Second Laboratory Demonstration of US EPA LC-MS/MS Methods

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Presented at 2015 WCTOW, Calgary AB

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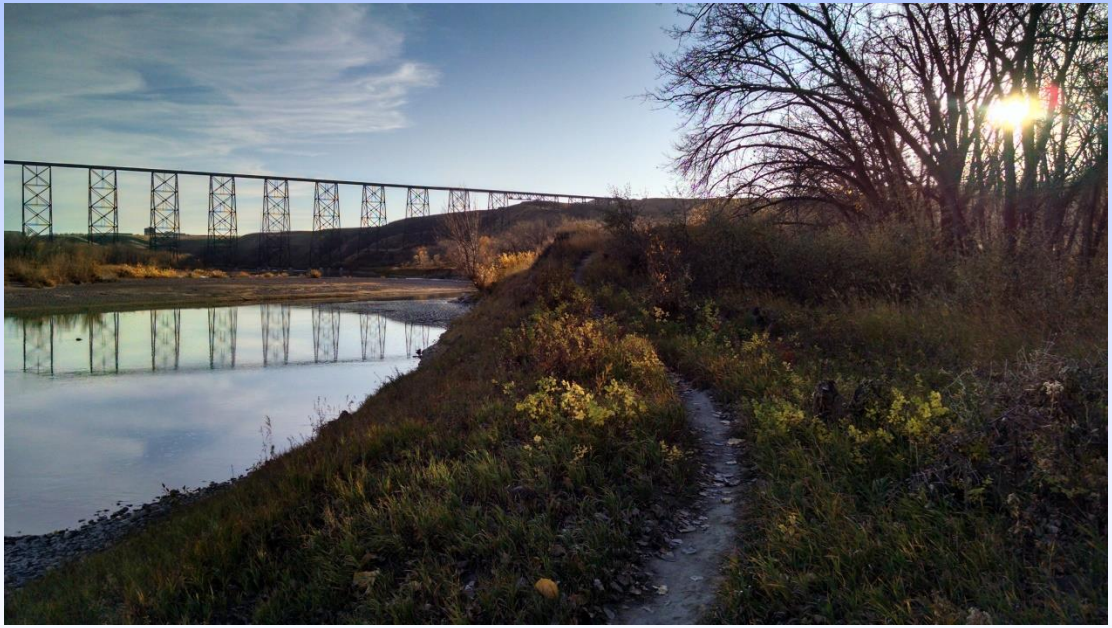
- LC and GC-MS and MS/MS
  - Method Development
  - Training
- Quality Systems
  - QMS Manual & Document
  - Method Validation



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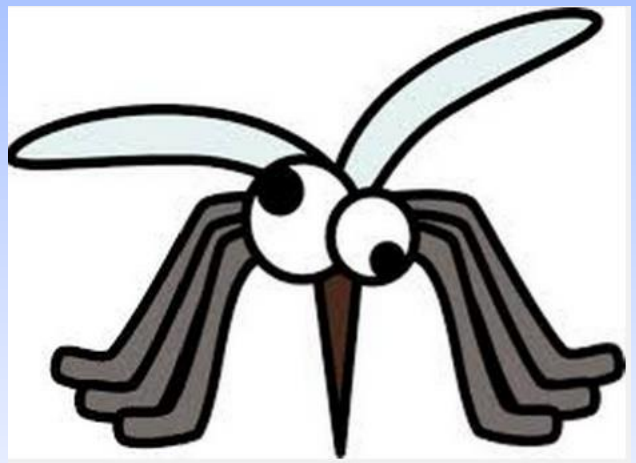
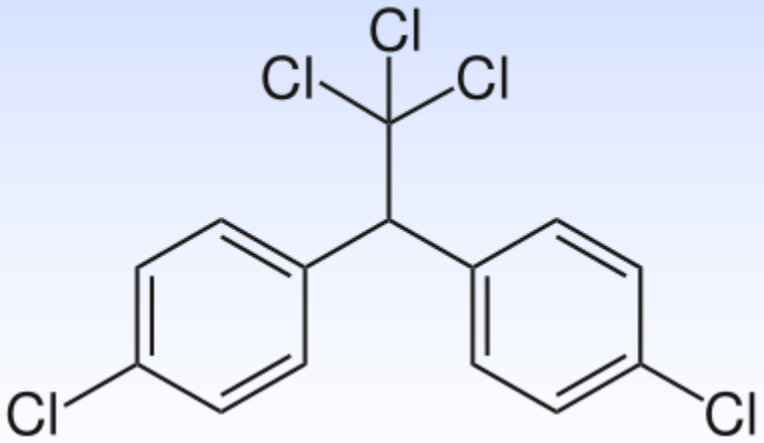
Get your Geek on… 70's style



A mosquito was heard to complain
that a chemist had poisoned his brain.
The cause of his sorrow
was para-Dichloro-
diphenyltrichloroethane



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US EPA Second Lab Demonstration

- Requires Initial Demonstration of Capability
  - Low system background
  - Precision
  - Accuracy
  - Detection Limits
    - MRL (Min Reporting Limit)
    - DL (Detection Limit)
    - LCMRL (Lowest Concentration Min Reporting Level)

US EPA – Initial Demonstration

TABLE 11. INITIAL DEMONSTRATION OF CAPABILITY QUALITY CONTROL REQUIREMENTS

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
Sect. 9.2.1 and 9.3.1	Initial Demonstration of Low System Background	Analyze LRB prior to any other IDC steps.	Demonstrate that all method analytes are below 1/3 the MRL and that possible interferences from extraction media do not prevent the identification and quantification of method analytes.
Sect. 9.2.2	Initial Demonstration of Precision (IDP)	Analyze four to seven replicate LFBs fortified near the midrange calibration concentration.	%RSD must be <20%
Sect. 9.2.3	Initial Demonstration of Accuracy (IDA)	Calculate average recovery for replicates used in IDP.	Mean recovery ± 30% of true value
Sect. 9.2.4	Minimum Reporting Limit (MRL) Confirmation	Fortify, extract and analyze seven replicate LFBs at the proposed MRL concentration. Calculate the Mean and the Half Range (HR). Confirm that the upper and lower limits for the Prediction Interval of Result (Upper PIR, and Lower PIR, Sect. 9.2.4.2) meet the recovery criteria.	Upper PIR ≤ 150% Lower PIR ≥ 50%
Sect. 9.2.5 and 9.3.7	Quality Control Sample (QCS)	Analyze a standard from a second source, as part of IDC.	Results must be within 70-130% of true value.
Sect. 9.2.6	Detection Limit (DL) Determination (optional)	Over a period of three days, prepare a minimum of seven replicate LFBs fortified at a concentration estimated to be near the DL. Analyze the replicates through all steps of the analysis. Calculate the DL using the equation in Sect. 9.2.6.	Data from DL replicates are not required to meet method precision and accuracy criteria. If the DL replicates are fortified at a low enough concentration, it is likely that they will not meet precision and accuracy criteria.

NOTE: Table 11 is intended as an abbreviated summary of QC requirements provided as a convenience to the method user. Because the information has been abbreviated to fit the table format, there may be issues that need additional clarification, or areas where important additional information from the method text is needed. In all cases, the full text of the QC in Section 9 supersedes any missing or conflicting information in this table.

US EPA Second Lab Demonstration

- Why participate?
  - Sucker for punishment
  - Acknowledgement

13.3 SECOND LABORATORY DEMONSTRATION – Performance of this method was demonstrated by multiple laboratories, with results similar to those reported in Section 17. The authors wish to acknowledge the assistance of the analysts and laboratories for their participation in the multi-laboratory verification studies: 1) Don Noot and Ralph Hindle of Vogon Laboratory Services Ltd., Cochrane, AB, Canada and 2) Karen A. Randazzo, Kevin Durk, and Amanda Comando of Suffolk County Water Authority, Hauppauge, NY.

  - Someone pays you

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Acronyms

CCC – continuing calibration check

DL – detection limit

FD – field duplicates

IDC – initial demonstration of capability

IS – internal standard

LFB – laboratory fortified blank

LFSM - laboratory fortified sample matrix

LFSMD - laboratory fortified sample matrix duplicate

LRB – laboratory reagent blank

LCMRL – lowest concentration minimum reporting level

MRL – minimum reporting level

PDS – primary dilution standard

QCS – quality control sample

RW – reagent water

SSS – stock standard solution

SUR - surrogate

NOTE: In order to obtain meaningful percent recovery results, correct the measured values in the LFSM and LFSMD for the native levels in the unfortified samples, even if the native values are less than the MRL. This situation and the LRB are the only permitted uses of analyte results below the MRL.

