ABSTRACT

Determination of Pharmaceuticals and Personal Care Products in Fish Using High Performance Liquid Chromatography-Tandem Mass Spectrometry and Gas Chromatography-Mass Spectrometry

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Labeled as emerging organic contaminants, pharmaceuticals and personal care products (PPCPs) have been the focus of global environmental research for over a decade. PPCPs have caused widespread concern due to their extensive use. As PPCPs were designed to correct, enhance, or protect a specific physiological or endocrine condition, their target effects in humans and/or farm stocks are relatively well understood and documented. However, there is limited knowledge about their unintended effects in the environment.

To address the occurrence, distribution and fate of PPCPs in the environment, efficient and reliable analytical methods are needed. The relatively low concentration, high polarity, and thermal lability of some PPCPs, together with their interaction with complex environmental matrices, makes their analysis challenging. Sample preparation followed by GC or HPLC separation and mass spectrometry (MS) detection has become the standard approach for evaluating PPCPs in environmental samples.

PPCPs have been widely reported in water, sediment and biosolids, but reports of their occurrence in aquatic organisms have been limited by the difficulty of analysis. Herein, we report the first HPLC-MS/MS screening method for the analysis of 23 pharmaceuticals and 2 metabolites representing multiple therapeutic classes in fish tissues. The developed methodology was successfully applied to assess the occurrence of target analytes in fish collected from 8 locations throughout the United States (6 effluent-dominated rivers and two reference sites). A complementary GC-MS method was developed for the analysis of 12 additional compounds belonging to either personal care product or industrial use compound classes in fish muscle. This approach was also applied to screen for target analytes in fish collected from a regional effluent-dominated stream.

Determination of Pharmaceuticals and Personal Care Products in Fish Using High Performance Liquid Chromatography-Tandem Mass Spectrometry and Gas Chromatography-Mass Spectrometry

by

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A Dissertation

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ABBREVIATIONS

AHTN	tonalide
API	atmospheric pressure ionization
APs	alkylpenol surfactants
AZ	Arizona
BP-3	benzophenone-3
BCF	bioconcentration factor
CCV	continuous calibration verification
CP	chlorophene
desser	desmethylsertraline
DL	detection limit
diclo	diclofenac
%D	percent difference
EDC	endocrine disrupting compound
EHMC	ethylhexyl methoxyciannamate
EI	electron impact
ESB	Environmental Specimen Bank
ESI	electrospray ionization
FL	Florida
fluox	fluoxetine
GC	gas chromatography

GPC	gel permeation chromatography
gem	gemfibrozil
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography-tandem mass spectrometry
HHCB	galaxolide
HRMS	high resolution mass spectrometry
HAc	0.1 M acetic acid buffer
ibupro	ibuprofen
IL	Illinois
IS	internal standard
keto	ketoprofen
K_{OW}	octanol-water partition coefficient
LCS	laboratory control sample
LOD	limit of detection
LOQ	limit of quantitation
4-MBC	4-methylbenzylidene camphor
MDL	method detection limit
MeCN	acetonitrile
MeOH	methanol
MK	musk ketone
MM	musk moskene
MS	mass spectrometry
MS/MSD	matrix spike and matrix spike duplicate

MTC	methyl-triclosan
MT	musk tibetene
MX	musk xylene
na	non applicable
napro	naprofen
nd	non detected
NM	New Mexico
NP	nonylphenol
NPE1	4-nonylphenolmonoethoxylate
NPE2	4-nonylphenoldiethoxylate
NPE3	4-nonylphenoltriethoxylate
NPE4	4-nonylphenoltetraethoxylate
nor	norfluoxetine
OC	octocrylene
OP	octylphenol
OPE	octylphenolethoxylate
OWCs	Organic wastewater contaminants
PA	Pennsylvania
PCBs	Polychlorinated Biphenyls
PCP	personal care product
PQL	quantitation threshold defined at 2 to 5 times above MDL
PO_4	0.1 M Phosphate buffer
PPCPs	pharmaceuticals and personal care products

QA/QC	quality assurance/ quality control
QAPP	quality assurance project plan
%R	percent recovery
RPD	relative percent difference
RSD	relative standard deviation
RRF	relative response factor
SEC	size exclusion chromatography
ser	sertraline
SIM	selected ion monitoring
SMs	synthetic musks
sp	species
SPE	solid phase extraction
TCS	triclosan
TFA	0.1 M trifluoroaceticacid buffer
4-t-op	4-tert-octylphenol
TX	Texas
UV	ultra-violet
UVFs	ultra-violet filters
WWTP	wastewater treatment plant

CHAPTER ONE

Introduction

Pharmaceuticals and Personal Care Products

During the last three decades, research in ecotoxicology has been focused almost exclusively on conventional pollutants, especially those highly toxic and/or carcinogenic pesticides and industrial intermediates exhibiting persistence in the environment. Another diverse group of bioactive chemicals receiving comparatively little attention as potential environmental pollutants includes both human and veterinary pharmaceuticals and active ingredients in personal care products (collectively called PPCPs). The term PPCPs refers not only to prescription drugs and biological medicines, but also diagnostic agents, food with medicinal effects, fragrances, sun-screens agents, and numerous other compounds. Today about 3000 different pharmaceuticals are being used in medicines such as painkillers, antibiotics, contraceptives, beta-blockers, lipid regulators, tranquilizers, and impotence drugs. During and after treatment, humans and animals excrete a combination of intact and metabolized pharmaceuticals. Consequently, many bioactive compounds enter wastewater and receiving bodies without any test for specific environmental effects. In addition, chemicals that compose personal care products also number in the thousands. The world's population consumes enormous quantities of skin care products, dental care products, soaps, sunscreen agents, and hair styling products. PPCPs are typically classified as emerging contaminants due to the paucity of information available for these compounds in comparison to conventional pollutants.

Occurrence and Pathway to the Environment

PPCPs, either in their native form or as metabolites, are continuously introduced into wastewater via excreta, disposal of unused or expired products, or directly from commercial discharges. Because most wastewater treatment processes do not effectively remove all PPCPs, they are subsequently introduced into the environment in wastewater treatment plant (WWTP) effluents. Discharge of PPCPs into the environment via this pathway is dependent upon human use, compound-specific pharmacokinetic and physicochemical properties, and the specific wastewater treatment process(es) employed at a particular site.[1] Alternative pathways also exist for direct introduction of PPCPs into the environment. For example, PCPs can be released directly into recreational waters (e.g., sunscreen) or volatilized into air (e.g., musks). Pharmaceuticals can also be directly introduced into surface waters via run-off from agricultural areas that utilize veterinary therapeutics.[2, 3] Because of this direct release they can bypass possible degradation in wastewater treatment plants (WWTPs).

Much of the scientific attention given to these emerging contaminants has resulted from an absence of aquatic life based regulations for surface waters.[4] Numerous studies have reported occurrence data for surface waters, wastewater, soil, sediment, and biosolids. These reports have been summarized in recent reviews.[5-9] In contrast, relatively few studies have documented the occurrence of PPCPs in aquatic organisms. Such data are necessary to promote ecological and human health risk assessments documenting potential consequences of environmental PPCP exposures. Recent reports from our laboratory[10] and others[11-31] have demonstrated that continuous introduction of PPCPs into surface waters generates the ideal exposure condition for accumulation of

contaminants and their metabolites in aquatic biota and emphasize the necessity of research focused on understanding partitioning, fate and secondary effects of these compounds in aquatic systems.

PPCPs in Aquatic Organisms: State of the Science

A comprehensive summary of proven analytical methodologies and environmental occurrence data for PPCPs in aquatic organisms, primarily fish, is presented in Table 1.1.[10-37] These data definitively demonstrate that the release of PPCPs into aquatic ecosystems results in accumulation of a variety parent chemicals and/or metabolites. Tissue concentrations vary by compound and generally range from a few tenths of a ng to a few μg per g tissue on a wet-weight basis. The highest concentrations of accumulated contaminants have been reported for PCPs, especially fragrance compounds. For example, galaxolide has been detected at concentrations up to 6400 ng/g wet weight.[18] Relatively high concentrations of the surfactant metabolite nonylphenol-monoethoxylate have also been reported (ca. 250 ng/g wet weight).[22, 23] In contrast, environmental concentrations of accumulated pharmaceuticals are typically lower, ranging from 0.1 to approximately 15 ng/g wet weight, and have been shown to be variable within different tissues dissected from a single organism.[10, 25]

Note that some concentrations given in Table 1.1 have been normalized based on lipid content of the organism. Such normalization stems from the historical viewpoint that accumulated organic compounds are most likely partitioned into fatty tissues (i.e., lipids). This view is supported by multiple observations of a predictive correlation between octanol-water partition coefficients (K_{OW}) and bioconcentration of contaminants

Table 1.1. Occurrence and analytical methodologies for PPCPs in aquatic organisms

Group	sample and target analytes	environmental concentration	method	reference
PCP/ UV-filter	Swiss lake fish, BP-3, OC, EHMC, 4-MBC and MTCS	Low conc. detected 4-MBC ↑ 166, BP-3 ↑ 123, EHMC ↑ 64, OC ↑ 25 ng/g lipid weight	20 g of fillet homogenized with Na_2SO_4, mixed with 150 ml CH_2Cl_2/cyclohexane 1:1, transferred to a glass column and row extract cleaned up by GPC, silica chromatography, GC-MS	Balmer et al. *Environ. Sci. Technol*. **2005**, *39*, 953-962
PCP/ UV-filter	Swiss river fish, OC and 4-MBC	4-MBC ↑ 1800, OC ↑ 2400 ng/g lipid weight	10-25 g of fillet suspended in 100 ml of water and blended, 2 L separation funnel with 2 ml $K_2C_2O_4$ 35%, 100 ml ethanol, 50ml diethylether, 70 ml *n*-pentane, GPC, silica gel clean up, GC-MS	Buser et al. *Environ. Sci. Technol*. **2006**, *40,* 1427-1431
PCP/ UV-filter	laboratory fish, 4-MBC, OC, BP-3 and EHMC	na	10 g of fillet homogenized in a blender, and extracted by soxhlet using a solvent mixture of 200 ml *n*-hexane/acetone (9/1, v/v), GPC, SPE, GC-MS	Meinerling et al. *Anal. Bioanal. Chem.* **2006**, *386*, 1465-1473
PCP/ anti microbial	fish, TCS and its 3-chlorinated derivates	na	10 g homogenized in MeCN, hexane and water washed, mixed with NaOH, NaCl and Hexane, extracted with HCl-hexane, dehydrated and saponificated, SPE, GC-MS	Okumura et al. *Anal. Chim. Acta,* **1996**, *325*, 175-184
PCP/ anti microbial	Swiss river and lake fish, MTCS	↑ 35 ng/g wet weight	25 g of fillet homogenized with Na_2SO_4, mixed with 150 ml CH_2Cl_2/cyclohexane 1:1, GPC, silica chromatography, GC-MS	Balmer et al. *Environ. Sci. Technol.* **2004**, *38,* 390-395

Table 1.1. Occurrence and analytical methodologies for PPCPs in aquatic organisms (cont.)

Group	sample and target analytes	environmental concentration	method	reference
PCP/ anti microbial	German ESB bream, TC, CP, and MTCS	1994-2003, TCS, 0.29-1.7; MTC, 3.8-26.1; CP, 0.31-2.9 ng/g	1-2.5 g mixed with Na_2SO_4, accelerated solvent extracted with cyclohexane at 100 °C, 14 MPa, GPC, silica gel, GC-MS/MS	Bohemer et al. *Organohalogen Compd*. **2004**, *66* 1516-1521
PCP/ musk	laboratory fish, HHCB, AHTN, ATII,ADBI, DPMI, AHMI, AETT, MK, MM, MX AND MT	na	100g of fish from whole homogenate closed-loop stripped in 400 ml of water + 80 ml of NaOH (1M) + 50 g $NaSO_4$, 50 °C, stirred and N_2 introduced to the soln., musks collected in a SPE cartridge, 24h, GC-MS	Osemwengie et al. *J. Chromatogr. A* **2003**, *993*, 1-15
PCP/ musk	Nevada carp, blood, 4-amino-MX	4-amino-MX: 6.0-30.6 ng/g in carp hemoglobin	blood samples centrifuged at 3k g for 10 min at 4°C, red blood cell separated from plasma, washed, lysed, centrifuged, dialyzed for 72h, alkaline hydrolyzed, GC-SIM-MS	Mottaleb et al. *J. Anal. Toxicol.*. **2004**, 28, 581-586
PCP/ musk	Japan coastal water marine mammals and shark, AHTN, HHCB, MX, MK and MA	HHCB: blubber ↑149; shark liver ↑48 ng/g. AHTN and nitro musks below DL	1-4 g tissue ground with $NaSO_4$, soxhlet extracted with CH_2Cl_2-Hexane (8:1) 7h, GPC, silica gel, GC-MS	Nakata et al. *Environ. Sci. Technol*. **2005**, *39,* 3430-3434
PCP/ musk	trout Danish fish, HHCB, AHTMI, ATII ADBI, AHMI, MK, MM, MA, MX and MT	median: yr/MX, HHCB: 1999/0.5, 5.0; 2003-2004/nd, 1.2 ng/g wet weight	10 g of fillet homogenate extracted by ultraturrax for 3 min at 12,000 rpm with 100 ml of acetone: pentane (1:3) and 12 g Na_2SO_4, GPC SPE, GC-HRMS	Duedahl-Olesen et al. *Chemosphere* **2005**, *61*, 422-431

Table 1.1. Occurrence and analytical methodologies for PPCPs in aquatic organisms (cont.)

Group	sample and target analytes	environmental concentration	method	reference
PCP/ musk	German ESB mussel and bream, HHCB, AHTN, AHMI, AETT, ADBI, MK and MX	HHCB, AHTN: mussel 0.5-1.7, 0.4-2.5; bream 545-6400, 48-2130 ng/g wet weight	1-5 g of sample, Na_2SO_4 added, accelerated solvent extracted (*n*-hexane, 80 °C, 14 MPa, 10 min), GPC, activated silica gel, GC-MS/MS	Rudel et al. *J. Environ. Monit.* **2006**, *8*, 812-823
PCP/ musk	Alpine lake fish, ADBI, AHMI, AHTM ATII, HHCB, MK and MX	AHTN: 20-54; HHCB: 42-230; MX 1.3-12; and MK: 2.0-2.9 ng/g lipid weight	different fillet species homogenized in 300 g pools, GPC, GC-MS	Schmid et al. *Chemosphere* **2007**, *67*, S16-S21
PCP/ musk	marine Ariake Sea organisms, AHTM, MX HHCB, MK and MA	clam: HHCB 258-2730 ng/g lipid weight, AHTN smaller	1-4 g tissue ground with $NaSO_4$, soxhlet extracted with CH_2Cl_2-hexane (8:1) 7h, GPC, silica gel, GC-MS	Nakata et al. *Environ. Sci. Technol*. **2007**, *41,* 2216-2222
PCP/ musk	MA, IL and CO fish and mussel, MX, HHCB, AHTN, ADBI AHMI, ATII and MK	average: mussel-HHCB 14.9, AHTN 10.0; fish-HHCB 1.12 ng/g wet weight	aliquot mixed with 30g Na_2SO_4, pressurize fluid extracted with CH_2Cl_2 at 13.79 MPa 1-4 h, SEC, SPE 5% deactivated alumina, GC-MS	Peck et al. *Anal. Bioanal. Chem.* **2007**, *387*, 2381-2388
EDC/ surfactant	laboratory fish and shellfish, OP and NPs	na	5 g in MeCN, lipids eliminated with hexane-MeCN, MeCN fraction evaporated, reconstituted in hexane, SPE cleaned up, GC-MS	Tsuda et al. *J. Chromatogr. B* **1999**, *723*, 273-279

Table 1.1. Occurrence and analytical methodologies for PPCPs in aquatic organisms (cont.)

Group	sample and target analytes	environmental concentration	method	reference
EDC/ surfactant	laboratory fish and shellfish, NP, NPE1, NPE2 and 4-t-OP	na	5 g in MeCN, lipids eliminated with hexane-MeCN, MeCN fraction evaporated, reconstituted in hexane, SPE cleaned up, HPLC fluorescence	Tsuda et al. *J. Chromatogr. B* **2000**, *746*, 305-309
EDC/ surfactant	Michigan fish, NP and NPE1-NPE3	NP 3.3-29.1 ng/g wet weight, rest below MDL	20 g into 2L flask with 20 g NaCl, 3 ml H_2SO_4, steam distillated 3h, concentrated to1 ml of isooctane HPLC- fluorescence clean up, GC-MS	Keith et al. *Environ. Sci. Technol*. **2001**, *35*, 10-13
EDC/ surfactant	Adriatic Sea seafood, NP, OP, OPE, and NPE1-NPE4	NP ↑ 696, OP ↑ 18.6, OPE ↑ 0.43 ng/g lipid weight	no skin or internal organs, 100 g of homogenized sample from each pool, 1-1.5 g, lipid removed with a mixture MeCN: 0.1 M NaOH 1:1, acidified and put into organic solvent, GC-MS	Ferrara et al. *Environ. Sci. Technol*. **2001**, *35,* 3109-3112
EDC/ surfactant	Las Vegas Bay of lake Mead carp, NP and NPE1-NPE3	average NP 184, NPE1 242 ng/g wet weight	20 g + 350 ml of water, blended, mixed with 20 g NaCl, 3 ml H_2SO_4, steam distillated 3h, concentrated to 1 ml in isooctane, HPLC-fluorescence, GC-MS	Snyder et al. *Environ. Toxicol. Chem.* **2001**, *20*, 1870-1873
EDC/ surfactant	German ESB mussel and bream, NP NPE1 and OPE1	bream 1994: NP ↑ 112, NPE1 ↑ 259, OP ↑ 5.5, OPE1 ↑ 2.6; mussel: NP ↑ 41 n/g wet weight	1 g, digested with 25% $(CH_3)_4NOH$, 60 °C for 1h, 10 ml of hexane and centrifuged, organic layer dried, 50 ul of Grignard reagent added, silica gel clean up and GC-MS	Wenzel et al. *Environ. Sci. Technol*. **2004**, *38,* 1654-1661
Pharma ceuticals/ anti-inflamatory	laboratory fish diclofenac	na	organs kept at -20 °C until analyzed, clean up procedure included SPE (Extrelut NT 20) and methylation with TMSH, GC-MS	Schwaiger et al. *Aquat. Toxicol.* **2004**, *68*, 141-150

Table 1.1. Occurrence and analytical methodologies for PPCPs in aquatic organisms (cont.)

Group	sample and target analytes	environmental concentration	method	reference
Pharma ceuticals/ anti-depressants	Texas muscle, brain & liver fish, fluoxetine and sertraline, and its metabolites	mean (brain): fluox 1.58, nor 8.86, ser 4.27 desser 15.6 ng/g wet w. brain conc.>liver>muscle	tissue homogenized, 1:5 diluted with PO_4 0.1 M pH 6, vortexed 10 min, ice cold MeCN added, centrifuged at 820 g 5 min, evaporated, reconstituted, SPE, and GC-MS	Brooks et al. *Environ. Toxicol. Chem.* **2005**, *24*, 464-469
Pharma ceuticals/ antibiotics	laboratory fish, eight veterinary anti-biotics representing three classes	na	5 g of homogenized fish mixed 50 ml MeCN, centrifuged at 1700 g for 1 min, supernatant through 15 g Na_2SO_4, filtrated, 5 ml of HAc, SPE, HPLC-MS and HPLC	Stubbings et al. *Anal. Chim. Acta.* **2005**, *547*, 262-268
Pharma ceuticals/ atilipemic	laboratory fish blood, gem	na	10 µl of plasma, acidified to pH 2 with H_2SO_4, SPE, eluted with 1 ml of ethanol, HLC-MS	Mimeault et al. *Aquat. Toxicol.* **2005**, *73*, 44-54
Pharma ceuticals/ antibiotics	laboratory fish, four tetracycline antibiotics	na	1 g of fillet, 0.01 M EDTA added (pH 4), pureed for 5 min, kept in the dark for 30 min at 4 °C, centrifuged 16k rpm for 5min, supernatant extracted in a monolithic capillary column, HPLC-UV	Wen et al. *Talanta* **2006**, *70*, 153-159
Pharma ceuticals/ anti-depressants	Canada fish, fluox, paroxetine, and norfluoxetine	three analytes at conc. ↑ 1 ng/g wet weight	3 g of ground fish pressurize liquid extracted with MeOH, rotary evaporated, SPE, eluted and HPLC-APCI-MS/MS	Chu et al. *J. Chromatogr. A.* **2007**, *1163*, 112-118

Table 1.1. Occurrence and analytical methodologies for PPCPs in aquatic organisms (cont.)

Group	sample and target analytes	environmental concentration	method	reference
Pharma ceuticals/ analgesics & antilipemic	laboratory fish blood, plasma, ibupro, napro, diclo, keto, and gem	na	300 µl plasma with 900 µl of HCOOH 1% extracted with SPE, eluted 1 ml MeOH, dried, 1 ml acetone, dried again, derivatized and GC-MS	Brown et al. *Environ. Toxicol. Pharma.* **2007**, *24*, 267-274
Pharma ceuticals/ anti-depressants	laboratory fish, fluox and norfluoxetine	na	50 mg fish body, 250 µl Na_2CO_3, 250 µl H_2O, and 500 µl of 1:1 n-hexane-diethylether added, homogenized, centrifuged, liquid extraction, derivartization, GC-MS	Nakamura et al. *Chemosphere* **2007**, *in press*

EDC, endocrine disrupting compound; na, non-applicable; BP-3, benzophenone-3; OC, octocrylene; ↑, up to; EHMC, ethylhexyl methoxycinnamate; 4-MBC, 4-methylbenzylidene camphor; MTCS, methyl-triclosan; GPC, gel permeation chromatography; SPE, solid phase extraction; TCS, triclosan; ESB, Environmental Specimen Bank; CP, chlorophene; DL, detection limit; nd, non detected; HHCB, galaxolide; AHTN, tonalide; MX, musk xylene; MK, musk ketone; MA, musk ambrett; ADBI, celestolide; AHMI, fixolide; ATII, traseolide; DPMI, cashmeran; AETT, versalide; MT, musk tibetene; MM, musk moskene; HRMS, high resolution mass spectrometry; NP, nonylphenol; OP, octylphenol, OPE, ethylphenol ethoxylate; NPE1, 4-nonylphenolmonoethoxylate; NPE2, 4-nonylphenoldiethoxylate; NPE3, 4-nonylphenoltriethoxylate; NPE4, 4-nonylphenoltetraethoxylate; SEC, size exclusion chromatography; 4-t-OP, 4-tert-octylphenol; MDL, method detection limit; fluox, fluoxetine; nor, norfluoxetine; ser, sertraline; desser, desmethylsertraline; ibupro, ibuprofen; napro, naprofen; diclo, diclofenac; keto, ketoprofen; gem, gemfibrozil.

from aqueous solution.[38-41] However, it is important to point out that these correlations were developed for neutral molecules possessing relatively large values of K_{OW} (e.g., log $K_{OW} > 4$). The physicochemical properties of PPCPs do not always conform to this stipulation. This is especially true for some pharmaceuticals which are expected to be charged at environmentally-relevant pH. Consequently, the historical models that have proven to be effective at predicting accumulation of pesticides or PCBs may not be applicable to partitioning of PPCPs in aquatic systems. Nevertheless, both normalized and un-normalized data continue to appear in literature.

An important analytical observation from Table 1.1 is that while methodologies for determination of PPCPs in aquatic organisms are numerous, each is limited in scope. That is, each methodology focuses on a select group of compounds, typically from the same analyte class. Additionally, it is important to note that the number of reported methodologies for assessment of pharmaceuticals is relatively small compared to the number reported for assessment of PCPs.

Mass Spectrometry and Environmental Analysis

Gas chromatography-mass spectrometry (GC-MS) was the primary analytical tool used to assess the environmental occurrence of PPCPs in initial studies. The popularity of GC-MS in early work was due to its widespread availability and historical use in contract service laboratories. The availability of electron-impact spectral libraries was also seen as a plus, increasing confidence in analyte identification, and the distinctive non-polar operating range of GC-MS was consistent with analysis of most PCPs. In contrast, the use of GC-MS for analysis of pharmaceuticals, which are relatively polar compared to PCPs, typically requires derivatization prior to analysis. These reactions are

often unpredictable for complex samples and can limit the quality of quantitative data. Consequently, liquid chromatography-mass spectrometry (LC-MS) has become the technique of choice for analyzing pharmaceuticals in environmental samples.

Numerous studies have demonstrated the distinct advantages of LC-MS for analysis of pharmaceuticals. The LC-MS approach enables identification and quantification without derivatization, and typically results in lower detection limits (below 1 ng/L and 1 ng/g for liquid and solid samples, respectively) and better precision than comparable GC-MS methodologies. In environmental applications, LC is typically combined with tandem MS (i.e., MS/MS) to promote enhanced selectivity and sensitivity for target analytes. In a routine MS/MS analysis, a molecular ion is selected and subsequently fragmented to produce one or more distinctive product ions that enable both qualitative and quantitative monitoring.

It is important to note, however, that LC-MS is not exempt from limitations. One of the limitations of LC-MS is that atmospheric pressure ionization (API) processes are influenced by co-extracted matrix components. Matrix effects typically result in suppression or less frequently enhancement of analyte signal. There have been a number of methods proposed to compensate for matrix effects, including the method of standard addition,[42-46] surrogate monitoring,[47, 48] and isotope dilution.[45, 46, 49-53] Although isotope dilution is the most highly-recommended approach,[45, 53] isotopically-labeled standards are not always readily available.[49, 54] An alternative approach involves the use of an appropriate internal standard (i.e., a structurally-similar compound expected to mimic the behavior of a target analyte(s)) with or without matrix-matched calibration. However, a given internal standard is typically effective over a limited retention time window.[55]

Accordingly, the use of more than one internal standard is recommended to compensate for matrix effects throughout the chromatographic run. Finally, it is important to point out that strategies to compensate for matrix effects should take into account the variability of matrix within each set of samples to be analyzed (e.g., river water, WWTP effluent, sediment extracts, fish, etc.).

Quality Control and Quality Assurance

Due to potential regulatory implications, environmental analyses typically include rigorous quality assurance and quality control (QA/QC) metrics to confirm reliability of analytical data. Initial method validation provides essential performance parameters, such as method recoveries, precision, and limits of detection (LODs). Recurring analysis of quality control (QC) samples (e.g., method blanks, matrix spikes, and laboratory control samples) is not only important to verify performance of the method over time, but also to assess potential matrix effects. Considering the unpredictable nature of matrix interference in LC-MS analysis and the lack of effective strategies to deal with this difficulty, it has become imperative to use QA/QC data to document and qualify analytical results. This is especially important when reporting concentrations at or near the limit of detection for a given analytical method.

Scope of the Dissertation

In order for the reader to appreciate the broader context of experimental work described herein, it is important to discuss the chronological development of research focused on accumulation of PPCPs in aquatic organisms that has occurred over the previous five years. Our efforts in this area were initiated in the later part of 2002, as

occurrence of PPCPs in surface water and wastewater was frequently being reported. As demonstrated by the publication dates shown in Table 1.1, surfactants were the only PPCPs that had been shown to accumulate in aquatic organisms at this time. However, there was increasing interest in assessment of a larger group of PPCP analytes, and methodologies for determination of musk fragrances in tissues were beginning to appear in literature. In the summer of 2003, a collaborative study, led by Dr. Bryan Brooks and involving additional Baylor researchers (including this author) and co-workers from the City of Denton Watershed Resources Program and Federal Aviation Administration Civil Aerospace Medical Institute, established for the first time that select human pharmaceuticals (i.e., antidepressants) could also be accumulated in fish residing in surface waters impacted by wastewater treatment effluent. Although resulting data did not appear in the primary literature until 2005, results of this benchmark study led to the primary research question that ultimately shaped this dissertation. Namely, whether alternative pharmaceuticals were also accumulated in fish residing in effluent-dominated streams?

The critical first step in being able to address this question was the development of suitable analytical methodology to monitor pharmaceuticals in fish tissue. While numerous methods for monitoring pharmaceuticals in water were available in literature, none had been reported that were applicable to tissues. In an effort to obtain maximum information with minimum analytical effort, the decision was made to focus on the development of a broad screening method, incorporating analytes with diverse physicochemical properties and belonging to multiple therapeutic classes.

The development and application of the first liquid chromatography-tandem mass spectrometry (LC-MS/MS) screening method for pharmaceuticals in fish is described in Chapter 2. Key steps in method development were identification of a suitable extraction solvent for recovery of 25 target compounds from fish muscle tissue and implementation of a quantitative protocol that compensated for observed matrix interference on electrospray ionization. The environmental relevance of the analytical approach was assessed by screening fish collected from a regional effluent-dominated stream (i.e., a stream significantly impacted by wastewater effluent). This initial study not only confirmed our previous report on antidepressants, but also resulted in the detection of three novel contaminants. This work was recently published in the American Chemical Society journal *Analytical Chemistry*.

During the course of method development for pharmaceuticals, it was determined that a multi-residue method for screening PCPs in fish tissue would also be a novel contribution to the literature. In Chapter 3, GC-MS methodology, employing selected ion monitoring (SIM), for simultaneous determination of 10 extensively used PCPs and 2 alkylphenol surfactants in fish is described. This work represents the first approach enabling routine monitoring of select UV-filters, fragrances, surfactants, an antimicrobial agent and an insect repellent in a single chromatographic run. The method was also applied to assess the presence of PCPs in the fish collected from a regional effluent-dominated stream that were also analyzed by LC-MS/MS (see above). Four compounds were detected at concentrations in general agreement with literature values determined using methods designed for a select group of compounds. It is important to note that much of this work was performed in collaboration with Dr. Mohammad A. Mottaleb.

In the spring of 2006, our group received notification that it had been selected to carry out analytical activities affiliated with the first National Pilot Study of PPCPs in Fish sponsored by the United States Environmental Protection Agency. That our laboratory was selected in an open competition further supports the novelty of work reported in Chapters 2 and 3. All sampling activities related to the pilot study were performed by personnel from TetraTech, Inc., and whole fish were sent to Baylor on dry ice for sample compositing and analysis. This study enabled assessment of PPCP accumulation in fish collected from 6 sites across the United States (5 effluent-dominated streams and one reference site). Results of LC-MS/MS screening analyses affiliated with this study are reported in Chapter 4 and clearly demonstrate: 1) that accumulation, and thereby exposure, of pharmaceuticals is likely limited to surface waters that are impacted by wastewater effluents and 2) that accumulated concentrations in fish are variable, depending on the type of process used to treat wastewater at a given site. Analytical observations resulting in slight modification of the method described in Chapter 2 are also presented.

CHAPTER TWO

Analysis of Pharmaceuticals in Fish Using Liquid Chromatography-Tandem Mass Spectrometry*

Introduction

The occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has received broad interest over the last decade.[1, 2, 56, 57] PPCPs have been increasingly detected in water, wastewater, soil, sediments, and biosolids. More recently, reports from our laboratory[10] and others[12, 13, 15, 17, 28] have demonstrated that environmental exposures to PPCPs may result in accumulation of parent compounds and/or their metabolites in tissues of aquatic organisms. These reports have heightened interest in secondary effects of PPCPs and impart a sense of urgency to research focused on understanding fate and partitioning of these compounds in aquatic systems.

Analytical methodologies for determination of PPCPs in water, sediment and biosolids are numerous and have been summarized in recent reviews.[7-9, 58] Due to the complexity of environmental samples, analyses typically employ detailed sample preparation followed by chromatographic separation of analytes and mass spectrometry detection. While methods focused on a single compound or unique compound class (e.g., antibiotics) continue to be reported,[59-63] increasing emphasis on simultaneous analysis of compounds with dissimilar physicochemical properties is evident in recent literature.[8, 48, 64-67] This shift in philosophy stems from a desire to gain diverse knowledge with minimal analytical expenditure.

At present, analytical methodologies for determination of PPCPs in aquatic organisms are numerous, but lack in scope of analytes. Procedures for measuring select compounds in fish tissues have been reported for diclofenac,[37] two antidepressants and their active metabolites,[10] 4 UV filters and methyl-triclosan,[12, 13] 12 musk fragrances,[15, 17] four tetracycline antibiotics,[26] and 8 veterinary antibiotics representing three structural classes.[27] The general approach employed for analysis of personal care products involved extraction of homogenized tissue with nonpolar solvents, followed by successive size-exclusion and silica gel cleanup procedures prior to gas chromatography- mass spectrometry (GC-MS) analysis.[12, 13, 15, 17] In contrast, pharmaceuticals were extracted from tissue using relatively polar solvents (i.e., aqueous buffer or acetonitrile), and extracts were cleaned up by solid phase extraction prior to GC-MS,[10, 37] HPLC[26, 27] or LC-MS[27] analysis.

