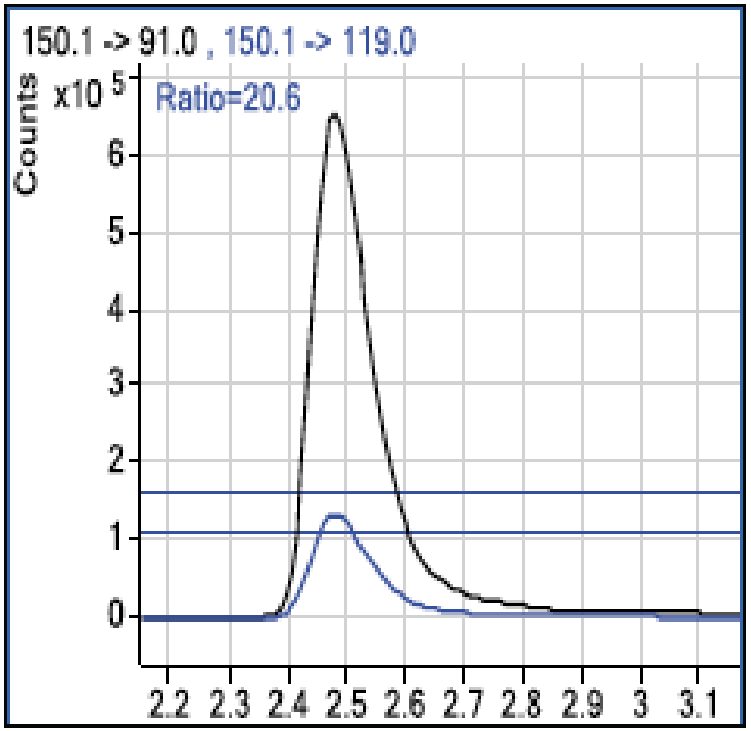
Drugs	Retention time (min)	Precursor ion *m/z*	Precursor ion Frag (V)	Target ion *m/z*	Target ion Col (V)	Qualifier ion *m/z*	Qualifier ion Col (V)
Methamphetamine	2.66	150.1	87	119	8	91	16
Methamphetamine-d_8	2.61	158.2	92	124.1	8	93	20
MDMA	3.92	194.1	92	163	8	105	24
MDMA-d_5	3.88	199.1	97	165	8	107	24
Morphine	1.45	286.2	166	181	36	165.1	44
Morphine-d_6	1.44	292.2	166	181	36	153.1	48
6-Acetylcodine	6.90	342.2	161	225	28	165.1	60
6-Acetylcodine-d_6	6.90	345.2	162	225.1	29	115	60
Norketamine	6.20	224	92	207	5	125	25
Norketamine-d_4	6.20	228.1	102	211	8	129	28

Highlight—LC-MS/MS for Conf./Quant. (5)

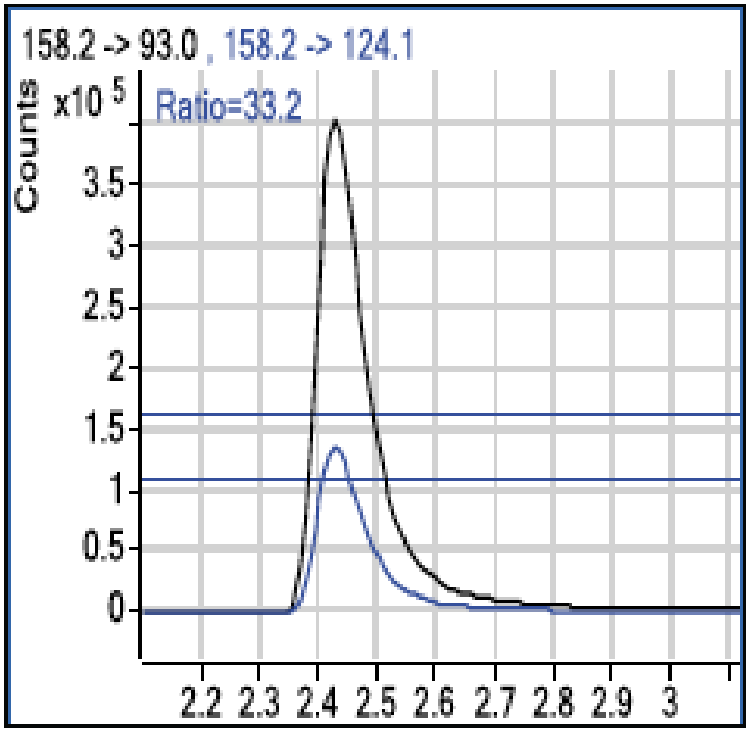
J Anal Toxicol **41**:679–687 (2017)

