



AXYS Analytical Services Ltd.

**METHOD VALIDATION FOR THE
ANALYSIS OF PHARMACEUTICALS AND
PERSONAL CARE PRODUCTS IN
BIOLOGICAL TISSUES**

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ISO 17025 CERTIFIED



Outline

- ❑ **Method Developed for 104 PPCP compounds in Biological Tissues**
- ❑ **Lessons from method development experiments**
- ❑ **Method Performance**
- ❑ **Target compound occurrence data in mussels from a marine coastal environment**



Characteristics of Analytical Method

- Large List of Target Analytes
- DLs in low ng/g range
- Positive Identification/Accurate Quantification
- Validated by Accepted Procedures
- Rugged
- Based closely on EPA Method 1694



EPA Method 1694

**PPCPs by
LC-MS/MS
73 Compounds
18 labelled
Surrogates**

**List 1 PPCPs
Acidic Extraction
+ve ESI
49 Compounds
10 Surrogates**

**List 2 TCY's
Acidic Extraction
+ve ESI
14 Compounds
1 Surrogates**

**List 3 PPCPs
Acidic Extraction
-ve ESI
6 Compounds
6 Surrogates**

**List 4 PPCPs
Basic Extraction
+ve ESI
4 Compounds
2 Surrogates**

Target Analytes

AXYS EPA Method 1694 (Modified)

**PPCPs by
LC-MS/MS**
121 Compounds
49 labelled
Surrogates

List 1 PPCPs
Acidic Extract
+ve ESI
49 Compounds
11 Surrogates

List 2 TCY's
Acidic Extract
+ve ESI
14 Compounds
1 Surrogates

List 3 PPCPs
Acidic Extract
-ve ESI
12 Compounds
9 Surrogates

List 4 PPCPs
Basic Extract
+ve ESI
14 Compounds
11 Surrogates

List 5 PPCPs
Acidic Extract
+ve ESI
32 Compounds
17 Surrogates

PPCP Standards and Surrogates

- Non-labeled standards for each target compound
- Stable isotope labeled standards where ever possible used as internal standards
 - Minimum 3 MU (i.e. d3)
 - Track performance accurately
 - Allows recovery correction
 - Room left in instrument method to allow addition of more internal standards
- Labeled injection standards to quantify recovery of internal standards
 - $^{13}\text{C}_3$ Atrazine (Lists 1,2,5 + Hormones by LC/MS/MS)
 - $^{13}\text{C}_6$ 2,4,5 Trichlorophenoxyacetic acid (List 3)
 - d3 Amitriptyline (List 4)



List 3 – PPCPs by Acidic Extraction / - ESI EPA Method 1694 List 3 plus Additions (yellow)

TARGET	LABELED STADARD
Gemfibrizol	d6 Gemfibrizol
Ibuprofen	13C3 Ibuprofen
Naproxen	13C d3 Naproxen
Triclocarban	13C6 Triclocarban
Triclosan	13C12 Triclosan
Warfarin	d5 Warfarin
Glyburide	d3 Glyburide
Glipizide	d11 Glipizide
Furosemide	13C d3 Naproxen
Hydrochlorothiazide	13C d3 Naproxen
2 – Hydroxy – Ibuprofen	13C3 Ibuprofen
Bisphenol A	d6 Bisphenol A

List 4 PPCPs by Basic Extraction / +ESI EPA Method 1694 List 4 plus Additions (yellow)

TARGET	LABELLED STANDARD
Albuterol	d3 Albuterol
Cimetidine	d3 Cimetidine
Metformin	d6 Metformin
Ranitidine	d3 Albuterol
Amphetamine	d5 Amphetamine
Atenolol	d7 Atenolol
Atorvastatin	d3 Cimetidine
Clonidine	d4 Clonidine
Codeine (previously APOS, List 1)	d6 Codeine
Cotinine (previously APOS, List 1)	d3 Cotinine
Enalapril	d5 Enalapril
Hydrocodone	d3 Hydrocodone
Oxycodone	d6 Oxycodone
Triamterene	d4 Clonidine