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Accuracy and Precision

- For drinking water methods, typically performed on fortified LFBs (Reagent Water)
- Precision must have %RSD ≤ 20%
- Accuracy must be ± 20% of the true value

LCMRL Calculations

- Lowest Concentration Minimum Reporting Level
- LCMRL is defined as the lowest spiking concentration at which recovery of between 50 and 150% is expected 99% of the time by a single analyst
- Requires minimum of 4 replicates at each of 7 fortification levels in matrix (drinking water), plus 4 LRBs

LCMRL Calculations

- Calculations are determined by entering values in EPA-supplied LCMRL Calculator[4,]
- Takes into account both precision and accuracy
- When the LCMRL value is not bracketed by the fortified sample levels, the calculator gives a warning that an additional set of replicates will be needed

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Prediction Interval of Results

Precision at the DL

- EPA requirement: lower and upper PIR
- limits set to 50 and 150%, respectively
- seven replicates at the proposed MRL
- both recovery and standard deviation are used to calculate the PIR
- as recovery deviates from 100%, the precision, (SD) must decrease in order to pass (see next slide).
- in fact, at 100% recovery, the maximum SD is 12.6%.
- this includes the entire method… *at the detection limit!*

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Prediction Interval of Results

Combined table showing combined Rec% and SD that pass.						
	0	5	12.4	12.5	12.6	15
50	Pass					
60	Pass					
70	Pass	Pass				
80	Pass	Pass				
90	Pass	Pass				
100	Pass	Pass	Pass	Pass	Pass	
110	Pass	Pass				
120	Pass	Pass				
130	Pass	Pass				
140	Pass					
150	Pass					
160						

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Method Flexibility

Typically, allowed to change:

- LC column
- LC gradient (but not mobile phase)
- MS/MS conditions

<u>Not allowed to change:</u>

- Sample collection and preservation
- Sample preparation
- QC requirements

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A Couple Examples

- US EPA 509
  - ethylenethiourea (ETU)
  - direct-injection using ESI and triple quadrupole MS/MS
- US EPA 543
  - Selected organic chemicals
  - Online SPE with ESI and triple quadrupole MS/MS

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US EPA 509

- EPA 509.1 is a draft method using electrospray LC-MS/MS for the analysis of Ethylenethiourea (ETU) in finished drinking water
- ETU is a degradation product of dithiocarbamate fungicides
- Samples are injected directly without prior extraction or concentration
- Method uses ETU-d4 as ISTD

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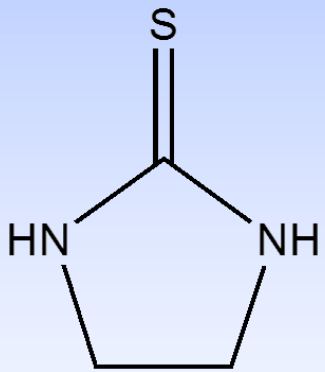
- EPA 509.1 compounds

Compound	Type	CAS No.	FWgt
Ethylenethiourea (ETU)	Target	96-45-7	102.0
ETU-d_4	Internal Standard	352431-28-8	106.1



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Instrumentation

HPLC	Mass Spectrometer
1260 ALS	6460 Triple Quadrupole
1290 Binary Pump	Agilent Jet Stream ESI Source
1290 TCC	MRM, positive mode

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Chromatographic Parameters

Parameter	Value
Column	Zorbax SB-Aq 3.0 x 150 mm 3.5 µm Part No. 863954-314
Injection Volume	60 µL
Mobile Phase	A = 1 mM Ammonium fluoride B = MeOH
Elution	0 %B isocratic
Flow Rate	0.5 mL/min
Column Temperature	40 ºC

Note: methanol is used for flushing the column after a set of samples has been analyzed

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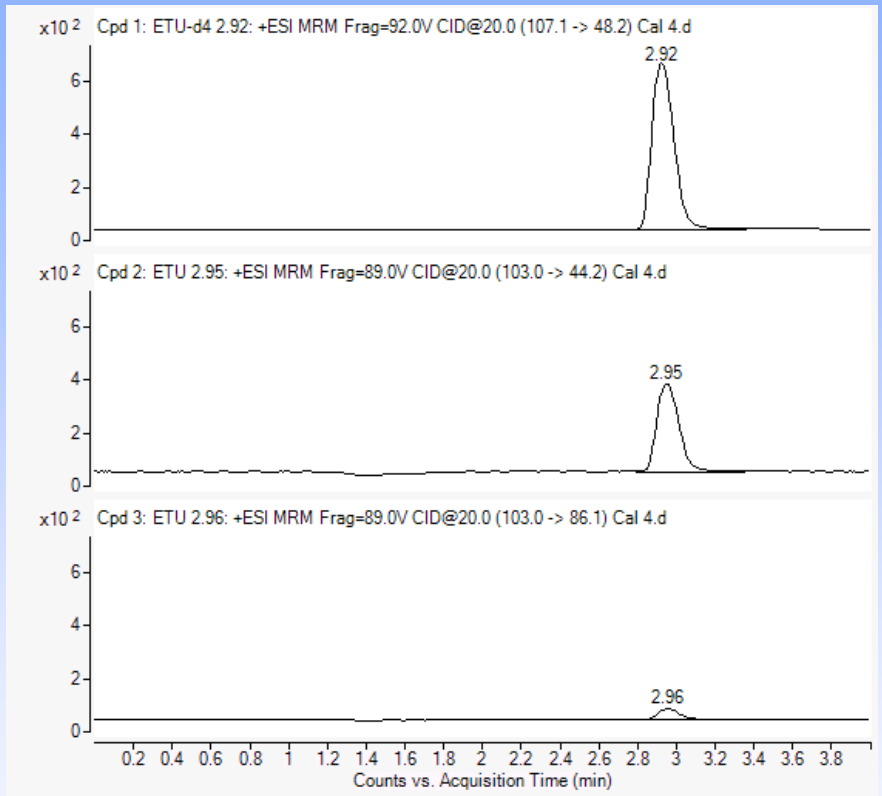
MS Source Parameters

Parameter	Value
Drying Gas Temp (ºC)	200
Drying Gas Flow (L/min)	4
Nebulizer (psi)	40
Sheath Gas Temp. (ºC)	380
Sheath Gas Flow (L/min)	12
Capillary Voltage (V)	2000
Nozzle Voltage (V)	0

MRM Parameters

Compound Name	Precursor	Product	Fragmentor	CE	Polarity	Type
ETU	103.0	86.1	89	20	Positive	Target
ETU	103.0	44.2	89	20	Positive	Target
ETU-d4	107.1	48.2	92	20	Positive	ISTD

Mid-Level calibrator



Zorbax SB-C18; 3.0 x 150 mm 3.5 u
A = 1 mM NH_4F
B = MeOH
Flow = 0.5 mL/min @ 40 °C
Isocratic @ 0 %B
Run time = 4 min

Top trace – MRM chromatogram for ISTD

Bottom traces – MRM chromatograms for target

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Calibration Levels

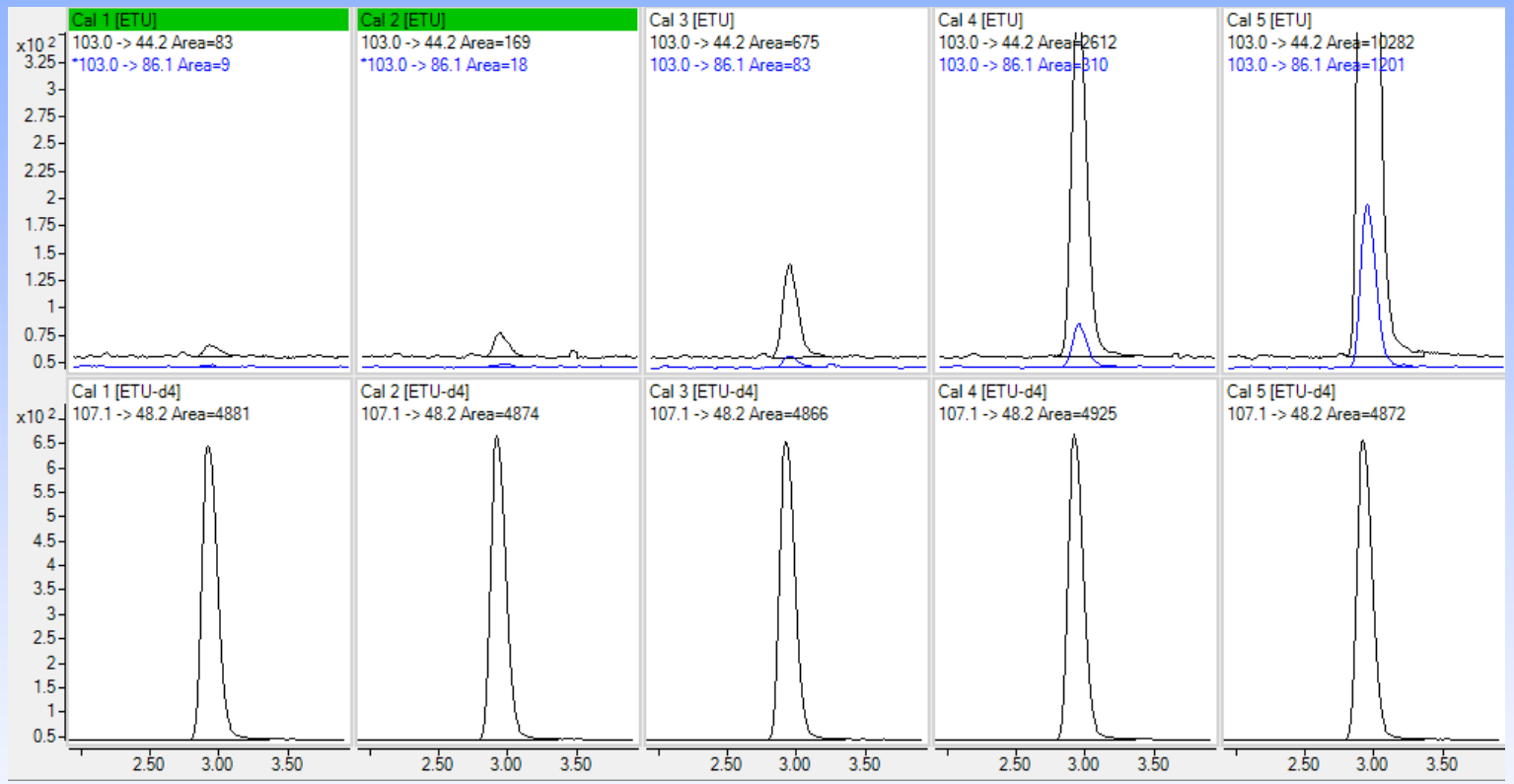
- ISTD: ETU-d4 added to each sample at 0.27 ng/mL