Dynamic Multiple Reaction Monitoring Chromatogram

methamphetamine



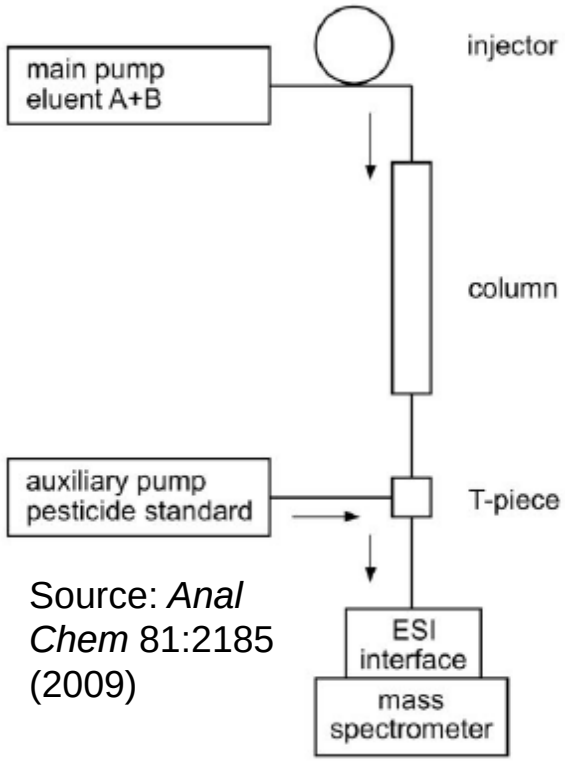
methamphetamine-d_8



J Anal Toxicol **41**:679–687 (2017)

Evaluation of Matrix Effect —Post-column infusion—

- Post-column infusion system
  - Infusion syringe pump delivers analyte (to be evaluated) standard to the LC column eluent via a mixing tee (relative intensity profile A)
  - Inject extract of drug-free samples (5 sources) into LC-MS/MS system (relative intensity profile B)
- Evaluation
  - A>B: ion suppression; A<B: ion enhancement
  - Matrix effect (%): [1 – (A – B) / A] × 100
  - Matrix effect: 61% – 102%
- Deuterated internal standards
  - Matrix effects compensated

