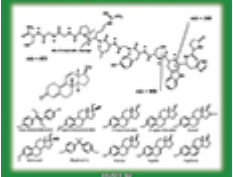
ACS SYMPOSIUM SERIES 1126

Evaluating Veterinary Pharmaceutical Behavior in the Environment



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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

Veterinary pharmaceutical use has expanded significantly in the last few decades due in part to the increased desire for animal protein in the human diet. Thus, there is a need to improve livestock productivity and health. Economically viable increases in animal protein production is made possible by maintenance of large assemblages of livestock in small areas, also known as concentrated animal feeding operations (CAFOs). Anabolic steroids promote muscle growth and allow increased protein production. Antibiotics and other pharmaceuticals prevent diseases in livestock housed within CAFOs. These examples illustrate a few reasons that veterinary pharmaceuticals have become a critical component of modern agricultural practice. The use of veterinary pharmaceuticals for pet health care and for management of wildlife species is also increasing. This book addresses veterinary pharmaceutical use in livestock and wildlife.

The distribution of concentrated feeding operations across the U.S.A. is extensive. Poultry operations are primarily located in the Southeast, while swine production facilities are mostly found in upper-Midwest, North Carolina, and Oklahoma. Beef cattle feedyards are predominantly located in the Central U.S. from Texas to Kansas. Aquaculture adds a new facet to the evaluation pharmaceutical use during food-animal production and is concentrated in states along the Gulf, mid-Atlantic, and West Coasts. Thus, many states in the U.S. contain either large numbers of CAFOs or expansive operations with large numbers of animals, terrestrial or aquatic.

The presence of veterinary pharmaceuticals in runoff from CAFOs has been known for many years. Off-site movement of these compounds has largely been attributed to CAFOs in areas of the U.S. that receive moderate to heavy rainfall. Control and mitigations strategies have been developed and implemented for many areas. Emerging information suggests that veterinary pharmaceuticals may also be transported via particulates or aerosols. Although the issue of odor from CAFOs has been evaluated for some time, aeolian transport of veterinary pharmaceuticals from CAFOs is a newly investigated topic.

In order to properly assess different modes of veterinary pharmaceutical transport, transformation, and resulting effects, studies must screen soil, water, sediment, air and particulate matter samples for veterinary pharmaceuticals. For these evaluations to be meaningful, they must use appropriately rigorous sampling and analysis methods. Many of these methods are recent developments or are still under development. Laboratory and field technique development and validation often require painstaking and lengthy evaluations to insure that high quality data are generated. As such, few integrated studies have been implemented to sample, analyze and evaluate effects of veterinary pharmaceuticals in the environment.

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Scientists, engineers, and public policy experts from academia, government and industry gathered in Denver Colorado at the 242nd National Meeting of the American Chemical Society (ACS) to present and to evaluate information regarding veterinary pharmaceuticals occurrence and behavior in the environment. This book includes contributions from several of the groups that presented information in that forum. The symposium was coordinated by the Divisions of Environmental Chemistry and Agricultural Chemistry. Speaker participation was facilitated by funds from the U.S. Department of Agriculture and an Innovation Award from the Divisional Activities Committee of the ACS.

Techniques presented in the Symposium, spanned a wide range of topics including regulatory processes, environmental sampling, chemical analysis, biochemical assays, biological effects, transport, transformation, and environmental fate.

One of the major foci of this symposium was the distribution and fate of antibiotics and steroidal growth promoters that are used in concentrated feeding operations. It is important that the fate and effects of these veterinary pharmaceuticals in the environment are understood more thoroughly. If environmental systems are not at risk then efforts can be focused on other areas of inquiry. As always, if any adverse effects are identified, risk benefit analyses will be needed to determine the best course of action for mitigating potential adverse effects. Many techniques presented in this text can be used as the basis for integrated assessments of environmental fate and effects of veterinary pharmaceuticals.

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Chapter 1

Mechanisms of Anabolic Steroid Action in Bovine Skeletal Muscle

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Both androgenic and estrogenic steroids significantly enhance feed efficiency, rate of gain, and muscle growth of feedlot cattle; and they have consequently been used as growth promoters in the beef cattle industry for nearly 60 years. This review summarizes research on the biological mechanisms of anabolic steroid-enhanced muscle growth in feedlot cattle. Emphasis is placed on the role of insulin-like growth factor-1 (IGF1), muscle satellite cells and specific growth factor and steroid receptors. Treatment of feedlot steers with a combined estradiol (E2) and trenbolone acetate (TBA) implant results in an increased number of muscle satellite cells, increased expression of IGF1 mRNA in muscle tissue and increased levels of circulating IGF1. Similarly, treatment of bovine satellite cell (BSC) cultures with either TBA or E2 results in increased expression of IGF1 mRNA, increased rates of proliferation and protein synthesis, and decreased rates of protein degradation. Effects of E2 on cultured BSC are mediated at least in part through the classical E2 receptor (estrogen receptor-α , ESR1), the IGF1 receptor (IGFR1) and the G-protein coupled estrogen receptor (GPER1), formerly known as G-protein coupled receptor 30 (GPR30). The effects of TBA appear to be primarily mediated through the androgen receptor. Despite their widespread use relatively little is known about the biological mechanism by which androgenic and estrogenic steroids enhance rate and efficiency of muscle growth in cattle and more research is necessary to delineate the mechanism of action of these widely used growth promoters.

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Introduction

Because both androgenic and estrogenic steroids significantly enhance feed efficiency, rate of gain, and muscle growth of feedlot cattle, these compounds have been widely used as growth promoters in the beef cattle industry for nearly 60 years. Yet, relative little is known about the biological mechanisms by which these compounds enhance rate and efficiency of muscle growth in cattle. Combined estrogen and androgen implants are more effective than either androgens or estrogens alone in stimulating muscle growth of steers (*1–3*); and, consequently, use of these combined implants has increased in recent years.

It is well established that E2 affects cellular functions by binding to estrogen receptor –α (ESR1) and –β (ESR2) (*4*). These receptors affect cells via classical genomic mechanisms involving binding of the receptor–ligand complex to specific response elements in the regulatory region of numerous genes (*4*). Additionally, the ER–ligand complex may activate various intracellular signaling pathways such as the Raf-1/MAPK kinase (MEK)1/2/ERK1/2 and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways via nongenomic mechanisms (*4*, *5*). A G-protein-coupled receptor (**GPR30**), more recently referred to as G protein-coupled estrogen receptor-1 (**GPER-1**) (*6*), that binds E2 has recently been identified and this receptor reportedly regulates some of the actions of E2 in some cell types (*7–9*). Androgens bind to the intracellular androgen receptor (AR) and affect cellular function via both genomic and nongenomic mechanism (*10–12*).

Despite the well documented effectiveness of anabolic steroid implants in increasing bovine muscle growth and the information on the mechanism of action in other species, the mechanisms responsible for the very significant and economically important anabolic effects of androgenic and estrogenic steroids on bovine muscle growth have not been extensively studied. Consequently, the purpose of this review is to focus on the specific mechanism(s) involved in estrogen and androgen-stimulated muscle growth in beef cattle.

Role of Satellite Cells in Muscle Growth

The number and size of the muscle fibers (cells) present in muscle tissue play a significant role in determining the rate and efficiency of muscle growth and feed conversion. Muscle fiber number in meat-producing animals is essentially fixed at birth and is determined prenatally by a complex interaction of proliferation and differentiation of embryonic myogenic cells. Consequently, postnatal muscle growth results from hypertrophy of existing muscle fibers. This fiber hypertrophy requires an increase in the number of myonuclei present in the fibers; however, the nuclei present in muscle fibers are unable to divide so the nuclei must come from outside the fiber. Specialized muscle stem cells known as satellite cells provide the nuclei needed to support postnatal muscle fiber hypertrophy (*13*, *14*) and are critically important in determining the rate and extent of muscle growth. Thus, proliferation and differentiation of satellite cells play crucial roles in determining

the rate and efficiency of muscle growth. Rate and efficiency of muscle growth are also significantly impacted by the balance between muscle protein synthesis and degradation rates. More rapid rates of synthesis coupled with decreased degradation rates result in increased rate and efficiency of muscle growth. Consequently, factors that affect satellite cell proliferation and differentiation, and/or muscle protein synthesis and degradation rates potentially have a major impact on rate and efficiency of muscle growth.

Effect of E2 and TBA on Satellite Cell Number in Bovine Muscle

The semi-membranous muscles of yearling steers implanted with a combined trenbolone acetate (**TBA**) and estradiol-17β (**E2**) implant contain a greater number of satellite cells than do the corresponding muscles of nonimplanted steers (*15*). Additionally, many studies in both humans and animals have shown that testosterone treatment increases muscle fiber diameter and the number of myonuclei present in muscle fibers in a dose-dependent manner (*16–18*). Subsequent studies showed that testosterone treatment caused a dose-dependent increase in the absolute number of satellite cells and the percentage of satellite cells relative to myofiber nuclei present in muscle (*18*). Given the significance of satellite cells in postnatal muscle growth described above, these results indicate that anabolic-steroid-induced muscle growth in humans, laboratory animals, and meat-producing animals may result at least partially from an anabolic steroid-induced increase in the number of muscle satellite cells available to fuse with existing muscle fibers.

Effect of Anabolic Steroids on Circulating and Muscle IGF1 Levels

Insulin-like growth factor-1(IGF1) and insulin-like growth factor -2 (IGF2) significantly affect muscle growth. IGF1 or IGF2 receptor deficient mice die shortly after birth because their muscles are not sufficiently developed to inflate their lungs (*19*, *20*). In contrast, mice over expressing IGF1 in skeletal muscle have larger muscle fibers and increased muscle strength (*21–23*). Additionally, IGF1 stimulates rates of proliferation, differentiation and protein synthesis and decreases rate of protein degradation in cultured bovine satellite cells. In few of the positive effects of IGF1 on muscle growth and satellite cell proliferation and differentiation, it may be significant that circulating IGF1 levels are increased approximately 2 fold in steers and sheep treated with a combined TBA and E_2 implant (*3*, *24–26*). However, because studies in which hepatic IGF1 production has been knocked out, resulting in a 75% reduction in circulating IGF1 level, suggest that hepatic sources of circulating IGF1 may not play a major role in muscle growth (*27*, *28*), local production of IGF1 in skeletal muscle is currently thought to play a predominant role in supporting normal muscle growth through autocrine and(or) paracrine mechanisms (*27*). In yearling steers, treatment with

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a combined TBA and E_2 implant has been shown to increase IGF1 mRNA levels in longissimus muscle (*24–26*, *29*). Evaluation of the time course of changes in muscle IGF1 mRNA levels relative to the time of TBA and E_2 implantation showed that muscle IGF1 mRNA levels were increased by 7 d post-implantation and continued to increase to 3 times pre-implant levels 28 d after implantation (*26*). In contrast, trenbolone acetate and E_2 implantation did not affect myostatin, IGFBP-3 or hepatocyte growth factor mRNA levels in longissimus muscles (*26*). In vitro, treatment of cultured bovine satellite cells with either E2 or TBA significantly increases IGF1 mRNA expression (*30*). Based on these studies it appears that anabolic steroid treatment increases muscle IGF1 levels and it is likely that this may be at least partially responsible for the increased number of satellite cells, increased myofiber nuclei, increased hypertrophy and increased muscle growth observed in anabolic steroid treated animals and humans.

In addition to the effects of combined E2/TBA implants on muscle IGF1 expression in steers, other studies have shown that treatment with E2 alone also increases the IGF1 mRNA level in the longissimus muscle of steers (*31*). Studies evaluating the mechanism by which E2 stimulates expression of IGF1 mRNA have shown that, even though the IGF1 gene does not contain a traditional estrogen response element (**ERE**) in its regulatory region, E2 stimulation of IGF1 mRNA expression can occur via a pathway involving the interaction of the ESR1/E2 complex with the AP-1 enhancer (*32*). In non-muscle tissues studied to date, E2 stimulation of IGF1 mRNA expression via this mechanism is abrogated by treatment ICI 182 780 (ICI) (an E2 antagonist the interferes with binding of E2 to ESR1 and ESR2) (*33–36*). In contrast, our data show that ICI does not suppress E2-stimulated IGF1 mRNA expression in BSC cultures, suggesting that E2 stimulation of IGF1 mRNA expression in bovine muscle may occur via receptors and/or mechanisms that differ from those in other tissues studied to date.

In addition to the classical estrogen receptors, G-protein-coupled receptor 30 (**GPR30**) (*9*), more recently referred to as G protein-coupled estrogen receptor-1 (**GPER-1**) (*6*) reportedly plays a role in mediating the actions of estrogen (*8*). Muscle tissue contains GPER-1 mRNA (*37–39*) and immunohistochemical studies have localized GPR30 receptor protein in skeletal muscle cells (*40*). We have shown that the specific GPER-1 agonist, G1 (*41*), stimulates IGF1 mRNA expression by cultured BSC (*42*), strongly indicating that GPER-1 plays a role in E2-stimulated IGF1 expression in these cells. Surprisingly, proliferation of cultured BSC is not stimulated by G1, and E2-stimulated proliferation is suppressed by ICI (*43*), suggesting that the effect of E2 on proliferation of cultured BSC occurs via different mechanisms than does stimulation of IGF1 mRNA expression.

IGF1 mRNA levels also are significantly increased in skeletal muscle of humans treated with testosterone (*44*, *45*) and in castrated rats treated with either testosterone or 5α-dihydrotestosterone (DHT) (*46*). Similarly, comparison of IGF1 mRNA levels in the splenius muscle of castrated and intact twin lambs showed higher levels of IGF1 mRNA in the muscle of intact sheep (*47*). Trenbolone acetate (a testosterone analog that is not subject to aromatization) also increases IGF1 mRNA expression in cultured BSC (*30*) and this increase is suppressed by flutamide, an inhibitor of the AR.

Effect of TBA and E2 on Proliferation of Cultured BSC

In bovine satellite cells (**BSC**) cultured in the presence of 10% fetal bovine serum (**FBS**), E2 treatment increases IGF1 mRNA level (*42*, *43*). However, under culture conditions in which E2 treatment does not increase expression of IGF1, IGF2 or IGF1 receptor (IGFR1), E2 treatment still stimulates proliferation in cultured BSC (*43*). These data indicate that, in addition to stimulating BSC proliferation by increasing IGF1 expression via interaction with the GPER-1 receptor, E2 may stimulate rate of proliferation through interaction with other receptors such as estrogen receptor –α (ESR1) (*42*, *43*). This possibility is further supported by the observation that ICI 182 780 (an estrogen receptor blocker) suppresses E2-stimulated BSC proliferation while actually stimulating IGF1 mRNA expression in cultured BSC (*43*). Additionally, E2-stimulated proliferation is completely abolished in BSC cultures in which ESR1 expression has been silenced by treatment with ESR1 specific siRNA (*48*). These data strongly suggest that ESR1 is required in order for E2 to stimulate proliferation of cultured BSC. In addition, the fact that treatment with ESR1 siRNA completely suppresses the ability of E2 to stimulate proliferation suggests that ESR2 (estrogen receptor-β) is not involved in E2 stimulated BSC proliferation. E2-stimulated proliferation of BSC also is suppressed by silencing expression of IGFR1 (*48*), suggesting that this receptor is required in order for E2 to stimulate proliferation of cultured BSC. Studies in cultured human breast cancer cell lines indicating that binding of the E2-ESR1 complex to the intracellular β subunit of IGFR1 may stimulate proliferation by activating the IGFR1 tyrosine kinase (*49*, *50*) provide a possible explanation for these observations. Additional studies are required to determine if a similar mechanism functions in bovine satellite cells.

TBA also stimulates proliferation of cultured BSC and this stimulation is completely suppressed by treatment with flutamide. Recently, an androgen response element (**ARE**) has been identified in the promoter region of the IGF1 gene (*51*), suggesting that androgen receptor – ligand complex may interact with this ARE to stimulate transcription of the IGF1 gene.

Both the Raf-1/MAPK kinase (MEK)1/2/ERK1/2, and the phosphatidylinositol 3-kinase/Akt pathways play significant roles in proliferation and differentiation of myogenic cells. The MEK1 inhibitor, PD98059, suppresses both E2- and TBA-stimulated proliferation of cultured BSC (*43*). Similarly wortmannin, an inhibitor of the PI3K/AKT pathway, suppresses both E2- and TBA-stimulated proliferation of BSC (*43*). Incubation of control cultures with either PD98059 or wortmannin did not decrease proliferation rate establishing that the suppression of both E2- and TBA-stimulated proliferation is not simply a general suppression of proliferation (*43*). These results indicate that both the Raf-1/MAPK kinase (MEK)1/2/ERK1/2 and the phosphatidylinositol 3-kinase/Akt pathways play a necessary role in E2- and TBA-stimulated BSC proliferation. Since IGF1 activates these pathways, this result is consistent with the potential role of IGF1 in E2- and TBA-stimulated proliferation in BSC cultures. However, it should be noted that the ESR1–ligand complex is reportedly able to phosphorylate and activate IGFR1 (*52*). Additionally,

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both E2 and testosterone have been shown to effect the Raf-1/MAPK kinase (MEK)1/2/ERK1/2 and the phosphatidylinositol 3-kinase/Akt pathways in numerous cell types (*53*, *54*). Therefore, at least some of the effects of PD98059 and wortmannin on E2- or TBA-stimulated proliferation in BSC cultures may reflect activation of Raf-1/MAPK kinase (MEK)1/2/ERK1/2 and the phosphatidylinositol 3-kinase/Akt pathways, respectively, by E2 or TBA independent of IGF1. Consequently, while it is significant that E2- and TBA-stimulated proliferation in BSC cultures is suppressed by inhibitors of these pathways, it is not possible to unequivocally establish a role of IGF1 in steroid-enhanced proliferation based on these data. However, the ability of both E2 and TBA to stimulated proliferation of cultured BSC is consistent with the increased satellite cell number observed in steers receiving a combined TBA/E2 implant and supports the hypothesis that increased satellite cell proliferation is at least partially responsible for the enhanced muscle growth seen in steers receiving steroid implants.

Effect of E2 on Protein Synthesis and Degradation in Cultured BSC

In addition to stimulating satellite cell proliferation, it seems likely that the muscle growth enhancing effects of E2 might include alterations in the rate of muscle protein synthesis and protein degradation. While in vivo studies have indicated that E2 affects protein synthesis and/or protein degradation rate in muscle, results from in vitro studies have been inconsistent and have often not shown effects of E2 treatment on these parameters (*55*–*58*). Treatment of fused bovine satellite cell (BSC) cultures for 6 h with E2 causes a concentration dependent increase in protein synthesis rate between 0.01 and 10 nM E2 ($p < 0.05$) with 10 nM E2 causing a 1.7-fold increase in synthesis rate ($p < 0.05$) compared to cultures receiving no E2 treatment (*59*). Treatment of fused BSC cultures with concentrations of E2 between 0.01 and 10 nM for 24 h results in a concentration dependent decrease in protein degradation rate ($p < 0.05$) (*59*). Protein degradation rate in cultures treated for 24 h with 10 nM E2 was approximately 72% of that in cultures incubated in same medium without added E2 (*59*). These data establish that E2 directly affects both protein synthesis and protein degradation rate in fused BSC cultures.

ICI 182 780 (a pure estrogen receptor agonist which suppresses E2's ability to bind to the classical E2 receptors, ESR1 and ESR2) suppresses the ability of E2 to stimulate protein synthesis and decrease protein degradation rates in fused BSC cultures, indicating that binding of E2 to ESR1 and/or ESR2 is necessary in order for E2 to affect protein synthesis and degradation rates in these cultures. In addition to the classical estrogen receptors (ESR1 and ESR2), as indicated above, recent reports have indicated that G protein coupled receptor 30 (GRP30) (*9*) (GPER-1), may play a role in mediating the actions of estrogen (*8*). However, G1 had no effect on rate of protein synthesis or protein degradation in fused BSC cultures (*59*), strongly suggesting that the GPER-1 receptor is not involved in the effects of E2 on either protein synthesis or degradation in fused BSC cultures.

Effect of TBA on Protein Synthesis and Degradation in Cultured BSC

Treatment of fused BSC cultures for 6 h with TBA causes a concentration-dependent increase in protein synthesis rate between 0.01 and 10 nM TBA with 10 nM TBA causing a 1.7-fold increase in synthesis rate compared with cultures receiving no TBA treatment (*60*), while similar treatment results in a 70% reduction in protein degradation rate (*60*). Flutamide, an inhibitor of androgen binding to the andogen receptor) suppresses the ability of TBA to stimulate rate of protein synthesis and to inhibit rate of protein degradation in BSC cultures (*60*) indicating that binding of TBA to the androgen receptor is necessary for TBA to affect protein synthesis and protein degradation rates in these cultures.

Summary

It is well established that anabolic steroids provide a significant economic benefit by enhancing both rate and efficiency of muscle growth in cattle; and, thus, these compound are widely used as growth promoters in the beef cattle industry. However, the mechanisms by which anabolic steroids enhance bovine muscle growth remain unclear. Questions remain as to the relative importance of genomic versus non-genomic effects of steroids on muscle growth. The relative importance of increased satellite cell numbers, increased circulating IGF1 and increased production of IGF1 in muscle tissue also remains unclear. Additionally, the involvement of specific growth factor receptors and of various intracellular signaling pathways and of cross-talk between various receptors and pathways in anabolic steroid-enhanced muscle growth needs to be delineated. In summary, it is clear that anabolic steroid-enhanced bovine muscle growth involves a complex interaction of numerous pathways and receptors. Continued research is necessary to understand mechanism involved in this complex process. In the future, the fundamental information generated by this research will help in developing safe and effective strategies to increase rate and efficiency of muscle growth in beef cattle.

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Chapter 2

Techniques for Characterization of Particulate Matter Emitted from Animal Feeding Operations

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In agricultural air quality studies, characterization of particulate matter (PM) emitted from animal feeding operations (AFOs) are of growing interest. Due to a lack of reference methods for AFO PM studies, significant variations in PM measurements exist among studies. Moreover, due to inherent limitations, different PM measurement techniques may give different results when used to sample PM in the same environment. A given technique may also demonstrate different magnitudes of sampling errors when exposed to different environmental conditions (e.g., humidity, etc.) or to PM with different characteristics (e.g. particle size, volatility, etc.). This chapter examines the scientific principles of various PM measurement techniques such that strengths and limitations of the techniques are revealed. Knowledge about causative factors associated with the limitations will lead to the appropriate selection of specific techniques to achieve the research goals for different sampling scenarios.

Introduction

As the world's demand for meat, milk, and egg production increases, animal feeding operations (AFOs) have become highly industrialized. While AFOs contribute significantly to the food supply, emissions of particulate matter (PM) and gaseous pollutants from AFO facilities pose adverse impacts on local environments and human health (*1*–*4*). In recent years, air emissions from

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AFOs have become a serious political and environmental problem (*5*, *6*). In the United States (U.S.), increasingly stringent local, state, and federal air pollution regulations are likely to occur (*5*). The sustainability and growth of the AFO will likely depend on addressing health and environmental concerns.

Under the 1970 Clean Air Act Amendments, the U.S. Environmental Protection Agency (EPA) established the National Ambient Air Quality Standards (NAAQS) to protect public health and the environment. For PM regulation, total suspended particulate (TSP) was first introduced into the NAAQS in 1971 (*7*). Since then, the U.S. EPA revised the NAAQS-PM standard by replacing TSP with PM_{10} in 1987 and by adding a new PM indicator, $PM_{2.5}$, in 1997 (*7*). PM_{10} and $PM_{2.5}$ were defined as PM with aerodynamic equivalent diameter (AED) smaller than, or equal to, 10μm (≤ 10μm) and 2.5μm (≤ 2.5μm), respectively. The current NAAQS for PM_{10} and $PM_{2.5}$ were revised in 2006, in which the PM_{10} 24-hr average concentration is limited to 150 μg/m^3 and the $PM_{2.5}$ 24-hr and annual average concentrations are limited to 35 μg/m^3 and 15 μg/m^3, respectively (*7*, *8*). In implementation of the NAAQS, a set of federal reference method (FRM) and federal equivalent method (FEM) PM samplers and monitors were developed and established for ambient air quality monitoring (*9–14*).

As a criteria pollutant, PM has been a research topic for decades in numerous AFOs air quality studies. One highlight of these studies is the National Air Emission Monitoring Study (NAEMS), under which baseline emissions of PM (i.e., TSP, PM_{10} and $PM_{2.5}$), ammonia, hydrogen sulfide, and carbon dioxide were determined based on continuous measurements of the pollutant concentrations and the AFO house ventilation airflow rates for two years from 2007 to 2009 (*15–19*). The main purpose of the study was to provide the U.S. EPA baseline emissions of the target pollutants for determination of new regulations under the EPA's Air Quality Compliance Agreement for AFOs (*6*). Specifically, for PM emission determination in NAEMS, concentrations of PM in the exhaust air and the inlet air of the monitored houses were simultaneously measured with tapered element oscillating microbalances (TEOMs) for house exhaust PM and Beta attenuation PM monitors (Beta Gauge) for house inlet PM. Due to lack of existing standardized methods for AFO PM emission measurements, selection of measurement methods and selection of the PM monitors for the NAEMS was overseen and approved by the U.S. EPA (*16*, *20*). The main reason for using TEOM and Beta Gauge was the fact that these two types of ambient PM monitors could provide continuous and semi-continuous PM concentration measurements with sufficient temporal resolution to better understand the sources and causes of elevated PM concentrations.

Among different AFO PM studies, various measurement techniques have been used for in-house concentration measurements, emission determination, and ambient upwind-downwind measurements to quantify fate and transport of emissions (*21–42*). Due to a lack of reference methods for measurements of PM in different sampling scenarios, significant variations in PM emission rate and characteristics have been observed among different studies (*21–32*). In most cases, ambient PM monitors were used to measure PM concentrations in confined animal buildings, where the PM concentrations were high, particle sizes were large, and the environments were harsh with high levels of gas pollutants and

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humidity (Figure 1). Many of the ambient PM monitors were not intended for use in such environments. It has been reported that there is an inherent sampling error associated with the FRM PM_{10} and $PM_{2.5}$ samplers due to the interaction of the samplers' design performance characteristics and the source-specific particle size distribution (PSD) of the PM being sampled by the samplers (*43–46*). In addition, particle-bound water and semi-volatile components of PM are also causes of PM measurement bias in AFO environments (*40*). The magnitude of the bias is dictated by the analytical methods for PM mass measurements as well as by the environmental conditions (e.g. temperature, relative humidity (RH), etc.) (*40*). As a result, different PM measurement techniques (samplers or monitors) may give different results when sampling in the same AFO environment. A given technique may also demonstrate different magnitudes of sampling errors when exposed to different environmental conditions or to PM with different characteristics (e.g. PSDs, volatility, etc.). Knowledge about the characteristics of the PM to be sampled and the environmental conditions is essential for accurate and consistent mass concentration measurements. These PM characteristics include (1) PM physical properties (i.e. mass concentration, PSD, particle density, and particle shape), and (2) chemical speciation (i.e. ion species, organic carbon / element carbon, and elemental compositions). This chapter aims to provide fundamental knowledge about strengths and limitations of various measurement techniques that have been used or may be used to determine physical and chemical properties of AFO PM. Through examination of the scientific principles in the design of various techniques, factors affecting measurement results under different AFO environmental conditions are revealed.

Physical Characterization of AFO PM

Studies of health impacts, emission estimation, fate and transport, and new control technologies require knowledge of characteristics of the PM in question. These characteristics include physical properties and chemical compositions. Characterization of physical properties of PM includes assessment of PM mass or number concentrations, analyzing PSD, determining particle densities, and examining particle shapes.

Mass Concentration Measurements

In AFO air quality studies, measurements of PM concentrations have been primarily focused on TSP, PM_{10} and $PM_{2.5}$. Although the FRM and FEM PM samplers were designed for ambient PM monitoring, they have also been used in AFO PM emission studies to measure PM concentrations both inside and outside AFO facilities. Other techniques used for AFO PM concentration measurements include cascade impactors, filtration, and optical particle counters.

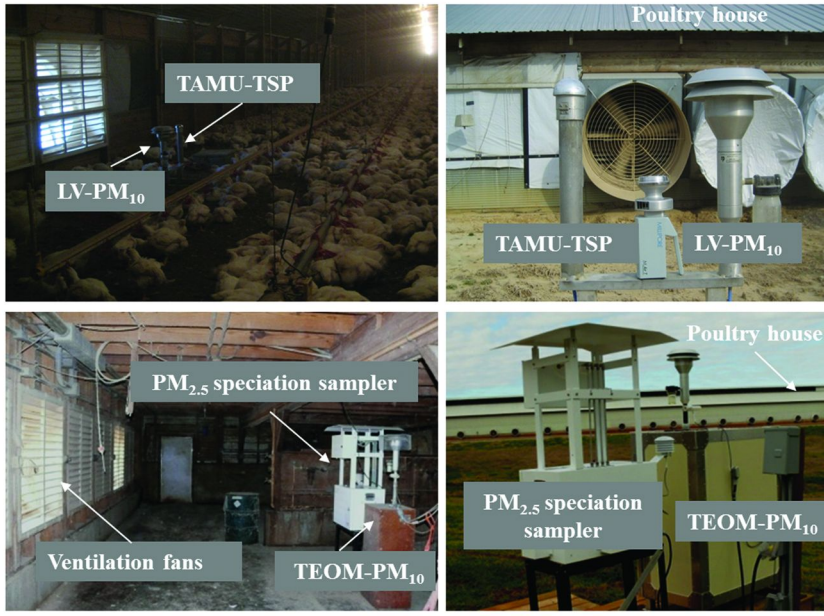

Figure 1. Some applications of ambient PM samplers in AFO PM studies (16, 17, 24, 34).

Total Suspended Particulate Samplers

Federal Reference Method TSP Sampler

The FRM TSP sampler is one type of high-volume PM sampler. It was designed to collect particles smaller than or equal to 100 μm in AED. As defined in the 40 CFR Part 50 Appendix B, the TSP sampler *"draws a measured quantity of ambient air into a covered housing and through a filter during a 24-hr sampling period"* (*13*). The sampler flowrate and the geometry dictate the sampler's particle collection efficiency.

As shown in Figure 2, the FRM TSP sampler has a roof mounted on the sampling housing wall to form an air inlet gap. The opening area of the inlet gap should be sized to provide a particle capture air velocity in the range of 20–35 cm/s at an operational flowrate in the range of 1.1–1.7 m^3/min (*13*). These capture velocities provide the nominal cutpoint of the sampler at 25-50 μm AED.

The collection filter media should be glass fiber or other relatively inert nonhygroscopic materials with dimensions of 20.3 ± 0.2 cm x 25.4 ± 0.2 cm. The filter should be conditioned before and after sampling for a minimum of 24

hours in controlled conditions at 15°C–30°C with less than ±3°C variation and less than 50% RH with less than ±5% variation. The analytical balance should have a sensitivity of 0.1mg (*13*).

In AFO air quality studies, the FRM TSP sampler has been used to measure PM concentrations inside AFO buildings and at ambient location in the vicinity of the AFO facilities (*33*, *34*). While the high-volume sampler was able to provide sufficient PM loading on the filter in a short sampling period for the follow-up analysis, it was difficult to maintain a constant flowrate over the sampling duration, especially in high PM concentration environments (e.g., AFO houses) (*41*). As the PM mass quickly deposited on the filter through high-volume sampling air flow, the pressure drop across the filter increased rapidly, consequently causing significant reduction of the sampling air flow. Thus, special care in maintaining constant air flow needs to be taken when this sampler is used to measure PM concentration in AFO facilities where the PM concentration is quite high.

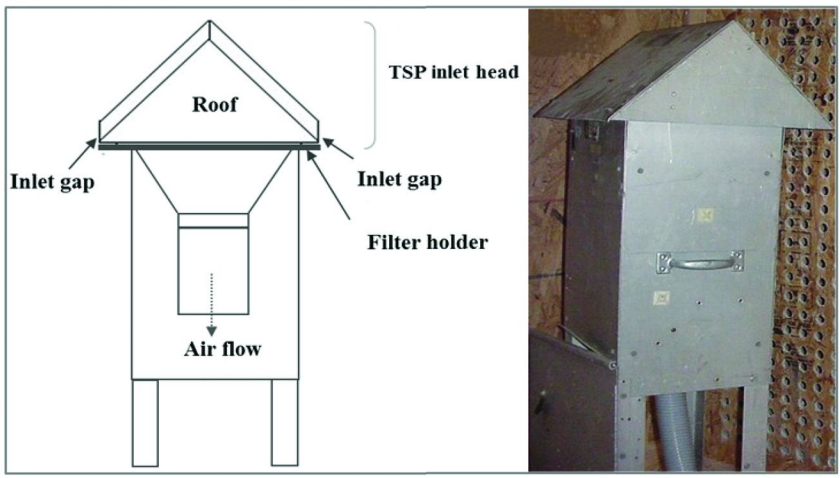

Figure 2. FRM high-volume TSP sampler in shelter.

The TAMU Low-Volume TSP Sampler

The measurement and control of sampling air flowrate are critical to the accuracy of PM sampling. While it is difficult to maintain constant air flow for high-volume TSP sampler, low-volume PM samplers have less of such problem. It has become more common to use low-volume samplers for PM measurements. The Texas A&M University (TAMU) low-volume TSP sampler is one type of low-volume sampler designed by the Center for Agricultural Air Quality Engineering and Science (CAAQES) at TAMU to measure TSP in high concentration environments. This sampler was originally designed under

the guidelines for FRM TSP sampler in 40 CFR Part 50 Appendix B (*13*) with operational flowrate of 16.7 L/min. Evaluation of the TAMU sampler suggested that there was no significant difference in performance between the FRM TSP sampler (high-volume) and the TAMU TSP sampler (low-volume) (*41*). In addition, the TAMU TSP sampler provided more robust and more accurate flowrate control, allowing for operation in high PM concentration for longer periods of time. Figure 3 shows the TAMU TSP sampler head (left), the flow system diagram (middle), and an application of this sampler in a poultry house PM sampling (right).

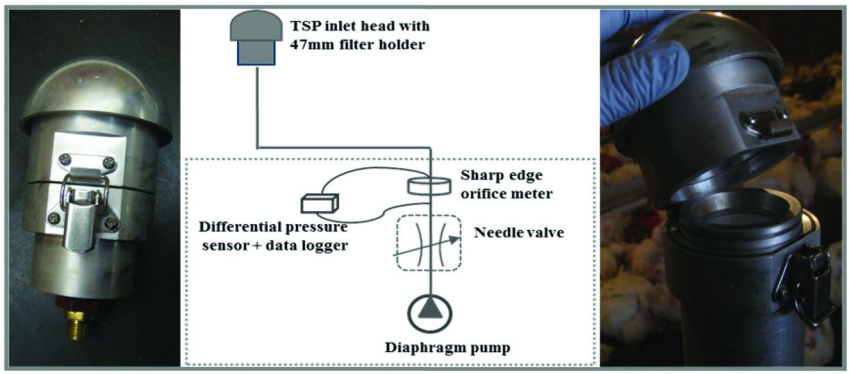

Figure 3. TAMU low-volume TSP sampler (33, 34, 41).

The UIUC Isokinetic TSP Sampler

Estimation of PM emissions from animal housing systems requires measurements of PM concentrations near the exhaust ventilation fans. The air flow profile near the exhaust fans often changes dynamically in speed and direction due to changes in ventilation stage settings under different housing and weather conditions. Under hot weather conditions, more ventilation fans operate to remove excessive heat in the house, thus causing higher air flow velocity near the ventilation exhaust. The design performance of the FRM TSP sampler was defined under wind speeds of 1.3-4.5 m/s (*13*). When the house ventilation air speed exceeds these FRM TSP sampler wind speed limits, the performance of the TSP sampler may be compromised. In this case, an isokinetic sampling approach may be considered to take representative PM samples through the exhaust stream.

The UIUC isokinetic TSP sampler was designed by researchers at University of Illinois at Urbana-Champaign (UIUC) to take PM samples in confined animal house systems where high ventilation air flowrate exist (*38*, *42*, *47*). As shown in Figure 4, the UIUC TSP sampler consists of an array of isokinetic TSP sampling heads along with an array of critical venturis to control air flow through the heads. The critical venturi ensures consistent sampling flowrate as long the pressure drop

across the sampling head is maintained above the critical pressure. The whole sampling system was designed to have a capacity of up to 50 sampling heads to conduct simultaneous measurements at 50 various spatial locations (*38*, *42*, *47*).

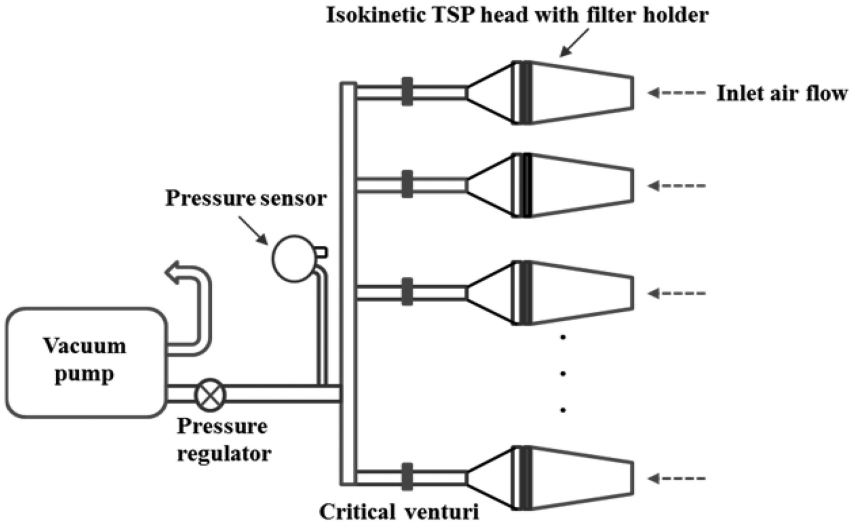

Figure 4. UIUC isokinetic TSP sampler (38, 42, 47).