List 5 PPCPs by Acidic Extraction / + ESI

TARGET	LABELLED STANDARD	TARGET	LABELLED STANDARD
Alprazolam	d5 Alprazolam	Amlodipine	d5 Norfluoxetine
Fluocinonide		Norfluoxetine	
Trenbolone		Paroxetine	d5 Paroxetine
Trenbolone Acetate		Desmethyl -diltiazem	d4 Promethazine
Amitriptyline	Promethazine		
Betamethasone	d6 Amitriptyline	10 Hydroxy Amitriptyline	d7 Propanolol
Verapamil		Norverapamil	
Benzoyllecgonine		d8 Benzoyl - ecgonine	
Benztropine	d3 Benztropine	Prendinisone	
Cocaine	d3 Cocaine	Propranolol	
DEET	d7 DEET	Sertraline	
Diazepam	d5 Diazepam	Propoxyphene	
Hydrocortisone	d4 Hydrocortisone	Simvastatin	
Fluticasone propionate	d7 Metoprolol	Valsartan	
Metoprolol		Meprobamate	
Methyl - prednisolone	d2 Methyl - prednisolone	Theophylline	13C15 N2 Theophylline



OVERVIEW of PPCP ANALYSIS SOLIDS and AQUEOUS

Sample – 2 X 1 gram wet solids or 2 X 1L
Acidic Fraction to pH 2 + EDTA stabilizer, Basic Fraction to pH 10

Respective Fractions – Add Labeled Standards
Acidic Fraction – 2X phosphate buffer/CAN; ACN
Basic Fraction – 2x NH₄OH; ACN

SPE Cartridge Cleanup (1 gram Waters Oasis HLB)
Add labeled standards

ANALYSIS by LC/MS/MS (x5)
ACIDIC EXTRACT – Neg. Ionization, Tetracyclines, Pos. Ionization
BASIC EXTRACT – Pos. Ionization

Modification of PPCP ANALYSIS

Tissue Matrix – Extended EPA 1694 List

Sample – 2 X 5 gram wet wt. tissue
Acidic Fraction – phosphate buffer (pH 2)
Basic Fraction – NH₄OH (pH 10)

Respective Fractions – Add Labeled Standards
Acidic Extraction – 2X phosphate buffer; ACN
Basic Fraction – 2X NH₄OH; ACN

SPE Cartridge Cleanup (1 g Waters Oasis HLB)
Add labeled standards

ANALYSIS by LC/MS/MS (X5)
ACIDIC EXTRACT – Neg. Ionization, 3 X Pos. Ionization,
BASIC EXTRACT – Pos. Ionization

Method Development Focus

- Extraction
 - How do you know if it's working?
 - Spiked tissues not representative
- Cleanup
 - Quality of Chromatography (Peak Shape, Interferences)
 - MS/MS response: suppression/enhancement
- LC/MS/MS Analysis
 - Use procedures of Method 1694



Initial Test Results

Analyte	List	Issue
Fluoroquinolones (all floxacin)	List1	Low recovery in acetonitrile, recovery good in acid fraction
Tetracyclines	List2	Low recovery in acetonitrile, recovery good in acid fraction
Triclosan and Triclocarban surrogate	List3	Relatively low recovery in catfish. Mussel samples are no affected.
Cimetidine surrogate	List4	Single digit recovery
Ranitidine	List4	Low recovery
Atorvastatin	List4	Very low recovery
Valsartan	List5	Relatively low recovery in 1 of 2 spiked mussel samples

Optimization of Modified PPCP Extraction Tissue Matrix – Extended EPA 1694 List

Sample – 2 x 1-5 gram wet wt. tissue
2 x tissue + labeled standards + ACN

Acidic Fractions – 2X Phosphate buffer extraction (pH 2)
Basic Fraction – 2X NH₄OH extraction (pH 10)
Add supernatants to ACN extract

SPE Cartridge Cleanup (1 g Waters Oasis HLB)
Add labeled standards

ANALYSIS by LC/MS/MS (5X – all lists)
ACIDIC EXTRACT – Neg. Ionization, 3x Pos. Ionization
BASIC EXTRACT – Pos. Ionization



