AXYS Analytical Services Ltd.

"EXPERIENCES in ANALYSIS of PPCPs and STEROIDS/HORMONES in POTW BIOSOLIDS"

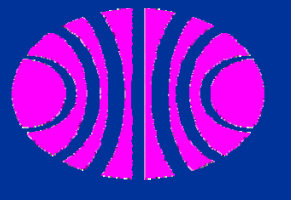
Richard Grace, Coreen Hamilton,
Million Woudneh, Blair Surridge
Brian Fowler
AXYS Analytical Services Ltd.
Sidney, BC, Canada

NELAP ACCREDITED

ISO 17025 CERTIFIED

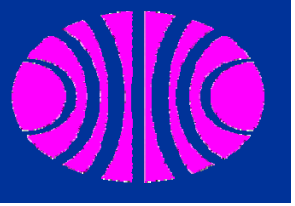
AGENDA

- Overview of Analytical Methods
  - PPCP Method
  - Hormone / Sterol Method
- Analytical Challenges
  - Clean Matrices vs. POTW Matrices
  - Positive Identification / False Positives and Suppression
  - Range of Analyte Concentration
  - Sample Handling / Preservation



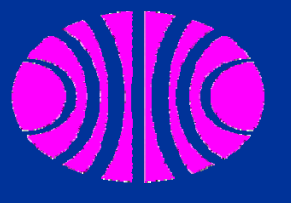
PURPOSE of ANALYSIS

- Ultra- Trace DL Targets (pg to ng)
- PPCP Method
  - Tool to Investigate Levels and Occurrence of PPCPs in Environment
  - 73 Targets
- HORMONE / STEROL Analysis
  - Tool to Investigate Levels and Occurrence of Hormones and Sterols in Environment
  - 27 Targets
  - Source Info / Fingerprinting



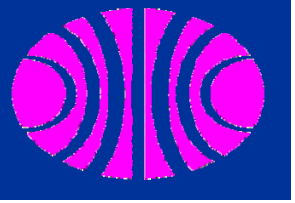
COMMON to BOTH ANALYSIS

- Developed as per EPA 1600 Series Methods
  - Performance based
  - Multi Matrix purpose and validation
  - Validated by Federal Register 40 CFR, Part 136, Appendix B
- Utilize labeled surrogate addition at start of process, recovery corrected vs. surrogates
- Validated for Positive Identification as part of large analyte panel
- Focus on native compounds, not degradation products or metabolites



Rationale for Analyte Selection

- Current or Historic Use / Occurrence
- Availability of Standards and Appropriate Surrogates
- Capture by SPE Cartridge (PPCP)
- Performance in Derivatization Process (Hormones and Sterols)
- Analyte Stability (Matrix and Instrument)
- Appropriate Monitoring Ions



BATCH QC

- Max. Batch Size (including QC) is 20 samples (8 to 14 is more common)
- Each Batch contains:
  - method blank
  - duplicate
  - Bracketing Cal.
  - Instrument Blank
  - MS/MSD, SRM,CRM included by request and applicability

OVERVIEW of PPCP ANALYSIS in POTW BIOSOLIDS

Sample – 2 X 1 gram wet solids
Acidic Fraction to pH 2 + EDTA stabilizer, Basic Fraction to pH10

Respective Fractions – Add labeled Surrogates
Acidic Fraction – 3X ultrasonic extraction with phosphate buffer/ACN
Basic Fraction – 3x ultrasonic extraction with H2O/NH4OH

SPE Cartridge Cleanup (1 gram Waters Oasis HLB)
SPE Steps – condition, load, rinse, elute, reduce, transfer to MeOH
Add recovery standards

ANALYSIS by LC/MS/MS (x4)
ACIDIC EXTRACT – Neg. Ionization, Tetracyclines, Pos. Ionization
BASIC EXTRACT – Pos. Ionization