Federal Reference Methods and Federal Equivalent Methods for PM_{10}, $PM_{2.5}$, and $PM_{10-2.5}$ Monitoring

Federal Reference Method PM_{10} Samplers

The goal of a PM_{10} sampler is to accurately measure the concentration of suspended particles smaller than or equal to 10μm (≤ 10μm) in the atmosphere. However, due to lack of a reference standard for particles suspended in the atmosphere, no calibration standards for suspended PM_{10} particle mass exist. Currently, the EPA defines accuracy for PM measurements in terms of the agreement between a candidate sampler and a reference sampler under standardized conditions for sample collection, storage, and analysis (*9*, *11*).

The FRM for PM_{10} include low-volume and high-volume sampling designs (*14*). As shown in Figures 6-7, the FRM PM_{10} sampler heads use the inertial separation principle to collect large particles while allowing smaller particles to penetrate the impaction zone (Figures 5-6) or the cyclonic separation zone (Figure 7) to the collection filter. The low-volume PM_{10} head has a single impaction channel and operates at a flowrate of 16.7 L/min, whereas the high-volume PM_{10} heads have either a multi-channel PM_{10} pre-separator or a cyclonic pre-separator (*48*). The high-volume PM_{10} samplers typically operate at a flowrate of 1.13-1.70

m^3/min. Filters used for PM_{10} sampling should be conditioned before and after sampling for a minimum of 24 hours in controlled conditions at 15°C–30°C with less than ± 3°C variation and 20- 45% RH with less than ±5% variation. The analytical balance for high-volume sampler should have a sensitivity of 0.1mg (*11*).

According to 40 CFR Part 53 (*9*), the FRM PM_{10} samplers have a cutpoint of 10 ± 0.5 μm AED, at which size the sampler has a 50% collection efficiency. Although the slope of the sampler's collection efficiency curve is not explicitly stated, the required collection efficiencies over different size ranges are defined in the 40 CFR Part 53 so that the collection and penetration efficiency curves may be developed accordingly (*9*).

Ideally, the PM_{10} samplers should produce accurate measurements of PM less than or equal to 10μm (≤10μm). However, the samplers are not perfect, and errors exist. These errors are caused by the established tolerances for sampler performance characteristics as well as the interaction between the sampler performance characteristics and the PSD of the PM being sampled (*43*). Buser et al. (*44*, *45*) reported that if the mass median diameter (MMD) of the PM to which the sampler is exposed to is smaller than 10μm, then under-sampling occurs in PM_{10} measurements by the sampler, whereas if the MMD of the PM is greater than 10μm, over-sampling occurs. AFO PM typically has PSDs with MMDs in the range of 15-25μm and geometric standard deviations (GSDs) in the range of 1.5-2.5. When a FRM PM_{10} sampler is exposed to an AFO PM source having a MMD of 20μm, it could overestimate PM_{10} by as much as 181-343% (*45*). These sampling errors compromise equal regulation among differing industries.

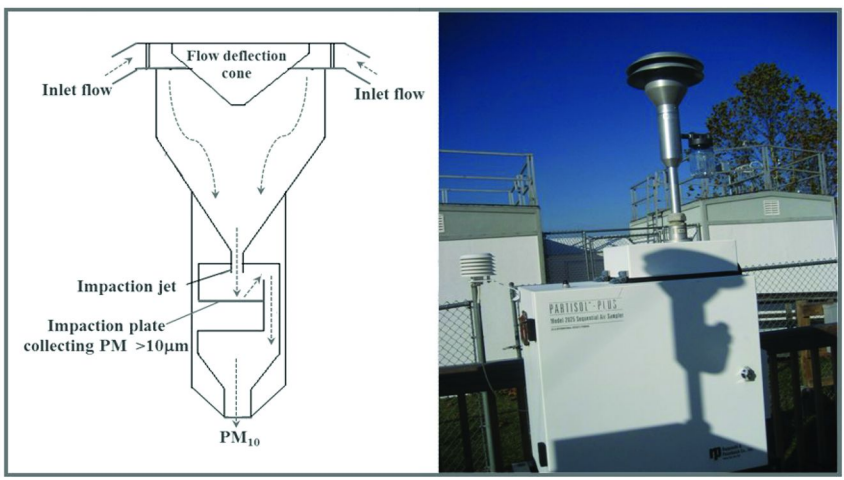

Figure 5. FRM low-volume PM_{10} sampler (right) with single channel impaction head (left).

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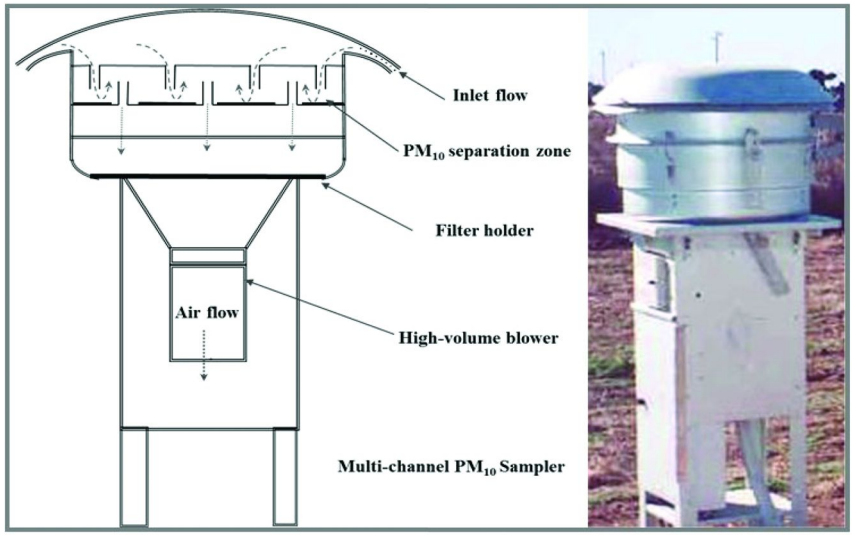

Figure 6. FRM high-volume PM_{10} sampler (right) with multi-channel impaction head (left) (48).

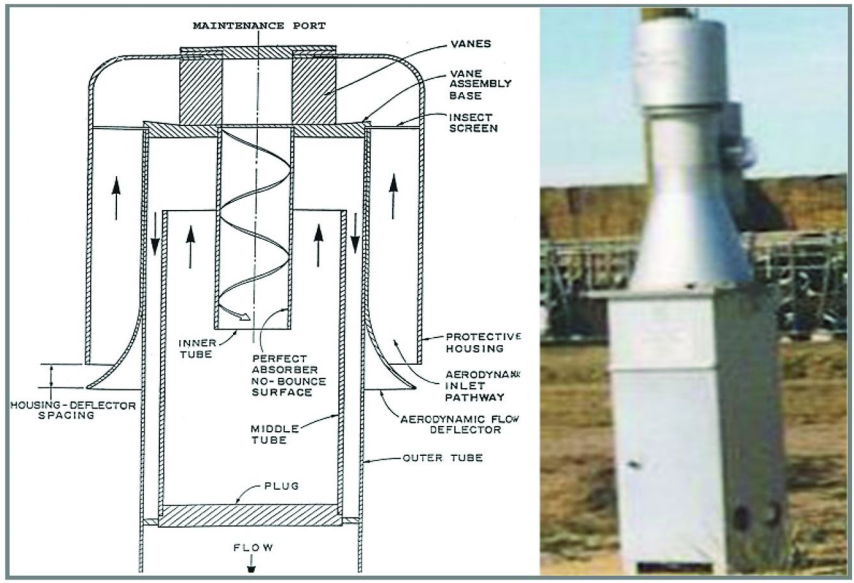

Figure 7. FRM high-volume PM_{10} sampler (right) with cyclonic separation head (left) (48).

The FRM $PM_{2.5}$ sampler operates at a flowrate of 16.7 L/min (low-volume). It consists of a FRM PM_{10} head, a FRM $PM_{2.5}$ head named Well-type Impactor Ninety Six (WINS) (*49*), a 47mm polytetrafluoroethylene (PTFE) filter, and a flow control system (Figure 8). The basic design of the FRM $PM_{2.5}$ sampler is given in 40 CFR Part 50, Appendix L (*12*). Performance specifications for FRM $PM_{2.5}$ samplers are listed in 40 CFR Parts 53 and 58 (*9*, *50*). The cutpoint for the FRM $PM_{2.5}$ head (WINS) is 2.5 ± 0.2 µm AED. No slope values for the sampler are listed in 40 CFR Part 53. PTFE filters used for $PM_{2.5}$ sampling should be conditioned before and after sampling for a minimum of 24 hours in controlled conditions at 20°C–30°C with less than ±2°C variation and 30–40% RH with less than ±5% variation. The analytical balance should have a readability of ±1µg (*12*).

In addition to the FRM designation for $PM_{2.5}$ sampler, there are three classes of FEM $PM_{2.5}$ sampler designations (i.e. Class I, Class II, and Class III). All the FEMs must provide results comparable to FRM measurements and similar $PM_{2.5}$ measurement precision. An increase in equivalency designation from Class I to Class II to Class III indicates a greater deviation from the FRM, thus requiring more extensive testing for equivalency verification (*9*).

Class I FEMs correspond to candidate samplers that have only minor deviations from the reference method and must undergo the same testing as the FRM candidate sampler. Class II FEMs use 24-hour integrated filter collection techniques that rely on gravimetric analysis but have significant design or performance deviations from the FRM. Very sharp cut cyclone (VSCC) $PM_{2.5}$ head (*51*) is one type of Class II FEM that has been widely used for $PM_{2.5}$ sampling (Figure 9). Class II FEM candidate samplers must undergo more extensive testing than the FRM or Class I FEMs. Class III FEMs further deviate from the FRM but still provide mass concentration measurements of $PM_{2.5}$ comparable to the FRM. Non-filter based techniques and continuous or semi-continuous monitors fall under Class III FEM designation. Class III FEMs may be required to undergo any or all of the testing required for validation as an FRM, Class I FEM, or Class II FEM, as well as additional testing specific to the sampling technology (*9*, *51*).

The aforementioned inherent sampling error in FRM PM_{10} sampler measurements also exists in the FRM and FEM $PM_{2.5}$ measurements. These errors are caused by the established tolerances for sampler performance characteristics and interaction of the sampler performance characteristics and the PSD of the PM being sampled. If the MMD of the PM that the sampler is exposed to is smaller than 2.5µm, under-sampling occurs in $PM_{2.5}$ measurements by the sampler, whereas if the MMD of the PM is greater than 2.5µm, over-sampling occurs (*46*).

Federal Reference Methods for $PM_{10\text{-}2.5}$ Monitoring

By definition, $PM_{10\text{-}2.5}$ (i.e., coarse PM) is PM with AED in the range of 2.5-10µm. Monitoring $PM_{10\text{-}2.5}$ is a more challenging task because it requires simultaneous separation of particles greater than 10µm and smaller than 2.5µm.

The FRM for $PM_{10-2.5}$ consists of two identical, collocated FRM samplers with one measuring PM_{10} and the other one measuring $PM_{2.5}$. The mass concentration of $PM_{10-2.5}$ is determined by subtracting measurements of the PM_{10} sampler by that of the $PM_{2.5}$ sampler ($PM_{10-2.5}$= PM_{10} - $PM_{2.5}$) (*52*).

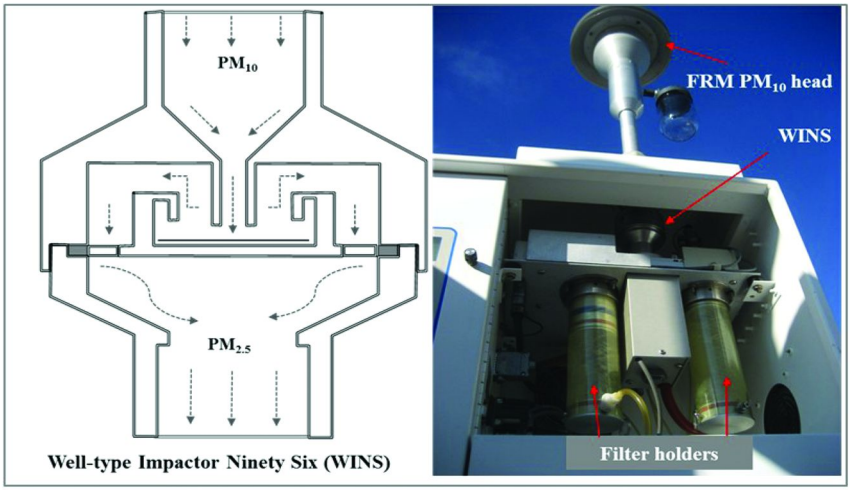

Figure 8. FRM $PM_{2.5}$ sampler (right) with a FRM PM_{10} head and a WINS impactor $PM_{2.5}$ head (left).

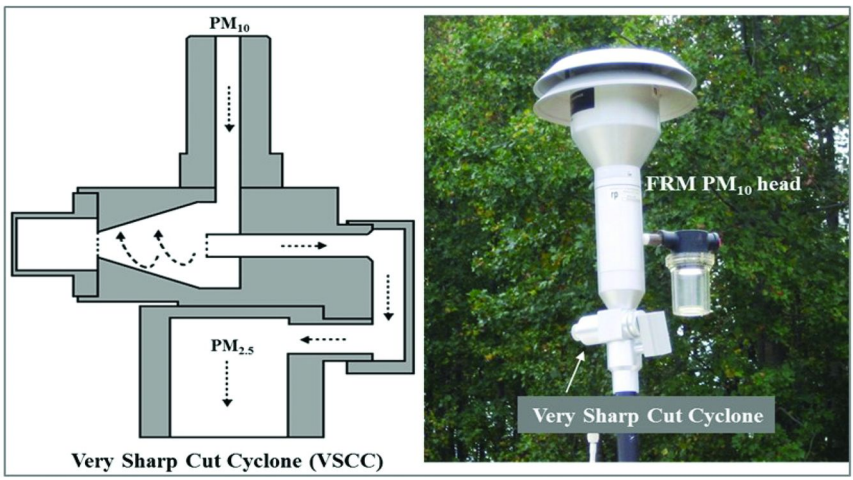

Figure 9. FEM $PM_{2.5}$ sampler (right) with a FRM-PM_{10} head and VSCC $PM_{2.5}$ head (left).

The filter-based FRMs and FEMs for ambient PM (i.e. TSP, PM_{10}, $PM_{2.5}$, $PM_{10-2.5}$) monitoring require a temperature and RH controlled filter conditioning/weighing room as well as intensive quality control. Moreover, these filter-based, time-integrated measurement methods cannot provide sufficient temporal resolution to better understand the sources and causes of elevated PM concentrations (*51*). With the advancement of continuous mass measurement techniques, continuous *in situ* PM monitoring has become a reality. Up to now, two types of automated mass measurement techniques have received EPA's approval for FEM designation for PM_{10} measurements: the TEOM technique and the Beta Gauge technique (Beta Attenuation Monitor, or, BAM) (Figure 10). The TEOM monitor operates on a unique microbalance system. This microbalance system continuously measures changes in the oscillating frequency of a hollow tapered tube as sample particles are collected on the filter that is attached to one end of the tube. The Beta Gauge technique uses the Beta radiation (e.g., Carbon 14) absorption (attenuation) principle to determine particle mass collected on the filter. Although TEOM and BAM have been approved as FEMs only for PM_{10} measurement, both of them have been used for $PM_{2.5}$ and TSP measurements as well.

In order to minimize the particle-bound water effect on mass measurements, the continuous *in situ* PM monitors (i.e., TEOM and Beta Gauge) operate with an internal heating mechanism to remove particle-bound water. The default temperature setting of a TEOM is 50 °C, and Beta Gauges often operate under slightly lower temperatures than TEOMs. It should be noticed that the internal heating mechanism of TEOMs and Beta Gauges can cause mass losses of semi-volatile PM components. Consequently, accuracies of PM measurements by these two techniques are compromised when they are used in environments with high levels of ammonia nitrate and/or organic PM masses. PM in AFO facilities possibly contains semi-volatile compounds and moisture due to high levels of RH and gas pollutants. In a poultry PM study, it was observed that TEOMs gave significantly lower values of both PM_{10} and $PM_{2.5}$ mass concentrations in comparison to low-volume filter-based PM samplers (*40*).

In addition to using the aforementioned PM monitors in AFO PM studies, various personal PM monitors and optical particle counters have also been used to determine concentrations of PM in various size fractions of interest in AFO facilities (*27*, *28*, *31*, *32*). While personal PM monitors provide good estimations of possible exposure by workers, they do not necessarily produce accurate measurements of concentrations of PM in air. Optical particle counters have been used in numerous AFO PM studies due to their ease of use (*27*, *28*, *31*, *32*) and their ability to provide both size and concentration measurements. However, these types of monitors do not provide PM measurements in AED. They require knowledge of particle shape and density to convert the measured sizes into AEDs and the measured number concentrations into mass concentrations. Moreover, frequent calibrations are needed when the optical particle counters are exposed to high PM concentration environments as in AFO housing systems. More discussion about the optical particle counters is in the next section of this chapter.

Figure 10. Automated PM monitors: FEM TEOM-PM_{10} (left) & BAM-$PM_{2.5}$ (PM_{10} head + VSCC) (right).

Particle Size Distribution Measurements

Particle size distribution governs the aerodynamic behavior of particles suspended in the air. The size distributions of AFO PM have been investigated for decades (*53–57*). However, the reported PSDs are often not comparable due to the limitations of different measurement techniques used in the studies. Although various techniques are available, there is no single method agreed upon for measuring the PSDs of PM with different physical characteristics (*42*, *55*). In general, the existing techniques for PSD measurement can be classified into different categories according to the principles applied in measurement and analysis.

Aerodynamic PSD Measurement Techniques

Cascade Impactors

The cascade impactor method is the most commonly used technique for measuring PSD in AED. It uses the inertial removal principle to separate PM into different size classes at different impaction stages, which are connected in series (Figure 11) (*58*). In a cascade impactor, each stage has a unique impaction nozzle size and cut-off size for separation; thus, each stage only collects particles corresponding to its cut-off size. The PSD may be computed based on mass fraction collected at each of the impaction stages. This technique measures a

PSD in AED, but it is only applicable for large particles in the micron range or above due to the lack of inertial force acting on small particles (e.g. submicron particles). Limitations of this technique also include inter-stage particle losses, incidence of particle bounce off from impaction plates or collected PM, and small number of size classes (*58*).

Figure 11. An eight-stage cascade impactor.

Aerodynamic Particle Sizers (APS)

Aerodynamic particle sizers measure aerodynamic diameter (AED) of individual particles using the time-of-flight principle (*42*, *58*). This technique provides real-time and high-resolution measurement of PSD in AED, and it has a particle size measurement range of 0.5-20 μm. The upper cut-off size of 20 μm limits the instruments' application for AFO PM study since they cut off much of the size distributions of PM in AFO facilities (*42*). Moreover, the instruments are usually calibrated using spherical particles with known densities, and they don't necessarily size well with particles having irregular shapes and large densities.

Optical PSD Measurement Techniques

Optical Particle Counters

Optical particle counters use the light scattering principle to detect particle size and number concentrations of PM being sampled. They are used more frequently for measuring particle sizes than concentrations. Although they can provide real-time size measurements of airborne particles, they are not intended for use on larger particles or in environments with high levels of PM and gaseous pollutants.

For PSD measurements, the optical particle counters measure particle sizes in equivalent spherical diameters (ESD), not AEDs as defined by NAAQS and FRMs. In order to convert ESD to AED, knowledge of particle shape factor and particle density is required (*58*). The conversion of ESD to AED is done by the following equation (*58*):

$$\mathrm{AED} = \mathrm{ESD} \times \sqrt{\frac{\rho_P}{\chi}} \tag{1}$$

where ρ_p is the particle density measured by a pycnometer and χ is the shape factor of the particle in question.

Research has revealed that the majority of AFO PM originates from feed, manure, bedding materials, feather, and skin, and the AFO PM particles are not spherical in shape (*42*, *56*). Figure 12 shows the particle image of a broiler (meat bird chicken) PM sample that was examined under an Environmental Scanning Electron Microscope with an Energy Dispersive X-ray system (E-SEM/EDX). It would be reasonable to assume that irregular shapes compromise particle size measurements by optical particle counters. In addition, the accuracy of optical particle counter measurements may also be comprised when they are used in high PM concentration environments where particle coagulation may occur. Consequently, extreme caution needs to be taken when optical particle counters are used for AFO PM studies.

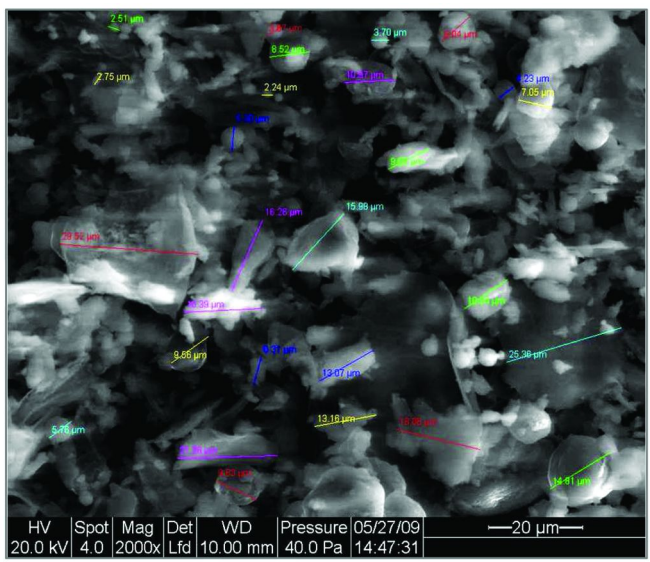

Figure 12. E-SEM/EDX image showing particle size and shape of a PM sample taken in a commercial broiler house.

In Evaluating Veterinary Pharmaceutical Behavior in the Environment; Cobb, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2013.

Light Scattering/Laser Diffraction Particle Sizers

The light scattering and laser diffraction particle size analyzers (Figure 13) use the particle light scattering principle for size measurements. Large particles scatter the light at small angels and small particles scatter the light at large angels when exposed to beams of light; thus, the scattering patterns of particles may be linked to particle sizes for measurements. The light scattering method may provide reliable PSD measurements with broad size ranges, from sub-micrometers to millimeters. On the other hand, the this technique is typically limited to measuring particles greater than 0.3 μm due to reduced detection efficiency with smaller particle size and errors caused by particle shape and refractive index variations (*42*, *58*).

In particle size measurements of airborne particles, light scattering analyzers don't provide real-time PSD measurements; they measure PSDs from PM samples taken using gravimetric collection techniques. In this method, PM samples need to be extracted from filter mediums into a solvent for PSD analysis. Consequently, this method is only suitable for insoluble particles. In addition, like the optical particle counter, the light scattering particle analyzers also provide PSD measurements in ESD, not AED. Conversion of ESD to AED is also needed for the PSD measurements by this technique.

Figure 13. LS 13 320 multi-wave length laser diffraction particle size analyzer.

Electrical Sensing Zone Technique

The electrical sensing zone (ESZ) technique, also known as the Coulter Counter method, applies the electrical resistance principle to measure PSD in an electrolyte solution (*58*). As shown in Figure 14, particles suspended in the electrolyte solution are forced to pass through a small orifice causing conductance changes in the solution. This change is a function of particle size and is then translated to particle volume measurements. The ESZ method measures a single particle's volume and provides high resolution and reproducibility for individual particle size assessments in ESD (*58*). The resultant PSD measurements by this technique also need to be converted into AED using equation 1 with known particle shape factor and density.

The Coulter Counter method is only suitable for insoluble particles that can be dispersed in an electrolyte solution and still retain their original integrity. As lighter scattering analyzers, Coulter Counter analyzers do not provide real-time PSD measurements; they measure PSDs of PM samples extracted from filters collected in the field.

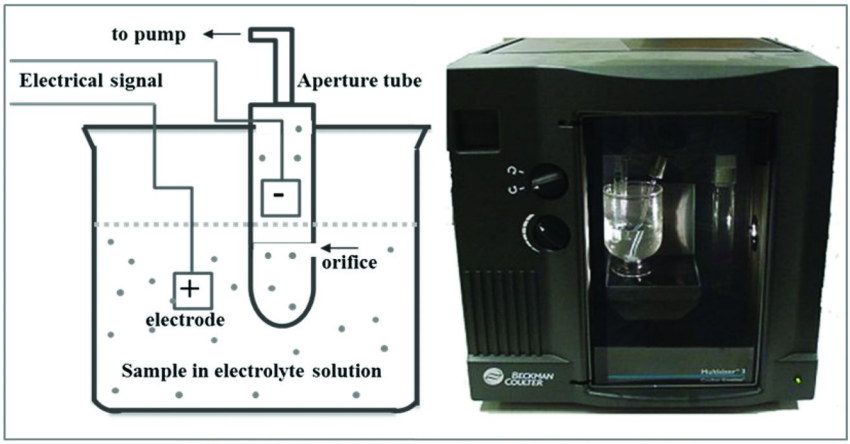

Figure 14. Coulter Counter Multi-Sizer3 (58).

Other Techniques

Other techniques of PSD measurement that have not been intensively used for AFO PM studies include electrical mobility analyzers and electron microscopy.

Electrical mobility analyzers depend on the electric mobility of the particles in an electrical field at a given voltage to measure particle sizes. They only work well for particles with good mobility. A commonly used electrical mobility analyzer is the differential mobility analyzer (DMA). It first uses the aerosol electrical mobility property to collect particles of different sizes and then counts these particles with a condensation nuclei counter (CNC). The CNC uses the aerosol condensation property to promote the growth of those particles for counting (*58*). The DMA+CNC analyzer measures particles of 0.005-1μm, and it is not suitable for use in AFO harsh environments with high level of PM and gas pollutants.

The electron microscopy method can provide both particle size and morphology information. However, this method for determining PSD is slow and cannot provide sufficient statistical representation of particle measurements to derive a PSD for a PM sample. It is not suitable for large sample size PSD analysis.

Chemical Speciation of Fine Particulate Matter

While physical characterizations of AFO PM have been studied for decades, chemical speciation of fine PM is a more recent research topic in AFO PM studies (*59–61*). Chemical speciation provides characterization of ions, carbon, and trace elements of PM. This characterization will lead to a better understanding of the sources of $PM_{2.5}$ and formation of secondary PM. The approaches used in PM chemical speciation include both field sampling and laboratory analysis.

Figure 15. $PM_{2.5}$ speciation samplers (62).

$PM_{2.5}$ Chemical Speciation Samplers

The full set of chemical speciation analysis includes examination of ions, carbon species, and trace elements. It requires PM samples to be collected on the appropriate filter medium to allow these chemical analyses. It is obvious that the single channel FRM ambient PM samplers are not capable of supporting characterization of all the chemical species. Samplers with multiple channels and filters have been designed for PM speciation sampling. Figure 15 shows some commercially available $PM_{2.5}$ speciation samplers (*62*).

Although the existing speciation samplers differ in designs, they all have at least three channels/cartridges to house three types of filters. Quartz filters are used for organic carbon (OC) and element carbon (EC) analysis, Nylon filters are used for ion (cations and anions) analysis, and Teflon filters are used for trace element analysis. In the Nylon cartridge, denuders of sodium carbonate or magnesium oxide are typically used upstream of the filter to remove acidic gases such as nitric acid. For AFO PM chemical speciation studies, a high level of ammonia gas is a concern and need to be removed from the sampling stream before it gets onto the Nylon filter for particle phase ion analysis. To remove ammonia from the AFO environment, acidic coating solutions need to be applied to the denuder (*59*, *60*).

$PM_{2.5}$ Chemical Analysis

Ions

Anion and cation analyses include the measurement of nitrate, sulfate, ammonium, sodium, potassium, and others. Sampling is usually carried out using Nylon filters. To avoid high backgrounds of sodium and other common ions, commercially purchased Nylon filters need to be washed and dried prior to sampling. PM on filters is quantitatively extracted into an aqueous medium which is subsequently analyzed by ion chromatography (IC). There may be extraction issues with the larger size fractions ($PM_{10\text{-}2.5}$), which may contain a high proportion of crustal minerals. Extraction procedures need to ensure adequate extraction efficiency for all target ions that might be present in the larger particulates (*63*).

Organic Carbon and Elemental Carbon

Particulate matter collected on Quartz filters may be analyzed for OC/EC using the Thermal–Optical Carbon Aerosol Analyzer. This analyzer uses a variation of the NIOSH 5040 thermo-optical analysis method to obtain carbon content measurements. Since this method does not directly account for the mass of hydrogen, oxygen, and any other elements contained in the particulate OC, corrections need to be made on measured OC. Typically, $PM_{2.5}$ has a ratio of OC to measured carbon in the neighborhood of 1.4, while coarse organic material, which may consist of mold spores and other biogenic material, can have a ratio in excess of 2.0. PM_{10} and $PM_{10\text{-}2.5}$ may have variable mixtures of carbon-containing particulates, which can contribute uncertainty to the calculation of mass for the carbonaceous PM. Another issue affecting OC measurement is the so-called organic carbon artifact, which is thought to be the result of adsorption of volatile organic compounds (VOCs) on the Quartz filter material. The magnitude of the organic carbon artifact depends on a number of variables, including season of the year and sampler type. Such variables need to be taken into account when calculating the net OC mass in the different particle size fractions (*63*, *64*).

Energy-dispersive X-Ray Fluorescence (EDXRF) analysis provides a rapid and cost-effective means for analyzing filter samples for a wide array of chemical elements. Forty-eight elements (Na through Pb) can be analyzed by EDXRF. Teflon filters used for the gravimetric analysis are used for the EDXRF analysis. There may be some issues with larger particulate size fractions that arise from the absorption of X-rays by larger particles. This self-absorption effect is most pronounced with lighter elements and can significantly bias measurements of crustal elements such as silicon and aluminum. Appropriate correction factors need to be applied to the data based on the mean particle diameter (*60*, *63*).

Summary

In AFO PM studies, ambient PM samplers and monitors have been widely used to measure PM concentrations in confined animal buildings where the PM concentrations are high, particle sizes are large, and the environments are harsh with high levels of gas pollutants. Many of the ambient PM monitors are not intended for use in such hostile environments. Consequently, AFO PM studies may suffer from lack of sufficient understanding of measurement techniques and their limitations. This chapter examines the scientific principles of various PM measurement techniques such that factors affecting measurement results are identified. Knowledge about strengths and limitations associated with each of the techniques will lead to the appropriate selection of a specific technique to achieve the research goals in AFO air quality studies.

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Chapter 3

Identification and Quantitation of Veterinary Compounds by Mass Spectrometry

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The analysis of compounds from veterinary sources has employed traditional wet chemistry, immunoassay and chromatographic methods. These older methods are valuable in classifying unknown compounds. With these tools and the guidance of a veterinary pathologist it is possible to narrow the focus of a chemical search to a particular class of compounds. Current requirements for lower detection limits, identification and confirmation require the use of mass spectrometry detection in order to provide legally defensible data. Two hyphenated techniques that meet these requirements are Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS). Tandem mass spectrometry (MS/MS or MS^2) offers even greater sensitivity and selectivity to provide the highest confidence in small molecule analyses. The future will include greater reliance on Time-of-Flight (TOF) and Fourier Transform (FT) mass spectrometry. Several of these instruments are currently available which incorporate higher mass resolution and software to "de-convolute" mass spectral information to identify unknowns. These techniques are financially out of the reach of many labs. However, just like LC-MS was once viewed as an exotic technique, so too will these state-of-the-art instruments come down in price as the technique gains more widespread acceptance.

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Identification of Unknown Compounds with Mass Spectrometry

The AOAC International (formerly the Association of Official Analytical Chemists) is one of the oldest organizations to establish standard methods of analysis. The AOAC Int. provides a compendium of approved and tested methods (*1*). The methods are rugged and are used for the proximate analysis of nutrients in feed for example. Many are wet chemistry methods that produce color changes or a have a titratable end result.

These methods were written when the level of concern was measured in percentage (%) of an analyte. Fats, protein, fiber and other major nutrients are all % concentration measurements which do not require a sensitive detection limit. The AOAC methods can provide general information but there are serious problems that can arise. This happens when the feed is adulterated or worse when inexpensive substances are used that are misidentified as nutrients in the simple proximate analyses.

This is a problem that has existed for decades and is the basis of the beginnings of the AOAC Int. How can scientists test the nutritive value of a feed? Unscrupulous providers of animal feeds have continued to locate substances which can be used to provide false positive results in the standard method of testing for protein in the feed (*2*). The Kjeldahl analysis is an oxidative method used to measure the amount of nitrogen in a sample. It has been recognized by fraudulent suppliers that inexpensive material could be used as an adulterant to provide an elevated measure of nitrogen. The classic example was the use of ground chicken feathers. This material provides nitrogen but it is in a form that provides no nutritive value in the feed.

In early 2007 a situation occurred when small companion animal cats and dogs started to become sick across the United States. This was a puzzle for more than two months as laboratories struggled to identify the problem. Animals continued to get ill and deaths were reported. Necropsy (post mortem exam of an animal) indicated that there were pathological abnormalities in the kidneys of the dead animals (*3*, *4*).

In late March, Direct Analyais in Real Time (DART) mass spectrometry was used to identify melamine and a few lesser contaminants in pet food. Product recalls of pet food began in earnest in early April and analytical testing focused on melamine only. Early versions of the FDA melamine procedure relied on methanol extraction and detection of the trimethyl silyl (TMS) derivative of melamine by GC-MS. Several of the recalled samples were positive for melamine.

Due to the low toxicity of melamine, there was still a determined if not frantic activity to isolate and identify the agent responsible for the animal sickness and disease. The FDA provided a lead role and it is informative to see how this investigation proceeded according to Dr. Greg Mercer of the FDA (*5*).

On 17 April the FDA investigators obtained a rice protein sample that was "suspicious." This particular sample was providing very low recovery results for melamine. The recoveries were in the range of 10% for this sample but were typically in the 80 - 120% range for all other samples. This was very puzzling at the time. The GC-MS library had spectra for melamine but not for many TMS derivatives.

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The GC for underivatized melamine is poorly resolved. The software library had melamine but not any spectra for the trimethyl silyl (TMS) derivatives which chromatograph much better. Later, another FDA researcher, Dr. Kevin Mulligan sent some spectra for the TMS derivatives of cyanuric acid, ammeline and ammelide, the lesser contaminants. On re-inspection of the data huge amounts of cyanuric acid (~ 7%) were observed. The "eureka moment" occurred when Dr. Mercer recognized that all of the samples that had high amounts of cyanuric acid showed poor recovery for melamine. He hypothesized that the cyanuric acid was forming a complex with the melamine.

The next day, in order to prove this hypothesis, work was begun on trying to "solvate" the proposed complex. Several different solvent mixtures were used and it was quickly observed that adjusting the pH of the extracting solvent resulted in a huge increase in melamine. The optimized solvent was found in using diethyl amine as an organic base in the acetonitrile/water solvent. In the end samples that had previously been reported at part per million levels of melamine were found to actually contain percentage levels of the material (*6*).

After this extensive investigation, the case was traced to Chinese importers using the highly nitrogenated compounds to deceive the Kjeldahl protein test. It is suspected that the use of melamine had been going on for some time but it was only when the Chinese began using a more impure source of melamine that contained higher levels of cyanuric acid, ammeline and ammelide that the problems were noticed. Unfortunately, the mixture of melamine and cyuranic acid forms an insoluble crystal which causes extensive renal damage in the kidneys of animals resulting in their sickness and death.

Due to the recognition of this problem, other methods have since been developed to analyze a sample for melamine adulteration (*7*, *8*). The problem is so widespread that companies have been formed to specialize in the analysis of proteins (*9*). This European company uses mass spectrometry exclusively to fully characterize samples for protein.

Using adulterants as a protein is an old and profitable fraud. Simple wet chemistry detection methods clearly are not up to the task of this complex analytical challenge.

Tools for Unknown Chemical Identification

It is clear that mass spectrometry is an important tool in the investigation of unknown chemicals. The use of chemical tests still remains very important to try and "classify" unknowns. In order to use a mass spectral method one needs to know something about the nature of the unknown chemical. The Hazchem kit has been a valuable resource for many years for first responders to test unknown chemicals (*10*). The kit allows the user to learn about the solubility of the unknown and do some simple chemical tests to classify the unknown. This process helps to narrow down the wide range of possibilities.

Infrared and Raman spectroscopy are tools which can help this process as well. Several manufactures have field portable instruments which have internal libraries of several thousand compounds to compare spectra to (*11*). It is important to

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remember that these tools have several inherent problems in identifying unknown chemicals. First of all, the compound needs to be pure. Even when material is directly from the manufacture, infrared spectra can be misleading.

This is the case of for sodium cyanide (NaCN). NaCN is toxic and is of concern because of its widespread availability and potential use as an agricultural terrorism agent. The material is 97% pure when purchased. The other 3% is starting material, sodium carbonate. While most chemists would recognize the triple bond absorbance at 2000 to 2200 cm^{-1}, the strong absorbances for sodium carbonate at 1410 and 860 cm^{-1} obscure this in a straight library search (*12*). This clearly demonstrates that all library searches must be critically reviewed.

To be useful, modern Fourier Transform (FT) Infrared Spectra (IR) require one or two spectral subtractions. Typically, water needs to be subtracted and followed by the major identified component. This will leave a spectrum that has a much better chance of being correctly identified by the library comparison program.

With chemical classification and a tentative library match, unknowns can then be evaluated for the appropriate mass spectral analysis using gas chromatography or liquid chromatography as the "inlet." For liquid chromatography conditions for ionization can also be better understood.

Mass spectrometry is a powerful tool for the identification of a compound based on the mass spectrum as a "fingerprint" that uniquely identifies a compound. Preliminary information is required about the physical characteristics of a compound in order to use mass spectrometry effectively in many cases.

Mass Spectrometers

The first separation by mass was recorded by Sir Joseph John Thomson of the University of Cambridge and this eventually led to him being awarded the1906 Nobel Prize in Physics (*13*). This discovery enabled both chemists and biologists to use the separation of chemicals by mass. There are several different configurations of mass spectrometers (*14*). The earliest configuration was a magnetic sector. Time-of-flight was invented next followed by the cyclotron style. Quadrupoles, a popular MS method have several configurations (linear quadrupole, linear quadrupole ion trap, and three dimensional quadrupole ion trap). Fourier Transform (FT) of ion cyclotron resonance has brought renewed interest into this mass spectral method.