Herein, we report the first multi-residue screening method for pharmaceuticals representing multiple therapeutic classes in fish tissue. This protocol enables simultaneous monitoring of 25 compounds using LC-MS/MS. Key steps in method development involved optimizing extraction of acidic, basic, and neutral analytes from 1-gram tissue homogenates and using matrix-matched calibration to compensate for observed matrix interference. As compared to previous methods for analysis of PPCPs in fish tissue, developed methodology offers relatively simple sample preparation in that tissue extracts are centrifuged and directly injected into the LC-MS/MS, following reconstitution in chromatographic mobile phase. The method was subsequently applied to assess the occurrence of target analytes in environmental samples. Four

pharmaceuticals were detected in all analyzed specimens, and accumulation of three of these compounds in fish tissues is reported here for the first time.

Experimental Section

Chemicals

All chemicals were reagent grade or better, obtained from commercial vendors, and used as received. The positive ESI internal standards 7-aminoflunitrazepam-d_7, and fluoxetine-d_6 (100.0 µg/ml in acetonitrile), surrogates (100.0 µg/ml in acetonitrile) acetaminophen-d_4, and diphenhydramine-d_3, and reference standards (1000.0 µg/ml in MeOH): fluoxetine, norfluoxetine, sertraline, codeine, diphenhydramine, propranolol and ibuprofen were purchased as certified analytical standards (Cerilliant Corporation, Round Rock, TX). Atenolol was purchased in solid form (99% purity), also from Cerilliant. The negative ESI internal standard meclofenamic acid and reference standards: 1,7 dimethylxanthine, acetaminophen, caffeine, miconazole, carbamazepine, erythromycin, gemfibrozil, trimethoprim, diltiazem, cimetidine, warfarin, thiabendazole, sulfamethoxazole, lincomycin, metoprolol, tylosin, clofibric acid were purchased in the highest available purity (Sigma-Aldrich, Milwaukee, WI). Surrogates (100.0 µg/ml in acetonitrile) carbamazepine-d_{10} and ibuprofen-$^{13}C_3$ were purchased from Cambridge Isotopes Lab. Inc., Andover, MA. Distilled water was purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond UV water purification system.

Sample Collection and Preservation

Pecan Creek and Clear Creek (two streams located in Denton County, TX, USA) were chosen for field sampling activities. Clear Creek is not impacted by effluent

discharges and is routinely used as a local reference stream by the City of Denton, Texas Watershed Protection program. In contrast, annual flows in Pecan Creek are comprised almost entirely of effluent discharge from the Pecan Creek Water Reclamation Plant. Effluent-dominated streams are likely worse case scenarios for investigating environmental exposures to PPCPs. Because these streams receive limited upstream dilution, wastewater contaminants may be considered 'pseudopersistent', and resident organisms may receive continuous life-cycle exposures. Fish (*Lepomis* sp.) were sampled from Pecan Creek (n = 11) and Clear Creek (n = 20) to serve as test and reference specimens, respectively. The approximate size of fish collected from these sites was similar and ranged from 8.8 cm to 11.5 cm (total length) and 29.4 g to 49.0 g. Lateral fillets were dissected from fish collected at both sites and homogenized using a Tissuemiser (Fisher Scientific, Fair Lawn, NJ) set to rotate at 30,000 rpm. Pecan creek homogenates were stored individually, while Clear Creek homogenates were composited into a single sample. All tissues were stored at –20 °C prior to analysis. No target analytes were detected in the Clear Creek composite. Accordingly, this tissue is hereafter referred to as 'clean'.

Analytical Sample Preparation

Approximately 1.0 g tissue was combined with 8 ml extraction solvent (see Fig. 2.2 for tested solvent compositions) in a 20 ml borosilicate glass vial (Wheaton; VWR Scientific, Rockwood, TN), and the mixture was homogenized using a Tissuemiser (Fisher Scientific, Fair Lawn, NJ) set to rotate at 30,000 rpm. Five surrogates were added to each sample: acetaminophen-d_4 (454 ng), fluoxetine-d_6 (636 ng), diphenhydramine-d_3 (8.9 ng), carbamazepine-d_{10} (38.5 ng) and ibuprofen-$^{13}C_3$ (789 ng). Samples were shaken

vigorously and mixed on a rotary extractor for five minutes. Following extraction, samples were rinsed into 50-ml polypropylene copolymer round-bottomed centrifuge tubes (Nalge Company; Nalgene® Brand Products, Rochester, New York) using 1 ml extraction solvent and centrifuged at 16,000 rpm for 40 min at 4 °C. The supernatant was decanted into 18-ml disposable borosilicate glass culture tubes (VWR Scientific, Rockwood, TN), and the solvent was evaporated to dryness under a stream of nitrogen at 45 °C using a Zymark Turbovap LC concentration workstation (Zymark Corp., Hopkinton, MA). Samples were reconstituted in 1 ml of mobile phase, and a constant amount of the internal standards 7-aminoflunitrazapam-d_7 (100 ng) and meclofenamic acid (1000 ng) was added. Prior to analysis, samples were sonicated for 1 min and filtered using Pall Acrodisc hydrophobic Teflon Supor membrane syringe filters (13 mm diameter; 0.2-µm pore size; VWR Scientific, Suwanee, GA).

LC-MS/MS Method

A Varian ProStar Model 210 binary pump equipped with a Model 410 autosampler was used in this study. Analytes were separated on a 15 cm × 2.1 mm (5 µm, 80 Å) Extend-C18 column (Agilent Technologies, Palo Alto, CA) connected with an Extend-C18 guard cartridge 12.5 mm x 2.1 mm (5 µm, 80 Å) (Agilent Technologies, Palo Alto, CA). A binary gradient consisting of 0.1% (v/v) formic acid in water and 100% methanol was employed to achieve chromatographic separation and is defined in Table 2.1. Additional chromatographic parameters were as follows: injection volume, 10 µl; column temperature, 30 ºC; flow rate, 350 µl/min. Eluted analytes were monitored by MS/MS using a Varian model 1200L triple-quadrupole mass analyzer equipped with an electrospray interface (ESI).

Table 2.1. HPLC gradient elution profile

	Mobile phase composition, %	
Time (min)	0.1 % formic acid	Methanol
0	93	7
2	93	7
7	85	15
12	85	15
21	52	48
28	52	48
34	41	59
45	2	98
50	2	98
51	93	7
65	93	7

To determine the best ionization mode (ESI + or −) and optimal MS/MS transitions for target analytes, each compound was infused individually into the mass spectrometer at a concentration of 1 μg/ml in aqueous 0.1% (v/v) formic acid at a flow rate of 10 μL/min. All analytes were initially tested using both positive and negative ionization modes while the first quadrupole was scanned from *m/z* 50 to [M + 100]. This enabled identification of the optimal source polarity and most intense precursor ion for each compound. Once these parameters were defined, the energy at the collision cell was

varied, while the third quadrupole was scanned to identify and optimize the intensity of product ions for each compound. Additional instrumental parameters held constant for all analytes were as follows: nebulizing gas, N_2 at 60 psi; drying gas, N_2 at 19 psi; temperature, 300 °C; needle voltage, 5000 V ESI+, 4500 V ESI-; declustering potential, 40 V; collision gas, argon at 2.0 mTorr.

Extraction Recoveries

Two groups of control samples prepared from 'clean' tissue were employed to determine extraction efficiency for target analytes. Group 1 samples were spiked with internal standards and each analyte, while group 2 samples were spiked with internal standards only. Both groups of samples were carried through the sample preparation procedure described above. Following syringe filtration, group 2 samples were spiked with the same amount of each analyte added to group 1. All samples were analyzed by LC-MS/MS, and individual analyte recoveries were calculated using the following equation:

$$\text{recovery} = \frac{A_{X1}/A_{IS1}}{A_{X2}/A_{IS2}} \times 100\% \qquad (2.1)$$

where A_{X1}, A_{IS1}, A_{X2} and A_{IS2} represent peak areas for the analyte (X) and internal standard (IS) in groups 1 and 2, respectively.

Results and Discussion

LC-MS/MS Methodology

Three factors were considered in selecting target analytes (Table 2.2): *i*) number of prescriptions dispensed in the United States during 2005,[68] *ii*) variability in structure,

physicochemical properties and therapeutic use, and *iii*) relative frequency of occurrence in soils, sediments and biosolids. Excluding potential ion-exchange phenomena, the physicochemical properties favoring compound partitioning from water to solid environmental matrices may also promote accumulation of water-borne chemicals in aquatic biota via diffusion across biological membranes. Additionally, compounds residing in sediment may be taken up by aquatic organisms via ingestion. Furlong et. al. summarized results from several U.S. Geological Survey occurrence studies targeting PPCPs in environmental matrices and demonstrated that the frequency of detection for fluoxetine in analyzed sediment, soil and biosolid samples (64-100%) was much higher than in water (5%).[69] This general trend was observed for seventeen additional compounds assessed in their work. Since fluoxetine was previously shown by our group to accumulate in fish tissues,[10] it seemed reasonable to target compounds with a similar occurrence pattern in screening activities.

Compound-dependent mass spectrometry parameters were investigated by direct infusion of individual analytes into the electrospray source. Optimized MS/MS transitions and collision energies employed for detection and quantitation of each analyte are provided in Table 2.2, along with the molecular structure and most common therapeutic use for each analyte. With the exception of erythromycin, selected precursors represent the molecular ion $[M + H]^+$ or $[M - H]^-$ for each analyte. The most abundant precursor for erythromycin was found to be the $[M + H - H_2O]^+$ ion at *m/z* 716, consistent with previous observations.[48, 58] Selected product ions represent the most abundant fragment observed for each precursor at the noted collision energy.

Table 2.2. Analyte-dependent mass spectrometry parameters for target analytes

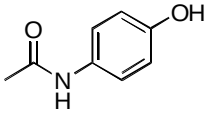
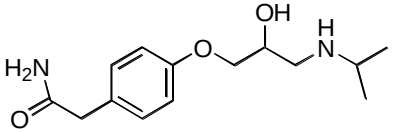
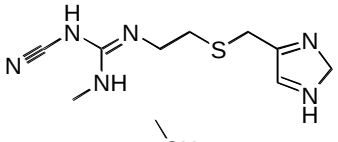
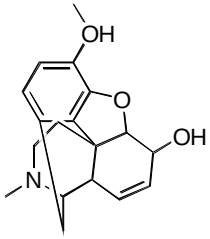
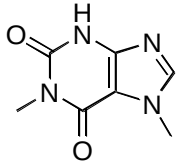
Compound	use	structure	precursor ion	collision energy (eV)	product ion	pKa[a]
ESI POSITIVE ANALYTES						
Acetaminophen	analgesic		152 $[M + H]^+$	-11.0	110	9.86
Atenolol	anti-hypertension		267 $[M + H]^+$	-21.5	145	9.16
Cimetidine	anti-acid reflux		253 $[M + H]^+$	-13.5	159	7.07
Codeine	analgesic		300 $[M + H]^+$	-38.0	215	8.25
1,7-dimethylxanthine	caffeine metabolite		181 $[M + H]^+$	-15.5	124	8.50

Table 2.2. Analyte-dependent mass spectrometry parameters for target analytes (cont.)

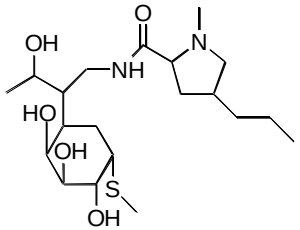
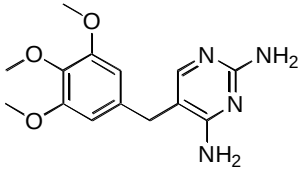
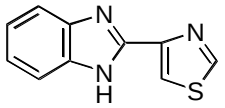
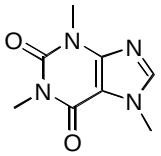
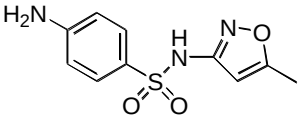
Compound	use	structure	precursor ion	collision energy (eV)	product ion	pKa[a]
Lincomycin	antibiotic		407 $[M + H]^+$	-15.5	359	8.78
Trimethoprim	antibiotic		291 $[M + H]^+$	-17.5	261	7.20
Thiabendazole	antibiotic		202 $[M + H]^+$	-23.0	175	
Caffeine	stimulant		195 $[M + H]^+$	-16.0	138	
Sulfamethoxazole	antibiotic		254 $[M + H]^+$	-13.0	156	5.81

Table 2.2. Analyte-dependent mass spectrometry parameters for target analytes (cont.)

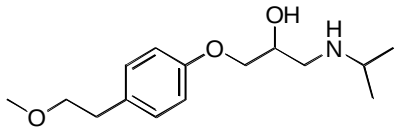
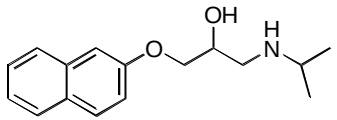
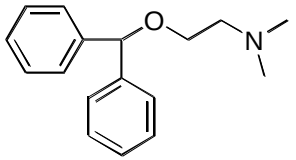
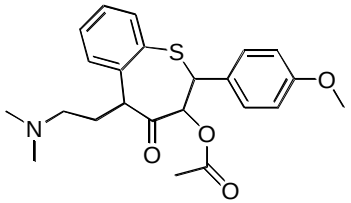
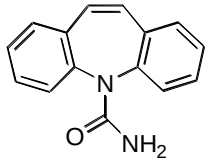
Compound	use	structure	precursor ion	collision energy (eV)	product ion	pKa[a]
Metoprolol	anti-hypertension		268 $[M + H]^+$	-15.5	191	9.17
Propranolol	anti-hypertension		260 $[M + H]^+$	-11.0	116	9.14
Diphenhydramine	antihistamine		256 $[M + H]^+$	-11.5	167	8.76
Diltiazem	anti-hypertension		415 $[M + H]^+$	-22.0	178	8.94
Carbamazepine	anti-seizure		237 $[M + H]^+$	-13.5	194	

Table 2.2. Analyte-dependent mass spectrometry parameters for target analytes (cont.)

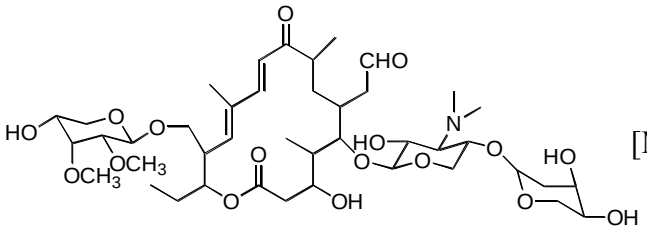
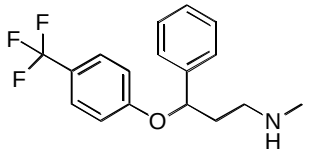
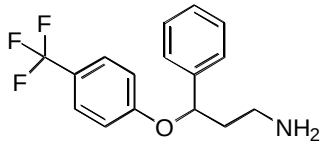
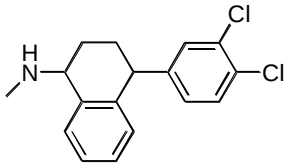
Compound	use	structure	precursor ion	collision energy (eV)	product ion	pKa[a]
Tylosin	antibiotic		916 $[M + H]^+$	-31.5	174	7.39
Fluoxetine	antidepressant		310 $[M + H]^+$	-6.0	148	10.1
Norfluoxetine	fluoxetine metabolite		296 $[M + H]^+$	-4.5	134	9.05
Sertraline	antidepressant		306 $[M + H]^+$	-11.0	275	9.47

Table 2.2. Analyte-dependent mass spectrometry parameters for target analytes (cont.)

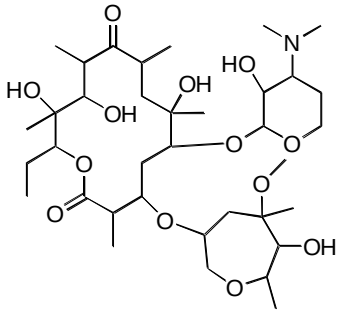
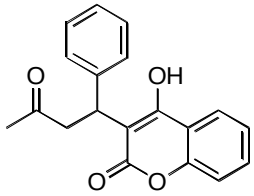
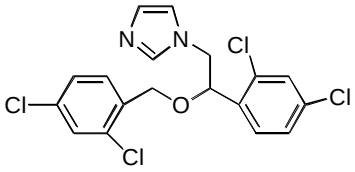
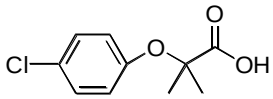
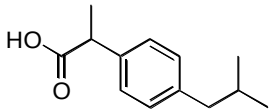
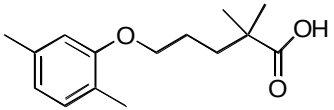
Compound	use	structure	precursor ion	collision energy (eV)	product ion	pKa[a]
Erythromycin	antibiotic		716 $[M + H - H_2O]^+$	-18.0	558	8.16
Warfarin	anti-coagulant		309 $[M + H]^+$	-14.0	163	4.50
Miconazole	antibiotic		417 $[M + H]^+$	-27.5	161	6.67

Table 2.2. Analyte-dependent mass spectrometry parameters for target analytes (cont.)

Compound	use	structure	precursor ion	collision energy (eV)	product ion	pKa[a]
ESI NEGATIVE ANALYTES						
Clofibric Acid	antilipemic		213 $[M - H]^-$	15.4	127	3.18
Ibuprofen	analgesic		205 $[M - H]^-$	7.0	161	4.41
Gemfibrozil	antilipemic		249 $[M - H]^-$	13.0	121	4.75

[a] Calculated values obtained from the SciFinder database (© 2006 American Chemical Society).

Once suitable MS/MS transitions were identified for each analyte, an aqueous mixture of reference standards was employed to optimize chromatographic parameters. A non-linear gradient consisting of 0.1% (v/v) formic acid and methanol resulted in near-baseline resolution of the majority of analytes in approximately 50 minutes (Fig. 1.1). A 15-minute isocratic hold (93:7 formic acid-methanol) was added to the end of each run to allow for column equilibration between injections. While the majority of analytes were eluted as single peaks, erythromycin was consistently eluted as two partially-resolved peaks. Similar chromatographic behavior for erythromycin has been observed previously and attributed to differing retention characteristics for presumed sterioisomers.[48, 70]

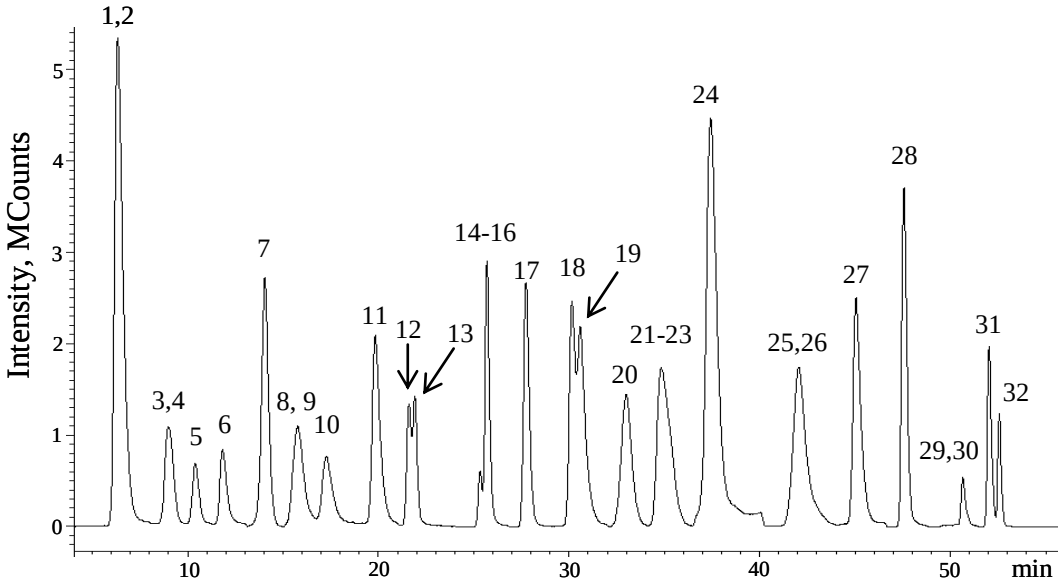


Figure 2.1. Time-schedule chromatogram of a spiked 'clean' muscle sample collected from Clear Creek. Peak identifications are as follows: (1) acetaminophen, (2) acetaminophen-d_4, (3) atenolol, (4) cimetidine, (5) codeine, (6) 1,7-dimethylxanthine, (7) lincomycin, (8) trimethoprim, (9) thiabendazole, (10) caffeine, (11) sulfamethoxazole, (12) 7-aminoflunitrazepam-d_7 (+IS), (13) metoprolol, (14) propranolol, (15) diphenhydramine, (16) diphenhydramine-d_3, (17) diltiazem, (18) carbamazepine, (19) carbamazepine-d_{10}, (20) tylosin, (21) fluoxetine, (22) fluoxetine-d_6, (23) norfluoxetine, (24) sertraline, (25) erythromycin, (26) clofibric acid, (27) warfarin, (28) miconazole, (29) ibuprofen, (30) ibuprofen-$^{13}C_3$, (31) meclofenamic acid (-IS), (32) gemfibrozil.

Additionally, isotope effects on retention behavior were observed for carbamazepine-d_{10} and fluoxetine-d_6. As evident in Figure 2.1 (peaks 18 and 19), the observed retention time for carbamazepine-d_{10} (30.08 min) was shorter than that observed for carbamazepine (30.53 min) by almost 30 seconds. Though not evident in Figure 2.1 due to co-elution of norfluoxetine (35.13 min), a 20-second difference in retention time was also observed for fluoxetine-d_6 (34.58 min) relative to that observed for fluoxetine (34.93 min). These differences are admittedly small but were very reproducible, and observed behavior for these analytes is consistent with previous studies demonstrating stronger retention for unlabeled compounds than deuterated analogs in reverse-phase chromatography.[71-73] Presumably, isotope effects were not observed for acetaminophen (peaks 1 and 2) and diphenhydramine (peaks 15 and 16) due to a lower degree of deuterium substitution and decreased resolution at shorter retention times.

Extraction of Target Analytes from Fish Tissue

Due to considerable variation in lipophilicity and pK_a among pharmaceuticals, a systematic study of extraction behavior was conducted with the goal of identifying a single solvent system affording optimized recoveries for the full range of analytes from 'clean' muscle tissue. Ten solvents, differing in pH and/or polarity, were tested. Mean recoveries (n=3) were calculated for individual analytes in each solvent system and are tabulated in Table 2.3. These data are summarized in Fig. 2.2, where individual analyte recoveries were averaged for each solvent system. 'Error bars' in this plot represent one standard deviation from the average and provide an assessment of variability among mean recoveries for individual analytes. While these data have no statistical relevance,

Table 2.3. Individual extraction recoveries (%) for tested solvent systems

Analyte	Average (n = 3) plus minus one standard deviation									
	CH_2Cl_2 -C_6H_{14}	CH_2Cl_2 -MeOH	MeOH -MeCN	TFA pH 2.4 -MeOH	HAc pH 4 -MeOH	HAc pH 4 -MeCN	PO_4 pH 6 -MeOH	TFA pH 2.4	HAc pH 4	PO_4 pH 6
Acetaminophen	8 ± 2	78 ± 22	85 ± 17	89 ± 4	92 ± 4	102 ± 4	98 ± 7	79 ± 6	61 ± 10	68 ± 8
Atenolol	25 ± 6	73 ± 11	85 ± 3	84 ± 5	97 ± 4	109 ± 2	97 ± 7	81 ± 6	71 ± 6	73 ± 8
Cimetidine	14 ± 1	79 ± 22	89 ± 9	92 ± 7	95 ± 4	108 ± 3	95 ± 8	77 ± 7	68 ± 11	71 ± 8
Codeine	100 ± 21	64 ± 25	90 ± 18	82 ± 4	86 ± 5	101 ± 3	90 ± 9	70 ± 4	45 ± 14	61 ± 7
1,7-Dimethylxanthine	9 ± 4	84 ± 10	93 ± 2	93 ± 5	92 ± 2	95 ± 7	92 ± 7	78 ± 5	60 ± 5	63 ± 8
Lincomycin	26 ± 1	65 ± 9	76 ± 8	86 ± 4	90 ± 5	98 ± 2	92 ± 3	73 ± 6	67 ± 5	72 ± 6
Trimethoprim	88 ± 26	68 ± 30	79 ± 9	90 ± 4	95 ± 3	104 ± 5	86 ± 11	66 ± 3	60 ± 3	65 ± 7
Thiabendazole	57 ± 15	65 ± 32	78 ± 9	82 ± 3	84 ± 4	97 ± 7	88 ± 4	54 ± 4	40 ± 6	45 ± 9
Caffeine	98 ± 8	80 ± 11	92 ± 2	84 ± 4	85 ± 3	96 ± 8	86 ± 7	80 ± 6	60 ± 9	67 ± 6
Sulfamethoxazole	11 ± 3	67 ± 11	78 ± 2	81 ± 3	79 ± 3	81 ± 6	84 ± 8	61 ± 4	42 ± 5	53 ± 7

Table 2.3. Individual extraction recoveries (%) for tested solvent systems (cont.)

Analyte	Average (n = 3) plus minus one standard deviation									
	CH_2Cl_2 -C_6H_{14}	CH_2Cl_2 -MeOH	MeOH -MeCN	TFA pH 2.4 -MeOH	HAc pH 4 -MeOH	HAc pH 4 -MeCN	PO_4 pH 6 -MeOH	TFA pH 2.4	HAc pH 4	PO_4 pH 6
Metoprolol	98 ± 31	65 ± 28	71 ± 13	73 ± 3	91 ± 4	96 ± 6	91 ± 6	69 ± 3	65 ± 8	66 ± 7
Propranolol	49 ± 8	47 ± 9	64 ± 14	44 ± 11	89 ± 3	90 ± 12	73 ± 4	27 ± 2	49 ± 10	36 ± 6
Diphenhydramine	35 ± 5	57 ± 6	68 ± 16	35 ± 8	75 ± 6	86 ± 11	83 ± 5	36 ± 4	47 ± 10	42 ± 9
Diltiazem	39 ± 12	50 ± 20	70 ± 1	18 ± 2	92 ± 3	100 ± 9	73 ± 4	45 ± 3	59 ± 9	50 ± 9
Carbamazepine	80 ± 9	70 ± 11	82 ± 4	83 ± 5	87 ± 3	98 ± 11	92 ± 6	47 ± 4	35 ± 6	39 ± 12
Tylosin	56 ± 13	40 ± 6	54 ± 9	61 ± 6	31 ± 4	60 ± 5	82 ± 4	65 ± 3	42 ± 9	64 ± 9
Fluoxetine	26 ± 4	47 ± 20	62 ± 16	18 ± 4	72 ± 1	66 ± 9	57 ± 1	8 ± 1	21 ± 10	16 ± 5
Norfluoxetine	24 ± 9	27 ± 13	58 ± 14	21 ± 4	71 ± 3	64 ± 10	53 ± 1	5 ± 1	19 ± 8	13 ± 4
Sertraline	27 ± 10	37 ± 14	54 ± 14	20 ± 4	59 ± 11	44 ± 11	42 ± 2	5 ± 1	10 ± 4	15 ± 5

Table 2.3. Individual extraction recoveries (%) for tested solvent systems (cont.)

Analyte	Average (n = 3) plus minus one standard deviation									
	CH_2Cl_2 -C_6H_{14}	CH_2Cl_2 -MeOH	MeOH -MeCN	TFA pH 2.4 -MeOH	HAc pH 4 -MeOH	HAc pH 4 -MeCN	PO_4 pH 6 -MeOH	TFA pH 2.4	HAc pH 4	PO_4 pH 6
Erythromycin	79 ± 33	56 ± 10	70 ± 30	78 ± 5	91 ± 4	101 ± 10	92 ± 2	66 ± 4	63 ± 5	64 ± 9
Clofibric Acid	8 ± 1	75 ± 15	97 ± 30	89 ± 3	60 ± 8	93 ± 10	83 ± 7	63 ± 11	28 ± 1	46 ± 5
Warfarin	23 ± 9	75 ± 22	97 ± 23	90 ± 7	64 ± 2	85 ± 9	89 ± 8	53 ± 11	14 ± 2	56 ± 3
Miconazole	13 ± 4	16 ± 5	64 ± 9	20 ± 8	60 ± 1	15 ± 2	35 ± 1	7 ± 1	22 ± 1	5 ± 2
Ibuprofen	7 ± 3	75 ± 16	83 ± 19	86 ± 3	55 ± 10	64 ± 12	83 ± 5	55 ± 11	10 ± 1	43 ± 5
Gemfibrozil	12 ± 2	75 ± 17	73 ± 15	87 ± 1	60 ± 10	59 ± 6	80 ± 2	40 ± 10	11 ± 3	28 ± 6

All solvent systems were prepared by dissolving equal volumes of liquid in a binary mixture unless noted otherwise. Solvent system notations are as follows: HAc, 0.1 M acetic acid buffer; MeCN, acetonitrile; TFA, 0.1 M trifluoroacetic acid buffer; MeOH, methanol; PO_4, phosphate buffer; CH_2Cl_2, dichloromethane; C_6H_{14}, hexane.

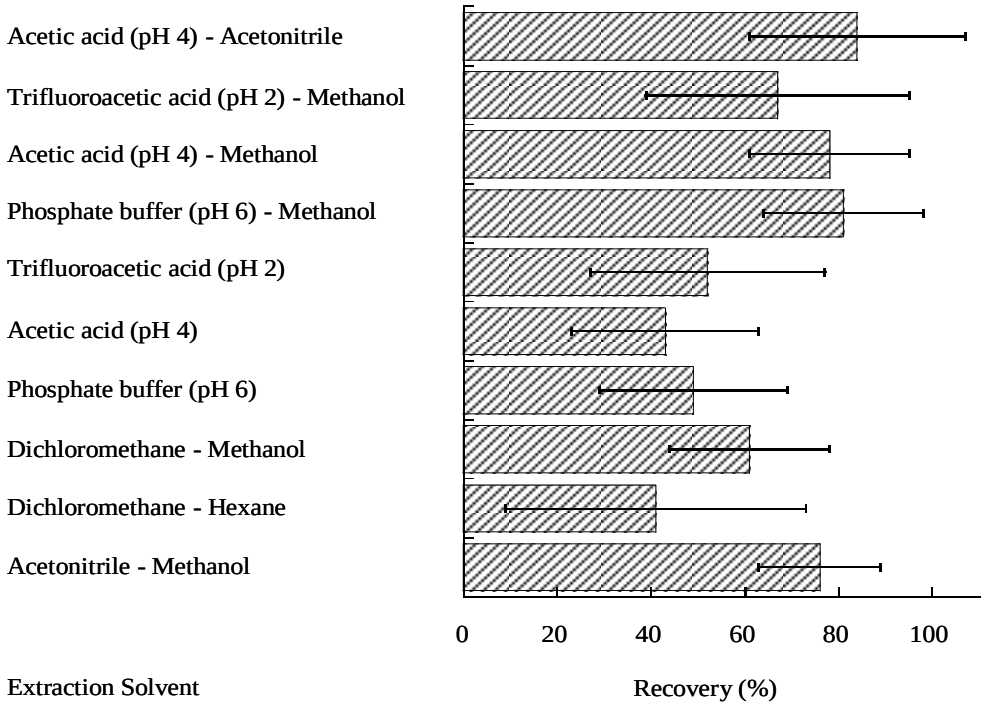


Figure 2.2. Average recoveries observed for extraction of target analytes from 'clean' muscle tissues using noted solvents. Solvents were prepared by combining equal volumes of liquid in a binary mixture. Nominal aqueous concentrations of acetic acid, trifluoroacetic acid and phosphate buffers were 0.1 M

they clearly provide a convenient metric for comparing overall solvent performance (i.e., the most effective solvents are those displaying maximum recovery and minimum 'error').

In general, moderate polarity solvents were found to be most effective at removing target analytes from tissue. Among tested organic solvents, efficiency increased with increasing polarity (i.e., dichloromethane-hexane < dichloromethane-methanol < acetonitrile-methanol). However, aqueous solvents (the most polar solvents tested) resulted in relatively poor extraction efficiency. Aqueous-organic mixtures proved to be efficient over the entire range of investigated pH conditions (pH 2.4-6), and with exception of tylosin and miconazole, substitution of acetonitrile for methanol had a negligible effect on solvent performance when combined with acetic acid.

Since most analytes included in this study are basic (Table 2.2) and expected to be protonated at pH ≤ 6, it is not surprising that pH had little effect on recovery as charted in Figure 2.2. Recovery of acidic analytes into these solvents was expected to decrease with decreasing pH. However, mean recoveries for clofibric acid, ibuprofen and gemfibrozil were greater for pH 2.4 trifluoroacetic acid-methanol and pH 6 phosphate buffer-acetonitrile than for pH 4 acetic acid-methanol (Table 2.3). The origin of this behavior is presently unknown.