PPCP Standards and Surrogates

- Non-labeled standards for each target compound
- Stable isotope labeled standards where ever possible used as internal standards
  - 17 labeled standards (13C, 2D or 15N) for acidic ESI+compounds
  - Labeled (13C) thiabenzadole and sulfamethazine for tetracyclines
  - Exact labeled analogue for all 6 Acidic ESI - compounds
  - Labeled (2D) albuterol and metformin for basic compounds
- Labeled injection standards (13C Atrazine or 2,4,5-TPP) to quantify recovery of internal standards

PPCP QA ACCEPTANCE CRITERA

- EPA Tier 1 Validation Protocols for Single Laboratory Method for IPR, MS/MSD, and MDL criteria
- Duplicate Criteria – 40% RPD for Analytes 10X above MDL
- Bracketing Cal. – 40% RSD for targets detected
- Cal. Ver – 25% RSD for native analytes with exact labeled standards, 35% for others
- Linearity (ICAL) – 25% RSD for native analytes that have exact labeled standards, 35% for others

PPCP Analyte List
ESI – LC/MS/MS
Acidic Extraction / + ve Ionization

ACETOMINOPHEN	CLAXACILLIN
AMPICILLIN	CODEINE
AZITHROMYCIN	COTININE
CAFFEINE	DEHYDRONIFEDIPINE
CARBADOX	DEPHENIHYRDAMINE
CARBAMAZEPINE	DILTIAZEM
CEFOTAXINE	DIGOXIN
CIPROFLAXIN	DIGOXIGENIN

PPCP Analyte List
ESI – LC/MS/MS
Acidic Extraction / + ve Ionization (cont.)

ENROFLAXIN	NORGESTIMATE
ERYTHROMYCIN – H2O	OFLOXACIN
FLUMEQUINE	ORMETOPRIM
FLUOXETINE	OXACILLIN
LINCOMYCIN	OXOLINIC ACID
LOMOFLOXACIN	PENICILLIN G
MICONAZOLE	PENICILLIN V
NORFLOXACIN	ROXITHROMYCIN

PPCP Analyte List
ESI – LC/MS/MS
Acidic Extraction / + ve Ionization (cont.)

SARFLOXACIN	TRIMETHOPRIM
SULFACHLOROPYRIDAZINE	TYLOSIN
SULFADIAZINE	VIRGINIAMYCIN
SULFADIMETHOXINE	1,7 DIMETHYLXANTHINE
SULFAMERIZINE	
SULFAMETHAZINE	
SULFAMETHAZOLE	
THIABENDAZOLE	

PPCP Analyte List
"Tetracyclines" by ESI - LC/MS/MS
Acidic Extraction / - ve Ionization

Chlortetracycline	Doxycycline
4–Epichlorotetracycline	Oxytetracycline
Anhydrochlortetracycline	4 – Epitetracycline
4-Epianhydrochlor-tetracycline	4 - Epianhydrotetracycline
Isochlortetracycline	Anydrotetracycline
Democlocycline	Mincycline

PPCP Analytes by ESI – LC/MS/MS

ACIDIC EXTRACTION / -VE IONIZATION MODE	BASIC EXTRACTION / + VE IONIZATION MODE
WARFARIN	ALBUTEROL
IBUPROFEN	CIMETIDINE
GEMFIBROZIL	METFORMIN
NAPROXEN	RANITIDINE
TRICLOSAN	
TRICLOCARBAN	

OVERVIEW of HORMONES/STEROLS ANALYSIS in POTW BIOSOLIDS

SAMPLE SIZE = 1 GRAM WET
(after discarding "debris")

Surrogate Addition, dry with Sodium Sulfate
Soxhlet 18 hrs. 40:60 Hexane:Acetone
DCM rinses added to extract, reduce

Solvent Exchange to 5% Toluene in Hexane
Clean-Up – Layered Alumina / Florisil Column - Concentrate
Derivatize with 99:1 BSTFA;TMCS Reagent to produce Trimethylsilyl Ethers