For many years the standard for analysis of organic compounds was the "hyphenated" technique of gas chromatography-mass spectrometry (GC-MS). The invention of fused silica columns greatly improved chromatographic resolution which made this technique more popular (*15*). The most widely used configuration of GC-MS is with the quadrupole mass spectrometer. These mass specs offer solid performance and provided standard, library searchable spectra with electron impact ionization.

A problem arose when these bench top mass spectrometers were compared to electron capture detection (ECD). The MS detection was noticeably less sensitive. To make up for this the manufactures began to market software that would focus

on specific ions in a time window. Manufactures named this capability differently but the standard today is Selected Ion Monitoring (SIM).

SIM provides much better sensitivity but now the "full scan" finger print mass spectrum is not available. By picking the larger ions in a mass spectrum and limiting the allowed ratio of the ions it was possible to provide a detection of a compound that was more reliable than the older ECD. Unfortunately, the SIM method still had a problem of both positive and negative interferences which could obscure compound identification in complex mixtures. This is the result of a low (unit mass) resolution system that allows matrix ions to interfere in the required ion ratio specification.

One approach to solving this problem is by using quadrupole ion trap systems. With ions trapped, a more sensitive signal could be obtained. The early ion traps had trouble with reproducible quantitation. This was solved with the invention of the automatic gain control (AGC) (*16*).

The ion trap effectively concentrates the ion of interest while expelling other ions creating greater signal to noise. Another feature of the ion trap is the ability to run SIM alternating with full scan. With this capability, higher sensitivity SIM can be used for target compounds while retaining the full scan mass spectrum for unknown compounds.

A compound must be volatile for GC-MS and this technique is widely used for volatile and many semi-volatile analyses. Compounds that are not volatile can be derivatized in order to make them amenable to gas chromatography. This takes time and introduces another variable into the analysis. While derivatization is still performed, many compounds are more appropriate for use in a liquid chromatograph.

LC-MS is becoming the dominant method of analysis for all but volatile compounds. The price of LC-MS instrumentation has dropped and the range of compounds that can be analyzed has grown. LC-MS is used for many different classes of compounds today and manufacturers offer a range of mass spectrometer configurations. LC-MS is also desirable because there are three main types of ionization; electro spray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). Three ionization modes offer wide applicability to a broad range of compounds (*17*).

The standard LC-MS has given way to LC-MS/MS in many cases. Here compounds are ionized, sent in the mass spectrometer where a specific ion is isolated, then subjected to a collisional dissociation (CD) and finally scanned out "reaction ions" are produced. This three dimensional mass spectrum allows for a very sensitive detection. This is extremely important in analyses with legal implications (*18*). Due to the high sensitivity coupled with production of mass spectral fragments, highly complex determinations can be accomplished with LC-MS/MS. MS/MS does require known analytes because a window must be set up which limits the usefulness of this technique for investigation of unknowns.

A report has been made about using the ion trap LC-MS/MS for the detection of "unknowns (*19*)." It should be pointed out this report might more appropriately be described as the detection of compounds from a list of 359 potential targets. While this is certainly a good selection of compounds, a true unknown is quite a different situation and is not well addressed by this method.

Manufactures have now begun to offer mixtures of other platforms in LC-MS. The Orbitrap from ThermoFisher is one example. It is a high resolution, high sensitivity instrument. Figure 1 is a diagram of the Orbitrap design (*20*).

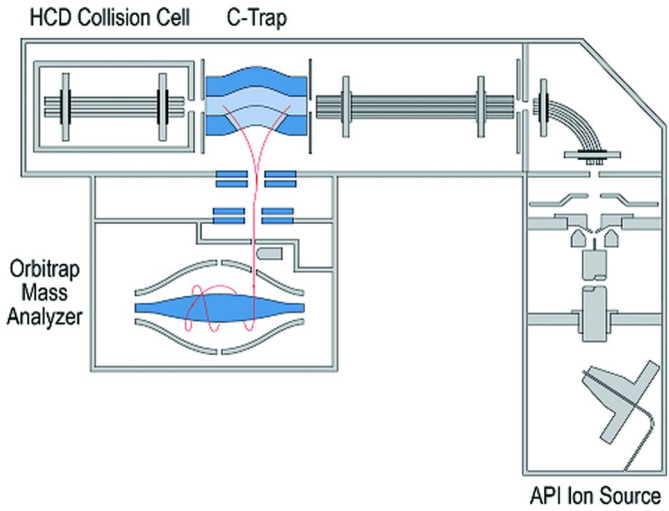

Figure 1. Reproduced with permission from ThermoFisher, all rights reserved.

This instrument incorporates the power of the FT mass spectrometer. One recent report demonstrates the utility of this technology. Here it was used for the analysis of a range of veterinary drugs in fish (*21*).

High resolution instruments have one inherent difficulty. Currently, this instrument is financially out of the reach of many laboratories. This was the case with standard LC-MS when compared to GC-MS. Over time the price of LC-MS has fallen and this will likely be the case with the higher resolution instruments as well.

No discussion of mass spectrometers and unknowns would be complete without including the previously mentioned Direct Analysis in Real Time (DART) (*22*). The DART system uses ambient ionization to produce mass spectrums of virtually any ionizable substance. This mass spectral technique is opening new frontiers in the analysis of unknowns. A recent review provides more complete information on this technology (*23*).

Veterinary Toxicology Employs Mass Spectrometry

In the world of chemistry all unknowns can be broken down to three categories: animal, mineral or vegetable. That is to say something that makes an animal sick can be related to a biological pathogen, a mineral origin toxicant such as an element or anion (arsenic or nitrate for example) or an organic compound, many of which are plant derived such as the opiates. For the analysis of a true unknown to begin an analytical laboratory needs information to determine what to analyze for.

A veterinary pathologist is the key to effective identification of chemicals responsible for animal disease and death. Either from the examination of specimen or symptoms of the animal the pathologist directs the chemical investigation. In the case of a dead animal a post mortem necropsy examination is performed. A pathologist will examine the carcass for abnormalities. The pathologists then can order specific tests for pathogens, histological samples and toxic chemicals The goal is to identify and assign a cause of death. In this section, the focus is on toxic chemicals which may be identified as a cause of death (*24*).

It is important to understand that it impossible to "test for toxic chemicals." Once there is some pathological basis, then a focused chemical investigation can take place. There are simply too many biological pathogens, inorganic and organic chemicals available to try and determine a toxic agent without some guidance or focus. While a "shotgun" approach may yield results, the chances of successfully identifying a responsible chemical are very low.

In order to test for toxic levels of a chemical, wet chemistry tests are frequently used. A simple test for toxic herbicide paraquat can easily be done by wet chemistry for instance (*25*). This test is guided by the veterinary pathologist who requests the test based on extensive bleeding observed in animal lung tissue. Paraquat and the related quaternary ammonium salt diquat cause the capillaries in the lungs to rupture. Blood in the lungs is a strong indication of this type of agent. The test for quaternary ammonium herbicides is requested by a pathologist to provide further evidence of the diagnosis of ingestion of this class of toxic herbicides. In this case the test is not for an unknown chemical but to verify identity of a suspected class of chemicals.

Enzyme-linked immune-sorbent assays (ELISA) can be used for screening purposes and are available for a wide range of chemicals. These tests require knowledge of what chemical is of interest as well. While sensitive and easy to use, ELISA tests have some inherit difficulties. The kits are biological and require that they be kept in a refrigerator. Test kits have only 6 months to a year shelf life before they expire. The user must also remember to use caution with ELISA tests because both positive and negative interferences can mask true results.

Mass Spectrometry of Metals

The analysis of toxic metals is a concern in veterinary situations. Inductively coupled plasma-mass spectrometry (ICP-MS) can be used to scan a sample for all of the toxic metals. This requires some type of digestion or extraction.

An orthogonal technique that may also be considered is X-ray Florescence Detection (XRF). This technique is very useful to screen samples for toxic levels of metals and metalloids. Several manufactures offer field portable units. These instruments can provide accurate semi-quantitative information within minutes about the toxic levels of elements in a sample (*26*).

The best feature of XRF is the ability to do analysis with no sample preparation. Samples are placed in disposable weighting boats and analyzed directly. This is a real advantage when rapid analysis of a large number of samples is required. Field analysis can also be accomplished on site and this saves significant amounts of time, transportation and analytical costs.

ICP-MS is clearly much more sensitive and has unsurpassed capability to perform quantitation. This platform has another interesting application. After the Japanese nuclear power plant in Fukushima began leaking, there were many countries interested in measuring potential radiation contamination in their citizens, animals and food supplies. ICP-MS played a role because of the sensitivity of the instrument to uranium. U has a large atomic mass and there is little background interference. While most radiation contamination is measured with scintillation counting, ICP-MS can measure low part per trillion levels of uranium-235 and 238 in urine samples. Uranium 234 requires pre-concentration, however (*27*).

Anion Mass Spectrometry

Toxic levels of anions are another analyte of concern in veterinary toxicology. Traditional methods of anion analysis are done with ion selective electrodes. These are widely used both in the lab and for field measurements. Laboratory based analysis has focused on either auto analyzers which use a wet chemistry color reaction or ion chromatography. Ion chromatography has employed several types of detection with electro-chemical (EC) detection used commercially for many years (*28*).

The EC detection is sensitive and reproducible but a problem arises when complex sample matrices are tested. In these cases EC, as with many single dimension detectors, have been superseded by mass spectrometric methods. A mass spectrum provides not only a detection method but information about the mass of the ion detected as well. This is important to reduce false positive data. Another method for anion analysis that has seen interest is the combination of capillary electrophoresis with mass spectrometry (CE-MS) (*29*).

The requirement for defensible data has shown that it is becoming more difficult to get analytical data accepted unless it has the confirmatory second dimension of a mass spectrum. In mass spectral detection, it is more than a simple signal at a particular time. The mass spectral signal contains mass information in a second dimension. This has proven valuable in the assignment of the identity of a particular anion as well as in low level quantification.

One anion analyte that has had much interest is perchlorate. This contaminant came from the manufacture and use of high energy munitions (*30*). Perchlorate was believed to be very reactive and not a problem in the environment. This has

been shown not to be the case. When analyzing for perchlorate in the environment research has proven it to be widespread (*31*). The anion has been reported in water, plants and animals (*32*).

The early analysis employed simple anion chromatography. The EC and other single amplitude type of detectors were used extensively. In more complex matrices there were reports of extremely high concentrations of perchlorate. This quickly led to the demand for a mass spectral detection to confirm these reports (*33*).

Example of Mass Spectrometry for Veterinary Use

Antibiotics are often used to treat infections in animals that are raised for food. Many shrimp are now farm raised and the close quarters can be a problem if an infection begins. Treating the pond with antibiotics is done but the problem of the shrimp retaining traces of these compounds is an area of veterinary concern. One group has provided a mass spectral method to measure chloramphenicol at 0.3 ppb (*34*).

Another example of the use of mass spectrometry is the determination of presence and concentration of growth hormones used to increase the weight of animals sold for meat. Animal food products are a multi-billion dollar a year industry (*35*). Modern animal husbandry has used knowledge of growth hormones and growth promoters to increase weight, muscle mass, and texture in meat products. These substances are carefully regulated and many countries outside the USA prohibit the use of these compounds. Thus analytical chemistry has been called upon to provide detection and quantitation of these substances (*36*). Growth promoters are very powerful with trenbolone being a synthetic compound that is typical of this class of growth promoters. (Figure 2)

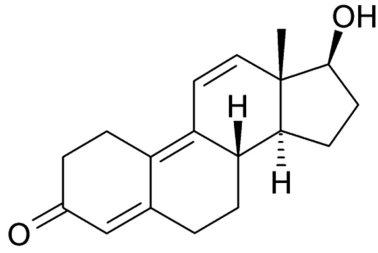

Figure 2

Animals are very sensitive to this compound and it can provide effects at near 5 ng/kg in the tissue of cattle (*37*). Because these compounds are so effective, highly sophisticated methods are required to detect the compounds (*38*). Wet chemistry or spectrometric methods simply do not have the sensitivity at the required detection limit.

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There is also the added difficulty of "designer" compounds or new mixtures of growth promoters. Thus analysis of meat for these compounds is an extremely difficult task. Couple that with an industry that is trying to protect its growth promoter formula and one ends up with varying mixtures and compounds being used as growth promoters. The analytical task is difficult when the compounds are known but becomes much more challenging to nearly impossible if the compounds have been modified even slightly. This is the area that mass spectrometry is most effective where a full scan of the compound can be obtained. The instruments with the highest sensitivity are capable of meeting this challenge but as was pointed out, these are expensive tools.

To further compound the chemical analysis is the use of well-known substances such as bacitracin as a growth promoter. These antibacterials are used to enhance growth by controlling low level bacterial infection and the compounds pose no risk to human health (*39*). However, low level veterinary use may lead to antibacterial resistant strains of bacteria in the environment and this is clearly a problem that is of universal interest.

Veterinary Pharmacology and Humans

It is not always obvious if the "cart is in front of the horse" when trying to find references for mass spectral method development. Veterinary pharmaceutical analysis can be critical for the development of analysis of compounds in humans. Sometimes the veterinary side leads and other times the human analytical methods are first.

The FDA has many roles in the United States. One area is to develop guidelines for both human and veterinary safety and efficacy (*40*). The use of pharmecuticals in animals covers the same type of use as that in humans. Thus the mass spectral analysis of these compounds can be used as a model for human monitoring as well. In 2001 a general guidance document was developed by the FDA for Bioanalytical Method Validation (*41*). Then in 2003 the FDA prepared a guideline in the use of mass spectrometry for the conformation of compounds in veterinary samples (*42*). Subsequently, the FDA has developed a 2012 guidline for the analysis of residues in food (*43*).

The connection between mass spectrometry for the determination of compounds and both human and veterinary applications is clear. When investigating mass spectrometry as an analytical method for particular classes of compounds it is prudent for veterinary researchers to consider the human clinical literature. There are many examples that can demonstrate this. One specific example comes from the use of mass spectrometry to identify steroidal compounds from newborn human infants that have genetic metabolic disorders such as congenital adrenal hyperplasia (CAH) (*44*). This analytical information can quickly lead veterinary researchers to methods for steroid mass spectral analysis in animals.

Veterinary pharmaceutical analysis by mass spectrometry is an important area for development but the funding has lagged behind the human clinical area. The International Federation for Animal Health (IFAH) has compiled data showing,

"the global market for human drugs is worth 40-times more than its veterinary counterpart, while sales of the world's leading pharmaceutical brand are 13-times higher than those of the best-selling animal health product. The gulf in spending power between the two sectors is equally spectacular. There is a 30-fold difference between the research budgets of the respective market leaders, for example, and the top-ranked human pharmaceutical company employs more research scientists than the world's 20 leading animal health businesses put together (*45*)."

With such a gap in funding, the list of potential examples of human-clinical mass spectral methods that can be employed for veterinary use is long. From the determination of drugs of abuse in urine to the analysis of samples for antibiotics, the veterinary, food and human applications are all important areas to review before trying to devolop mass spectrometry techniques. Since funding is so heavily weighted to the human-clinical side it is important for veterinary researchers to survey this literature prior to trying working to develop new mass spectral analytical methods for veterinary use.

Conclusion

Those with interest in veterinary samples will need to rely more on the application of mass spectrometric techniques. Many times a literature review of human-clinical mass spectral techniques may result in obtaining proven methods that are applicable to veterinary problems.

Older techniques still remain important in many areas of analysis. However, as problems continue to proliferate in adulteration, contamination and fraud mass spectrometers are invaluable in this research.

Finally, the plethora of mass spectrometers and techniques has opened the door to lower detection limits with greater confidence in the data. The latest development in high resolution instruments adds greater ability to perform very low concentration determinations in complex matrices. The major drawback is the expense of this latest generation of instruments. As legally defensible data requirements proliferate, it may necessitate a transition to these new instruments. Still, for many applications there are more affordable options with instruments such as the ion trap which can provide excellent data at a lower cost.

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Chapter 4

An Isotope-Dilution Standard GC/MS/MS Method for Steroid Hormones in Water

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An isotope-dilution quantification method was developed for 20 natural and synthetic steroid hormones and additional compounds in filtered and unfiltered water. Deuterium- or carbon-13-labeled isotope-dilution standards (IDSs) are added to the water sample, which is passed through an octadecylsilyl solid-phase extraction (SPE) disk. Following extract cleanup using Florisil SPE, method compounds are converted to trimethylsilyl derivatives and analyzed by gas chromatography with tandem mass spectrometry. Validation matrices included reagent water, wastewater-affected surface water, and primary (no biological treatment) and secondary wastewater effluent. Overall method recovery for all analytes in these matrices averaged 100%; with overall relative standard deviation of 28%. Mean recoveries of the 20 individual analytes for spiked reagent-water samples prepared along with field samples analyzed in 2009–2010 ranged from 84–104%, with relative standard deviations of 6–36%. Detection levels estimated using ASTM International's D6091–07 procedure range from 0.4 to 4 ng/L for 17 analytes. Higher censoring levels of 100 ng/L for bisphenol A and 200 ng/L for cholesterol and 3-beta-coprostanol are used to prevent bias and false positives associated with the presence of these analytes in blanks. Absolute method recoveries of the IDSs provide sample-specific performance information and guide data reporting. Careful selection of labeled compounds for use as IDSs is important because both

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inexact IDS-analyte matches and deuterium label loss affect an IDS's ability to emulate analyte performance. Six IDS compounds initially tested and applied in this method exhibited deuterium loss and are not used in the final method.

Introduction

An increasing number of scientific investigations have documented the potential of estrogenic hormones to affect the endocrine systems of exposed organisms at extremely low doses; in some cases at less than 1 nanogram per liter (ng/L) (*1*–*5*). The primary pathways by which steroid hormones are introduced to the environment include discharge of municipal and industrial wastewater and runoff from agricultural operations, although a large variety of anthropogenic sources has been considered (*6*). These compounds can occur in the environment at concentrations exceeding published lowest-observable effects concentrations, especially in treated wastewater effluents and surface waters that receive discharge from wastewater-treatment plants (WWTPs) (for example, see (*7*, *8*)).

Collaborative studies by the U.S. Geological Survey (USGS) (*9*, *10*) and studies by others, for example (*11*, *12*), have shown that fish living downstream from some WWTP discharges have abnormal development of sex organs, and that exposure to natural and synthetic estrogens is likely to play a role in the induction of such abnormalities. These effects, known as endocrine disruption, can be manifested in several different ways including inappropriate expression of vitellogenin (an egg yolk protein) by males or juveniles, demasculinization of secondary sex characteristics, suppression of gonadal development, suppression of sperm development, and the formation of intersex gonadal tissue, which occurs when both male and female reproductive germ tissue are present in the same individual.

Estrogens have been most commonly linked to biological effects in the environment. In particular, the principal human estrogen, 17-beta-estradiol (17β-estradiol), its metabolite estrone, and the synthetic pharmaceutical 17-*alpha*-ethynylestradiol (17α-ethynylestradiol) (*13*). Kidd and others (*14*) observed the total collapse of a fathead minnow population in a lake exposed to 6 ng/L of 17α-ethynylestradiol. Mixtures of estrogens may even act additively or synergistically (*15*, *16*), and consideration of all compounds possible that have known disrupting activity of the endocrine system is ideal. Although there is less direct evidence of this activity in the environment, androgens and progestins can induce biological effects by similar nuclear receptor-mediated modes-of-action and might exert similar disruptive effects at low concentrations (*17*, *18*).

As a result of these observations, there has been considerable interest for analytical methods to measure these compounds at environmentally relevant concentrations, in part, to (1) further understand their presence and distribution in the environment, (2) examine their role in inducing deleterious effects on wildlife, and (3) assess the efficacy of their removal from waste streams using various treatment technologies. To meet this need, the USGS National Water Quality Laboratory (NWQL) developed an isotope-dilution gas

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chromatography–tandem-quadrupole mass spectrometry (GC/MS/MS) method to analyze for a suite of 20 target compounds (referred to as "analytes"; Figure 1) in filtered (method 2434) or unfiltered (method 4434) water. Laboratory processing of samples by either method is identical; the only difference is whether or not samples are filtered (preferably in the field) before extraction.

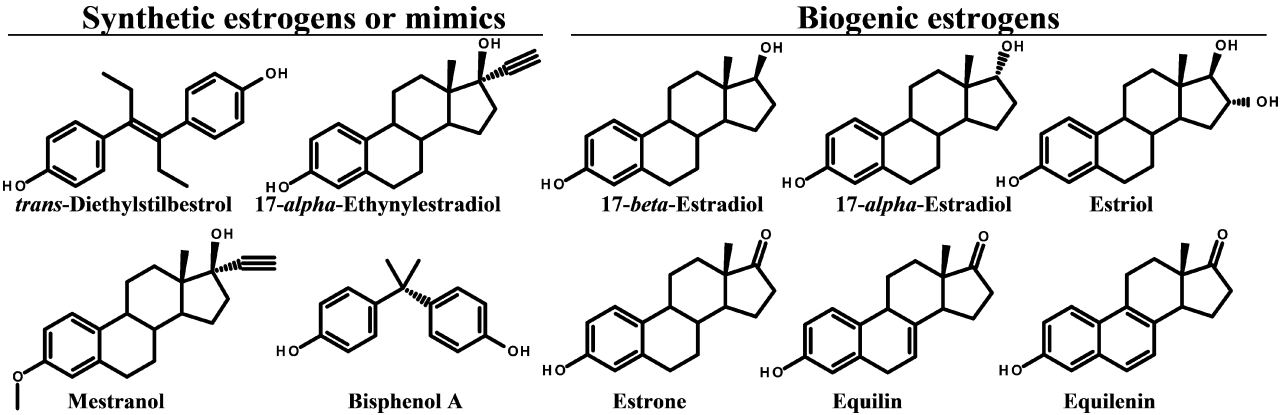
Eighteen of the method analytes share a common polycyclic steroid backbone and differ only in saturation or substitution. These include 6 natural and 3 synthetic estrogens, 6 natural androgens, 1 natural and 1 synthetic progestin, and 2 sterols. The industrial chemical bisphenol A (BPA) and the pharmaceutical *trans*-diethylstilbestrol, which themselves are structurally similar, do not share structural similarity with the steroid hormones but are known to act as endocrine system modulators (*19*, *20*).

Eleven of the 13 natural hormones included in the method are excreted by humans in free or conjugated forms in urine and feces (*21*). Two others, equilin and equilenin, are equine hormones that are isolated and administered pharmaceutically during estrogen-replacement therapy (*22*). The four synthetic hormones have human pharmaceutical uses, although diethylstilbestrol use is limited due to its undesirable teratogenic side effects (*19*). Only the strongly estrogenic (endrocrine disrupting) *trans*-isomer of diethylstilbestrol is determined by the method; *cis*-diethylstilbestrol, which is not strongly estrogenic, is not determined.

The two sterols, cholesterol and 3-beta-coprostanol (3β-coprostanol), typically are present at high concentrations in waters receiving substantial WWTP discharges, runoff from fields with applied biosolids, and runoff from animal feeding operations. They are included mainly as potential indicators of fecal-source (coprostanol) and sewage-source (both sterols) contamination (*23*) and not as primary target analytes for this method, which was designed specifically to determine substantially lower concentrations of the steroid hormones. The method determines the free (non-conjugated) forms of the analytes. Most of the glucoronide conjugates of the steroids are converted to the free form during WWTP processes, whereas sulfate conjugates are partially (35–88%) deconjugated (*24*).

The method analyzes for the seven hormones [17β-estradiol, 17α-ethynylestradiol, estriol, estrone, equilin, 4-androstene-3,17-dione (androstenedione), and testosterone] included for Screening Survey monitoring under the revisions to the Unregulated Contaminant Monitoring Regulation (UCMR 3) for public water systems (*25*).

This chapter summarizes the method procedures and performance data obtained using primary validation matrices: reagent water, wastewater-affected surface water, and primary (no biological treatment) and secondary wastewater effluent. Performance data also are summarized for method application to a variety of other (primarily unfiltered) water matrices. Advantages and limitations of isotope-dilution quantification (IDQ) as applied to this method are described, including observations of isotope-label instability for some previously used isotope dilution standard (IDS) compounds. Also, presented are results of a multi-concentration detection level determination and a summary of sample and extract holding-time experiments.



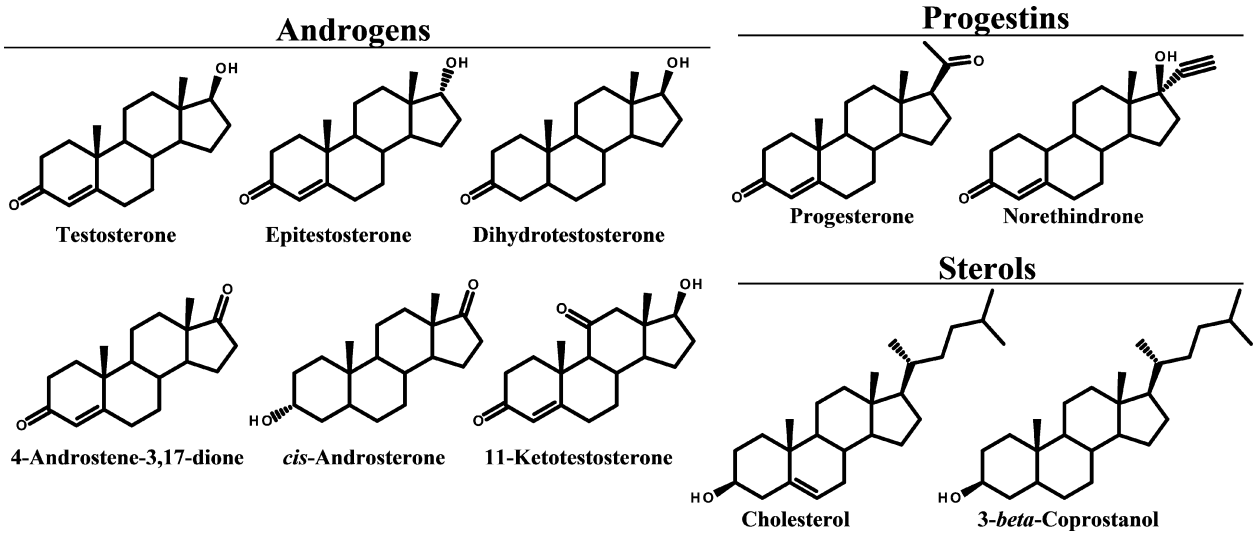


Figure 1. Structures of method analytes.

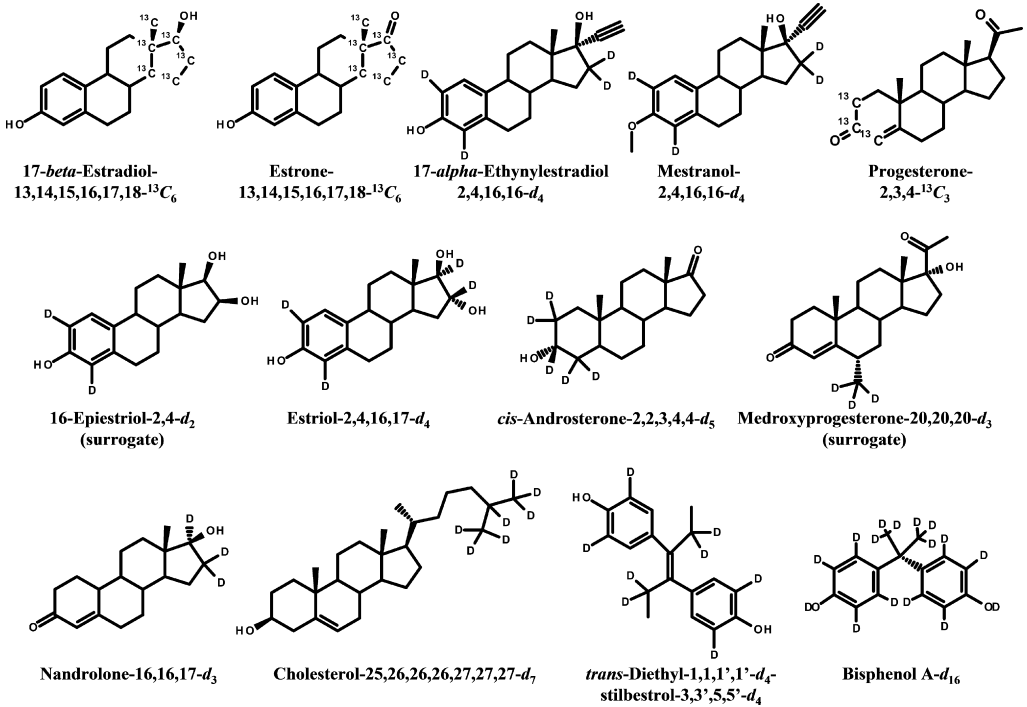


Figure 2. Structures of the isotope-dilution standard and surrogate compounds.

Summary of Method

Methodological details, including commercial sources of supplies, reagents, and method compounds are provided in Foreman and others (*26*). Field-filtered (method 2434) or unfiltered (method 4434) water samples are collected and processed using USGS protocols for organic contaminants (section 5.6.1.F of (*27*)), except that the samples are contained in 0.5-L high-density polyethylene (HDPE) bottles which facilitates freezer storage of samples prior to extraction. Samples suspected to contain residual chlorine include 100 mg/L of ascorbic acid in the sample bottle.

Laboratory processing of filtered or unfiltered samples is identical. Samples not processed within 3 days at the NWQL are held frozen at ≤–5°C until the day preceding extraction, then allowed to thaw at room temperature. Samples are fortified with approximately 100 mg/L of sodium chloride specifically to improve recoveries for the sterols only from reagent water matrices, and with 100 ng/L (10,000 ng/L for cholesterol-d_7) of the deuterium- and ^{13}C-labeled compounds that are used as IDS or surrogate compounds (Figure 2. Note: the IDS names used elsewhere in this chapter typically do not include the label-substitution numbering shown in Figure 2).

The sample is extracted by solid-phase extraction (SPE) by passing it through a 47-mm diameter multigrade glass-fiber filter (GFF; Whatman Inc., Piscataway, N.J., GMF150, cat. no. 1841–047) positioned over a glass-fiber filter disk that is embedded with octadecyl surface-modified silica (C18 disk; Supelclean™ ENVI™-18 SPE Disk; Sigma-Aldrich Corp., St. Louis, MO, cat. no. 57171). The GFF/C18 disks are contained in a custom-fabricated 530-mL capacity, pressurized stainless-steel extractor (Martin Enterprises, Lakewood, CO, cat. no. SSFT500mlSet) (Figure 3).

Following compound isolation, the GFF/C18 disks are rinsed with 10 mL of 25% methanol in reagent water to remove polar compounds that interfere with instrumental analysis. Dry nitrogen gas (N_2) is passed through the GFF/C18 disks to remove residual water, and the method compounds are eluted with 40 mL of methanol. The eluent is evaporated to dryness at 25°C with N_2 and reconstituted in 2 mL of a 5% methanol in dichloromethane solution.

The extract is transferred to a 1-g Florisil SPE column (Biotage, LLC, Charlotte, NC, cat. no. 712–0100–C), and the analytes are eluted with 25 mL of 5% methanol in dichloromethane solution. The eluent is reduced in volume, transferred to a 5-mL reaction vial, and evaporated to dryness with N_2. Processing of multi-level calibration standards contained in reaction vials is included beginning at this evaporation step. Alcohol (C–OH) and ketone (C=O) functional groups on the analytes and IDS compounds are derivatized to trimethylsilyl ether or trimethylsilyl-enol ether analogs to increase compound volatility and minimize compound interactions with active sites in the gas-chromatography system. Derivation is accomplished by (1) addition of 200 µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide activated with 2-(trimethylsilyl)ethanethiol and ammonium iodide to the dried extract, and (2) heating of this MSTFA solution to 65°C for 1 hour (h). The MSTFA solution also

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contains cholestane-2,2,3,3,4,4-d_6 (cholestane-d_6) and chrysene-d_{12} as injection internal standards. This reconstituted extract is transferred to a vial (Wheaton Science Products, Millville, NJ, cat. no. 225326) for analysis.

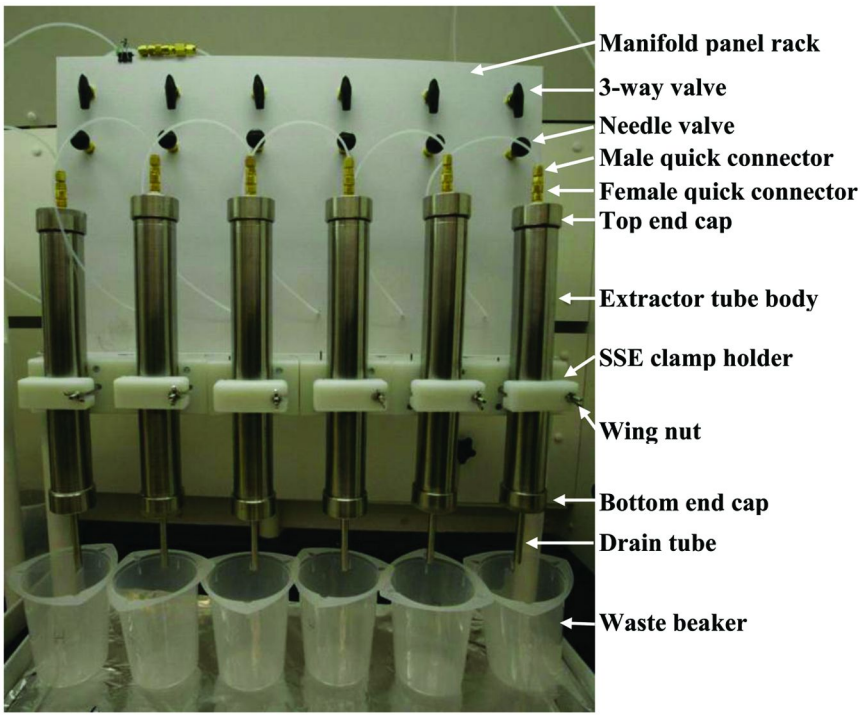

Figure 3. Stainless-steel extractor (SSE) tubes mounted on a 6-position manifold panel rack.

Method compounds are separated by gas chromatography (model 6890 GC with 7673B autosampler; Agilent Technologies, Santa Clara, CA) by using a 30-m by 0.25-mm internal diameter capillary column coated with 0.25-µm film thickness of Rxi®-XLB stationary phase (Restek Corp., Bellefonte, PA, cat. no. 13726) and a multi-ramp temperature program. Analytes are detected by tandem-quadrupole mass spectrometry by monitoring the product ions of three specific precursor-to-product ion transitions (Table I). Positive analyte identification requires the presence of at least two unique transition product ions, with ion ratios not deviating from those in a standard by more than specified tolerances (*26*, *28*).

All 20 method analytes are quantified relative to a specific IDS (Table II; structures in Figure 2) by using an IDQ procedure that automatically corrects for procedural losses in the reported analyte concentration by correction relative to the absolute recovery of the IDS. Ten deuterium- or ^{13}C-labeled isotopes found suitable for use as IDS compounds were used during method validation, seven

of which were exact isotopic analogs of the method analytes (shown in bold in Table II). Note: the exact isotopic analogs estriol-2,4,16,17-d_4, cis-androsterone-2,2,3,4,4-d_5, and progesterone-2,3,4-$^{13}C_3$ became commercially available since obtaining the validation data summarized in this chapter, and they have been added to the method as the exact IDS compound used for the quantification of estriol, cis-androsterone, and progesterone, respectively (see Table II and the "Use of Isotope-Dilution Standards" section). The remaining method analytes also are quantified using isotope dilution by using one of the IDS compounds that has similar related chemical functionality but is not a direct isotopic analog of the analyte (Table II and Figure 2).

The recoveries (in %) of the IDS compounds are reported along with the analyte concentrations for each sample. However, these IDS measurements reflect sample-specific absolute recoveries achieved during sample preparation and are only corrected for injection variability during quantification by using the injection internal standard compounds chrysene-d_{12} or cholestane-d_6. Analyte concentrations (or method recoveries in matrices fortified with method analytes) are automatically corrected for procedural losses by using IDQ.

In addition to the quality-control (QC) performance information provided by the sample-specific IDS recoveries, method performance also is monitored by using both laboratory reagent-water blank and spike samples that are prepared and analyzed with each set of (typically 10) field samples. Additional quality assurance (QA) samples that might be analyzed with the sample set include field-submitted blank samples, and field replicate samples, some of which can be fortified with the method analytes as matrix-spike samples.

Results and Discussion

Method Development Strategies

The method incorporates some chemical and instrumental analysis strategies that contribute to enhanced specificity, selectivity, and reliability, especially in difficult matrices. First, samples undergo chemical derivatization with activated MSTFA before GC/MS/MS analysis, which makes the method compounds less polar and more volatile and, thus, readily amenable to GC separation. These higher molecular weight trimethylsilyl ether or enol-ether derivatives also produce characteristic ions of higher mass-to-charge ratio (*m/z*) that typically make identification and quantitation less susceptible to interference.

Second, the application of tandem-quadrupole mass spectrometry (MS/MS) in comparison to single-quadrupole MS dramatically improves the specificity of the analysis and decreases the likelihood of false positives. The method described here evolved from a USGS method developed by Barber and others (*29*) that used single-quadrupole mass spectrometry, and substantial improvements in selectivity and achievable detection levels were obtained by using MS/MS. Indeed, over the last decade, many methods for determining steroid hormones by either GC or liquid chromatography used either MS/MS (for example, (*7*, *8*, *30–36*)) or high-resolution mass spectrometry (for example, (*37*, *38*)) for their high specificity of qualitative identification and low detection-level capabilities.

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Finally, analyte concentrations are determined by using isotope-dilution quantification, a procedure also applied in some U.S. Environmental Protection Agency (USEPA) methods—for example, method 8290A for polychlorinated dioxins and furans (*39*) and methods 1698 and 539 for selected steroid hormones (*36*, *38*). The IDQ procedure corrects the analyte concentration for procedural losses due to many factors, including incomplete extraction, partial extract spills, low derivatization yield, matrix interferences, instrumental signal suppression or enhancement, or other mechanisms.