Sample Size Data Showing Suppression

AXYS ID	Mussel Tissue	Mussel Tissue	Mussel Tissue
Matrix	Reference Sample	Reference Sample	Reference Sample
Conc Units	ng/g	ng/g	ng/g
SAMPLE SIZE	5.000 g	2.54 g	1.000 g
ALBUTEROL	ND 0.06	ND 0.118	ND 0.3
AMPHETAMINE-1	ND 0.597	ND 2.562	ND 1.805
ATENOLOL-1	0.805	ND 0.236	ND 0.6
ATORVASTATIN-1	ND 0.3	ND 0.591	ND 1.5
CIMETIDINE	ND 0.12	ND 0.236	ND 0.6
CLONIDINE-1	ND 0.3	ND 0.591	ND 1.5
CODEINE-1	ND 0.988	ND 1.181	ND 3.000
COTININE	ND 0.3	ND 0.591	ND 1.5
ENALAPRIL-1	0.211	ND 0.118	ND 0.3
HYDROCODONE-1	ND 0.3	ND 0.591	ND 1.5
METFORMIN	ND 0.6	ND 1.181	ND 3.000
OXYCODONE-1	ND 0.12	ND 0.236	ND 0.6
RANITIDINE	ND 0.416	ND 0.236	ND 0.6
TRIAMTERENE-1	ND 0.06	ND 0.118	ND 0.3

AXYS ID		Mussel Tissue	Mussel Tissue	Mussel Tissue
Matrix		Reference Sample	Reference Sample	Reference Sample
Conc Units		ng/g	ng/g	ng/g
SAMPLE SIZE		5.000 g	2.54 g	1.000 g
Labeled Standards	% Recovery Specs			
D3-ALBUTEROL	20-140	32.5	35.5	77.3
D6-METFORMIN	3-130	5.9	7.4	12.7
D3-COTININE-1	50-140	135.9	81.1	125.8
D3-CIMETIDINE-1	15-130	82.6	41.8	62.3
D5-ENALAPRIL-1	50-130	83.6	76.1	95.0
D6-OXYCODONE-1	50-150	63.6	V 45.059	81.8
D4-CLONIDINE-1	50-130	V 166.651	100.4	129.4
D5-AMPHETAMINE-1	20-130	V 17.212	V 9.127	25.1
D6-CODEINE-1	50-130	50.7	V 30.185	75.6
D3-HYDROCODONE-1	50-130	61.1	V 38.322	60.8
D7-ATENOLOL-1	50-130	71.8	V 41.296	89.0

PPCPs – Suppression and Enhancement

- **Key effect in PPCP analysis by LC/MS/MS**
 - Wide variety in sediments, biosolids, wastewater
 - Varies widely by target analyte

- **Controls**
 - Limit sample size
 - Increase capacity of clean-up (SPE type and size)

- **Monitoring and Correcting**
 - Labeled Injection Standards to Monitor Internal Standard Response
 - Dilution and re-injection when suppression or enhancement suspected
 - Secondary Transitions if Available / appropriate



Alligators at the Door – Exploring the Limits of the Methods



PPCP Method QC Notes

- **Reference Matrix Selection (IPRs, MS/MSD)**
 - 2 mussel species – variation in matrix may limit sample size (1 – 5 grams wet)
 - 2 clean fish tissues (ling cod, salmon)
- **Alignment with EPA 1694 QC Specs.**
 - Most 1694 QC specs. applicable
 - Exception – recovery criteria – tighter, low RPD, but may be marginally outside of EPA 1694 recovery ranges
- **DL and % RPD Notes**
 - 90% of compounds 0.1 to 10 ng/g range
 - 10% of compounds 10 – 40 ng/g range
 - < 20% RPD between method variations, MS/MSDs, duplicates, dilutions



OPR Specifications List 1 (17/49)

Analyte	Mean (% Rec)	SD	Below Existing Spec (%)	Above Existing Spec (%)
AZITHROMYCIN	125.3	62.9	0	20
CARBADOX	36.8	13.6	20	0
CLARITHROMYCIN	120.7	65.3	0	20
CLOXACILLIN	133.4	50.2	0	40
DEHYDRONIFEDIPINE	107.9	31.1	0	20
DIPHENHYDRAMINE	90.1	24.1	20	0
DIGOXIN	144.4	53.1	0	20
DIGOXIGENIN	105	49.5	0	20
MICONAZOLE	48.8	50.2	60	0
NORGESTIMATE	42.2	42.2	60	0
OXACILLIN	111	33.8	0	20
PENICILLIN V	117.7	51.7	0	20
ROXITHROMYCIN	93.7	42.8	0	20
SULFADIAZINE	139.1	63.8	0	20
SULFAMERAZINE	115.8	21.8	0	20
SULFANILAMIDE	112.5	81.8	0	20
TYLOSIN	92.3	44.2	20	20
Surrogates				
13C2-ERYTHROMYCIN-H2O	122.1	52.8	0	40