Calibration Level	Conc (ng/mL)
1	0.004
2	0.008
3	0.020
4	0.067
5	0.200
6	0.667
7	2.000

Calibration Levels 1-5

ETU Quantifier + Qualifier chromatograms

ETU-d_4 ISTD chromatograms



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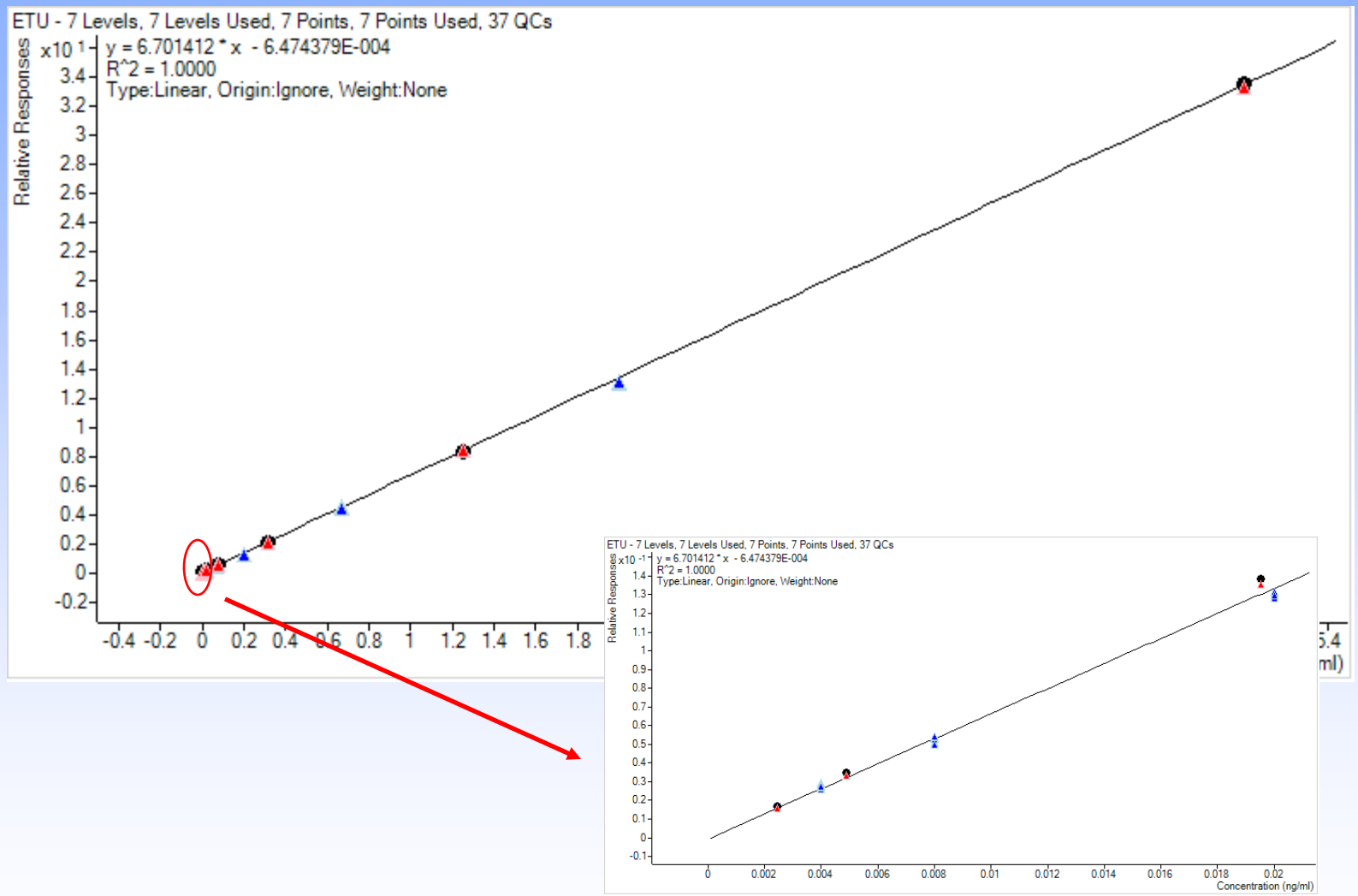
Linearity



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Section 9.2 - Initial Demonstration of Capability (IDC)

Exp Conc (ng/mL)	0.004	0.008	0.020	0.0667	0.200	0.667	2.000
Spike Replicate 1	111.1%	101.6%	98.7%	99.4%	96.3%	99.0%	97.0%
Spike Replicate 2	105.9%	94.1%	97.2%	97.1%	97.3%	98.8%	97.6%
Spike Replicate 3	105.5%	102.3%	97.9%	96.6%	96.7%	101.4%	97.0%
Spike Replicate 4	102.6%	100.1%	96.2%	99.5%	96.4%	99.0%	96.9%
Spike Replicate 5	99.6%	100.2%			96.1%		
Spike Replicate 6	98.9%	100.1%			96.9%		
Spike Replicate 7	112.4%	96.4%			96.9%		
Accuracy	**105.2%**	**99.3%**	**97.5%**	**98.1%**	**96.7%**	**99.6%**	**97.1%**
Precision	**5.0%**	**3.0%**	**1.1%**	**1.5%**	**0.4%**	**1.3%**	**0.3%**

3 levels were spiked with 7 replicates each, to ensure that an MRL could be properly determined, as described in the method.

All levels met Accuracy (± 20%) and Precision (≤ 20%) requirements.

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LCMRL and DL Results - ETU

Parameter	LCMRL	DL	Precision
EPA	6.1	2.8	6.8
Vogon	5.3	1.2	5.0

Vogon spikes at 4 ng/L
n = 7; t-stat = 3.365 at 99% confidence level

EPA spikes at 10 ng/L

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Lower and Upper PIR

Compound	HR_{PIR} (ng/L)	Lower PIR Limit	Upper PIR Limit	PIR Result
ETU	0.8	84.4%	126.0%	Pass

Seven replicates at or below proposed MRL
Calculate Mean and Standard Deviation (S) of replicates

$HR_{PIR} = 3.963 \times S$

Upper PIR Limit ≤ 150% Recovery
(Mean + HR_{PIR}) ≤ 150%

Lower PIR Limit ≥ 50% Recovery
(Mean - HR_{PIR}) ≥ 50%

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Summary

- Use of an Agilent 1290/6460 LC-MS/MS for the analysis of ETU in drinking water can help laboratories meet the stringent QC requirements of EPA Draft Method 509.1
- DLs can be lower than EPA levels, with LCMRL at 5.2 and DL at 1.2 ng/L, respectively
- Method %RSDs ranged from 0.3 – 5.0% including all fortification levels from 4 to 2,000 ng/L

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EPA 543 - Online SPE

- Some benefits of online SPE:
  - reduced labor costs through automation,
  - a high degree of precision as each sample is processed by the instrument and not a person,
  - improved sample turnaround due to vastly reduced sample preparation time,
  - reduced materials costs as the online SPE cartridges can be used for many samples (likely hundreds)
  - directly compatible with reversed phase LC
  - seamless incorporation of online SPE into Agilent LC-MS/MS systems using the Flexible Cube module

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Online SPE

- Online SPE can provide outstanding detection limits
- For example, in terms of the amount of target compound on-column:
  - online SPE using a 1.8 mL of sample volume

    is equivalent to processing

    360 mL sample by offline SPE
    concentrating the eluant to 1 mL
    and injecting 5 µL

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Materials and Equipment

- 6 mL sample vials with screw caps and pre-slit septa
- Agilent Bond Elut Online SPE, PLRP-S, 2.1x12.5 mm, 15-20 µm
- Water & ACN, Caledon HPLC grade
- Agilent Poroshell 120 PhenylHexyl, 3.0 x 100mm, 2.7µm
- Agilent 1260 / 1290 LC system
- Agilent 6460 QQQ, ESI with Agilent Jet Stream Technology

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Materials and Equipment

- Specific LC equipment used for automated online SPE:
  - Agilent 1260 Standard Autosampler (G1329B)
    - expanded injection range including a 900 µL metering head and 900 µL loop capillary, and multi-draw option including a 900 µL loop after the needle seat
    - two trays, each holding 15 - 6 mL vials