Use of Isotope-Dilution Standards

The method uses IDQ to enhance the accuracy of determined analyte concentrations by the addition of IDS compounds to all samples just before extraction, and to the calibration standards just before derivatization of the calibration standards and sample extracts. The IDS compounds (Figure 2) are direct or structurally similar stable-isotope labeled analogs of method analytes (Figure 1). Chemical behavior during sample preparation and analysis is considered to be nearly identical between the analyte and its corresponding IDS, especially when using an exact isotopic analog of an analyte. Thus, IDS recovery can be used as a proxy for absolute analyte recovery by the method.

Table II lists all the compounds determined by this method and which IDS is used for relative quantification for each method analyte. For 10 analytes (7 analytes for the validation data presented in this chapter as noted in Table II and (*26*)), an exact *d*- or ^{13}C-labeled isotopic analog is used for calibration and quantification. For the remaining analytes, an exact isotopic analog was either unavailable or unusable because of *d*-label instability, insufficient number of *d* atoms, inadequate standard purity, or prohibitive cost. For these remaining analytes, chemically similar IDS analogs are used for IDQ. The use of a non-exact isotopic analog for IDQ of an analyte can introduce some bias (either positive or negative) in the determined concentration of the corresponding analyte compared with use of an exact isotopic analog, because the absolute recovery of the IDS through the procedural steps might not exactly match the absolute recovery of the determined analyte. However, based on method performance results including those summarized in this chapter, analyte quantification typically was improved by applying the IDQ procedure in comparison to the traditional approach of quantifying analytes using an injection internal standard procedure alone. Analyte method recoveries obtained by using IDQ were consistently closer to the desired 100% optimum in comparison to the lower absolute recoveries observed for the IDS compounds in the validation matrices (see "Method Performance from Primary Validation Matrices" section).

Evaluation of Other Candidate IDS Compounds

Several exact-analog IDS compounds tested were deemed unsuitable for use. Most IDSs were received with ≥97% chemical purity and isotopic enrichment levels of approximately 98-atom percent. As a result, some quantity of the

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unlabeled analyte was always present in the labeled analog. This quantity decreases substantially as the number of labeled sites on the compound increases. It was determined that exact d_2 and $^{13}C_2$ IDS analogs of method analytes were not suitable for this analysis and that use of d_3 and $^{13}C_3$ IDS compounds would be marginal. Indeed, estriol-d_3 initially was used in the method, but had to be fortified into samples at 10% of the concentration used for the other hormone IDS compounds to minimize unlabeled estriol signal. To eliminate risk of false estriol signal from estriol-d_3 use, the stereoisomer 16-epiestriol-d_2 was substituted as the IDS for quantifying estriol for the validation results presented in this chapter; more recently, estriol-d_4 was substituted as the IDS for estriol (Table II). The IDS 17β-estradiol-2,4,16,16-d_4 initially was tested and used; however, although substantially more expensive, 17β-estradiol-$^{13}C_6$ was substituted for 17β-estradiol-2,4,16,16-d_4 because of improved purity. Equilin-2,4,16,16-d_4 initially was rejected because its derivative shares the same nominal parent mass (416.3 atomic mass units) as, and co-elutes with, the unlabeled 17β-estradiol derivative under the GC conditions used. Regardless of this interference issue, equilin-2,4,16,16-d_4 ultimately would have been rejected because of deuterium-label instability as discussed in the next section.

All exact-analog IDS compounds used in this method for which data are provided in this chapter had four or more labeled sites and were found to have acceptable purity. Use of 16-epiestriol-d_2, medroxyprogesterone-d_3, and nandrolone-d_3 containing less than four label positions was possible because the corresponding unlabeled compounds are not determined as method analytes. Although only 3 mass units higher than progesterone, progesterone-$^{13}C_3$ has been found sufficiently pure (no progesterone is detected at the IDS fortification concentration) to use as the exact-analog IDS for quantifying progesterone, which likely will provide improved IDQ accuracy for progesterone compared with that obtained using medroxyprogesterone-d_3 as its IDS (Table II; see "Surface Water" section). Our tests highlight the importance of considering the amount of labeling and purity when selecting a compound for use as an IDS.

Observations of Deuterium-Label Loss for Some IDS Compounds

Six exact-analog compounds initially tested and used in the method (4-androstene-3,17-dione-2,2,4,6,6,16,16-d_7; dihydrotestosterone-1,2,4,5a-d_4; estrone-2,4,16,16-d_4; norethindrone-2,2,4,6,6,10-d_6; testosterone-2,2,4,6,6-d_5, and progesterone-2,2,4,6,6,17a,21,21,21-d_9) were found to be susceptible to *d*-loss due to deuterium-hydrogen exchange. This exchange occurred in methanol extracts of environmental samples at one or more labeled positions on alpha-carbons adjacent to ketone functionalities through keto-enol tautomerization. The enolization process also led to double bond rearrangement resulting in *d*-losses even at the 6 position for 4-androstene-3,17-dione-d_7; norethindrone-d_6; testosterone-d_5, and progesterone-d_9. The amount of loss increased dramatically if the extracts were heated above ambient temperature during evaporation steps. Loss also occurred (at slower rates) even in IDS solutions in methanol stored primarily at –15°C (*40*).

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Loss of *d* labels results in low-biased recovery for the IDS (because only the expected IDS precursor ion (*m/z*) is monitored during MS/MS), which results in high biased analyte concentrations. Further, substantial loss of multiple labels from these IDSs can result in the detection of the precursor ion for the corresponding target analyte. The resulting "analyte" signal observed for a sample might be a partial (if some native analyte also is present in the sample) or complete false positive, and the resultant determined analyte concentration will be biased high by the low-biased IDS recovery when using IDQ. Therefore, these six isotopes were removed from the method beginning with samples prepared in 2010. The validation data summarized in this chapter do not include use of these six IDS compounds, except for the analyte holding-time studies that were conducted just before their elimination. Deuterium-hydrogen exchange was not observed for the deuterium-labeled IDS compounds listed in Table II because their *d*-labels are not situated adjacent to a ketone functional group.

It is noted that deuterium-hydrogen exchange might occur for these six isotopes, or structurally similar isotopes, used in other IDQ methods for hormones that use protic solvents; for example, USEPA method 1698 uses norethindrone-d_6 and progesterone-d_9 (*38*). Careful consideration of label type, position, and stability, along with isotope purity, is vital when evaluating any labeled-compound as an IDS or surrogate candidate, especially for methods that quantify analytes at extremely low concentrations in a wide variety of matrices.

Method Performance from Primary Validation Matrices

Unfiltered replicate samples from four validation matrices were used to test method performance: (1) reagent water, (2) surface water collected downstream from a WWTP discharge, (3) secondary WWTP effluent, and (4) primary WWTP effluent collected prior to any biological treatment. The non-reagent-water matrices were selected in part because they were collected from a location affected by municipal wastewater discharge (the surface water) or were from a specific treatment section of the WWTP flow path. They provide performance data for difficult sample matrices likely to be submitted for analysis. Unfiltered waters were used because they provide a more challenging test matrix than filtered water and presumably are a better indicator of overall method performance. Matrix collection is described in Foreman and others (*26*), and outlined in the subsections that follow. Several method analytes (especially cholesterol and 3β-coprostanol) are moderately hydrophobic and partition to suspended particles; thus, procedures were used to ensure optimal homogeneous splitting of sample replicates.

Replicates of each matrix were fortified with 17 of the method analytes to assess analyte recovery at 10 ng/L (also referred to as the "low" level) and 100 ng/L (the "high" level) fortification concentrations assuming a 0.5-L sample volume. Bisphenol A was added at 10-times higher and cholesterol and 3β-coprostanol at 100-times higher concentrations than the other analytes because these three analytes tend to occur in the environment at substantially higher concentrations and because they are blank limited (as described in the "Assessment

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of Blank Contamination and Determination of Detection and Reporting Levels for Blank-Limited Analytes" section). Fortification at sufficiently high levels relative to the ambient concentration reduces the uncertainty in compound recovery calculations. However, in a number of cases (especially for the primary effluent matrix), the ambient analyte concentrations approached or exceeded the fortification concentration by a substantial amount. In addition, four unspiked (ambient) replicates of each of the three non-reagent-water (field) sample matrices were analyzed to determine whether any method analytes were present and their concentrations. The validation samples were fortified with the isotope dilution standard compounds at 100 ng/L, except for cholesterol-d_7, which was added at 10,000 ng/L. The exception was the secondary effluent matrix, where bisphenol A-d_{16} was inadvertently spiked at a concentration 100 times higher than intended (see "Secondary Wastewater Effluent" section).

Mean analyte method recoveries (those based on analyte concentrations determined by the IDQ procedure) and relative standard deviations (RSDs) of recovery at the low- and high-fortification levels in the four test matrices are given in Tables III and IV, respectively, along with mean absolute recoveries and RSDs of the IDS compounds. In several matrices, the unfortified samples had detectable "ambient" concentrations of analytes (Table V) that complicate the recovery calculation. Analyte recovery in fortified samples is calculated by subtracting the mean ambient concentration from the determined concentration in the fortified sample and dividing by the fortification concentration.

Mean recovery of the IDS compound provides an estimate of absolute analyte recovery because the IDSs are quantified using a traditional injection internal standard approach. For example, if estrone-$^{13}C_6$ recovery is 50% in a given sample, then only half of its total fortified mass was in the final extract when analyzed by GC/MS/MS. By inference, the expected absolute recovery of estrone in this sample would be the same (50%) as estrone-$^{13}C_6$. Only half the mass of estrone originally in the sample would be in the final extract when analyzed. The IDQ procedure compensates for procedural losses in analyte mass in calculation of the analyte's sample concentration. If the IDS and analyte exactly emulate each other in procedure performance (absolute recovery) in a sample, then the analyte's IDQ-based method recovery in the sample should be near 100% (distributed around 100% based on sample replicates). Poor emulation of the analyte's performance by the IDS in a sample will produce a substantial bias (positive or negative) in analyte method recovery from the 100% ideal recovery.

Comparisons of analyte method recoveries relative to IDS absolute recoveries provide an assessment of the application of the IDQ procedure in this method when using both exact and non-exact isotopic analogs (Table II). Plots of relations between analyte method recoveries and IDS absolute recoveries for 12 representative analytes in the four validation matrices described in this section are shown in Figure 4 (see plots for all analytes in (*26*)). In these plots, recovery data are excluded for analytes having a mean ambient concentration in the matrix that exceeded 100% of the fortification concentration.

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Table I. Parameters for multiple-reaction monitoring (MRM) analysis of derivatized method compounds and other compounds used in the tandem mass-spectrometry acquisition method. [Precursor ion (boldface value) is the same for each MRM transition (except 11-ketotestosterone) and is the molecular ion (M^+) for most compounds. The transition product quantitation (Quant) ion and the primary (Q1) and secondary (Q2) qualifier ions are shown along with the argon gas collision energy (CE) used for each transition. Additional qualifier ion information are provided in (*26*). Compounds are listed in ascending gas chromatography retention time. eV, electron volts; IDS, isotope-dilution standard; IIS, injection internal standard; min, minutes; nd, not determined; RT, retention time; --, not monitored; ion values in atomic mass units]

Analyte, IDS, surrogate or IIS	*RT (min)*	*M^+*	*Precursor ion*	*Quant ion*	*CE (eV)*	*Q1 ion*	*CE1 (eV)*	*Q2 ion*	*CE2 (eV)*
Bisphenol A-d_{16} [a]	10.62	386.3	**368.2**	197.1	20	296.1	20	--	--
Bisphenol A	10.75	372.2	**357.2**	191.1	18	175.1	15	251.1	15
Diethylstilbestrol-d_8	12.57	420.3	**420.3**	374.2	22	220.1	18	--	--
trans-Diethylstilbestrol	12.65	412.2	**412.2**	217.1	18	179.1	20	231.2	18
cis-Androsterone-d_5	17.39	439.3	**439.3**	334.2	12	244.1	19	--	--
cis-Androsterone	17.46	434.3	**434.3**	329.3	14	239.2	18	169.2	20
Chrysene-d_{12} (IIS)	18.12	240.2	**240.2**	240.2	2	236.2	2	--	--
Epitestosterone	20.61	432.2	**432.2**	301.2	17	327.2	19	209.2	19
17α-Estradiol	21.16	416.3	**416.3**	285.2	18	326.2	6	232.2	15
Nandrolone-d_3	21.35	421.3	**421.3**	194.3	15	182.3	14	--	--
Dihydrotestosterone	21.49	434.3	**434.3**	195.2	16	143.2	16	187.2	15
4-Andostene-3,17-dione [b]	21.97	430.3	**430.3**	260.2	14	169.2	20	209.2	14
Estrone-$^{13}C_6$	22.11	420.3	**420.3**	314.3	17	404.3	17	--	--

Analyte, IDS, surrogate or IIS	*RT (min)*	*M^+*	*Precursor ion*	*Quant ion*	*CE (eV)*	*Q1 ion*	*CE1 (eV)*	*Q2 ion*	*CE2 (eV)*
Estrone	22.11	414.2	**414.2**	155.2	17	309.3	17	231.2	21
Testosterone	22.64	432.3	**432.3**	209.2	14	301.2	18	327.2	14
Equilin	22.93	412.2	**412.2**	182.1	23	231.2	23	307.2	16
17β-Estradiol-$^{13}C_6$	22.99	422.3	**422.3**	288.3	15	332.3	15	--	--
17β-Estradiol	22.99	416.3	**416.3**	285.3	16	232.2	15	129.1	15
11-Ketotestosterone	24.00	**518.3**[c]	**503.3** [d]	323.2	12	169.1	15	503.3	10
Norethindrone	25.31	442.3	**442.3**	302.3	10	287.2	17	194.2	19
Mestranol-d_4	25.38	386.3	**371.3**	195.1	16	169.1	16	--	--
Mestranol	25.50	382.2	**367.2**	193.1	16	167.1	16	173.1	16
Equilenin	26.31	410.2	**395.2**	305.2	8	279.2	12	213.2	17
17α-Ethynylestradiol-d_4	26.78	444.3	**429.3**	195.2	19	233.2	19	--	--
17α-Ethynylestradiol	26.90	440.3	**425.2**	193.2	19	231.2	19	205.2	17
Cholestane-d_6 (IIS)	29.00	378.4	**378.4**	121.1	20	223.2	5	--	--
Estriol-d_4	29.32	508.3	**508.3**	314.2	11	300.2	16	--	--
Estriol	29.44	504.3	**504.3**	311.3	15	296.2	15	270.2	18
Progesterone-$^{13}C_3$	30.50	461.3	**461.3**	447.2	8	357.2	4	446.2	8
Progesterone	30.50	458.3	**458.3**	157.2	19	353.3	12	235.2	13

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Table I. (Continued). Parameters for multiple-reaction monitoring (MRM) analysis of derivatized method compounds and other compounds used in the tandem mass-spectrometry acquisition method. [Precursor ion (boldface value) is the same for each MRM transition (except 11-ketotestosterone) and is the molecular ion (M^+) for most compounds. The transition product quantitation (Quant) ion and the primary (Q1) and secondary (Q2) qualifier ions are shown along with the argon gas collision energy (CE) used for each transition. Additional qualifier ion information are provided in (*26*). Compounds are listed in ascending gas chromatography retention time. eV, electron volts; IDS, isotope-dilution standard; IIS, injection internal standard; min, minutes; nd, not determined; RT, retention time; --, not monitored; ion values in atomic mass units]

Analyte, IDS, surrogate or IIS	*RT (min)*	*M^+*	*Precursor ion*	*Quant ion*	*CE (eV)*	*Q1 ion*	*CE1 (eV)*	*Q2 ion*	*CE2 (eV)*
16-Epiestriol-d_2	30.79	506.3	**506.3**	388.3	8	326.3	10	--	--
Medroxyprogesterone-d_3	33.25	563.3	**563.3**	318.3	16	331.3	14	--	--
3β-Coprostanol	34.75	460.4	**370.4**	215.2	8	257.2	10	313.2	8
Cholesterol-d_7	36.12	465.4	**375.4**	346.2	9	255.2	9	--	--
Cholesterol	36.25	458.4	**368.4**	339.2	9	255.2	9	159.1	10

[a] Derivative M^+ is d_{14}. [b] For 4-andostene-3,17-dione, the more responsive 430.3-to-234.2 precursor-to-product ion transition initially was selected as the quantitation ion, but was subsequently omitted because of interferences observed in some matrices. [c] Precursor ion used for transitions to Q2 product ion. [d] Precursor ion used for transitions to Quant and Q1 product ions.

Table II. Method analyte and the corresponding isotope-dilution standard (IDS) used for its quantification. [Table sorted based on IDS grouping; exact isotopic analogs of analytes used for the validation data presented in this chapter are shown in bold]

Method analyte	*Isotope-dilution standard*[a]
17α-Ethynylestradiol	**17α-Ethynylestradiol-d_4**
17α-Estradiol	17β-Estradiol-$^{13}C_6$
17β-Estradiol	**17β-Estradiol-$^{13}C_6$**
Equilenin	17β-Estradiol-$^{13}C_6$
Bisphenol A	**Bisphenol A-d_{16}**
3β-Coprostanol	Cholesterol-d_7
Cholesterol	**Cholesterol-d_7**
cis-Androsterone	*cis*-Androsterone-d_5 [b]; Nandrolone-d_3
trans-Diethylstilbestrol	**Diethylstilbestrol-d_8**
Estriol	Estriol-d_4 [c]; 16-Epiestriol-d_2
Equilin	Estrone-$^{13}C_6$
Estrone	**Estrone-$^{13}C_6$**
Progesterone	Progesterone-$^{13}C_3$ [d], Medroxyprogesterone-d_3
Mestranol	**Mestranol-d_4**
11-Ketotestosterone	Nandrolone-d_3
4-Androstene-3,17-dione	Nandrolone-d_3

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Table II. (Continued). Method analyte and the corresponding isotope-dilution standard (IDS) used for its quantification. [Table sorted based on IDS grouping; exact isotopic analogs of analytes used for the validation data presented in this chapter are shown in bold]

Method analyte	*Isotope-dilution standard*[a]
Dihydrotestosterone	Nandrolone-d_3
Epitestosterone	Nandrolone-d_3
Norethindrone	Nandrolone-d_3
Testosterone	Nandrolone-d_3

[a] All IDSs are quantified relative to injection internal standard chrysene-d_{12} except for cholesterol-d_7, estriol-d_4, and 16-epiestriol-d_2 that are quantified relative to cholestane-d_6. [b] *cis*-Androsterone-d_5 was implemented as the IDS for quantifying *cis*-androsterone for samples collected on or after October 1, 2011. For the validation data summarized in this report, the non-exact IDS analog nandrolone-d_3 was used as the IDS for quantifying *cis*-androsterone because *cis*-androsterone-d_5 previously was not available. [c] Estriol-d_4 was implemented as the IDS for quantifying estriol for samples collected on or after March 17, 2011. For the validation data summarized in this report, the stereoisomer 16-epiestriol-d_2 was used as the non-exact IDS analog for quantifying estriol because estriol-d_4 previously was not available. [d] Progesterone-$^{13}C_3$ was implemented as the IDS for quantifying progesterone for samples collected on or after March 1, 2012. For the validation data summarized in this report, the non-exact IDS analog medroxyprogesterone-d_3 was used as the IDS for quantifying progesterone because progesterone-$^{13}C_3$ previously was not available.

Reagent Water

Mean IDS recoveries in the reagent-water validation matrix ranged from 67 to 93%, with RSDs <7% (Tables III and IV). Mean method recoveries of the analytes in the reagent-water validation replicates ranged from 78–120%, with RSDs ≤12% for all analytes except equilin, which had an RSD of 22% in high-level spikes. These recoveries are within the target method performance range of 60–120% mean recovery and ≤25% RSD (see (*26*)).

A comparison between analyte and IDS recoveries for these reagent-water validation replicates illustrates the expected differences between absolute IDS recovery and the corresponding analyte's method recovery obtained by using the IDQ procedure (Figure 4). At least theoretically, each analyte's method recovery always will be greater than the IDS's absolute recovery (for an exact isotopic IDS) and is expected to be near 100% if the analyte and corresponding IDS emulate each other in absolute recovery during sample preparation and analysis. For example, the mean absolute recovery of mestranol-d_4 in the low-level reagent-water spikes was 74%, whereas the mean method recovery for mestranol analyte was 98% (Table III). These expected recovery differences between analytes and IDS compounds warrant consideration by data users when interpreting method performance data. All analyte method recoveries fell within 60–120% for all reagent-water validation replicates (Figure 4; see also (*26*)), except for equilin in one replicate that was biased high (Figure 4D), and bisphenol A in six low-level replicates that ranged between 120 and 130% (Figure 4I). The high bias in BPA recovery in the low-level replicates is likely from unaccounted for contamination introduced during sample preparation for this blank-limited compound (see "Assessment of Blank Contamination and Determination of Detection and Reporting Levels for Blank-Limited Analytes" section).

Surface Water

Replicate samples of the surface-water matrix used for method validation were collected on April 14, 2010, from Rapid Creek about 50 meters downstream from a WWTP outfall near Rapid City, SD., and had a pH of 8.16 and specific conductance of 930 microsiemens/cm at 25°C; concentrations of total suspended solids and dissolved organic carbon (DOC) were not determined. Mean IDS recoveries in the surface-water matrix spikes ranged from 46 to 88%, with RSDs <12%, except for medroxyprogesterone-d_3 that had mean recoveries <32% and RSDs as high as 44% and diethylstilbestrol-d_8 that had mean recoveries <24% (RSDs <7%) (tables III–V). Indeed for these two IDSs plus bisphenol A-d_{16} and nandrolone-d_3, the recovery bias in the surface-water validation matrix was distinctly low.

Mean recoveries for most analytes ranged from 71 to 144%, with RSDs <17%. Recoveries for cholesterol and 3β-coprostanol were more variable in the low-level spikes because of high mean ambient concentrations (Table V) that were >1,400% of the fortification level of 1,000 ng/L. Progesterone was poorly recovered (<12% mean) in the surface-water matrix (Tables III and IV). Progesterone's loss in this matrix was substantially greater in most replicates

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than that for its corresponding IDS, medroxyprogesterone-d_3 (Figure 4L), indicating that the IDQ procedure using this non-exact IDS analog did not adequately compensate for the amount of progesterone loss in this matrix. This is one example of the limitation of the IDQ procedure when non-exact isotopic analogs are used; the absolute recovery of the analyte is not well emulated by its corresponding IDS. Nevertheless, IDQ resulted in lower negative bias of progesterone concentrations in these surface-water matrix spikes than provided by traditional quantification procedures.

Medroxyprogesterone-d_3 and especially progesterone had poor recoveries in this matrix and some other field matrices tested outside of the validation study (see "Isotope-Dilution Standard Absolute Recoveries in Laboratory Reagent-Water Spike and Blank Samples, and in Field-Sample Matrices" section), the cause for which is unknown. In addition, the half-life of the di-(trimethylsilyl)-derivative of progesterone was determined to be about 3.5 days at ambient temperature (data not shown); although not specifically characterized, the half-life is substantially longer when the extract is stored at <–15°C. Derivatized medroxyprogesterone-d_3 appears to be stable at ambient temperature. When non-exact IDSs are selected, it is beneficial to consider and attempt to match as closely as possible the relative reactivities of the analyte and its corresponding IDS.

Based on its observed performance characteristics (*26*), all progesterone concentrations are qualified as estimated. Although progesterone concentrations might be biased low in some matrices, it has been retained as an analyte in this method because of evidence that progesterone might have environmental effects at concentrations substantially lower than those for some other method analytes (*41*). It has been consistently detected using this method in influent or primary effluent samples collected from WWTPs. Substitution of exact analog progesterone-$^{13}C_3$ (Table II) is expected to improve quantitative accuracy of progesterone by this method.

The mean recovery of diethylstilbestrol (75%) in the surface-water matrix is lower than in the other validation matrices (Tables III and IV; Figure 4J), but is well within the target performance range of 60–120% mean recovery and demonstrates the advantage and applicability of the IDQ procedure even when the IDS recovery is low (in this case, about 20% recovery for diethylstilbestrol-d_8 at both spiking levels).

Secondary Wastewater Effluent

Replicates samples of the secondary wastewater effluent matrix used during method validation were collected on March 11, 2010, from a WWTP in New York (identified as NY3 by Phillips and others (*42*)), and had a pH of 6.9 (data provided by the plant operator on a separate aliquot); concentrations of DOC and total suspended solids were not determined, although total suspended solids from effluents samples collected monthly by the plant operator are normally <4 mg/L. Use of ascorbic acid was not necessary for this matrix because ultra-violet treatment is used instead of chlorination for disinfection by this WWTP. Mean IDS recoveries in these secondary wastewater effluent matrix spikes ranged from

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53 to 110%, with RSDs <14% (Tables III and IV). RSDs for most of the IDS compounds in the unspiked (ambient) replicates were somewhat higher than in the spiked replicates but were still <23% (Table V).

Mean recoveries for most analytes ranged from 75 to 117%, with RSDs <15%. Bisphenol A-d_{16} was spiked in error at 100 times the normal fortification level in both the low and high spikes, and this error probably produced the high biased recoveries (means of about 170%) for bisphenol A, which are not plotted in Figure 4I. Equilin had unusually high recoveries (>220% mean) at both spike levels (Tables III and IV), which is not readily explained because no interference was apparent from the GC/MS/MS analysis and equilin was not detected in the unspiked ambient replicate samples for this matrix (Table V). The IDS estrone-$^{13}C_6$, which is used to quantify equilin, had highly reproducible recoveries of about 78% in these spikes (Figure 4D). In previous performance testing in various matrices (not presented), equilin was found to have more variable recoveries; thus, all equilin concentrations are qualified as estimated.

Primary Wastewater Effluent

Replicate samples of the primary wastewater effluent matrix used for method validation were collected June 29, 2010, from a WWTP in New York (site identified as NY2-I and referred to as an "influent" sampling location in (*42*)). The sampling location was after partial particle removal by sedimentation from the incoming WWTP flow but before any biological or other treatments. This was the most challenging validation matrix examined due to the presence (observational only) of high amounts of dissolved, colloidal, and particulate organic matter. In many cases, the presence of high concentrations of method analytes in the ambient replicates relative to the fortification levels (10 and 100 ng/L) confounded or prevented accurate calculation of analyte recovery. However, use of this type of matrix was considered an important test of method performance because municipal wastewater can be a major source of steroids to the environment, depending on level of treatment and other operational conditions (for example, treatment bypass of wastewater during storm events (*43*)). Primary effluent contains elevated concentrations of steroid hormones compared to secondary-treated effluent because the particle-removal and biological processes used by many WWTPs as secondary treatment remove a substantial fraction of trace organic compounds, including estrogens and especially androgens and progestins that are present in primary effluent (*44*). Studies designed to test the efficiency of engineered technologies for removal of the method analytes during the WWTP processes will necessarily examine this type of complex sample matrix; for example, see (*45*) and references therein.

Mean IDS recoveries in the spiked primary wastewater effluent replicates ranged from 41 to 88%, with RSDs less than 23% (Tables III and IV). The estrogen isotopes 17β-estradiol-$^{13}C_6$, estrone-$^{13}C_6$, and 17α-ethynylestradiol-d_4, had somewhat lower recoveries in this complex matrix compared to the other matrices (Figures 4A, C, K). High ambient concentrations (Table V) precluded reporting recoveries for bisphenol A, testosterone, and progesterone in the low-level spikes

and for 3β-coprostanol, androstenedione, cholesterol, and cis-androsterone in the low- and high-level spikes. Mean recoveries for those analytes with ambient concentrations less than 25% of the fortification level ranged from 64 to 149% (RSDs <19%), with only 17α-estradiol (149% in low-level spikes, 138% in high-level spikes) having mean recoveries outside the target performance range of 60–120% (Figure 4B).

Also shown in Tables III and IV (in bold and bold italics type) are recoveries and RSDs for several analytes that had sizable ambient concentrations. Although recovery variation as described by the RSD was substantially greater than 25% for some of these analytes, especially in the low-level spikes, mean recoveries ranged from 46 to 147% in this complex matrix and were within the target performance range of 60–120% for most of the analytes, demonstrating reasonable method performance at both low- and high-fortification levels in the presence of substantial co-extracted organic material.

Analyte Variability in Unspiked Validation Matrices

Table V shows mean ambient concentrations and RSDs of analytes determined in the quadruplicate samples for the three non-reagent-water (unspiked) validation matrices. Concentrations for some analytes were in the low range of the method (near detection levels), whereas others were in the upper range (100–1000 ng/L for most analytes), especially in the primary wastewater effluent matrix. Indeed, 3β-coprostanol, cholesterol, androstenedione, and cis-androsterone concentrations were determined using dilutions of the primary wastewater effluent matrix extracts. The RSDs were ≤27% for all analytes in these matrices, except 3β-coprostanol in the secondary wastewater effluent matrix.

Comparison of Validation Results

Recoveries in all matrices were within the target performance range of 60–120% for most analytes (Tables III and IV; Figure 4). There were significant, albeit small, differences in recovery performance between matrices. For each method analyte, pairwise comparisons were made of the performance for each possible pair of matrices and fortification level based on individual sample recoveries using the nonparametric Wilcoxon rank-sum test (Helsel and Hirsch, 2002). The IDS compounds were used to evaluate relative performance between matrices because IDS recovery was not biased by ambient concentrations. Reagent water showed the best overall performance relative to the other matrices; out of 60 possible IDS comparisons in paired matrices, recovery was as good or better in reagent water than the other matrix 86% of the time (p-value <0.05). The three field matrices were compared to one another, excluding reagent water (40 possible comparisons). The secondary wastewater effluent had the best average IDS recovery (as good or better recovery in 77% of these possible comparisons), followed by surface water (70%), and the primary wastewater effluent (50%).

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Table III. Mean recovery and variability of the method analytes fortified at low levels in replicate samples of reagent water, surface water, secondary wastewater effluent, and primary wastewater effluent. [N, number of replicates; NR, not reported because ambient concentrations >300% of fortified amount, which produced substantially skewed recoveries; RSD, relative standard deviation. Some values might have additional bias due to concentrations in the ambient sample from 25–150% (bold values), from 151–300% (*bold italicized* values), or >300% (*bold italicized* values with footnote) of the fortified amount. Fortification level was 10 ng/L for 17 analytes, 100 ng/L for bisphenol A, 320 or 1,000 ng/L for 3*β*-coprostanol, and 1,000 ng/L for cholesterol, assuming a 0.5-L sample volume. Isotope-dilution standards fortified at 100 ng/L, except cholesterol-d_7 at 10,000 ng/L. Shown are method recoveries for analytes and absolute recoveries for isotope dilution standards, see text.]

Compound	*Reagent water (N = 9)*		*Surface water (N = 8)*		*Secondary waste-water effluent (N = 9)*		*Primary wastewater effluent (N = 8)*	
	Mean (percent)	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*
				Method analytes				
11-Ketotestosterone	100	3.2	83.4	16.3	106	2.9	***145*** [a]	***33.1*** [a]
17α-Estradiol	103	1.6	103	7.8	107	5.5	149	8.3
17α-Ethynylestradiol	98.7	3.5	82.2	9.3	92.8	1.6	86.4	13.0
17β-Estradiol	102	1.9	92.3	8.4	103	4.4	**94.3**	**10.8**
3β-Coprostanol	95.7	2.9	***113*** [a]	***65.1*** [a]	**89.9**	**21.0**	NR	NR
Androstenedione	98.3	2.7	109	9.9	97.3	4.4	NR	NR
Bisphenol A	120	6.4	72.5	8.0	**171** [b]	**5.7**	NR	NR
Cholesterol	83.1	2.3	***174*** [a]	***39.9*** [a]	88.1	15.1	NR	NR
cis-Androsterone	96.6	2.7	118	8.7	80.2	4.9	NR	NR

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Table III. (Continued). Mean recovery and variability of the method analytes fortified at low levels in replicate samples of reagent water, surface water, secondary wastewater effluent, and primary wastewater effluent. [N, number of replicates; NR, not reported because ambient concentrations >300% of fortified amount, which produced substantially skewed recoveries; RSD, relative standard deviation. Some values might have additional bias due to concentrations in the ambient sample from 25–150% (bold values), from 151–300% (*bold italicized* values), or >300% (*bold italicized* values with footnote) of the fortified amount. Fortification level was 10 ng/L for 17 analytes, 100 ng/L for bisphenol A, 320 or 1,000 ng/L for 3β-coprostanol, and 1,000 ng/L for cholesterol, assuming a 0.5-L sample volume. Isotope-dilution standards fortified at 100 ng/L, except cholesterol-d_7 at 10,000 ng/L. Shown are method recoveries for analytes and absolute recoveries for isotope dilution standards, see text.]

Compound	*Reagent water (N = 9)*		*Surface water (N = 8)*		*Secondary waste-water effluent (N = 9)*		*Primary wastewater effluent (N = 8)*	
	Mean (percent)	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*
	Method analytes							
Dihydrotestosterone	98.0	4.4	107	13.9	96.0	6.5	***85.9*** [a]	***102*** [a]
Epitestosterone	97.5	1.8	121	6.7	104	3.1	***126*** [a]	***27.9*** [a]
Equilenin	93.5	5.7	92.6	11.6	82.8	6.7	63.8	18.1
Equilin	91.3	9.5	107	9.1	287 [c]	10.0	***120*** [a]	***84.5*** [a]
Estriol	88.4	3.1	75.1	11.0	88.3	2.9	***75.5*** [a]	***224*** [a]
Estrone	103	2.2	**93.5**	**8.1**	103	4.8	***110*** [a]	***38.4*** [a]
Mestranol	97.8	3.6	93.3	7.0	101.7	4.2	82.3	9.3
Norethindrone	98.3	3.7	99.4	9.2	95.5	4.2	97.4	9.1
Progesterone	90.8	3.2	8.9	25.3	75.4	5.2	NR	NR

Compound	Reagent water (N = 9)		Surface water (N = 8)		Secondary waste-water effluent (N = 9)		Primary wastewater effluent (N = 8)	
	Mean (percent)	RSD (percent)	Mean (percent)	RSD (percent)	Mean (percent)	RSD (percent)	Mean (percent)	RSD (percent)
Method analytes								
Testosterone	97.5	4.1	104	8.7	94.0	4.4	NR	NR
trans-Diethylstilbestrol	95.2	3.5	76.1	9.2	93.3	2.5	88.1	6.9
Isotope-dilution standards								
16-Epiestriol-d_2	78.3	6.0	88.4	6.3	80.1	4.7	87.1	11.5
17α-Ethynylestradiol-d_4	80.8	4.4	77.8	4.1	76.1	4.0	66.5	10.8
17β-Estradiol-$^{13}C_6$	80.1	3.6	66.1	4.5	72.0	4.1	57.5	10.8
Bisphenol A-d_{16}	81.5	4.4	58.9	6.2	52.9	6.5	65.9	22.6
Cholesterol-d_7	78.6	3.8	71.6	6.1	72.9	13.7	44.4	9.1
Diethylstilbestrol-d_8	67.3	5.7	19.8	5.2	55.2	5.7	83.6	10.8
Estrone-$^{13}C_6$	79.6	4.9	77.5	5.6	78.6	3.5	65.1	9.9
Medroxyprogesterone-d_3	78.4	4.8	26.5	38.7	110	6.3	82.8	8.1
Mestranol-d_4	74.5	4.5	70.7	3.5	70.3	4.7	67.4	7.4
Nandrolone-d_3	81.4	4.5	55.0	7.8	85.0	3.7	61.2	10.6

[a] Values provided to show recovery and RSD even when the ambient or interference concentration exceeded 300% of amount fortified. [b] High bias in the bisphenol A recovery likely due to fortification of bisphenol A-d_{16} at 100-times normal level in error for the secondary wastewater effluent. [c] Equilin recovery in the secondary wastewater effluent was unexpectedly high for unknown reasons; no interference was noted.

Table IV. Mean recovery and variability of the method analytes fortified at high levels in replicate samples of reagent water, surface water, secondary wastewater effluent, and primary wastewater effluent. [See table III headnote. Fortification level was 100 ng/L for 17 analytes, 1,000 ng/L for bisphenol A, 3,200 or 10,000 ng/L for 3β-coprostanol, and 10,000 ng/L for cholesterol assuming a 0.5-L nominal sample volume.]

Compound	*Reagent water (N = 9)*		*Surface water (N = 8)*		*Secondary waste-water effluent (N = 8)*		*Primary wastewater effluent (N = 8)*	
	Mean (percent)	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*
				Method analytes				
11-Ketotestosterone	104	6.5	70.6	11.9	117	6.8	**87.7**	**6.1**
17α-Estradiol	101	7.1	104	5.8	114	3.7	138	5.0
17α-Ethynylestradiol	97.8	4.8	81.5	2.3	94.8	3.3	97.8	3.0
17β-Estradiol	104	6.9	94.4	6.0	108	4.6	94.6	4.4
3β-Coprostanol	107	6.2	82.3	6.7	141	6.4	NR	NR
Androstenedione	100	5.9	124	5.3	106	2.3	NR	NR
Bisphenol A	98.1	6.2	82.8	3.5	168 [a]	13.6	**101**	**7.9**
Cholesterol	102	5.7	78.8	4.1	108	5.9	NR	NR
cis-Androsterone	91.2	5.7	144	8.9	96.6	3.2	NR	NR
Dihydrotestosterone	94.9	5.3	116	8.6	106	2.5	***88.1***	***9.9***
Epitestosterone	96.4	5.5	131	6.4	109	2.1	**110**	**2.6**
Equilenin	78.4	12.0	92.8	4.6	82.4	7.1	103	6.7

Compound	*Reagent water (N = 9)*		*Surface water (N = 8)*		*Secondary waste-water effluent (N = 8)*		*Primary wastewater effluent (N = 8)*	
	Mean (percent)	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*
				Method analytes				
Equilin	118	22.4	122	3.3	222 [b]	8.9	**147**	**11.7**
Estriol	92.4	4.8	79.9	3.3	89.3	2.9	***106***	***29.7***
Estrone	100	4.1	93.2	3.9	105	4.0	**116**	**8.0**
Mestranol	98.8	5.8	92.1	4.3	104	3.8	92.9	3.1
Norethindrone	103	5.4	96.6	5.2	99.6	5.4	102	2.7
Progesterone	88.8	6.7	11.9	40.0	78.6	4.9	**45.9**	**35.6**
Testosterone	99.2	5.7	122	7.1	98.8	3.1	***103***	***9.2***
trans-Diethylstilbestrol	92.4	4.6	74.7	2.6	98.7	3.0	95.1	3.2
				Isotope-dilution standards				
16-Epiestriol-d_2	88.5	5.5	87.7	4.2	77.2	6.7	88.0	7.8
17α-Ethynylestradiol-d_4	89.4	3.4	79.1	2.3	72.0	4.3	59.1	3.9
17β-Estradiol-$^{13}C_6$	88.4	5.2	67.4	5.2	69.7	3.3	51.8	5.9
Bisphenol A-d_{16}	93.0	6.2	58.5	5.1	56.5	13.9	79.6	4.8
Cholesterol-d_7	70.0	5.6	87.5	6.4	62.7	10.8	41.3	14.0
Diethylstilbestrol-d_8	76.6	3.8	21.9	6.5	55.9	10.0	73.5	4.7

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Table IV. (Continued). Mean recovery and variability of the method analytes fortified at high levels in replicate samples of reagent water, surface water, secondary wastewater effluent, and primary wastewater effluent. [See table III headnote. Fortification level was 100 ng/L for 17 analytes, 1,000 ng/L for bisphenol A, 3,200 or 10,000 ng/L for 3β-coprostanol, and 10,000 ng/L for cholesterol assuming a 0.5-L nominal sample volume.]