Matrix Effects

It is widely recognized that co-extracted matrix components can affect analyte ionization in analyses employing electrospray interfaces.[9, 48, 55] Accordingly, an approach reported by Vanderford et al.[48] was employed to evaluate matrix effects for extraction solvents promoting recovery > 60% in Figure 2.2. 'Clean' muscle tissue (1 g) was extracted, centrifuged, and reconstituted in 0.1% formic acid. Extracts were spiked with a known amount of each analyte prior to analysis. Aqueous formic acid (0.1% v/v) was also spiked with the same concentration of target compounds and analyzed as a matrix-free reference sample. Concentrations of analytes derived from an internal standard calibration curve prepared using standards constituted in 0.1 % formic acid are tabulated in Table 2.4.

As expected, the degree of matrix interference was found to depend on both analyte and extraction solvent. Co-extracted matrix components were found to have minimal effect on the analytical response of early-eluting analytes (retention time < 25 min). In contrast, matrix suppression was observed for most other ESI+ analytes;

Table 2.4. Observed matrix effects for extracted tissue samples

Analyte	Conc. in water ng/ml	PO_4 pH 6 -MeOH, ng/g	HAc pH 4 -MeOH, ng/g	HAc pH 4 -MeCN, ng/g	TFA pH 2.4 -MeOH, ng/g	MeOH -MeCN, ng/g
Acetaminophen	220	186(-15%)	194(-12%)	197(-11%)	154(-30%)	195(-11%)
Atenolol	110	141(29%)	117(7%)	118(8%)	98(-11%)	102(-7%)
Cimetidine	60	51(-16%)	57(-5%)	63(5%)	53(-11%)	53(-11%)
Codeine	330	330(~0%)	335(2%)	331(~0%)	299(-9%)	326(-1%)
1,7-dimethylxanthine	40	41(3%)	42(4%)	43(7%)	38(-4%)	39(-2%)
Lincomycin	220	239(9%)	228(4%)	231(5%)	201(-8%)	224(2%)
Trimethoprim	90	97(-8%)	87(-3%)	87(-3%)	81(-10%)	80(-11%)
Thiabendazole	90	83(-8%)	84(-6%)	84(-7%)	81(-10%)	86(-4%)
Caffeine	210	222(6%)	214(2%)	228(8%)	223(-6%)	228(8%)
Sulfamethoxazole	85	66(-23%)	81(-5%)	81(-4%)	75(-12%)	76(-11%)
Metoprolol	85	83(-2%)	78(-9%)	81(-5%)	80(-6%)	72(-15%)

Table 2.4. Observed matrix effects for extracted tissue samples (cont.)

Analyte	Conc in water ng/ml	PO_4 pH 6 -MeOH, ng/g	HAc pH 4 -MeOH, ng/g	HAc pH 4 -MeCN, ng/g	TFA pH 2.4 -MeOH, ng/g	MeOH -MeCN, ng/g
Propranolol	40	32(-19%)	35(-11%)	30(-24%)	31(-23%)	19(-52%)
Diphenhydramine	4	3.3(-17%)	3.2(-20%)	3.1(-21)	3.1(-23%)	2.2(-45%)
Diltiazem	6	6.3(4%)	5.8(-4%)	5.6(-6%)	5.7(-5%)	4.0(-34%)
Carbamazepine	40	31(-22%)	36(-11%)	35(-12%)	35(-12%)	35(-12%)
Tylosin	210	344(64%)	237(13%)	248(18%)	261(24%)	262(25%)
Fluoxetine	220	92(-58%)	141(-36%)	99(-55%)	72(-67%)	91(-58%)
Norfluoxetine	200	69(-65%)	116(-42%)	87(-56%)	53(-74%)	81(-60%)
Sertraline	220	47(-78%)	117(-47%)	63(-71%)	31(-86%)	93(-58%)
Erythromycin	200	510(155%)	1000(400%)	2422(1111%)	1476(638%)	1934(867%)
Clofibric acid	70	165(136%)	38(-46%)	53(-25%)	238(240%)	42(-39%)
Warfarin	40	20(-49%)	31(-22%)	29(-27%)	18(-55%)	25(-38%)

Table 2.4. Observed matrix effects for extracted tissue samples (cont.)

Analyte	Conc in water ng/ml	PO_4 pH 6 -MeOH, ng/g	HAc pH 4 -MeOH, ng/g	HAc pH 4 -MeCN, ng/g	TFA pH 2.4 -MeOH, ng/g	MeOH -MeCN, ng/g
Miconazole	400	43(-89%)	87(-78%)	73(-82%)	19(-95%)	196(-51%)
Ibuprofen	1600	4045(152%)	1271(-20%)	1289(-19%)	5491(243%)	859(-46%)
Gemfibrozil	200	307(54%)	129(-35%)	119(-41%)	275(38%)	110(-45%)

Values in parenthesis represent the relative percent difference in concentrations. Negative sign inside parenthesis indicates suppression of the signal. Solvent system notations are as follows: PO_4, phosphate buffer; MeOH, methanol; HAc, 0.1 M acetic acid buffer; MeCN, acetonitrile; TFA, 0.1 M trifluoroacetic acid buffer.

especially for fluoxetine, norfluoxetine, sertraline and miconazole. Exceptions include tylosin and erythromycin, for which significant signal enhancements were observed in a number of cases. Data for clofibric acid, ibuprofen and gemfibrozil in Table 2.4 are misleading for extracts resulting from extraction with phosphate buffer-methanol and trifluoroacetic acid-methanol. In each of these cases data in Table 2.4 suggest an apparent signal enhancement. In fact, the analytical response for these analytes was suppressed in all cases, but more pronounced suppression of the ESI– internal standard (meclofenamic acid) in these extracts resulted in calculated concentrations exceeding the reference condition.

Matrix effects identified in Table 2.4 collectively demonstrate that accurate quantitation of analytes in tissue extracts is not feasible using calibration standards prepared in aqueous formic acid. Common approaches for dealing with matrix interference include spiking each sample with a known amount of labeled analyte(s) prior to analysis (i.e., isotope dilution), employing the method of standard additions, or using matrix-matched calibration standards. While isotope dilution is perhaps the best approach for compensation of matrix interference in analyses employing mass spectrometry,[53] cost and limited availability of labeled standards are problematic for broad screening methods. A primary limitation for standard addition methods is related to sample mass and/or volume, which is often limited in tissue analyses. Furthermore, quality assessment of standard addition data is difficult to monitor using standard QA/QC performance metrics. In contrast, matrix-matched calibration is relatively simple to implement, provided that ‘clean’ reference tissue is available.

For reasons discussed above, matrix-matched calibration was employed to minimize matrix interference in the analysis of environmental samples. Calibration standards were prepared by adding a known amount of each target analyte and five labeled surrogates to 1 g 'clean' muscle tissue. Tissues were homogenized and carried through the entire sample preparation procedure prior to analysis. Acetic acid (pH 4)-methanol was selected as the extraction solvent, since observed matrix effects were minimized in this extract (Table 2.4). Linear calibration curves (r^2 > 0.99 for concentration ranges specified in Table 2.5) were constructed by plotting the response factor for each analyte versus tissue spiking levels (ng/g tissue) and used to determine analyte concentrations in all subsequent analyses.

Analytical Performance Metrics

Analyte-specific limits of detection (LOD), limits of quantitation (LOQ) and method detection limits (MDL) are defined and reported in Table 2.5. Although LOD and LOQ are recognized performance metrics within academic circles, MDL is more appropriate for establishing detection thresholds in environmental analyses with potential regulatory implications. LOD[74] and LOQ[75] are derived from analyses of a 'blank' sample and thus, do not account for potential matrix effects. In contrast, MDL is derived from replicate analyses of a matrix spike and represents the lowest concentration of analyte that can be detected in a given matrix with 99% confidence that the concentration is non-zero.[76]

As shown in Table 2.5, MDLs for fish tissue were typically higher than either LOD or LOQ, irrespective of the level of matrix interference identified in Table 2.4. These results clearly suggest that use of LOD and LOQ as detection and quantitation

Table 2.5. Retention time (t_R), investigated linear range, LOD, LOQ, and MDL for target analytes in fish muscle tissue

Analyte	t_R, min	linear range, ng/g	LOD,[b] ng/g	LOQ,[c] ng/g	MDL,[d] ng/g
Acetaminophen	6.4	3.12-400	0.30	0.99	4.40
Atenolol	9.1	1.25-160	0.48	1.62	1.48
Cimetidine	8.9	0.625-80	0.24	0.81	1.04
Codeine	10.4	4.69-600	1.07	3.55	6.11
1,7-dimethylxanthine	11.8	0.625-80	0.17	0.58	1.02
Lincomycin	14.0	3.12-400	0.63	2.09	5.53
Trimethoprim	15.8	1.25-160	0.79	2.63	2.15
Thiabendazole	15.6	1.25-160	0.14	0.47	2.63
Caffeine	17.2	3.12-400	0.34	1.15	3.93
Sulfamethoxazole	19.8	1.25-160	0.23	0.76	2.29
Metoprolol	21.9	1.25-160	0.25	0.85	2.50
Propranolol	25.3	0.625-80	0.01	0.03	1.07
Diphenhydramine	25.8	0.0625-8	0.01	0.03	0.05
Diltiazem	27.7	0.094-12	0.04	0.13	0.12
Carbamezepine	30.6	0.625-80	0.03	0.12	0.54
Tylosin	32.9	3.12-400	1.18	3.93	5.02
Fluoxetine	34.9	4.69-600	0.76	2.54	6.73
Norfluoxetine	35.2	3.12-400	0.32	1.08	2.90
Sertraline	37.2	3.12-400	0.21	0.71	3.57

Table 2.5. Retention time (t_R), investigated linear range, LOD, LOQ, and MDL for target analytes in fish muscle tissue[a] (cont.)

Analyte	t_R, min	linear range, ng/g	LOD,[b] ng/g	LOQ,[c] ng/g	MDL,[d] ng/g
Erythromycin	42.0	3.12-400	0.85	2.84	6.42
Clofibric Acid	42.4	1.25-160	0.10	0.32	2.69
Warfarin	45.0	0.625-80	0.09	0.29	0.86
Miconazole	47.5	3.12-400	0.39	1.32	10.8
Ibuprofen	50.6	25-3200	3.14	10.4	45.9
Gemfibrozil	52.5	3.12-400	0.25	0.85	6.68

[a] 'Clean' tissues employed in the determination of these parameters were extracted using a 1:1 mixture of 0.1 M acetic acid buffer (pH 4) and methanol. See text for details. [b] Limit of detection (LOD), calculated as three times the standard deviation in the background signal. [c] Limit of quantitation (LOQ), calculated as ten times the standard deviation in the background signal. [d] Method detection limit (MDL), determined by multiplying the one sided 99 percent student's t-statistic ($t_{0.99}$) by the standard deviation from eight replicates analysis of spiked sample.

thresholds in practical applications of this method could lead to acceptance of questionable data. For this reason, MDLs were employed as a single detection/quantitation threshold in screening analyses (see below). Additionally, it is important to note that relative standard deviations derived from replicate analyses of the matrix spike (data not shown) were ≤ 6% for all analytes, demonstrating excellent reproducibility.

Analysis of Environmental Samples

In order to confirm the utility of LC-MS/MS methodology for analysis of environmental samples, fish were sampled approximately 650 m downstream from the

effluent discharge into Pecan Creek and screened for target analytes. Four compounds were detected in all analyzed specimens at concentrations exceeding statistically-derived MDLs (Table 2.6). Identification of analytes in environmental samples was confirmed by monitoring two fragment ions and comparing their retention time and relative intensity with a spiked sample. Representative reconstituted ion chromatograms are shown in Figure 2.3. Note that collision induced dissociation of norfluoxetine produced only one fragment ion of sufficient intensity to be observed under these conditions.

Fluoxetine and sertraline have been detected previously in fish from Pecan Creek.[10] However, it is not surprising that these compounds were not detected in this study, as reported concentrations in fish muscle (1.1 ng/g and 0.34 ng/g, respectively) fall below MDLs defined in Table 2.5. While norfluoxetine has also been observed previously in fish tissues,[10] accumulation of diphenhydramine, diltiazem and carbamazepine is reported here for the first time.

Table 2.6. Concentrations of analytes (ng/g tissue) detected in muscle tissues from fish collected in Pecan Creek, Denton County, TX, USA

Analyte	Range (n = 11)	Mean (n = 11)
Diphenhydramine	0.66 – 1.32	0.96
Diltiazem	0.11 – 0.27	0.21
Carbamazepine	0.83 – 1.44	1.16
Norfluoxetine	3.49 – 5.14	4.37

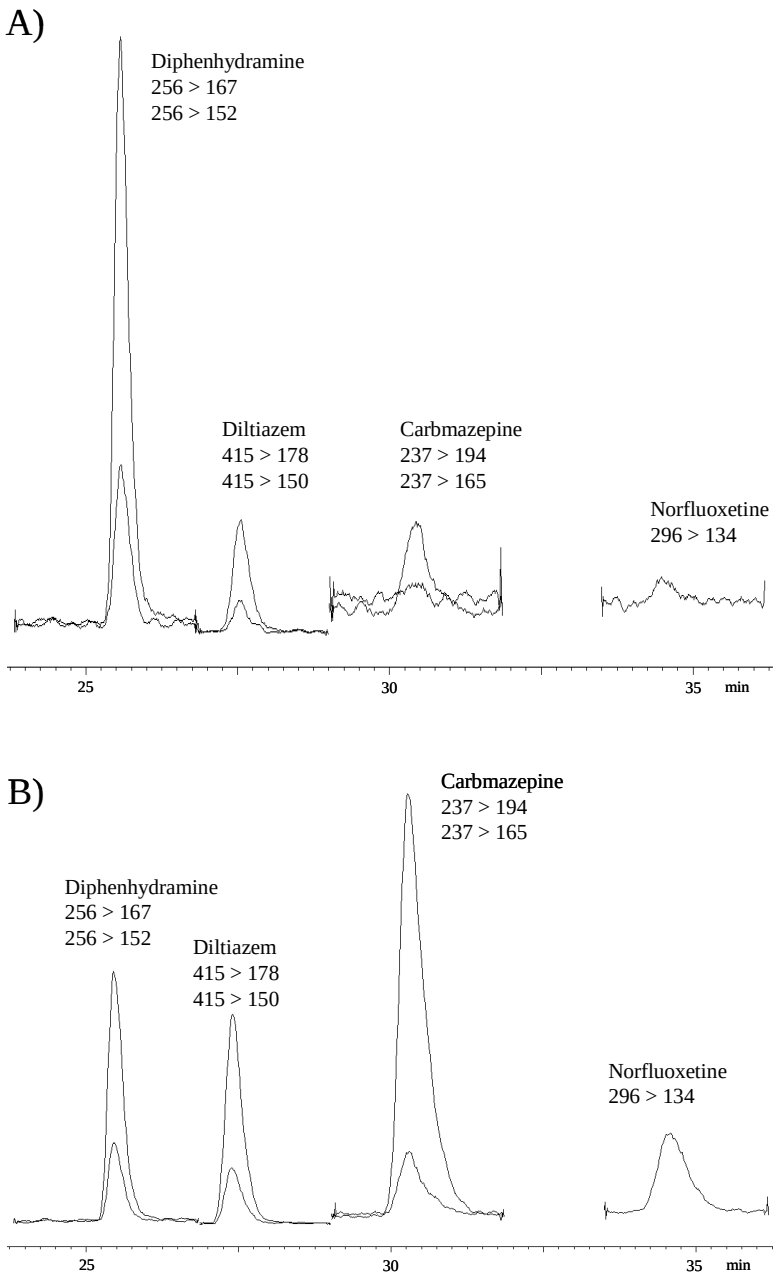


Figure 2.3. LC-MS/MS reconstituted ion chromatograms displaying analyte-specific quantitation and qualifier ions for (A) a tissue extract from a fish (*Lepomis sp.)* collected in Pecan Creek and (B) an extract from 'clean' tissue spiked with known amounts of diphenhydramine (1.6 ng/g), diltiazem (2.4 ng/g), carbamazepine (16 ng/g) and norfluoxetine (80 ng/g). The higher *m/z* fragment is more intense in all cases

Duplicate analysis of a matrix spike prepared from a Pecan Creek tissue specimen was conducted to assess method accuracy. Analyte spiking levels in this sample corresponded to the upper third of the calibration range for each analyte (ca. 15 × MDL). Excepting fluoxetine, norfluoxetine and sertraline, mean spike recoveries ranged from 88%-120% (data not shown), demonstrating that target compounds can be quantified with acceptable accuracy in environmental samples. Relative percent difference for duplicate analyses was ≤ 16% for all compounds. It is important to point out that positive bias was observed for clofibric acid and ibuprofen (recovery = 186% and 145%, respectively) when matrix-matched internal standard calibration was employed. However, improved accuracy for these analytes was achieved (97% recovery in both cases) by using a matrix-matched external standard calibration curve (i.e., a plot of analyte peak area versus tissue spiking level). Improvements in surrogate recovery for ibuprofen-$^{13}C_3$ in unspiked samples were also observed using the external standard approach. These results suggest that meclofenamic acid is not a suitable internal standard for clofibric acid and ibuprofen in environmental samples.

Mean recoveries for fluoxetine, norfluoxetine and sertraline were less than quantitative in the matrix spike (44%, 64%, and 46%, respectively), and surrogate recoveries for fluoxetine-d_6 in unspiked samples ranged from 60-97%. Matrix-matched external standard calibration did not significantly improve accuracy for these compounds. However, quantitation of fluoxetine based on isotope dilution with fluoxetine-d_6 resulted in 110% recovery for the matrix spike. It is reasonable to expect that additional compounds, not present in 'clean' tissue extracts, may be present in Pecan Creek samples since this stream is significantly impacted by wastewater effluent and previous studies

have demonstrated that wastewater contaminants not classified as PPCPs are accumulated in fish residing in effluent-dominated ecosystems.[77] Therefore, it is possible that compounds not monitored in this study co-elute with target antidepressants and result in unforeseen matrix effects. To the extent that this proves to be a general occurrence in future applications of reported methodology, it may become necessary to employ isotope dilution for accurate quantitation of these compounds.

CHAPTER THREE

Development of a Gas Chromatography-Mass Spectrometry Screening Method for Simultaneous Determination of Select UV Filters, Synthetic Musks, Alkylphenols, an Antimicrobial Agent, and an Insect Repellent in Fish

Introduction

The occurrence of personal care products (PCPs), such as ultra-violet filters (UVFs), synthetic musks (SMs), antimicrobials, and insect repellents has been increasingly reported in literature.[1, 2, 57, 78] Much of the recent scientific attention given to these emerging contaminants has resulted from an absence of aquatic life based regulations for surface waters. Of particular relevance are effluent-dominated streams, which represent "worse case scenarios" for studying PCPs and other organic wastewater contaminants.[79] In these streams, even compounds with relatively short environmental half-lives may be considered pseudopersistent due to their continuous introduction from a WWTP. As a result, organisms residing in these aquatic systems receive continuous exposures to wastewater-derived contaminants over their entire life cycle.[79] Recent reports from several research groups[12, 14, 17, 28] have demonstrated that environmental exposure to PPCPs results in accumulation of parent compounds, their metabolites, or both in tissues of aquatic organisms. More significantly, a series of studies has also identified that nitromusk fragrances are not only accumulated but are subsequently metabolized to reactive intermediates that form covalent protein adducts.[28, 80]

Alkylphenol surfactants (APs) represent a second class of organic wastewater contaminants that has also received broad coverage in literature. These compounds are

ubiquitous constituents of industrial products such as, emulsifiers, paints, detergents and other cleaning products. They are perhaps the most widely-studied wastewater contaminants due to their ability to promote endocrine disruption in aquatic organisms.[81] Multiple studies have demonstrated their occurrence in a variety of environmental matrices, including tissues.[21-24, 82-84] Continued investigation of partitioning and accumulation for these and alternative organic wastewater contaminants (OWCs) is critical to comprehensive assessment of potential consequences of environmental exposures.

Analytical protocols for determination of OWCs in water, soil, sediment, and biosolids are numerous and have been summarized in recent reviews.[6, 9, 85] While targeted analytical methods for determination of UVFs,[86-88] SMs,[67, 89-93] APs,[82-84] antimicrobials,[94, 95] and insect repellents[96, 97] continue to be reported, an increasing trend in environmental chemistry is the development of protocols affording simultaneous analysis of compounds belonging to different classes.[67, 78, 98-100] It is important to note that due to similarities in physicochemical properties, PCPs and APs are amenable to assessment using a single analytical method. [78] In contrast, methods for determination of OWCs in tissue continue to focus almost exclusively on a specific class of analytes. For example, protocols have recently appeared for 4 UV filters and methyl-triclosan,[12, 13, 32] 12 musk fragrances,[16-20] 9 alkylphenol surfactants,[21, 23, 24, 36] and chlorophene, triclosan and its 3-chlorinated derivatives.[11, 14, 33] The first multi-residue approach for determination of pharmaceuticals in fish using LC-MS/MS was described in Chapter 2.

In this chapter, a complementary GC-MS method affording simultaneous analysis of 3 UVFs, 5 SMs, 2 APs, the antimicrobial agent triclosan and the insect repellent *m*-

toluamide is described. An important step in method development that imparts a distinct advantage, as compared to previous methods for analysis of similar OWCs in fish, was alleviation of gel-permeation chromatography from the sample preparation protocol. Centrifuged extracts were simply cleaned using silica gel, reconstituted in hexane, and injected directly into the GC-MS. Target analytes were monitored with the mass analyzer operated in selected ion monitoring (SIM) mode. Initial method validation involved determination of statistically-derived MDLs using spiked muscle tissue. The method was subsequently applied to assess the occurrence of target analytes in environmental samples. Four compounds were detected at concentrations in general agreement with literature values determined using methods designed for a single class of compounds.

Experimental Section

Chemicals and Materials

All chemicals were reagent grade or better, obtained from commercial vendors, and used as received. The reference standards, benzophenone, 4-methylbenxylidine camphor, *m*-toluamide, *p*-octylphenol, galaxolide, tonalide, musk xylene, musk ketone, triclosan, octocrylene, and *p*-nonylphenol, and surrogate standards, 2,2'-dinitrobiphenyl, pentachloronitrobenzene, and benzophenone-d_{10} were purchased in the highest available purity from Sigma-Aldrich (Milwaukee, WI, USA). Celestolide was obtained from Cambridge Corporation (San Diego, CA, USA). Surrogate standards 3,3',4,4'-tetrachlorobiphenyl-$^{13}C_{12}$ (PCB # 77) and *p*-n-nonylphenol-$^{13}C_6$ were acquired from Cambridge Isotopes Labortatories, Inc. (Andover, MA, USA). The internal standard mirex was purchased from Cerilliant Corporation (Round Rock, TX, USA). The

derivatizing agent N-methyl-N-(trimethylsilyl)trifluoracetamide (MSTFA) was obtained from VWR Scientifics (Irving, TX, USA). Silica gel (grade 60, 70-230 mesh, 60 Å), *n*-hexane (HPLC grade), and acetone (spectrophotometric grade) were also obtained from Sigma-Aldrich Inc. Distilled water was purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond UV water purification system.

Sample Collection and Preservation

Sampling activities related to this work were described in detail in Chapter 2. It is important to recall that annual flows in Pecan Creek are comprised almost entirely of effluent discharge from the Pecan Creek Water Reclamation Plant. In contrast, Clear Creek is not impacted by effluent discharges. Fish (*Lepomis* sp.) were sampled from Pecan Creek (n = 11) and Clear Creek (n = 20). Tissue from the Clear Creek composite was used for initial evaluations of method performance, determination of statistically-derived MDLs, and as control matrix in the analysis of Pecan Creek samples. The same 11 Pecan Creek fish analyzed in Chapter 2 were also employed here to evaluate the environmental relevance of developed methodology.

Determination of Tissue Lipid Content

Lipid content was determined for each fish collected from Pecan Creek using a modified literature procedure.[101] Approximately 2 grams of homogenized muscle tissue were taken from each fish and mixed with 15 ml 1:1 hexane-dichloromethane (50% v/v) in a glass vial. Samples were re-homogenized using a Tissuemiser (Fisher Scientific) set to rotate at 30,000 rpm. Homogenized samples were then equilibrated on a rotary extractor (15 rotations/min) for 18 h at 35 ^{o}C. The phases were separated, and the

resulting organic layer was transferred to a Pyrex test tube using disposable borosilicate glass pipettes. The tissue residue was rinsed with an additional 15 ml solvent, and the extracts were combined in a single test tube. Residual water was removed by passing extracts through laboratory assembled glass columns packed with anhydrous Na_2SO_4 and collected in a pre-weighed test tube. The solvent was evaporated to dryness under a stream of nitrogen at 45 ^{o}C and dried to constant weight in a vacuum oven at 40 °C. Lipid content was calculated by dividing the weight of extracted residue by the wet weight of tissue prior to extraction. (This procedure was carried out by Dr. Mohammad A. Mottaleb and Mr. Christopher Pankow.)

Extraction of Target Analytes

Approximately 1.0 g of tissue was combined with 10 ml of acetone in a 20 ml VWR Wheaton borosilicate glass vial (Rockwood, TN, USA), and the mixture was homogenized using a Tissuemiser set to rotate at 30 000 rpm. Five surrogates were added to each sample as an acetone solution: PCB # 77 (200 ng), *p*-n-nonylphenol-$^{13}C_6$ (80 ng), 2,2'-dinitrobiphenyl (500 ng), pentachloronitrobenzene (500 ng), and benzophenone-d_{10} (120 ng). Samples were then shaken vigorously and mixed on a rotary extractor for 5 min. Following extraction, samples were transferred into 50-ml polypropylene copolymer round-bottomed centrifuge tubes (Nalgene, Rochester, NY, USA) using 1 ml of acetone as a rinse and centrifuged at 16000 rpm for 40 min at 4°C. The supernatant was then transferred into VWR Scientific 18-ml disposable borosilicate glass tubes (Irving, TX, USA), and the solvent was evaporated to dryness under a stream of nitrogen at 40 ^{o}C using a Zymark Turbovap (Hopkinton, MA, USA). Finally, samples were reconstituted in 200 µl of 65:35 hexane-acetone (v/v).

Sample Clean-up and Derivatization

One gram of silica gel (Sigma-Aldrich Inc) was stirred in 4 ml hexane and the slurry was transferred to a borosilicate glass Pasteur pipette. Approximately 10 mg of glass wool was used as a porous plug, keeping silica confined to the pipette. Silica-gel columns were preconditioned with 8 ml of 65:35 hexane-acetone (v/v). The extract was loaded onto the column using a second Pasteur pipette, and analytes were eluted using 10 ml of the hexane-acetone mixture. The eluate was subsequently collected and evaporated to dryness at 40 °C under dry nitrogen. Residues were again dissolved in 200 µl of solvent mixture, and 100 µl of MSTFA derivatizing agent was added. The resulting mixture was transferred into a GC vial and heated in an oven at 60 ^{o}C for 45 min. Subsequently, the mixture was evaporated to dryness and reconstituted in 180 µl of *n*-hexane. Finally, a constant amount of the internal standard mirex (200 ng) was added prior to GC-MS analysis.

GC-MS Analysis

A Varian CP-3800 GC system equipped with a Varian CP-8400 auto sampler was used in this study. Analytes were separated on a 30 m x 0.25 mm I.D. x 0.25 µm film thickness, XTI-5 capillary column (VWR Scientific, West Chester, PA, USA) using the following temperature program: initial temperature, 100 °C, ramped to 180 °C at 15 °C/min, held for 5 min, ramped to 290 °C at 6 °C/min, and held for 31 min. Helium was used as carrier gas at a constant flow rate of 1 ml/min. Injections of 1.0 µl were made using splitless mode and an injection port temperature of 275 °C. The transfer line was kept at 280 °C. Eluted analytes were monitored by electron-impact (EI) ionization mass spectrometry in SIM mode using a Varian model 1200L quadrupole mass spectrometer.

A representative total ion chromatogram is shown in Figure 3.1, and ions (*m/z*) used for quantitation and identification of each target analyte are shown in Table 3.1. Mass spectrometry parameters held constant for all analytes were as follows: ion source temperature, 250 °C; manifold temperature, 40 °C; EI ionization energy, 70 eV; scan time, 35 min.

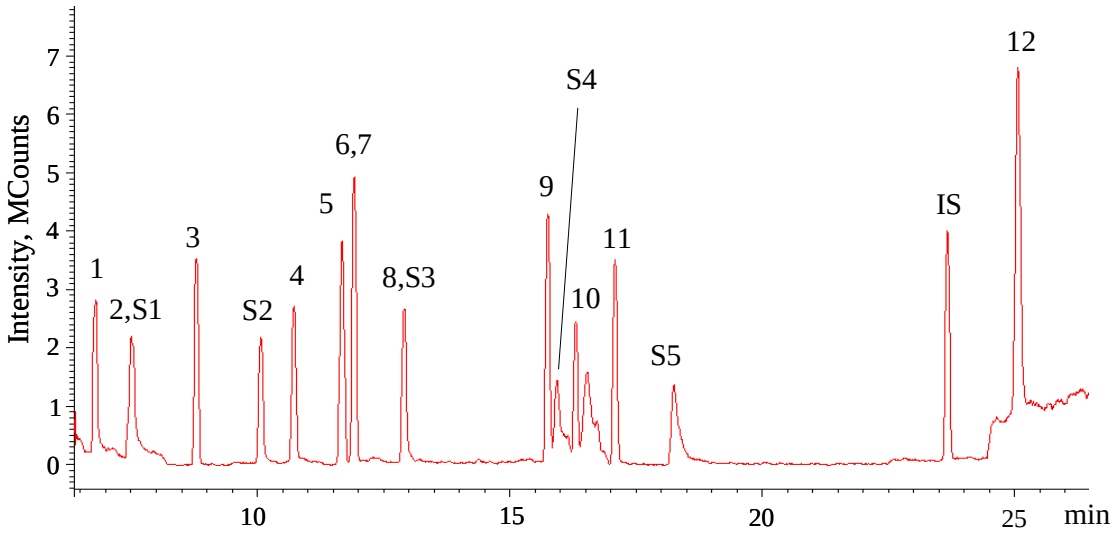


Figure 3.1.Time-scheduled chromatogram of a calibration standard.
Key: (1) *m*-toluamide, (2) Benzophenone, (S1) Benzophenone-d_{10}, (3) Celestolide, (S2) pentachloronitrobenzene, (4) *p*-octylphenol, (5) galaxolide, (6) Tonalide, (7) musk xylene, (8) *p*-nonylphenol, (S3) *p*-n-nonylphenol-$^{13}C_6$, (9) 4-methylbenxylidine camphor, (S4) 2,2'-dinitrobiphenyl, (10) musk ketone, (11) triclosan, (S5) PCB # 77, (IS) mirex, and (12) octocrylene.

Quantitation

Ten calibration standards were prepared by dissolving varying concentrations of all target analytes and surrogates in acetone. Calibration curves were constructed for each analyte by plotting the response factor (i.e., peak area of analyte divided by the peak area of internal standard) versus analyte concentration (ng/μl). Calibration data were fit

Table 3.1. Brand and IUPAC names, use, group, structure, CAS number and SIM ions for selected target analytes

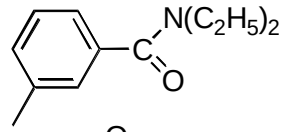
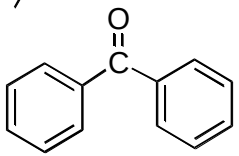
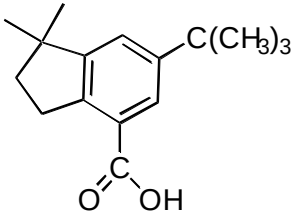
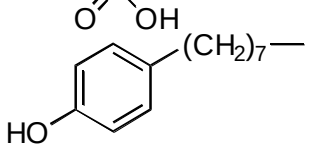
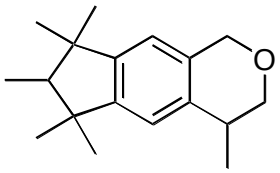
Analyte	use	group	chemical structure	CAS Number	SIM ions
m-Toluamide (N,N-diethyl-m-methylbenzamide)	insect repellent			134-62-3	91, **119,** [a] 190
Benzophenone (diphenyl ketone)	sun screen	UVF		119-61-9	77, 105, **182**
Celestolide (4-Acetyl-1,1-dimethyl-6-tert-butylindane)	fragrance	SM		13171-00-1	173, **229**, 244
p-Octylphenol	surfactant	AP		1806-26-4	165, 180, **278**
Galaxolide (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl cyclo-penta[g]-2-benzo pyrane)	fragrance	SM		1222-05-5	213, **243**, 258

Table 3.1. Brand and IUPAC names, use, group, structure, CAS number and SIM ions for selected target analytes (cont.)