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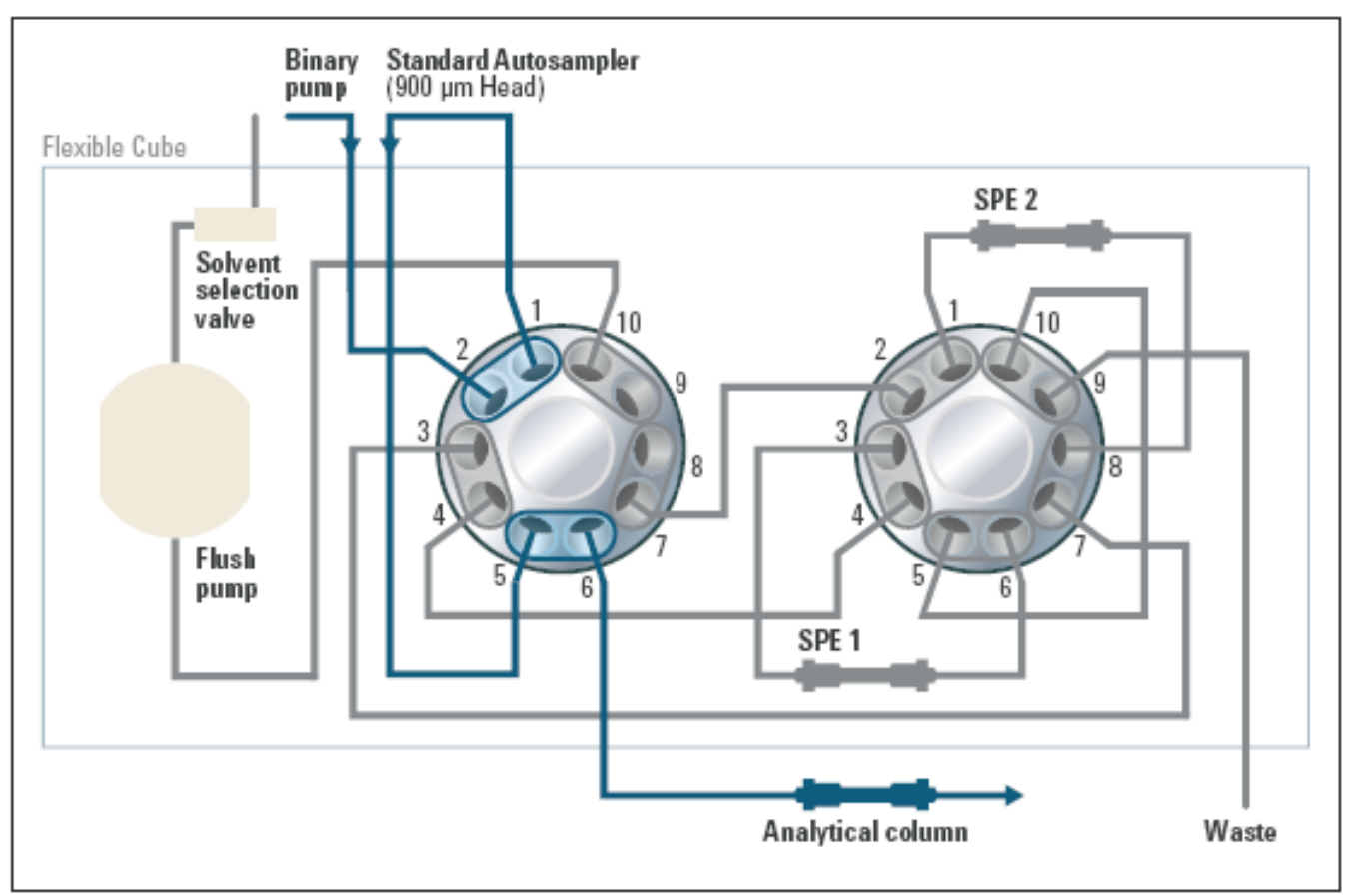
Materials and Equipment

- Specific LC equipment used for automated online SPE:
  - Agilent 1290 Flexible Cube
    - single piston pump and solvent selection valve allows use of three different solvents for SPE conditioning, sample loading, flushing & re-equilibration
    - left valve provides for direct injection to the analytical column or online SPE
    - right valve allows two SPE cartridges to be mounted
      - maximizes sample processing and use of the mass spectrometer (see next slide)

Flexible Cube Setup

Left Valve – showing direct injection to analytical column



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Materials and Equipment

- Agilent 1290 Flexible Cube Operation
  - sample is loaded on the 1st SPE cartridge where the target compounds are concentrated
  - the right valve rotates and the target compounds are backflushed off the SPE cartridge onto the analytical column for separation and detection by the mass spectrometer
  - during backflushing of the 1st cartridge, the 2nd cartridge undergoes flushing with a strong solvent (e.g. ACN) and then re-equilibration to initial sample conditions (e.g. buffered H_2O)
  - the next sample injection uses the 2nd cartridge for analysis while the 1st cartridge undergoes flushing / re-equilibration

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Flexible Cube – valves & cartridges

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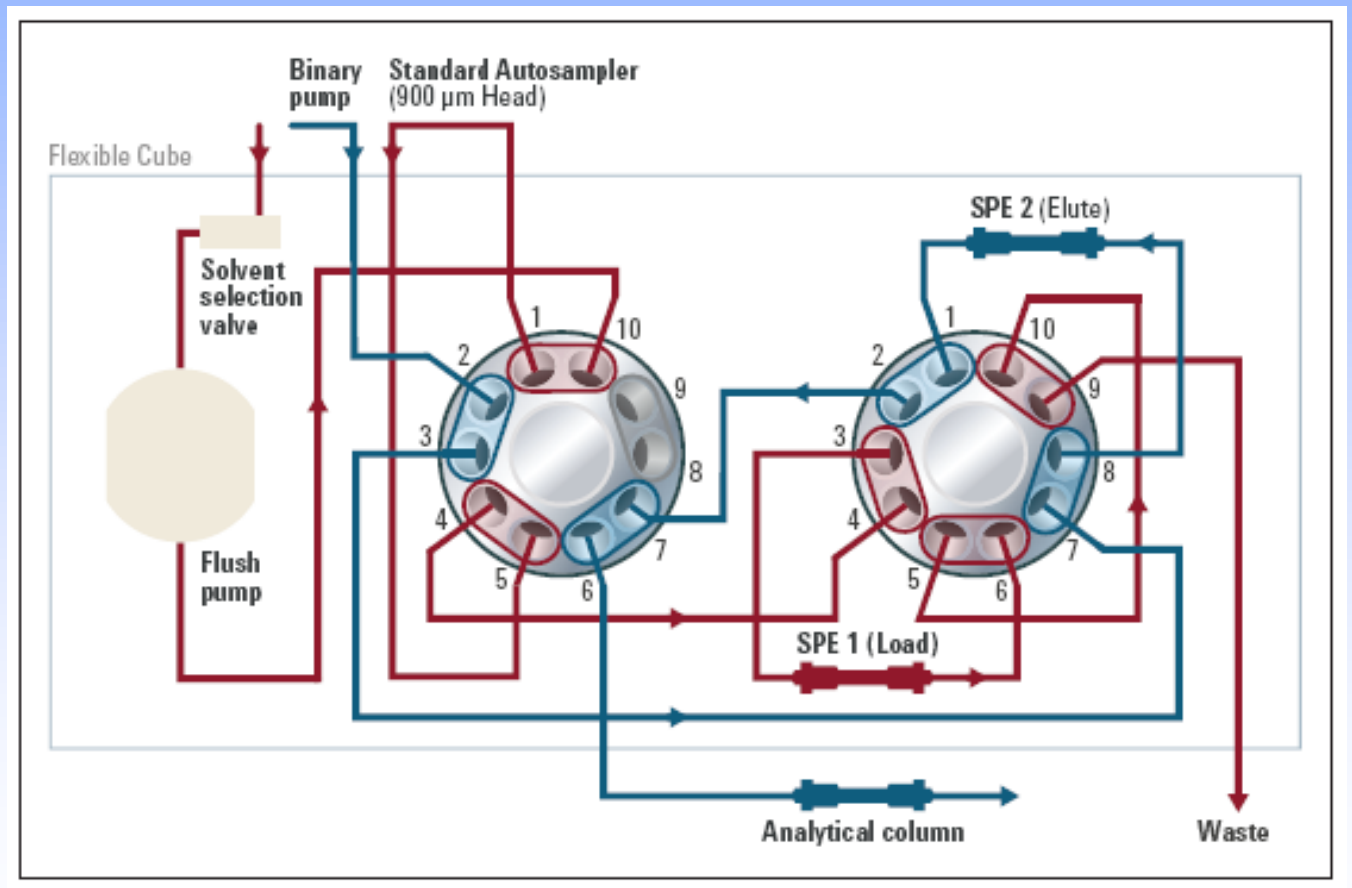
Flexible Cube Setup

