Determination of Urinary Catecholamines and Metanephrines in a single run

Analysis by offline SPE and LC-MS/MS for Clinical Research

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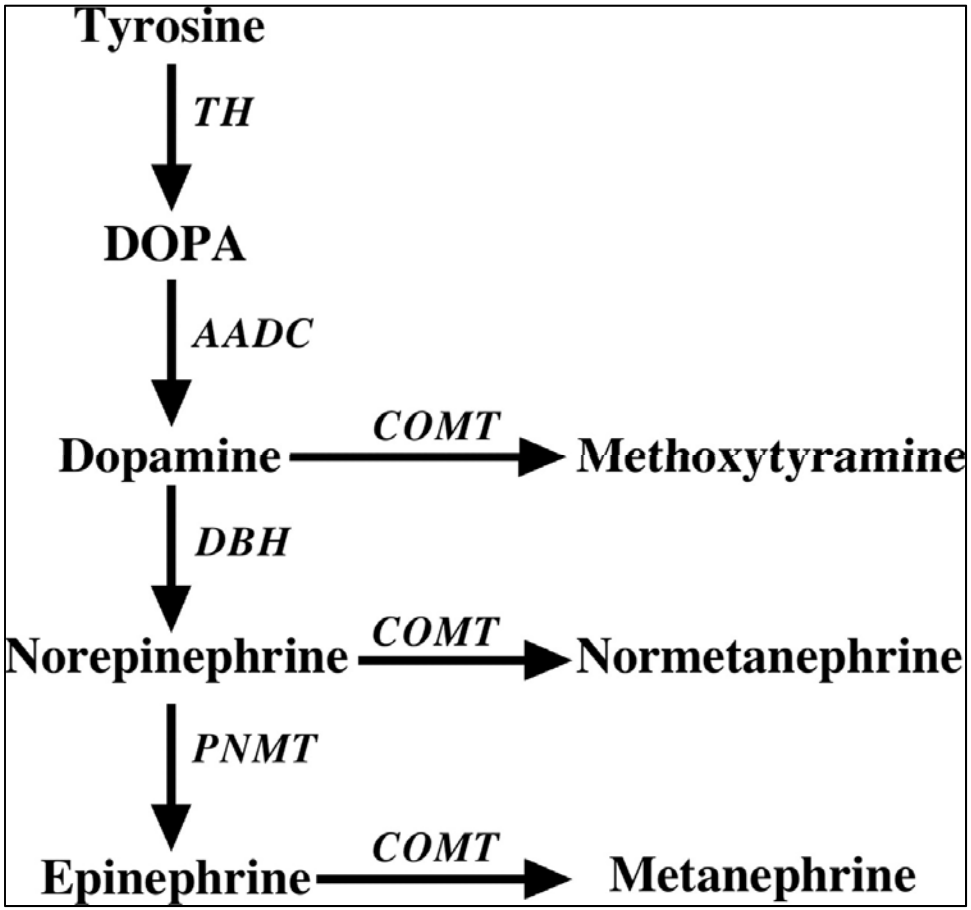
Objectives

In this presentation, we will be discussing:

- A four minute method for quantifying catecholamines and their metabolites: epinephrine, norepinephrine, dopamine, metanephrine normetanephrine and 3-methoxytyramine
- The use of offline solid phase extraction (SPE) for simultaneous extraction of all six analytes from urine
- The chromatographic separation of all six analytes with conditions compatible with LC-MS/MS
- Typical method performance results
- Tips and tricks

Pathways of catecholamine synthesis and O-methylation



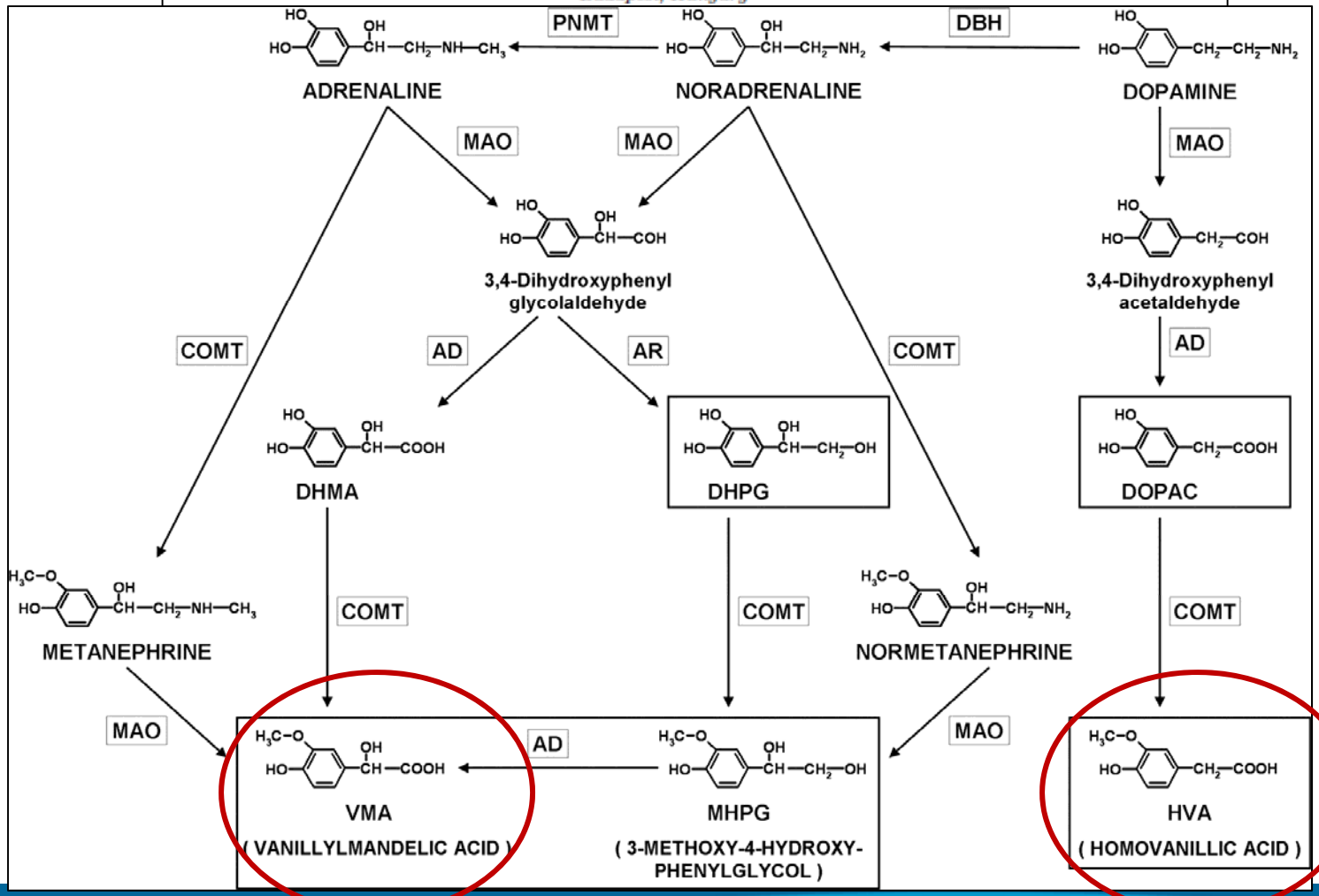
Eisenhofer G et al. JCEM 2005;90:2068-2075

Catecholaminergic Systems in Stress: Structural and Molecular Genetic Approaches

Physiol Rev 89: 535–606, 2009;
doi:10.1152/physrev.00042.2006.

RICHARD KVETNANSKY, ESTHER L. SABBAN, AND MIKLOS PALKOVITS

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic; Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York; and Neuromorphological and Neuroendocrine Research Laboratory, Semmelweis University and Hungarian Academy of Sciences, Budapest, Hungary



Molecular Genetics and Metabolism

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Minireview

A new perspective on the treatment of aromatic L-amino acid decarboxylase deficiency

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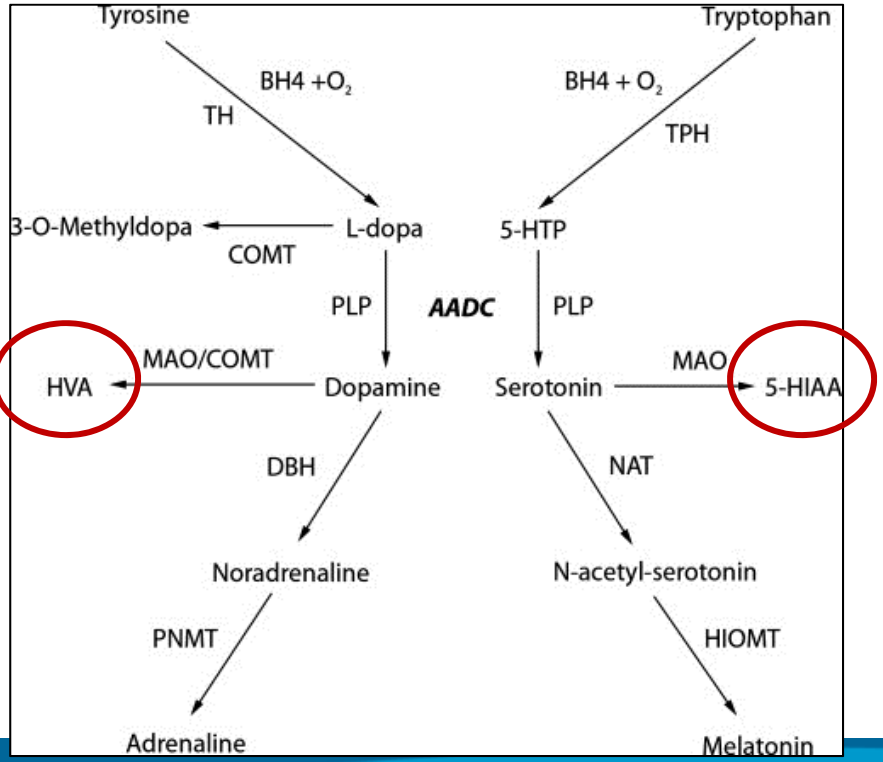


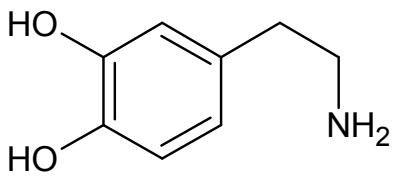
Which ones and in which matrix urine or plasma?

- Free catecholamines and metanephrines in urine
- Total catecholamines and metanephrines in urine
- Free catecholamines in plasma
- Free metanephrines in plasma
- VMA in urine
- HVA in urine
- 5-HIAA in urine

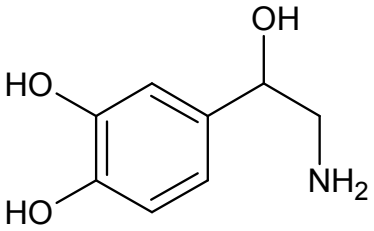
Which ones and in which matrix urine or plasma?