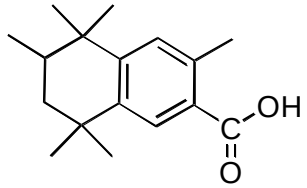
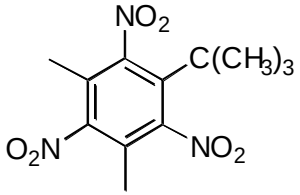
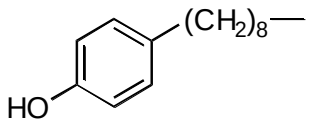
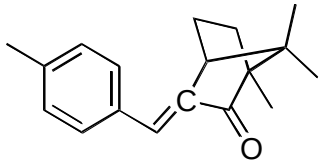
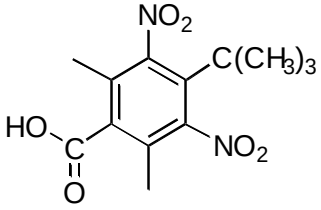
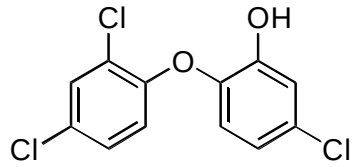
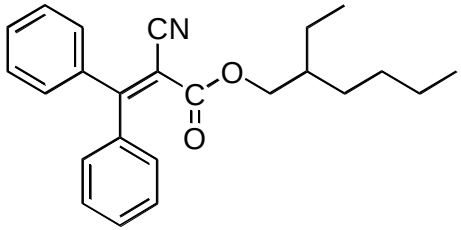
Analyte	use	group	chemical structure	CAS Number	SIM ions
Tonalide (7-acetyl-1,1,3,4,4,6-hexamethyltetralin)	fragrance	SM		21145-77-7	201, **243**, 258
Musk xylene (1-tert-butyl-3,5-dimethyl-2,4,6-trinitrobenzene)	fragrance	SM		81-15-2	**282**, 283, 297
p-Nonylphenol	surfactant	AP		104-40-5	149, 179, **292**
4-MBC (4-methylbenzylidine camphor)	sun screen	UVF		36861-47-9	115, 211, **254**
Musk ketone (4-aceto-3,5-dimethyl-2,6-dinitro-tert-butylbenzene)	fragrance	SM		81-14-1	217, **261**, 366

Table 3.1. Brand and IUPAC names, use, group, structure, CAS number and SIM ions for selected target analytes (cont.)

Analyte	use	group	chemical structure	CAS Number	SIM ions
Triclosan (4-chloro-2-hydroxyphenyl-2,4-dichlorophenyl ether)	anti-microbial			3380-34-5	200, **345**, 362
Octocrylene (2-ethylhexyl-2-cyano-3,3-diphenylacrylate)	sun screen	UVF		6197-30-4	177, 249, **361**

[a] Bold print indicates m/z used for quantitation. UVF, ultra-violet filter; SM, synthetic musk; AP, alkylphenol.

to a linear regression forced through the origin, resulting in correlation coefficients (r^2) exceeding 0.99 in all cases. In the analysis of unfortified control matrix (i.e., unspiked tissue from Clear Creek), benzophenone, galaxolide and triclosan were detected at average concentrations (n = 3) of 24.1, 12.4 and 12.1 ng/g, respectively, with less than 12% RSD. Therefore, measured concentrations in fortified control samples used to determine MDLs and monitor method performance in the analysis of Pecan Creek samples were corrected by subtracting the corresponding blank values.

Quality Control

In the analysis of samples from Pecan Creek, method performance was monitored via analysis of a procedural blank, laboratory control sample, and matrix spike-matrix spike duplicate pair. Surrogates were added to all tissue samples prior to extraction. No analytes were detected in the blank, and recoveries for the laboratory control sample and MS/MSD pair were acceptable for all analytes. (MS/MSD recoveries are presented in Table 3.4 and discussed in more detail below.) Additionally, surrogate monitoring revealed that the RSDs of mean recoveries calculated for each analyte varied between 2 and 13 percent, indicating a controlled method throughout analysis.

Results and Discussion

Method Validation

Analyte recoveries and method detection limits (MDLs) were evaluated using spiked muscle tissue derived from fish collected in Clear Creek and are reported in Table 3.2. Due to variability in physicochemical properties of target analytes included in this work (Table 3.1), the choice of extraction solvent was an important consideration. The

following solvents were evaluated in initial studies: *n*-hexane, dichloromethane, acetone, acetonitrile, methanol, 1:1 *n*-hexane-acetone, 1:1 methanol-acetonitrile, and 1:1 methanol-acetate buffer (pH 4). When dichloromethane or hexane was used, either alone or in combination, the tissue did not mix well with the solvent and formed a sticky dispersed residue, resulting in poor reproducibility for replicate extractions. Among remaining solvent systems tested, acetone was selected as optimal for extracting all target compounds from fish muscle tissue. Mean recoveries in reference fish ranged from 87 to 114 % with RSDs < 13 %, demonstrating efficient extraction and good reproducibility. Statistically-derived MDLs, determined using an EPA-approved protocol,[76] were < 10 ng/g for most analytes, and as low as 2.4 ng/g for *p*-nonylphenol. MDL was employed as a single detection/quantitation threshold in subsequent screening analyses of Pecan Creek samples.

Analysis of Environmental Samples

In order to confirm the utility of GC-MS methodology for analysis of environmental samples, eleven fish were sampled ~650 m downstream from the effluent discharge into Pecan Creek and screened for target analytes. Four compounds were detected in all analyzed specimens at concentrations exceeding statistically derived MDLs (Table 3.3). Average concentrations of benzophenone, galaxolide, tonalide, and triclosan in tissues were 57 ng/g, 1020 ng/g, 58 ng/g, and 21 ng/g, respectively. Detected concentrations of benzophenone, galaxolide, and tonalide were consistent with previous studies. For example, Balmer et al. found concentrations of benzophenone up to 123 ng/g in fish from Swiss lakes.[12] Boehmer et al. detected triclosan in bream from the German Environmental Specimen Bank (ESB) at concentrations up to 1.7 ng/g,[14] and its

Table 3.2. Retention time, investigated linear range, spiking level, average recovery and MDL for target analytes in fish muscle tissue

Analyte	retention time, min	linear range, ng/µl	spiking level,[a] ng/g	recovery,[b] %	MDL,[c] ng/g
m-Toluamide	6.79	0.003 - 6.0	12	110 ± 10	3.48
Benzophenone	7.51	0.004 - 8.0	16	101 ± 5	7.46
Celestolide	8.77	0.005 - 9.0	18	97 ± 3	4.03
p-Octylphenol	10.73	0.006 - 12.0	24	114 ± 12	2.92
Galaxolide	11.66	0.008 - 16.0	32	105 ± 6	9.05
Tonalide	11.88	0.006 - 12.0	24	87 ± 9	4.81
Musk xylene	11.89	0.012 - 24.0	48	102 ± 3	7.29
p-Nonylphenol	12.97	0.003 - 6.0	12	111 ± 7	2.38
4-MBC	15.79	0.015 - 30.0	60	99 ± 3	5.34
Musk ketone	16.28	0.048 - 96.0	192	101 ± 4	16.9
Triclosan	17.04	0.006 - 12.0	24	98 ± 4	5.33
Octocrylene	24.92	0.036 - 72.0	144	98 ± 2	16.6

[a] Spiked control matrix from Clear Creek were employed in the determination of recovery and MDL. [b] Reported values represent mean recovery plus or minus one standard deviation (n = 11). Spike concentrations for determination of recovery were ca. 10 times MDL. [c] Determined by multiplying the one-sided Student's t-statistic at the 99% confidence limit times the standard deviation observed for eight replicate analyses of control matrix spiked at the noted concentrations.

metabolite, methyl-triclosan, was commonly found at concentrations up to 35 ng/g in fish from Swiss rivers and lakes.[11] Ruedel et al. reported galaxolide and tonalide concentrations ranging from 545-6400 ng/g and 48-2130 ng/g, respectively, in bream

Table 3.3. Occurrence of target analytes in fish collected from Pecan Creek, Denton County,TX, USA

			Analytes average concentrations (ng/g)			
Fish ID	weight (g)	lipid content (%)	benzophenone	galaxolide	tonalide	triclosan
A	30.1	0.23	37	462	33	22
B	42.5	0.40	79	1415	69	19
C	49.0	0.16	79	747	35	20
D	35.5	na[a]	41	606	39	19
E	46.9	0.20	46	419	26	18
F	33.6	0.42	56	919	50	17
G	31.2	na	50	940	58	23
H	29.4	na	90	1317	82	31
I	33.6	na	44	989	70	20
J	39.3	0.45	44	1739	97	19
K	37.2	0.75	63	1664	76	19

[a] na, non-applicable. Values of lipid of content could not be measured in replicates D, G, H and I due to limited tissue mass.

from the German ESB.[18] In order to confirm the accuracy of observed concentrations in Pecan Creek tissues, duplicate matrix spikes (MS/MSD) were included in the analytical batch. Analyte spiking levels in MS/MSD samples corresponded to the middle of the calibration range for each analyte (~ 12 x MDL). Mean spike recoveries for not only detected analytes but also all other monitored compounds ranged from 93 to 135% (Table

3.4), suggesting that target analytes were quantified with acceptable accuracy in Pecan Creek samples. Also note that values of relative percent difference (RPD) for MS/MSD samples were generally less than 20%, demonstrating acceptable precision.

Table 3.4. Matrix spiked and matrix spiked duplicate performance

Analyte	amount spiked ng/g	sample conc. ng/g	MS conc. ng/g	MSD conc. ng/g	MS % recovery	MSD % recovery	% RPD
m-Toluamide	41	nd	44	46	108	114	5
Benzophenone	87	41	125	124	97	95	2
Celestolide	47	nd	57	63	121	134	11
p-Octylphenol	34	nd	39	33	115	96	18
Galaxolide	106	606	732	748	119	135	12
Tonalide	56	38.9	108	103	124	114	9
Musk xylene	85	nd	103	94	121	110	10
p-nonylphenol	28	nd	34	30	122	108	12
4-MBC	62	nd	67	70	107	113	5
Musk ketone	197	nd	184	209	93	106	13
Triclosan	62	19.3	84	99	103	128	21
Octocrylene	194	nd	187	198	97	102	6

MS, matrix spiked; MSD, matrix spiked duplicate; RPD, relative percentage difference; nd, non detected

Identification of detected analytes was confirmed by comparing relative ion abundance ratios and retention times observed for environmental samples with those observed for standards spiked in control matrix. Three ions (one quantitation and two

qualifiers) were used for monitoring each analyte in all analyses (Table 3.1). The criteria imposed for positive identification were matching retention times and 15-percent agreement in relative ion abundance ratios. Representative ion chromatograms and mass spectra are shown in Figure 3.2 for galaxolide, displaying excellent agreement in both retention time and relative ion abundance.

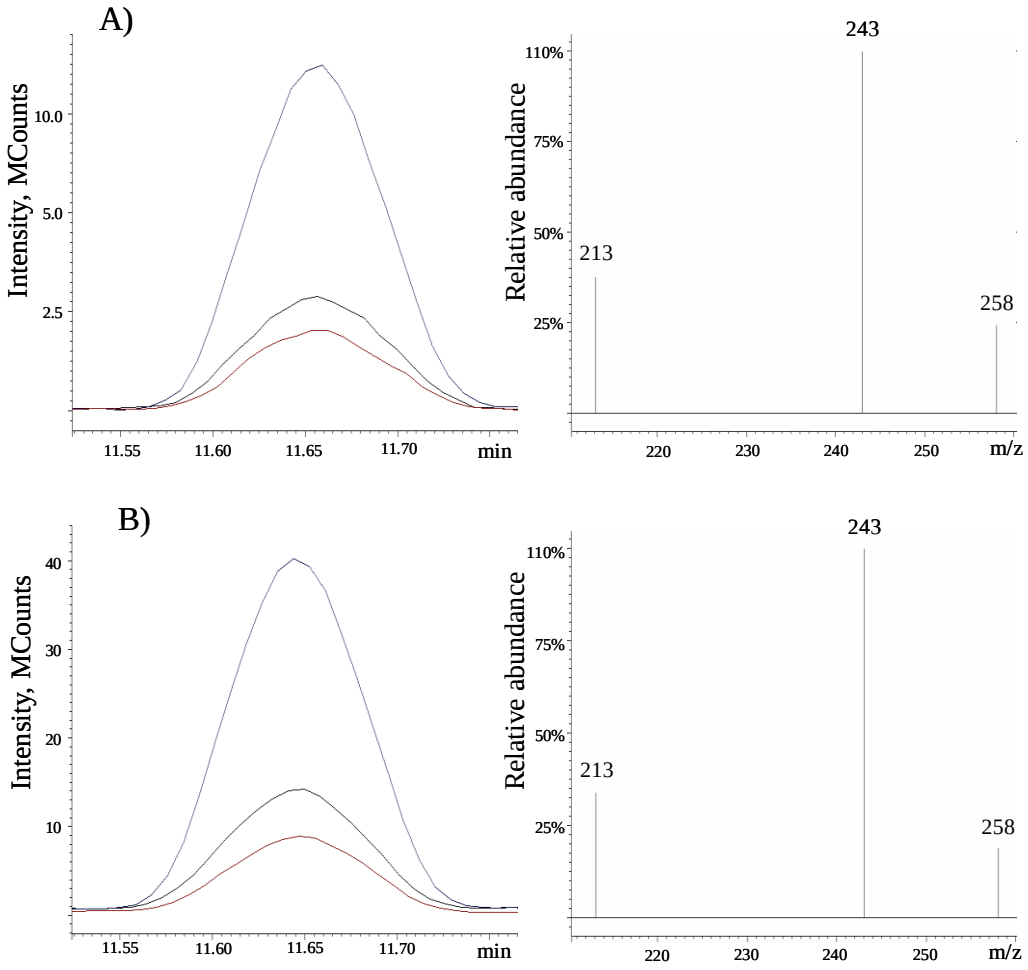


Figure 3.2. GC-SIM-MS reconstituted ion chromatogram and mass spectra for galaxolide in (A) fortified reference fish containing 120 ng/g galaxolide and (B) environmental sample collected from an effluent-dominated stream.

An interesting observation from Table 3.3 is that although analyses reported in Chapters 2 and 3 were conducted on identical tissues collected from the same fish, the

RSDs for detected concentrations of personal care products (Chapter 3) were significantly higher than those observed for pharmaceuticals (Chapter 2). RSDs calculated using data from Table 3.3 were 32%, 45%, 40%, and 19% for benzophenone, galaxolide, tonalide, and triclosan, respectively. In contrast, RSDs for detected pharmaceuticals in Chapter 2 were 26%, 36%, 17% and 14% for diphenhydramine, diltiazem, carbamazepine and norfluoxetine, respectively. Previous investigations of nonpolar organic contaminants have identified useful correlations between accumulation and octanol-water partition coefficients, which may also be roughly correlated with lipid content.[38-41]

Concentrations of detected personal care products in the present study are plotted versus lipid content in Figure 3.3. It can be seen in Figure 3.3 (A) that galaxolide has an apparent tendency to display higher concentrations with increasing lipid content. Nakata et al. also attributed large variations in galaxolide concentrations between individuals of the same species to differences in lipid content among analyzed tissues.[16] Although less clear, the tonalide data in Figure 3.3 (B) demonstrate a similar trend. In contrast, triclosan concentrations did not show monotonic dependence with lipid content, and it was difficult to discern a definitive trend for benzophenone due to the relatively high concentration observed in the sample with lowest lipid content. All concentrations were subsequently normalized by lipid content (i.e., ng/g lipid) in an effort to further clarify possible correlations. In the case of galaxolide and tonalide, RSDs for normalized concentrations improved to 36% and 29%, respectively. On the other hand, RSDs for benzophenone and triclosan were increased to 70% and 56% respectively. This analysis clearly suggests that observed accumulation trends for some PPCPs may not be

rationalized using historical partitioning models developed using data for alternative pollutants.

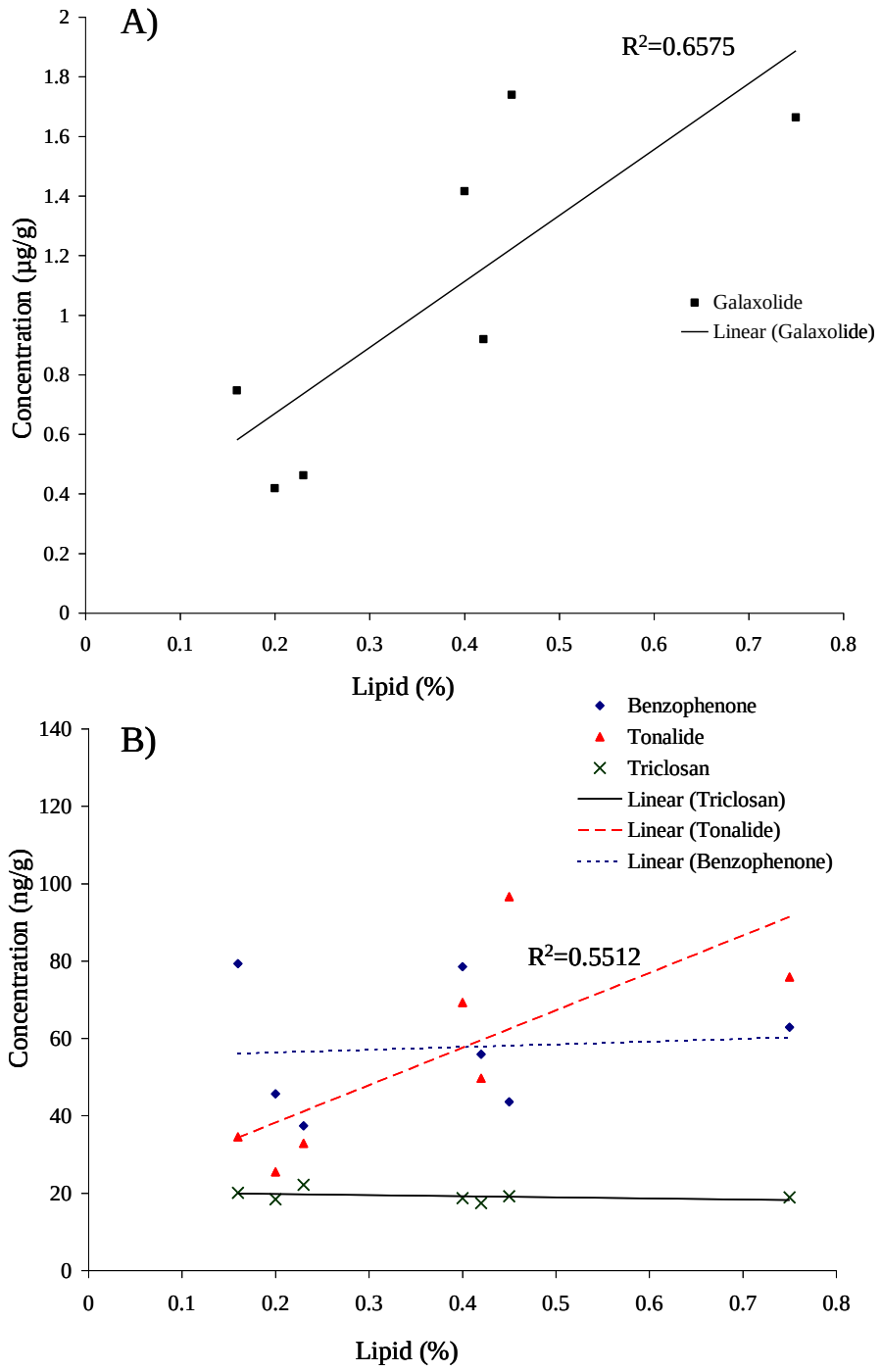


Figure 3.3. Plots of detected tissue concentrations (Denton, TX) versus lipid content for (A) galaxolide and (B) benzophenone, tonalide and triclosan

Conclusions

A multi-residue method has been developed and validated for the determination of select personal care products and alkylphenols in fish muscle tissue. The method was successfully applied to fish collected from an effluent-dominated stream and resulted in the detection of 4 contaminants. It is important to point out that analyzed tissues were derived from lateral fillets, excluding skin and belly flap, and that the reported approach may not be applicable to alternative tissues. While the elimination of GPC from the sample preparation protocol is noted advantage of reported methodology, the author concedes that analyzed tissues had a significantly lower lipid content than specimens analyzed in other studies. Accordingly, GPC may become necessary in future applications of this approach for more fatty tissues.

CHAPTER FOUR

Multi-residue Screening of Pharmaceuticals in Fish - A National Pilot Study in the US

Introduction

In Chapter 2, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) screening method was developed targeting 23 pharmaceuticals and 2 metabolites with differing physicochemical properties in fish muscle tissue. It was found that tissue extracts influenced the LC-MS/MS response for several analytes, therefore matrix-matched calibration standards were employed to determine analyte concentrations in environmental samples. This method was subsequently used to screen for target analytes in fish from one effluent-dominated stream in central Texas.

In this chapter, developed LC-MS/MS methodology was applied to assess the occurrence of target analytes in fish collected from six locations throughout the United States (5 effluent-dominated streams and one reference site). This work was conducted as part of the first national pilot study of pharmaceuticals in fish tissue sponsored by the U.S. Environmental Protection Agency. The analytical method was slightly modified to overcome limitations related to matrix effects that were noted in Chapter 2, and the new approach was successfully applied to both fillet and liver tissues. Concentrations of detected pharmaceuticals varied with geographic location and tissue type and site-to-site variations are rationalized in terms of the type of wastewater treatment process that was operational at each location. While findings in this study add definitive support to the work reported in Chapter 2, it is important to note that one novel contaminant was also

identified in liver specimens from two different locations. Additionally, the analysis of multiple biological tissues derived from diverse fish species collected at different locations enabled an expanded assessment of matrix effects on the observed analytical response for target analytes. Perhaps the most significant finding in this work was the absence of a correlation between tissue concentrations and lipid content.

Background on Wastewater Treatment

Pharmaceuticals, either in their native form or as metabolites, are continuously introduced to sewage waters through excreta and disposal of unused or expired drugs. Previous work has demonstrated that the ability of wastewater treatment processes to remove pharmaceuticals is compound-specific and variable, depending on the treatment process employed in a particular study. For example, in one study comparing removal efficiencies for a variety of wastewater-derived contaminants, percent removal observed for clofibric acid ranged from 34-51%, while that observed for ibuprofen was near quantitative (90%).[102] Also, it has been demonstrated that removal of β-blockers in secondary wastewater treatments employing activated sludge was only 8%, while that observed for biological filtration was 60%.[103] Finally, it is important to appreciate that the rate of removal for some compounds depends not only on the nature of the treatment process, but also on other factors like, hydraulic retention time,[103] seasonal temperature,[59] and potentially the age of activated sludge. Compounds which persist following municipal wastewater treatment are typically discharged to surface waters and have potential to accumulate in aquatic organisms.

A brief discussion of municipal sewage treatment is included here to provide the reader with pertinent background to understand the rationalization of analytical data

reported in this chapter. A majority of wastewater in the United States is subjected to primary and secondary treatments at municipal facilities. Primary treatment involves mechanical separation of fats, oils, and grease as well as rocks, gravel, and other solids from sewage. In secondary treatment, the primary effluent is routed over a bed of coarse stones or plastic media covered with biomass (i.e., bacteria, protozoa, and fungi). At this stage in the treatment process soluble organic compounds (including select pharmaceuticals) are absorbed, digested, and metabolized by microorganisms into more stable inorganic and oxidized organic forms that resist further metabolism. An additional tertiary treatment capable of degrading pharmaceuticals that are not removed by biological degradation is also employed at some but not all sewage treatment facilities because these processes (i.e., activated carbon adsorption, membrane filtration, ozonation or photocatalysis) are relatively expensive and are still largely viewed as emerging technologies.[104]

Experimental Section

Chemicals

All chemicals were reagent grade or better, obtained from commercial vendors, and used as received. The positive ESI internal standards 7-aminoflunitrazepam-d_7, and fluoxetine-d_6 (100.0 µg/ml in acetonitrile), surrogates (100.0 µg/ml in acetonitrile) acetaminophen-d_4, and diphenhydramine-d_3, and reference standards (1000.0 µg/ml in MeOH): fluoxetine, norfluoxetine, sertraline, codeine, diphenhydramine, propranolol and ibuprofen were purchased as certified analytical standards (Cerilliant Corporation, Round Rock, TX). Atenolol was purchased in solid form (99% purity), also from Cerilliant.

The negative ESI internal standard meclofenamic acid and reference standards: 1,7 dimethylxanthine, acetaminophen, caffeine, miconazole, carbamazepine, erythromycin, gemfibrozil, trimethoprim, diltiazem, cimetidine, warfarin, thiabendazole, sulfamethoxazole, lincomycin, metoprolol, tylosin, clofibric acid were purchased in the highest available purity (Sigma-Aldrich, Milwaukee, WI). Surrogates (100.0 µg/ml in acetonitrile) carbamazepine-d_{10} and ibuprofen-$^{13}C_3$ were purchased from Cambridge Isotopes Lab. Inc., Andover, MA.

Study Site Selection

All aspects of the pilot study were carried out using protocols defined in a publically accessible Quality Assurance Project Plan (QAPP).[105] Information pertinent to analytical work is summarized below. All sampling activities related to the pilot study were performed by personnel from TetraTech, Inc, and whole fish were sent to Baylor on dry ice for sample compositing and analysis. Six geographic locations (five effluent-dominated streams and one reference site) were selected as sampling sites. Five streams utilized in this pilot study had a viable fish population of resident species which spend most of its life stages within effluent-dominated waters. These sites were targeted in mid- to large-sized cities representing diverse geographic regions of the country. Information on WWTP design capacity, average discharge, and in-stream waste concentration was collected for each site through publically-accessible data (i.e., NPDES permits, WWTP websites, and USGS flow data) and through phone calls to state officials and permitting agencies. The site selection criteria used was:

- High effluent flow versus ambient flow
- High population density

- Large fraction of elderly residents
- Large volume of PPCP sales/consumption
- Fish availability

Demographic information for each of these sites is summarized in Table 4.1. Fish were also collected from the Gila River (New Mexico, USA). The Gila River is not impacted by wastewater effluent discharges; therefore no accumulation of pharmaceuticals was expected in fish collected from this site. Accordingly, these fish were included in the study as a reference condition and also used to provide a clean control matrix.

Sampling and Preservation

In order to maintain consistency with existing EPA programs focused on accumulation of contaminants in fish tissue, this pilot study included composite sampling of fish fillets and fish livers from each sample site. Six composite samples were prepared at each site. At least three adult individuals were collected per composite such that the combined biomass of the specimens was adequate to provide sufficient tissue for lipid determination and complementary screening analyses for personal care products and pharmaceuticals, respectively. Each sample consisted of adult-sized fish that are typically consumed by wildlife and humans. All fish used to prepare a single composite were from the same species, were collected at the same time, and were of similar size such that the smallest individual in a composite was no less than 75% of the total length of the largest individual. The sampling period was from summer to early fall, since lipid content is usually highest and water levels lowest at that time. Species were identified by experienced personnel as soon as fish were removed from the collection device.

Table 4.1. Sampling locations

	Sites				
	IL	AZ	FL	PA	TX
City	Chicago	Phoenix	Orlando	West Chester	Dallas
Facility name	Northside WRD	91st Avenue WWTP	Orlando-Iron Br Fac	Taylor Run WWTP	Dallas WWTP
Treatment	advanced secondary	secondary	advanced treatment I	secondary	tertiary
Receiving water name	North shore channel	Salt river	Little econlo-ckhatchee	Taylor run	Trinity river
County name	Cook	Maricopa	Seminole	Chester	Dallas
Population	5,376,741	1,418,041	442,542	17,701	3,500,000
% effluent	100 %	100 %	64 %	36 – 86 %	100 %
% 65 & older	10.3	8.1	11.3	9.0	8.1
Median income	$38,625	$41,207	$35,732	$37,803	$43,324

Individuals of the target species were rinsed with distilled water to remove any foreign material from the external surface. Each fish within the target species was weighed and measured to determine total body mass (g) and total body length (mm). Each fish selected for the composited sample was individually wrapped in cleaned, (rinsed in methylene chloride and dried at 450 °C for a minimum of one hour), extra-heavy aluminum foil and placed between two pieces of food-grade plastic that were subsequently sealed on each end with a nylon cable. Samples were placed on dry ice and

shipped to Baylor via next-day air. Upon receipt, all samples were catalogued and stored at –20 °C prior to dissection and homogenization. Freezer temperature was recorded daily to ensure proper storage conditions.

Preparation of Composite Tissue Specimens

Samples were composited using the "batch" method, in which like tissues from all individual specimens that comprise a composite are homogenized together, regardless of each individual's respective portion (as opposed to the "individual" method, in which equal weights of each specimen are combined). For preparation of composite samples, each fish was rinsed with distilled water, scaled, and fillet (including skin and belly flap) and liver tissues were dissected independently (tissue dissections were carried out by Ms. Laura Dobbins). Each fillet was cut into approximately 2.5 cm cubes using high-quality stainless steel aluminum scissors. Individual cubes were combined and frozen (at –20 °C) prior to being ground to a fine powder using a high speed blender. Note that successive addition of small amounts of dry ice during grinding was critical in maintaining the consistency of frozen tissues and greatly simplified this aspect of sample preparation. The ground sample was then divided into quarters, opposite quarters were combined, and the resulting halves were mixed together by hand. After applying this procedure to at least three fish from one site, portions were combined again by dividing them into quarters and mixing halves to prepare a composite. This procedure was applied to prepare six different composite samples for each field site. Liver samples did not require the preparation of frozen cubes or the use of a high speed blender for homogenization. Instead, livers corresponding to each composite (at least three livers per composite) were combined in a clean glass container and homogenized using a

Tissuemiser (Fisher Scientific, Fair Lawn, NJ) set to rotate at 30,000 rpm. (Preparation of composite samples was carried out in collaboration with Dr. Mohammad A. Mottaleb and Ms. Pilar Perez-Hurtado).

Determination of Lipid Content

Lipids were extracted from three replicate fillet tissues (ca. 2 g) using 15 ml of 1:1 dichloromethane:hexane (v/v). Each mixture was homogenized for 3 min using a Tissuemiser (Fisher Scientific), and the vials were subsequently placed in an incubator for 24 h at 35 °C and agitated by gentle end-over-end rotation. Following extraction, solid anhydrous sodium sulfate was added to each sample (g Na_2SO_4 = 2 × g tissue), and the mixture was filtered through Grade 415 Filter Paper. The solid residue was then washed with an additional 15 ml of 1:1 dichloromethane:hexane, and the combined filtrate for each sample was collected in a pre-weighed test tube. The solvent was evaporated for 8 h at 45 °C using a Zymark Turbovap LC Concentration Workstation. After evaporation, each residue was dried to constant weight in a vacuum oven at 40 °C. Lipid content was determined gravimetrically by weighing three replicates of each sample. (This experiment was conducted by Ms. Pilar Perez-Hurtado.)

This procedure was modified slightly to determine lipid content of liver specimens. Approximately 2 g liver tissue was combined with 15 ml of 1:1 dichloromethane-hexane and ca. 2 g solid Na_2SO_4 in a borosilicate glass vial. Samples were sonicated for 30 min in an ultrasonic water bath at room temperature. The vials were subsequently placed in an incubator for 24 h at 35 °C and agitated by gentle end-over-end rotation. Following extraction, samples were centrifuged at 16,000 rpm for 40 min at 4 °C to ensure complete phase disengagement. The supernatant was collected by

filtration, and the solid residue was washed with an additional 15 ml of 1:1 dichloromethane:hexane that was also passed through the filter and collected in a pre-weighed test tube. The solvent was evaporated and residues were dried to constant weight in a vacuum oven at 40 °C. Due to limited sample mass, triplicate measurements were made for only one composite from each sampling site. All other determinations were based on a single measurement. (This experiment was conducted in collaboration with Ms. Pilar Perez-Hurtado.)