Isotope-dilution standards								
Estrone-$^{13}C_6$	90.0	3.6	78.9	4.9	78.2	3.3	61.5	4.8
Medroxyprogesterone-d_3	91.5	8.0	15.6	43.7	104	11.0	84.8	6.8
Mestranol-d_4	79.8	5.2	71.6	3.6	67.1	4.0	59.2	3.9
Nandrolone-d_3	86.2	2.6	45.9	11.8	81.3	3.5	60.0	4.2

[a] High bias in the bisphenol A recovery likely due to fortification of bisphenol A-d_{16} at 100-times normal level in error for the secondary wastewater effluent. [b] Equilin recovery in the secondary wastewater effluent was unexpectedly high for unknown reasons; no interference was noted.

Table V. Bias and variability of method analyte concentrations and isotope-dilution standard recoveries in unspiked, quadruplicate samples of validation matrices from surface water, and secondary- and primary-wastewater effluent. [nd, not detected; ng/L, nanograms per liter; RSD, relative standard deviation; <, less than; --, not applicable

Compound	*Surface water*		*Secondary wastewater effluent*		*Primary wastewater effluent*	
	Mean (ng/L)	*RSD (percent)*	*Mean (ng/L)*	*RSD (percent)*	*Mean (ng/L)*	*RSD (percent)*
			Method analytes			
11-Ketotestosterone	nd	--	nd	--	40.7	7.9
17α-Estradiol	0.1	18.2	nd	--	nd	--
17α-Ethynylestradiol	nd	--	nd	--	nd	--
17β-Estradiol	0.6	18.2	nd	--	9.4	7.1
3β-Coprostanol	13,920	7.8	147 [a]	69.9	816,100 [b]	15.1
Androstenedione	2.0	18.6	1.2 [c]	15.3	424 [b]	12.6
Bisphenol A	14.7 [a]	1.5	25.8 [a,d]	7.9	705	11.2
Cholesterol	17,060	6.7	113 [a]	14.5	1,249,000 [b]	10.6
cis-Androsterone	1.9	14.8	<2.4 [c]	1.6	2,315 [b]	27.1
Dihydrotestosterone	nd	--	nd	--	127	4.8
Epitestosterone	nd	--	nd	--	47.7	3.0
Equilenin	nd	--	nd	--	nd	--

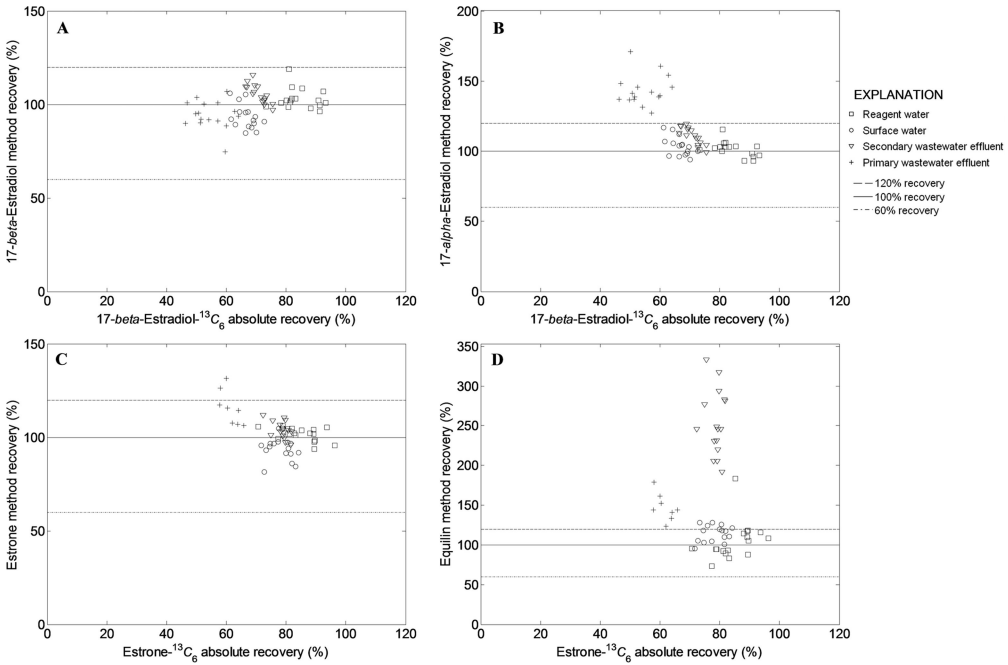
Continued on next page.

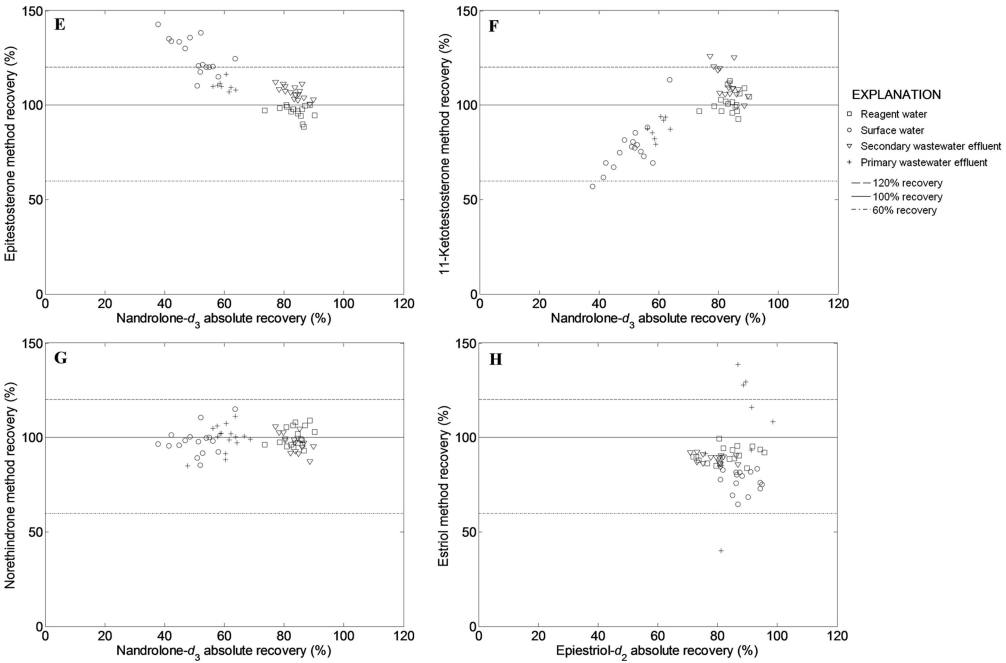
Table V. (Continued). Bias and variability of method analyte concentrations and isotope-dilution standard recoveries in unspiked, quadruplicate samples of validation matrices from surface water, and secondary- and primary-wastewater effluent. [nd, not detected; ng/L, nanograms per liter; RSD, relative standard deviation; <, less than; --, not applicable

Compound	*Surface water*		*Secondary wastewater effluent*		*Primary wastewater effluent*	
	Mean (ng/L)	*RSD (percent)*	*Mean (ng/L)*	*RSD (percent)*	*Mean (ng/L)*	*RSD (percent)*
Method analytes						
Equilin	2.6 [c]	15.0	nd	--	<57 [c]	10.6
Estriol	1.9	14.0	nd	--	234	4.4
Estrone	4.8	9.4	nd	--	54.9	7.2
Mestranol	nd	--	nd	--	nd	--
Norethindrone	nd	--	nd	--	0.6	21.3
Progesterone	nd	--	nd	--	36	25.6
Testosterone	0.5 [c]	--	nd	--	171	3.6
trans-Diethylstilbestrol	0.5 [c]	19.9	nd	--	nd	--
Isotope-dilution standards						
Compound	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*
16-Epiestriol-d_2	94.4	4.0	85.2	11.1	99.3	1.8
17α-Ethynylestradiol-d_4	82.0	3.4	91.4	16.7	82.0	7.7

Isotope-dilution standards						
Compound	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*	*Mean (percent)*	*RSD (percent)*
17β-Estradiol-$^{13}C_6$	69.8	3.7	92.6	21.6	71.3	8.4
Bisphenol A-d_{16}	61.9	1.4	87.6 [d]	20.1	88.4	8.2
Cholesterol-d_7	77.0	8.5	52.2	12.3	48.5	9.3
Diethylstilbestrol-d_8	23.9	5.3	74.9	22.4	99.1	5.9
Estrone-$^{13}C_6$	82.1	3.9	101	18.1	79.2	12.3
Medroxyprogesterone-d_3	31.2	21.2	101	5.0	89.5	2.1
Mestranol-d_4	74.5	4.2	85.2	17.4	81.0	8.2
Nandrolone-d_3	59.1	6.8	101	16.4	79.8	8.1

[a] The mean ambient concentration was used for correction of matrix spike recoveries even though its value is less than the analyte's minimum reporting level. [b] Extract dilution was required to quantify the analyte in replicates from the primary wastewater effluent. [c] Mean unspiked concentration shown was used for background correction of spike recoveries, but the concentration in one or more of the unspiked replicates was not reported because mass spectral qualification criteria were not met to confirm analyte presence. [d] Bisphenol A-d_{16} was fortified at 100-times normal level in error for one replicate of the secondary wastewater effluent. The high-biased bisphenol A and bisphenol A-d_{16} values from that replicate were omitted from the calculation of the mean.





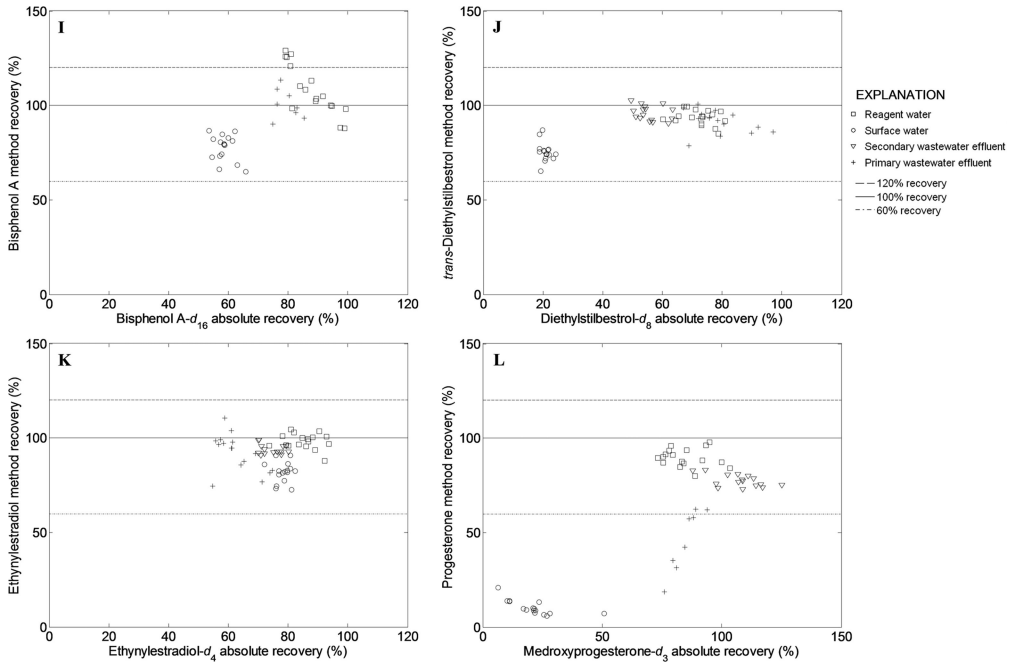


Figure 4. Relation between analyte method recoveries and isotope-dilution standard absolute recoveries in spiked replicates of reagent water, surface water, secondary wastewater effluent, and primary wastewater effluent validation matrices. Samples with mean ambient analyte concentrations that exceeded the fortified concentrations were excluded (see text).

The pattern of higher reagent-water recoveries compared to other three matrices was not as consistent for the analytes as for the IDS compounds. This is likely due to two factors. First, certain analytes were present in the ambient samples at levels comparable to or sometimes exceeding the fortification concentrations (Table V), which adds uncertainty to recovery calculations. Second, analyte concentrations are calculated relative to the IDS compounds, so there is a possibility of some analyte bias (positive or negative) if the performance of the analyte is not exactly emulated by (is better or worse than, respectively) the corresponding IDS compound (Figure 4). For example, the somewhat higher mean recoveries for epitestosterone (121% in low-level spikes and 131% in high-level spikes) in the surface-water matrix indicate that this analyte experienced less absolute mass loss during sample preparation relative to its corresponding non-exact androgen IDS compound, nandrolone-d_3 (Figure 4E). Conversely, 11-ketotestosterone, also quantified versus nandrolone-d_3, exhibited correspondingly lower biased recoveries in these surface-water replicates (Figure 4F). Interestingly, absolute recoveries for the synthetic progestin norethindrone were well emulated by the androgen IDS nandrolone-d_3, because norethrindrone method recoveries clustered near 100% in all matrices (Figure 4G). Overall, the IDQ procedure provided acceptable method recoveries for most analytes in these four validation matrices.

When all the recovery data are aggregated by matrix, overall mean IDS recovery in the validation matrices is highest in reagent water, followed by secondary wastewater effluent, primary wastewater effluent, and surface water: overall mean range of 61–82%; overall RSD range of 10–38%. The overall mean IDS recovery in the surface-water matrix is biased low by a matrix effect producing low medroxyprogesterone-d_3 and diethylstilbestrol-d_8 recoveries (Tables III and IV). Again, the magnitude of the observed differences between the four matrices generally was small.

Omitting recovery data for analytes that have mean ambient concentrations in the matrix greater than 300% of the fortification concentration, overall mean recovery of the analytes in the four matrices ranged from 93 to 110% (overall RSDs of 10–37%). Although the differences between matrices were generally less than 20%, statistical analysis shows that the highest overall mean recovery for the analytes was in the secondary wastewater effluent matrix, followed by the primary wastewater effluent matrix, and the reagent water, with the lowest overall recovery in the surface-water matrix. Further comparative discussion is presented in (*26*).

Long-Term Estimates of Method Performance

Although the method-validation tests provided a consistent and comparable treatment using four different sample matrices, longer-term assessment of method performance was available from QA/QC data associated with field samples prepared and analyzed during application as research methods to support a variety of USGS field projects. Data for six analytes and five IDSs presented in this section are associated with 247 filtered-water samples analyzed by method 2434 and 578 unfiltered-water samples analyzed by method 4434 during 2009–2010.

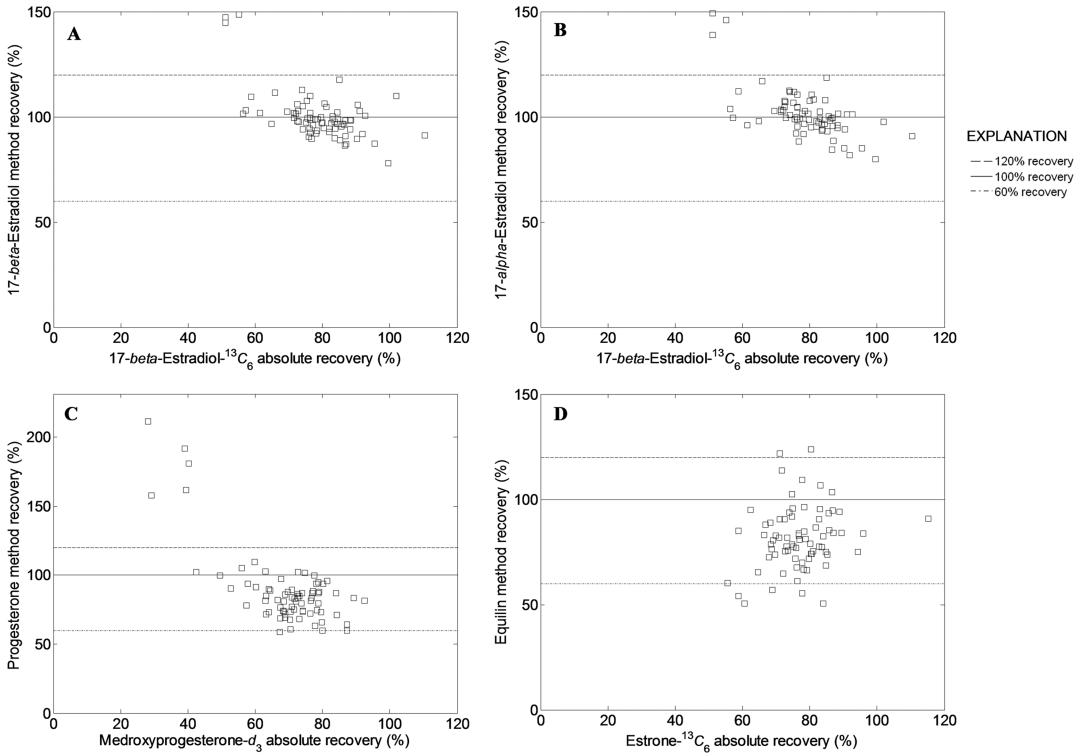
Data for the remaining method analytes and IDS compounds are from samples (92 samples by method 2434 and 316 samples by method 4434) prepared in 2010 only, following the implementation of substitute IDS compounds for reasons described in the "Use of Isotope-Dilution Standards" section. The performance data are provided from laboratory reagent-water spike (LRS) and laboratory reagent-water blank (LRB) samples analyzed with each set of 10 (or more) environmental samples. In addition, IDS recoveries provide a measure of method performance (comparable to surrogate recoveries) in the wide variety of sample matrices submitted for analysis that ranged from source groundwater to WWTP influent and animal feeding operation matrices. All samples were fortified with approximately 100 ng/L of nine IDS compounds and 10,000 ng/L of cholesterol-d_7, assuming a 0.5-L sample volume.

Analyte Method Recoveries in Laboratory Reagent-Water Spike Samples

In addition to the fortified reagent-water replicates analyzed as a specific method validation matrix, as many as 113 laboratory reagent-water spike samples were analyzed in conjunction with custom sample analyses; these samples provide an estimate of method performance in this reagent matrix over an extended time period. All LRSs were fortified with 25 ng/L of 17 method analytes, 250 ng/L of bisphenol A, 800 or 2,500 ng/L of 3β-coprostanol, and 2,500 ng/L of cholesterol, assuming a 0.5-L sample volume. These fortification concentrations fall between those used for the low-level (10 ng/L for most analytes) and high-level (100 ng/L) reagent-water validation replicates.

Analyte method recoveries in the LRSs relative to IDS absolute recoveries are shown in Figure 5 for eight representative analytes (see plots and statistical summaries for all analytes in (*26*)); these plots also include recoveries for the reagent-water validation replicates shown in Figure 4. Mean analyte recoveries in the LRSs ranged from 84 to 104% and generally are similar to those obtained from the low- and high-level reagent-water validation replicates (Tables III and IV). The RSDs were ≤25% for all analytes in the LRSs except estriol (28%), 11-ketotestosterone (29%), and progesterone (36%), and, not unexpectedly, were greater than the RSDs from the reagent-water validation tests.

Although summaries using parametric statistics are the primary comparative performance descriptors, several nonparametric statistical descriptors also were calculated including: (1) median recoveries, which compare well with the mean recoveries for many compounds; and (2) relative F-pseudosigma (RF_σ), a "robust" indicator of relative variation based on the interquartile range of the data about the median (*46*). Unlike RSD, RF_σ is not strongly influenced by extreme outliers in the data distribution. If the distribution is Gaussian, or nearly so, then RSD and RF_σ are expected to be similar in magnitude because the variation will be (nearly) symmetric about the mean and median, which themselves should be (nearly) identical. The RF_σ values were <16% for all analytes in the LRSs and are substantially less than the corresponding RSDs for estriol and 11-ketotestesterone that were strongly influenced by unusually low recoveries in as many as five LRS samples (Figures 5F and H).



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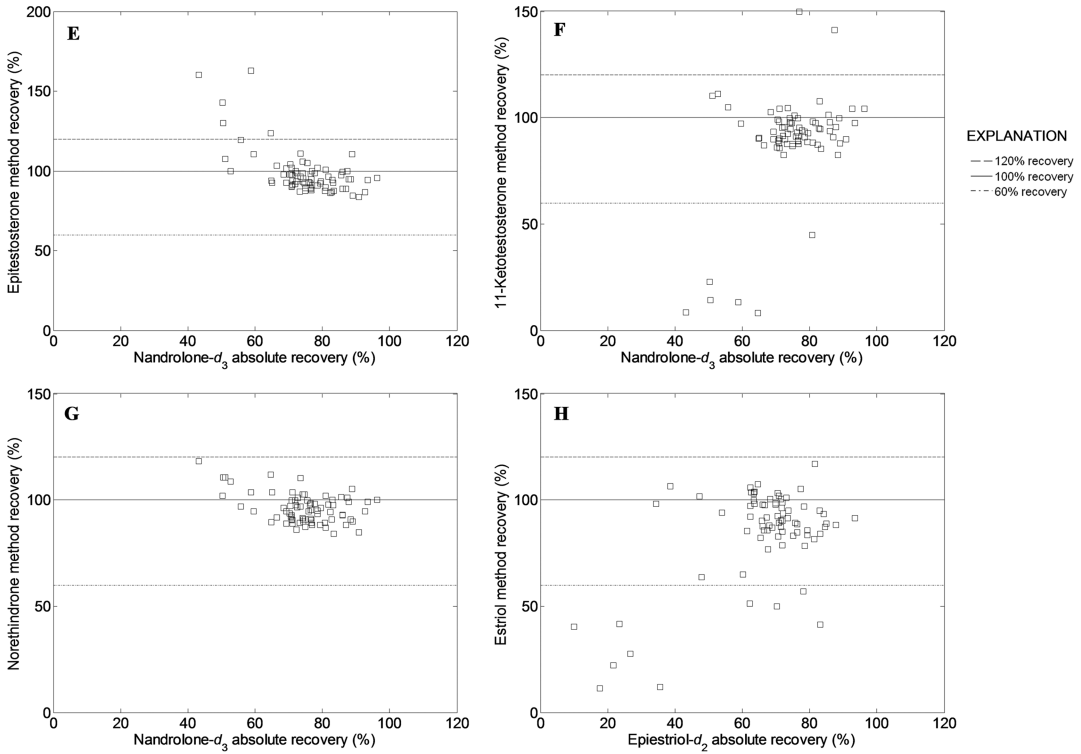

Figure 5. Relation between analyte method recoveries and isotope-dilution standard absolute recoveries for eight analytes in laboratory reagent-water spike samples prepared in 2010.

The reason for the low estriol and 11-ketotestesterone recoveries in several LRS samples (and four matrix-spike samples; see "Compound Recoveries in Other Spiked-Matrix Samples" section) is unknown, but might be attributed to either incomplete recovery during Florisil SPE or derivatization limitations. Estriol was found to elute incompletely (15% maximum estriol retention) from a larger 2-g Florisil cleanup column used in a complementary method developed at the NWQL for the determination of steroid hormones in solids (method description summarized in (*47*)) when using 25 mL of a 5% methanol in dichloromethane solution. Although it can not be completely discounted, the extensive loss of estriol and 11-ketotestosterone in the few LRS (and lab matrix spike) samples probably did not occur when using the smaller 1-g Florisil SPE cleanup columns in this water method.

Most analytes and IDS compounds contain one or (more commonly) two C–OH or C=O functional groups (Figures 1 and 2) that are converted to trimethylsilyl derivatives upon reaction with MSTFA, whereas estriol and 16-epiestriol-d_2 have three C–OH groups and 11-ketotestosterone has two C–OH groups and one C=O group that require conversion to trimethylsilyl derivatives. Thus, it is possible that derivatization of all three functional groups on estriol and 11-ketotestosterone was incomplete in these particular samples compared to their corresponding IDS compounds (16-epiestriol-d_2 and nandrolone-d_3, respectively) and compared to the other analytes and their corresponding IDS compounds. If derivatization of the analyte is complete in the calibration standards but not in a given sample matrix, whereas derivatization of the corresponding IDS is complete in both the calibration standards and the given sample matrix, then the analyte's performance is not being well emulated by its IDS in that sample matrix and the determined analyte concentration (or recovery) in that sample will be biased low. Improvements in matrix-specific method recoveries for estriol are expected by the recent substitution of the exact isotopic analog estriol-d_4 for 16-epiestriol-d_2 (see Table II).

Isotope-Dilution Standard Absolute Recoveries in Laboratory Reagent-Water Spike and Blank Samples, and in Field-Sample Matrices

Mean IDS recoveries in the laboratory reagent-water spike and blank (LRS/B) samples range from 64 to 81% (Table VI), and are similar overall with those obtained for the reagent-water validation matrix (especially the low-level spikes) shown in Tables III and IV. Mean recoveries were somewhat lower in the LRS/B samples for 16-epiestriol-d_2, medroxyprogesterone-d_3, and nandrolone-d_3 compared to the reagent-water validation matrix (Table III–IV). The RSDs in the LRS/B samples were ≤21% except for 16-epiestriol-d_2 (27%), and greater than observed for the reagent-water validation.

A summary of IDS recoveries in all ambient field-sample matrices that were analyzed by method 4434 is shown in Table VI (values in bold). Mean IDS recoveries for field samples by this method ranged from 48 to 84% (and were similar between methods 2434 and 4434 for most IDS compounds; see method

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2434 data and performance comparison discussion in (*26*)). Mean IDS recoveries in field samples generally were similar to those observed for LRS/B samples, although variability in the field-sample matrices was greater than observed with the reagent-water (LRS/B) samples for some IDSs. The "All field matrices" summary largely reflects recoveries from surface-water matrices for five IDSs, and from surface water and WWTP effluent matrices for the other IDSs.

Table VI also summarizes IDS recoveries grouped by water matrix (medium) type. For most samples, the water matrix classification listed is based on the USGS National Water Information System (NWIS) medium code used by field staff (see definitions in (*48*)). Three matrix types without unique NWIS medium codes suspected to be "more complex" were grouped separately: (1) WWTP influent (includes primary effluent) samples, (2) hog manure slurry samples, and (3) surface-water samples believed to have been affected by a hog manure-waste spill event. The matrix types listed are not represented for all 10 IDS compounds because samples for some matrix types were prepared before implementation of substitute IDS compounds as described in the "Use of Isotope-Dilution Standards" section.

Mean IDS recoveries were greater than 60% (with many in the range of 70 to 90%) in most matrices and generally were of a similar magnitude between matrix types (or between methods 2434 and 4434 within a matrix type; see (*26*)). Similarly, RSDs were ≤25% for most matrices and of comparable magnitude for many matrix types regardless of method. These data indicate that the analytical method is applicable to diverse water matrix types. However, recoveries were substantially lower or more variable, or both, for one or more IDS compounds in some matrix types, especially those having a more "complex" makeup (higher amounts of suspended solids, DOC, and coextracted organic matter compared to other matrices), including WWTP influent (especially unfiltered samples), hog manure slurry, and sludge samples. In addition, 16-epiestriol-d_2 had somewhat lower recoveries in the groundwater matrix for method 2434, and medroxyprogesterone-d_3 had lower recoveries (with high variability) in the surface-water matrices for method 4434 because of unusually low (<20%) or no recovery in some samples. The relatively low number of "complex" matrix samples might be influencing their resultant statistics, but clearly the method does not work as well for these matrices compared to less "complex" ones.

The assumption in this IDQ-based method is that the absolute recovery of progesterone in a given sample matrix is closely emulated by the recovery of medroxyprogesterone-d_3. As noted previously in the "Surface Water" section for the surface-water validation matrix, recoveries of medroxyprogesterone-d_3 were particularly low in some surface-water matrices. Based on the validation and lab matrix-spike (see next section) test samples, the absolute medroxyprogesterone-d_3 recovery was similar to the absolute progesterone recovery in some matrices. When they differed, the progesterone absolute recovery typically was less than the medroxyprogesterone-d_3 recovery (sometimes substantially lower), leading to progesterone method recoveries that remained well below the 100% optimum recovery even when using IDQ. Only occasionally was this condition reversed, leading to a high biased progesterone recovery. As a consequence, reported progesterone concentrations might be biased low in a given sample matrix, but

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are less likely to be biased high. For this and other reasons noted previously, progesterone concentrations are qualified as estimated.

Compound Recoveries in Other Spiked-Matrix Samples

Various field-water matrices were spiked just before extraction with the analytes and IDS compounds at the same fortification concentrations as used for the LRSs to assess method recovery performance from environmental waters. These included 5 filtered and 18 unfiltered samples. Sample details and recovery data are provided in (*26*). Comparisons of analyte method recoveries relative to IDS absolute recoveries for the matrix spike samples are shown in Figure 6 for eight representative compounds. Mean analyte recoveries in the 23 matrix-spike samples ranged from 66% (progesterone) to 141% (3β-coprostanol). RSDs were ≤25% for all but six analytes. High ambient concentrations disproportionally bias some of these means, especially for cholesterol and 3β-coprostanol. Median recoveries range from 70% (progesterone) to 105% (epitestosterone). For most analytes, mean and median recoveries differed by no more than 5%. The RF_σ values were <22% for all analytes except equilin (36% RF_σ), which had a broad recovery range (41–174%; Figure 6D), and progesterone (65% RF_σ), which had an even broader recovery range (0–271%; Figure 6C). Two non-detections and all other low (<46%) progesterone recoveries occurred in the unfiltered surface-water matrices. As noted in the "Surface Water" section, the cause of these progesterone losses is unknown. The recovery of progesterone's IDS, medroxyprogesterone-d_3, was relatively low in some of these samples, but not in others. Loss of progesterone in these matrices clearly was not well emulated by this non-exact IDS analog. More variable matrix-spike recoveries also might be expected for progesterone because it has a wide range of potential transformation pathways compared to other steroids (*49*).

11-Ketotestosterone and estriol had unusually low recoveries (<31%) in the same four unfiltered samples (Figures 6F and 6H), comprising three different matrix types. Recoveries of their corresponding IDSs likewise clearly did not emulate the analytes' absolute recoveries in these matrices because the analyte method recoveries were lower than the IDS abolute recoveries. Possible reasons for these low recoveries were described in the "Analyte Method Recoveries in Laboratory Reagent-Water Spike Samples" section.

Although method recoveries for most analytes in the spiked matrices were within a range of 80–120%, the matrix-spike recovery results highlight that using an isotope-dilution quantification procedure still might be insufficient to compensate for matrix-specific performance limitations. This is especially the case for those analytes not having exact isotopic analogs. More importantly, these results emphasize the importance of including matrix-spike samples as a QA component in environmental studies for steroid hormones and other compounds.

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Table VI. Bias and variability of recoveries for isotope-dilution standard compounds from laboratory reagent-water spike and blank samples and by water matrix type from ambient field samples collected in 2009–2010 and analyzed by unfiltered-water method 4434. [N, number of samples; RFσ, relative F-pseudosigma; RSD, relative standard deviation; WWTP, wastewater treatment plant; %, percent; --, not applicable]

Water matrix	*N*	*Mean (%)*	*RSD (%)*	*Median (%)*	*RF_σ (%)*	*Minimum (%)*	*Maximum (%)*
16-Epiestriol-d_2							
Lab spikes and blanks	103	63.8	27.2	66.1	12.6	10.0	93.4
Blended, untreated water supply	40	74.3	18.8	78.5	12.7	34.8	94.5
Effluent, not landfill	48	76.7	27.1	80.7	13.4	23.8	117
Groundwater	8	71.9	11.8	70.1	7.7	63.9	90.5
Hog manure slurry	1	12.6	--	--	--	--	--
Surface water	199	74.1	17.7	75.9	16.1	37.2	104
Treated water supply	7	77.7	14.0	77.3	2.1	61.4	98.5
WWTP influent/primary effluent	13	79.1	47.5	69.1	53.7	24.3	154
All field matrices	**316**	**74.6**	**22.0**	**76.9**	**17.1**	**12.6**	**154**
17α-Ethynylestradiol-d_4							
Lab spikes and blanks	228	78.4	10.7	78.2	10.0	53.7	101
Blended, untreated water supply	73	83.1	16.6	80.1	10.0	51.8	128
Effluent, not landfill	151	81.6	20.1	79.8	15.9	41.4	135
Groundwater	27	70.5	14.4	72.5	11.0	48.3	90.3

Water matrix	*N*	*Mean (%)*	*RSD (%)*	*Median (%)*	*RF_σ (%)*	*Minimum (%)*	*Maximum (%)*
Hog manure slurry	3	65.6	13.9	66.9	10.0	55.9	74.1
Hog manure spill impacted stream	16	77.9	14.1	79.5	11.9	58.7	96.6
Sludge	3	48.1	21.8	45.7	16.6	39.0	59.5
Surface water	236	77.3	15.8	79.1	13.6	48.2	119
Treated water supply	14	86.1	16.8	89.7	18.0	51.3	103
WWTP influent/primary effluent	52	85.6	27.1	79.7	35.6	40.4	148
All field matrices	**578**	**79.7**	**19.3**	**79.1**	**14.9**	**39.0**	**148**
17β-Estradiol-$^{13}C_6$							
Lab spikes and blanks	103	77.8	14.9	78.3	13.4	46.0	110
Blended, untreated water supply	40	77.4	6.2	77.8	3.5	62.6	88.3
Effluent, not landfill	48	83.5	14.0	82.2	11.5	61.5	111
Groundwater	8	76.0	17.0	75.0	16.6	57.7	92.0
Hog manure slurry	1	2.4	--	--	--	--	--
Surface water	199	71.1	18.9	73.8	21.1	38.2	98.4
Treated water supply	7	79.4	13.7	77.7	10.5	60.6	93.7
WWTP influent/primary effluent	13	70.1	29.5	64.2	27.3	45.8	118
All field matrices	**316**	**73.8**	**19.0**	**76.8**	**17.7**	**2.4**	**118**

Continued on next page.

Table VI. (Continued). Bias and variability of recoveries for isotope-dilution standard compounds from laboratory reagent-water spike and blank samples and by water matrix type from ambient field samples collected in 2009–2010 and analyzed by unfiltered-water method 4434. [N, number of samples; RFσ, relative F-pseudosigma; RSD, relative standard deviation; WWTP, wastewater treatment plant; %, percent; --, not applicable]

Water matrix	*N*	*Mean (%)*	*RSD (%)*	*Median (%)*	*RF_σ (%)*	*Minimum (%)*	*Maximum (%)*
Bisphenol A-d_{16}							
Lab spikes and blanks	228	81.4	13.3	82.4	10.4	27.3	115.5
Blended, untreated water supply	73	91.9	17.7	91.5	12.6	48.0	132
Effluent, not landfill	151	86.4	22.6	85.6	24.7	43.3	151
Groundwater	27	78.0	18.4	79.2	10.3	52.3	125
Hog manure slurry	3	81.3	61.2	105	22.6	24.1	114
Hog manure spill impacted stream	16	102	14.9	97.1	19.5	81.3	127
Sludge	3	23.1	124	8.6	222	4.5	56.1
Surface water	236	86.2	19.0	88.8	22.6	53.8	145
Treated water supply	14	94.3	14.3	95.4	7.5	61.6	123
WWTP influent/primary effluent	52	53.0	72.4	55.2	79.9	0.2	156
All field matrices	**578**	**83.9**	**27.6**	**87.1**	**24.1**	**0.2**	**156**
Cholesterol-d_7							
Lab spikes and blanks	228	67.8	13.6	67.7	12.9	17.4	97.7
Blended, untreated water supply	73	73.7	10.1	74.1	7.9	57.5	88.7

Water matrix	*N*	*Mean (%)*	*RSD (%)*	*Median (%)*	*RF_σ (%)*	*Minimum (%)*	*Maximum (%)*
Effluent, not landfill	151	69.1	17.7	69.5	17.5	41.7	100
Groundwater	27	68.6	11.2	68.9	8.0	47.2	81.9
Hog manure slurry	3	31.3	43.2	37.8	24.2	15.8	40.5
Hog manure spill impacted stream	16	67.1	16.1	66.7	16.8	48.4	84.3
Sludge	3	28.8	51.8	25.7	42.3	15.7	45.0
Surface water	236	73.9	12.9	75.6	12.3	48.2	108
Treated water supply	14	71.2	10.7	73.3	9.1	57.2	85.6
WWTP influent/primary effluent	52	51.1	39.6	51.6	51.4	17.7	103
All field matrices	**578**	**69.6**	**19.5**	**72.3**	**15.2**	**15.7**	**108**
Diethylstilbesterol-d_8							
Lab spikes and blanks	228	65.1	14.0	64.3	13.8	38.4	93.4
Blended, untreated water supply	73	41.5	29.8	40.4	32.5	19.0	80.6
Effluent, not landfill	151	70.8	24.6	68.9	23.3	13.5	109
Groundwater	27	58.5	21.6	58.1	15.4	33.7	99.2
Hog manure slurry	3	63.8	50.1	53.8	42.4	38.1	99.6
Hog manure spill impacted stream	16	44.2	58.2	37.0	66.2	16.0	92.4
Sludge	3	39.1	98.2	31.7	88.6	4.9	80.6

Continued on next page.