- **Free catecholamines and metanephrines in urine**
- **Total catecholamines and metanephrines in urine**
- Free catecholamines in plasma (in progress)
- Free metanephrines in plasma (in progress)
- VMA in urine (in progress)
- HVA in urine (in progress)
- 5-HIAA in urine (in progress)

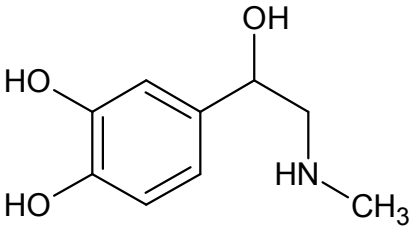
Compound structures



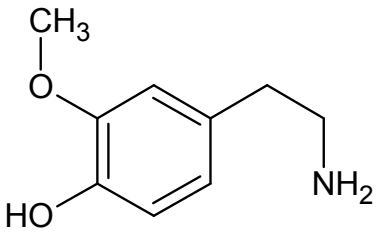
Dopamine (D)
C8H11NO2
Neutral Mass: 153.08



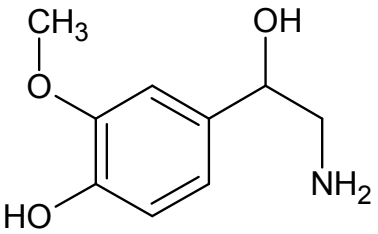
Norepinephrine (NE)
C8H11NO3
Neutral Mass: 169.07



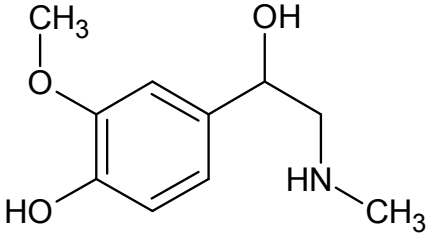
Epinephrine (E)
C9H13NO3
Neutral Mass: 183.09



3-Methoxytyramine (3-MT)
C9H13NO2
Neutral Mass: 167.09

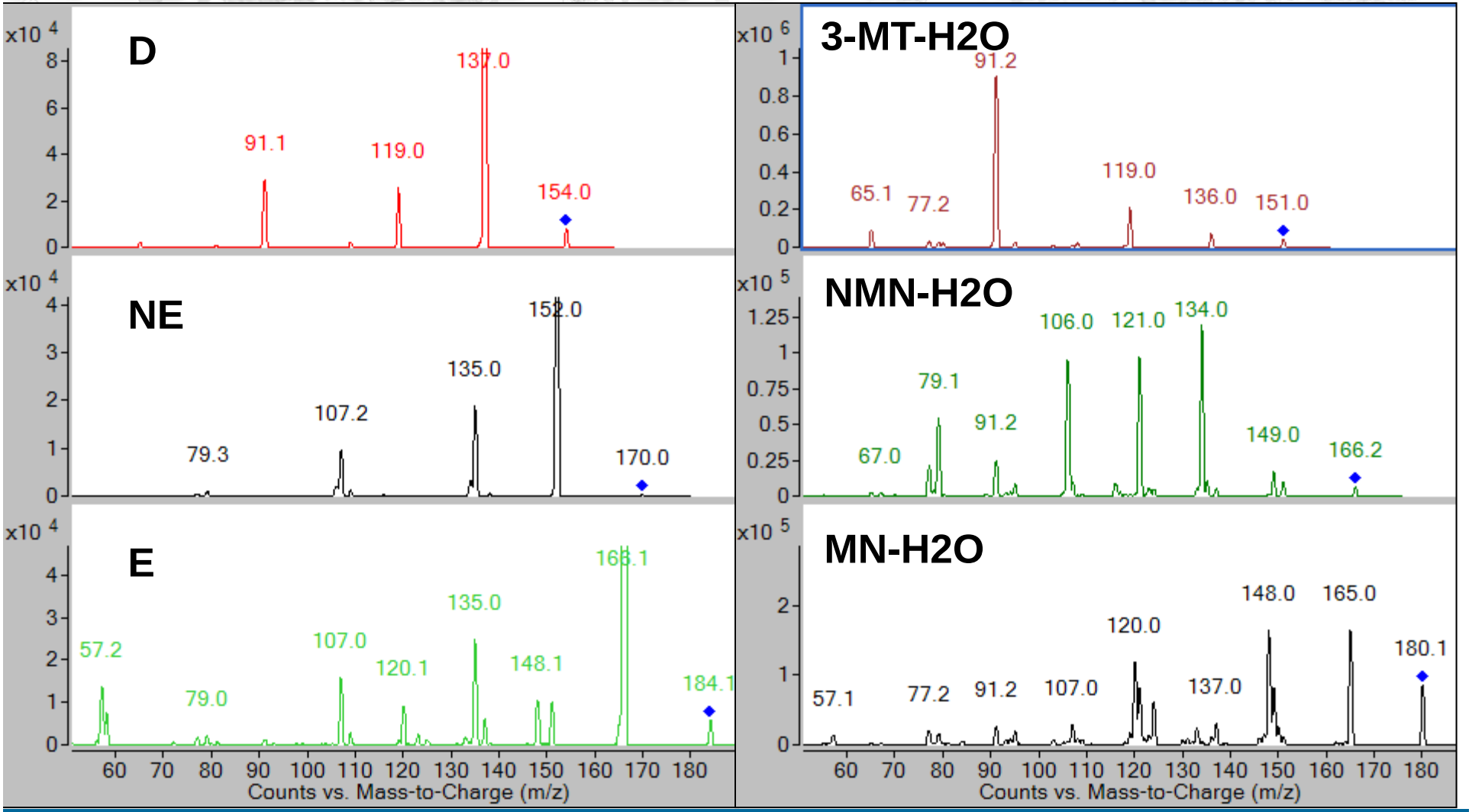


Normetanephrine (NMN)
C9H13NO3
Neutral Mass: 183.09



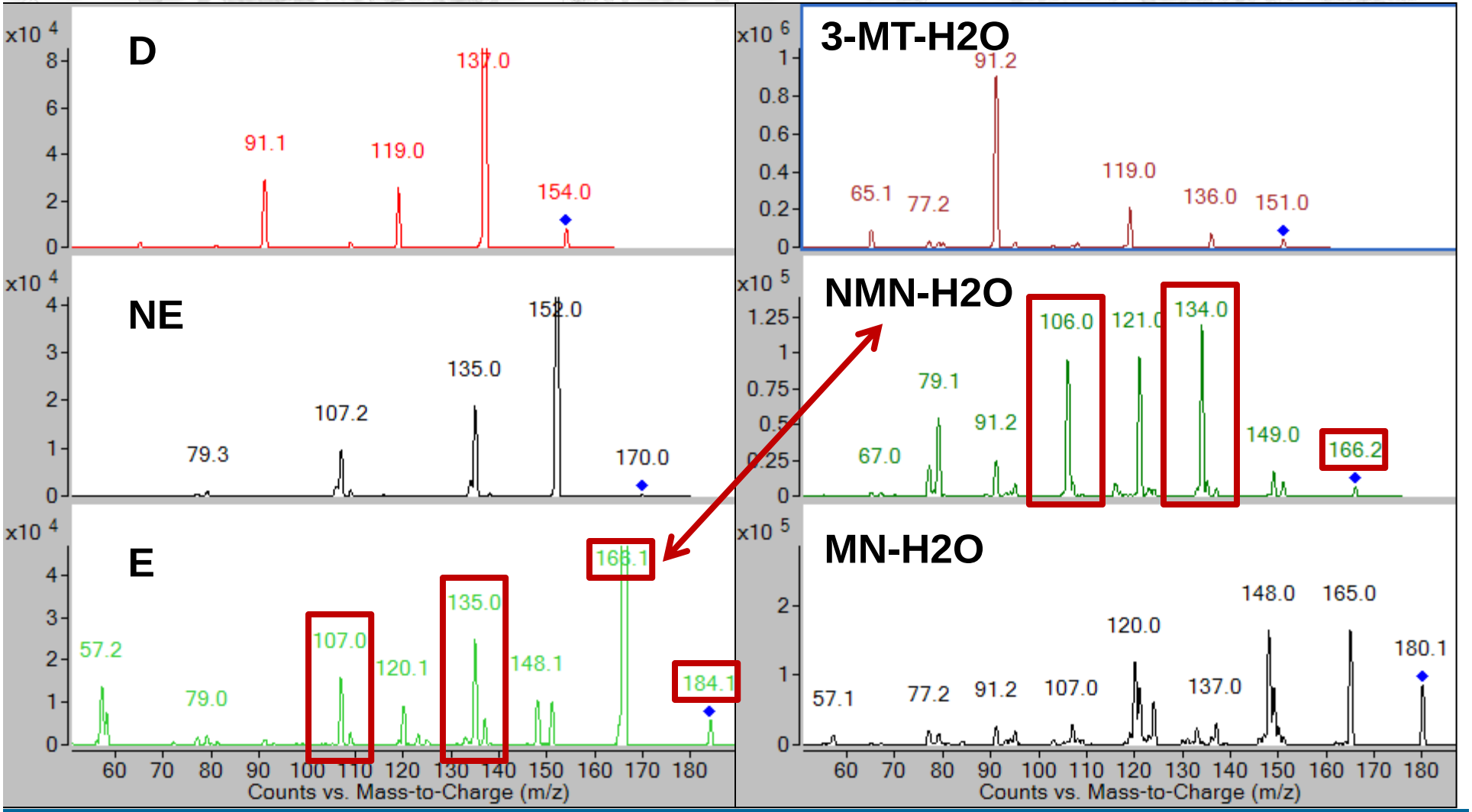
Metanephrine (MN)
C10H15NO3
Neutral Mass: 197.11

Product Ion Scans (MS/MS)



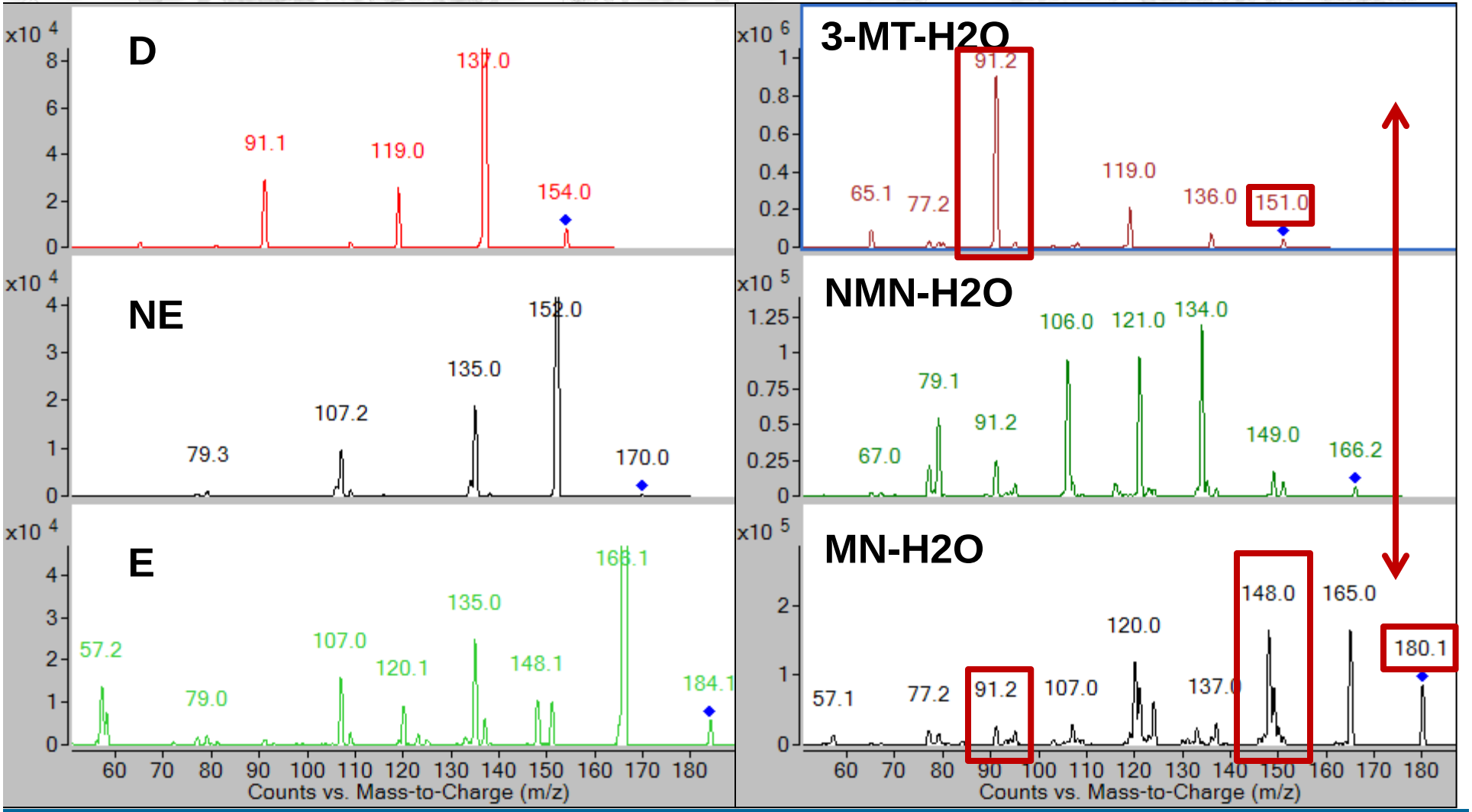
Product Ion Scans (MS/MS)

Must separate E and NMN by chromatography



Product Ion Scans (MS/MS)