Analytical Sample Preparation

Each analytical sample, either 1.0 g fillet composite or 0.5 g liver composite, was placed into a 20-ml borosilicate glass vial (Wheaton; VWR Scientific, Rockwood, TN). Four surrogates were added to each sample, acetaminophen-d_4 (500 ng), diphenhydramine-d_3 (10 ng), carbamazepine-d_{10} (40 ng) and ibuprofen-$^{13}C_3$ (840 ng). 8 ml of extraction solvent (1:1 mixture of 0.1 M aqueous acetic acid and methanol) was added to the sample and the mixture was sonicated for 15 min at 25 °C. Following extraction, samples were rinsed into 50-ml polypropylene copolymer round-bottomed centrifuge tubes (Nalge Company; Nalgene® Brand Products, Rochester, New York) using 1 ml extraction solvent and centrifuged at 16,000 rpm for 40 min at 4 °C. The supernatant was decanted into 18 ml disposable borosilicate glass culture tubes (VWR Scientific, Rockwood, TN), and the solvent was evaporated to dryness under a stream of nitrogen at 45 °C using a Zymark Turbovap LC concentration workstation (Zymark Corp., Hopkinton, MA). Samples were reconstituted in mobile phase (1 ml for fillet composites and 0.5 ml for liver composites) and a constant amount of the internal standards 7-aminoflunitrazapam-d_7 (100 ng), fluoxetine-d_6 (585 ng), and meclofenamic

acid (995 ng) was added (half of these amounts were added to liver composites). Prior to analysis, samples were sonicated for 1 min and filtered using Pall Acrodisc hydrophobic Teflon Supor membrane syringe filters (13 mm diameter; 0.2-µm pore size; VWR Scientific, Suwanee, GA).

HPLC-MS/MS Analysis

All samples were screened for 24 target analytes using the HPLC-MS/MS approach described in Chapter 2. It is important to point out that fluoxetine-d_6 replaced 7-aminoflunitrazepam-d_7 as the internal standard for quantification of fluoxetine, norfluoxetine and sertraline. Similarly, 7-aminoflunitrazepam-d_7 replaced meclofenamic acid as the internal standard for quantification of ibuprofen. These changes were made in an effort to more effectively account for expected matrix effects on the analytical response for these analytes. Finally, note that clofibric acid was dropped from the analyte list to overcome a persistent instrumental limitation that prohibited the use of positive and negative electrospray ionization within the same chromatographic segment.

Calibration standards were prepared in fillet or liver control matrix, as appropriate, and carried through the entire sample preparation protocol prior to analysis. Note that in the preparation of calibration samples, varying concentrations of all target analytes and surrogates were added prior to extraction. Calibration curves were constructed from a minimum of five points by plotting the ratio of observed peak areas for the analyte and internal standard, respectively, versus analyte concentration. Calibration data were subjected to a linear regression that was forced through the origin, and the resulting equation was used to calculate analyte and surrogate concentrations in all subsequent analyses. This approach resulted in regression coefficients (r^2) exceeding

0.99 for all target compounds. Calibration plots employed for quantification of target compounds in both fillet and liver tissues are included in the Appendix (Figures A.1 to A.14). Analyte-specific MS/MS transitions monitored for quantitation were given in Chapter 2 (Table 2.2). Qualifier transitions monitored for detected analytes in environmental samples were as follows: diphenhydramine, *m/z* 256 > 152; diltiazem, *m/z* 415 > 150; carbamazepine, *m/z* 237 > 165; fluoxetine *m/z* 310 > 44; sertraline *m/z* 306 > 159, gemfibrozil, 249 > 127. Note that collision-induced dissociation of norfluoxetine results in only one product ion of sufficient intensity to be observed in environmental samples.[25] Therefore, a qualifying transition was not confirmed for this analyte.

The determination of experimentally-derived method detection limits (MDLs) for fish muscle tissue was discussed in Chapter 2, and it was initially assumed that these previously determined values would be appropriate for establishing detection and quantitation thresholds for the present study. Recall that the MDL corresponds to the lowest concentration of analyte that can be reported in a defined matrix with 99% confidence that the concentration is non-zero.[76] For data qualification purposes, a more conservative quantitation threshold for each analyte (PQL) was set 2 to 5 times above MDL. However, since analyte identification was supported by both chromatographic retention time and the relative response of two MS/MS product ions, each target compound was calibrated at or near the MDL and quantitative information for all positive identifications are reported. Over the course of this investigation it was determined that matrix interference encountered in the analysis of liver samples was more pronounced

Table 4.2. Low calibration level, MDL and PQL for target analytes in fillet and liver tissues

	Fillet			Liver	
Analyte	Low cal level, ng/g	MDL,[a] ng/g	PQL,[b] ng/g	Low Cal level, ng/g	MDL, ng/g
Diphenhydramine	0.10	0.05	0.25	0.10	0.26
Diltiazem	0.10	0.12	0.25	0.10	0.26
Carbamazepine	0.80	0.54	2.00	0.80	1.86
Warfarin	0.80	0.86	2.00	2.00	2.70
Cimetidine	2.00	1.04	5.00	5.00	5.18
Propranolol	2.00	1.07	5.00	2.00	3.77
1,7 Dimethylxanthine	2.00	1.10	5.00	2.00	5.84
Atenolol	2.00	1.48	5.00	12.5	12.86
Trimethoprim	3.20	2.15	8.00	3.20	8.00
Sulfamethoxazole	3.20	2.29	8.00	3.20	13.95
Metoprolol	3.20	2.50	8.00	3.20	8.90
Thiabendazole	3.20	2.63	8.00	3.20	7.84
Norfluoxetine	3.20	2.90	8.00	20.0	15.31
Sertraline	3.20	3.56	8.00	20.0	17.29
Caffeine	6.00	3.93	15.0	6.00	25.47
Acetaminophen	6.00	4.40	15.0	15.0	34.28
Fluoxetine	6.00	6.74	15.0	15.0	12.41
Tylosin	8.00	5.02	20.0	8.00	34.67

Table 4.2. Low calibration level, MDL and PQL for target analytes in fillet and liver tissues (Cont.)

	Fillet			Liver	
Analyte	Low cal level, (ng/g)	MDL,[a] (ng/g)	PQL,[b] (ng/g)	Low Cal level, (ng/g)	MDL, (ng/g)
Lincomycin	8.00	5.53	20.0	50.0	56.14
Codeine	8.00	6.11	20.0	20.0	31.49
Erythromycin	8.00	6.42	20.0	8.00	43.03
Gemfibrozil	8.00	6.68	20.0	8.00	24.82
Miconazole	12.0	10.83	30.0	na	na
Ibuprofen	60.0	45.96	150	60.0	172.81

[a] 'Clean' tissues employed in the determination of liver and fillet method detection limits (MDLs). Samples were extracted using a 1:1 mixture (v/v) of 0.1 M acetic acid buffer (pH 4) and methanol. See text for details. MDLs determined by multiplying the one sided 99 percent student's t-statistic ($t_{0.99}$) by the standard deviation from eight replicates analysis of spiked sample. [b] PQL was assigned in this study as two to three times the MDL values (depending upon lower calibration point behavior in the matrix).

than that observed for fillet tissues. Consequently, independent MDLs were determined for liver using an identical approach to that employed previously for fillet specimens. Adjusted MDLs and calibration limits for liver are reported in Table 4.2 along with the calibration limits, MDLs, and PQLs noted above for fillet tissues.

Starting with fillet specimens, all composite tissues were grouped into batches of less than 20 samples. Each batch contained one blank (i.e., control matrix spiked with surrogates only) and two laboratory control samples (LCS 1 and 2; control matrix spiked with surrogates and a known amount of each target analyte). LCS1 was a low-level control sample spiked with analyte concentrations corresponding to the PQL values

reported in Table 4.2, while LCS2 was spiked with analyte concentrations that were approximately 10-fold greater than the lowest concentration calibration standard. Composite samples from no more than two sites were also included in each batch, and one composite sample from each site was randomly selected and used to prepare two identical matrix spikes (i.e., one matrix spike-matrix spike duplicate pair, MS/MSD). Spike concentrations employed in the preparation of MS/MSD samples were equivalent to concentrations added to LCS2. Each batch was analyzed independently, and once fillet analyses were complete, an identical approach was employed for analysis of liver tissues.

Quality Control

The following quality control criteria were used throughout the study to monitor performance of the employed analytical method. Acceptable initial calibration required that the relative standard deviation of independent relative response factors (RRF defined in equation 4.1 below) for each analyte and surrogate was less than 30%.

$$RRF = \frac{A_x}{A_{is}} \times \frac{C_{is}}{C_x} \tag{4.1}$$

In this equation A_x and A_{is} are the peak areas for the target chemical and internal standard, respectively, and C_x and C_{is} are their respective concentrations in each calibration standard. The high-level laboratory control sample (LCS2) was not only employed to evaluate analyte and surrogate recoveries as defined in more detail below, but also doubled as a continuing calibration verification (CCV) sample in each analytical batch. Instrument calibration was evaluated by monitoring the percent difference (%D) between the RRF calculated for LCS2 and the average RRF from initial calibration data.

$$\%D = \frac{\left(RRF_{obs} - RRF_{nom}\right)}{RRF_{nom}} \times 100 \tag{4.2}$$

In this equation RRF_{obs} corresponds to LCS2 and RRF_{nom} is the average RRF resulting from initial calibration. A result ≤ 25% demonstrated acceptable calibration for continued sample analysis. Method performance was monitored on a continuing basis by evaluating analyte and surrogate recoveries (%R) in laboratory control samples (LCS 1 and 2) and matrix spikes (MS/MSD).

$$\%R = \frac{C_x}{C_s} \times 100 \tag{4.3}$$

In this equation C_x and C_s represent the observed sample concentration and spiking level, respectively. For the purposes of this study, recoveries ranging from 60% to 150% were deemed acceptable. Note that the same criteria were used to evaluate surrogates in every sample. Precision was evaluated by monitoring the relative percent difference (RPD) between duplicate matrix spikes:

$$RPD = \frac{\left|C_1 - C_2\right|}{\left(C_1 + C_2\right)/2} \times 100 \tag{4.7}$$

where C_1 and C_2 are the first and second of 2 measurements. In general, observed RPDs were less than 15%.

Results and Discussion

Analytical Observations from Control Samples

Results for initial and continuing calibration verification data in fillet tissues demonstrated acceptable performance with one exception. Evaluation of initial calibration data revealed that the RSDs of RRFs for 27 of 28 target compounds ranged

from 3 to 17% with an average of 9%. In contrast, the initial RSD observed for miconazole fell outside the 30% quality assurance threshold. Visual inspection of chromatograms demonstrated that miconazole response was suppressed to a greater extent than anticipated in this control matrix. Therefore, the calibration range for this analyte was adjusted to exclude concentrations that did not resolve from the baseline (i.e., the two lowest concentration calibration standards). As a result, the lowest concentration calibrated for miconazole was 75 ng/g, and the RSD improved to 27%. Initial calibration data for fillet analyses is included in the Appendix Table A.1. Percent difference (%D) for all continuing calibration verification samples ranged from -24% to 19%, demonstrating adherence to established criteria for continued sample analysis (see Table A.2 for representative CCV data, corresponding to the first analytical batch of fillet tissues).

Matrix suppression of analyte response and/or increased background required that the calibration range for a larger number of compounds be adjusted in analyses of liver tissues. Due to matrix suppression similar to that described above for miconazole, an analytical response for acetaminophen, codeine, cimetidine, fluoxetine, norfluoxetine, sertraline, and warfarin was not observed in one or more low concentration standards prepared in liver. Accordingly, these standards were eliminated in the construction of calibration curves for these analytes (Table A.3). In contrast, visual inspection of chromatograms for atenolol revealed significant peak-shaped responses of approximately equal area for samples spiked with 2, 5, and 12.5 ng of this compound. A peak-shaped response of similar magnitude was observed at the atenolol retention time upon subsequent analysis of a liver blank. However, the qualifier ion for atenolol was not

observed in the blank, confirming that atenolol was not present (i.e., the observed response was due to an unidentified compound native to control matrix). As a result, the three lowest concentrations were eliminated from atenolol calibration curves to overcome background interference. A similar situation required that the two lowest calibration levels for lincomycin be eliminated. As demonstrated in Figure 4.1, lincomycin was unresolved from a background peak eluting at slightly shorter retention time, and the concentration of the unidentified component was of sufficient magnitude to mask the lincomycin response at concentrations below 50 ng/g. A non-peak shaped background response was present in chromatograms for miconazole of sufficient magnitude to prohibit quantitative (or even qualitative) analysis of calibration data for this analyte in liver extracts.

After making the noted adjustments, initial calibration criteria were satisfied for all analytes (except miconazole) in liver tissue. As demonstrated in Table A.3, the RSDs of RRFs for target compounds ranged from 6 to 21% (with an average of 12%). In continuing analyses, observed values of %D for CCV samples ranged from -19 % to 23% with three exceptions. The %D for lincomycin was -29% and -38% in the IL and TX batches, respectively, and %D for norfluoxetine was -28.6 % in the TX batch. Although these values fall outside the CCV control limit specified for the pilot study (±25%), it was determined that corrective action (i.e., recalibration and repeated analysis of samples from the IL and TX batches) was not necessary since lincomycin was not detected in environmental samples and recoveries calculated for norfluoxetine in control samples (LCS 1 and 2) corresponding to the TX batch fell within the 60-150% limit.

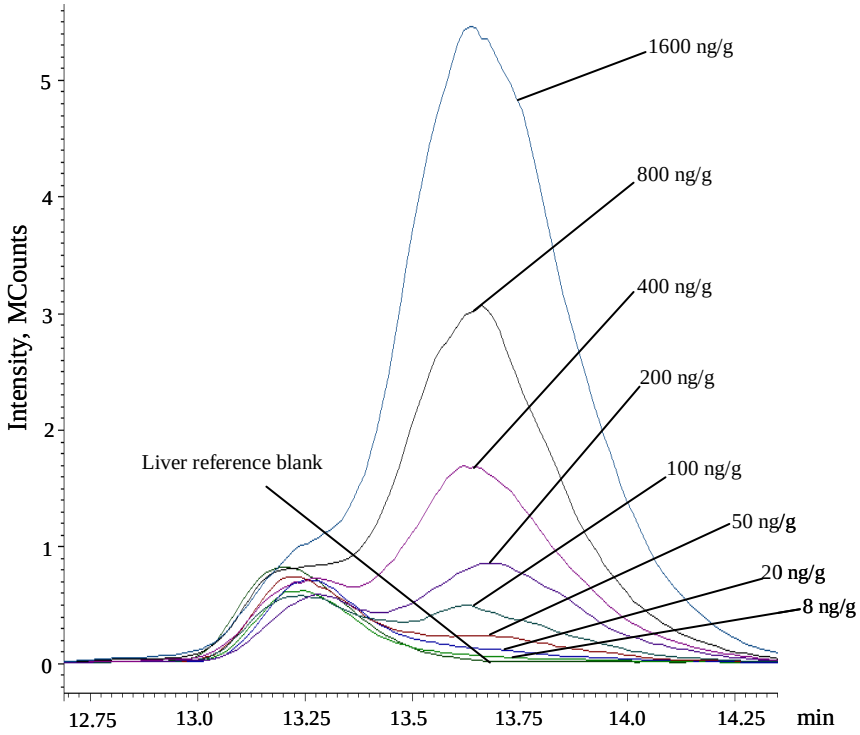


Figure 4.1. Overlaid MS/MS chromatograms for lincomycin calibration standards and liver control matrix

Surrogate and Control Sample Data

Surrogate monitoring was employed to evaluate method performance for each individual sample. A representative surrogate report corresponding to the first analytical batch is provided in Table A.4. Surrogate recoveries for samples derived from muscle tissue were within defined control limits (60-150%) and fell into the following ranges: acetaminophen-d_4, 87 – 110%; diphenhydramine-d_3, 63 – 80%; carbamazepine-d_{10}, 63 – 112%; and ibuprofen-$^{13}C_3$, 84 – 137%. It is noteworthy that recoveries for diphenhydramine-d_3 exhibited low bias. The origin of this behavior is presently unknown. Surrogate recoveries in liver samples also demonstrated acceptable performance with one exception: acetaminophen-d_4, 112 – 150 % (except for TX batch);

diphenhydramine-d_3, 71 – 120 %; carbamazepine-d_{10}, 75 – 119 %; and ibuprofen-$^{13}C_3$, 63 – 138 %. Acetaminophen-d_4 recoveries in the TX batch ranged from 131 to 234 %. However, acceptable recoveries were observed for acetaminophen-d_4 in CCV and LCS samples and for unlabeled acetaminophen in MS/MSD samples from this batch. Thus, it is possible that acetaminophen-d_4 experienced unique matrix interference in samples derived from the TX site.

Two laboratory control samples (LCS 1 and 2) were also included in each analytical batch to monitor instrumental performance. Representative control sample data corresponding to the first analytical batch is shown in Tables A.5 and A.6 for LCS 1 and 2, respectively. The LCS1 sample was employed to demonstrate quantitative accuracy near the MDL, and LCS2 demonstrated accuracy near the middle of the calibration range. Recoveries for all but two analytes in LCS1 samples prepared from muscle tissue ranged from 84 to 119%. The range of recoveries observed for erythromycin and miconazole in these samples was somewhat broader (88 – 136% and 86 – 147%, respectively) yet still within control limits. A similar trend was observed for LCS2 samples prepared from muscle tissue. Recoveries ranged from 84 – 120% for all compounds, excepting erythromycin (92 – 133%) and miconazole (105 – 133%).

In general, control sample data also validated quantitative accuracy for determination of analyte concentrations in liver extracts. With the exception of atenolol and lincomycin, observed recoveries for LCS1 and LCS2 samples ranged from 85 – 148% and 75 – 136%, respectively. As noted above, a background signal native to control matrix prohibited quantitation of atenolol and lincomycin at LCS1 spiking levels.

In LCS2 samples, lincomycin recoveries ranged from 94 – 133% while recoveries for atenolol still exhibited high bias 113 – 155%.

Matrix Spike Data

Matrix spike samples (i.e., MS/MSD samples) were included in each analytical batch to evaluate the influence of sample matrix on the analytical response for each analyte. Average MS/MSD recoveries observed for muscle and liver samples from each site are reported in Table 4.3. Consistent with matrix effect data presented in Chapter 2, these data clearly demonstrate that variability of sample matrix between sites had a negligible effect on the analytical response of early eluting analytes. That is, observed analyte recoveries in the present study were essentially quantitative for compounds eluting at retention times ≤ 30 minutes (i.e., all compounds preceding and including carbamazepine in Table 4.3). In contrast, with the exception of ibuprofen, observed recoveries for all analytes eluting at longer retention time fell outside of control limits (60-150%) in one or more MS/MSD samples (these occurrences are denoted in italics in Table 4.3). In general, matrix effects were more pronounced in liver extracts than in extracts derived from fillet tissue. Note that the data in Table 4.3 are essentially normalized to the NM sample, since this matrix was also employed for instrument calibration. Signal enhancement relative to the NM reference condition was the effect observed for the majority of influenced analytes, resulting in increased recoveries by a factor of ca. 2-6. Suppression was also observed in some cases (e.g., tylosin in both fillet and liver samples from the IL site and warfarin in the fillet sample from AZ). Interestingly, gemfibrozil response was suppressed in the fillet extract from IL but

Table 4.3. Average matrix spike recoveries (n = 2) for target analytes

	Fillet / liver					
Analyte	IL	NM	AZ	FL	PA	TX
Acetaminophen	92/97	94/89	99/94	89/110	87/100	84/125
Atenolol	98/109	109/88	120/114	105/96	107/92	105/120
Cimetidine	89/107	101/105	119/112	99/107	101/106	99/118
Codeine	100/97	102/107	114/89	98/98	103/102	103/112
1,7 dimethylxanthine	102/117	94/110	105/110	91/106	92/101	90/129
Lincomycin	108/96	96/124	111/86	98/103	99/101	93/104
Trimethoprim	108/109	110/109	117/113	107/108	110/119	114/115
Thiabendazole	117/102	104/107	110/108	117/101	112/93	114/116
Caffeine	112/106	99/111	102/105	95/109	100/105	94/109
Sulfamethoxazole	104/85	101/111	97/84	90/107	89/95	90/108
Metoprolol	112/100	103/99	111/94	89/87	103/89	97/86
Propranolol	105/99	100/95	111/106	98/87	100/104	103/108
Diphenhydramine	104/88	97/88	94/69	87/89	102/114	90/105
Diltiazem	98/75	95/100	104/103	93/89	93/95	92/105
Carbamazepine	100/81	90/97	85/97	98/83	90/80	86/80
Tylosin	*9/53*[a]	113/111	118/78	113/99	100/106	110/99
Fluoxetine	90/144	92/105	97/*271*	105/*349*	94/*362*	100/*335*
Norfluoxetine	138/92	99/115	108/*197*	*152/350*	125/*247*	134/*398*
Sertraline	119/96	91/120	99/*172*	144/*407*	93/*473*	126/*584*

Table 4.3. Matrix spike average recoveries (n = 2) for selected analytes (Cont.)

	Fillet / liver					
Analyte	IL	NM	AZ	FL	PA	TX
Erythromycin	68/115	127/108	135/139	*160/240*	*197/297*	*174*/123
Warfarin	94/84	91/85	*50*/78	110/92	93/69	79/76
Miconazole	*353*/na[b]	125/na	107/na	*414*/na	*296*/na	*199*/na
Ibuprofen	114/112	96/83	99/95	85/94	106/76	104/102
Gemfibrozil	*42/172*	88/106	126/*246*	149/*527*	94/*218*	86/*166*

[a] italic numbers represent values outside control limit (60 - 150%); [b] a non-peak shaped background response was present in chromatograms for miconazole of sufficient magnitude to prohibit quantitative (or even qualitative) analysis of calibration data for this analyte.

enhanced to varying degrees in liver extracts. These results were somewhat surprising but clearly demonstrate that extracts from fish collected at different sites exert variable influence on electrospray ionization, despite the fact that they were derived from a single biological tissue (i.e., muscle or liver).

Variation of sample matrix between sites also resulted in a noticeable shift in retention time for some analytes. Average retention times observed in analyses of fillet and liver MS/MSD samples are plotted as a function of collection site in Figure 4.2. Calculated RSDs for analyte retention times plotted in Figure 4.2A were ≤ 1% in all cases, demonstrating that retention times were essentially constant in analyses of fillet extracts, independent of collection site. Similar RSDs were observed for most compounds in analyses of liver extracts as well. However, RSDs calculated using data

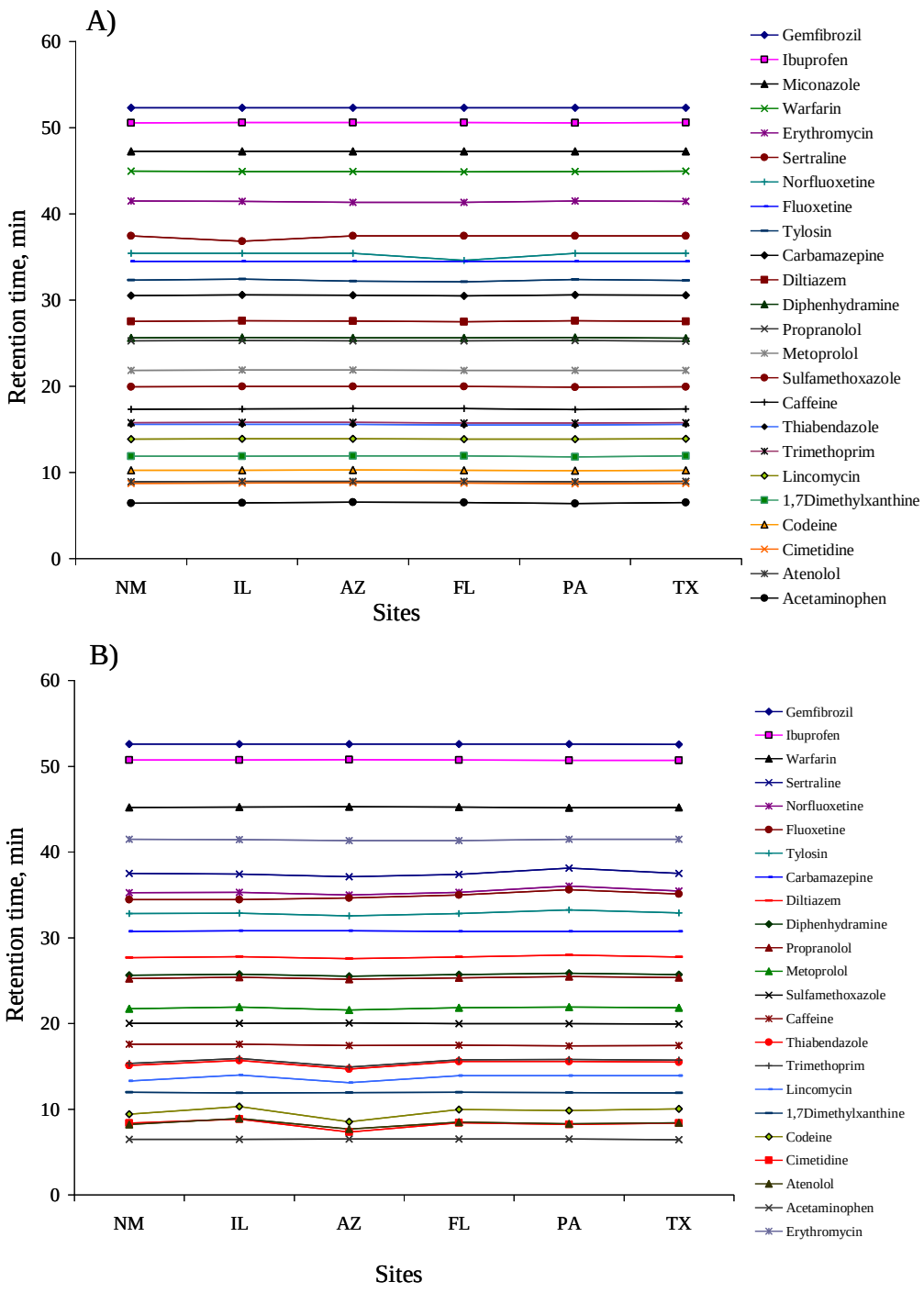


Figure 4.2. Average retention times observed for MS/MSD samples in A) fillet and B) liver tissue extracts

plotted for atenolol, cimetidine, codeine, lincomycin, trimethoprim and thiabendazole in Figure 4.2B ranged from 2 – 7%, demonstrating that co-extracted matrix components

derived from liver had a pronounced effect on the chromatographic behavior of these analytes. This can be attributed to the presence of co-eluting compounds that either interact with target analytes in the mobile phase or compete for stationary-phase adsorption sites. It is important to mention that within the same matrix, analyte retention times of atenolol, cimetidine, codeine, lincomycin, trimethoprim and thiabendazole were constant.

Environmental Occurrence

Analytical concentrations of pharmaceuticals detected in fillet and liver tissues from each sampling location are summarized in Tables 4.4 and 4.5, respectively. Plus-or-minus values presented in these tables represent one standard deviation from the mean concentration observed upon screening independent composite samples from each site. Accordingly, the standard deviation provides a qualitative estimate of the range of concentrations detected at each site rather than an estimate of analytical variability. Data for independent composites corresponding to each collection site are included in Tables A.7– A.12. It is important to point out that no analytes were detected in fish sampled from the NM site, definitively demonstrating that environmental accumulation of these compounds is a consequence of point-source distribution of pharmaceuticals to surface waters.

Diphenhydramine, diltiazem, carbamazepine, fluoxetine, norfluoxetine, and sertraline were detected at concentrations above MDLs in all 6 composite fillet samples from one or more study site(s) (Table 4.4), confirming results included in Chapter 2 and previous reports citing accumulation of these compounds in fish tissues.[10, 25] Inspection

Table 4.4. Average concentrations of pharmaceutical (ng/g tissue) detected in 6 independent fillet composites from each sampling site

					Mean concentrations ± SD, (ng/g)[a]						
Site	sp[b]	length (cm)	weight (kg)	lipid % mean ± SD	diphenhy-dramine	diltiazem	carbama-zepine	fluoxetine	norflu-xetine	sertraline	gemfibrozil
NM	*ss*	33-34	0.7-0.9	4.9 ± 1.6	nd	nd	nd	nd	nd	nd	nd
IL	*lmb*	27-36	0.4-0.9	2.3 ± 0.6	1.38 ± 0.27	0.13 ± 0.02	2.3 ± 0.5	nd	2.3 ± 0.4[c]	2 ± 1[c]	nd
AZ	*carp*	35-55	0.8-2.0	3.9 ± 0.8	1.20 ± 0.12	0.04 ± 0.01[c]	N.D	1.5 ± 0.4[c]	3 ± 1	5 ± 1	nd
FL	*b*	34-44	0.4-2.2	1.0 ± 0.7	nd	nd	nd	nd	nd	nd	nd
PA	*ws*	29-36	0.3-0.6	1.9 ± 0.4	1.74 ± 0.42	0.13 ± 0.04	0.3 ± 0.1[c]	3 ± 1	4 ± 1	11 ± 5	nd
TX	*smb*	43-60	1.4-4.6	2.2 ± 1.1	nd	nd	nd	nd	1.5 ± 0.3[c,d]	nd	nd

[a] Reported analytes were detected in 6 of 6 composite samples for each site ($n = 6$) unless otherwise noted. See Tables A.7-A.12 for details.
[b] sp, species; *ss*, sonora sucker; *lmb*, large mouth bass; *b*, bowfin; *ws*, white sucker; *smb*, small mouth buffalo
[c] reported concentration < MDL and/or lowest calibration standard reported in Table 4.2
[d] analyte detected in 2 of 6 composite samples ($n = 2$)

Table 4.5. Average concentrations of pharmaceuticals (ng/g tissue) detected in 6 independent liver composites from each sampling site

					Mean concentrations (n = 6) ± SD, (ng/g)						
Site	sp	length (cm)	weight (kg)	% lipid mean ± SD	diphenhy-dramine	diltiazem	carbama-zepine	fluoxetine	norfluo-xetine	sertraline	gemfibrozil
NM	ss	33-34	0.7-0.9	4.9 ± 2.5	nd	nd	nd	nd	nd	nd	nd
IL	lmb	27-36	0.4-0.9	2.2 ± 0.4	6.97 ± 2.09	0.71 ± 0.17	6.0 ± 1.2	19 ± 4[c]	73 ± 47	84 ± 52	nd
AZ	carp	35-55	0.8-2.0	11.6 ± 2.1	6.72 ± 2.34	0.28 ± 0.09	0.3 ± 0.1[a]	nd	30 ± 11	71 ± 17	70 ± 14
FL	b	34-44	0.4-2.2	2.9 ± 1.6	nd	nd	0.3 ± 0.1[a,d]	nd	49 ± 23	13 ± 5[a,d]	nd
PA	ws	29-36	0.3-0.6	4.7 ± 0.9	10.21 ± 1.35	0.69 ± 0.07	0.6 ± 0.1[a]	70 ± 7	38 ± 5	380 ± 120	19 ± 7[a,b]
TX	smb	43-60	1.4-4.6	8.1 ± 2.7	0.47 ± 0.31	0.03 ± 0.01[a]	0.18 ± 0.03[a]	13 ± 1[b]	16 ± 56[a]	8.5 ± 39[a]	nd

sp, species; *ss*, sonora sucker; *lmb*, large mouth bass; *b*, bowfin; *ws*, white sucker; *smb*, small mouth buffalo. See Tables A.7-A.12 for details.
[a] reported concentration < MDL and/or lowest calibration standard reported in Table 4.2
[b] analyte detected in 2 of 6 composite samples
[c] analyte detected in 3 of 6 composite samples
[d] analyte detected in 5 of 6 composite samples

of Table 4.5 demonstrates that this statement also applies to liver data, with the additional detection of one novel contaminant. Although one previous report has demonstrated bioconcentration of gemfibrozil in a laboratory setting,[31] the present study represents the first example of environmental accumulation for this compound. In general, concentrations of detected analytes were increased and more variable in liver relative to fillet tissues. This finding is consistent with results reported in a previous investigation of environmental accumulation focused on antidepressants,[10] but the origin of this behavior is presently unknown.

In a recent study citing bioaccumulation of alternative organic wastewater compounds, Barber et al. rationalized higher analyte concentrations in same samples as being a consequence of higher lipid content.[77] Such rationalization is based on a collection of studies demonstrating a correlation between experimentally-derived bioconcentration factors and analyte-specific physicochemical properties, most commonly the octanol-water partition coefficient (K_{OW}). Multiple observations of increased bioaccumulation with increasing K_{OW} have led to the supposition that organic contaminants are most likely partitioned to fatty tissues (i.e., lipids.) Consequently, it is expected that higher lipid content will promote increased concentrations of accumulated chemicals. Recall that this trend was roughly demonstrated for galaxolide and tonalide in Chapter 3 (Figure 3.2). Occurrence data in Tables 4.4 and 4.5 corresponding to samples from TX, PA, FL, and AZ are consistent with this trend. However, increased liver concentrations were also observed in samples collected from the IL site, despite the fact that lipid content for muscle and liver tissues from this site was essentially identical. This result leads to divergent hypotheses that either data observed for tissues from the IL site

are anomalous or that conventional wisdom relevant to environmental accumulation of these pharmaceuticals is flawed.