Table VI. (Continued). Bias and variability of recoveries for isotope-dilution standard compounds from laboratory reagent-water spike and blank samples and by water matrix type from ambient field samples collected in 2009–2010 and analyzed by unfiltered-water method 4434. [N, number of samples; RFσ, relative F-pseudosigma; RSD, relative standard deviation; WWTP, wastewater treatment plant; %, percent; --, not applicable]

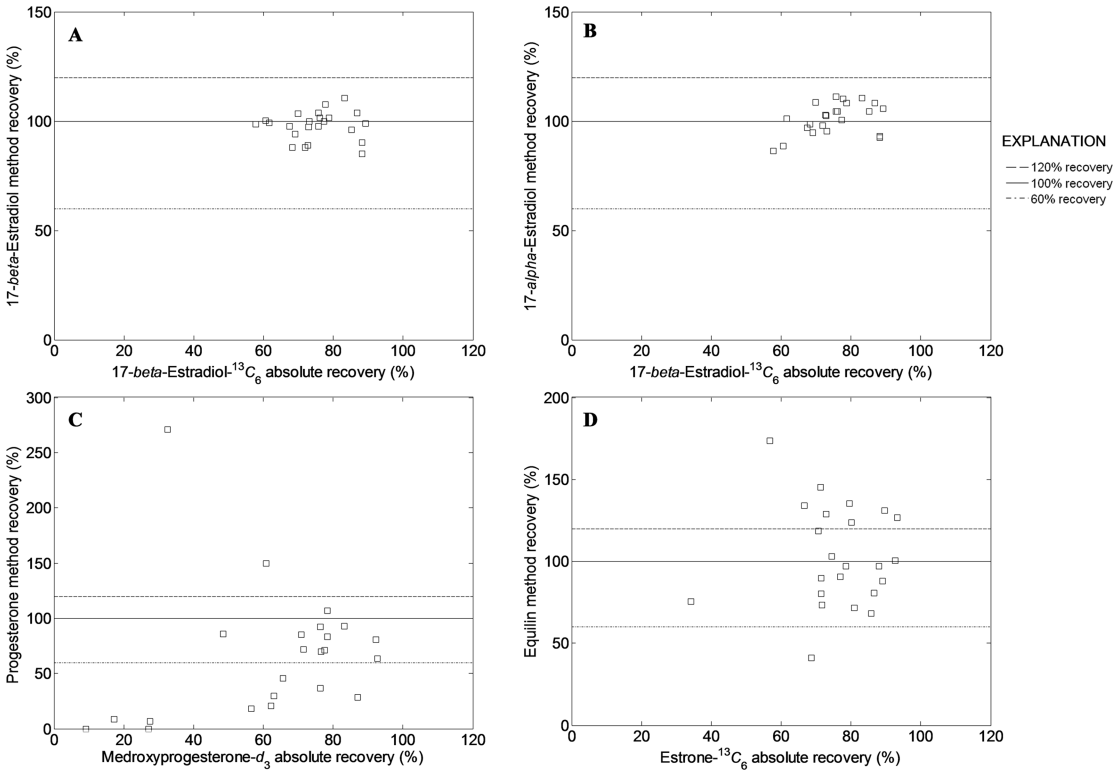
Water matrix	*N*	*Mean (%)*	*RSD (%)*	*Median (%)*	*RF_σ (%)*	*Minimum (%)*	*Maximum (%)*
Surface water	236	49.3	34.9	47.8	40.6	16.1	85.2
Treated water supply	14	65.9	19.8	63.6	25.5	47.4	85.5
WWTP influent/primary effluent	52	74.5	30.8	71.6	23.7	25.5	134
All field matrices	**578**	**57.1**	**37.1**	**56.7**	**39.3**	**4.9**	**134**
Estrone-$^{13}C_6$							
Lab spikes and blanks	103	75.6	12.6	75.1	12.0	51.9	115
Blended, untreated water supply	40	79.3	6.5	79.4	5.3	61.3	93.9
Effluent, not landfill	48	89.0	27.0	89.7	17.9	0.0	127
Groundwater	8	74.5	27.5	78.2	14.7	34.1	97.5
Hog manure slurry	1	87.3	--	--	--	--	--
Surface water	199	77.3	16.0	78.7	13.0	49.6	104
Treated water supply	7	80.2	19.0	78.5	11.8	53.1	100
WWTP influent/primary effluent	13	51.7	48.7	58.6	30.9	4.0	93.4
All field matrices	**316**	**78.3**	**21.0**	**79.6**	**13.0**	**0.0**	**127**
Medroxyprogesterone-d_3							

Water matrix	*N*	*Mean (%)*	*RSD (%)*	*Median (%)*	*RF_σ (%)*	*Minimum (%)*	*Maximum (%)*
Lab spikes and blanks	103	69.8	20.8	71.7	14.1	20.9	95.5
Blended, untreated water supply	40	47.6	50.2	45.9	56.7	0.7	94.8
Effluent, not landfill	48	88.1	26.1	89.7	20.0	18.8	128
Groundwater	8	75.9	21.3	78.7	17.1	48.5	95.1
Hog manure slurry	1	12.9	--	--	--	--	--
Surface water	199	37.9	65.7	36.0	86.3	0.0	114
Treated water supply	7	77.9	19.3	76.4	10.4	50.2	98.5
WWTP influent/primary effluent	13	30.2	95.0	19.0	172	0.5	83.0
All field matrices	**316**	**48.2**	**63.8**	**46.8**	**77.8**	**0.0**	**128**
Mestranol-d_4							
Lab spikes and blanks	228	73.5	10.2	73.3	9.2	50.9	98.1
Blended, untreated water supply	73	80.3	10.6	78.9	9.1	56.1	105
Effluent, not landfill	151	78.1	17.7	77.5	13.1	45.5	125
Groundwater	27	72.2	12.7	72.0	11.7	51.6	85.6
Hog manure slurry	3	61.1	34.4	51.2	27.7	46.9	85.2
Hog manure spill impacted stream	16	77.2	9.0	79.0	9.0	64.3	87.7
Sludge	3	49.9	34.1	56.6	21.0	30.6	62.6

Continued on next page.

Table VI. (Continued). Bias and variability of recoveries for isotope-dilution standard compounds from laboratory reagent-water spike and blank samples and by water matrix type from ambient field samples collected in 2009–2010 and analyzed by unfiltered-water method 4434. [N, number of samples; RFσ, relative F-pseudosigma; RSD, relative standard deviation; WWTP, wastewater treatment plant; %, percent; --, not applicable]

Water matrix	*N*	*Mean (%)*	*RSD (%)*	*Median (%)*	*RF_σ (%)*	*Minimum (%)*	*Maximum (%)*
Surface water	236	76.3	14.9	77.8	12.4	50.5	125
Treated water supply	14	79.2	13.7	84.0	10.9	49.5	91.7
WWTP influent/primary effluent	52	85.2	26.5	82.4	24.1	50.0	166
All field matrices	**578**	**77.8**	**17.3**	**77.9**	**12.7**	**30.6**	**166**
Nandrolone-d_3							
Lab spikes and blanks	103	73.2	15.1	73.8	9.3	43.3	96
Blended, untreated water supply	40	66.3	19.3	68.3	14.0	21.7	85.6
Effluent, not landfill	48	91.4	31.2	89.3	25.0	0.6	147
Groundwater	8	77.1	30.4	84.5	10.4	30.3	104
Hog manure slurry	1	0.7	--	--	--	--	--
Surface water	199	61.4	26.0	62.6	31.9	7.1	92.5
Treated water supply	7	83.0	19.6	84.9	8.4	49.9	101
WWTP influent/primary effluent	13	67.2	66.3	64.4	49.4	5.1	170
All field matrices	**316**	**67.5**	**34.0**	**67.4**	**27.6**	**0.6**	**170**



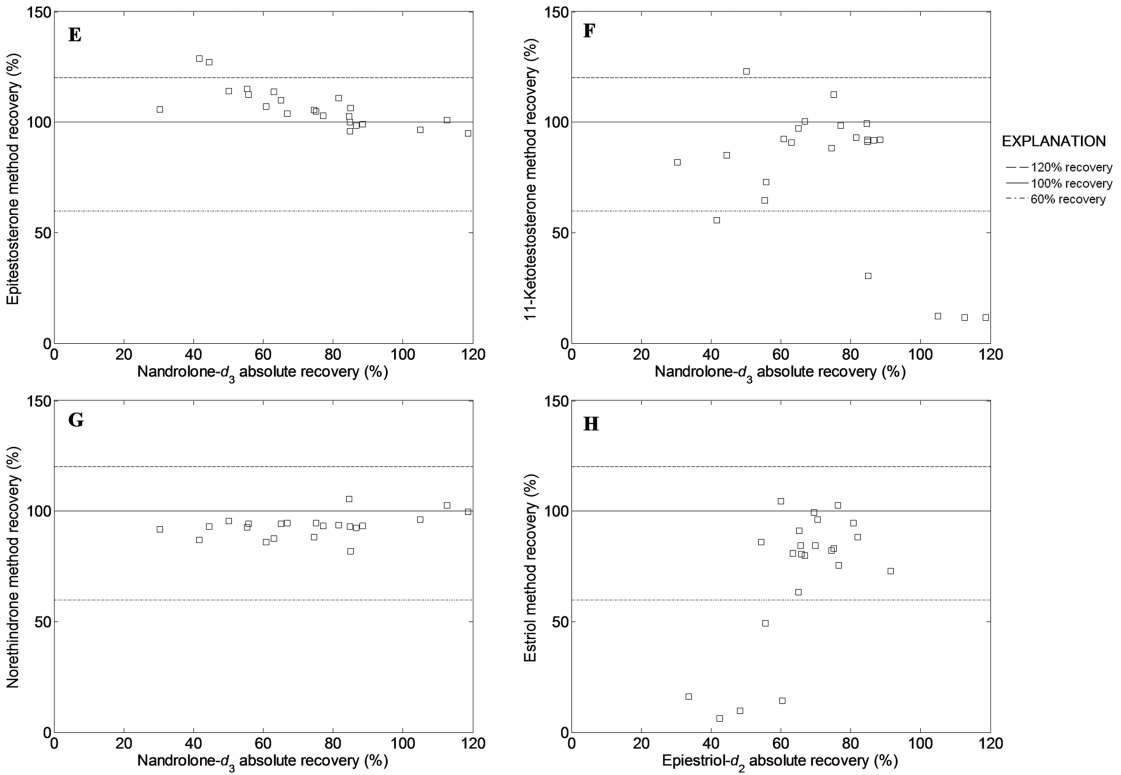


Figure 6. Relation between analyte method recoveries and isotope-dilution standard absolute recoveries for eight analytes in laboratory matrix-spike samples.

Cholesterol-d_7 Recoveries in Nonsalted Reagent Water

Sodium chloride (salt) is added to all sample matrices before extraction, and is done specifically to improve the absolute recoveries of cholesterol-d_7, cholesterol, and 3β-coprostanol only in reagent-water matrices. In method testing and sample analyses before 2009, recoveries of cholesterol-d_7 were found to be especially poor (about 10% on average) in reagent-water-based matrices, including all lab and field-blank samples and reagent water spike samples.

Loss of these three sterol compounds in unsalted reagent-water samples occurs because of their incomplete isolation on the GFF/C18 disks (referred to as sorbent breakthrough) during the SPE step (see test details and data in (*26*)). Mean recoveries for cholesterol-d_7, cholesterol, and 3β-coprostanol in extracted water filtrate that had passed through the GFF/C18 disks ranged from 50–63%, revealing that the majority of these three compounds are not retained by the disks when extracting these compounds from reagent water. No more than 2% of any other analyte or IDS was observed in the filtrate, demonstrating excellent SPE efficiency from reagent water by the disks for the non-sterol compounds. Mean recoveries of the sterols in the standard 40-mL methanol elution of the disks ranged from 34 to 45%, whereas recoveries for the other 17 analytes and 11 IDSs tested were 97% or greater in this standard elution volume.

Three other components of sample extraction were tested to assess possible sterol losses and showed no substantial correlation. (1) The GFF/C18 disks wash step contained no analytes or IDS compounds at recoveries greater than 1% (cholesterol the most). This step, which removes some unwanted coextracted matrix before the final disk elution step, does not reduce sterol recoveries. (2) Following the standard elution of the GFF/C18 disks with 40 mL of methanol, the disks were eluted with 20 mL of dichloromethane (DCM) to see if an alternative strong solvent was required to provide complete compound elution. No more than 3.4% (cholesterol-d_7) of any analyte or IDS compound was found in this eleunt, indicating that the standard methanol elution volume was sufficient to achieve high recovery of the method compounds from the disks. (3) The glass sample bottles (used instead of HDPE bottles at that time) were rinsed with 20 mL of DCM to check for compounds that adhere to the bottle surface, which might be expected for lower solubility compounds, such as cholesterol and 3β-coprostanol, and has been observed for pesticides (for example, dichlorodiphenyltrichloroethane [DDT] and permethrin) with low water solubility (*50*). This bottle rinse contained no more than 3% (again cholesterol-d_7) of any analyte or IDS compound.

One possible mechanism for the substantial sorbent breakthrough by the sterols might be related in part to their water solubility. Based on partition theory, compounds like cholesterol and 3β-coprostanol that have lower water solubility compared to the other method analytes are predicted to partition strongly from water to solid sorbents such as C18 (*51*, *52*), and, likewise, to suspended particles, colloids, and dissolved organic matter (*53*). Thus, good isolation (high sorbent retention and little sorbent breakthrough) on C18 of these sterols during extraction of spiked reagent water might be expected. However, partitioning from water to any sorbent requires that the compound be truly "dissolved" in the water.

The substantial amount of sorbent breakthrough by the sterols during SPE of non-salted reagent water indicates that the cholesterol and 3β-coprostanol might not be completely dissolved. If a substantial portion of these analytes partitions to the small amount of DOC (typical DOC concentration is less than 0.016 mg/L in the reagent water) or to any fine particulate matter in the reagent water, or both, then this non-dissolved portion will not be available to partition to the C18 sorbent and also might not be physically "captured" by either the C18 disk matrix or the GFF that overlays the disk.

Landrum and Giesy (*54*) observed substantial breakthrough on an Amberlite™ XAD-4 sorbent column of benzo(a)pyrene, a compound with very low water solubility, when spiked into reagent water. Breakthrough was worse when DOC was added to the water because this compound partitions to the DOC that is not well retained by the sorbent. De Llasera and others (*55*) also noted increasing breakthrough of moderately polar pesticides on C18 sorbent with increasing sample DOC concentration. Foreman and Foster (*50*) observed 14% breakthrough using stacked 10-g C18 SPE sorbent columns for DDT in reagent water, whereas pesticides with somewhat higher water solubilities (for example, dichlorodiphenyldichloroethene (DDE) and atrazine) exhibited no breakthrough. Based on these findings, samples with high concentrations of DOC or fine colloids might lead to incomplete isolation (breakthrough) on C18 of the sterols (and possibly other method analytes) during extraction. Indeed, somewhat lower and more variable cholesterol-d_7 recoveries were observed in matrices with higher suspended solid and DOC concentrations (WWTP influent and primary effluent, sludge, hog manure slurry samples) compared with other matrices (Tables III–VI). Nevertheless, cholesterol-d_7 recoveries even in these complex matrices (salted or not) usually were substantially greater than the recoveries obtained from non-salted reagent water.

The addition of NaCl to reagent water appears inconsequential to recoveries of the other method analytes based on IDS recoveries before and after salt use was implemented in January 2009. The actual mechanism underlying the improved sorbent retention of sterols when extracted from reagent water containing salt is unknown. Increasing the ionic strength of aqueous-phase solutions by salt addition is known to increase the retention of ionic compounds on C18 (*56*).

Assessment of Blank Contamination and Determination of Detection and Reporting Levels for Blank-Limited Analytes

Inadvertent contamination of samples with analytes might occur because (1) many are common biogenic compounds, (2) some are used pharmaceutically or in personal-care products, and (3) bisphenol A has extensive commercial applications (*57*). The scope of analyte contamination potential is monitored by using laboratory- and field-blank samples. In addition, consideration of blanks is an integral component in the determination of detection levels, especially for blank-limited analytes as addressed in this section.

Bisphenol A, cholesterol, and 3β-coprostanol are ubiquitous low-level procedural (sample preparation) contaminants that were detected frequently in laboratory and field reagent-water blank samples. Data for these three blank-limited analytes are reported using a minimum reporting level (MRL) convention (*58*), a threshold below which no value is reported. Table VII summarizes concentration data for these three analytes for laboratory reagent-water blank (LRB) samples and HDPE bottle lot-check samples (prepared identical to LRBs) from 2009, LRB samples from 2010, and the combined data for 2009–2010.

These blank data were used to estimate detection levels for the blank-limited analytes by using the standard deviation of the blank sample data in the simple parametric-based detection estimate equation described in the USEPA's method detection limit (MDL) procedure (*59*), where MDL is calculated as:

$$\mathrm{MDL} = s \times t \qquad (1)$$

where:

s = standard deviation of the determined concentrations, in ng/L

t = Student's t-value at the 99% confidence level ($\alpha = 0.01$) and N minus one degrees of freedom, where N is the number of samples.

The USEPA's MDL is defined (*59*) as "…the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than *zero* and is determined from analysis of a sample in a given matrix containing the analyte." The USEPA's MDL procedure estimates the analyte's MDL concentration (1) using the standard deviation of concentration data obtained from replicate samples ($N \geq 7$) that are fortified with the analyte at a concentration that is within five times the determined MDL and, (2) by assuming that the resultant variation from these (typically few) replicates is adequately represented by a parametric (Student's t) distribution that is centered at zero concentration. For analytes with frequent detections in blanks, the use of variation data obtained from blank replicates provides a more direct (no spiking required) and accurate determination of the detection level because it better represents the "true" variation in the blank distribution in comparison with an assumed variation derived from low-concentration spike samples that are used to calculate the MDL.

More importantly, analytes that are blank limited commonly have blank-concentration distributions that are not centered on zero, but are offset to a higher concentration. Also shown in Table VII are "mean-offset MDLs" that are calculated by adding the mean laboratory blank concentration to the MDL calculated using equation 1. The mean-offset MDL accounts for the fact that the blank distribution is not centered on zero concentration and, thus, represents the estimated minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration in the sample is greater than that in the *blanks*.

Table VII. Statistical summary of concentrations and estimated method detection limits for bisphenol A, cholesterol, and 3β-coprostanol from laboratory reagent-water blank (LRB) samples and bottle lot-check samples from 2009, LRB samples from 2010, and combined blanks from 2009–2010. [MDL, method detection limit; MRL, minimum reporting level; N, number of blank samples; ng/L, nanograms per liter; C99, 99th percentile concentration; %, percent; --, not applicable]

			Concentration in LRB samples									
Analyte	*Year*	*N*	*Mean* (ng/L)	*Standard deviation [s]* (ng/L)	*RSD* (%)	*Mini-mum* (ng/L)	*Median* (ng/L)	*C99* (ng/L)	*Maxi-mum* (ng/L)	*MDL* (ng/L)	*Mean-offset MDL*[a] (ng/L)	*MRL applied* (ng/L)
Bisphenol A	2009	129	15.9	31.5	199	0.0[b]	5.3	172	208	74	90	100
	2010	52	4.0	2.6	66	1.6	3.1	12.4	13.6	6.3	**10**	200
	2009–10	181	12.5	27.1	218	0.0	4.0	155	208	64	76	--
Cholesterol	2009	124	30.5	14.6	48	0.0[b]	28.3	74.1	90.9	34	65	2,000
	2010	52	36.0	27.6	77	1.3	30.3	147	198	66	**102**	200
	2009–10	176	32.1	19.5	61	0.0	28.9	92.9	198	46	78	--
3β-Coprostanol	2009	124	14.8	10.8	73	0.0[b]	19.8	28.7	28.9	26	40	2,000
	2010	52	19.1	21.8	114	2.7	14.4	117	135	52	**71**	200
	2009–10	176	16.1	15.0	93	0.0	18.5	55.7	135	35	51	--

[a] Mean-offset MDL = Mean + MDL. The value in **bold** from 2010 was used as the applied detection level value in table VIII. [b] Numbers of non-detects (no value) reported in 2009 were 2 for bisphenol A, 5 for cholesterol, and 39 for 3β-coprostanol.

Bisphenol A

Bisphenol A (BPA) is used in the synthesis of polycarbonate polymers and epoxy resins that are used in a diverse array of products or applications that might be encountered in either the laboratory or field environment (*57*, *60*). BPA residues in these products and applications provide a potential source for sample contamination.

BPA concentrations in 2009 blanks were greater, and considerably more variable, than those in 2010 blanks, indicating a apparent reduction in BPA contamination sources beginning in 2010. This possibly was attributable to minor method changes including implementation of substitute IDS compounds; however, the source of BPA contamination is unknown. Estimated MDLs and mean-offset MDLs for BPA based on the standard deviation of 2009, 2010, and combined blank sample data are shown in Table VII.

Based on sparse prior blank data, an MRL of 100 ng/L was applied to samples prepared and analyzed in 2009. The mean-offset MDL calculated from 2009 laboratory blank data was less than this 100 ng/L censoring limit. Nevertheless, BPA's MRL applied to samples prepared and analyzed in 2010 was raised to 200 ng/L as a precaution against false positives based on the magnitude of the 99th-percentile concentration (172 ng/L) from the 2009 blanks. Based on 2010 laboratory blank data, the MRL for BPA was lowered to 100 ng/L in Oct. 2011. This MRL is 10 times greater than the 2010 mean-offset MDL of 10 ng/L, but was applied as a conservative reporting level partly in consideration of field-blank data for BPA as described in (*26*).

Cholesterol and 3β-Coprostanol

Cholesterol is a primary sterol produced by animals that, contrary to widespread misinformation stating otherwise, also is produced by and present in plants, although at substantially lower concentrations than in animals (*61*). Cholesterol is present in human skin flakes and occurs at substantial concentrations in both indoor and outdoor dust (*62*). It is important to avoid use of paper products during field or laboratory processing of these samples because these products can contain cholesterol. The analyte 3β-coprostanol is a fecal sterol that, coupled with other sterol concentration information, is used as an indicator of human fecal-waste sources (*63*). 3β-Coprostanol likewise is associated with dust, which might be a potential contamination source when collecting water samples downwind from biosolids-treated fields or animal feeding operations.

Mean (or median) LRB concentrations for cholesterol were similar for 2009 and 2010; as was the case for 3β-coprostanol (Table VII). Limited testing revealed that these two sterols are introduced to LRB sample extracts primarily during the post-extraction evaporation steps, likely from introduction of dust particles during the three solvent-evaporation steps. Based on prior blank data for cholesterol and 3β-coprostanol, a conservative MRL value of 2,000 ng/L for each sterol was applied to samples prepared and analyzed in 2009. Very low cholesterol-d_7 recoveries in non-salted LRBs analyzed before 2009 resulted in a high bias artifact

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in sterol concentrations in the LRBs because the IDS and sterol analytes did not emulate each other during sample preparation. The two sterols contaminate the extract after the extraction step, whereas the cholesterol-d_7 loss occurred during the extraction step. Data collected in 2009 showed dramatically lower cholesterol or 3β-coprostanol concentrations in the LRBs (Table VII), which was a direct consequence of the much improved cholesterol-d_7 recovery achieved during the SPE isolation step because of NaCl addition to the LRB (and all) samples before extraction. Thus, the MRLs for these two sterols were lowered to 200 ng/L for samples analyzed starting in 2010.

11-Ketotestosterone

Tetradecamethylcycloheptasiloxane ($C_{14}H_{42}Si_7O_7$) with a mass-to-charge ratio (*m/z*) of 518.1315 is thought to interfere with determination of the derivatized 11-ketotestosterone at near instrument detection level concentrations. This "siloxane" compound is believed to come from thermal decomposition of the polydimethylsiloxane stationary phase of the gas chromatographic capillary column or possibly from the silicone septum used for the GC or reaction vials. This compound also is present in various personal care, household, and other silicone containing products (see, for example, (*64*) and references therein). In the ion source, this siloxane compound can lose a methyl (CH_3) group to form an ion (*m/z* = 503.1081) that, if present, produces some positive signal bias in the precursor ion used for 11-ketotestosterone (*m/z* = 503.3) under the low-resolution MS/MS conditions used in this method. The level of resulting interference in the monitored 11-ketotestosterone product quantitation ion (*m/z* = 323.2) averages about 0.3 ng/L, with a calculated mean-offset MDL of about 0.8 ng/L (maxiumum of about 1.2 ng/L) as established from analysis of >200 blank samples [see Table 23 in (*26*)]. Therefore, a MRL of 2 ng/L is used for 11-ketotestosterone. Alternative precursor ions for 11-ketotestosterone are at least 10 times less responsive compared to the *m/z* 503.3 ion.

Laboratory Reagent-Water Blank and Field-Blank Sample Results

The concentrations of analytes detected in LRB samples and in field-blank samples submitted for methods 2434 and 4434 are summarized in Table 24 of Foreman and others (*26*). BPA, cholesterol, and 3β-coprostanol were "detected" in all or nearly all LRB and field-blank samples from 2009–10 (N=200). The percentage of these detections that exceeded the applied mean-offset MDL (2010 value in bold in table VII) ranged from 5.5% for 3β-coprostanol to 11% for BPA. Thus, inclusion of a mean-offset to the MDL still was insufficient to achieve the desired 1% maximum false positive risk. These percentages would be greater if the lower non-offset MDL values were applied. However, only one field-blank value (a method 2434 sample) for BPA and one LRB value for cholesterol exceeded the higher applied MRL censoring concentrations. Comparative high BPA concentrations for the field blanks submitted for filter-water method 2434

(443 ng/L maximum, N=15) compared to unfiltered method 4434 (53 ng/L maximum; N=70) and the LRBs (30 ng/L maximum, N=115) indicate that the field-filtration steps might be an additional source of BPA contamination.

For the remaining 17 analytes, either they were not detected in the blanks or they were detected infrequently; all but three of these detections were less than the applied detection level (or less than the MRL for 11-ketotestosterone). One LRB value for ethynylestradiol (0.57 ng/L, N=115) and two method 4434 field-blank values for cis-androsterone (1.5 ng/L maximum, N=70) exceeded the applied detection levels of 0.4 ng/L (see next section).

Detection Levels for Nonblank-Limited Analytes

Estimated detection levels for the 16 analytes that are not blank-limited were determined by using the multi-concentration spiking procedure in ASTM International's Standard Practice D6091–07 for the determination of the interlaboratory detection estimate (IDE; (*65*)). The IDE procedure has several key advantages in comparison to the widely used USEPA MDL procedure (*59*). In particular, the IDE procedure simplifies detection-estimate determinations for multi-analyte methods with varying analyte responses (typical for many organic methods) because the IDE is designed as a multi-concentration procedure. Thus, it does not require the cumbersome, successive iterative determinations of the MDL if the original (and subsequent, typically lower) spiking level used in the MDL procedure is not between 1 and 5 times the determined MDL value. In addition, the IDE procedure considers (through three model scenarios) changes in the standard deviation with concentration to determine several detection-related parameters, whereas the MDL procedure assumes a constant standard deviation from the spiking level down to zero concentration (see (*65*) and references therein).

The IDE procedure and associated DQCALC software (*66*) also calculate the USEPA MDL value for each spiking level, and Currie's critical level (Lc) that, like the MDL, is the estimated concentration where the predicted risk of false positives is no more than 1% in the tested matrix (spiked reagent water for this study). Theoretically, Lc and MDL values will be nearly identical; however, they might differ because the IDE procedure uses a spike concentration relative to determined concentration (calibration-like) model to estimate Lc (and IDE). Furthermore, the IDE procedure performs the calculations assuming no change in standard deviation ("constant" model) or changes in standard deviation with concentration based on three model options: "straightline," "exponential," and a "hybrid" model developed by Rocke and Lorenzato (*67*) (*65*).

The IDE is defined in Standard Practice D6091-07 as "the lowest concentration at which there is 90% confidence that a single measurement from a laboratory selected from the population of qualified laboratories represented in an interlaboratory study will have a true detection probability of at least 95% (5% false negative risk) and a true nondetection probability of at least 99% (1% false positive risk when measuring a blank sample)" (*65*). For the analytical method presented in this report, the IDE procedure was applied as, and the determined IDE value used as, an intralaboratory detection estimate. As such, the IDE should

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be approximately equal to the NWQL's laboratory reporting level (LRL) value, which Childress and others (*58*) note is calculated by multiplying the determined MDL (or long-term method detection level) value by a minimal factor of 2 (see additional discussion in Appendix C of (*68*)).

Eight replicate reagent-water samples (about 450 mL) were spiked at about 0.1, 0.4, 1, 2, 3, and 4 ng/L of each analyte (BPA was 10 times higher, and cholesterol and 3β-coprostanol were 100 times higher at each level) as shown in Table VIII, and were fortified with the normal amount of the IDSs. Unspiked (0 ng/L of analytes) replicates also were included. All replicates were prepared and analyzed by the method in a manner identical to field samples in three independent sample preparation and analysis sets. Use of independent sets is important partly because instrument detection performance varies with time based on the level of GC and MS maintenance. Inclusion of this variation provides more realistic detection level estimates (*58*).

A summary of some detection- and quantitation-related parameters calculated using the DQCALC software is shown in Table VIII. Results for the four blank-limited compounds (BPA, cholesterol, 3β-coprostanol, and 11-ketotesterone) are included in this summary for comparison with the blank-based mean-offset MDL estimates shown in Table VII. For most analytes, there were no detections in the 0-ng/L (unspiked) replicates and no or very few detections in the 0.1-ng/L replicates; these concentration levels were omitted from calculations. For several analytes, the 0.4-ng/L level also was omitted because of no or few detections. One unusually high value at the 0.4-ng/L level was omitted for several analytes. The number of determined values used in the calculation at each spiking level is provided in Table VIII, along with the number of these values where the analyte did not meet secondary ion qualifying criteria. These "non-qualified" values were included in the calculation to provide at least four spiking levels for inclusion in the models, but are an indication of the concentration level where reliable qualitative "detection" might not always be high. All calculations are based on the determined concentration from the quantitation ion response that might be substantially greater than the responses for the two secondary ions that are used to ensure qualitative identification of the analyte in samples.

Estimated Lc and IDE concentrations calculated using each of the four standard deviation models noted previously are shown in Table VIII, along with a notation of the model having the best "fit" parameters under the "standard deviation model" column. Values of Lc shown in bold are similar from two or more models and are considered reasonable estimates of Lc. Also shown is the calculated USEPA MDL at each spike level, along with a notation of whether the MDL is considered "valid" by the DQCALC software. The criteria for "validity" are a MDL concentration that is no greater than the corresponding spiking level but not less than 20% of the spike level, and a minimum of seven values at the spike level. (Note: the DQCALC software simply considers the number of values [including zero] in determining this "minimum," not the number of actual analyte "detections" that meet identification criteria).

The "valid" MDL value shown in bold in Table VIII was similar to the bolded Lc values. Note that the determined MDL can vary substantially based on the spiking level, with generally increasing MDL as the spike concentration increases.

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This change in MDL value based on spike level highlights the requirement within the USEPA MDL procedure to perform iterative determinations of the MDL at successively decreasing spiking level to ensure that the determined MDL value is within 1 to 5 times the spike level as mentioned previously. This iterative process can result in a nearly equivalent number of total measurements to estimate the MDL value as is required by the IDE procedure; yet, the IDE procedure provides a more pratical approach for determining detection levels, especially for multi-analyte methods that often have subtantially different instrument detector response characteristics.

Also shown in Table VIII are the detection and reporting levels applied to the validation data in this chapter and to data for field samples prepared and analyzed beginning in 2010 (or earlier for select analytes). These interim detection and reporting levels initially were estimated using calibration and earlier performance data. Data for 16 analytes are reported using the NWQL's laboratory reporting level (LRL) convention, with concentration data less than the detection level provided for this "information rich" mass spectrometry method, while data for blank-limited compounds 11-ketotestosterone, bisphenol A, cholesterol, and 3β-coprostanol are reported using the minimum reporting level (MRL) convention, which does not permit reporting of data below the MRL (*58*). The USEPA minimum level value, defined as 3.18 times the MDL, is only calculated by DQCALC when the MDL is deemed "valid," and is shown in Table VIII for comparison with the IDE and the applied interim reporting level.

For many analytes, the Lc concentrations estimated using two or more of the standard deviation models were similar and also compared well with the lowest "valid" MDL concentration (values in bold in Table VIII). For 8 of the 16 analytes reported using the LRL convention, these Lc and corresponding valid MDL values agreed well with (that is, were within 1.5 times) the interim detection level concentrations applied to the performance data in this chapter. For the other 8 analytes, the Lc and MDL value were somewhat (more than 1.5 times) greater or less than the interim applied detection levels. An exception was progesterone, with Lc and MDL values estimated as 1 ng/L or less compared to the 4-ng/L applied interim detection level that was used because its quantitation ion is substantially more responsive than its secondary ions (at least six non-qualified values occurred even at the 2.2-ng/L spike level) and because of its more variable method performance in some matrices. In general, the Lc and MDL values were similar to or somewhat greater than the Lc and MDL values determined before 2009 when using the original 13 IDSs (data not shown), indicating that GC/MS/MS instrumental sensitivity was slightly better in that earlier test for several analytes, a typical scenario for mass spectrometric (and other chromatographic-based) instrumentation.

The Lc and MDL values calculated for the blank-limited analytes BPA, cholesterol, and 3β-coprostanol were similar to or lower than one or more of the MDL and mean-offset MDL values determined using the larger number of reagent-water blanks (Table VII) and were at least four times lower than the applied MRLs. Lc and MDL values for 11-ketotestosterone were greater than the mean-offset MDL from blanks (0.77 ng/L) and similar to the MRL of 2 ng/L.

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Table VIII. Estimates of Currie's critical level (Lc), the intralaboratory detection estimate (IDE), and the U.S. Environmental Protection Agency (USEPA) method detection limit (MDL) and minimum level (ML) concentrations for method analytes using ASTM standards D6091–07 (*65*) and D7510–10 (*66*). [N, no; ng/L, nanograms per liter; SD, standard deviation at spike level; Y, yes; --, not applicable; values in bold considered to exhibit good agreement for Lc by different models and with the estimated MDL]

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Reporting level (ng/L)*
11-Ketotestosterone	Constant	**1.4**	2.5	0.4	0.32	7	4	1.0	N	--	0.8[j]	2
	Straight line	**1.4**	2.4	1.1	0.65	8	3	1.9	N	--		
	Exponential	**1.2**	2.2	2.2	0.70	8	0	**2.1**	Y	6.7		
	Hybrid	**1.4**	2.4	3.2	0.51	8	0	1.5	Y	4.9		
				4.3	0.45	8	0	1.4	Y	4.3		
17α-Estradiol	Constant	1.1	1.8	0.4	0.36	8	1	1.1	N	--	0.4	0.8
	Straight line	**0.66**	1.3	1.1	0.25	8	0	**0.74**	Y	2.4		
	Exponential	**0.71**	1.3	2.2	0.40	8	0	1.2	Y	3.8		
	Hybrid	**0.76**	1.4	3.2	0.32	8	0	0.97	Y	3.1		

Method analyte	Lc and IDE summary[a]			USEPA MDL and minimum level summary[a]							Applied levels[b]	
	Standard deviation model[c]	Lc (ng/L)	IDE[d] (ng/L)	Spike level[e] (ng/L)	SD (ng/L)	Number of values[f]	Non-qualified values[g]	MDL (ng/L)	"Valid" MDL?[h]	ML[i] (ng/L)	Detection level (ng/L)	Reporting level (ng/L)
				4.3	0.61	8	0	1.8	Y	5.8		
17β-Estradiol	Constant	0.76	1.3	0.4	0.30	8	1	0.90	N	--	0.4	0.8
	Straight line	**0.37**	0.70	1.1	0.13	8	0	**0.38**	Y	1.2		
	Exponential	**0.38**	0.70	2.2	0.11	8	0	0.33	N	--		
	Hybrid	**0.45**	0.79	3.2	0.35	8	0	1.0	Y	3.3		
				4.3	0.43	8	0	1.3	Y	4.1		
17α-Ethynylestradiol	Constant	0.94	1.6	0.4	0.17	7	1	0.54	N		0.4	0.8
	Straight line	**0.33**	0.68	1.1	0.18	8	0	**0.53**	Y	1.7		
	Exponential	**0.42**	0.80	2.2	0.32	8	0	0.96	Y	3.0		
	Hybrid	**0.45**	0.83	3.2	0.34	8	0	1.0	Y	3.2		
				4.3	0.50	8	0	1.5	Y	4.7		
3β-Coprostanol	Constant	48	82	0	9.2	8	0	**28**	N	--	71[j]	200

Continued on next page.