Must separate 3-MT and MN by chromatography



Final Method Gradient to Include 3-MT

- ✓ Need to keep resolution between E and NMN
- ✓ Also between MN and 3-MT

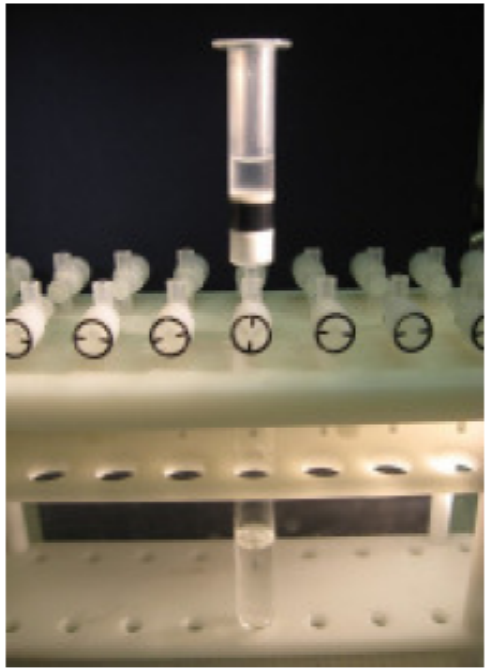
Internal Standards

Analyte	Internal Standard
Dopamine	Dopamine-D4
Norepinephrine	Norepinephrine-D6
Epinephrine	Epinephrine-D6
3-Methoxytyramine	3-Methoxytyramine-D4
Normetanephrine	Normetanephrine-D3
Metanephrine	Metanephrine-D3

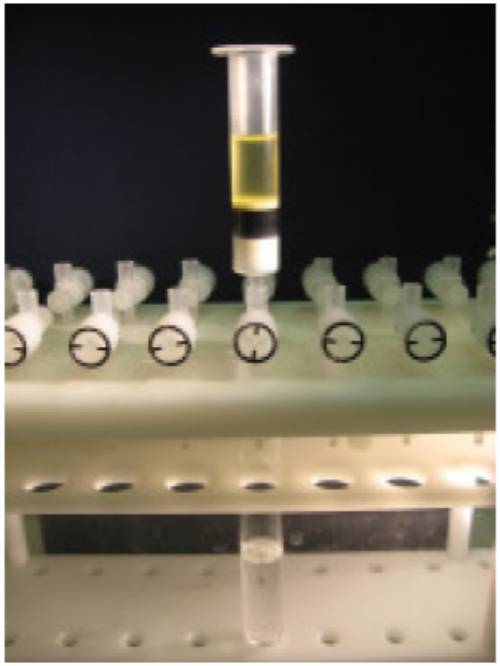
Sample preparation

- Calibrators are prepared with clean urine matrix from Golden West Biologicals
- Isotopically labelled Internal standards
- 24 hours collection of urine
- Native urine for free catecholamines and metabolites (typically for catecholamines)
- Acid-hydrolysed urine for total (typically for metanephrines)
- Solid phase extraction (SPE) is used to cleanup urine

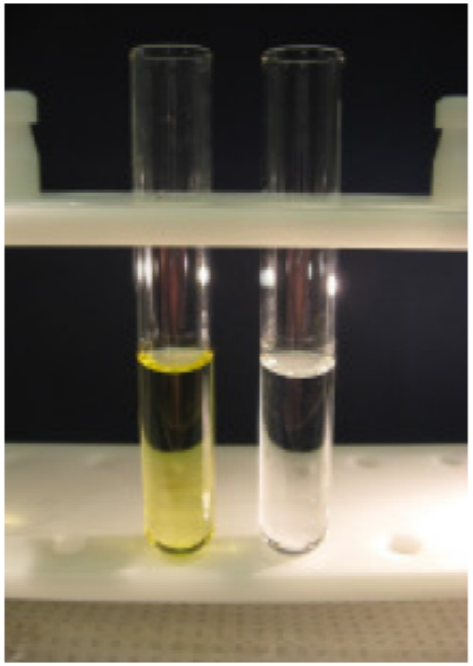
Solid Phase Extraction (SPE)



Conditioning



Clean-up



Before and after clean-up

Diphenyl boronate-catecholamine complex

"The diphenyl boronate forms a stable negatively charged complex (Fig. 1) with cis-hydroxyl groups of catecholamines, which is strongly retained on a C18 extraction sorbent when operating in alkali media. This allows for column washing with methanol-buffer solutions to remove interfering compounds without the loss of the catecholamines which are eluted by disrupting the complex under acid conditions".

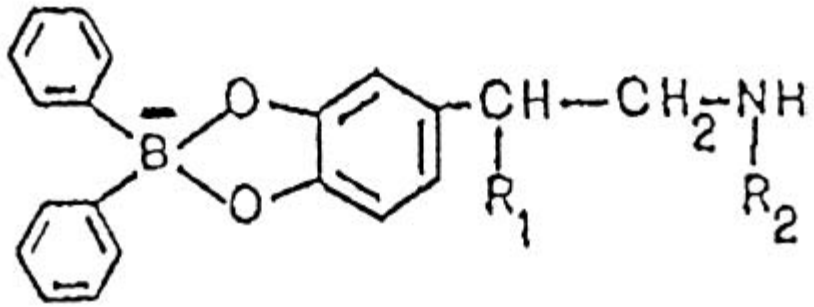


Fig. 1. Diphenyl boronate–catecholamine complex structure. (For noradrenaline: R1=OH, R2=H; for adrenaline: R_1=OH, R_2=CH_3; for dopamine: R_1=H, R_2=H). Adapted from Ref. [6].

[2] Talwar et al., Journal of Chromatography B, 769 (2002) 341–349

Catecholamines and metanephrines in a single run

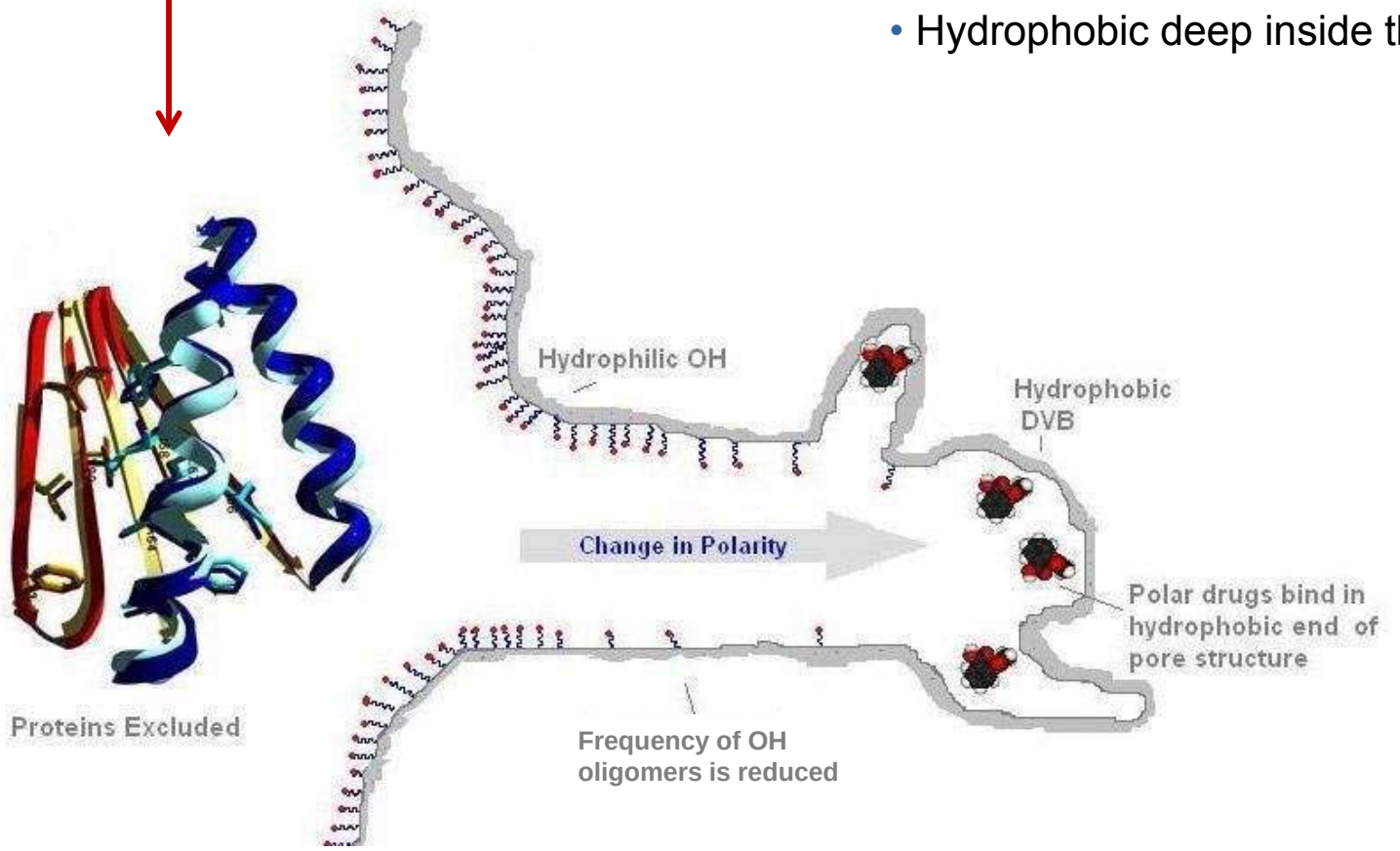
- One SPE cartridge is used to recover both catecholamines and metanephrines
- SPE Bond Elut Plexa was chosen for best recoveries
- Simple acid elution for direct injection into LC-MS/MS
- pH control for stabilization of catecholamines
- Metanephrines are also retained under the same conditions, even though they are methylated and do not contain the cis-diol moiety for the covalent linkage binding mechanism
- Studies have shown that metanephrines do have some affinity for this sorbent

[1] Ann Clin Biochem 2009; 46: 129–136. DOI: 10.1258/acb.2008.008180

Bond Elut Plexa
A Unique "Polar Enhanced" Polymeric Sorbent

- Unique chemistry with Polarity gradient:
  - Hydrophilic OH ligands on the exterior
  - Frequency of OH is reduced as getting inside
  - Hydrophobic deep inside the pore

Going thru the Plexa cartridge

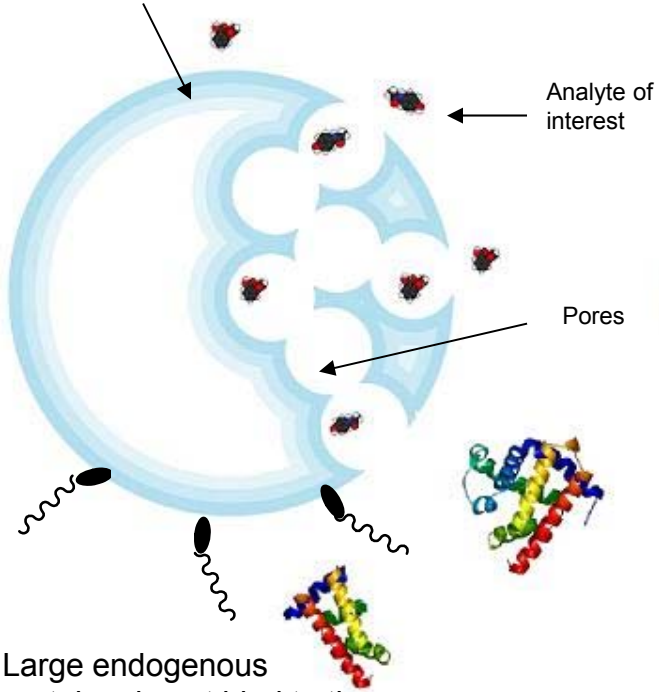


Plexa particle

Bond Elut Plexa – How does it works

Apply Sample

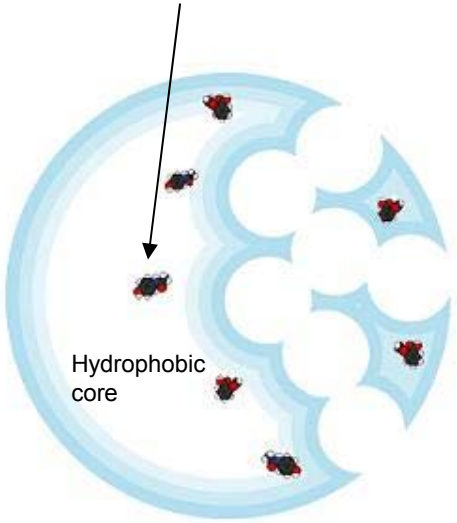
Water Rich Hydrophilic component allows excellent phase transfer of analytes into the polymer core



Large endogenous proteins do not bind to the surface of the polymer and cannot access pore structure.