Right Valve – showing loading of SPE cartridge #1



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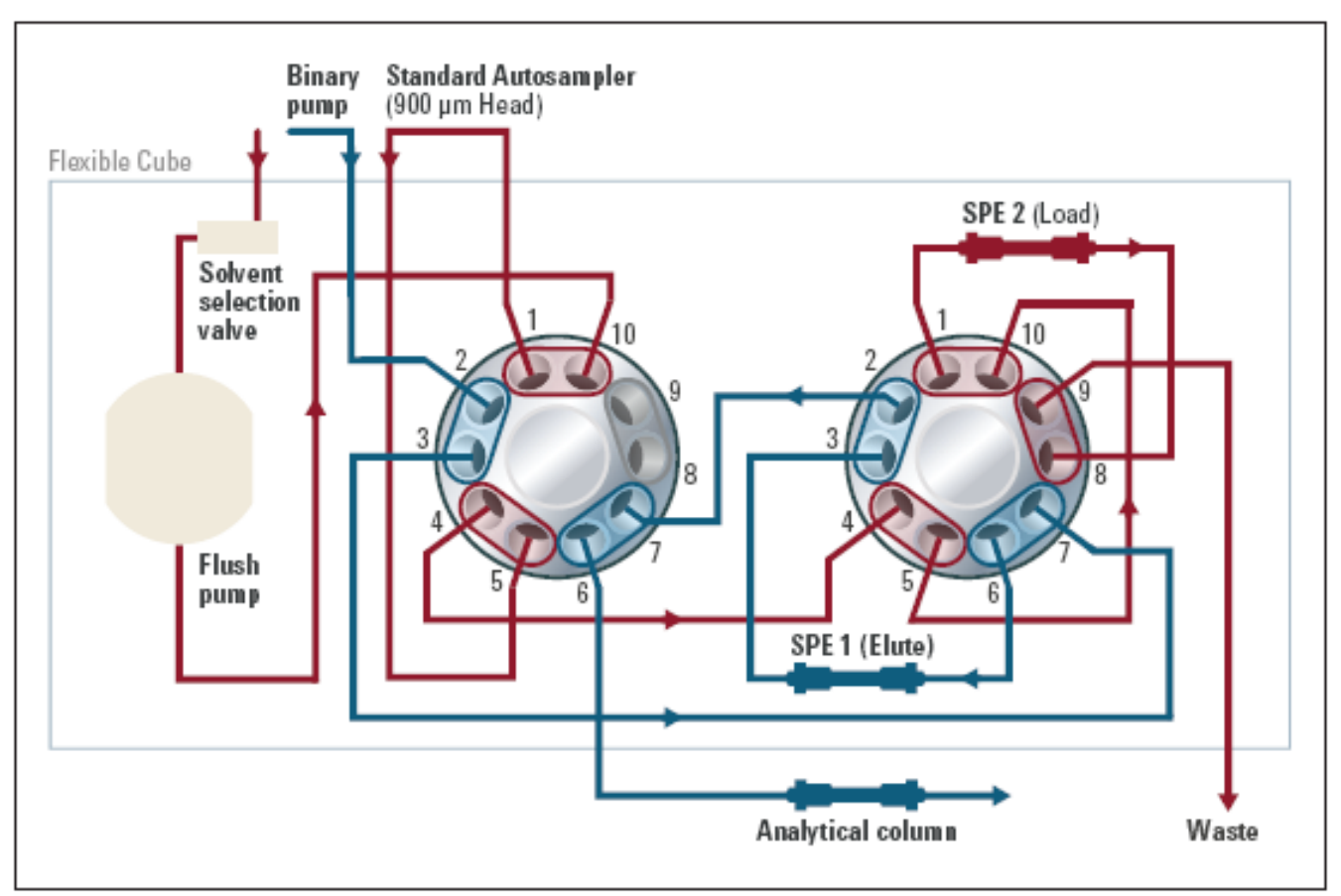
Flexible Cube Setup

Right Valve – showing elution of SPE cartridge #1



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USEPA 543

- USEPA Method 543 is an electrospray LC-MS/MS method for the analysis of seven pesticides and metabolites in finished drink water using online SPE
- Preserved water samples are fortified with internal standards and processed automatically by online SPE followed by LC-MS/MS analysis
- Precision of laboratory fortified blanks (LFB, reagent water) must have %RSD ≤ 20%
- Accuracy must be ± 30% of the true value

USEPA 543

Compound	CAS No.	Type
3-Hydroxycarbofuran	16655-82-6	Target
Bensulide	741-58-2	Target
Fenamiphos	22224-92-6	Target
Fenamiphos sulfone	31972-44-8	Target
Fenamiphos sulfoxide	31972-43-7	Target
Tebuconazole	107534-96-3	Target
Tebufenozide	112410-23-8	Target
Methomyl-$^{13}C_2$, ^{15}N		ISTD
Carbofuran-$^{13}C_6$		ISTD
Bensulide-d_{14}		ISTD

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USEPA 543

- Sample preservatives

Compound	Amount	Purpose
Trizma Preset Crystals	7.75 g/L	buffering reagent and removes free chlorine
2-Chloroacetamide	2 g/L	antimicrobial
Ascorbic Acid	100 mg/L	dechlorinating agent

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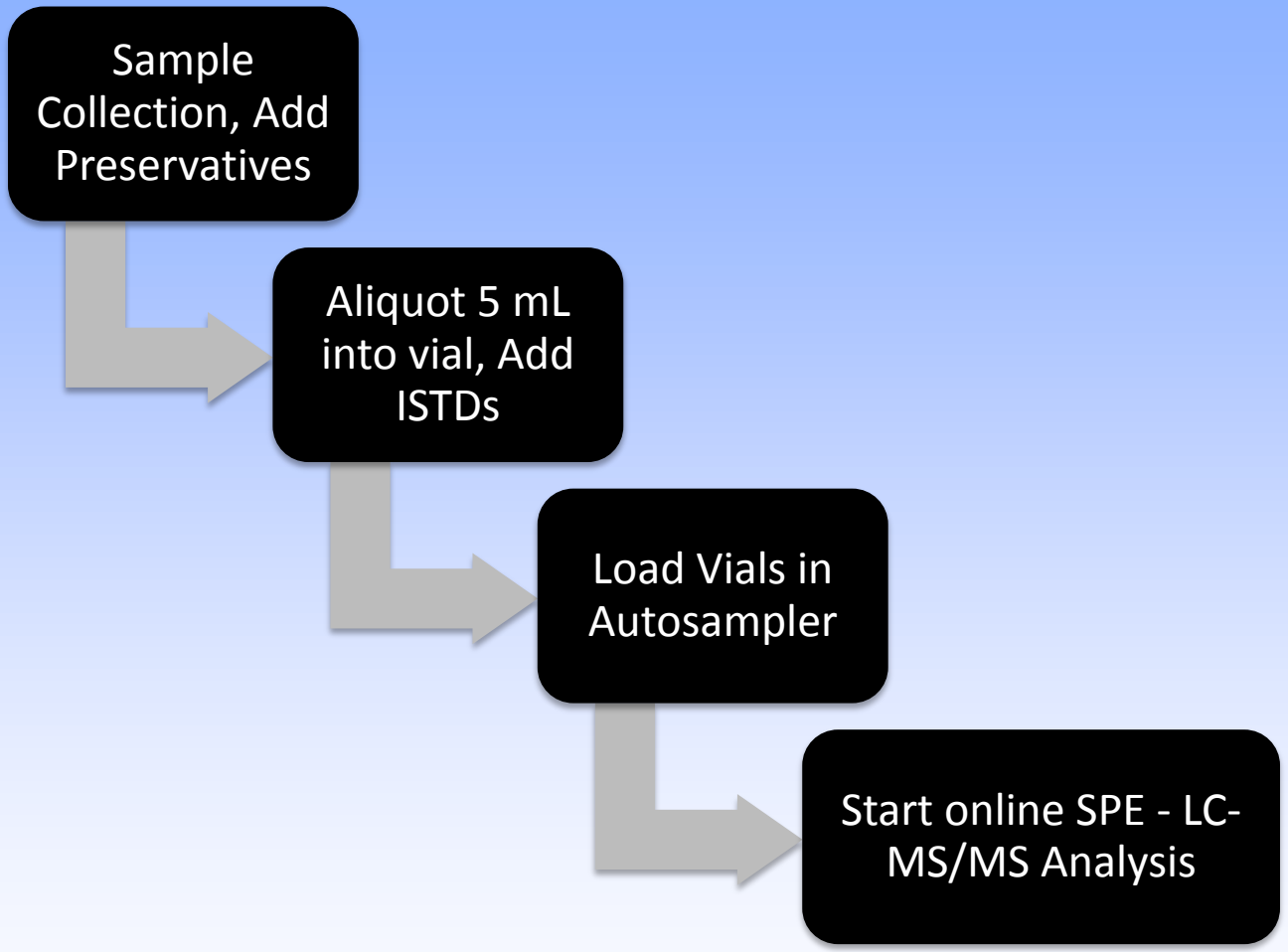
Method Summary



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Analytical Conditions

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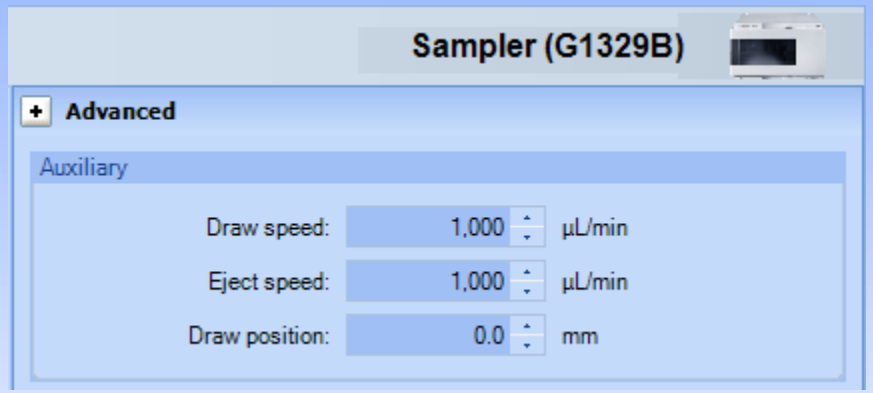
Autosampler Parameters

- Draw and Eject Speeds set to 1000 µL/min



- Use Injector Program

Function	Parameter
Draw	Draw 900 µL from sample
Eject	Eject to needle seat
Draw	Draw 900 µL from sample
Inject	

Flexible Cube Parameters

- Solvent A1 = 20 mM Ammonium Acetate*
- Solvent A2 = ACN

Time	Function / Parameter
0:00 min	pump 7.6 mL A1 at 2 mL/min
4:00 min	increase valve position
4:50 min	pump 6 mL A2 at 2 mL/min
8:00 min	pump 6 mL A1 at 2 mL/min