ANALYSIS by HRGC / HRMS
or GC/MS

HORMONES / STEROLS QA ACCEPTANCE CRITERA

- EPA Tier 1 Validation Protocols for Single Laboratory Method for IPR, MS/MSD, and MDL criteria
- Duplicate Criteria – 40% RPD for Analytes 10X above MDL
- Bracketing Cal. – 40% RSD for targets detected
- Cal. Ver – 25% RSD for native analytes with exact labeled standards, 35% for others
- Linearity (ICAL) – 25% RSD for native analytes that have exact labeled standards, 35% for others

Hormone Targets (*= dx surrogate)

ANDROSTENIDIENE	ESTRIOL
ANDROSTERONE	ESTRONE
COPROSTANOL	17 α ETHINYL ESTRADIOL *
DESOGESTRAL	MESTRANOL *
17 α DIHYDROEQUILIN	NORETHINDIONE *
EQUILENIN	NORGESTREL *
EQUILIN	PROGESTERONE *
17 α ESTRADIOL	TESTOSTERONE
17 β ESTRADIOL *	

Sterol Targets (*= dx surrogate)

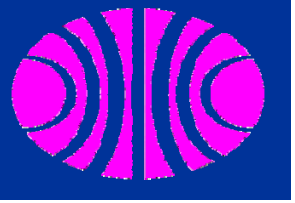
CAMPESTEROL	β – SITOSTEROL
CHOLESTEROL *	β - STIGMASTRANOL
DESMOSTEROL	STIGMASTEROL
EPICOPROSTANOL	
ERGOSTEROL	
β – ESTRADIOL – 3 - BENZOATE	

BIOSOLID DETECTION LIMITS

- PPCP in validation matrix (Peat Moss)
  - Acidic Extraction / + ve Ionization (0.1 to 50 ng/g)
  - Acidic Extraction / - ve ionization (1 to 98 ng/g)
  - Tetracyclines (2-23 ng/g)
  - Basic extraction / + ve ionization (1-56 ng/g)
  - Matrix may raise DLs 10X in biosolids, target specific
- Hormones and Sterols (10 – 2000 pg/g)
  - Hormones low, sterols high
  - LR GC/MS 5-50X higher

PPCPs - CLEAN MATRICES vs. POTW MATRICES

- Removal of Matrix Interference is Limiting DL and Method QC factor , not instruments
- Some Options in Removal of Matrix Interference
  - Limit sample size
  - Wet weight preferred as POTW Biosolids Solids up to 95% H2O with sig. matrix
  - SPE cartridge must capture targets and surrogates, excess matrix will compete
  - Increase run times, less polar mobile phases
- Representative Matrices for Validation not Available
  - Forces clean matrices DL, IPR validation (peat, sand)
  - Experience from Method QC required