Washing

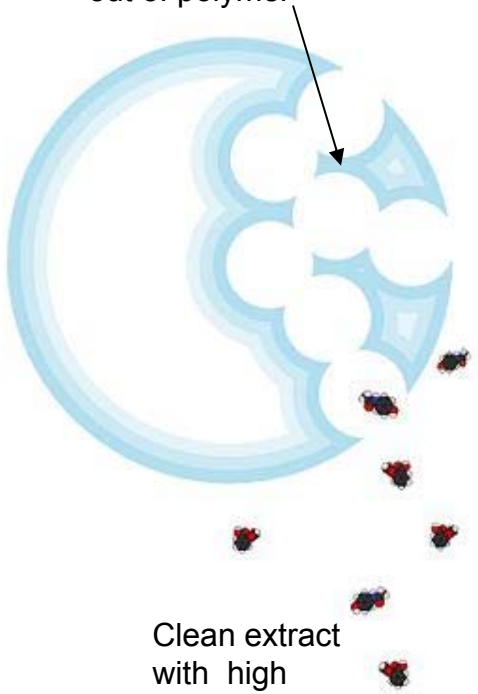
Analytes remain tightly bound in the hydrophobic core.



Interferences (lipids and proteins) washed away without into the analytes of interest

Elution

Specially engineered pore structure allows excellent mass transfer out of polymer



Clean extract with high recovery

Sample Preparation
Solid Phase Extraction (SPE)

Prepare complexed samples:

0.5 mL urine*, calibrators, QCs*
Add 40 µL of internal standards mix
Add 0.8 mL of complexing agent
Verify pH, must be between 7.5-9.5. If necessary adjust to pH 8.5 with NH4OH

Step 1: Condition SPE cartridge (Bond Elut Plexa, 30 mg, 3 mL) with:
1 mL of MeOH
1 mL of wash buffer 0.2 M NH4Cl-NH4OH

Step 2: Add complexed samples

Step 3: Wash with 1 mL of 5% MeOH wash buffer 0.2 M NH4Cl-NH4OH
Dry at full vacuum for 5 minutes

Step 4: Elute with 1 mL of 5% formic acid in water. Apply vacuum 5" Hg for 30 seconds
Transfer to autosampler vial

* Native for free catecholamines, hydrolyzed for total metanephrines
(add 25 µL HCl 6N, incubate at 90 deg. C for 25 min., cool at RT)

[1] Ann Clin Biochem 2009; 46: 129–136. DOI: 10.1258/acb.2008.008180
[2] Talwar et al., Journal of Chromatography B, 769 (2002) 341–349

Four Minute Method LC Conditions

Agilent 1290 LC

Analytical Column: Pursuit 3 PFP, 2 x 150 mm, 3 µm

Guard Column: Pursuit 3 PFP MetaGuard 2 mm

Columns Temp: 40 °C

Injection volume: 20 µL

Needle Wash: 1:1:1:1 MeOH:ACN:IPA:H_2O + 0.1% formic acid (20 sec)

Injector Temp: 4 °C

Mobile Phase:
A: 0.2% Formic Acid in Water
B: Methanol

Flow rate: 0.3 mL/min.

1290 Pump Gradient:

Time (min)	%B
0.0	2
0.5	2
1.5	60
4.0	60

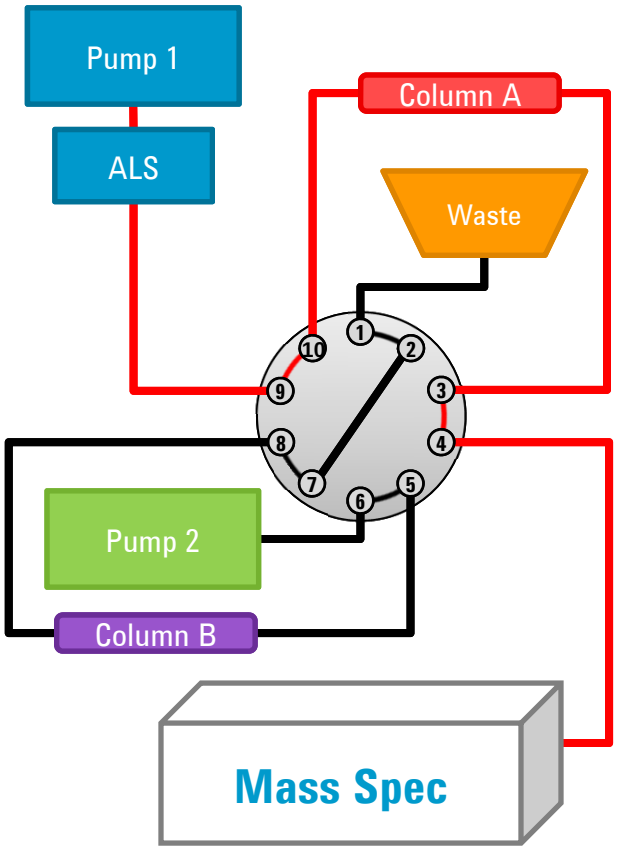
Stop time: 4 min.

Re-equilibration time: 3 min. (use automated column regeneration for increased throughput)

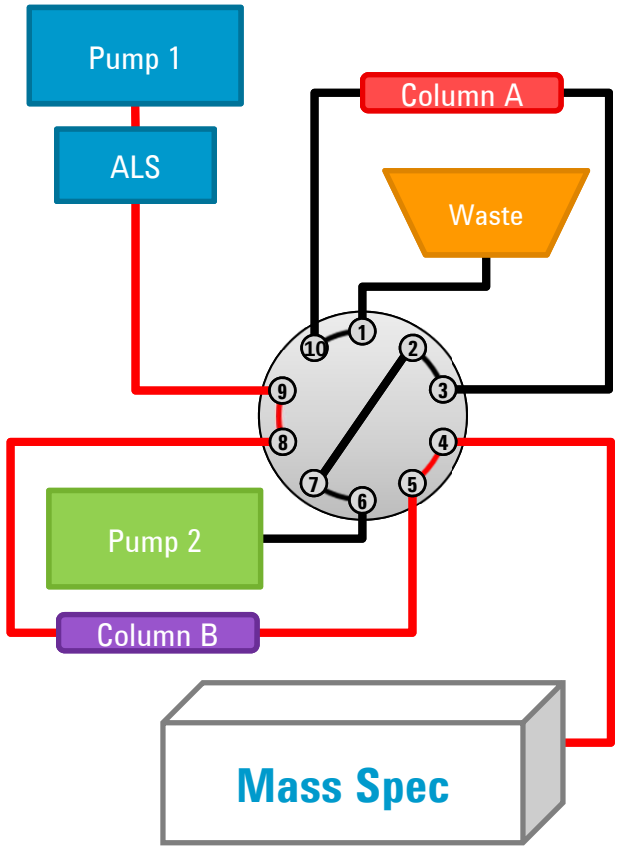
Automated Column Regeneration (ACR)

For increased throughput

Position 1 (Port 1 > 2)



Position 2 (Port 1 > 10)



QQQ MS/MS Method Conditions

Agilent 6460 QQQ Mass Spectrometer

Agilent 6460 QQQ MS/MS

Ion Source:	Agilent Jet Stream (AJS)
Ion Mode:	Positive Ionization

Ion source conditions

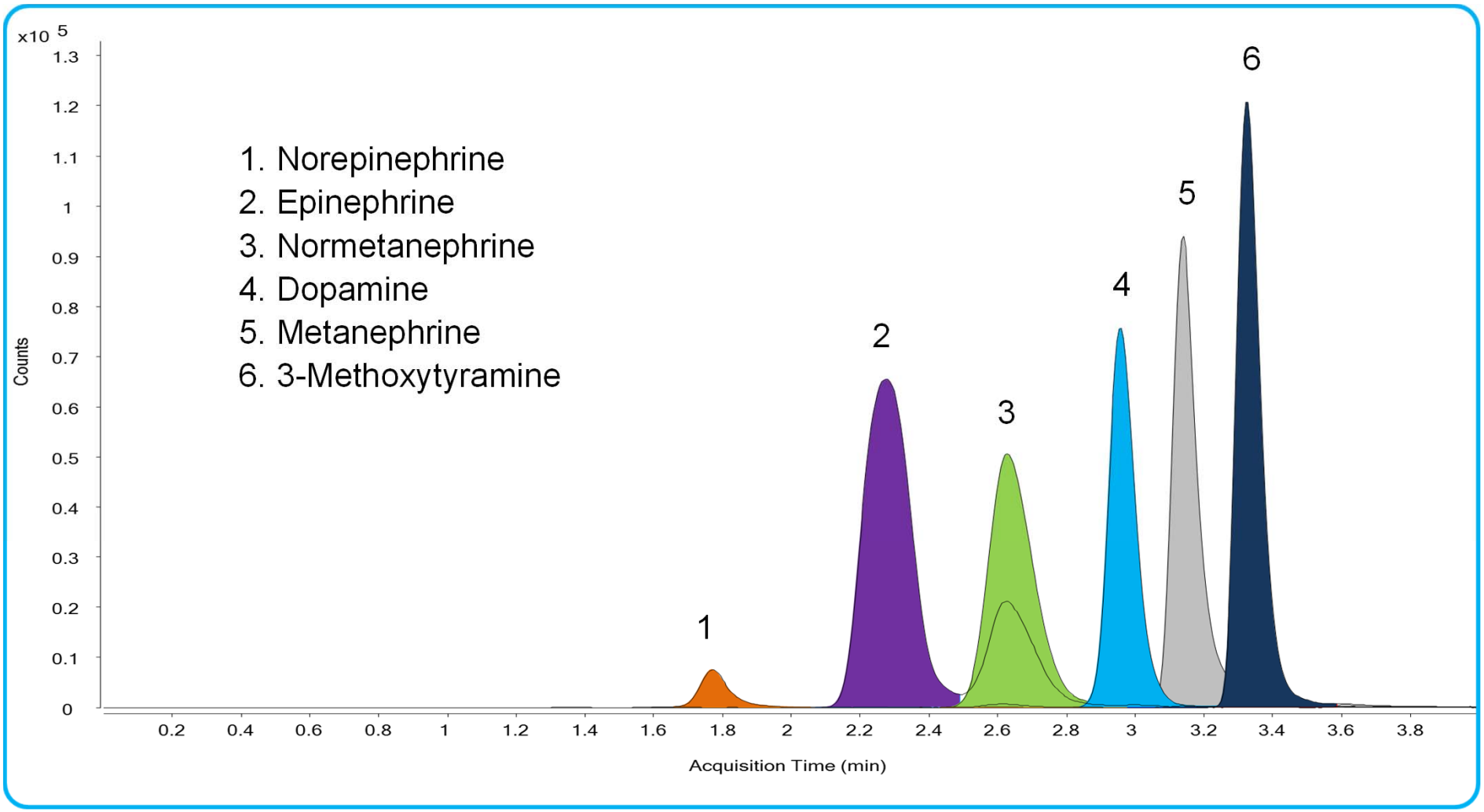
Drying gas temperature:	325°C
Drying gas flow:	5 L/min
Nebulizer pressure:	35 psi
Sheath gas temperature:	375°C
Sheath gas flow:	12 L/min
Capillary voltage:	3000 V
Nozzle voltage:	0 V
Q1/Q3 resolution:	0.7 unit
Δ EMV	200 V

MRM Transitions Table

Compound	Prec Ion	Prod Ion	Dwell	Frag (V)	CE (V)	CAV (V)
Dopamine*	154.1	137.1	20	75	8	3
Dopamine	154.1	91.1	20	75	28	3
Dopamine-D4	158.1	141.1	20	75	8	3
Norepinephrine*	170.1	152.1	20	65	4	5
Norepinephrine	170.1	107	20	65	20	5
Norepinephrine-D6	176.1	158.1	20	65	4	5
Epinephrine*	184.1	166.1	20	70	8	5
Epinephrine	184.1	107.1	20	70	24	5
Epinephrine-D6	190.1	172.1	20	70	8	5
3-Methoxytyramine*	151.1	91.1	20	135	20	3
3-Methoxytyramine	151.1	119	20	135	12	3
3-Methoxytyramine-D4	155.1	95.1	20	135	24	3
Normetanephrine*	166.1	134	20	105	16	3
Normetanephrine	166.1	106.1	20	105	20	3
Normetanephrine-D3	169.1	137.1	20	105	16	3
Metanephrine*	180.1	165.1	20	120	16	5
Metanephrine	180.1	148.1	20	120	16	5
Metanephrine-D3	183.1	168.1	20	120	16	5