In order to further investigate this issue, analytical concentrations of detected analytes were plotted versus lipid content. It is important to note that accumulation is expected to depend on the aqueous concentration of contaminants, which likely differ from site to site. Accordingly, it is only valid to inspect potential correlations for data corresponding to a single site. Representative plots for fillet and liver tissues corresponding to the IL site are given in Figure 4.3. Similar plots corresponding to other collection sites are shown in Figures A.15 – A.18. In no case, were analytical concentrations of detected analytes correlated with lipid content, clearly demonstrating that historical models developed for neutral, lipophilic contaminants are not likely to be useful in predicting accumulation trends for these and similar pharmaceuticals. Improved models are likely to include considerations of contaminant ionization state, differential metabolic and partitioning processes *in vivo*, and receptor-binding interactions. These factors may in part explain much of the variability observed in analyte concentrations between independent samples from the same site and between different biological tissues in the present study. Variability in tissue concentrations between sites is likely to be a function of the concentration of contaminants in the aqueous stream. While these concentrations are not yet available, they are expected to be dependent upon a number of site-specific factors listed in Table 4.1, including human population, the efficiency of employed wastewater treatment processes, and the percentage of stream flow represented by wastewater treatment effluent (i.e., % effluent). Average concentrations detected in

samples from all sites are displayed graphically in Figures 4.4 A and B for fillet and liver tissues, respectively. It is important to note that concentrations in Figure 4.4B have been

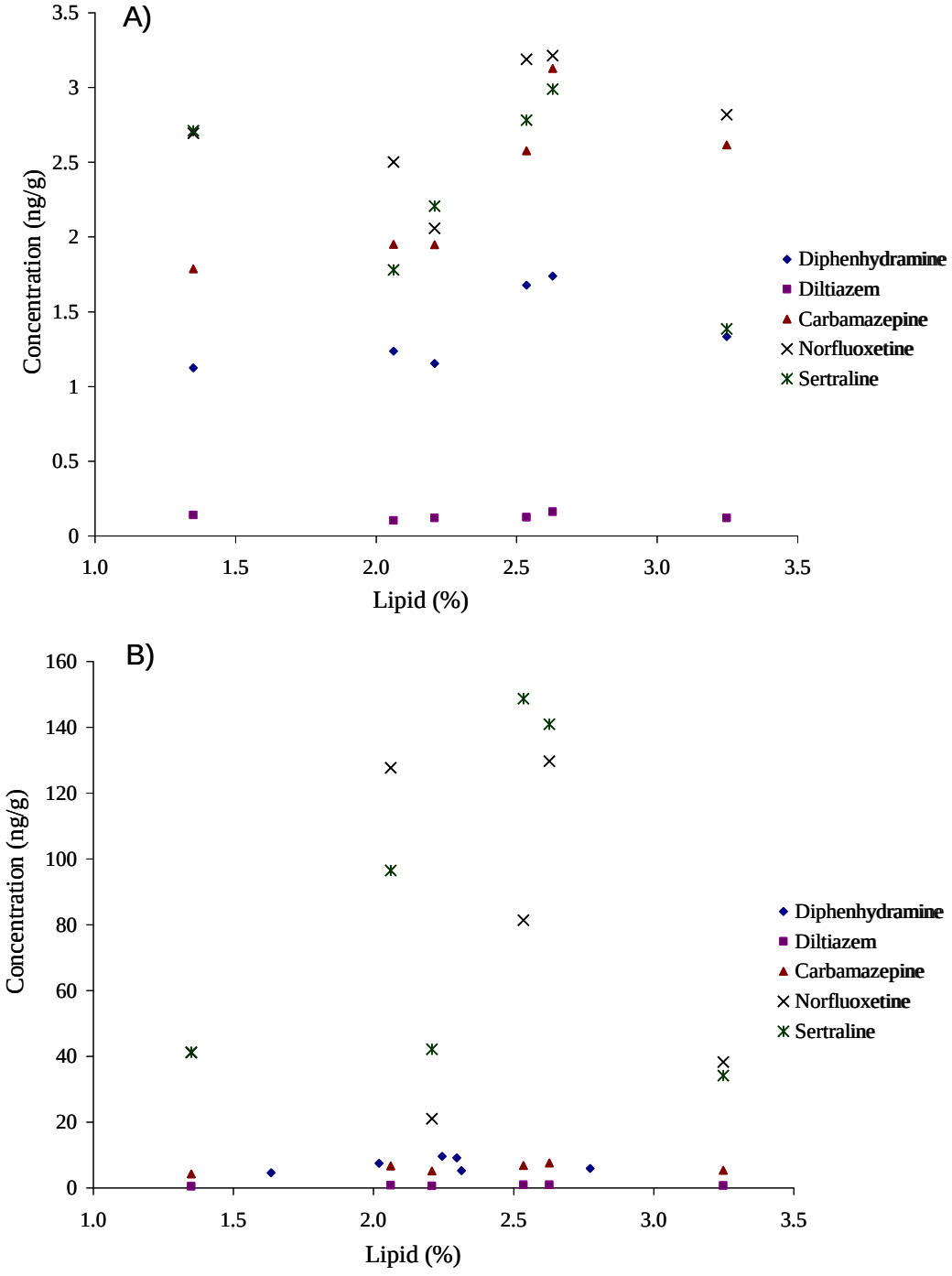


Figure 4.3. Plots of detected concentrations of pharmaceuticals versus lipid content for fish collected from IL; A) fillet and B) liver tissue.

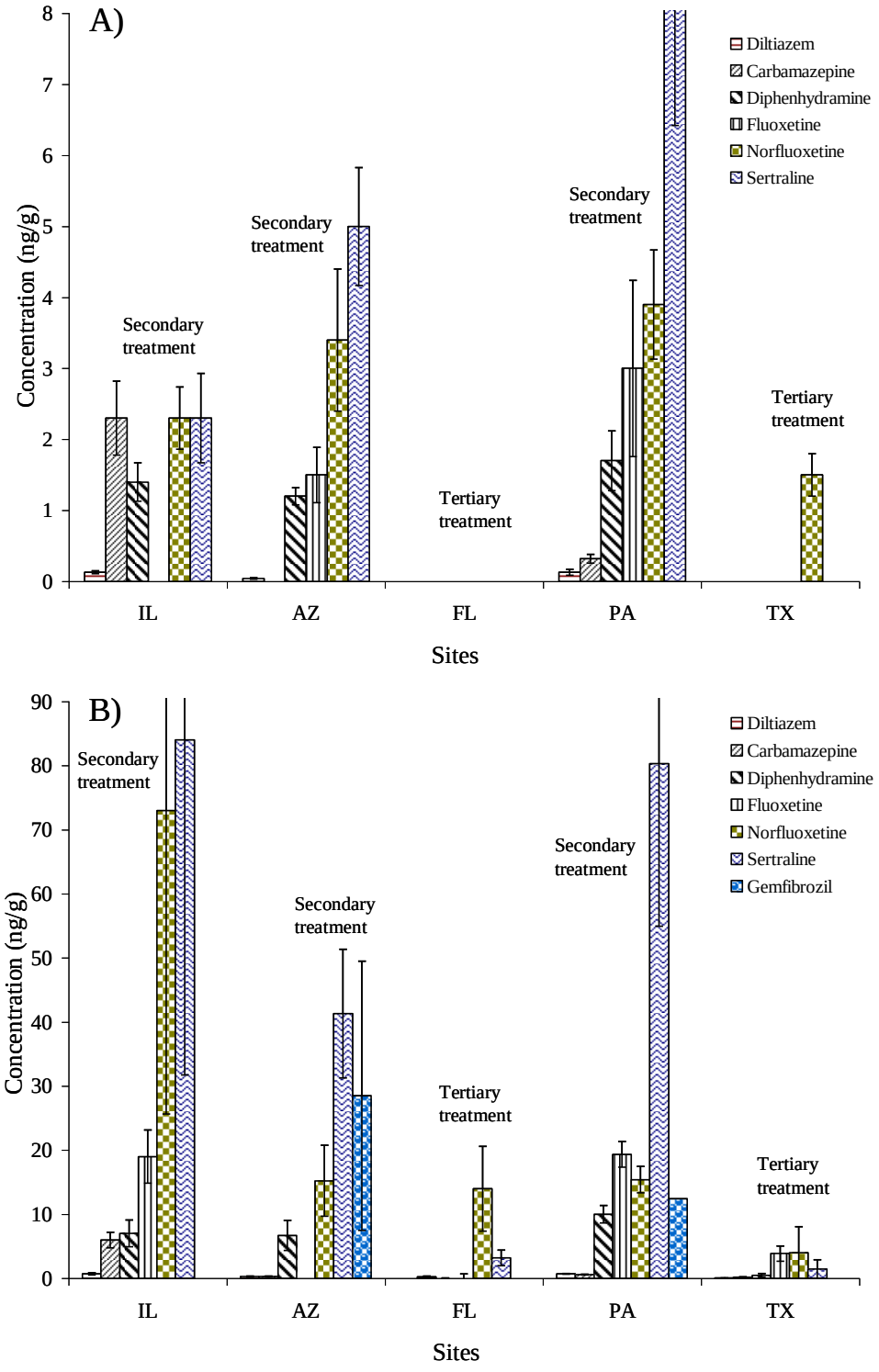


Figure 4.4. Average concentrations of pharmaceuticals detected in fish (A) fillet (B) liver tissues from each collection site

corrected to account for matrix enhancements noted in Table 4.3, as appropriate. These plots clearly demonstrate that the highest analyte concentrations were detected in samples from the IL, AZ, and PA sites. Inspection of detection frequency in Tables A.7 – A.12 revealed a similar trend. These findings do not appear to be strongly correlated with either human population or % effluent, as the PA site maintains the lowest population and lowest % effluent. However, the relatively low concentrations and frequency of detections observed for FL and TX sites are consistent with the application of more rigorous wastewater treatments. Tertiary treatments are employed at the FL and TX sites while secondary treatments are operable in IL, AZ, and PA. Observed variability between sites may also reflect differences in bioavailability of contaminants, but assessment of this factor is beyond the scope of the present study.

CHAPTER FIVE

Conclusions

The development and application of two complementary multi-residue screening methodologies, collectively assessing 37 target analytes in fish tissues, revealed a number of novel observations of significance. Work reported here demonstrated environmental accumulation of 11 chemicals (5 of which were novel) belonging to the PPCP class of emerging contaminants. This finding clearly establishes that environmental PPCP exposures are realistic and justifies continued efforts to understand PPCP fate in aquatic systems as well as effects of aquatic exposures. Of particular significance to environmental chemistry, much of the data reported here support the conclusion that traditional models for predicting contaminant partitioning to aquatic organisms are not likely to be applicable for many PPCP analytes. This is expected to be especially true for more polar compounds that may also be ionized in environmental systems.

Key factors promoting successful implementation of developed LC-MS/MS methodology for pharmaceuticals were diversion of chromatographic mobile phase away from the mass analyzer and implementation of matrix-matched calibration employing multiple internal standards. Although not explicitly stated in the body of this dissertation, chromatographic flow was not introduced into the electrospray source for the first 6 minutes of each chromatographic run. During this timeframe, all salts remaining in samples are expected to be eluted in reversed-phase separations and thus, are prevented from clogging the electrospray capillary or otherwise disturbing analyte ionization in the

electrospray source. When combined with the matrix-matched calibration approach described in Chapter 2, these procedures were successful in reducing the effect of sample matrix on observed analytical response for most analytes. However, even though multiple internal standards were employed to mimic the range of influences exerted by sample matrix, observed recoveries for matrix-spike samples reported in Chapter 4 demonstrated that matrix could alter the analytical response for select analytes by as much as a factor of 2-6. This finding supports the conclusion that isotope dilution should likely be incorporated for analyte quantitation to improve quality assurance in future applications of reported methodology.

A potential limitation of reported LC-MS/MS methodology is sensitivity. Many concentrations reported for fillet tissues in Chapter 4 required qualification (i.e., the detected concentration was at or below statistically-derived MDLs for each analyte). If more tissue were used in the extraction procedure (e.g., 10 g instead of 1 g of fillet), the concentration of analytes would be expected to increase in extracts. This would also likely result in a predictable decrease in MDL (10-fold for the example given above). In order to accommodate more tissue mass, it may also become necessary to incorporate more rigorous sample preparation. It is well known that gel permeation chromatography (GPC) helps to isolate analytes of interest away from large biomolecules and lipids that typically complicate analyses of biological samples.

Differences in accumulated concentrations of detected pharmaceuticals between sites were rationalized in Chapter 4 in terms of the type of wastewater treatment process employed. However, it is important to point out that this most likely reflects differing aqueous concentrations of target compounds in effluent receiving waters. Therefore it is

very important that aqueous concentrations be determined before drawing definitive conclusions. The availability of aqueous concentrations will also promote estimation of effective bioconcentration factors (BCF = concentration of analyte in tissue/concentration of analyte in water). Once calculated it should be interesting to examine potential correlations of these data with analyte physicochemical properties such as K_{OW}. This and similar comparisons may be useful in establishing whether PPCP accumulation is due primarily to partitioning of contaminants across membranes in direct contact with water (e.g., gills) or other factors such as ingestion of sediments or biomagnification.

APPENDIX

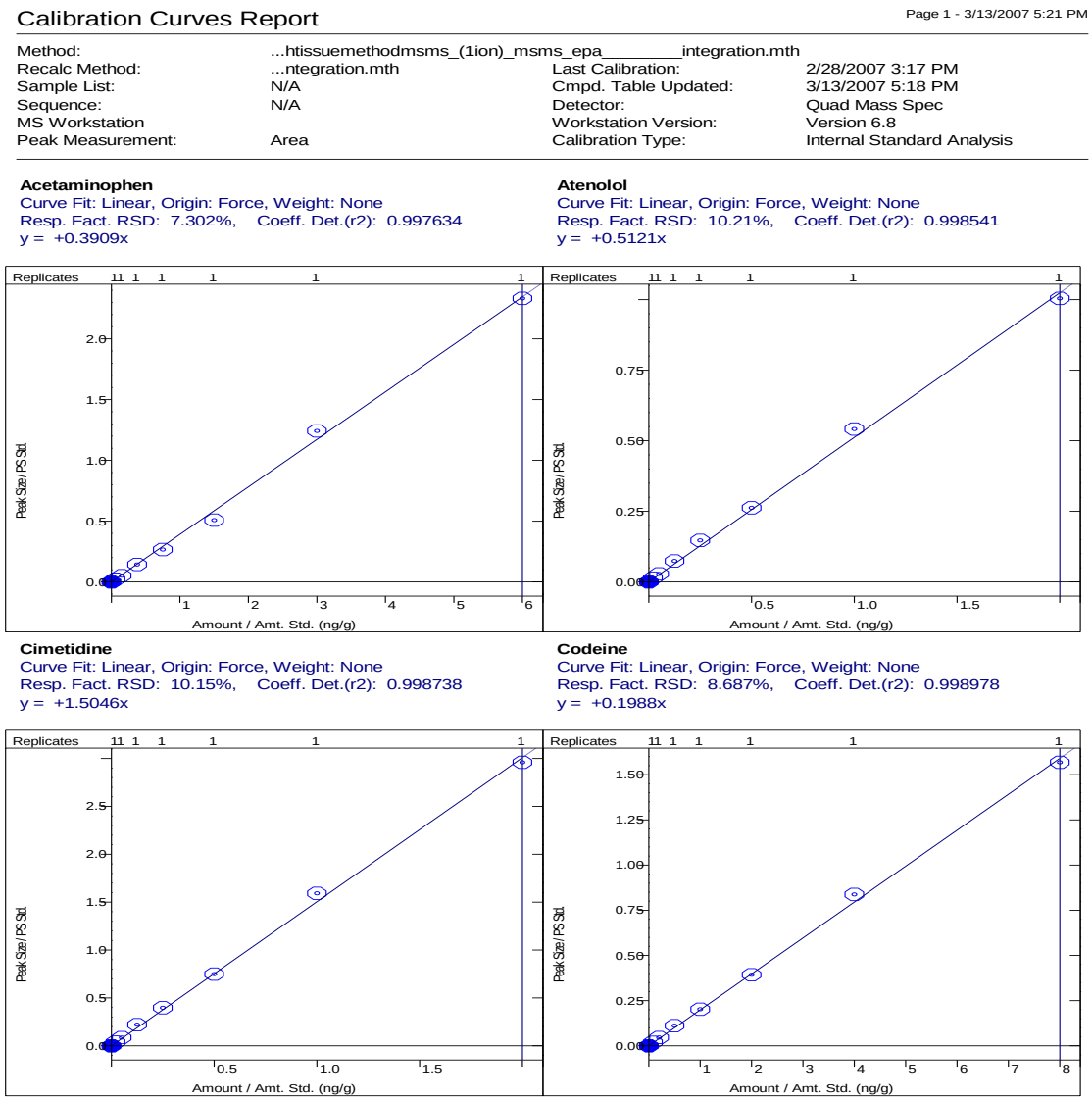


Figure A. 1. Calibration curves for acetaminophen, atenolol, cimetidine and codeine in fillet tissue

Calibration Curves Report

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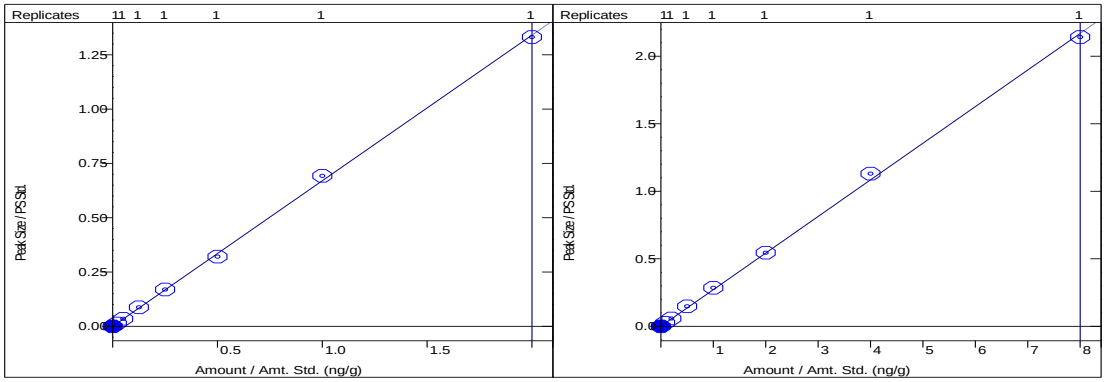
Recalc Method:	...ntegration.mth	Last Calibration:	2/28/2007 3:17 PM
Sample List:	N/A	Cmpd. Table Updated:	3/13/2007 5:18 PM
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

1,7Dimethylxanthine
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 3.156%, Coeff. Det.(r2): 0.999476
y = +0.6702x

Lincomycin
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 6.262%, Coeff. Det.(r2): 0.999338
y = +0.2711x



Trimethoprim
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 11.94%, Coeff. Det.(r2): 0.998146
y = +0.3331x

Thiabendazole
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 6.008%, Coeff. Det.(r2): 0.999549
y = +1.3077x

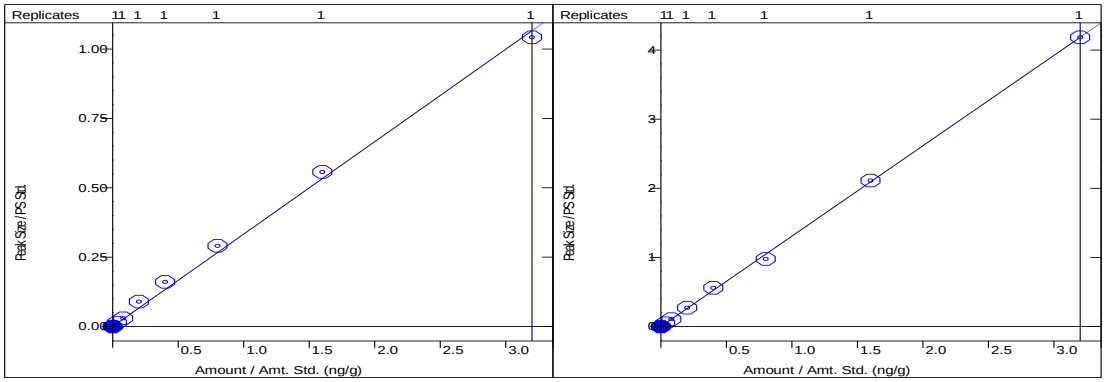


Figure A. 2. Calibration curves for 1,7-Dimethylxanthine, lincomycin, trimethoprim and thiabendazole in fillet tissue

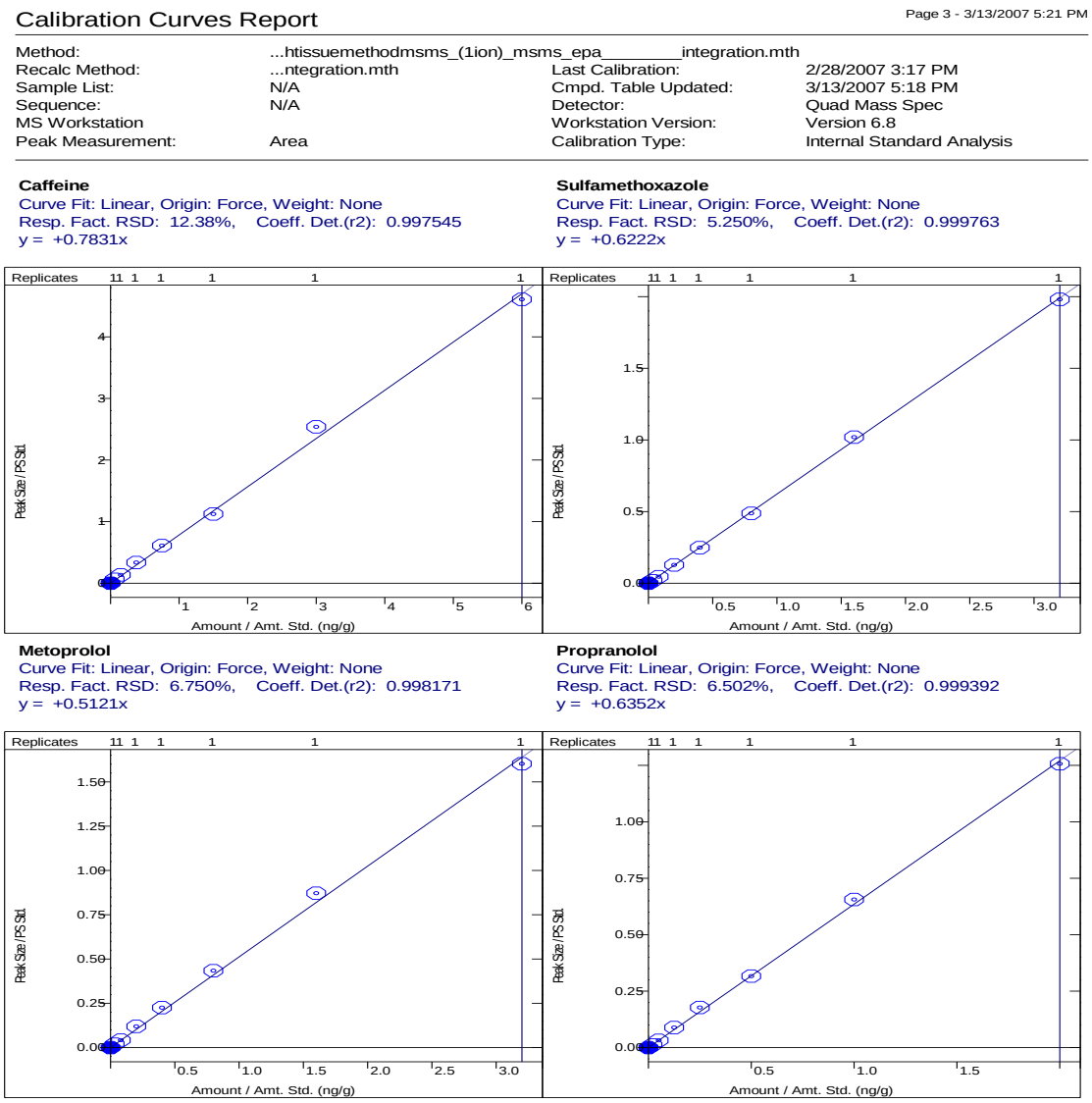


Figure A. 3. Calibration curves for caffeine, sulfamethoxazole, metoprolol and propranolol in fillet tissue

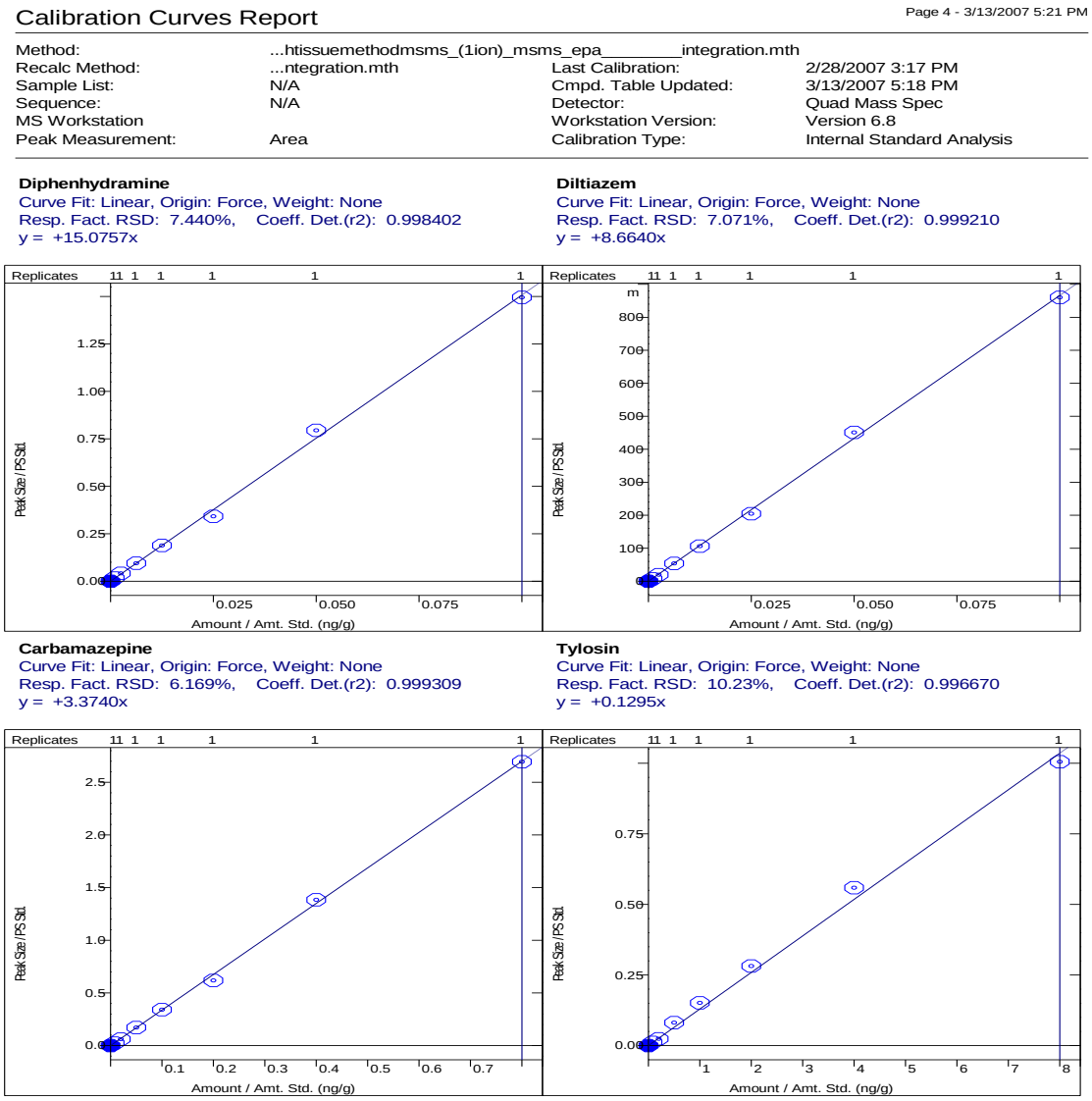


Figure A. 4. Calibration curves for diphenhydramine, diltiazem, carbamazepine and tylosin in fillet tissue

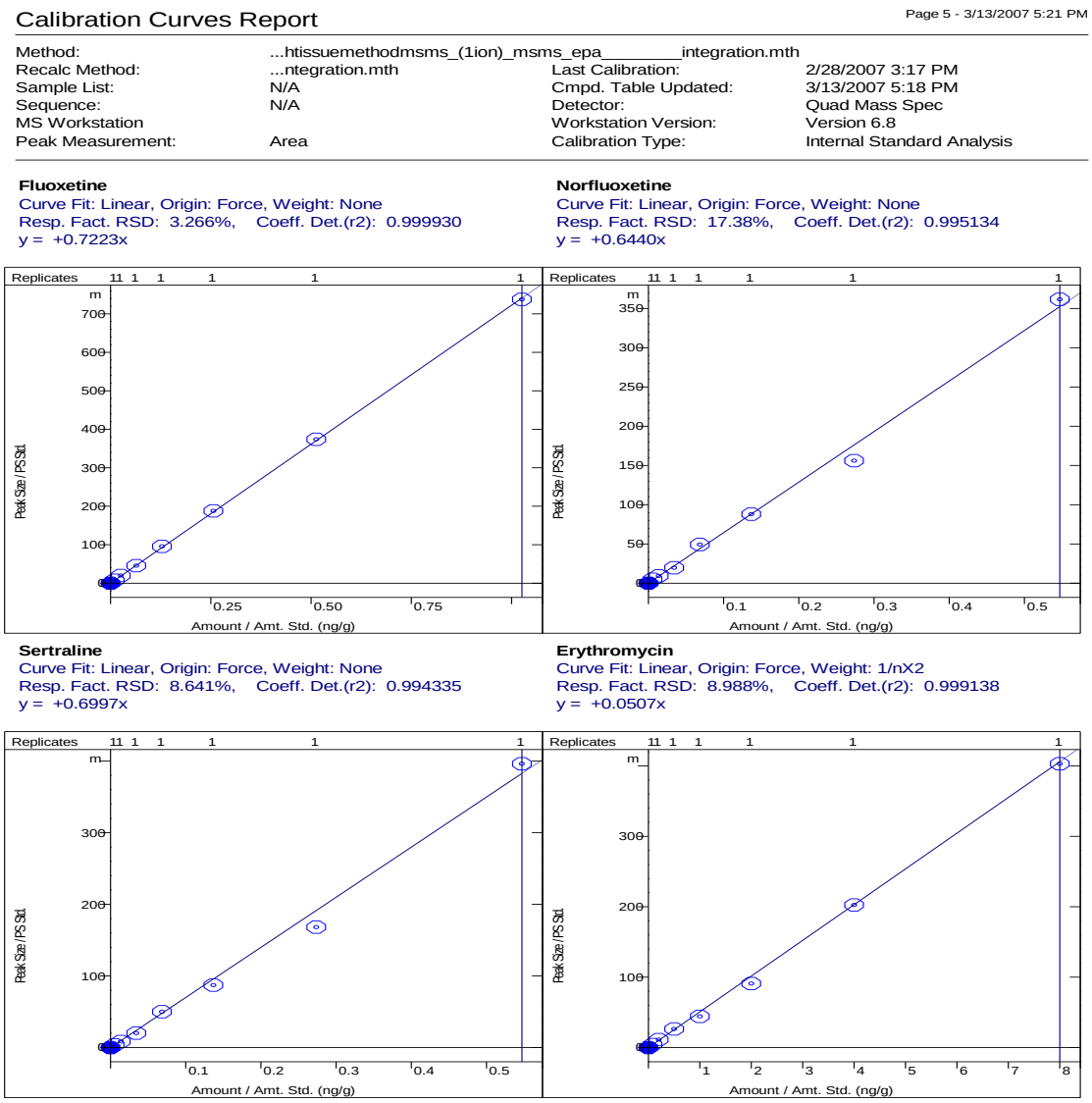


Figure A. 5. Calibration curves for fluoxetine, norfluoxetine, sertraline and erythromycin in fillet tissue