** prescribed by EPA*

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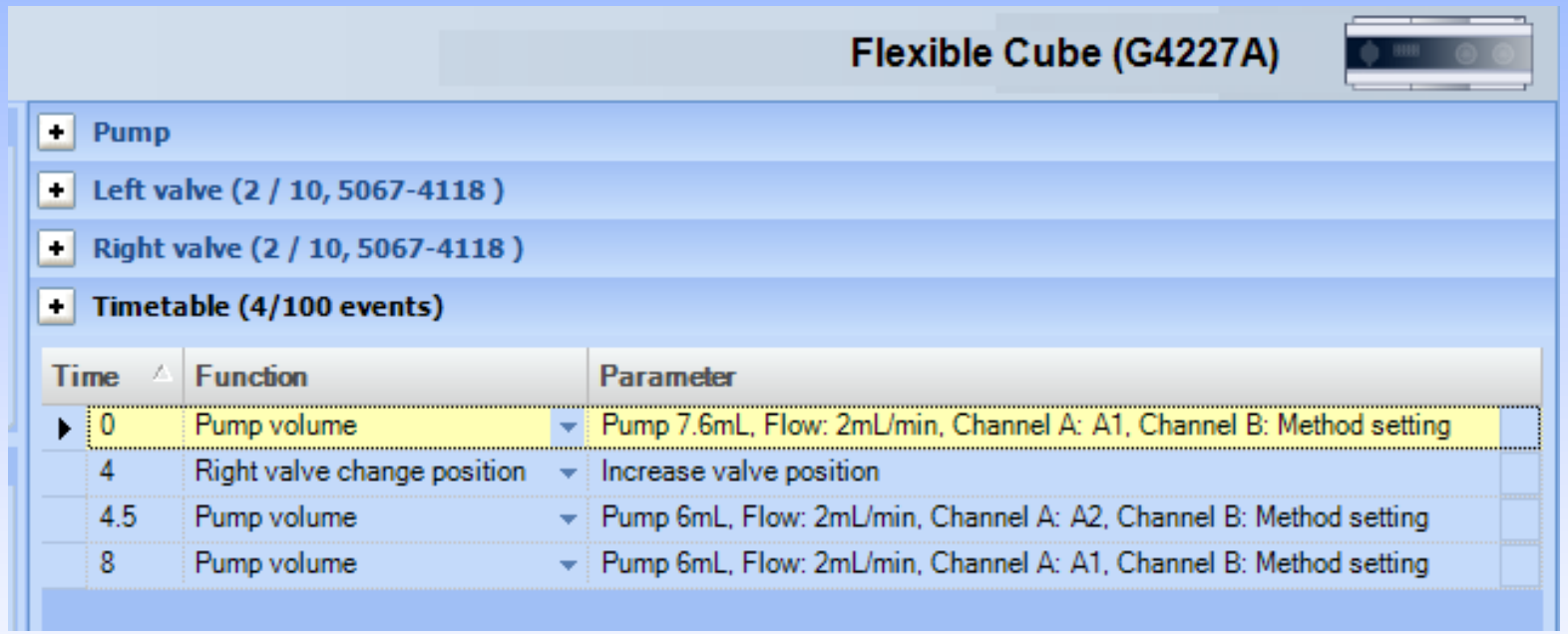
Flexible Cube Parameters

- MassHunter screen – Timetable



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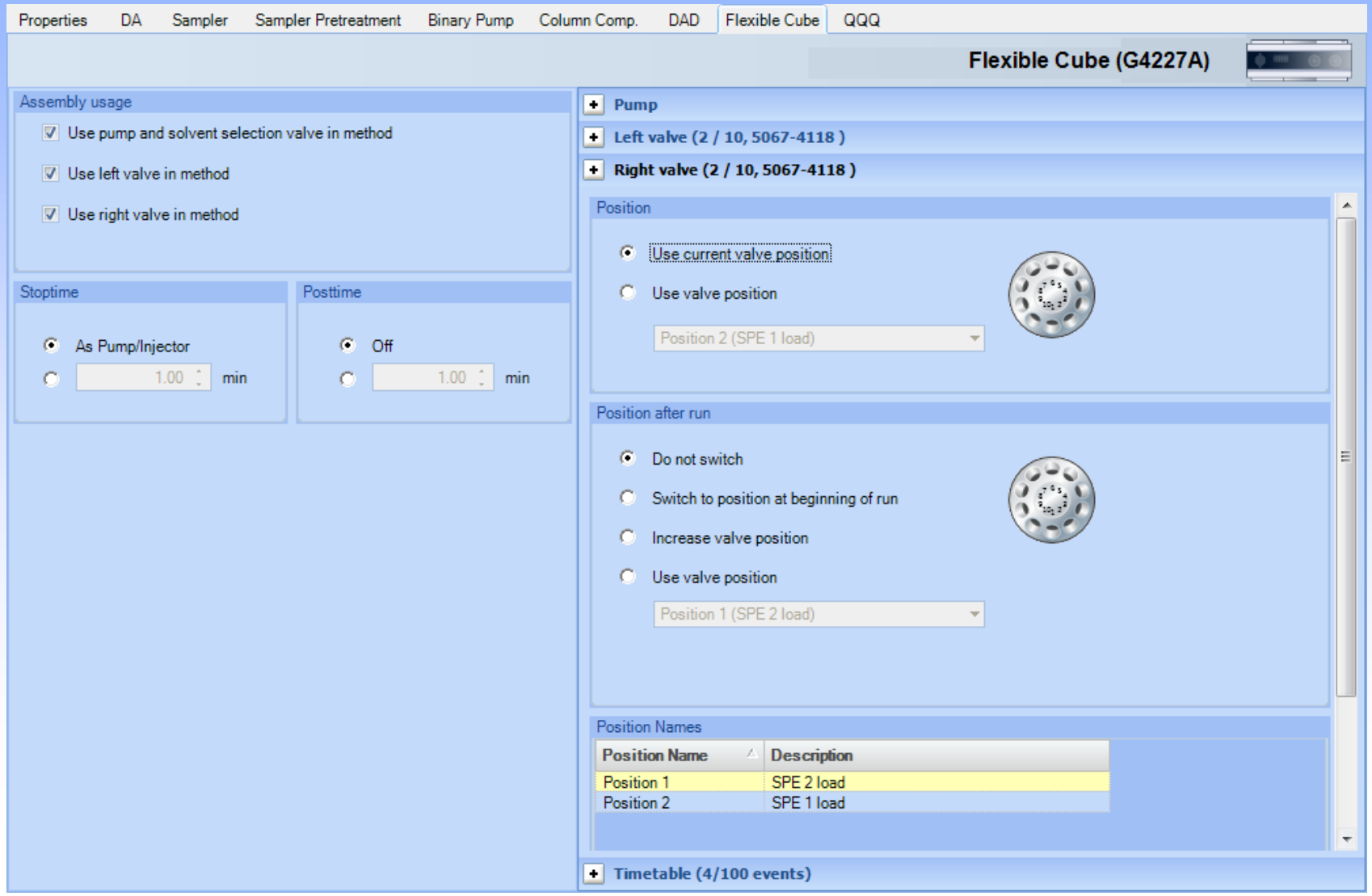
Flexible Cube Parameters

- MassHunter screen – right valve



Chromatographic Conditions

Parameter	Value
Column	Poroshell 120 PhenylHexyl, 3.0 x 100mm, 2.7µm
Mobile Phase	A = 20 mM ammonium acetate in water B = acetonitrile
Flow Rate	0.4 mL/min
Column Temperature	40 °C
Injection Volume	1800 µL

Binary Pump Parameters

Parameter	Time	%B	Flow (mL/min)
Mobile Phase Gradient	0:00 min 4:10 min 10:00 min	10 %B 10% B 98% B	0.4 mL/min 0.4 mL/min 0.4 mL/min
Stop Time	11.5 min		
Post Time	0 min (equilibration happens while next sample is being drawn)		

injection to injection time = 17 min

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MS Source Parameters

Parameter	Value
Drying Gas Temp (ºC)	300
Drying Gas Flow (L/min)	7
Nebulizer (psi)	35
Sheath Gas Temp. (ºC)	375
Sheath Gas Flow (L/min)	12
Capillary Voltage (V)	4500
Nozzle Voltage (V)	0

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Dynamic MRM Parameters

	Compound	Precursor	Product	Fragmentor (V)	Collision Energy (V)	Cell Acceleration (V)	Ret Time (min)	Ret Window	Polarity
*	Methomyl-13C2-15N	166.1	91.1	65	4	7	6.95	1.2	Positive
*	3-Hydroxycarbofuran	238.1	181.1	100	5	4	7.3	1.2	Positive
	3-Hydroxycarbofuran	238.1	163.1	100	8	4	7.3	1.2	Positive
	Fenamiphos sulfoxide	320.1	233	120	24	4	7.65	1.2	Positive
*	Fenamiphos sulfoxide	320.1	171.1	120	20	4	7.65	1.2	Positive
*	Fenamiphos sulfone	336.1	266.1	120	16	4	8.2	1.2	Positive
	Fenamiphos sulfone	336.1	188	120	24	4	8.2	1.2	Positive
*	Carbofuran-13C6	228.1	171.1	90	8	4	8.45	1.2	Positive
*	Fenamiphos	304.1	234.1	115	12	4	9.1	1.2	Positive
	Fenamiphos	304.1	217	115	20	4	9.1	1.2	Positive
*	Tebuconazole	308.2	70	130	20	4	9.25	1.2	Positive
*	Tebufenozide	353.2	297.1	68	4	4	9.55	1.2	Positive
	Tebufenozide	353.2	133.1	68	12	4	9.55	1.2	Positive
*	Bensulide-d14	412.2	364.1	80	0	4	9.85	1.2	Positive
	Bensulide	398.1	356	85	0	4	9.85	1.2	Positive
*	Bensulide	398.1	158	85	20	4	9.85	1.2	Positive
* denotes transition used for quantitation									