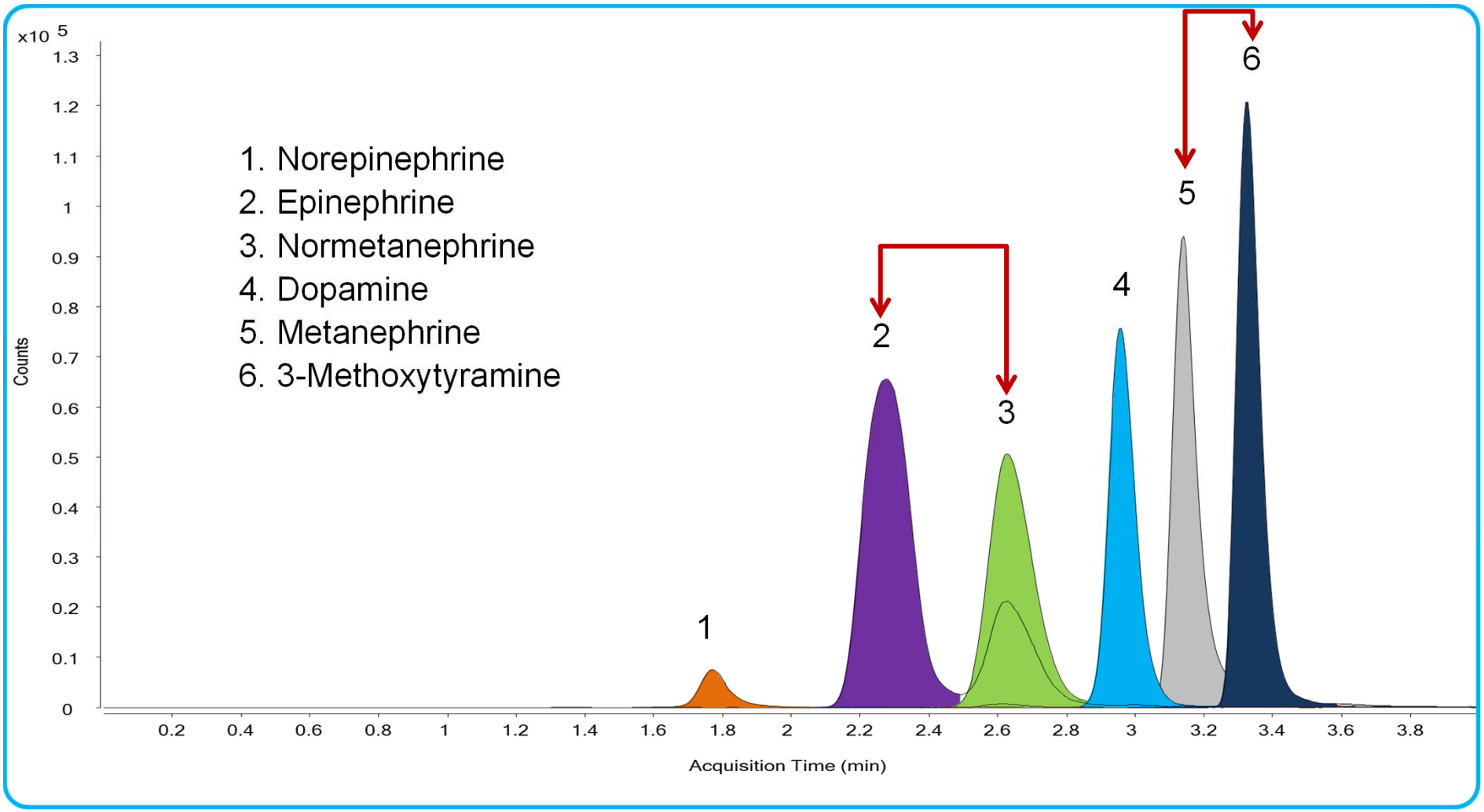
* = Quantification transition

Example chromatogram

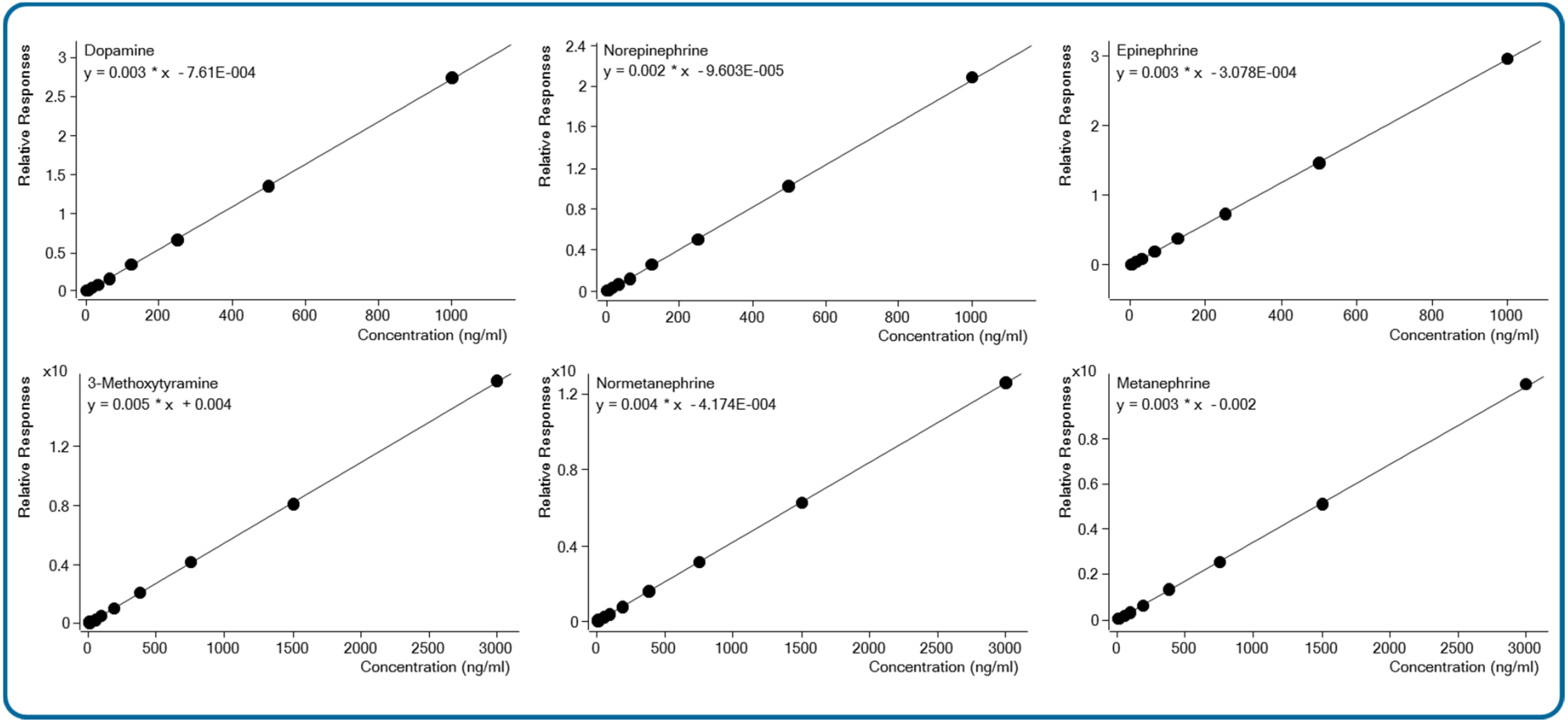


Example chromatogram

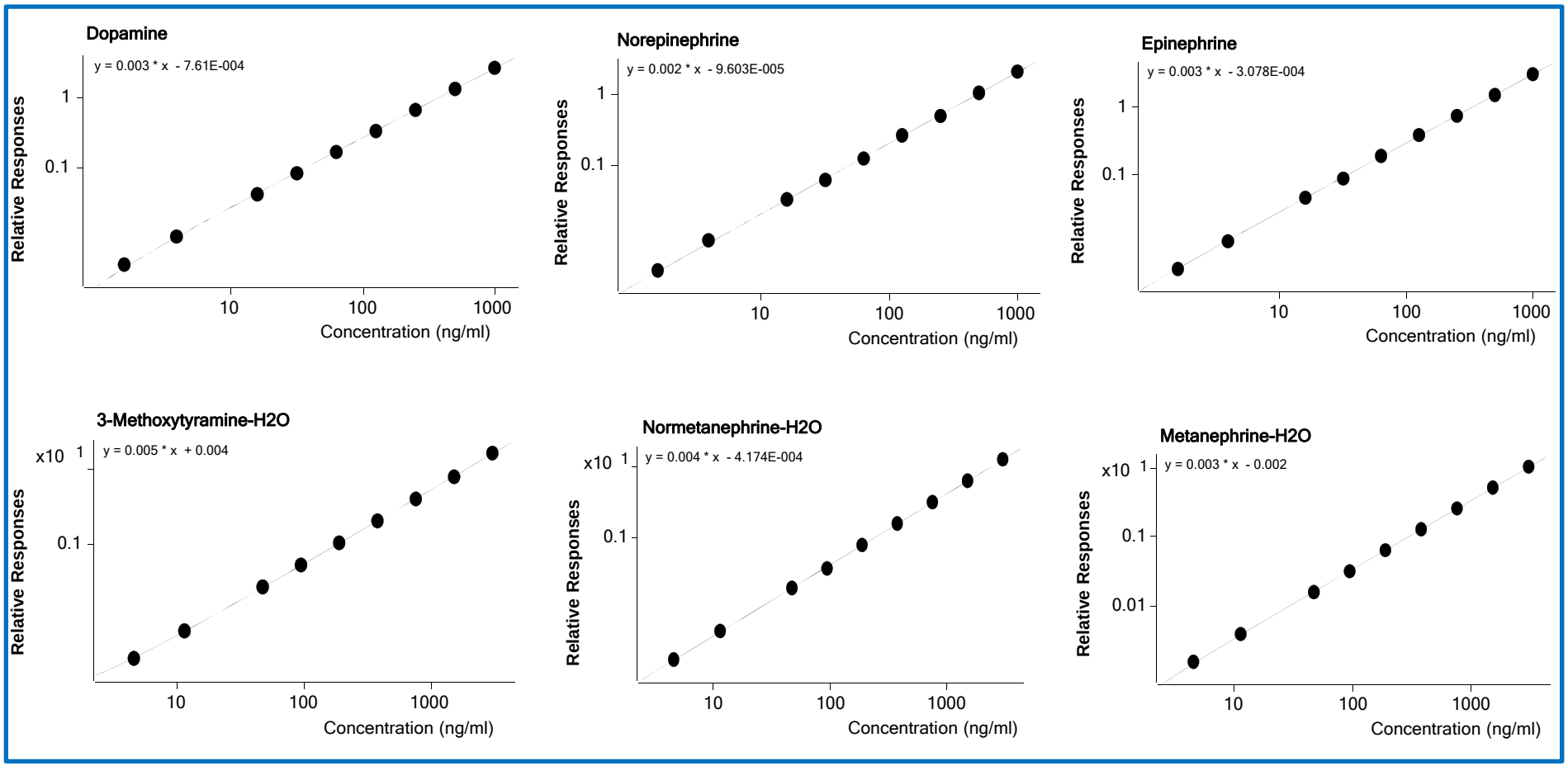
E/NMN and MN/3-MT Resolution is Critical



Calibration curves



Calibration curves in log scales



Results

Summary of Analyte Performance

Compound	R^2	Concentration (ng/mL)	Concentration (nmol/L)	Accuracy (%) n = 3	Intraday CV (%) n = 3	Interday CV (%) n = 5
Dopamine	0.9997	1.56	10.2	107.5	1.0	2.7
		62.5	408.0	99.1	1.7	2.0
		1000	6528.3	101.3	0.1	0.3
Norepinephrine	0.9999	1.56	9.2	102.9	0.9	5.4
		62.5	369.4	101.1	3.5	4.0
		1000	5910.9	101.1	0.6	0.6
Epinephrine	0.9998	1.56	8.5	101.6	4.3	2.7
		62.5	341.2	100.9	2.5	2.0
		1000	5458.4	100.3	0.4	0.3

Note: Signal to noise ratios and CVs indicate that LLOQs are lower than measured here for all analytes

Results

Summary of Analyte Performance

Compound	R^2	Concentration (ng/mL)	Concentration (nmol/L)	Accuracy (%) n = 3	Intraday CV (%) n = 3	Interday CV (%) n = 5
3-Methoxytyramine	0.9999	4.69	28	95.7	1.1	3.6
		187.5	1121.4	102.9	0.9	2.0
		3000	17942.1	100.0	0.2	0.3
Normetanephrine	0.9999	4.69	25.6	100.1	1.5	3.2
		187.5	1023.45	102.0	1.1	2.5
		3000	16375.2	100.7	0.2	0.2
Metanephrine	0.9999	4.69	23.8	100.5	0.3	2.8
		187.5	950.7	102.0	0.5	2.2
		3000	15210.6	100.8	0.1	0.2