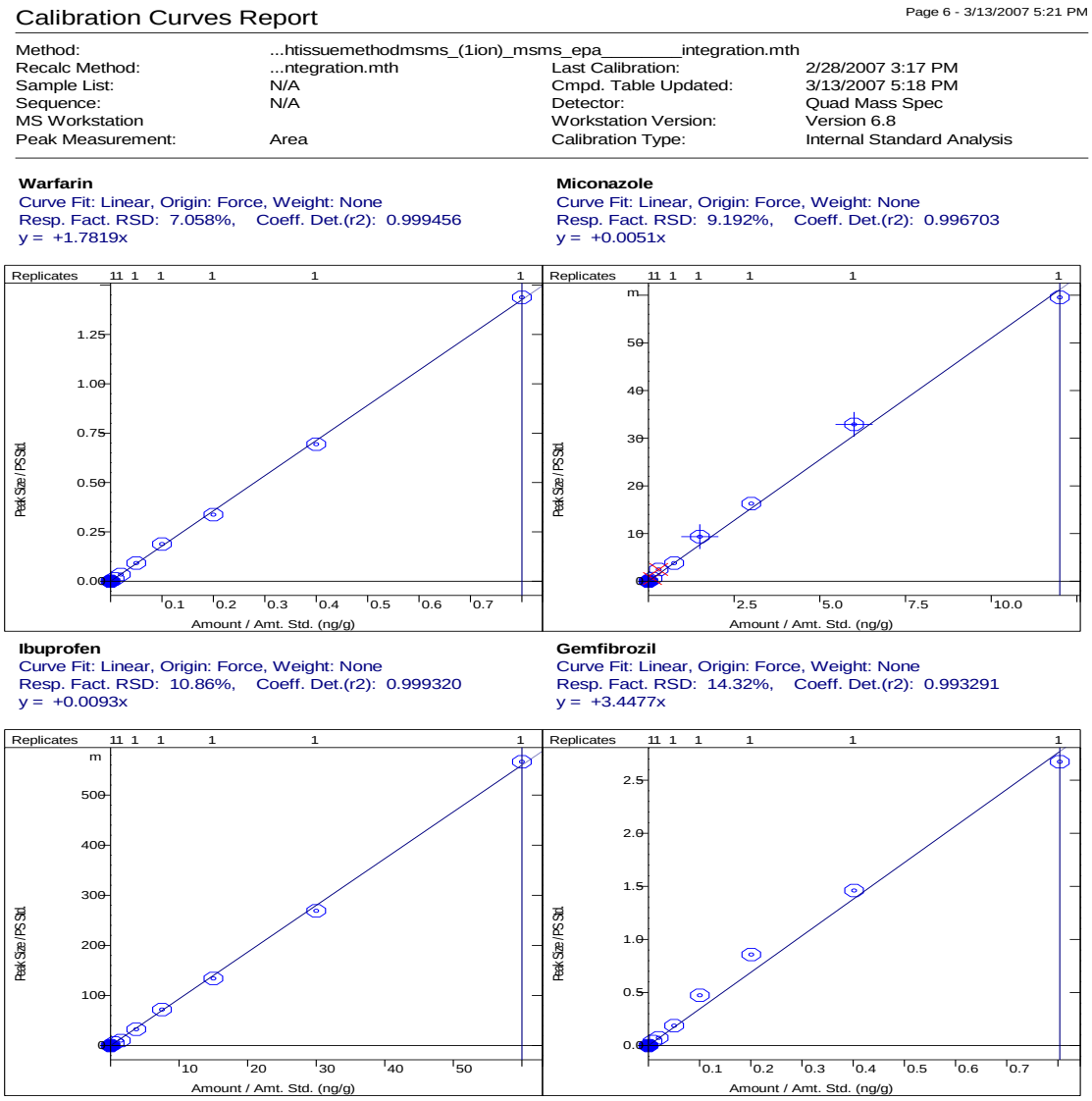


Figure A. 6. Calibration curves for warfarin, miconazole, ibuprofen and gemfibrozil in fillet tissue

Calibration Curves Report

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Recalc Method:	...ntegration.mth	Last Calibration:	2/28/2007 3:17 PM
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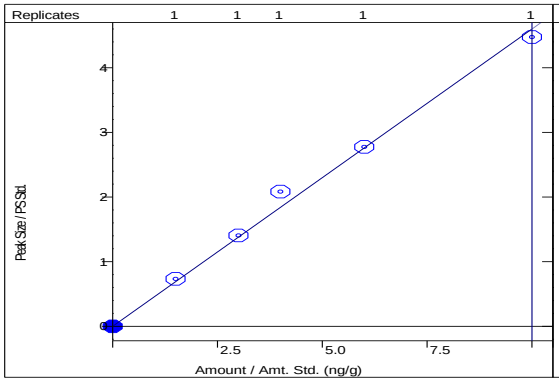
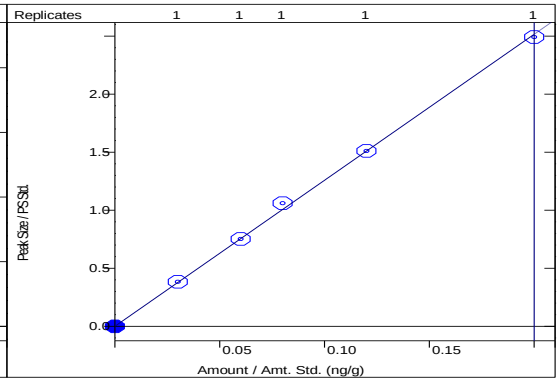
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

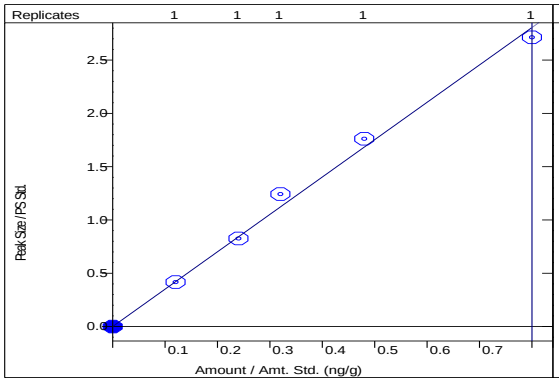
S1_AcetaminophenD4
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 5.961%, Coeff. Det.(r2): 0.995322
y = +0.4599x



S4_DiphenhydramineD3
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 2.522%, Coeff. Det.(r2): 0.999242
y = +12.5793x



S5_CarbamazepineD10
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 5.683%, Coeff. Det.(r2): 0.994596
y = +3.5067x



S2_Ibuprofen13C3
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 11.65%, Coeff. Det.(r2): 0.995171
y = +0.0105x

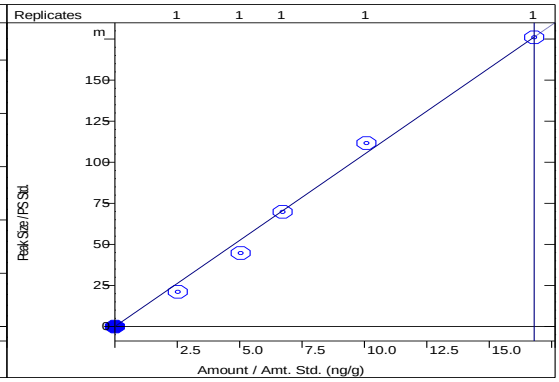


Figure A. 7. Calibration curves for acetaminophen-d_4, diphenhydramine-d_3, carbamazepine-d_{10} and ibuprofen-$^{13}C_3$ in fillet tissue

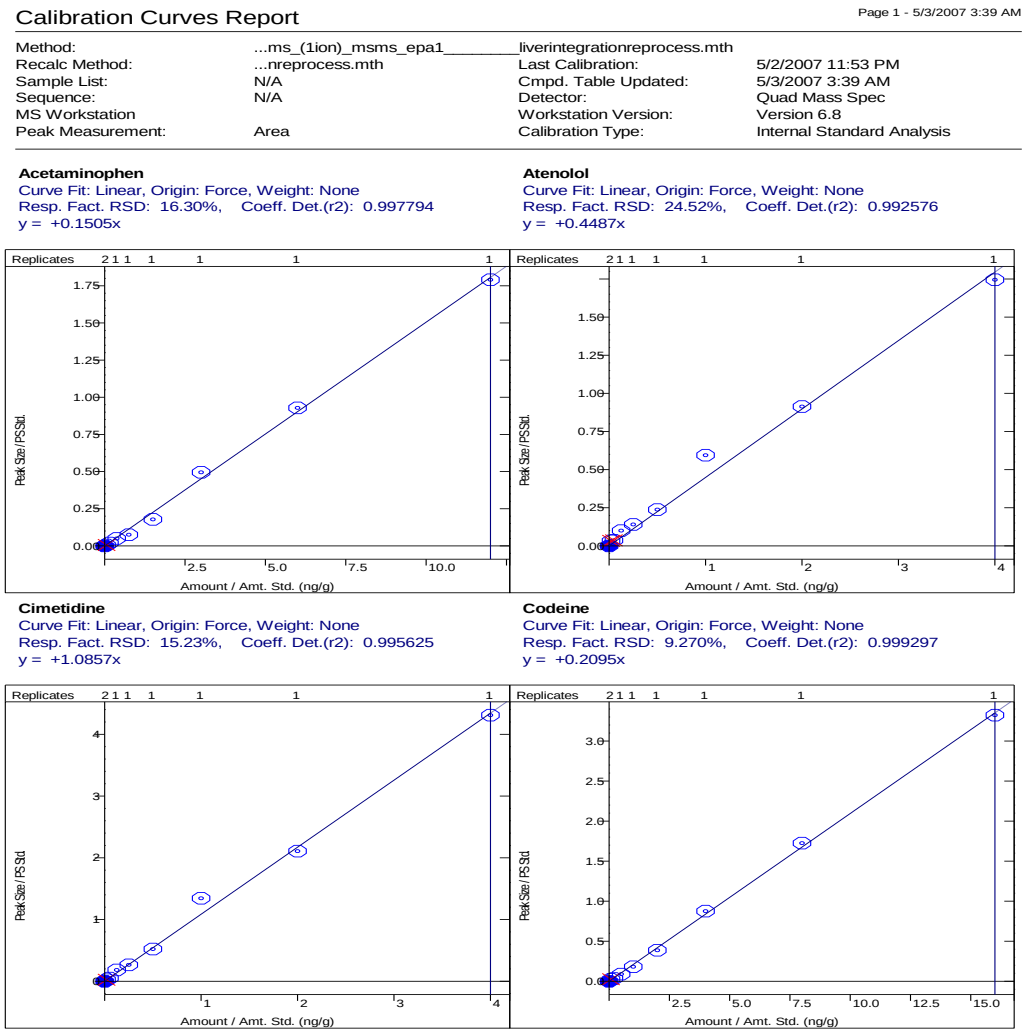


Figure A. 8. Calibration curves for acetaminophen, atenolol, cimetidine and codeine in liver tissue

Calibration Curves Report

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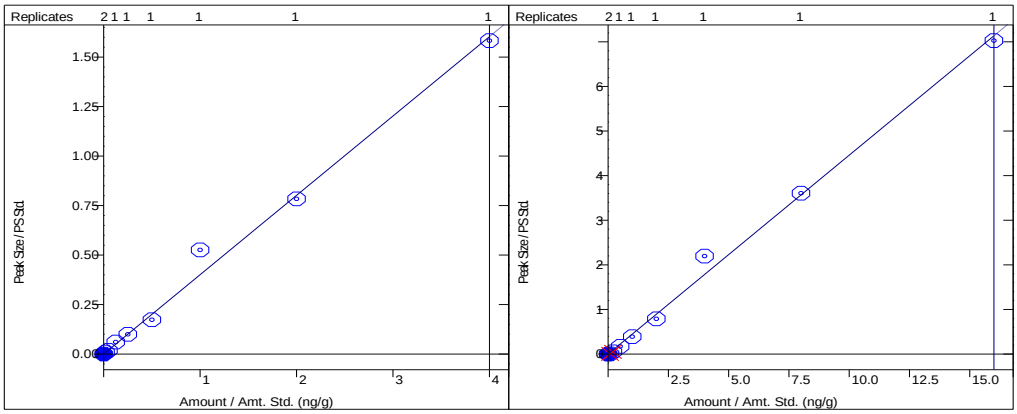
Sample List:	N/A	Cmpd. Table Updated:	5/3/2007 3:39 AM
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

1,7Dimethylxanthine
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 16.45%, Coeff. Det.(r2): 0.992753
y = +0.4008x

Lincomycin
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 16.34%, Coeff. Det.(r2): 0.995036
y = +0.4459x



Trimethoprim
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 12.11%, Coeff. Det.(r2): 0.995816
y = +0.4502x

Thiabendazole
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 9.673%, Coeff. Det.(r2): 0.996753
y = +1.3707x

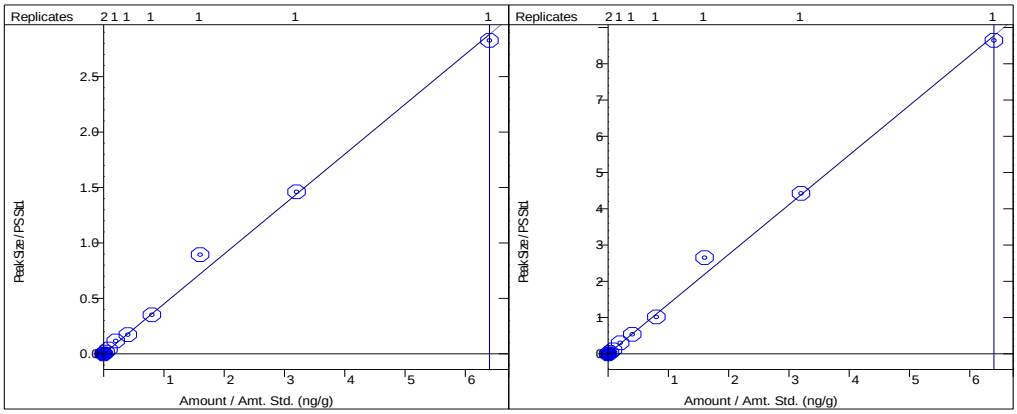


Figure A. 9. Calibration curves for 1,7-Dimethylxanthine, lincomycin, trimethoprim and thiabendazole in liver tissue

Calibration Curves Report

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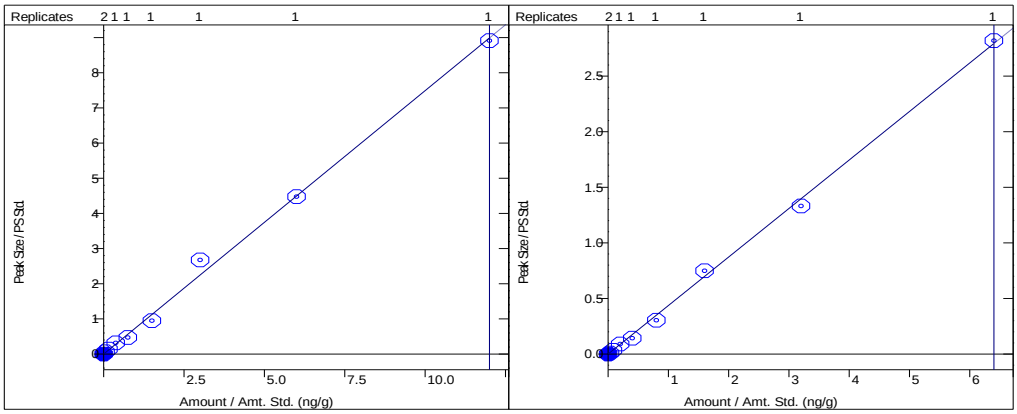
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

Caffeine
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 20.43%, Coeff. Det.(r2): 0.996841
y = +0.7491x

Sulfamethoxazole
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 14.49%, Coeff. Det.(r2): 0.998615
y = +0.4364x



Metoprolol
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 8.335%, Coeff. Det.(r2): 0.998897
y = +0.9027x

Propranolol
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 16.96%, Coeff. Det.(r2): 0.995025
y = +0.8942x

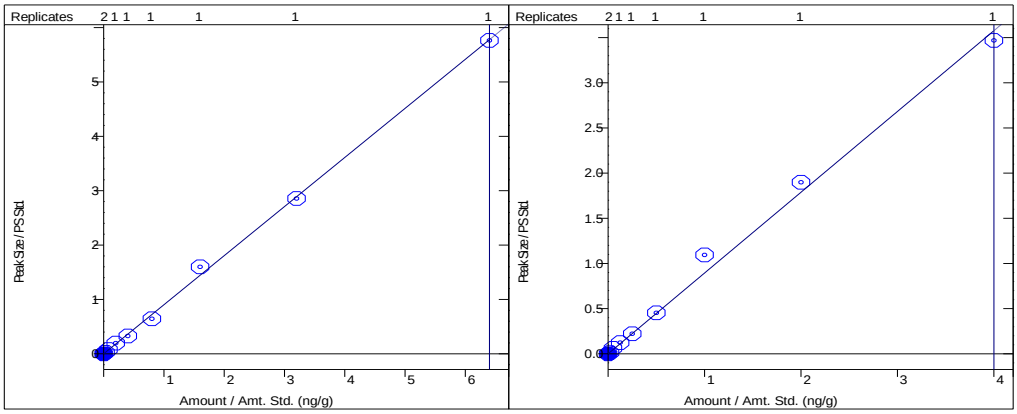


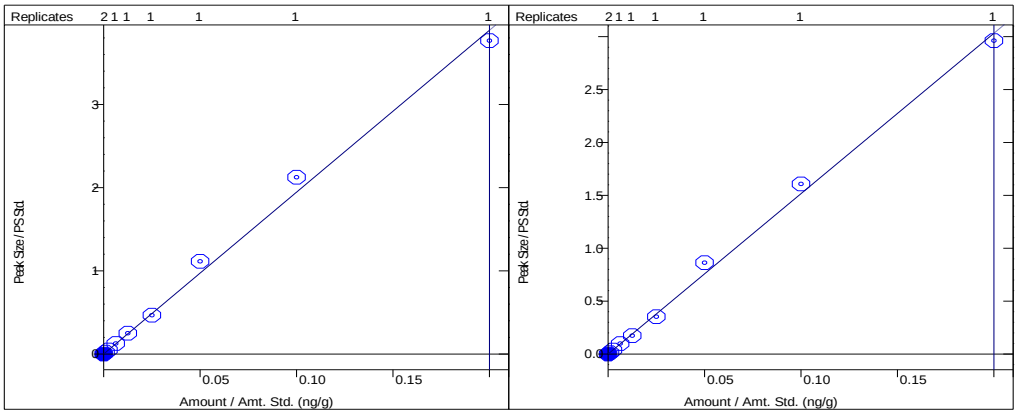
Figure A. 10. Calibration curves for caffeine, sulfamethoxazole, metoprolol and propranolol in liver tissue

Calibration Curves Report

Method:	...ms_(1ion)_msms_epa1_____liverintegrationreprocess.mth		
Recalc Method:	...nreprocess.mth	Last Calibration:	5/2/2007 11:53 PM
Sample List:	N/A	Cmpd. Table Updated:	5/3/2007 3:39 AM
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

Diphenhydramine
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 7.933%, Coeff. Det.(r2): 0.995349
y = +19.4583x

Diltiazem
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 8.787%, Coeff. Det.(r2): 0.997036
y = +15.1604x



Carbamazepine
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 10.61%, Coeff. Det.(r2): 0.998062
y = +8.0942x

Tylosin
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 16.87%, Coeff. Det.(r2): 0.993405
y = +0.4945x

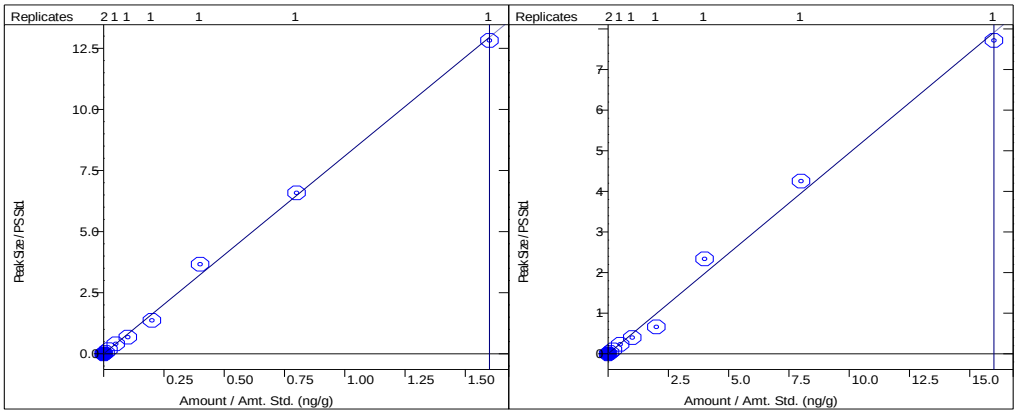
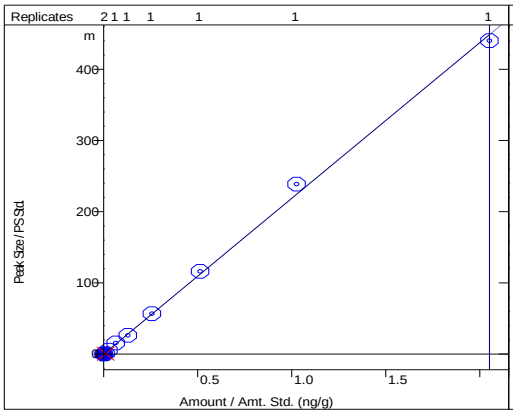


Figure A. 11. Calibration curves for diphenhydramine, diltiazem, carbamazepine and tylosin in liver tissue

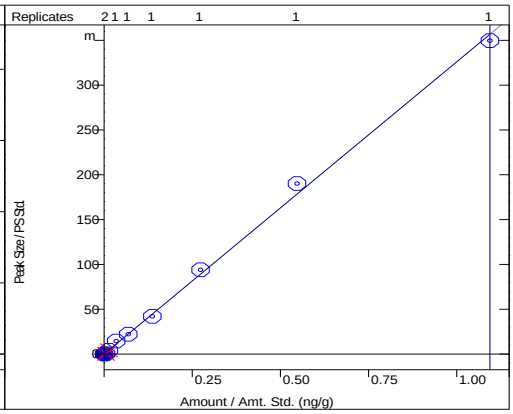
Calibration Curves Report

Method:	...ms_(1ion)_msms_epa1_____liverintegrationreprocess.mth		
Recalc Method:	...nreprocess.mth	Last Calibration:	5/2/2007 11:53 PM
Sample List:	N/A	Cmpd. Table Updated:	5/3/2007 3:39 AM
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

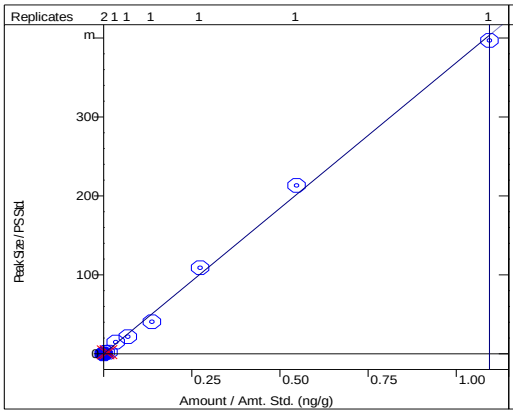
Fluoxetine
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 5.730%, Coeff. Det.(r2): 0.998344
y = +0.2187x



Norfluoxetine
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 12.28%, Coeff. Det.(r2): 0.997890
y = +0.3260x



Sertraline
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 14.26%, Coeff. Det.(r2): 0.997195
y = +0.3688x



Warfarin
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 9.760%, Coeff. Det.(r2): 0.997583
y = +4.4414x

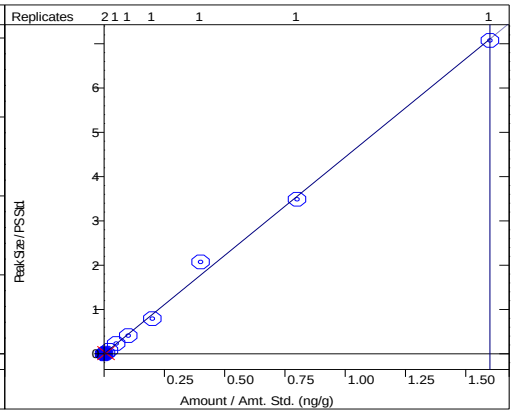
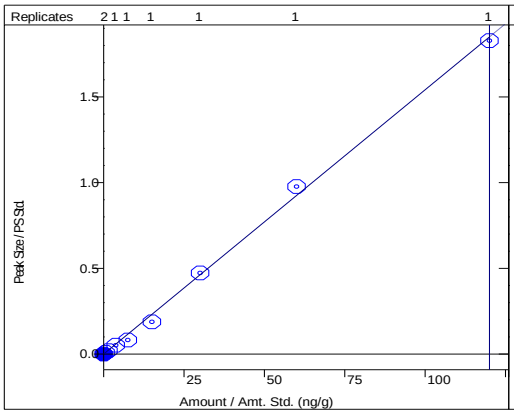


Figure A. 12. Calibration curves for fluoxetine, norfluoxetine, sertraline and warfarin in liver tissue

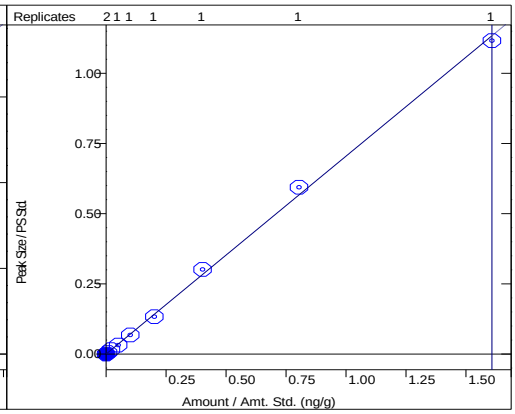
Calibration Curves Report

Method:	...ms_(1ion)_msms_epa1_____liverintegrationreprocess.mth		
Recalc Method:	...nreprocess.mth	Last Calibration:	5/2/2007 11:53 PM
Sample List:	N/A	Cmpd. Table Updated:	5/3/2007 3:39 AM
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

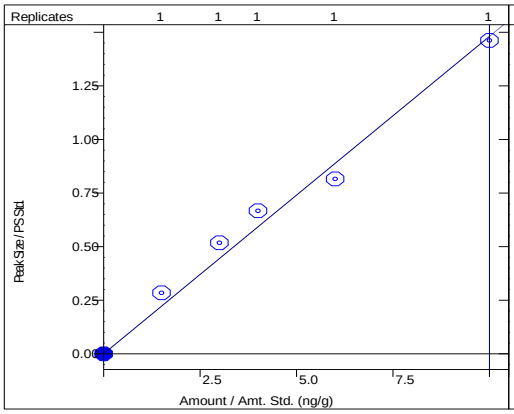
Ibuprofen
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 13.53%, Coeff. Det.(r2): 0.998056
y = +0.0154x



Gemfibrozil
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 7.170%, Coeff. Det.(r2): 0.998760
y = +0.7049x



S1_AcetaminophenD4
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 13.20%, Coeff. Det.(r2): 0.988646
y = +0.1481x



S4_DiphenhydramineD3
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 5.584%, Coeff. Det.(r2): 0.998015
y = +16.1121x

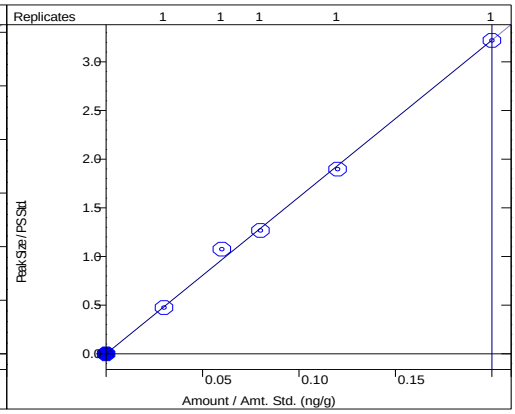


Figure A. 13. Calibration curves for ibuprofen, gemfibrozil, acetaminophen-d_4 and diphenhydramine-d_3 in liver tissue

Calibration Curves Report

Method:	...ms_(1ion)_msms_epa1_____liverintegrationreprocess.mth		
Recalc Method:	...nreprocess.mth	Last Calibration:	5/2/2007 11:53 PM
Sample List:	N/A	Cmpd. Table Updated:	5/3/2007 3:39 AM
Sequence:	N/A	Detector:	Quad Mass Spec
MS Workstation		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

S5_CarbamazepineD10
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 7.214%, Coeff. Det.(r2): 0.995446
y = +7.3840x

S2_Ibuprofen13C3
Curve Fit: Linear, Origin: Force, Weight: None
Resp. Fact. RSD: 11.58%, Coeff. Det.(r2): 0.986942
y = +0.0149x

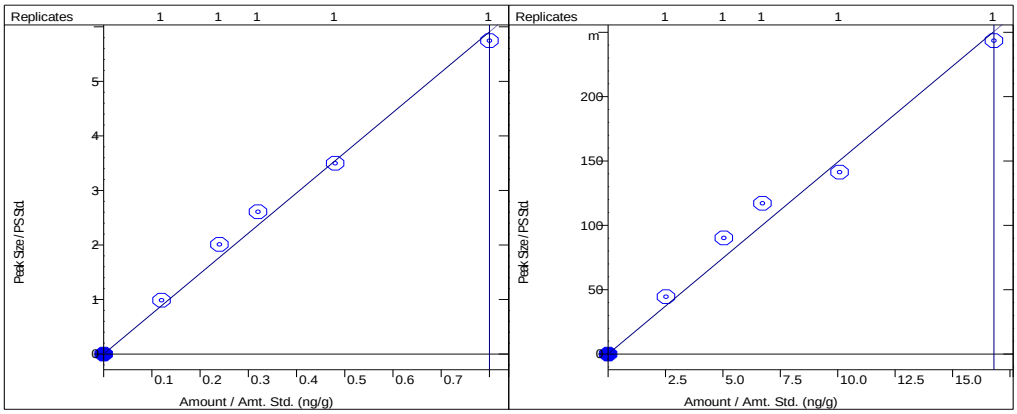


Figure A. 14. Calibration curves for carbamazepine-d_{10} and ibuprofen-$^{13}C_3$ in liver tissue

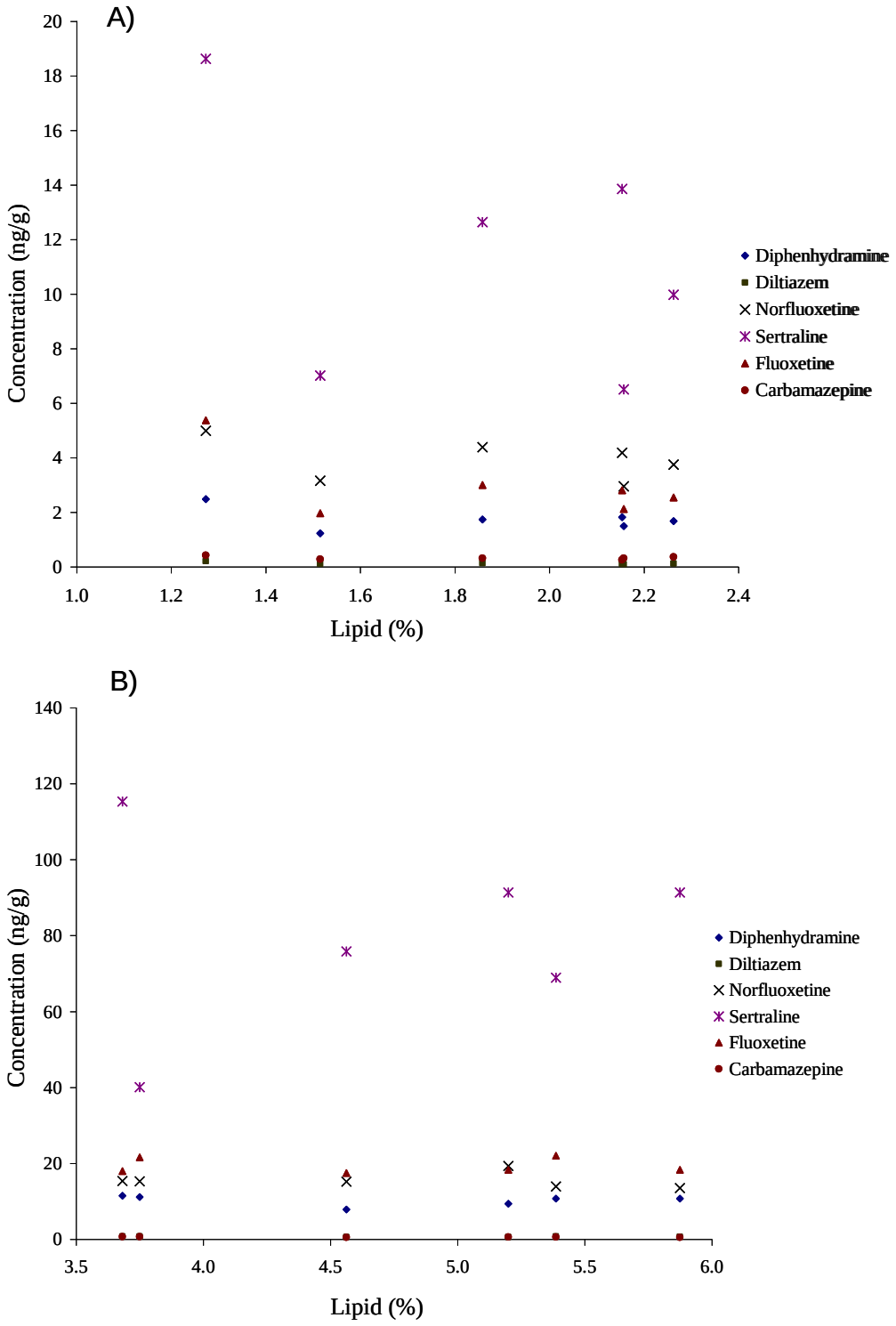


Figure A. 15. Plots of detected concentrations of pharmaceuticals versus lipid content for fish collected from PA; A) fillet and B) liver tissue