- Cycle Time set to 700 ms
- MS1 and MS2 resolutions set to Unit

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Chromatography

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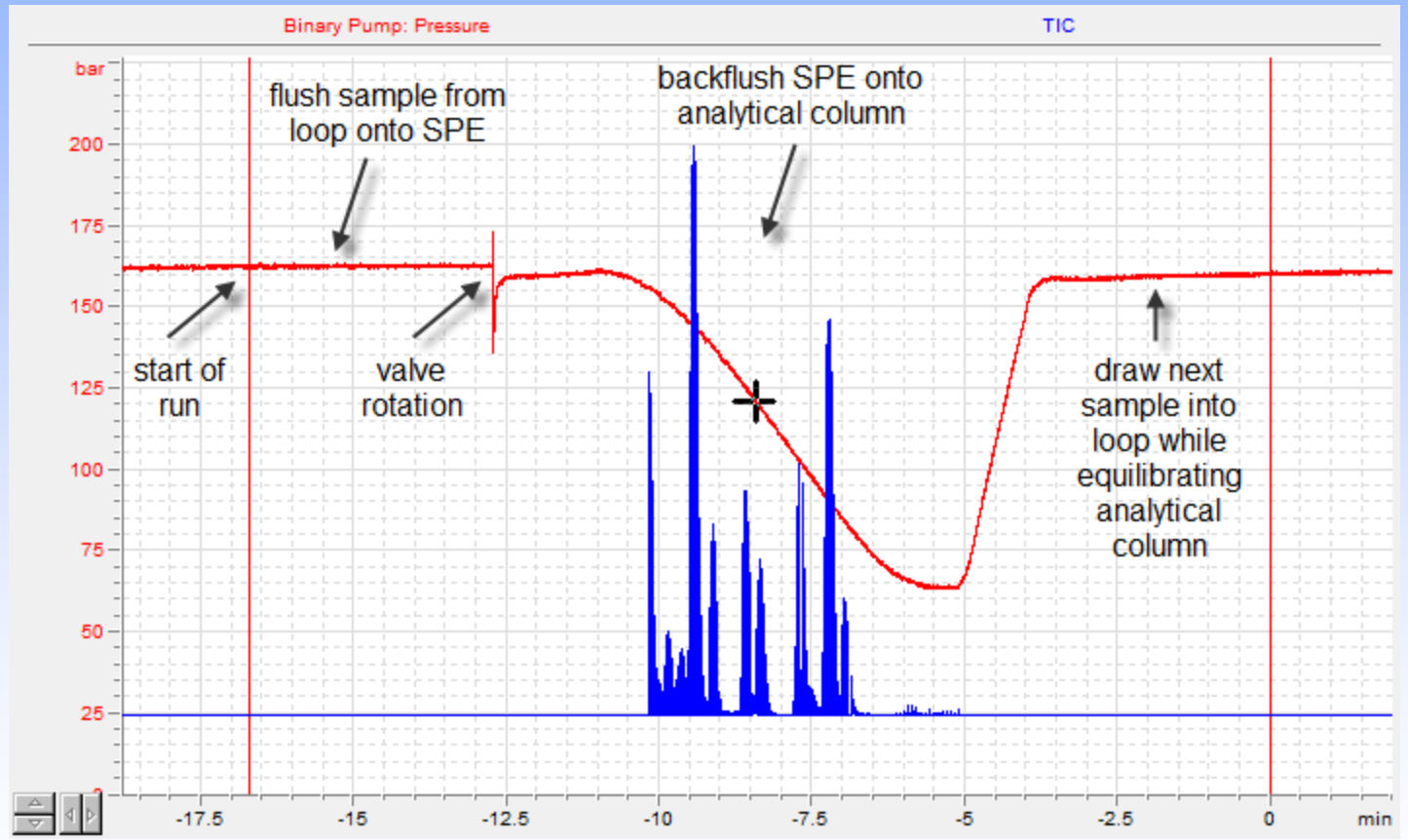
Chromatography

- TIC and pressure trace showing operation



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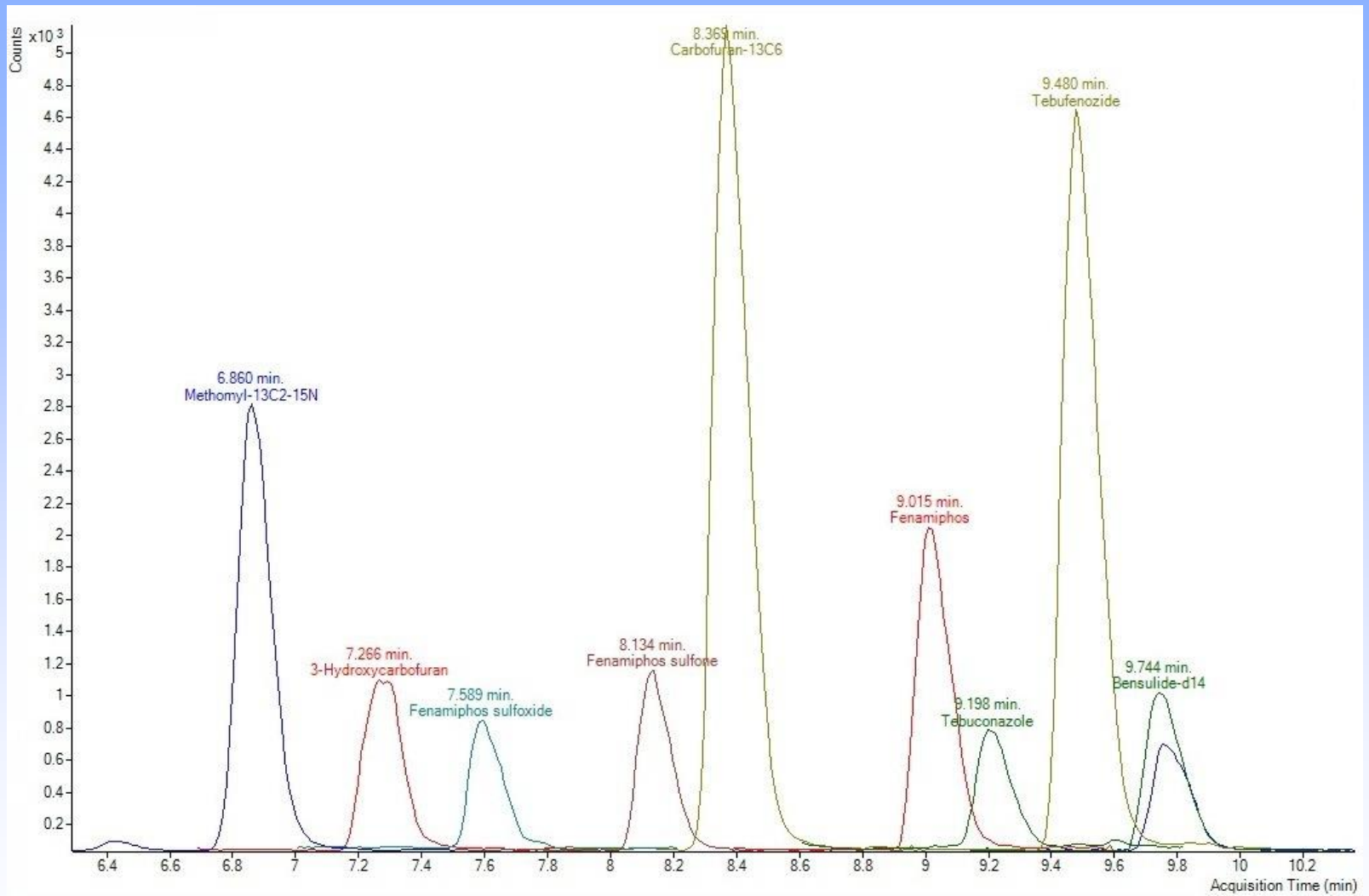
Chromatography



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Analytical Results

Results produced (accuracy, precision and LCMRL) were very similar to those produced by the USEPA.