Note: Signal to noise ratios and CVs indicate that LLOQs are lower than measured here for all analytes

Results

Inter-run Over 3 Days for Commercial QC (BioRad Lyphocheck)

Compound	Free/Total	Level 1			Level 2		
		Range (HPLC)	Measured	CV (%)	Range (HPLC)	Measured	CV (%)
Dopamine	Free	44.4 -75.0	61.4	3.4	377 – 629	509	2.8
Norepinephrine	Free	31.3 – 51.6	38.4	5.8	156 – 239	192	4.8
Epinephrine	Free	9.62 – 19.1	14.3	5.3	67.8 – 104	86.7	2
3-Methoxytyramine	Total	28.6 – 48.7	44.7	3.8	381 – 572	557.7	2.2
Normetanephrine	Total	220 - 366	300.7	2.4	1084 – 1630	1379.2	2.8
Metanephrine	Total	69.0 - 116	91.2	2	434 - 655	612	2.5

- All measurements are in **ng/mL**
- Bio-Rad QC material was used. Ranges provided were for free catecholamines and total metanephrines

Results

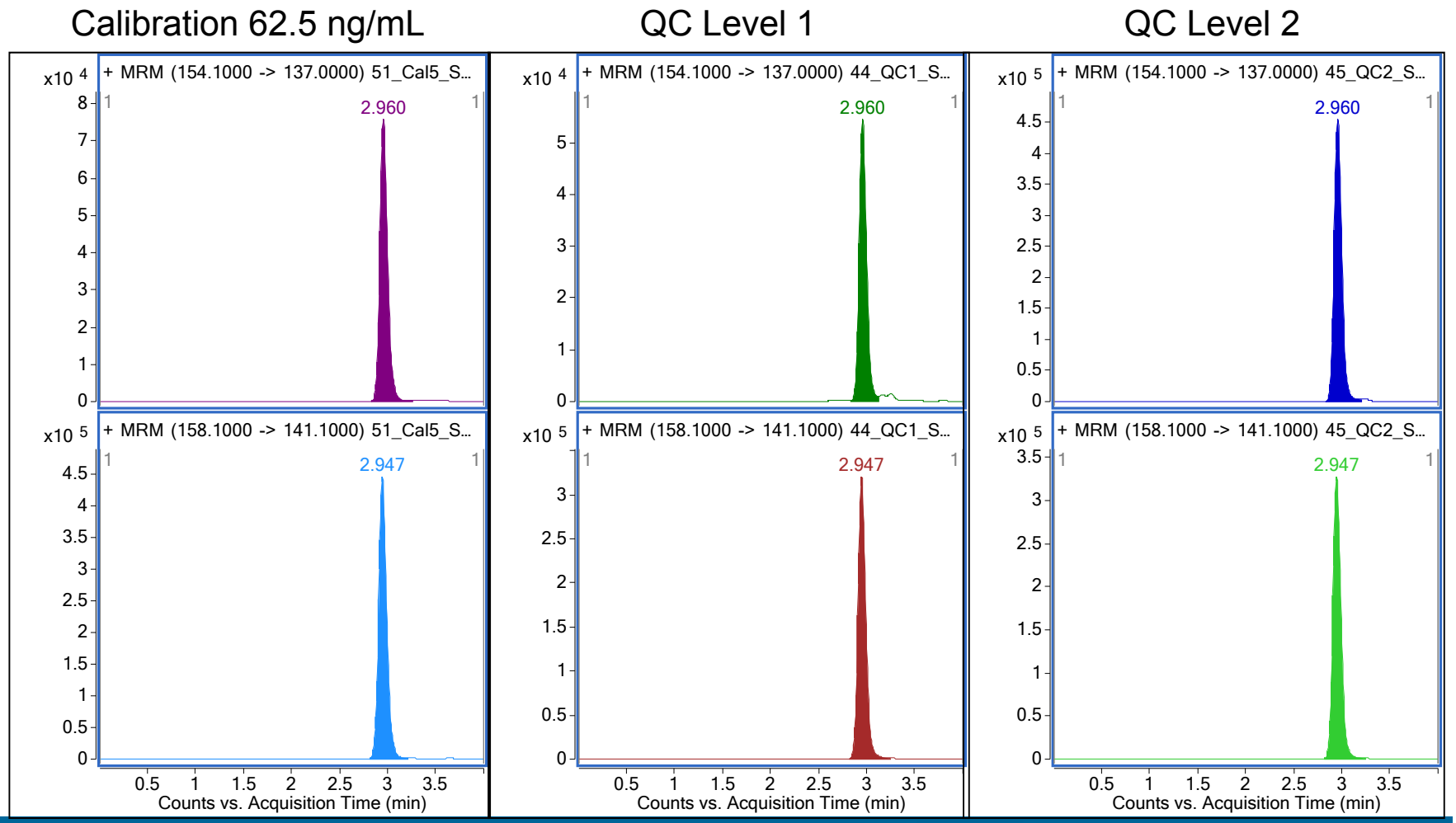
Inter-run Over 3 Days for Commercial QC (BioRad Lyphocheck)

Compound	Free/Total	Level 1			Level 2		
		Range (HPLC)	Measured	CV (%)	Range (HPLC)	Measured	CV (%)
Dopamine	Free	290-490	401	3.4	2465-4105	3323	2.8
Norepinephrine	Free	185-305	227	5.8	920-1410	1135	4.8
Epinephrine	Free	52.5-104	78	5.3	370-570	473	2
3-Methoxytyramine	Total	171-291	267	3.8	2280-3420	3335	2.2
Normetanephrine	Total	1200-2000	1641	2.4	5920-8900	7528	2.8
Metanephrine	Total	350-590	462	2	2200-3320	3103	2.5

- All measurements are in **nmol/L**
- Bio-Rad QC material was used. Ranges provided were for free catecholamines and total metanephrines

Example chromatogram

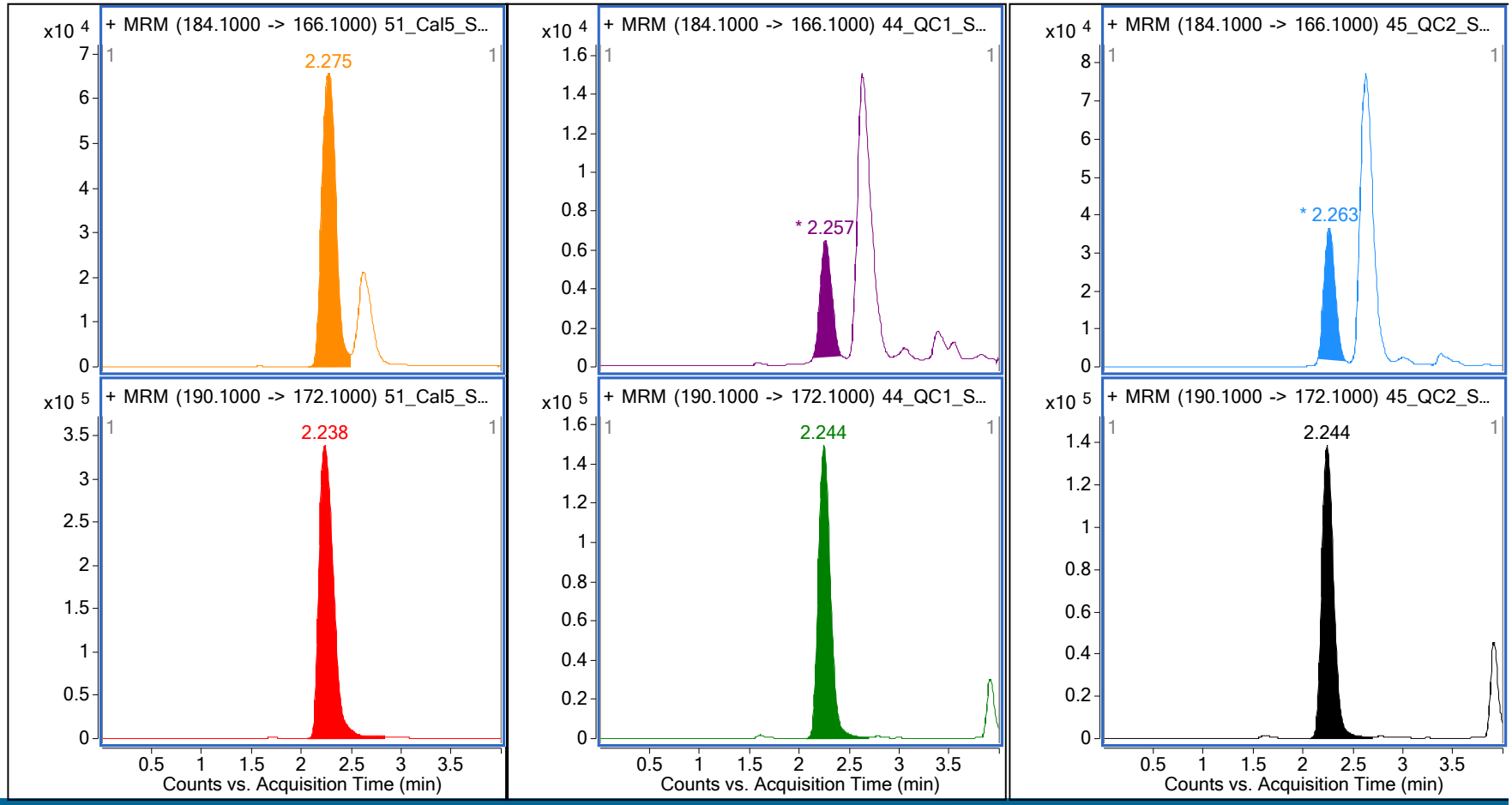
Dopamine and Dopamine-D4



Example chromatogram

Epinephrine and Epinephrine-D6

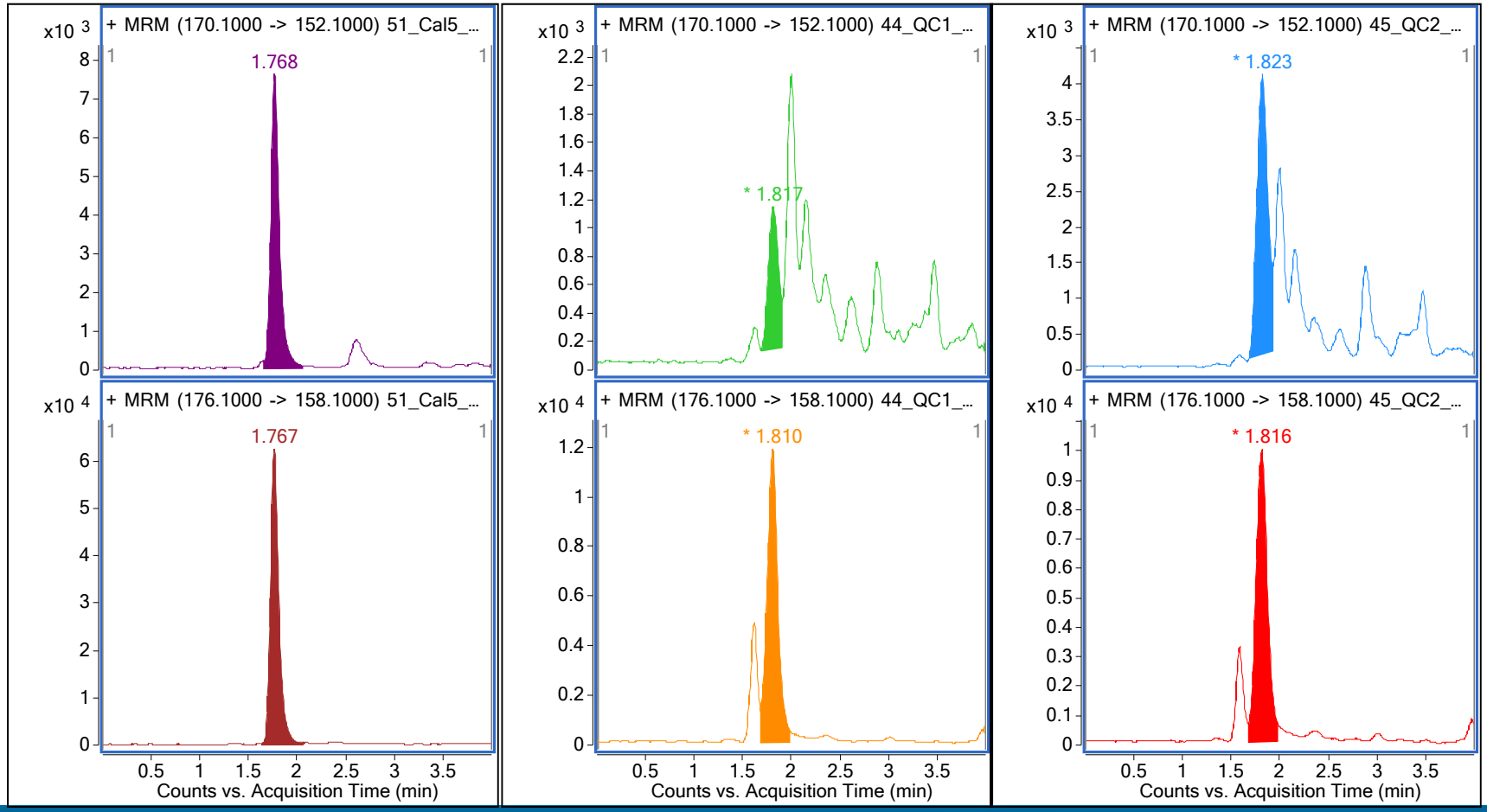
Separation from Normetanephrine and other interference is critical