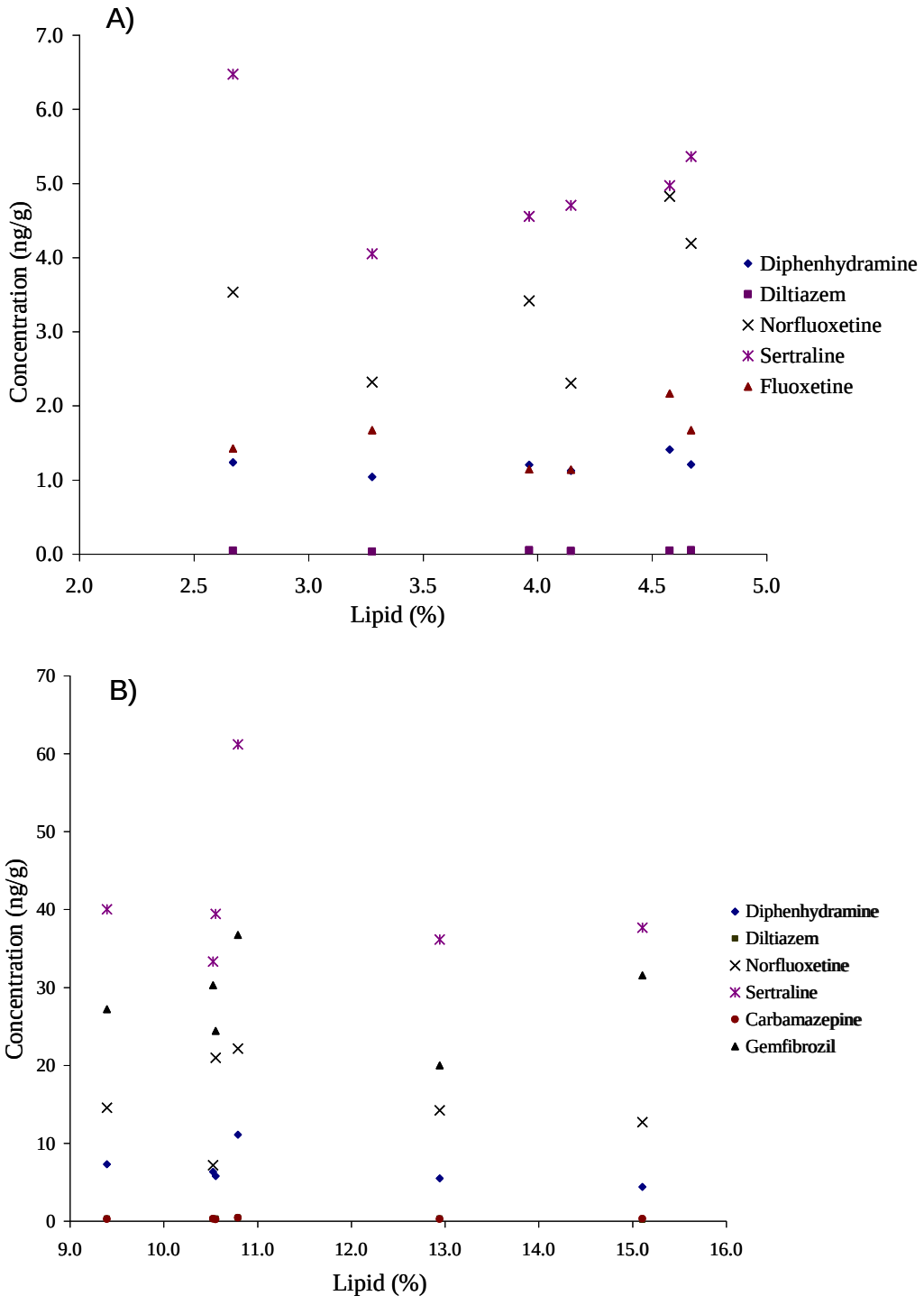


Figure A. 16. Plots of detected concentrations of pharmaceuticals versus lipid content for fish collected from AZ; A) fillet and B) liver tissue

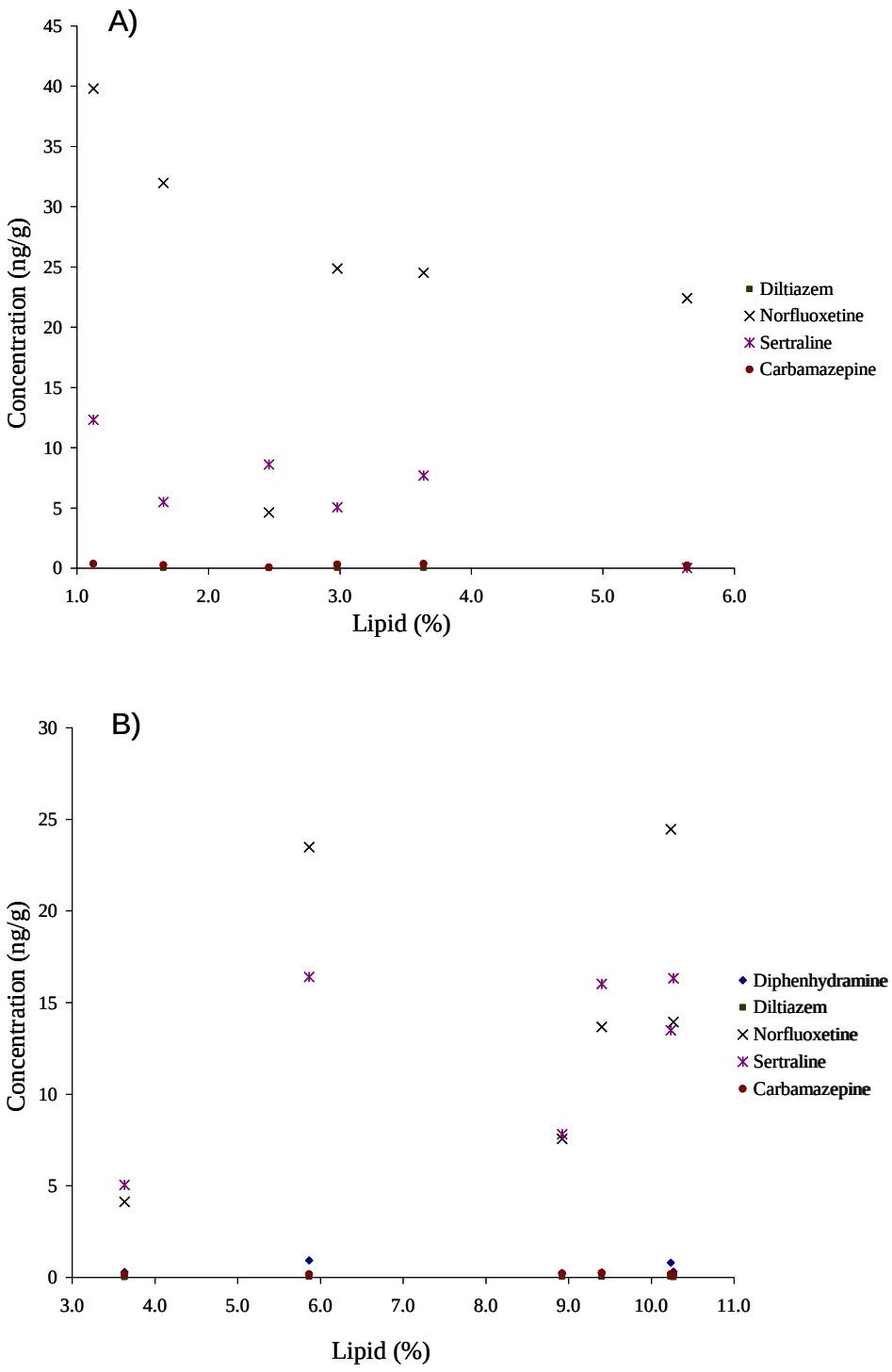


Figure A. 17. Plots of detected concentrations in liver of pharmaceuticals versus lipid content for fish collected from A) FL and B) TX

Table A.1. Fish fillet initial calibration data

Compound	RRF1[a]	RRF2	RRF3	RRF4	RRF5	RRF6	RRF7	$\overline{RRF}$ [b]	%[c] RSD
Acetaminophen	0.388	0.346	0.384	0.356	0.339	0.414	0.389	0.374	7.3
Atenolol	0.680	0.572	0.595	0.590	0.525	0.542	0.502	0.572	10.2
Cimetidine	1.952	1.729	1.770	1.591	1.498	1.593	1.480	1.659	10.2
Codeine	0.245	0.231	0.223	0.203	0.197	0.209	0.196	0.215	8.7
1,7Dimethylxanthine	0.663	0.696	0.702	0.679	0.642	0.693	0.666	0.677	3.2
Lincomycin	0.323	0.291	0.297	0.285	0.273	0.283	0.268	0.288	6.3
Trimethoprim	0.438	0.373	0.450	0.403	0.364	0.348	0.326	0.386	11.9
Thiabendazole	1.482	1.341	1.365	1.402	1.222	1.321	1.308	1.349	6.0
Caffeine	1.067	0.901	0.897	0.815	0.750	0.847	0.768	0.863	12.4
Sulfamethoxazole	0.555	0.574	0.639	0.621	0.610	0.637	0.619	0.608	5.2
Metoprolol	0.606	0.530	0.599	0.565	0.543	0.545	0.501	0.555	6.7
Propranolol	0.598	0.646	0.712	0.711	0.634	0.656	0.629	0.655	6.5
Diphenhydramine	17.111	16.688	15.225	15.101	13.681	15.899	14.955	15.523	7.4
Diltiazem	7.221	7.991	8.672	8.523	8.190	9.025	8.606	8.318	7.1
Carbamazepine	2.983	3.084	3.437	3.416	3.095	3.462	3.369	3.264	6.2
Tylosin	0.141	0.121	0.163	0.151	0.141	0.140	0.126	0.140	10.2
Fluoxetine	0.758	0.782	0.711	0.748	0.733	0.730	0.719	0.740	3.3
Norfluoxetine	0.922	0.685	0.577	0.722	0.644	0.571	0.661	0.683	17.4
Sertraline	0.695	0.621	0.591	0.733	0.638	0.615	0.725	0.660	8.6

Table A.1. Fish fillet initial calibration data (cont.)

Compound	RRF1[a]	RRF2	RRF3	RRF4	RRF5	RRF6	RRF7	$\overline{RRF}$ [b]	%[c] RSD
Erythromycin	0.054	0.057	0.053	0.044	0.045	0.051	0.050	0.051	9.0
Warfarin	1.515	1.705	1.848	1.884	1.687	1.735	1.798	1.739	7.1
Miconazole	na[d]	na	0.005	0.008	0.005	0.004	0.005	0.005	27.4
Ibuprofen	0.009	0.007	0.009	0.010	0.009	0.009	0.009	0.009	10.9
Gemfibrozil	4.807	3.674	3.749	4.724	4.274	3.638	3.329	4.028	14.3
Acetaminophend$_4$[e]	0.490	0.469	0.521	0.463	0.448			0.478	6.0
Diphenhydramined$_3$	12.800	12.547	13.263	12.595	12.462			12.734	2.5
Carbamazepined$_{10}$	3.480	3.445	3.886	3.672	3.393			3.575	5.7
Ibuprofen$^{13}C_3$	0.008	0.009	0.010	0.011	0.010			0.010	11.7

[a] RRF = (Area(sample)/(Amount(sample))/(Area(standard)/Amount(standard)); [b] average relative response factor for calibration standards spiked in reference fish; [c] relative standard deviation of response factor; [d] non-applicable, miconazole response was suppressed in the control matrix, therefore projected calibration range excluded concentrations that did not resolve from the baseline (i.e., calibration level one and two); [e] only five calibration levels were prepared for surrogates

Table A.2. Continuing calibration check for fillet in the Illinois batch

Compound	AvgRRF	RRF	%D	Max
Acetaminophen	0.374	0.366	2.1	25.0
Atenolol	0.572	0.560	2.2	25.0
Cimetidine	1.659	1.581	4.7	25.0
Codeine	0.215	0.202	6.1	25.0
1,7Dimethylxanthine	0.677	0.641	5.4	25.0
Lincomycin	0.288	0.277	3.9	25.0
Trimethoprim	0.386	0.382	0.9	25.0
Thiabendazole	1.349	1.260	6.6	25.0
Caffeine	0.863	0.809	6.3	25.0
Sulfamethoxazole	0.608	0.617	-1.4	25.0
Metoprolol	0.555	0.535	3.6	25.0
Propranolol	0.655	0.654	0.1	25.0
Diphenhydramine	15.523	15.781	-1.7	25.0
Diltiazem	8.318	8.440	-1.5	25.0
Carbamazepine	3.264	3.217	1.4	25.0
Tylosin	0.140	0.145	-3.4	25.0
Fluoxetine	0.740	0.728	1.6	25.0
Norfluoxetine	0.683	0.563	17.6	25.0
Sertraline	0.660	0.693	-5.0	25.0
Erythromycin	0.051	0.047	7.4	25.0

Table A.2. Continuing calibration check for fillet in the Illinois batch (cont.)

Compound	AvgRRF	RRF	%D	Max
Warfarin	1.739	1.863	-7.1	25.0
Miconazole	0.005	0.005	1.9	25.0
Ibuprofen	0.009	0.009	-5.6	25.0
Gemfibrozil	4.028	3.463	14.0	25.0
$Acetaminophend_4$	0.478	0.487	-1.9	25.0
$Diphenhydramined_3$	12.734	13.039	-2.4	25.0
$Carbamazepined_{10}$	3.575	3.583	-0.2	25.0
$Ibuprofen^{13}C_3$	0.010	0.011	-12.3	25.0

Table A.3. Fish liver initial calibration data

Compound	RRF1[a]	RRF2	RRF3	RRF4	RRF5	RRF6	RRF7	RRF8	R^2	$\overline{RRF}$ [b]	%RSD[c]
Acetaminophen		0.130	0.134	0.101	0.119	0.165	0.155	0.149	0.9978	0.136	16.3
Atenolol				0.562	0.476	0.595	0.457	0.436	0.9926	0.505	13.7
Cimetidine		1.040	1.473	1.066	1.045	1.345	1.054	1.078	0.9956	1.157	15.2
Codeine		0.175	0.180	0.182	0.194	0.219	0.216	0.208	0.9993	0.196	9.3
1,7Dimethylxanthine	0.523	0.373	0.489	0.400	0.347	0.526	0.392	0.396	0.9928	0.431	16.5
Lincomycin			0.346	0.391	0.395	0.549	0.451	0.439	0.9950	0.429	16.3
Trimethoprim	0.503	0.542	0.583	0.434	0.440	0.559	0.457	0.442	0.9958	0.495	12.1
Thiabendazole	1.247	1.375	1.524	1.347	1.274	1.659	1.383	1.351	0.9968	1.395	9.7
Caffeine	1.125	0.946	0.854	0.630	0.634	0.893	0.747	0.743	0.9968	0.821	20.4
Sulfamethoxazole	0.291	0.425	0.455	0.358	0.381	0.468	0.416	0.441	0.9986	0.404	14.5
Metoprolol	0.888	0.989	0.991	0.822	0.806	1.001	0.893	0.901	0.9989	0.911	8.3
Propranolol	0.678	1.220	0.996	0.890	0.909	1.095	0.950	0.867	0.9950	0.951	17.0

Table A.3. Fish liver initial calibration data (cont)

Compound	RRF1[a]	RRF2	RRF3	RRF4	RRF5	RRF6	RRF7	RRF8	R^2	$\overline{RRF}$ [b]	%RSD[c]
Diphenhydramine	23.385	19.758	20.403	20.113	18.718	22.296	21.256	18.839	0.9953	20.596	7.9
Diltiazem	13.206	15.281	15.929	13.980	14.148	17.288	16.086	14.816	0.9970	15.092	8.8
Carbamazepine	7.219	8.514	8.205	6.837	6.855	9.171	8.235	8.016	0.9981	7.881	10.6
Tylosin	0.424	0.486	0.471	0.401	0.332	0.585	0.532	0.482	0.9934	0.464	16.9
Fluoxetine		0.216	0.244	0.206	0.221	0.227	0.233	0.215	0.9983	0.223	5.7
Norfluoxetine			0.426	0.325	0.308	0.344	0.348	0.320	0.9979	0.345	12.3
Sertraline			0.437	0.318	0.297	0.399	0.390	0.363	0.9972	0.367	14.3
Warfarin		4.013	4.667	4.115	3.977	5.187	4.363	4.423	0.9976	4.392	9.8
Ibuprofen	0.016	0.013	0.014	0.011	0.013	0.016	0.016	0.015	0.9981	0.014	13.5
Gemfibrozil	0.695	0.794	0.640	0.679	0.663	0.750	0.739	0.694	0.9988	0.707	7.2
Acetaminophen-d_4	0.190	0.173	0.167	0.136	0.146				0.9886	0.162	13.2
Diphenhydramine-d_3	15.858	17.927	15.849	15.816	16.103				0.9980	16.311	5.6

Table A.3. Fish liver initial calibration data (cont)

Compound	RRF1[a]	RRF2	RRF3	RRF4	RRF5	RRF6	RRF7	RRF8	R^2	$\overline{RRF}$ [b]	%RSD[c]
Carbamazepine-d_{10}	8.236	8.383	8.157	7.290	7.185				0.9954	7.850	7.2
Ibuprofen-$^{13}C_3$	0.018	0.018	0.017	0.014	0.014				0.9869	0.016	11.6

[a] RRF = (Area(sample)/(Amount(sample))/(Area(standard)/Amount(standard)); [b] average relative response factor for calibration standards spiked in reference fish; [c] relative standard deviation of response factor; [d] non-applicable, miconazole response was suppressed in the control matrix, therefore projected calibration range excluded concentrations that did not resolve from the baseline (i.e., calibration level one and two); [e] only five calibration levels were prepared for surrogates

Table A.4. Illinois batch surrogate recoveries

	Surrogates				
Sample	S1	S2	S3	S4	total out
Blank	104	102	103	97	0
Control (PQL)	103	102	99	99	0
Control (LCS)	106	104	102	105	0
IL composite 1	94	104	101	136	0
IL composite 2	99	107	103	115	0
IL composite 3	97	104	99	135	0
IL composite 4	107	106	107	136	0
IL composite 4 (MS)	110	108	111	129	0
IL composite 4 (MSD)	103	112	105	131	0
IL composite 5	105	106	106	117	0
IL composite 6	106	117	104	111	0
Average	103	106	104	119	na
Std. Dev.	5	4	4	15	na

S1, acetaminophen-d_4; S2, diphenhydramine-d_3; S3, carbamazepine-d_{10}; S4, ibuprofen-$^{13}C_3$; quality control limits defined from 60 to 150%

Table A.5. Illinois batch LCS1 control sample

Compound	conc.	units	amount	accuracy	limits	status
Acetaminophen	14	ng/g	15	96 %	60 - 150	PASS
Atenolol	5	ng/g	5	104 %	60 - 150	PASS
Cimetidine	5	ng/g	5	104 %	60 - 150	PASS
Codeine	20	ng/g	20	101 %	60 - 150	PASS
1,7Dimethylxanthine	5	ng/g	5	102 %	60 - 150	PASS
Lincomycin	21	ng/g	20	104 %	60 - 150	PASS
Trimethoprim	9	ng/g	8	109 %	60 - 150	PASS
Thiabendazole	8	ng/g	8	101 %	60 - 150	PASS
Caffeine	15	ng/g	15	103 %	60 - 150	PASS
Sulfamethoxazole	8	ng/g	8	99 %	60 - 150	PASS
Metoprolol	8	ng/g	8	105 %	60 - 150	PASS
Propranolol	5.5	ng/g	5.0	109 %	60 - 150	PASS
Diphenhydramine	0.25	ng/g	0.25	101 %	60 - 150	PASS
Diltiazem	0.24	ng/g	0.25	97 %	60 - 150	PASS
Carbamazepine	1.9	ng/g	2.0	97 %	60 - 150	PASS
Tylosin	22	ng/g	20	108 %	60 - 150	PASS
Fluoxetine	15	ng/g	15	102 %	60 - 150	PASS
Norfluoxetine	8	ng/g	8	104 %	60 - 150	PASS
Sertraline	8	ng/g	8	104 %	60 - 150	PASS
Erythromycin	18	ng/g	20	88 %	60 - 150	PASS

Table A.5. Illinois batch LCS1 control sample (cont.)

Compound	conc.	units	amount	accuracy	limits	status
Warfarin	1.9	ng/g	2	98 %	60 - 150	PASS
Miconazole	26	ng/g	30	86 %	60 - 150	PASS
Ibuprofen	151	ng/g	150	101 %	60 - 150	PASS
Gemfibrozil	22	ng/g	20	109 %	60 - 150	PASS

Table A.6. Illinois batch LCS2 control sample

Compound	conc.	units	amount	accuracy	limits	status
Acetaminophen	70	ng/g	75	94 %	60 -150	PASS
Atenolol	27	ng/g	25	109 %	60 -150	PASS
Cimetidine	26	ng/g	25	105 %	60 -150	PASS
Codeine	101	ng/g	100	101 %	60 -150	PASS
1,7Dimethylxanthine	24	ng/g	25	96 %	60 -150	PASS
Lincomycin	102	ng/g	100	102 %	60 -150	PASS
Trimethoprim	46	ng/g	40	115 %	60 -150	PASS
Thiabendazole	39	ng/g	40	96 %	60 -150	PASS
Caffeine	78	ng/g	75	103 %	60 -150	PASS
Sulfamethoxazole	40	ng/g	40	99 %	60 -150	PASS
Metoprolol	42	ng/g	40	104 %	60 -150	PASS
Propranolol	25.8	ng/g	25.0	103 %	60 -150	PASS
Diphenhydramine	1.31	ng/g	1.25	105 %	60 -150	PASS
Diltiazem	1.22	ng/g	1.25	97 %	60 -150	PASS
Carbamazepine	9.5	ng/g	10.0	95 %	60 -150	PASS
Tylosin	112	ng/g	100	112 %	60 -150	PASS
Fluoxetine	76	ng/g	75	101 %	60 -150	PASS
Norfluoxetine	35	ng/g	40	87 %	60 -150	PASS
Sertraline	40	ng/g	40	99 %	60 -150	PASS
Erythromycin	93	ng/g	100	93 %	60 -150	PASS

Table A.6. Illinois batch LCS2 control sample (cont.)

Compound	conc.	units	amount	accuracy	limits	status
Warfarin	10.5	ng/g	10.0	105 %	60 -150	PASS
Miconazole	157	ng/g	150.	105 %	60 -150	PASS
Ibuprofen	743	ng/g	750	99 %	60 -150	PASS
Gemfibrozil	100	ng/g	100	100 %	60 -150	PASS

Table A.7. Concentrations of target analytes (ng/g tissue) in individual fillet and liver composites from New Mexico

	Composite							
	1	2	3	4	5	6		
Length range (cm)	39 – 40	40 – 44	40 – 44	40 – 44	36 – 40	33 – 41		
Weight range (kg)	0.83 – 0.89	1.1 – 1.3	1.0 – 1.2	0.86 – 1.0	0.67 – 0.75	0.65 – 0.88		
Lipid content, fillet / liver (%)	3.5 / 4.0	4.1 / 4.6	8.0/ 3.4	4.4 / 3.0	4.6 / 9.8	5.0 / 4.3		
Analytes	fillet / liver concentrations (ng/g)						average	%RSD
Diphenhydramine	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Diltiazem	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Carbamazepine	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Fluoxetine	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Norfluoxetine	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Sertraline	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Gemfibrozil	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na

Table A.8. Concentrations of target analytes (ng/g tissue) in individual fillet and liver composites from Illinois

	Composite							
	1	2	3	4	5	6		
Length range (cm)	27 – 29	30 – 35	32 – 35	31 – 36	34 – 36	34 – 36		
Weight range (kg)	0.38 – 0.46	0.48 - 0.76	0.72 – 0.87	0.48 – 0.82	0.75 – 0.99	0.76 – 0.83		
Lipid content, fillet / liver (%)	1.4 / 2.3	2.2 / 1.6	2.1 / 2.0	2.5 / 2.3	3.3 / 2.8	2.6 / 2.2		
Analytes	fillet / liver concentrations (ng/g)						average	%RSD
Diphenhydramine	1.12 / 5.20	1.15 / 4.54	1.24 / 7.46	1.68 / 9.13	1.33 / 5.91	1.74 / 9.59	1.38 / 6.97	19 / 30
Diltiazem	0.14 / 0.50	0.12 / 0.54	0.10 / 0.78	0.12 / 0.88	0.12 / 0.64	0.16 / 0.90	0.13 / 0.71	16 / 24
Carbamazepine	1.8 / 4.3	2.0 / 5.6	2.0 / 6.6	2.6 / 6.8	2.6 / 5.3	3.1 / 7.5	2.3 / 6.0	22 / 21
Fluoxetine	nd / nd	nd / nd	nd / 18	nd / 14	nd / nd	nd / 23	na / 19	na / 22
Norfluoxetine	3 / 41	2 / 21	3 / 128	3 / 81	3 / 38	3 / 129	2 / 73	16 / 65
Sertraline	3 / 41	2 / 42	2 / 96	3 / 148	1 / 34	3/ 141	2/ 84	27 / 62
Gemfibrozil	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na

Table A.9. Concentrations of target analytes (ng/g tissue) in individual fillet and liver composites from Arizona

	Composite							
	1	2	3	4	5	6		
Length range (cm)	47 – 49	38 – 42	35 – 41	40 – 42	37 – 46	52 – 55		
Weight range (kg)	1.6 – 1.9	1.1 – 1.2	0.81 – 0.96	0.92 – 1.13	1.4 – 2.0	1.9 – 2.6		
Lipid content, fillet / liver (%)	3.3 / 10	4.0 / 15	4.6 / 11	4.7 / 13	4.1 / 9.4	2.7 / 11		
Analytes	fillet / liver concentrations (ng/g)						average	%RSD
Diphenhydramine	1.04 / 6.32	1.20 / 4.38	1.41 / 5.79	1.21 / 5.48	1.12 / 7.28	1.24 / 11.1	1.20 / 6.72	10 / 35
Diltiazem	0.03 / 0.24	0.05 / 0.18	0.05 / 0.29	0.05 / 0.28	0.04 / 0.28	0.05 / 0.44	0.04 / 0.28	15 / 31
Carbamazepine	nd / 0.3	nd / 0.3	nd / 0.2	nd / 0.3	nd / 0.2	nd / 0.4	na / 0.3	na / 25
Fluoxetine	2 / nd	1 / nd	2 / nd	2 / nd	1 / nd	1 / nd	2 / na	25 / na
Norfluoxetine	2 / 14	3 / 25	5 / 41	4 / 28	2 / 29	4 / 44	3 / 30	29 / 36
Sertraline	4 / 57	5 / 65	5 / 68	5 / 62	5 / 69	7 / 105	5 / 71	17 / 24
Gemfibrozil	nd / 74	nd / 78	nd / 60	nd / 49	nd / 67	nd / 90	na / 70	na / 21

Table A.10. Concentrations of target analytes (ng/g tissue) in individual fillet and liver composites from Florida

	Composite							
	1	2	3	4	5	6		
Length range (cm)	52 – 59	44 – 49	38 – 41	38 – 40	34 – 38	37 – 42		
Weight range (kg)	1.3 – 2.2	0.84 – 1.1	0.62 – 0.66	0.56 – 0.63	0.38 – 0.52	0.48 – 0.89		
Lipid content, fillet / liver (%)	2.3 / 3.6	1.5 / 5.6	0.9 / 3.0	0.5 / 1.1	0.5 / 1.7	0.6 / 2.5		
Analytes	fillet / liver concentrations (ng/g)						average	%RSD
Diphenhydramine	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Diltiazem	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Carbamazepine	nd / 0.4	nd / 0.2	nd / 0.3	nd / 0.4	nd / 0.3	nd / nd	na / 0.3	na / 45
Fluoxetine	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na
Norfluoxetine	nd / 48	nd / 44	nd / 49	nd / 78	nd / 63	nd / 9	na / 49	na / 48
Sertraline	nd / 13	nd / nd	nd / 9	nd / 21	nd / 9	nd / 15	na / 13	na / 37
Gemfibrozil	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na

Table A.11. Concentrations of target analytes (ng/g tissue) in individual fillet and liver composites from Pennsylvania

	Composite							
	1	2	3	4	5	6		
Length range (cm)	31 – 34	29 – 32	34 – 36	29 – 33	28 – 33	30 – 32		
Weight range (kg)	0.39 – 0.43	0.33 – 0.42	0.52 – 0.59	0.33 – 0.40	0.31 – 0.46	0.32 – 0.41		
Lipid content, fillet / liver (%)	1.5 / 4.6	2.2 / 5.2	2.2 / 5.9	1.9 / 3.7	2.3 / 5.4	1.3 / 3.8		
Analytes	fillet / liver concentrations (ng/g)						average	%RSD
Diphenhydramine	1.23 / 7.86	1.49 / 9.40	1.82 / 10.7	1.74 / 11.4	1.67 / 10.73	2.48 / 11.2	1.74 / 10.21	24 / 13
Diltiazem	0.10 / 0.59	0.10 / 0.60	0.11 / 0.71	0.14 / 0.75	0.12 / 0.72	0.20 / 0.76	0.13 / 0.69	31 / 11
Carbamazepine	0.3 / 0.5	0.3 / 0.6	0.3 / 0.5	0.3 / 0.7	0.4 / 0.6	0.4 / 0.7	0.3 / 0.6	19 / 11
Fluoxetine	2 / 63	2 / 66	3 / 66	3 / 65	3 / 80	5 / 78	3 / 70	42 / 10
Norfluoxetine	3 / 38	3 / 48	4 / 33	4 / 38	4 / 34	5 / 38	4 / 38	20 / 10
Sertraline	7 / 359	7 / 432	14 / 432	13 / 545	10 / 326	19 / 190	11 / 380	40 / 32
Gemfibrozil	nd / na	nd / na	nd / 27	nd / na	nd / 27	nd / na	na / 27	na / 1.2

Table A.12. Concentrations of target analytes (ng/g tissue) in individual fillet and liver composites from Texas

	Composite							
	1	2	3	4	5	6		
Length range (cm)	56 – 60	50 – 54	48 – 49	48 – 56	43 – 45	45 – 47		
Weight range (kg)	3.5 – 4.6	2.3 – 2.8	2.0 – 2.3	2.4 – 2.9	1.4 – 1.7	1.6 – 1.8		
Lipid content, fillet / liver (%)	1.6 / 5.9	2.1 / 8.9	0.9 / 9.4	3.0 / 10	4.0 / 10	1.7 / 3.6		
Analytes	fillet / liver concentrations (ng/g)						average	%RSD
Diphenhydramine	nd / 0.93	nd / 0.23	nd / 0.26	nd / 0.80	nd / 0.31	nd / 0.28	na / 0.47	na / 66
Diltiazem	nd / 0.04	nd / 0.04	nd / 0.04	nd / 0.03	nd / 0.03	nd / 0.03	na / 0.03	na / 20
Carbamazepine	nd / 0.2	nd / 0.2	nd / 0.2	nd / 0.2	nd / 0.2	nd / 0.2	na / 0.2	na / 19
Fluoxetine	nd / 12	nd / nd	nd / nd	nd / 14	nd / nd	nd / nd	na / 13	na / 37
Norfluoxetine	nd / 46	2 / 15	nd / 27	nd / 48	nd / 27	1.3 / 8	2 / 29	0.3 / 56
Sertraline	nd / 28	nd / 13	nd / 28	nd / 23	nd / 28	nd / 9	na / 22	na / 39
Gemfibrozil	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	na / na	na / na

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