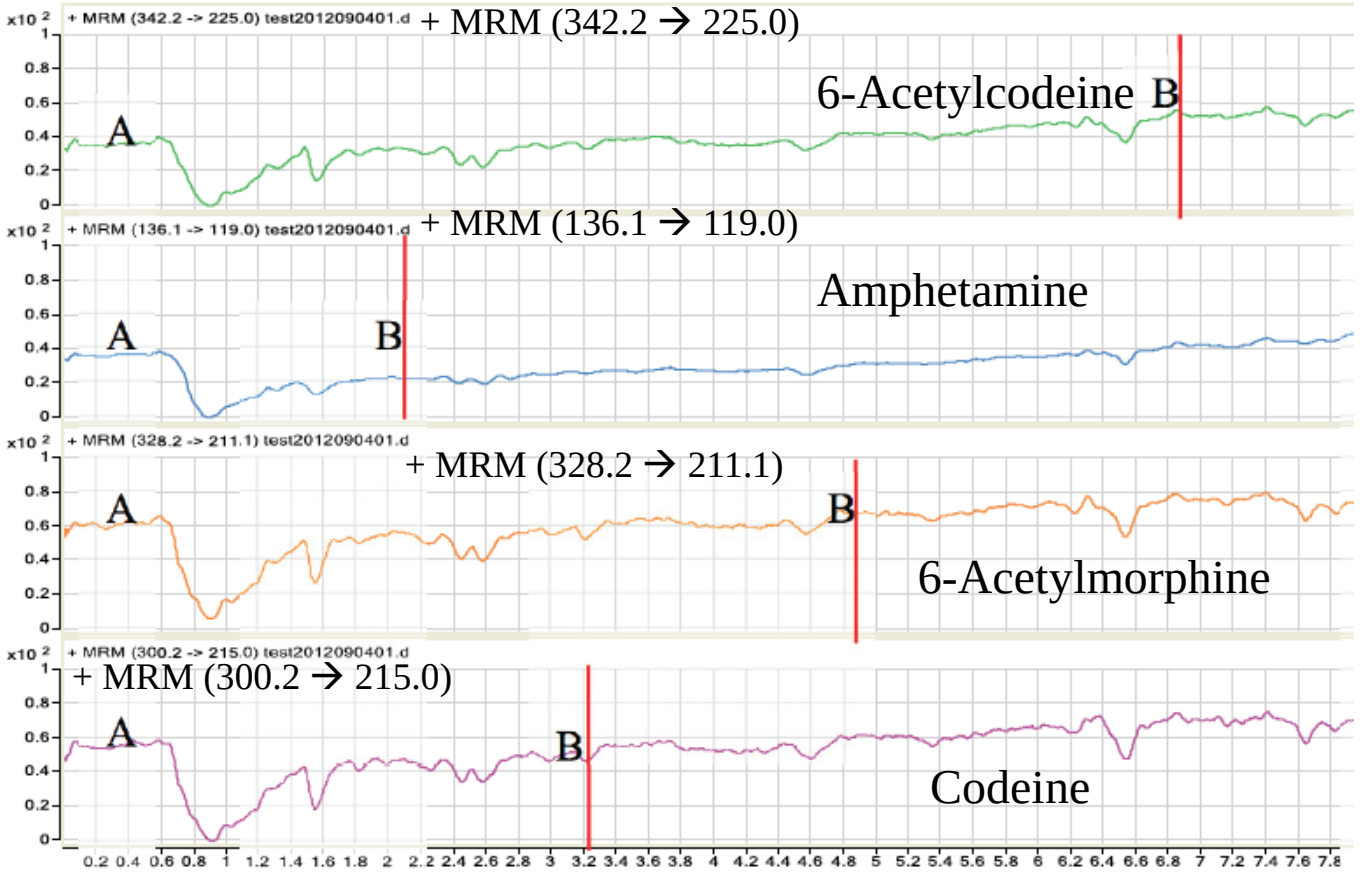


Source: *Anal Chem* 81:2185 (2009)

Highlight—LC-MS/MS for Conf./Quant. (7)

J Anal Toxicol **41**:679–687 (2017)

Evaluation of Matrix Effect —Post-column infusion—



Highlight—LC-MS/MS for Conf./Quant. (8)

J Anal Toxicol **41**:679–687 (2017)

Accuracy Data—250 ng/mL in Urine (*n* = 3)

Analyte	Mean ± SD	Accuracy	CV (%)
Amphetamine	248.9 ± 4.9	99.6	2.0
Methamphetamine	250.8 ± 8.4	100	3.3
MDA	246.7 ± 3.0	98.7	1.2
MDMA	244.0 ± 3.5	97.6	1.4
Morphine	249.2 ± 11	99.7	4.6
Codeine	246.4 ± 5.8	98.5	2.4
6-Acetylmorphine	252.5 ± 15	101	6.0
6-Acetylcodeine	249.7 ± 11	99.9	4.4
Ketamine	246.4 ± 3.9	98.6	1.6
Norketamine	243.5 ± 3.7	97.4	1.5

QuEChERS—Background

- Quick, Easy, Cheap, Effective, Rugged, and Safe—dispersive SPE
- 2001–2002: Developed by Michelangelo Anastassiades (postdoctoral fellow from Univ. of Hohenheim, Stuttgart, Germany) in USDA's ERRC (Lehotay's group)
- Developed for extracting basic/polar pesticide residues in plant materials
- Original and modified (with buffering salts) versions now widely used
- Drugs in blood: *Legal Medicine* 14:286 (2012)

QuEChERS—Procedure

- Extraction (step 1)
  - European standard: 2 mL acetonitrile, 1.3 g pre-packed extraction preparation (0.8 g $MgSO_4$, 0.2 g NaCl, 0.2 g sodium citrate and 0.1 g disodium citrate)
  - IFM: 1 mL Na_2CO_3/$NaHCO_3$ buffer (pH 9.5), 2 mL acetonitrile, 1.0 g pre-packed extraction preparation (0.8 g $MgSO_4$, 0.2 g NaCl)
- Purification (step 2)
  - 25 mg primary secondary amine-bonded silica, 25 mg C18EC, 150 mg $MgSO_4$

QuEChERS—Recovery

Drug	d-SPE (EU)	d-SPE (IFM)	Toxi-Tubes® A
Amphetamine	54.0 ± 0.6	55.4 ± 2.3	80.9 ± 3.0
Benzoylecgonine	42.2 ± 1.6	32.6 ± 1.9	5.5 ± 0.3
Cocaethylene	71.0 ± 1.5	78.2 ± 2.7	86.0 ± 3.3
Cocaine	70.4 ± 2.3	74.3 ± 2.5	88.9 ± 2.6
Codeine	52.8 ± 0.7	57.8 ± 3.6	87.8 ± 1.2
Hydrocodone	61.2 ± 1.3	66.3 ± 4.2	88.3 ± 1.6
MDA	59.0 ± 0.9	56.3 ± 2.7	82.6 ± 1.2
MDMA	63.6 ± 0.5	65.0 ± 2.1	86.2 ± 1.3
Methamphetamine	62.3 ± 0.8	64.5 ± 2.7	84.7 ± 3.8
Morphine	42.3 ± 1.2	39.9 ± 1.7	42.7 ± 1.5