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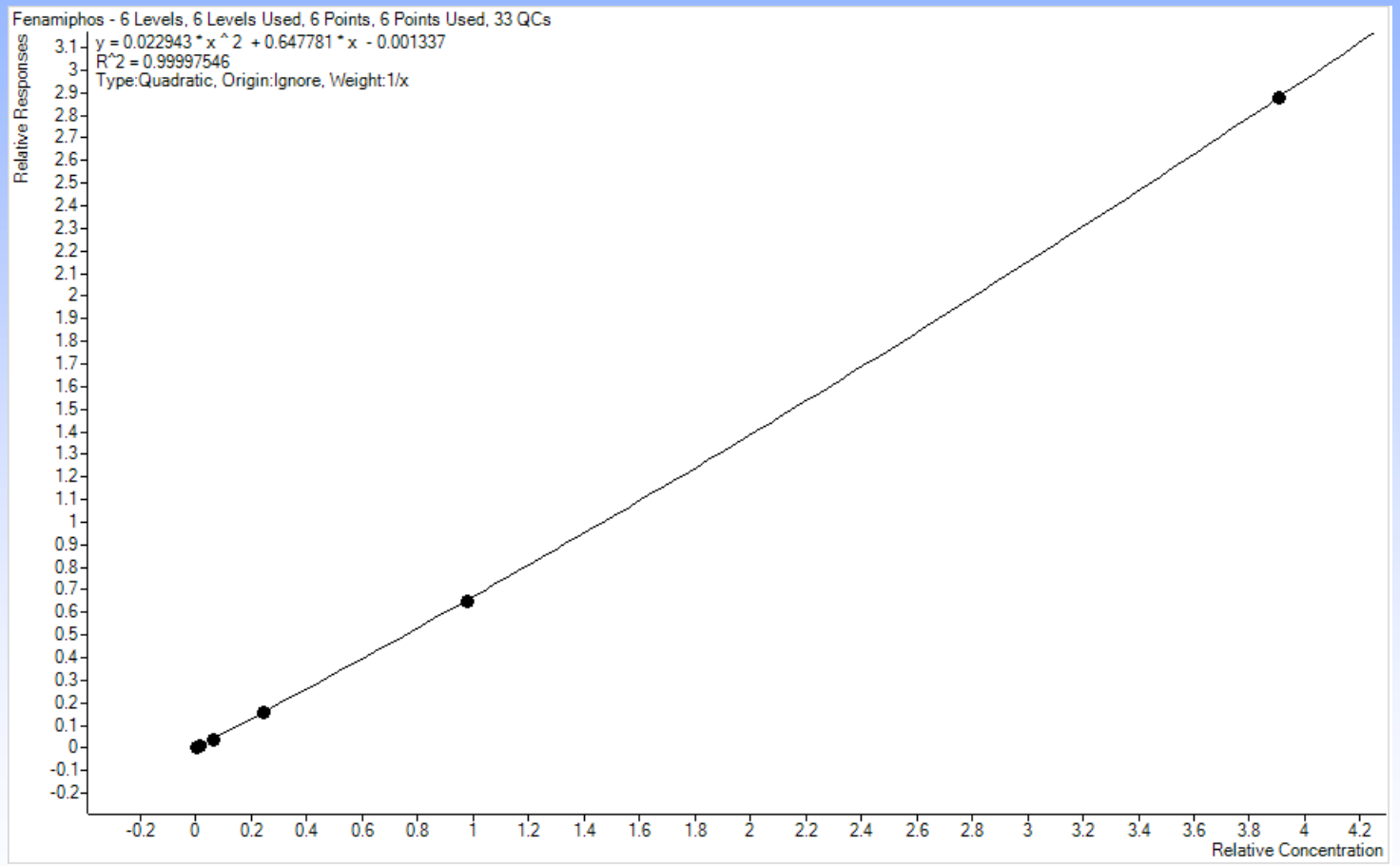
Direct Injection to SPE

- Comparison to determine “recovery” of the SPE process
- Spiked samples were analysed by direct injection at 10 µL as well as 1000 µL injections of a 100x dilution by online SPE
- Results were between 84 – 113% “recovery” (ESTD)
- Good recovery desired, although absolute recovery is not critical as calibrators are analysed using same SPE process

Calibration

- Compounds showed best fit using quadratic, 1/X weighting
- Fenamiphos example shown (typical), from 0.02 to 20 ng/L



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Calibration Range

- Compounds were analysed at different concentrations due to differences in sensitivity
- Calibration standards were prepared and down to levels where the compounds were not detected

Compound	Range (ng/L)
3-Hydroxycarbofuran	0.2 - 50
Bensulide	0.05 - 50
Fenamiphos	0.02 - 20
Fenamiphos sulfone	0.05 - 50
Fenamiphos sulfoxide	0.05 - 50
Tebuconazole	0.02 - 20
Tebufenozide	0.02 - 20

20 parts per quadrillion!

Accuracy and Precision

- Preserved tap water (surface water source) was fortified at a mid-level compared to the calibration curve range
- Seven fortified replicates were analysed to determine Accuracy and Precision
- Accuracy is presented as the average % recovery of all seven replicates
- Precision is presented as the % Relative Standard Deviation (%RSD)

Accuracy (%)

Compound	LFB		LFSM	
	VLS	EPA	VLS	EPA
3-Hydroxycarbofuran	102	100	103	111
Bensulide	104	95	97	88
Fenamiphos	100	105	100	97
Fenamiphos sulfone	100	101	96	95
Fenamiphos sulfoxide	99	102	105	102
Tebuconazole	94	112	96	103
Tebufenozide	102	101	96	105

** EPA data from fortifications at a similar concentration level, and from a surface water for LFSM*

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Precision (% RSD)

Compound	LFB		LFSM	
	VLS	EPA	VLS	EPA
3-Hydroxycarbofuran	1.7	4.1	4.2	3.5
Bensulide	3.4	2.9	2.4	12
Fenamiphos	3.7	3.7	3.8	6.7
Fenamiphos sulfone	3.4	4.4	1.7	1.8
Fenamiphos sulfoxide	2.7	4.0	1.7	4.0
Tebuconazole	2.0	5.4	5.1	9.8
Tebufenozide	4.0	3.6	3.9	8.3

** EPA data from fortifications at a similar concentration level, and from a surface water for LFSM*

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LCMRL Results (ng/L)

Compound	VLS	EPA
3-Hydroxycarbofuran	3.4	1.7
Bensulide	0.36	1.2
Fenamiphos	0.078	0.27
Fenamiphos sulfone	0.16	1.4
Fenamiphos sulfoxide	0.48	1.2
Tebuconazole	0.57	1.3
Tebufenozide	0.066	0.47

Note: the EPA method specifies use of Ammonium Acetate as the mobile phase. Better sensitivity using the Agilent LC-QQQ can be achieved by using Ammonium Formate (especially for 3-Hydroxycarbofuran).

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Discussion - Tips

- Understand the plumbing, remove some fittings to confirm flow
- Keep tubing lengths same between two cartridges
- Be sure not to overlap timing in Flexible Cube programming
- Ensure sufficient solvent levels for bottles used for Flexible Cube
- Be aware of possible overpressure issues with high flow rates
- Run H_2O blanks at start of worklist for both SPE cartridges to ensure they are flushed and equilibrated

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Conclusions – EPA 543

- Online SPE using Agilent Bond Elut SPE PLRP-S cartridges and an Agilent LC-MS/MS performs similarly to the USEPA results for method 543.
- The Agilent Flexible Cube incorporates seamlessly into the LC system and is controlled by MassHunter software. Online SPE provides many benefits for the analytical lab.
- The analysis method time (injection to injection) of less than 17 minutes (including online SPE), allows many samples to be automatically processed each day.

Conclusions - EPA Methods

- Acronyms
- Frustrating restrictions
  (moving towards performance based but…)
- Can you please calibrate your mass axis
  EPA 539: product ions of 144.7, 96.6…
- Working with EPA methods gets easier after you've done about three of them

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Acknowledgements

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- Trisa Robarge of Agilent Technologies for supplying the Bond Elute SPE PLRP-S cartridges
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- Jody Shoemaker of USEPA for provision of standards
- Ralph Hindle of Vogon Labs

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References

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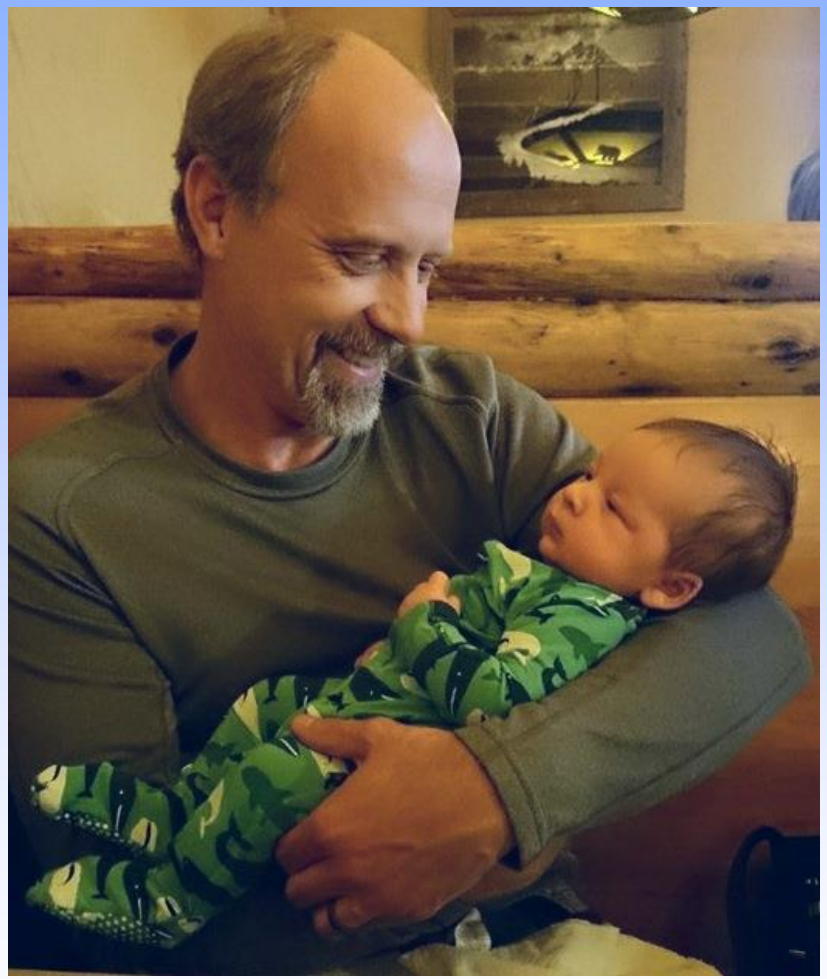
2. EPA Method 543 – Determination of Selected Organic Chemicals in Drinking Water by On-Line Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry (On-line SPE-LC/MS/MS); Version 1.0, March 2015

3. Sheher Bano Mohsin and Michael Woodman, Quantitation of Trace Level Emerging Contaminants in Water Using Online SPE with LC/MS/MS, Agilent Application Note 5991-2731EN, Oct. 2013.

4. Winslow, S. D.; Pepich, B. V.; Martin, J. J.; Hallberg, G. R.; Munch, D. J.; Frebis, C. P.; Hedrick, E. J.; Krop, R. A. *Environmental Science & Technology* **2006**, *40*, 281.

Thanks for your attention!



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