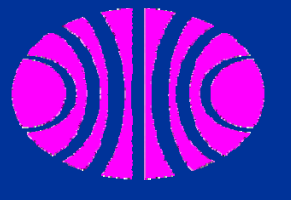


HORMONES and STEROLS - CLEAN MATRICES vs. POTW MATRICES

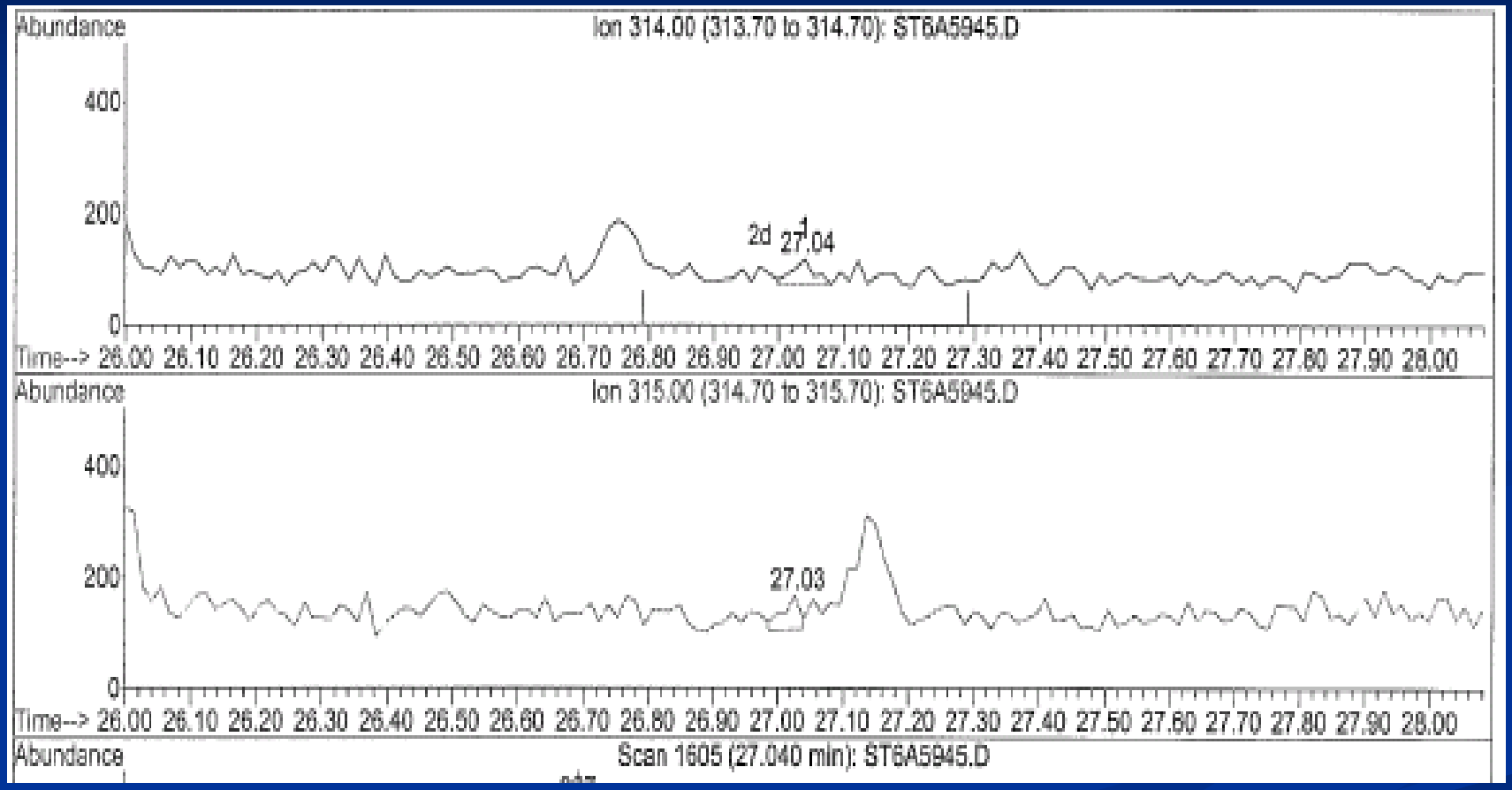
- Removal of matrix interference is limiting DL and Method QC factor

- Some Options in Removal of Matrix Interference
  - HRMS option to improve selectivity
  - Can be accomplished with LC/MS/MS if high level sterols not targeted with low level hormones
  - Water removal vital
  - Limited clean-up options for large target list

- Representative Matrices for Validation not Available
  - Clean Matrices used for DL, IPR validation (peat, sand)
  - Experience from method QC required



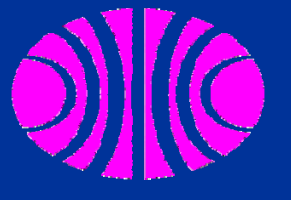
17 β Estradiol in Biosolids – GC/MS



- Native 17 β Estradiol – “NQ”

PPCPs – Suppression and Enhancement

- Key effect in PPCP analysis by LC/MS/MS
  - Wide variety in POTW biosolid chemistry / process
  - Varies widely by target
- Controls
  - Limit sample size
  - Increase capacity of clean-up (SPE type and size)
- Monitoring and Correcting
  - Recovery Standards to Monitor Surrogate Response
  - Dilution and re-injection when suppression or enhancement suspected