OPR Specifications List 3 (5/12)

Analyte	Mean (% Rec)	SD	Below Existing Spec (%)	Above existing Spec (%)
BISPHENOL A	80.9	12.6	20	0
FUROSEMIDE	114.3	24.4	0	20
HYDROCHLOROTHIAZIDE	38.3	15.7	80	0
2-HYDROXY-IBUPROFEN	180.5	49.6	0	80
TRICLOSAN	116.5	22.9	0	20
Surrogates				
D6-GEMFIBROZIL	47.2	26	60	0
13C-IBUPROFEN	57.9	26	60	0
13C6-TRICLOCARBAN	18.8	17.1	60	0
13C12-TRICLOSAN	22.9	21.3	60	0

OPR Specifications List 4 (2/14)

Analyte	Mean (% Rec)	SD	Below Existing Spec (%)	Above existing Spec (%)
Atorvastatin	108.2	21.5	0	20
Oxycodone	122.8	13.2	0	20
Surrogates				
d6-Codeine	59.7	27.1	20	0
d5-Enalapril	43.8	11.6	60	0

OPR Specifications List 5 (6/32)

Analyte	Mean (% Rec)	SD	Below Existing Spec (%)	Above existing Spec (%)
Benzotropine	123.7	5	0	20
DEET	111.8	19.1	0	20
Sertraline	48.3	28.3	60	0
Trenbolone acetate	61.2	25.9	60	0
Valsartan	44	12	100	0
Verapamil	133	22	0	40
Surrogates				
d2-Methylprednisolone	109.1	38.9	0	20
d7-Metoprolol	121.6	24.5	0	20

Compounds Not Well Recovered From Tissues

- Cimitedine
 - <10% Recovery
- Sertraline
 - <50% Recovery
- Valsartan
 - <50% Recovery



PPCP Occurrence Data

5 Samples Each Matrix

- Mussel Tissue
 - Sediments
 - Aqueous



17 PPCPs Detected in Mussels

Compound (# sites detected) (extended EPA 1694 compounds in red)	Use	ng/g wet wt	
		Max	Mean
Diethyl-3-methyl-benzamide, N,N- (DEET) (5)	insect repellent	14	7
Digoxigenin (3)	cardiac drug metabolite	10	5
Carbamazepine (5)	anticonvulsant	5	3
Amphetamine (3)	stimulant	4	1
Triclocarban (2)	antimicrobial	2	0.5
Sertraline (5)	antidepressant	1	0.5
Dehydronifedipine (5)	antianginal	0.7	0.4
Triamterine (3)	antihypertensive	0.6	0.2
Ranitidine (3)	antacid	0.4	0.2
Diphenhydramine (3)	antihistamine	0.3	0.2
Atenolol (1)	beta blocker	0.3	0.1
Cocaine (2)	stimulant	0.3	0.1
Amitryptiline (2)	antidepressant	0.2	0.1
Sulphamethiazole (1)	antibiotic	0.2	0.04
Erythromycin-H2O (4)	erythromycin metabolite	0.2	0.1
Enalapril (2)	antihypertensive	0.1	0.04
Diltiazem (2)	antianginal	0.1	0.04

11 PPCPs Detected in Sediment

Compound (# sites detected) (extended 1694 compounds in red)	Use	ng/g dry wt	
		Max	Mean
Ciprofloxacin (2)	antibiotic	680	400
Caffeine (3)	stimulant	38	18
Triclocarban (3)	antimicrobial	33	8
Trimethoprim (1)	antibiotic	18	3
Triamterene (5)	antihypertensive	11	3
Thiabendazole (2)	fungicide	9	2
Diethyl-3-methyl-benzamide, N,N- (DEET) (2)	insect repellent	3	1
Erythromycin-H2O (1)	erythromycin metabolite	3	1
Amphetamine (2)	stimulant	3	1
Sulphamethoxazole (1)	antibiotic	1	0.1
Cocaine (1)	stimulant	0.2	0.1

Not quantified (NQ) compounds occur in silicate (sand) and aluminosilicate (clay) matrices. This is a systemic interaction between the matrix and specific targets in list 1 and 5. Surrogates and natives are not recovered. Fluoroquinolone compounds are examples.