Table VIII. (Continued). Estimates of Currie's critical level (Lc), the intralaboratory detection estimate (IDE), and the U.S. Environmental Protection Agency (USEPA) method detection limit (MDL) and minimum level (ML) concentrations for method analytes using ASTM standards D6091–07 (*65*) and D7510–10 (*66*). [N, no; ng/L, nanograms per liter; SD, standard deviation at spike level; Y, yes; --, not applicable; values in bold considered to exhibit good agreement for Lc by different models and with the estimated MDL]

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Repo-rting level (ng/L)*
	Straight line	**21**	41	11	7.5	8	0	22	N	--		
	Exponen-tial	**21**	38	43	2.6	8	0	7.9	N	--		
	Hybrid	**24**	42	108	6.2	8	0	19	N	--		
				217	14	8	0	42	N	--		
				325	23	8	0	69	Y	218		
				434	17	8	0	52	N	--		
Androstene-dione	Constant	3.0	5.2	1.1	0.20	8	1	**0.61**	Y	1.9	0.4	0.8
	Straight line	-0.7[k]	-4.3	2.2	0.77	8	0	2.3	N	--		
	Exponen-tial	**0.42**	0.72	3.2	0.81	8	0	2.4	Y	7.8		

Method analyte	Lc and IDE summary[a]			USEPA MDL and minimum level summary[a]							Applied levels[b]	
	Standard deviation model[c]	Lc (ng/L)	IDE[d] (ng/L)	Spike level[e] (ng/L)	SD (ng/L)	Number of values[f]	Non-qualified values[g]	MDL (ng/L)	"Valid" MDL?[h]	ML[i] (ng/L)	Detection level (ng/L)	Reporting level (ng/L)
	Hybrid	0.0[k]	0.0	4.3	1.6	8	0	4.8	N	--		
Bisphenol A	Constant	4.3	7.5	1.1	0.43	8	0	1.3	N	--	10[j]	200
	Straight line	**1.6**	3.1	4.3	0.73	8	0	**2.2**	Y	7.0		
	Exponential	1.8	3.3	10.8	1.1	8	0	3.3	Y	10		
	Hybrid	**2.1**	3.6	21.7	1.1	8	0	3.2	N	--		
				32.5	1.9	8	0	5.8	N	--		
				43.4	2.0	8	0	6.1	N	--		
Cholesterol	Constant	**57**	98	0	19	8	0	57	N	--	102[j]	200
	Straight line	**43**	78	11	11	8	0	32	N	--		
	Exponential	**43**	78	43	16	8	0	48	N	--		
	Hybrid	**45**	79	108	14	8	0	**42**	Y	133		
				217	19	8	0	58	Y	184		
				325	18	8	0	53	N	--		

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Table VIII. (Continued). Estimates of Currie's critical level (Lc), the intralaboratory detection estimate (IDE), and the U.S. Environmental Protection Agency (USEPA) method detection limit (MDL) and minimum level (ML) concentrations for method analytes using ASTM standards D6091–07 (*65*) and D7510–10 (*66*). [N, no; ng/L, nanograms per liter; SD, standard deviation at spike level; Y, yes; --, not applicable; values in bold considered to exhibit good agreement for Lc by different models and with the estimated MDL]

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Repo-rting level (ng/L)*
				434	29	8	0	86	N	--		
cis-Androsterone	Constant	0.48	0.83	0.4	0.12	7	2	**0.37**	Y	1.2	0.4	0.8
	Straight line	**0.36**	0.63	1.1	0.15	8	0	0.45	Y	1.4		
	Exponen-tial	**0.36**	0.63	2.2	0.18	8	0	0.53	Y	1.7		
	Hybrid	**0.40**	0.69	3.2	0.19	8	0	0.56	N	--		
				4.3	0.19	8	0	0.57	N	--		
Dihydrotestos-terone	Constant	2.5	4.3	1.1	0.46	8	4	1.4	N	--	2	4
	Straight line	**1.8**	3.6	2.2	0.99	8	3	3.0	N	--		

Method analyte	Lc and IDE summary[a]			USEPA MDL and minimum level summary[a]							Applied levels[b]	
	Standard deviation model[c]	Lc (ng/L)	IDE[d] (ng/L)	Spike level[e] (ng/L)	SD (ng/L)	Number of values[f]	Non-qualified values[g]	MDL (ng/L)	"Valid" MDL?[h]	ML[i] (ng/L)	Detection level (ng/L)	Reporting level (ng/L)
	Exponential	1.6	3.5	3.2	0.70	8	1	**2.1**	Y	6.7		
	Hybrid	**2.1**	3.9	4.3	0.79	8	0	2.4	Y	7.6		
Epitestosterone	Constant	**0.99**	1.7	0.4	0.27	8	0	0.82	N	--	2	4
	Straight line	**0.79**	1.5	1.1	0.29	8	0	**0.85**	Y	2.7		
	Exponential	**0.79**	1.5	2.2	0.41	8	1	1.2	Y	3.9		
	Hybrid	**0.87**	1.5	3.2	0.34	8	0	1.0	Y	3.3		
				4.3	0.39	8	0	1.2	Y	3.8		
Equilenin	Constant	**1.3**	2.3	1.1	0.66	8	2[l]	2.0	N	--	1	2
	Straight line	**1.6**	2.6	2.2	0.48	8	0	**1.5**	Y	4.6		
	Exponential	**1.6**	2.6	3.2	0.55	8	0	1.6	Y	5.2		
	Hybrid	**1.3**	2.2	4.3	0.46	8	0	1.4	Y	4.4		
Equilin	Constant	**4.2**	7.2	1.1	1.4	8	6[m]	4.2	N	--	2	4

Continued on next page.

Table VIII. (Continued). Estimates of Currie's critical level (Lc), the intralaboratory detection estimate (IDE), and the U.S. Environmental Protection Agency (USEPA) method detection limit (MDL) and minimum level (ML) concentrations for method analytes using ASTM standards D6091–07 (*65*) and D7510–10 (*66*). [N, no; ng/L, nanograms per liter; SD, standard deviation at spike level; Y, yes; --, not applicable; values in bold considered to exhibit good agreement for Lc by different models and with the estimated MDL]

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Repo-rting level (ng/L)*
	Straight line	**3.7**	7.4	2.2	1.4	8	5[m]	4.3	N	--		
	Exponen-tial	**3.7**	7.6	3.2	1.8	8	4	5.5	N	--		
	Hybrid	**3.9**	8.0	4.3	1.5	8	0	**4.6**	N	--		
Estriol	Constant	**1.1**	2.0	0.4	0.53	8	1[l]	1.6	N	--	1	2
	Straight line	**0.92**	1.7	1.1	0.18	8	0	0.55	Y	1.7		
	Exponen-tial	**0.83**	1.5	2.2	0.37	8	0	1.1	Y	3.5		
	Hybrid	**0.93**	1.7	3.2	0.31	8	0	0.9	Y	3.0		
				4.3	0.55	8	0	1.7	Y	5.3		

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Reporting level (ng/L)*
Estrone	Constant	1.2	2.0	0.4	0.25	8	6	0.74	N	--	0.4	0.8
	Straight line	**0.64**	1.3	1.1	0.35	8	1	**1.0**	Y	3.3		
	Exponential	**0.67**	1.3	2.2	0.41	8	1	1.2	Y	4.0		
	Hybrid	**0.78**	1.4	3.2	0.65	8	0	1.9	Y	6.2		
				4.3	0.53	8	0	1.6	Y	5.0		
Mestranol	Constant	**0.96**	1.6	0.4	0.27	8	3	0.81	N	--	0.4	0.8
	Straight line	**0.76**	1.4	1.1	0.27	8	0	**0.82**	Y	2.6		
	Exponential	**0.77**	1.4	2.2	0.38	8	0	1.1	Y	3.6		
	Hybrid	**0.82**	1.4	3.2	0.29	8	0	0.88	Y	2.8		
				4.3	0.41	8	0	1.2	Y	3.9		
Norethindrone	Constant	1.2	2.1	0.4	0.40	8	0	1.2	N	--	0.4	0.8
	Straight line	**0.79**	1.6	1.1	0.28	8	0	**0.83**	Y	2.6		

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Table VIII. (Continued). Estimates of Currie's critical level (Lc), the intralaboratory detection estimate (IDE), and the U.S. Environmental Protection Agency (USEPA) method detection limit (MDL) and minimum level (ML) concentrations for method analytes using ASTM standards D6091–07 (*65*) and D7510–10 (*66*). [N, no; ng/L, nanograms per liter; SD, standard deviation at spike level; Y, yes; --, not applicable; values in bold considered to exhibit good agreement for Lc by different models and with the estimated MDL]

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Reporting level (ng/L)*
	Exponential	**0.84**	1.6	2.2	0.44	8	0	1.3	Y	4.2		
	Hybrid	**0.90**	1.7	3.2	0.36	8	0	1.1	Y	3.4		
				4.3	0.66	8	0	2.0	Y	6.3		
Progesterone	Constant	1.4	2.4	0.4	0.32	8	7	0.95	N	--	4	8
	Straight line	0.64	1.4	1.1	0.35	8	6	1.0	Y	3.3		
	Exponential	**0.78**	1.5	2.2	0.60	8	6	1.8	Y	5.8		
	Hybrid	**0.84**	1.6	3.2	0.28	8	0	**0.85**	Y	2.7		
				4.3	0.91	8	0	2.7	Y	8.7		
Testosterone	Constant	1.4	2.4	0.4	0.23	7	5	0.72	N	--	0.4	0.8

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Repo-rting level (ng/L)*
	Straight line	0.85	1.7	1.1	0.38	8	1	1.1	N	--		
	Exponen-tial	0.81	1.6	2.2	0.54	8	0	1.6	Y	5.2		
	Hybrid	1.01	1.8	3.2	0.63	8	0	1.9	Y	6.0		
				4.3	0.45	8	0	1.4	Y	4.3		
trans-Diethyl-stilbestrol	Constant	**0.51**	0.87	0.4	0.16	8	0	0.49	N	--	0.4	0.8
	Straight line	**0.34**	0.62	1.1	0.15	8	0	**0.46**	Y	1.5		
	Exponen-tial	**0.35**	0.62	2.2	0.08	8	0	0.23	N	--		

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Table VIII. (Continued). Estimates of Currie's critical level (Lc), the intralaboratory detection estimate (IDE), and the U.S. Environmental Protection Agency (USEPA) method detection limit (MDL) and minimum level (ML) concentrations for method analytes using ASTM standards D6091–07 (*65*) and D7510–10 (*66*). [N, no; ng/L, nanograms per liter; SD, standard deviation at spike level; Y, yes; --, not applicable; values in bold considered to exhibit good agreement for Lc by different models and with the estimated MDL]

	Lc and IDE summary[a]			*USEPA MDL and minimum level summary*[a]							*Applied levels*[b]	
Method analyte	*Standard deviation model*[c]	*Lc (ng/L)*	*IDE*[d] *(ng/L)*	*Spike level*[e] *(ng/L)*	*SD (ng/L)*	*Number of values*[f]	*Non-qualified values*[g]	*MDL (ng/L)*	*"Valid" MDL?*[h]	*ML*[i] *(ng/L)*	*Detection level (ng/L)*	*Repo-rting level (ng/L)*
	Hybrid	**0.38**	0.66	3.2	0.23	8	0	0.69	Y	2.2		
				4.3	0.23	8	0	0.68	N	--		

[a] Summary of calculations provided by the D7501-10 DQCALC software using an earlier version that includes the exponential model. See "Detection Levels for Non-Blank-Limited Analytes" section for additional information. [b] Detection and reporting levels applied to validation data provided in this report and for data reporting in 2010 (and earlier for select analytes). The laboratory reporting level convention is applied to all analytes, except 11-ketotestosterone, bisphenol A, cholesterol, and 3β-coprostanol, for which the minimum reporting level (MRL) convention is applied (*58*). [c] The software calculates detection estimates for the constant, straight-line, exponential, and hybrid (Rocke-Lorenzato) models of the variation of intralaboratory standard deviation with concentration (*65*). The model selected as preferred in DQCALC based on regression statistics and visual observation of plots in shown in italics. [d] The IDE acronym can denote an interlaboratory detection estimate, but was applied, and is referred to here, as the *intra*laboratory detection estimate. [e] For most analytes, there were no analyte detections at the 0-ng/L (unspiked) level and no or very few analyte detections at the 0.1-ng/L level; these levels were omitted from calculations. For several analytes, the 0.4-ng/L level also was omitted because of no or few detections. [f] Number of determined values at the spiking level. One unusually high value at the 0.4-ng/L level was omitted for several analytes based on Grubbs' outlier test. [g] Number of determined values used in the calculations where the qualifying ion criteria were not met for the analyte (see "Detection Levels for Non-Blank-Limited Analytes" section). [h] MDL considered valid in DQCALC if the number of spike observations is 7 or more, and if the MDL does not exceed the spike concentration or is not less than 20 percent of the spike concentration. [i] The USEPA minimum level is defined as 3.18 times the MDL and is only calculated by DQCALC when MDL is deemed valid. [j] Analyte reported using MRL (ML) convention. Blank-based mean-offset MDL shown for comparison with other values. [k] A substantial positive y-intercept can lead to less than zero (nonsensical) values. [l] One of the non-qualified values was zero; no quantifying ion detected.

The detection data presented in Tables VII and VIII, coupled with other method performance observations not presented here, either validated the continued use of the applied detection- and reporting-levels shown in Table VIII or resulted in those values being lowered by ½ for epitestosterone or raised by 2 times for equilin and testosterone (see Table 14 in (*26*)). In addition, the MRL for BPA was lowered to 100 ng/L as noted in the "Bisphenol A" section.

Holding-Time Experiments

Holding-time experiments were performed to test analyte stability in spiked reagent water stored refrigerated or frozen. A test of storage stability of dry extracts before derivatization also was conducted alongside the refrigerator and freezer sample-storage experiments because extracts prepared during those hold-time studies also were stored for similar (or less) periods of time as dry extracts before completing compound derivatization and analysis. All method compounds appear to be stable when stored as dry, underivatized extracts in silanized reaction vials for as long as 58 days in a freezer (about –15°C) (*26*).

The spiked-water tests were conducted using reagent water only; thus, the results might represent optimum stability compared to that obtainable with field matrices, especially matrices such as WWTP influent or primary effluent samples expected to have considerable microbiological activity. Except for the use of ascorbic acid as a dechlorination reagent, no other sample preservation reagent currently (October 2012) is prescribed for use for these methods. Schenck and others (*69*) have shown that chlorination of water removes as much as 98% of tested steroid hormones. Ascorbic acid has been found to be an effective dechlorination reagent for many steroids and hormones (and other pharmaceuticals and personal-care products) determined in chlorinated-water test samples by USEPA method 1698 (*38*, *70*), whereas sodium thiosulfate is the prescribed dechlorination reagent in new USEPA method 539 (*36*) that determines seven of the hormone analytes included in the NWQL method.

Several studies by others have shown that some of the method analytes had rapid loss or formation in tested environmental matrices stored refrigerated or even frozen, and that enhanced stability was obtained for some analytes by addition of certain preservative reagents including acids and biocides ((*70*) and references therein; (*71*, *72*)). USEPA method 539 prescribes addition of 2-mercaptopyridine-1-oxide sodium salt during sample collection to protect the seven hormone analytes determined by that method from microbial degradation. Limited testing of this preservation reagent using spiked reagent water samples resulted in no or very poor (<10%) recoveries for all 11 analytes that contain ketone functionality only (Figure 1), possibly because of reagent interference with the MSTFA derivatization step.

The procedure used for the holding-time experiments was based in part on the experimental and data-evaluation guidelines described in ASTM Standard Practice D4841–88 (*73*), although the number of day 0 replicates (four) was less than that prescribed for several analytes that have relative standard deviations (RSDs) greater than 9%. In each experiment, the analytes were fortified into the sample replicates at concentrations used to prepare the LRS samples and subsequently

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were treated as described in the sections that follow. The holding-time experiments were conducted before the IDS substitutions implemented in January 2010 (see "Observations of deuterium-label loss for some IDS compounds" section).

Analyte stability as a function of time was evaluated by comparison of the mean concentration for quadruplicate spiked test samples held for various storage periods relative to the mean concentration from quadruplicate spikes analyzed at day 0. Three comparative approaches were applied to the results to gage overall stability. This was done to minimize misinterpretation of a compound's stability from any one comparative approach because recovery variation for day 0 replicates (to which other storage times are compared) was minimal for some analytes in these tests and leads to statistical predictions of instability that are not reflected by a substantial change in concentration for the storage period. Analyte instability was defined to occur for a storage period if the following three test criteria were met: (1) a mean concentration for the storage period that was outside the tolerable range of variation (99-percent confidence interval) as defined in D4841–88 (*73*), (2) a *p*-value of 0.01 or less from a Student's *t*-test of mean concentration for the storage period compared to the day 0 mean, and (3) a modulus (absolute value) percentage change (PC) greater than 20 percent, where the PC between the mean concentration for a given hold time ($Mean_T$) and the mean at day 0 ($Mean_0$) is calculated as:

$$PC = 100 \times \frac{Mean_T - Mean_0}{Mean_0} \quad (2)$$

A substantial negative PC value indicates loss during storage compared to day 0, whereas a substantial positive PC indicates analyte formation. This 20 percent threshold also was used by the USEPA to indicate a substantial change in mean concentration in stability studies for hormones and other compounds (*70*). Procedural details and summarized data for the holding time experiments are provided in (*26*).

Method Analyte Stability in Refrigerated Reagent Water

Analyte-only stability in reagent-water samples held refrigerated for 1, 3, and 8 days was tested to simulate possible storage periods for samples received at the NWQL and placed in a refrigerator only before sample extraction, and for samples maintained under refrigerated conditions (near 4°C) by field staff before and during shipment to the NWQL of sample coolers containing water ice.

Only two analytes had clearly significant concentration changes in refrigerated reagent water through 8 days of storage. Mestranol showed the largest initial drop in concentration (PC of –29%) at day 1, but the loss rate appeared to rapidly diminish with further storage because its PC values at days 3 and 8 were no more than –40% suggesting most of the loss occured at warmer temperatures while the sample was cooling. Progesterone also appreared to undergo significant loss with a

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PC by day 8 of –42%. The analyte *cis*-androsterone had PC values <–25% at days 3 and 8, but the changes were not statistically significant because of the relatively large variability (22% RSD) at day 0. Similar holding-time experiments by the USEPA (*70*) found that cis-androsterone's concentration decreased (mean PC of about –42% on day 7) in "chlorinated effluent samples, held in HDPE bottles, dechlorinated with ascorbic acid, and stored at 4°C (for as much as 14 days), with no pH adjustment." Interestingly, that study reported a statistically significant loss (PC of –30%) for mestranol in day 7 test samples, but no apparent loss in day 14 samples compared to day 0 concentrations. That study also showed a non-statistically significant decrease in progesterone concentration (mean PC of about –37% by day 14).

Method Analyte Stability in Frozen Reagent Water

Analyte-only stability in reagent-water samples held frozen (–15±5°C) for 2, 7, 14, 21, and 56 days was tested to simulate some potential frozen-sample storage periods. Also, freezer storage was believed preferred for maintaining analyte concentrations or at least slowing analyte-loss processes relative to refrigerated conditions, especially for environmental samples.

Seventeen analytes, including *cis*-androsterone, had no statistically significant concentration changes in frozen reagent water through 56 days of storage. Mestranol concentrations initially dropped after 2 days of freezer storage (PC of –25%), but exhibited no further decrease for all other storage periods. The amount of mestranol loss for all frozen-sample storage periods is less than the loss observed after only one day of refrigerated storage, which suggests that most of the mestranol loss occurs when the water sample is warmer (for example during the initial sample cool-down or thaw periods) rather than at colder (freezer) temperatures. Cholesterol and 3β-coprostanol had significant decreases in concentration (PC ≤–32% for both) in all but the longest (day 56) frozen samples that might be related to sorption issues because of the decrease in their water solubilities with decreasing temperature (see previous description of sterol solubility/sorption issues in the "Cholesterol-d_7 Recoveries in Non-salted Reagent Water" section).

Equilin concentrations appeared to increase (PC ≤28%) in day 14–56 samples, although the mean equilin recoveries for these storage periods were no more than 113% and the changes were not significant. Progesterone concentrations were not significantly different for any storage period, although the PC at day 7 (–29%) was substantial. Substantially greater variability in determined concentrations by this method reduces the ability to detect statistically significant trends under all storage conditions for both equilin and progesterone.

These experiments indicate that freeze-storage of samples for at least 56 days does not significantly alter sample concentrations for most method analytes, especially relative to storage in a refrigerator. The possible exceptions are cholesterol and 3β-coprostanol because of presumed sorption losses, and mestranol because of rapid initial loss (day 1), the rate of which slows with additional freezer storage. Sample freezing is anticipated to reduce biotic activity

in sample matrices relative to refrigerated storage conditions. Thus, storage of samples in a freezer is prescribed as the standard storage condition for all samples, unless they can be extracted within 3 days of receipt. Freezer storage of samples also is prescribed for field samples that can not be shipped immediately from the field on water ice.

Reporting of Analyte Data Based on Isotope-Dilution Standard Performance

Assesment of sample-specific performance by use of the absolute recoveries of the IDS compounds provides a valuable tool for making decisions regarding the reporting and qualification of analyte concentrations in a given sample. Analyte concentration (analytical result) in a given sample is reported based on the sample-specific recovery of the corresponding IDS (see further discussion in (*26*)). For analytes with an exact isotopic analog (see Table II), the analyte concentration is reported as estimated (NWIS "E" remark code) if the recovery of its IDS is outside the 25–120% range. For those analytes that use a non-exact IDS analog, the analyte concentration is reported as estimated if recovery of its IDS is outside the 40–120% range. All values for equilin and progesterone are reported as estimated as noted previously. If the IDS recovery is less than 5%, the analyte concentration is not reported, regardless of whether the analyte is detected or not; instead an analyte or sample deletion code is applied. Note that although the IDS recovery might fall within the range where "E" coding is not required, the "E" code still might be applied for reasons other than IDS recovery performance (*58*).

Summary and Conclusions

The U.S. Geological Survey's National Water Quality Laboratory has developed a new analytical method for the determination of 20 steroid hormones and related compounds, many of which reportedly exhibit endocrine system modulating activity. The analytes include 6 natural and 3 synthetic estrogen compounds, 6 natural androgens, 1 natural and 1 synthetic progestin compound, and 2 sterols: cholesterol and 3-beta-coprostanol. These two sterols have limited biological activity but typically are abundant in wastewater effluents and serve as useful tracers. Bisphenol A, an industrial chemical used primarily to produce polycarbonate polymer and epoxy resins and that has been shown to have estrogenic activity, is also determined by the method. The method is applicable to a variety of filtered or unfiltered water-matrix types including groundwater, surface water, surficial runoff, and wastewater-treatment plant (WWTP) effluent and influent samples. However, method performance for some analytes might be outside the desired recovery range of 60–120%, and some analytes have more variable performance for some matrices including WWTP influents and primary effluents, biosolids runoff samples, animal-feeding operation waste lagoon samples, and other "complex" water samples.

Deuterium- or ^{13}C-labeled isotope-dilution standards (IDSs), all of which are direct or chemically similar isotopic analogs of the method analytes, are added to the samples before analyte isolation by C18 solid-phase extraction

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(SPE). The extacts are passed through a Florisil SPE column to remove some polar organic interferences in the extract. Method compounds in the samples and in associated calibration standards are derivatized to trimethylsilyl analogs that are separated by GC/MS/MS by monitoring the product ions of three specific precursor-to-product ion transitions (two transitions for the IDSs). All 20 method analytes are quantified relative to a specific IDS compound by using an isotope-dilution quantification (IDQ) procedure. The absolute recoveries of IDS compounds provide an indication of procedural performance for the specific sample in a manner comparable to surrogate compound recoveries. Reporting and result qualification of analyte data is based on sample-specific IDS-recovery information and performance criteria.

Method performance was tested by spiking replicates of the following sample validation matrices at 10 and 100 ng/L for most analytes: reagent water, surface water receiving WWTP effluent, and WWTP secondary and primary effluent. For most analytes, mean method recoveries in these matrices were within the desired recovery performance range of 60–120%; relative standard deviations of recovery typically were no more than 25%. Exceptions occurred in the field matrices (particularly the primary WWTP effluent matrix) for those analytes that had substantial ambient concentrations relative to the analyte fortification level, which leads to enhanced recovery bias, variability, or both. Matrix-spike samples of additional field matrices provided similar results to those obtained for the validation matrices. Progesterone had unusually low recoveries in some matrices (especially some surface waters). Equilin had more variable recoveries in spiked matrices. Thus, determined sample concentrations for these two analytes are reported as estimated only.

Bisphenol A, cholesterol, and 3-beta-coprostanol are sample preparation blank-limited analytes, and 11-ketotestosterone is an instrumental blank-limited analyte. Concentrations of these four analytes are reported using censoring levels above their blank-based detections levels to minimize the risk of false positivies and concentration bias. Detection levels estimates in reagent water for the 16 non-blank-limited analytes range from 0.4 to 4 ng/L.

Several deuterium-labeled compounds initially tested as candidate direct-analog IDS compounds were determined to be unacceptable because either they did not have sufficient chemical purity or were susceptible to deuterium loss (deuterium-hydrogen exchange) in protic solvents, which compromises accuracy of the IDQ procedure and is a source of potential analyte false positive detection. Thus, careful consideration of label type, position, and stability, along with isotope purity, is vital when evaluating any labeled-compound as an IDS or surrogate candidate. Several analytes had no exact IDS analog available or, if available, were not tested because of excessive cost or purity limitations. Additional labeled compounds might be added as method IDS compounds as they become available to further improve quantitative accuracy for those analytes described in this chapter that did not have exact isotopic analogs.

Holding-time experiments indicate acceptable analyte stability in reagent water stored refrigerated (4 degrees Celsius) for as long as 8 days and stored in a freezer (–15°C) for as long as 56 days. Freezer storage of samples before

extraction is used by the NWQL and encouraged for field storage to reduce microbiotic or other degradation processes.

Many of the method analytes are naturally occurring compounds and bisphenol A is a component of materials used in a variety of products. As such, the inclusion of field blanks during sampling is vital to assess the potential for unintended contamination of samples with these analytes. Likewise, the matrix-spike results highlight the importance of including field-submitted matrix-spike samples as a quality-assurance component in environmental studies that use this method.

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Chapter 5

Analysis of Veterinary Growth Promoters in Airborne Particulate Matter by Liquid Chromatography–Tandem Mass Spectrometry

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Veterinary growth promoters are widely used throughout the United States in livestock production. In beef production, steroid hormones are administered to an estimated 90% of cattle. These include the synthetic steroids trenbolone acetate and melengestrol acetate, as well 17β-estradiol. Given the endocrine-modulating activity of steroid growth promoters, a sensitive and reliable analytical method is needed to detect trenbolone, melengestrol acetate, estradiol and related residues in environmental matrices. We have developed a method that incorporates solid phase extraction (SPE) and liquid chromatography–tandem mass spectrometry for the simultaneous determination of trendione, trenbolone, melengestrol acetate, estrone, and estradiol in airborne particulate matter samples. Sample preparation involved liquid-solid extraction followed by cleanup with SPE

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cartridges. Analytes were separated using reversed-phase liquid chromatography. Utilizing a fast gradient, analysis time was under 11 minutes, but did not provide complete isomeric separation of 17β-trenbolone and 17α-trenbolone, or 17β-estradiol and 17α-estradiol. Column effluent underwent atmospheric-pressure chemical ionization (APCI) followed by detection using a triple-quadrupole mass spectrometer in SRM mode. The lower limit of quantitation (LLOQ) was ≤0.55 ng/filter for all compounds. Recoveries ranged from 93% - 116%, and coefficient of variation (CV) was ≤16.1% at all spiking levels.

Introduction

In the Unites States, veterinary growth promoter use has become common practice in beef cattle finishing. Trenbolone acetate (TBA), 17β-estradiol (17βE2), and melengestrol acetate (MGA) are three frequently used steroid growth promoters (*1*). Following application to cattle, the synthetic androgen TBA can be excreted as trendione (TbO), 17β-trenbolone (17βTb), and 17α-trenbolone (17αTb) with the majority excreted as 17α-trenbolone (*2*). 17βE2 follows the same pattern being excreted primarily as 17α-estradiol (17αE2), and only a small percentage as 17βE2 or estrone (E1) (*3*). MGA, a progestin, is primarily excreted as the parent compound (*4*).

After excretion, growth promoter metabolites can be transported into water bodies via runoff (*5–7*) or in discharged effluent; however, the majority of cattle in the US are located in semiarid climates in the states of Texas, Kansas, Nebraska, and Colorado (*8*). Research has demonstrated particulate matter (PM) emissions from beef cattle feedyards can serve as a route of TBA metabolite transport (*9*). To expand upon previous research, a method for the detection of androgenic, estrogenic, and progestagenic veterinary growth promoters was needed.

Steroid analysis is routinely performed using either gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS). GC-MS techniques require derivatizations to successfully detect steroids (*10–14*), and different derivatization techniques must be employed to derivatize TBA metabolites (*7*) versus most other steroids (*10–14*). LC-MS techniques employing either electronspray ionization (ESI) or atmospheric pressure chemical ionization (APCI) have been used in the analysis of both biological (*15–21*) and environmental (*22–26*) matrices. Multiple studies on steroid matrix effects using ESI or APCI (*27*, *28*) have displayed lower signal suppression using APCI, thus it was chosen as the ionization technique for this method.

The objective of this study was to develop a sensitive, simultaneous LC-MS/MS method for analysis of TBA metabolites, estrogens, and MGA in PM samples collected from beef cattle feedyards. Utilizing liquid-solid extraction, solid-phase extraction (SPE) clean-up, and LC-APCI-MS/MS analysis, good recovery and repeatability were achieved in low parts-per-billion concentrations.

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Experimental Section

Chemicals and Reagents

Steroid standards 17β-trenbolone (17βTb) (17β-hydroxyestra-4,9,11-triene-3-one, >98%)) and trendione (TbO) (estra-4,9,11-trien-3,17-dione, >98%) were purchased from Steraloids (Newport, RI, USA). 17α-trenbolone (17αTb) (17α-hydroxyestra-4,9,11-triene-3-one, >98%) and 17β-Estradiol-d5 (E2-d5) were from Cerilliant (Round Rock, TX, USA). Estrone (E1) (3β-Hydroxyestra-1,3,5(10)-trien-17-one, >98%), 17α-estradiol (17αE2) (17α-estra-1,3,5(10)-triene-3,17diol), 17β-estradiol (17βE2) (17β-estra-1,3,5(10)-triene-3,17diol), melengestrol acetate (MGA) (4,6-pregnadien-6-methyl-16-methylene-17-ol-3,20-dione acetate) and Florisil cartridges (6 ml, 1 g) were purchased from Sigma (St. Louis, MO, USA). Deuterated internal standards (ISTD) 17β-trenbolone-d3 (Tb-d3) and melengestrol acetate-d3 (MGA-d3) were obtained from RIVM (Bilthoven, The Netherlands). Water, acetone, methanol (MeOH), hexane and dichloromethane ($MeCl_2$) (all HPLC grade) and Zefluor 47mm filters were obtained from VWR (West Chester, PA, USA). Regenerated cellulose syringe filters were obtained from Phenomenex (Torrance, CA, USA).

Standard Solutions

Stock standard and ISTD solutions (1 mg/mL) were prepared in MeOH and stored at -20°C. Working standard solutions were prepared by dilution of the stock solution with methanol and were stored at -20°C.

Sample Collection

Air sampling was conducted at five commercial feedyards on the Southern High Plains, USA. Two separate sampling campaigns were conducted at each site in January 2011 and April 2011. Each campaign consisted of collocating two USDA-ARS designed low-volume total suspended particulate matter (TSP) samplers, one Thermo Scientific (East Greenbush, NY) low-volume ambient PM10 sampler, and one Thermo Scientific low-volume ambient very sharp cut cyclone (VSCC) PM2.5 sampler at four sites at a given feedyard. This required 8 TSP, 4 PM10, and 4 PM2.5 samplers deployed for each sampling campaign. All samplers utilized Zefluor 47mm filters for sample collection. The airflow rate for all low-volume (16.67 lpm) samplers was monitored and controlled by a system developed by the USDA-ARS Cotton Production and Processing Research Unit in Lubbock, TX. A total of 137 samples were collected from these sampling campaigns and analyzed for growth promoters.

Prior to deploying filters in the field, gravimetric analysis was conducted on all samples. Filters were conditioned in an environmental chamber (21 ±2°C; 35 ±5% RH) for 48 h prior to weighing. Samples were weighed in the environmental chamber on a Mettler MX-5 microbalance (Mettler-Toledo Inc., Columbus, OH) after being passed through an anti-static device (Mettler-Toledo Inc., Columbus, OH). After PM collection, filters were reconditioned for 24 h,

weighed, and mass of PM calculated. After gravimetric analysis, filters were stored in sealed polycarbonate containers at -80° C for a maximimum of 180 days before proceeding to extraction.

Sample Preparation

PM samples underwent shaking extraction followed by SPE clean-up prior to LC-MS analysis. Each step is detailed below.

Isotope addition

Each PM-laden filter was spiked with 2.5 ng of each Tb-d3, MGA-d3, and E2-d3 dissolved in 10uL µL of MeOH to allow analyte quantification by isotope dilution procedures. Spiked filters were kept in an open container at room temperature for 30 min before proceeding to extraction to facilitate complete evaporation of spiking solvents.

Extraction

Each PM-laden filter was placed into a 12 mL glass, screw top vial. 10 mL of MeOH was added to each vial and placed on rotary shaker. Samples were extracted for 30 min at 350 rpm. After extraction, samples were centrifuged at 2500 rpm for 5 min to allow particulates to settle. The supernatant was filtered through 0.45 µm regenerated cellulose filter, and the extraction process repeated. After filtration, combined extracts were evaporated to dryness on rotary evaporators before proceeding to SPE clean-up.

Solid-Phase Extraction Cleanup

Each extract was re-suspended in 5 mL of hexane:$MeCl_2$ (95:5, v/v). Florisil cartridges were conditioned with 5 mL of hexane: $MeCl_2$ (95:5, v/v) and extracts loaded onto cartridges. Each Florisil cartridge was washed with 5 mL of hexane:$MeCl_2$ (60:40, v/v) to remove interfering compounds. Analytes were eluted from Florisil cartridges in 5 mL (2 x 2.5 mL) of acetone:hexane (60:40, v/v). This eluate was evaporated to dryness under nitrogen stream at 35±5°C. Extracts were reconstituted in 500 µL MeOH:water (60:40, v/v), filtered through 0.2 µm regenerated cellulose syringe filter, and analyzed by LC-MS/MS.

LC-MS/MS Analysis

A Thermo Fisher LC-MS/MS system (San Jose, CA, USA) consisting of an Accela MS pump, PAL autosampler, a TSQ Access Max triple quadrupole mass spectrometer, and Xcalibur data system was used to identify and quantify steroids of interest. Separations were obtained using a 100 mm x 2.1 mm Kinetex c18 column with a 2.6 µm particle size (Phenomenex, Torrance, CA, USA) under gradient conditions. Flow rate was set at 0.4 mL/min with an injection volume of 25 µL. Gradient conditions with LC-MS grade water (solvent A) and LC-MS

grade MeOH (solvent B) were as follows: 40% solvent B (t=0 min) increased linearly to 70% (t=3.0 min, held for 2.5 min), increased to 100% (t=5.6 min, held for 1.4 min), and given 6 min to equilibrate to starting conditions. The α- and β- isomers of estradiol and trenbolone were not separated using this mobile-phase gradient. A fast gradient was applied in this method both to reduce analysis run time, and to effectively increase signal-to-noise (S/N) ratios for these "compounds" by combining the peak responses, if present in a sample, of the individual isomers. This approach does sacrifice potentially valuable information regarding the isomer-specific composition of the growth promoters in the PM samples. Thus, in all chromatograms, 17αTb and 17βTb are combined into one peak as total trenbolone (α-/β-Tb), and likewise, 17αE2 and 17βE2 are combined as total estradiol (E2). MS analyses were conducted with an atmospheric pressure chemical ionization (APCI) probe. Each compound was investigated in positive and negative selected ion monitoring (SRM) mode to determine the optimum ionization conditions. Only E1, E2, and E2-d3 were analyzed utilizing negative ionization mode. All other compounds were analyzed in positive ionization mode. Conditions were optimized to identify two or more product ions for each compound. Auxiliary and sheath nitrogen flow were set at 0-5 and 10-30 arbitrary units, respectively. Vaporizer temperature was set to 500°C. Optimum discharge current was determined at 4 μA and -20 μA for positive and negative mode, respectively. Capillary temperature was tested between 250-350°C, with 350°C chosen as the optimum value. Peak width of quadrupole Q1 and Q3 were set at 0.40. Tube lens voltage and collision energy (CE) are compound specific and were optimized for each compound (Table I).

Calibration curves were constructed using the ratio of target compound to ISTD as the response variable. Calibration curves included a solvent blank, zero sample (ISTD only), and six nonzero points. Points were fit with a linear curve using least-squares linear regression. Coefficient of determination (R^2) was >0.99 for all compounds. Matrix effects were investigated through comparison of calibration curves created by analyte addition to blank solvent then analyte addition to LG PM matrix extracts. In all compounds, both produced calibration curves of no significant difference.

Results and Discussion

Sample Preparation Optimization

Multiple extraction solvents were investigated to obtain optimum recovery of steroid growth promoters. Excellent recoveries were obtained with both acetonitrile and MeOH for all trenbolone compounds; however, MeOH provided better overall recovery for both estrogens and MGA. Up to four liquid-solid shaking extraction replicates were tested, with no significant increase in sample recovery after two 30 min extractions. Therefore, two extraction replicates were chosen for this method.

After extraction, sample matrix was very dirty, so multiple SPE techniques were investigated to purify extracts and ultimately increase analyte S/N ratios. Extract cleanup efficiency using SPE was assessed by examining resultant

improvement in analyte S/N ratios after spiked test extracts were passed through Oasis HLB, Florisil, and tandem HLB-Florisil cartridges. All sorbents provided substantial improvement in analyte S/N over uncleaned extracts. Florisil alone provided optimum S/N ratio of all target compounds, with no substantial improvement from the addition of HLB extraction prior to Florisil clean-up, thus Florisil SPE was chosen for this study.

Table I. Retention time (Rt), ion transitions, collision energy (CE), and tube lens settings for each compound

Analyte	*Rt (min)*	*Precursor ion $[M+H]^+$*	*Product ions (m/z)*	*CE (eV)*	*Tube Lens (V)*
Trendione	2.3	269.2	165.1	59	102
			192.2	35	102
			225.1[a]	21	102
α-/β-Trenbolone	2.4	271.2	107.2	30	96
			199.1[a]	22	96
			227.0	22	96
17β-Trenbolone-d3	2.4	274.1	199.1	24	106
			227.0[a]	21	106
Melengestrol acetate	3.8	397.2	279.1	19	87
			294.4	21	87
			337.1[a]	12	87
Melengestrol acetate-d3	3.8	400.2	282.1	19	87
			340.1[a]	12	87
Analyte	*Rt (min)*	*Precursor ion $[M-H]^-$*	*Product ions (m/z)*	*CE (eV)*	*Tube Lens (V)*
Estrone	2.7	269.1	143.0	40	121
			145.1[a]	60	121
α-/β-Estradiol	2.7	271.1	145.1[a]	43	110
			183.0	43	110
17β-Estradiol-d5	2.7	276.1	147.1	42	109
			187.0[a]	37	109

[a] Ion used for quantification (most abundant ion).