Example chromatogram

Norepinephrine and Norepinephrine-D6

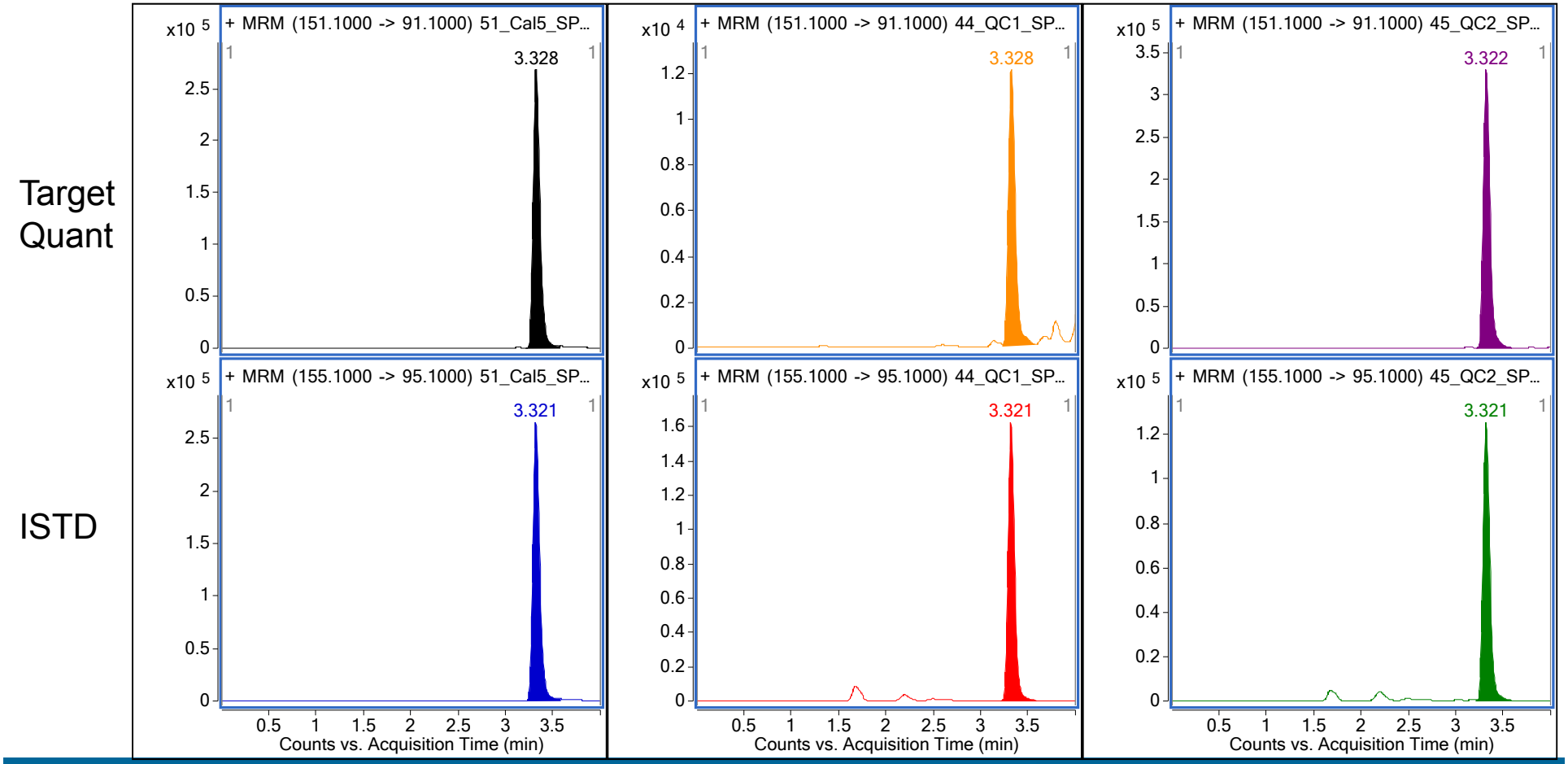
Separation from interferences is critical



Example chromatogram

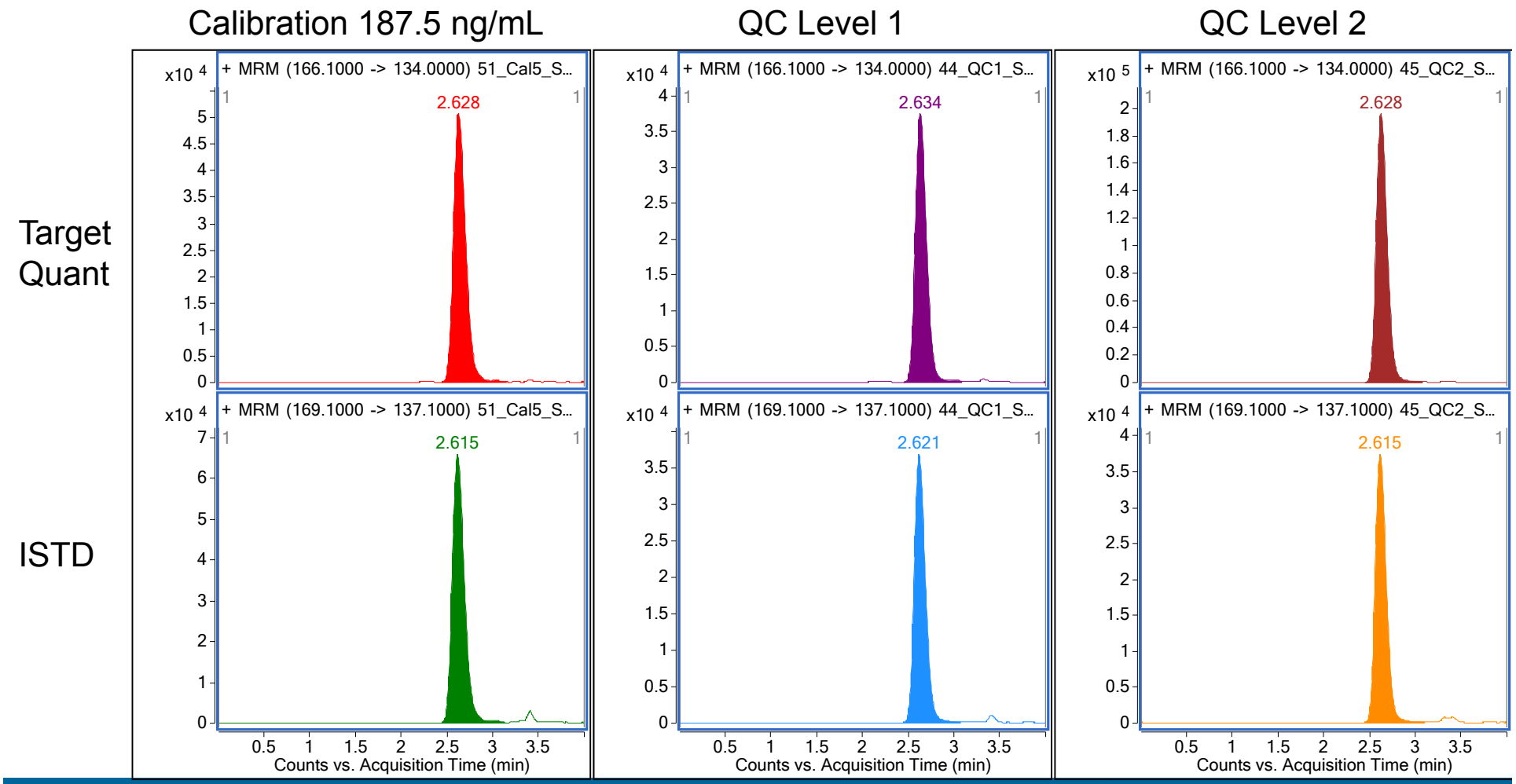
3-Methoxytyramine and 3-Methoxytyramine-D4

Separation from Metanephrine is critical



Example chromatogram

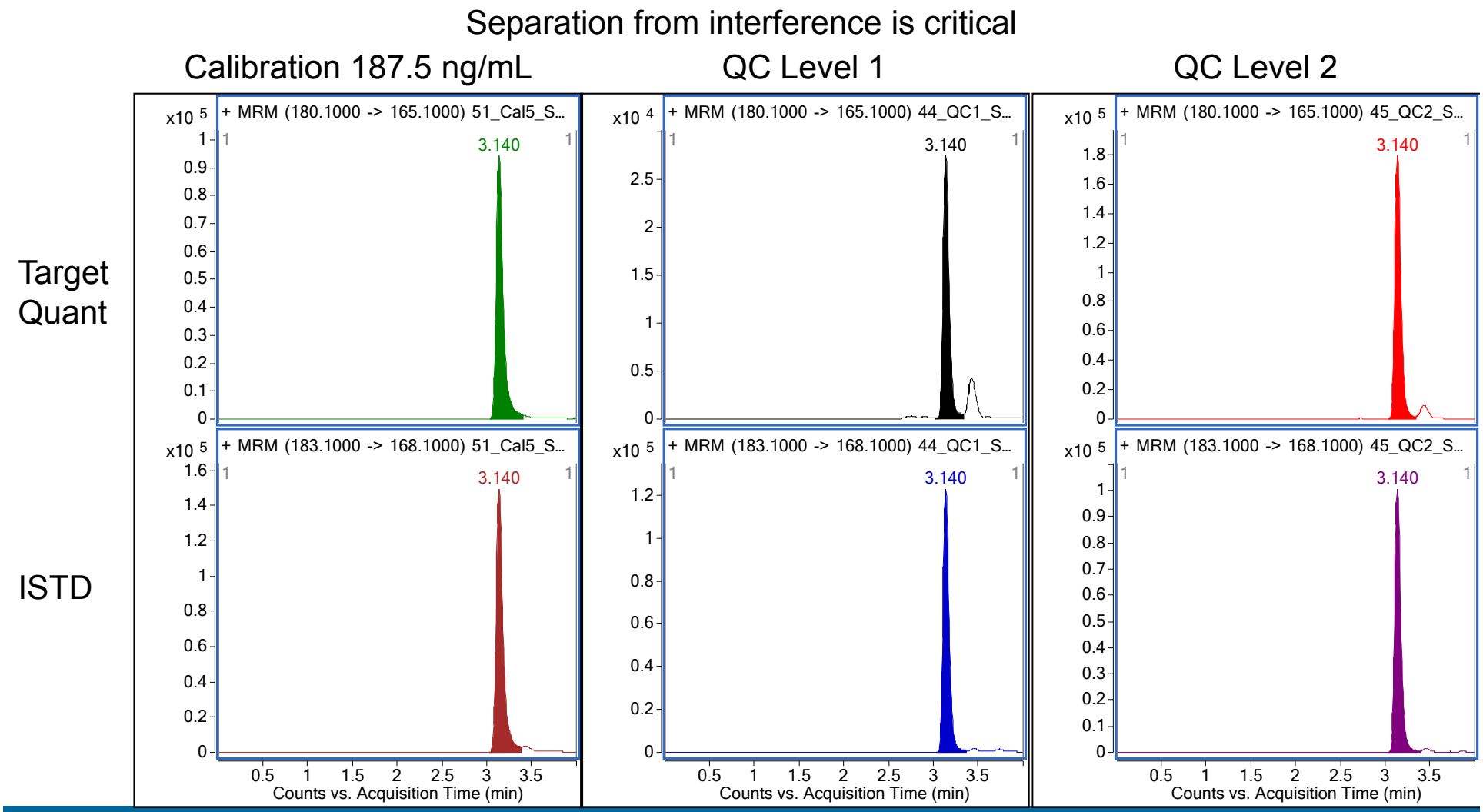
Normetanephrine and Normetanephrine-D3



Example chromatogram

Metanephrine and Metanephrine-D3

Separation from interference is critical



Results

Recoveries Observed Using SPE Procedure

Compound	Absolute Recoveries %* (n = 9)		Relative recoveries % With ISTDs corrections** (n = 9)		
	Average	SD	Range	Average	SD
Dopamine	73.5	2.4	95.0-103.4	100.0	2.5
Norepinephrine	112.5	4.9	99.0-102.4	100.0	1.0
Epinephrine	90.3	3.6	94.5-104.4	100.0	2.9
3-Methoxytyramine	53.2	3.6	94.4-102.5	100.0	3.0
Normetanephrine	88.7	7.5	97.3-102.0	100.0	2.0
Metanephrine	93.9	3.6	97.2-103.9	100.0	2.2