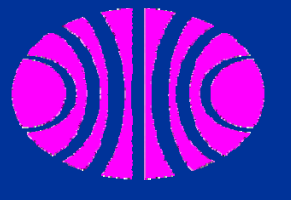


PPCPs – SUPPRESSION and ENHANCEMENT

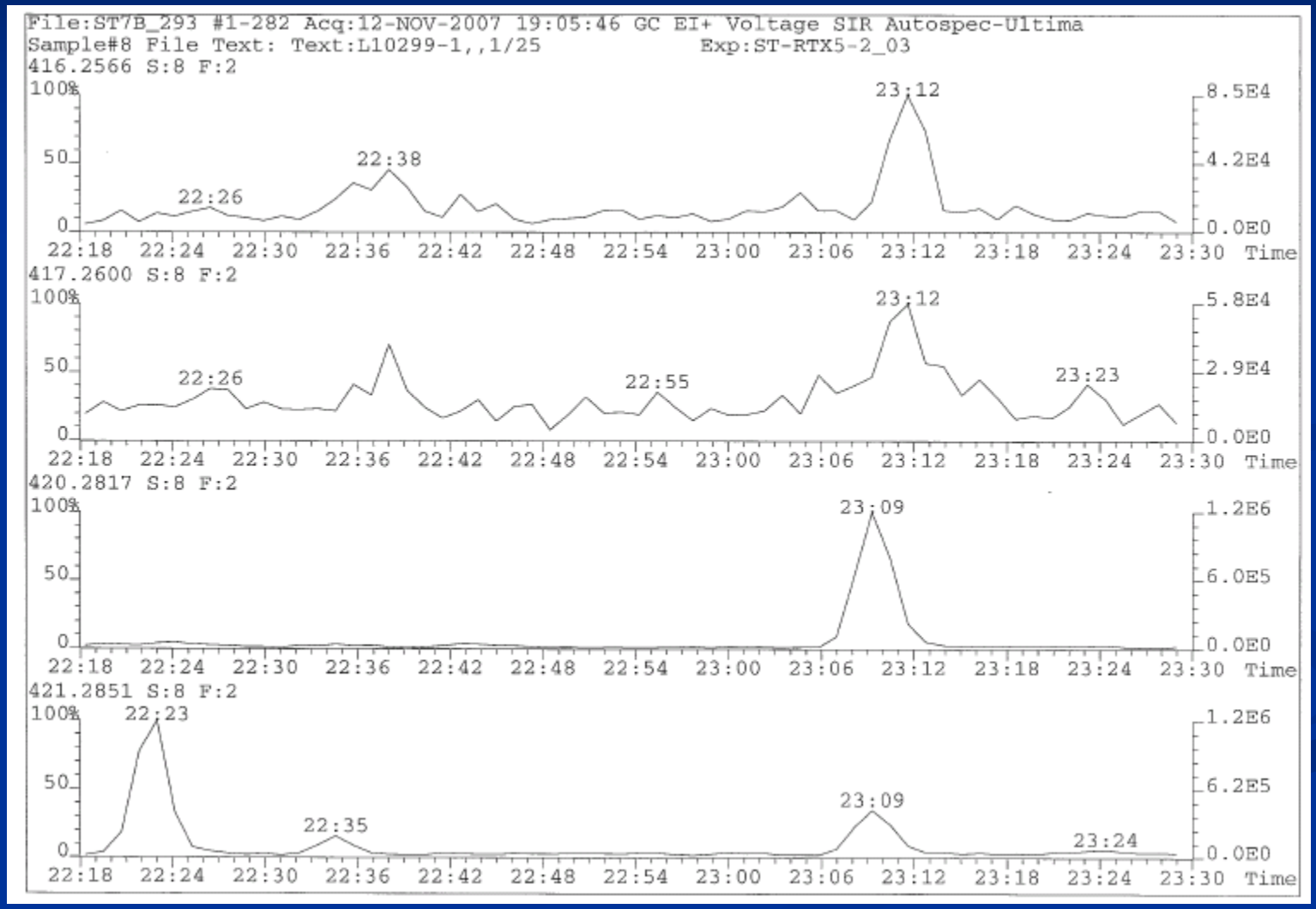
	Biosolid A		Biosolid B		Biosolid C	
Sample size	1.14 g	3.09 g	1.16 g	3.07 g	1.11 g	3.12 g
	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
Albuterol	0.2	0.1	0.2		0.4	
Cimetidine	11.9	10.3	34.1	29.6	0.2	0.3
Metformin		10.1	40.4	21.3	28.0	15.9
Ranitidine	1.2	1.1	3.6	3.0		
d3-Albuterol	95.6	78.3	85.9	67.5	119.0	125.6
d3-Cotinine	64.3	61.9	85.5	74.2	55.5	33.3
d6-Metformin	136.9	169.5	108.8	110.7	251.8	349.4