12 PPCPs Detected in All Waters

Compound (extended 1694 compounds in red)	Use	Units = ng/L	
		Max	Mean
Valsartan	antihypertensive	92	45
Sulfamethoxazole	antibiotic	67	26
Carbamazepine	anticonvulsant	44	18
Caffeine	stimulant	41	27
Gemfibrozil	antilipidemic	38	25
Atenolol	beta blocker	37	18
Meprobamate	antianxiety	36	20
Diethyl-3-methyl-benzamide, N,N-(DEET)	insect repellent	21	11
Erythromycin-H2O	erythromycin metabolite	12	4
Triamterine	antihypertensive	10	4
Benzoylecgonine	cocaine metabolite; analgesic	7	5
Diltiazem	antianginal	3	1

19 Other PPCPs Detected in Some Water Samples

Compound (# sites detected) (extended 1694 compounds in red)	Use	Units = ng/L	
		Max	Mean
Ibuprofen (1)	antiinflammatory	38	8
Metoprolol (3)	antianginal	26	6
Cotinine (4)	nicotine metabolite	25	11
Clarithromycin (2)	antibiotic	18	5
Sulphamethizole (1)	antibiotic	16	3
Amphetamine (2)	stimulant	10	4
Naproxen (1)	antiinflammatory	8	2
Hydrocodone (1)	analgesic	7	1
Trimethoprim (2)	antibiotic	4	1
Thiabendazole (1)	fungicide	3	0.5
Cocaine (4)	stimulant	2	1
Diphenhydramine (4)	antihistamine	2	1
Desmethyldiltiazem (2)	diltiazem metabolite	2	0.4
Dehydronifedipine (4)	antianginal	1	0.7
Albuterol (1)	antiasthmatic	1	0.2
Propoxyphene (2)	analgesic	0.7	0.2
Amitriptyline (2)	antidepressant	0.6	0.2
Diazepam (1)	antianxiety	0.5	0.1
10-hydroxy-amitriptyline (2)	amitriptyline metabolite	0.3	0.1

Analytes Detected in Mussel Tissue From a Marine Coastal Environment (n = 75)

- 14 - List 1 compounds
- 2 - List 3 compounds
- 8 - List 4 compounds
- 14 - List 5 compounds



List 1 Compounds

Analyte	Occurrence	Maximum Concentration (ng/g)
CAFFEINE	13	22.8
CIPROFLOXACIN	2	3
DIPHENHYDRAMINE	15	4.3
DILTIAZEM	2	0.2
ENROFLOXACIN	9	2.1
ERYTHROMYCIN-H2O	17	0.3
FLUOXETINE	4	0.8
LOMEFLOXACIN	46	22.5
MICONAZOLE	2	0.8
NORFLOXACIN	1	8.2
OFLOXACIN	5	3
SULFADIMETHOXINE	1	0.3
SULFAMETHAZINE	26	87.5
VIRGINIAMYCIN	38	10.2

List 3 Compounds

Analyte	Occurrence	Maximum Concentration (ng/g)
2-HYDROXY-IBUPROFEN	2	184
TRICLOCARBAN	10	31.2

List 4 Compounds

Analyte	Occurrence	Maximum Concentration (ng/g)
Albuterol	2	0.5
Amphetamine	7	3.7
Atenolol	4	1.4
Hydrocodone	1	2.7
Metformin	1	6.7
Oxycodone	1	2.8
Ranitidine	5	4.2
Triamterene	2	0.9

List 5 Compounds

Analyte	Occurrence	Maximum Concentration (ng/g)
Amitriptyline	12	0.8
Benzoylecgonine	1	0.1
Cocaine	31	0.9
DEET	71	1.5
Fluocinonide	1	7.1
10-hydroxy-amitriptyline	1	0.2
Methylprednisolone	11	30.9
Metoprolol	2	1.4
Norverapamil	1	0.1
Propoxyphene	2	0.1
Propranolol	1	1
Sertraline	36	6.5
Theophylline	25	506.9
Verapamil	1	0.1

Questions?