* ISTDs peak areas spiked in formic acid subjected to SPE compared with spiked formic acid without SPE

** Calculated concentrations with ISTDs peak area ratios corrections (with SPE) versus theoretical concentrations

Results

Matrix Effects Observed Using SPE Procedure

Compound	Matrix effects %* (n = 9)		Accuracies % With ISTDs corrections** (n = 9)		
	Average	SD	Range	Average	SD
Dopamine	102.1	3.9	95.4-102.5	100.0	2.3
Norepinephrine	30.1	5.2	94.7-104.4	100.0	3.6
Epinephrine	107.1	3.2	95.2-104.6	100.0	3.7
3-Methoxytyramine	88.2	8.5	95.4-104.0	100.0	3.1
Normetanephrine	93.8	3.1	96.4-101.8	100.0	2.8
Metanephrine	103.0	1.3	95.3-101.3	100.0	2.8

* ISTDs peak areas spiked in urine subjected to SPE compared with spiked formic acid subjected to SPE

** Calculated concentrations with ISTD corrections (urine with SPE) versus theoretical concentrations

Stability of extracted spiked urine

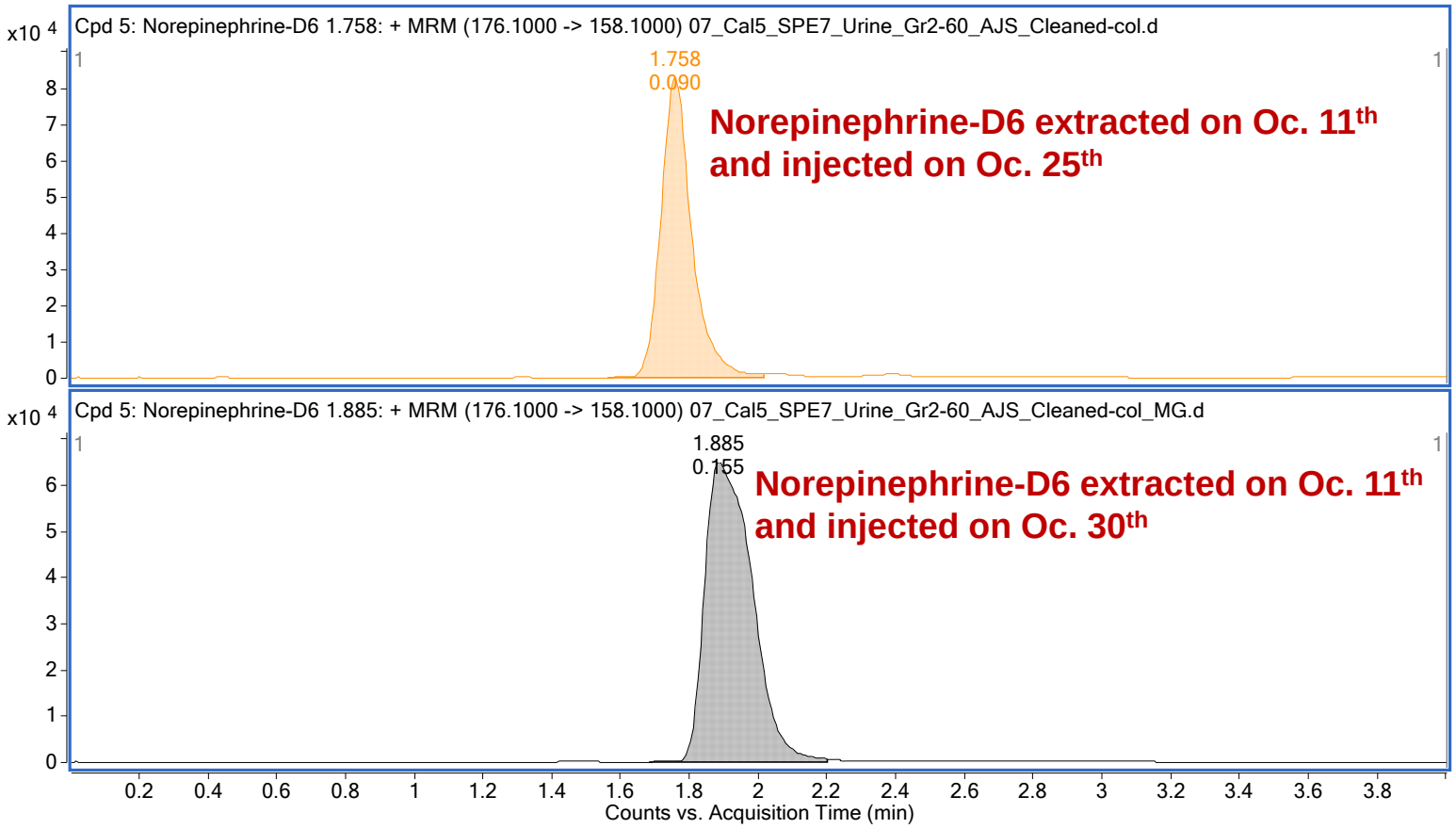
18 hours in Autosampler at 6ºC

Calibration Levels	Stability in Autosampler 18 hours at 6 ºC					
	% Difference					
	D	NE	E	3-MT	NMN	MN
1	0.7	-4.2	-1.2	-2.0	4.7	2.4
2	0.8	-2.8	0.7	1.5	0.4	0.5
3	0.0	-1.8	1.1	-0.4	1.3	0.4
4	-0.2	-4.6	1.9	-0.2	-0.8	0.3
5	-0.7	-0.9	1.7	-1.2	0.2	2.1
6	-0.8	0.6	1.1	0.6	0.9	-0.2
7	-0.4	-1.0	2.0	0.1	-0.5	-0.1
8	0.0	-1.5	0.9	1.0	-0.5	-0.6
9	-0.1	-0.3	0.7	-1.7	-0.6	-0.8

Stability of Extracted Spiked Urine

Urine Extracts Instability

Urine extracts kept at 4ºC



Tips and Tricks

- Mobile phase quality; grade and freshness
- Inline filter between needle seat and injection valve
- If sensitivity decreases, check cleanness of spray chamber and run Scan method to monitor background ions levels
- Use needle wash to reduce carryover
- Perform a wash method with 100% methanol at the end of a batch
- Use guard column and change when necessary
- Minimize extra column volume
- Delay volume adjustment

Conclusions

- A four minute method has been developed for quantifying catecholamines and their metabolites: epinephrine, norepinephrine, dopamine, metanephrine normetanephrine and 3-methoxytyramine for research
- Offline solid phase extraction (SPE) for simultaneous extraction of all six analytes from urine is shown with excellent recoveries
- Chromatographic separation of all six analytes with conditions compatible with LC-MS/MS have been developed
- Typical method performance results are well within acceptable criteria

References

[1] **Simultaneous measurement of urinary metanephrines and catecholamines by liquid chromatography with tandem mass spectrometric detection**
M J Whiting, Clinical Biochemistry and Pharmacology Laboratory, SA Pathology, Flinders Medical Centre, Bedford Park 5042, South Australia
Ann Clin Biochem 2009; 46: 129–136. DOI: 10.1258/acb.2008.008180

[2] **Extraction and separation of urinary catecholamines as their diphenyl boronate complexes using C solid-phase extraction 18 sorbent and high-performance liquid chromatography**
Dinesh Talwar*, Cathie Williamson, Allison McLaughlin, Alan Gill, Denis St.J. O'Reilly
Department of Clinical Biochemistry, *Macewen Building*, *Royal Infirmary*, *Glasgow G4 OSF*, *UK*
Journal of Chromatography B, 769 (2002) 341–349

[3] **Extraction of Catecholamines from Urine**
AN 1071A, Argonaut, Dr Wéber Consulting KFT,
www.weber.hu/PDFs/SPE/AN1071S_CatecholaminesUrine.pdf

Solutions preparations

Solutions preparations:	
0.2 % Formic acid in water:	2 mL of formic acid in 1 L water
5 % Formic acid in water:	50 mL of formic acid in 1 L water
HCl 0.1N in water:	830 uL of HCl 6N in 50 mL water
2 M NH4Cl-NH4OH buffer:	Dissolve 107g of NH4Cl in 1 L water and adjust pH to 8.5 with NH4OH (about 8 ml of NH4OH 50%).
	Store at 4 deg.C
Diphenyl-boronate complexing agent:	To 200 mL of 2 M NH4Cl-NH4OH buffer, add 400 mg of diphenylboronic acid ethanolamine ester
	and 1 g of disodium EDTA. Diphenylboronic acid does not dissolve easily and may require
	to mixing slowly overnight to dissolve completely. Adjust pH to 8.5 with NH4OH.
	Store at 4 deg.C and check pH before use
Wash buffer 0.2 M NH4Cl-NH4OH:	Add 50 mL of 2 M NH4Cl-NH4OH buffer to 450 mL of water. Add 250 mg EDTA.
	Adjust pH to 8.5 with NH4OH (about 3 ml of NH4OH 50%).
	Store at 4 deg.C and check pH before use
5% Methanol in wash buffer 0.2 M NH4Cl-NH4OH:	Add 25 mL of methanol to 475 mL of wash buffer 0.2 M NH4Cl-NH4OH.
	Adjust pH to 8.5 with NH4OH.
	Store at 4 deg.C and check pH before use

Supplies

Supplier:	Part number:	Description:
Agilent	12109303	SPE cartridges Bond Elut Plexa, 30 mg, 3 mL, 50/pk
Agilent	12234022	Vac Elut SPS 24 (SPE manifold)
Sigma	57120-U	SPE vacuum trap
VWR	54908-037	Vacuum Pressure Pump, Gast®
Agilent	5182-0868	Screwcap vials with septa, 500/pk
Agilent	A3051150X020	HPLC column Pursuit 3 PFP, 2 x 150 mm, 3 µm
Agilent	A3051MG2	Meta Guard column Pursuit 3 PFP, 2 mm, pk 3
Agilent	5067-4638	1290 Infinity Inline filter, 0.3 µM

Chemicals

Supplier:	Part number:	Description:
VWR	LC230-2.5	Burdick & Jackson Methanol LCMS grade
Sigma-Aldrich	94318	Formic acid
Golden West Biologics	MSG5000	DC Mass Spect Gold urine
Sigma-Aldrich	D9754-25G	2-Aminoethyl diphenylborinate
Biorad	376	Lyphochek Quantitative Urine control, Level 1 Normal
Biorad	377	Lyphochek Quantitative Urine control, Level 2 Abnormal
VWR	RC375032	Hydrochloric acid 6.0 N
VWR	CAJT0660-1	Ammonium Chloride

Standards

Cerilliant (http://cerilliant.com)

Item	Description
C-109	Catecholamine Mix 1 (Epinephrines), 1.0 mg/mL (as free base) of each component New
C-110	Catecholamine Mix 2 (Metanephrines), 1.0 mg/mL (as free base) of each component New
C-111	Catecholamine Metabolites Mix, 1.0 mg/mL of each component New
D-072	Dopamine-D_4 HCl, 100 µg/mL (as free base) New
D-081	Dopamine HCl, 1.0 mg/mL (as free base) New
E-077	(±)-Epinephrine-D_6, 100 µg/mL New
M-148	(±)-Metanephrine-D_3 HCl, 100 µg/mL (as free base) New
N-068	(±)-Normetanephrine-D_3 HCl, 100 µg/mL (as free base) New
N-069	(±)-Norepinephrine-D_6 HCl, 100 µg/mL (as free base) New

Cambridge Isotopes: PN: DLM2739-0.05g, Description: 3-methoxytyramine-d4 HCl

Medical Isotopes: PN: 5376, Description: 3-methoxytyramine HCl

Questions

Thank You!

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Agilent Technologies
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Clinical Applications Specialist

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