HORMONES and STEROLS
Avoiding False Positives / Negatives

- Use of internal labeled standards and relative recovery correction
- Blank acceptance criteria from Method QC (3X standard deviation, no blank correction)
- Monitor completeness of derivatization process natives and TMS products
- Increase resolution to reduce matrix effects
  - RP of GC/MS in method 100-400
  - RP of HRMS 5000 min.

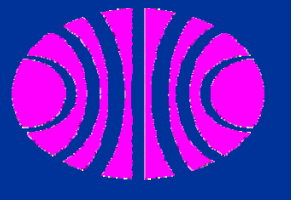


17 β ESTRADIOL by HRMS
Primary / Confirming Channels



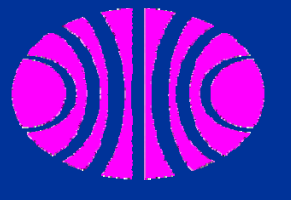
PPCPs – Concentration Range of Analytes

- High Levels produce need for dilution
- Create difficulties in MS/MSD for high level analytes
- Frequent detects above 1000 ng/g in Biosolids
  - Ciproflaxin, Oflaxicin, Triclocarban, Triclosin, ETC, Doxycycline
- Frequent detects above 1000 ng/L in POTW influent
  - Acetaminophen, Caffeine, Ibuprofen, Cotinine, Trimethoprim, Carbamazepine, Metformin, Sulfamethoxazole



HORMONES and STEROLS – Concentration Range of Analytes

- Hormones low level, many sterols very high level in POTW biosolids
- Affects blank acceptance criteria
  - 5-10 ng for most compounds
  - 500 for cholesterol and beta sitosterol
- Adjustments required to prevent “NQ” on high level sterols (cholesterol and beta sitosterol most frequent
- Extract split used to maintain QC
  - Over spike with Sterol surrogates at beginning of process
  - 1/20 split after clean-up
  - 2 instrument runs
  - Method QC adjusted to match



SAMPLE HANDLING / PRESERVATION

- Freeze biosolid samples immediately
  - Arrest Microbiological activity
  - No definitive studies indicating hold times
  - Glass containers (PPCP and Hormones/Sterols)
- Extract immediately on thawing
- POTW processes homogenize sample
  - Allows minimization of sample size
  - Sampling plan to determine repeatability
- Have a strategy for “free water”
  - Discard, 2 analysis, attempt to re-homogenize

CONCLUSIONS

- Viable low level detection methods available for large panels of PPCPs and Hormones / Sterols
- Basic methods applicable for environmental levels (water, soil, and sediment) and POTW matrices (Influent, Effluent,Biosolids)
- High variability in POTW sludge matrix effects and levels, much work required to characterize vs. specific attributes
- High variability requires robust methods and "coping" strategies

NEXT STEPS

- Current - Peer Review of method performed by EPA – Q3/4 2007
- Further processing of samples to develop further statistical information
- Detailed Information targeted for publication by AXYS clients
- Multi-Lab Validation?
- Metabolites and Degradation Products
- Questions?

ACKNOWLEGEMENTS

- AXYS Laboratory staff
  - 20 staff over 3 year period
- Those who have contributed to literature on this subject