Validation

To determine bias and variability in recovery of the developed LC-MS/MS method, 50 mg of lab generated (LG) PM was collected on a 47mm Zefluor filter and spiked with 0.75 ng, 1.25 ng, or 5.0 ng of each analyte, along with 2.5 ng of each ISTD. To create LG PM, soil from a small scale experimental feedyard without TBA use was collected. Soil was stored at room temperature for over 6 months prior to use in validations. To produce airborne PM, soil was sieved into an open, polypropylene container and agitated by shaking the container. A TSP sampler was then used to collect the airborne PM as it was generated. Spiked filters stood at room temperature for 30 min until all solvent had evaporated and were then processed according to the sample preparation procedure and LC-MS/MS analysis described above. This was done on three different days with seven replicates for each spiking level. For QC purposes, solvent blanks and filter blanks were included in all validation sample sequences and all subsequent environmental sample sequences. Target analytes were not detected in solvent blanks or filter blanks throughout the study. Recovery of compounds ranged from 93-116%, with CV ranging from 1.8-16.1% (Table II). All values were within acceptable ranges of variation (CV ≤ 20.0% for low level standards; CV ≤ 15.0% for middle to high level standards) according to FDA bioanalytical method development criteria (*29*). Figure 1 displays a chromatogram of 5 ng/mL standard and PM spiked at 1.25 ng/filter.

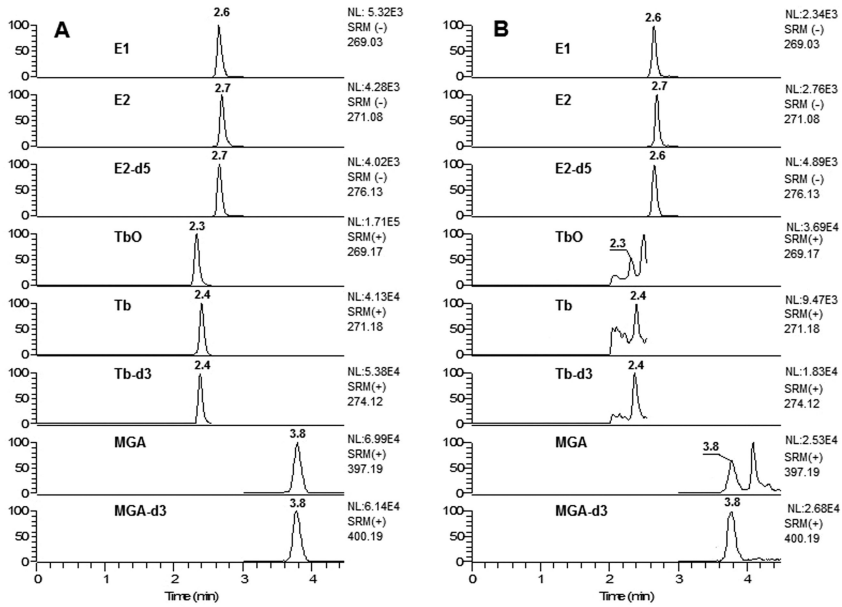

Figure 1. Chromatogram of A) 5 ng/mL standard; B) LG PM spiked at 1.25 ng/filter.

Table II. Performance of analytical method for growth promoters in 50 mg PM

Compound	*Spiked (ng/filter)*	*Recovery (%)*	*CV (%)*
Trendione	0.75	93.9	6.2
	1.25	101.9	5.5
	5.0	99.1	4.1
Trenbolone	0.75	102.8	15.2
	1.25	116.5	6.6
	5.0	99.5	1.8
Estradiol	0.75	112.3	11.0
	1.25	115.9	7.4
	5.0	112.3	7.8
Estrone	0.75	116.4	16.1
	1.25	105.8	14.8
	5.0	104.1	12.9
Melengestrol	0.75	112.2	8.5
Acetate	1.25	111.7	6.1
	5.0	106.3	4.3

Table III. Detection capabilities of target compounds through LC-APCI-MS/MS analysis

Analyte	*LOD (ng/filter)*	*LLOQ (ng/filter)*
Trendione	0.30	0.50
Trenbolone	0.35	0.55
Estrone	0.20	0.40
Estradiol	0.20	0.35
Melengestrol Acetate	0.35	0.55

Limits of detection (LOD) and the lower limit of quantification (LLOQ) were established through replicate injections of blank and spiked LG PM. Blank LG PM was analyzed to confirm method selectivity and ensure no co-eulting compounds. Using spiked LG PM, LOD was established as S/N = 3 for analyte quantitation ions, along with qualifier ions present in appropriate ratios (± 20% of spiked PM ratios). Likewise, LLOQ was established as S/N = 10 for quantitation ions, along

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with qualifier ions present in appropriate ratios (± 20% of spiked PM ratios). Of note, all LOD and LLOQ values were calculated on a per filter basis, not a ng/g of PM basis. As environmental PM samples will all vary in mass of PM collected, calculations based on ng/filter remove one additional variable, giving a truer representation of detection capabilities. LLOQ for all compounds were ≤0.55 ng/filter for all compounds and LOD ≤0.35 for all compounds (Table III).

Real Sample Analysis

This method was applied to 127 field collected samples. Field samples were collected from five separate commercial feedyards (see Sample collection section above). Samples were processed according to the described method. Of the target compounds, estradiol and estrone were detected most frequently and at the highest concentrations. Table IV presents the percentage of detections and range of concentrations in analyzed samples. Estrogen concentrations are further presented in Figure 2.

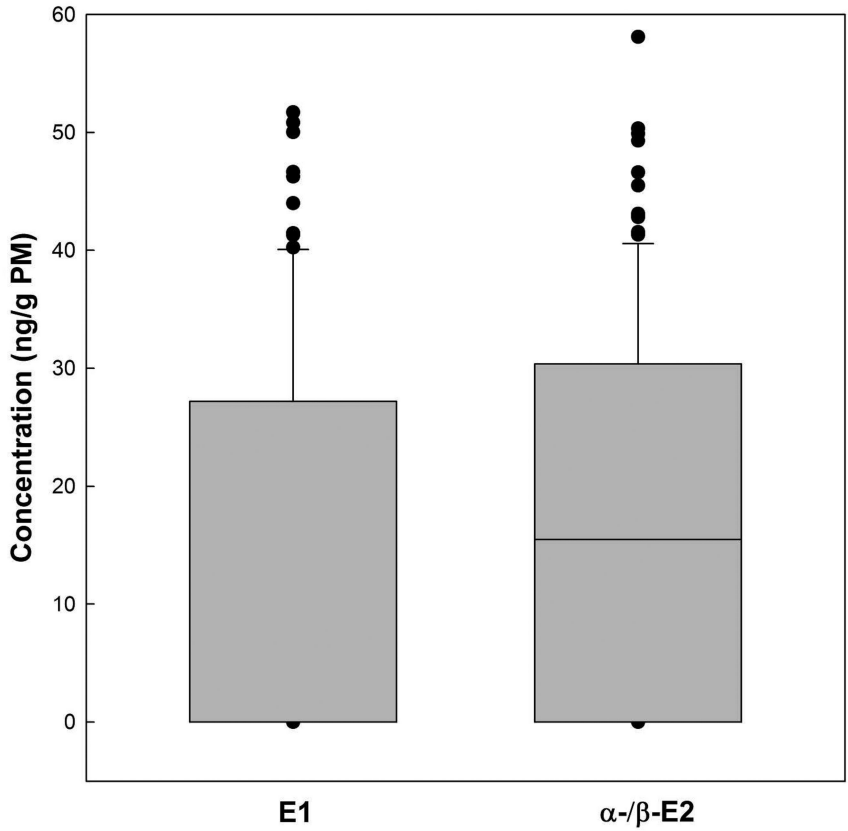

*Figure 2. Estrone (E1) and α-/β-estradiol (α-/β-E2) concentrations in PM samples (n = 127). All values reported in ng/g PM. If analytes were not detected, sample was assigned a value of ½*LOD.*

Table IV. Results of analysis of 127 real samples

Analyte	*Positive Detections (%)*	*Range*
Trendione	2	ND – 43 ng/g PM
Trenbolone	3	ND – 20 ng/g PM
Estrone	52	ND – 52 ng/g PM
Estradiol	62	ND – 58 ng/g PM
Melengestrol Acetate	7	ND – 36 ng/g PM

Experiments of sample degradation have begun by spiking filters and subjecting these to the sampling and processing procedure. Degradation during sample collection, during gravimetric analysis preparation, and during long term storage will be investigated. Preliminary experiments have been performed by spiking filters prior to deploying air samplers, then collecting samples of outdoor air for the appropriate amount of time. Degradation of trenbolone metabolites has been observed over the course of sample collection, and degradation of the other growth promoters may be occurring. Thus, compounds collected during h 1 of sampling could be degraded by h 48 when samples are collected and transported to the laboratory. These additional degradation experiments are ongoing and will provide additional detail to the accuracy of final detected growth promoter concentrations. If degradation is significant, procedures to further aid in mitigating degradation during sampling or processing will need to be implemented.

Conclusions

The aim of this study was to develop a specific, sensitive method for the simultaneous quantification of veterinary growth promoters and growth promoter metabolites, specifically trendione, trenbolone, estrone, estradiol, and melengestrol acetate, in airborne PM originating from beef cattle feedyards. Obtained data displayed satisfactory precision and accuracy using the described method. With the presence of veterinary growth promoters confirmed in a portion of PM samples, this method has application for future analysis of PM samples collected from agricultural facilities. Further research and understanding the stability of veterinary pharmaceuticals in the atmosphere and the mechanism of veterinary growth promoter transport will be critical in evaluating potential risks to human or ecological receptors.

Acknowledgments

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Chapter 6

Determination of an Immunocontraceptive Peptide in a Wildlife Vaccine Formulation

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Wildlife populations continue to grow despite the use of traditional management techniques. GonaCon™ Immunocontraceptive Vaccine is a vaccine used to reduce reproduction in mammalian species, including white-tailed deer (Odocoileus virginianus). The vaccine consists of synthetic gonadotropin releasing hormone (GnRH) conjugated to a mollusk hemocyanin (*Concholepas concholepas*) prepared as an emulsion with mineral oil to promote a prolonged immune response. Development of an analytical method for determination of the active ingredient in the vaccine formulation was complicated by the emulsion and conjugation of GnRH to the carrier protein. Breaking the emulsion was achieved chemically by addition of diethyl ether. The aqueous portion containing the GnRH conjugate was cleaved enzymatically with a protease (clostripain) at the arginine-proline site of its peptide sequence. Hydrolysis produced a diagnostic eight amino acid peptide fragment which was unique to GnRH and easily quantified by LC/MS/MS. Typical recoveries of fortified samples at the target concentration exceeded 90%.

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Introduction

Wildlife populations, in particular wild deer populations have been steadily increasing since the early 1900's when population estimations placed the number of wild deer in the US at approximately 500,000 (*1*). Restocking and conservation efforts were undertaken to replenish the populations. These efforts have been extremely successful to the point that a serious overpopulation problem now exists, particularly in urban and suburban areas (*2*). Current population estimates are in the neighborhood of 20 million individuals. Population densities can reach as much as 40 to 100 individuals per square mile in some urban and suburban areas (*3*). These high population densities can lead to severe wildlife management issues including increased deer-vehicle collisions (*4*), damage to ornamental plants or crops (*5*), degradation of habitat quality for other wildlife due to overgrazing (*6*, *7*), and possible disease transmission to other wild or domestic animals as well as human populations (*8*, *9*).

Management techniques typically employed to minimize deer-human conflicts in rural settings include culling, the use of scare devices, or exclusion fences. Firing high powered rifles in urban areas has very little support from members of the public who live in the areas where control is needed (*10*). Scare devices are either wholly ineffective or are only effective for a short time before the animals become habituated to them (*11*, *12*). Exclusion fences are not practical in many settings, can be cost prohibitive, and must be extremely tall in order to keep highly agile deer from jumping over them (*13*). Ecosystem controls such as the introduction of predators is not feasible in urban settings (*14*). A long-term management solution must include new tools in an integrated approach. A technique such as immunocontraception used to limit reproduction of local populations is such a tool.

GonaCon™ Immunocontraceptive Vaccine (hereafter GonaCon™) is an immunocontraceptive vaccine registered with the Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for use with female white-tailed deer 1 year of age or older (*15*). In addition to registration with EPA, use of this product requires registration within each state and approval by the State natural resource agency responsible for managing wildlife. Currently, it is only registered for use in New Jersey and Maryland.

GonaCon™ consists of a synthetic gonadotropin releasing hormone peptide (GnRH) conjugated to a mollusk hemocyanin (blue protein or BP). The conjugated protein is mixed with mineral oil and a surfactant to produce an emulsion (*16*, *17*). The final vaccine emulsion is delivered to the target animals via a 1 mL intra-muscular injection (*17*, *18*). The conjugated protein stimulates the production of antibodies that bind to endogenous GnRH. By binding to GnRH, the antibodies reduce GnRH's ability to stimulate the production of sex hormones such as estrogen, progesterone, and testosterone. As a result, all sexual activity is decreased and the animals remain in a nonreproductive state as long as a sufficient level of antibody activity is present.

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In order to support the continued registration of GonaCon™, an Enforcement Analytical Method is required by the EPA. The method needed to be accurate and reliable. It also needed to be sensitive enough to detect small differences in the active ingredient content of the final vaccine.

Methods

Reagents

Formic acid (LC-MS Grade), DL-dithiothreitol, 2,2,2-trifluoroethanol, and calcium chloride dihydrate were all obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile (Optima Grade), diethyl ether (anhydrous), sodium phosphate monobasic, and potassium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA). Clostripain (87 units/mg dry weight; Endoproteinase-Arg-C) was purchased from Worthington Biochemical (Lakewood, NJ). Gonadotropin releasing hormone (GnRH; [pE]HW SYG LRP GGC-CONH2; 95.42% pure) was synthesized by GL Biochem (Shanghai, China). Peptide-1 ([Pyr]HWSYGLR-Acid; 97.39% pure) was synthesized by Pi Proteomics (Huntsville, AL).

The mobile phase consisted of a mixture of 0.1% formic acid in deionized water and acetonitrile. Deionized water was purified using an E-Pure water purification system (Barnstead, Dubuque, IA). The sample extraction solution was prepared by combining approximately 0.36 g of sodium phosphate monobasic and 40 mg of DL-dithiothreitol in 60 mL deionized water. The pH was adjusted to approximately 7.6 with a solution of 1M potassium hydroxide. To this solution 5 mL of 2,2,2-trifluoroethanol was added before diluting to a final volume of 100-mL with deionized water. The enzyme activation solution was prepared by combining approximately 15 mg of calcium chloride dihydrate and 40 mg of DL-dithiothreitol. The solution was diluted to 100 mL with deionized water before use.

A concentrated stock solution of GnRH and Peptide-1 was prepared by weighing 1.000 mg of each and dissolving them in 5.00 mL of deionized water. Working standards, ranging in concentration from 10 ng/mL to 2 µg/mL, were prepared by dilution of stock solutions with a solution of 9:1 0.1% formic acid in deionized water:0.1% formic acid in acetonitrile. All standard solutions were stored at 5°C prior to use. A representative chromatogram is shown in Figure 1.

Sample Analysis

Breaking the Emulsion

GonaCon™ vaccine aliquots (0.5 g) were placed in 15-mL glass centrifuge tubes. One milliliter of extraction solution was added to each sample. The sample was vortexed to suspend the vaccine material followed by centrifugation at approximately 5,000 g for 2 minutes. Diethyl ether was used to break the emulsion and separate the mixture into an organic and aqueous layer. This was

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accomplished by adding three successive 5 mL aliquots of ether followed by a vortexing and centrifugation step to separate the organic and aqueous layers. The upper, organic layer was discarded and the aqueous layer was transferred to a 5-mL volumetric flask and diluted to produce a solution containing 5% trifluoroethanol in phosphate buffer.

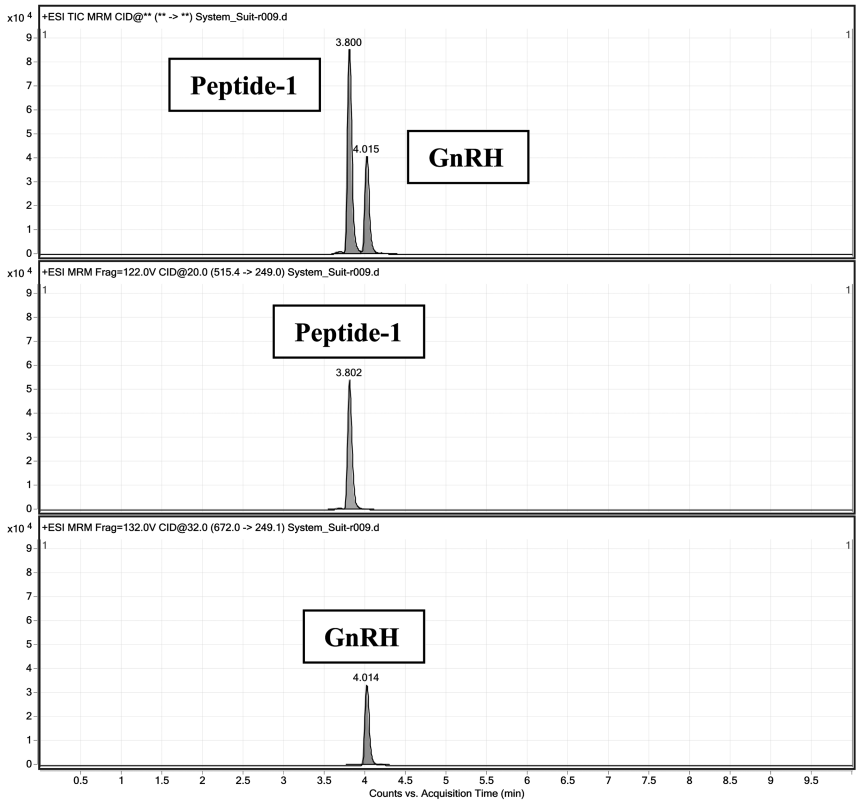

Figure 1. Chromatogram of a 500 ng/mL standard solution.

Enzymatic Cleavage

A solution of activated clostripain was prepared by weighing approximately 0.25 mg of clostripain into a 1.5-mL microcentrifuge tube and adding 1 mL of enzyme activation solution. A 50 µL aliquot of sample extract was removed and reacted with 50 µL of the clostripain solution in 1 mL of extraction solution to produce the peptide fragment. The enzyme reaction was conducted in a water bath held at approximately 25°C for two hours. Following completion of the reaction,

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1 mL of a solution of 8:2 0.1% formic acid in deionized water:0.1% formic acid in acetonitrile was added and the sample was centrifuged to remove any heavy protein fragments. This clarified solution was injected into the LC/MS/MS and the concentration of the diagnostic peptide fragment was quantified. Each sample was analyzed for both total GnRH content and free (or unconjugated) GnRH content by preparing a second sample which contained all of the components except the clostripain.

High-Performance Liquid Chromatography Tandem Mass Spectrometry

The analytical system consisted of an Agilent 1200 liquid chromatograph coupled to an Agilent 6410A-2K triple quadrapole mass spectrometer (Palo Alto, CA). Instrumental parameters are listed in Table I. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode (Table II). The precursor ion for both GnRH and Peptide-1 had a z value of 2. Therefore, the base peak for each was found at half the mass of the molecule. Following collision in the mass spectrometer, the product ions were found to have z values of 1. This led to a situation in which the product ions had larger m/z than the precursor ions for the quantification transition for each analyte. The proposed fragment ions can be seen in Figures 2 and 3. The m/z of the precursor ions are given in the lower left corner of each figure. The lines indicate the site of fragmentation for each product ion as well as the corresponding m/z value for each. The calibration standards (10 – 2,000 ng/mL) were injected in triplicate and subjected to a weighted quadratic regression. Quantification was accomplished by comparing analyte responses to the external standard calibration curve.

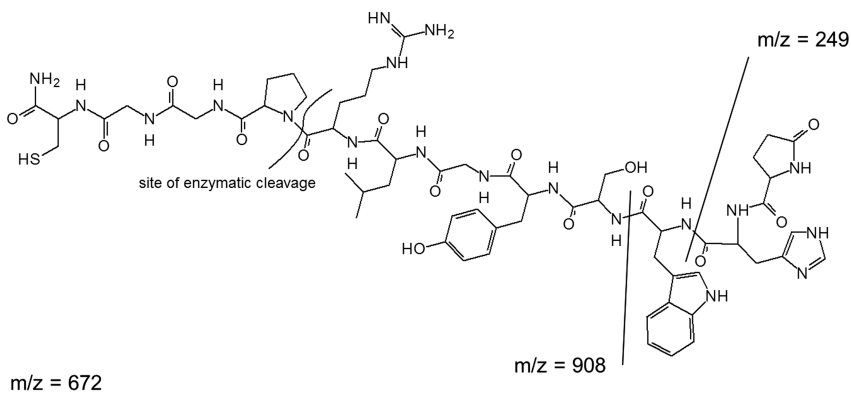

Figure 2. Mass fragmentation of GnRH.

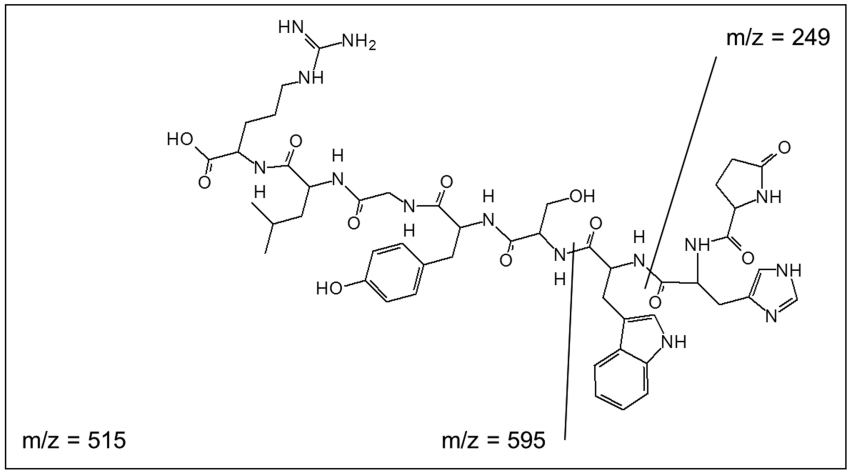

Figure 3. Mass fragmentation of Peptide-1.

Quality Control Samples and Fortification of Controls

A sham vaccine was prepared in the same manner as the GonaCon vaccine. This sham contained all of the components except GnRH and was prepared using the same protocols as the vaccine samples. The sham was fortified with a solution of GnRH in deionized water to produce fortification levels 50% above and below the target level for the vaccine (300 µg/mL). The quality control samples were extracted and analyzed using the same procedure described above.

Results and Discussion

Enzymatic Cleavage

The GonaCon™ vaccine is manufactured by conjugating the synthetic GnRH peptide to a mollusk hemocyanin protein via a sulfo-SMCC linker. Direct measurement of the fully conjugated protein was not possible due to the mass of the BP (approx.. 8 mDa). Attempts were made to analyze the final vaccine through the use of near infrared transflectance. This approach proved to be unfeasible possibly due to the high background created by the BP. The GnRH could easily be measured in solutions via HPLC, but not when linked to BP. The use of this linker produced a very stable and rugged link between the two

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components of the vaccine. However, this linkage is irreversible. Various solvents, acids, bases, and buffer treatments were investigated in an attempt to break this linkage. All efforts were unsuccessful. Clostripain is a proteinase isolated from *Clostridium histolyticum* which is highly specific for the carboxyl peptide bond of arginine and cleaves the GnRH at the cysteine residue in the conjugate (Figure 2) (*19*). This produces a smaller fragment of GnRH which has been called Peptide-1. Peptide-1 results from one GnRH molecule in a directly proportional relationship. Treatment of the sham vaccine with clostripain did not produce any measurable Peptide-1. By use of this enzyme, direct measurement of GnRH content in GonaCon vaccine was accomplished.

Table I. Instrument parameters

Parameter	*Conditions*		
Mobile Phase:	A = 0.1% formic acid in deionized water B = 0.1% formic acid in acetonitrile		
Gradient:	Time	% A	% B
	0.0	90	10
	1.0	90	10
	5.0	60	40
	5.5	10	90
Flow Rate:	0.5 mL/minute		
Injection Volume:	20 µL		
Column:	Agilent Rapid Resolution HT Zorbax SB-C3, 100 mm x 3.0 mm i.d., 1.8 µm		
Column Temperature:	80 °C		
Detector Gas:	Nitrogen; 350 °C; 12.0 L/minute		
Nebulizer Pressure:	40 psi		
Capillary Voltage:	4000 v		
MS1 Resolution:	Wide		
MS2 Resolution:	Unit		
Run Time:	10 minutes		
Post Time:	3 minutes		

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Table II. Multiple reaction monitoring transitions

Compound	*Transition*	*Dwell (ms)*	*Fragmentor (v)*	*Collision Energy (v)*
GnRH	672.0 → 908.3	75	132	24
	672.0 → 249.1	75	132	32
Peptide-1	515.4 → 595.3	75	122	22
	515.4 → 249.0	75	122	20

Additional problems were encountered while selecting the final extraction solution. The conjugated protein proved to be difficult to cleave with clostripain. Various buffers were used in an attempt to maximize the recovery of Peptide-1 from the GonaCon conjugate. It was hypothesized that the protein could be present in a folded conformation which might hinder the ability of the clostripain to cleave it. The use of 2,2,2-trifluoroethanol provided a minor denaturing effect which permitted the clostripain to cleave the GnRH. The enzyme system was optimized by investigation of the concentration of 2,2,2-trifluoroethanol which would permit the clostripain to still function while denaturing the conjugated GonaCon. A solution of 5% was found to be optimal. The time needed for the clostripain to cleave all of the GnRH present was also optimized. A reaction time of two hours was found to be sufficient for greater than 99% cleavage.

Table III. Analytical recoveries of free and total GnRH in sham vaccine quality control samples (n=7)

Fortification Level (μg/g)	*Range (%)*	*Mean (%)*	*Std. Dev. (%)*	*CV (%)*
Free GnRH				
150	91.3 - 103	99.3	4.1	4.1
450	94.5 - 101	98.2	2.3	2.3
Total GnRH				
150	106 - 127	116	11	9.5
450	110 - 124	117	6.2	5.3

Quality Control Results

Mean recoveries of free and total GnRH in sham vaccine (n=7) quality control samples were excellent (Table III).

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Method Limit of Detection

The method limit of detection (MLOD) was estimated from the mean chromatographic response at the retention time of the parent (GnRH) or product (Peptide-1) peptide plus three times the response standard deviation from seven replicate control samples. The mean and standard deviation for GnRH was measured and found to be 3.881±0.335 ng/mL. The mean and standard deviation for Peptide-1 was measured and found to be 1.577±0.458 ng/mL. Using these values, the MLOD for GnRH was estimated to be 2.1 µg/g and the MLOD for Peptide-1 was 1.6 µg/g.

GnRH Determination in GonaCon Vaccine

Six replicate samples of a formulated GonaCon vaccine with a target content of 300 µg/g were analyzed by the procedures outlined above. The range of observed values was 151 to 199 µg/g. The mean and standard deviation were 178 µg/g and 18 µg/g respectively.

Conclusion

A highly selective and sensitive method for the determination of GnRH in GonaCon vaccines has been detailed. The method is repeatable and accurate. Use of this method will permit further investigation of the manufacturing and storage stability of this important wildlife management tool.

Acknowledgments

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Chapter 7

Fate of Erythromycin in Sediment-Containing Surface Water Microcosms: How Does Aged Erythromycin in Sediment Influence Bioavailability?

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The detection of antibiotics in water and sediment systems is of concern due to the potential adverse effects which could be associated with their environmental fate. The central aim of this study was to evaluate the fate of erythromycin in microcosms consisting of pond water and submerged pond sediment. The first study examined the dissipation of erythromycin from spiked water and total recovery of [^{14}C]-erythromycin from water and sediment within microcosms ranged between 90.1% and 48% throughout the 63-day study. Erythromycin was reduced in surface water of sediment-containing systems by day 7, which corresponded to an increase of eryrthromycin detected in sediment. In the second study the availability of aged erythromycin was evaluated by incubating sediment with and without a manure amendment with [^{14}C]-erythromycin for 0, 1, 3, or 8 weeks; followed by assessing movement and availability of erythromycin in sediment microcosms after 1, 3, 7, and 14 days. Results indicated differences in residues from aged sediment, with and without manure additions, in extractable residues at day 7 and 14. The addition of manure resulted in greater extractable erythromycin from aged sediments than from sediments without manure. There was a greater release of erythromycin to the water overlying the manure-treated

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sediments with fresh and 1 week aged sediment than the unamended sediment after 1 and 2 weeks. The results from this experiment demonstrate the ability of manure to influence the fate of erythromycin in environmental matrices.

Keywords: Erythromycin; sediment-containing systems; manure; microcosms

Introduction

Antibiotics continue to be an emerging contaminant of concern due to their increase in usage and detection in the environment. Various classes of antibiotics have been found in environmental sampling studies, and concentrations have been measured in a broad range within various matrices including water, soil, sediment, and manure (*1–9*). The presence of these compounds in the environment could potentially affect many aspects of ecosystem function including alteration of bacterial populations leading to nutrient cycle impacts, potential adverse effects to aquatic and non-target organisms, and possibly influence human health (*10*, *11*). One environmental entry point of antibiotics is through land application of manure (*7*, *12*, *13*). Antibiotics are administered to livestock and poultry for treatment of infections as well as for disease prevention, growth promotion, and feed efficiency (*14*, *15*). The majority of the antibiotics administered to agricultural animals are excreted as parent compound, due to low absorption rates (*16*, *17*). Manure produced from these animals is ultimately applied to farmland by injection or waste incorporation as fertilizer to improve crop growth and development (*4*, *16*, *18*, *19*). Detection of antibiotics in water, sediment, soil, and manure samples has been prevalent over the past few years, with tetracyclines, sulfonamides, and macrolides being the most frequently detected antimicrobial compounds.

Macrolides, including erythromycin and tylosin, are one of the most frequently detected antibiotic classes in the environment. Erythromycin's structure is comprised of a 14-member lactone ring with two sugar groups with a molecular weight of 733.9, a pK_a of 8.8 and a K_{ow} of 3.06 (*20–23*). This antibiotic is effective against most gram-positive and some gram-negative bacteria, and its mode of action is through blocking elongation of peptide chains in the ribosome, which inhibits protein synthesis (*21*, *24*, *25*). Elimination of erythromycin occurs through bile and feces at a rate of 50-67% and with urinary excretion at 5-10% (*26*). The high excretion rates of erythromycin may allow for environmental entry of the compound through manure application to agricultural fields, which could enter water and sediment systems through runoff events.

The United States Geological Survey in a 2002 study found 48% of 139 streams tested contained antibiotics, and the second most frequently detected antibiotic from this study was erythromycin (*2*). Another study conducted in 2002 detected antibiotics in 31% of samples collected near swine farms and 67% of samples near poultry farms, with tetracyclines and macrolides (e.g.

erythromycin, tylosin) having the highest concentrations (*3*). One of the most prevalent macrolide antibiotics detected in water samples has been erythromycin, ranging in concentration between 50 ng L^{-1} and 300 ng L^{-1} (*2*, *3*).

In addition to the detection of antibiotics in surface waters, these compounds have also been found in sediment systems and manure slurries. Macrolides have been found in sediment samples with reported concentrations ranging between 2.1 μg kg^{-1} to 24.3 μg kg^{-1} (*5*). Erythromycin was detected in sediment samples ranging between 82 μg kg^{-1} to 128 μg kg^{-1}, which are markedly higher concentrations compared to water systems (50 ng L^{-1} to 300 ng L^{-1}), possibly due to the amount of aged residues that are sequestered within the sediments (*4–6*, *27*). In an environmental monitoring study erythromycin was determined to have the highest relative loss with greater than 50% loss due to runoff and erosion in a rainfall study compared to other antibiotics examined (*6*). The half-life of erythromycin in soil has been experimentally determined to be between 11.5 and 20 days (*7*, *28*). Another macrolide antibiotic found to have strong sorption in soils was tylosin, which demonstrated an affinity to adsorb to manure and sediments, as evidenced by recoveries of less than 2.5% from soil columns (*9*). It has been suggested that macrolide antibiotics, including erythromycin and tylosin, are adsorbed to clay particles, organic matter, or manure in soil, which reduce their degradation and leaching (*12*). These studies found macrolide antibiotics in multiple environmental matrices, with tylosin and erythromycin being widely detected. However, less information regarding erythromycin's environmental fate is available compared to tylosin and further research is needed to understand its behavior within the environment.

Erythromycin has the potential for transport in the environment through runoff from manure-treated fields leading to erythromycin's entry into water, which in turn leads to erythromycin in sediment where it may persist and age, and be bioavailable for uptake by terrestrial and aquatic organisms to some unknown extent Although few studies have focused on aged antibiotic residues in sediments, many studies have examined other organic compounds including herbicides and insecticides (*29–31*). Some herbicides and insecticides bioaccumulate in organisms within sediment and affect non-target organisms, but bioavailability of these aged residues within the environment is influenced by sediment characteristics (particles size, pH, clay content, and organic matter content) which affect adsorption and desorption rates of those compounds (*30*, *32*).

The overall aim of this study was to investigate the fate of erythromycin in a pond water and pond sediment microcosm through examination of its ability to bind to organic particulate matter and sediment, to partition between water and sediment, and of its abiotic and biotic degradation within the environment. This paper examines erythromycin's movement within water and sediment microcosms, specifically to improve the understanding of erythromycin's environmental fate and to simulate the impact of erythromycin run-off, which commonly occurs with manure from agricultural field application. Potential bioavailability of erythromycin residues in sediment were examined after aging for 0, 1, 3, or 8 weeks.

Materials and Methods

Chemicals

Acetonitrile (HPLC grade), acetic acid, ammonium acetate, sodium hydroxide, erythromycin, ashless cellulose powder, and Ultima Gold scintillation cocktail were purchased from Fisher Scientific (Pittsburgh, PA). Carbosorb E and Permafluor E+ scintillation cocktails were purchased from Perkin and Elmer (Waltham, MA). [^{14}C]-radiolabeled erythromycin was purchased from American Radiolabeled Chemicals (St. Louis, MO). The [^{14}C]-label was present in one of the methyl groups bonded to the nitrogen of the desosamine sugar of the erythromycin molecule.

Pond Water, Pond Sediment, and Manure Collection

Pond water and sediment were collected from the Iowa State University Horticulture Research Station (Gilbert, Iowa). Sediment was manually collected by inserting a soil auger 10 – 15 cm (depth) into the pond sediment. Sediment composition was determined as 60 % sand, 28 % silt, 12% clay, 2 % organic matter, and a pH of 8.1. Water had an alkalinity of 103 mg ml^{-1} and total hardness was 150 mg ml^{-1}. Sediment moisture was 47% prior to use. Water and sediment samples were transported to the lab and were stored at 4° C until use (< 7 days). Fresh manure was obtained from the Iowa State University Swine Nutrition Farm (Iowa State University) from antibiotic-free pigs on a corn-soybean-based diet. Manure characterization was completed by the Iowa State University Agricultural Waste Management Laboratory indicating a pH of 6.3 and containing: 33.6% total solids, 1.7% total Kjeldahl nitrogen, 1.1% total phosphorous,0.7% ammonia, and 0.4% dissolved reactive phosphorous. The collected manure was kept at 4°C until use (< 7 days).

Environmental Fate Experimental Design and Analysis

This study examined the fate of erythromycin entering a simulated pond in runoff from an upstream source. Four different microcosm treatments: pond water only (PW), pond water overlying pond sediment (PWS), autoclaved pond water overlying autoclaved pond sediment (APWS), and pond water with dilute swine manure overlying pond sediment (PWS+M). The APWS treatment aimed to measure sorption and non-biotic processes, while the PWS treatment focused on the combined impact of sediment sorption and biodegradation, and the PWS+M examined the impact of manure associated with runoff. The PW treatment was used to assess the impact of erythromycin degradation in water. All treatments utilized in this study were selected to investigate erythromycin's potential environmental fate and their detection in matrices (water, sediment, and manure), with the APWS treatment serving as a control.

One week prior to set-up 4 L of pond water and 1200 g of pond sediment were autoclaved three times at 121°C in one-hour cycles for use in the APWS treatment at one-day intervals. The PW treatment consisted of 200 ml of pond water, while the APWS treatment had 64.8 g (50 g dry wt.) autoclaved pond sediment and 185.2

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ml autoclaved pond water. For the PWS and PWS+M treatments the microcosm was comprised of 73.5 g (50 g dry wt.) pond sediment and 176.5 ml pond water. Microcosms were assembled in wide-mouth 470-ml jars (Ball Corp., Broomfield, CO), 50 g dry weight of sediment and 200 ml pond water per jar and were incubated for 7, 14, 28, or 63 days. Each jar served as a replicate with four replicates per treatment and timepoint.

Sediment was allowed to settle one hour prior to [^{14}C]-erythromycin addition. The treatment solution utilized in this study was comprised of labeled and non-labeled erythromycin which was added to each treatment replicate. Treatment spiking solution was prepared with 85 mg of non-labeled erythromycin to obtain a concentration of 0.425 mg ml^{-1} in a 200 ml volumetric flask and 171 µl of 0.1 mCi ^{14}C-radiolabeled erythromycin (specific activity of 55 µCi mmol^{-1}). Each treatment replicate received 2.35 ml of the treatment spiking solution yielding final concentrations of 5 mg L^{-1} and 0.201 µCi per jar.

For the PWS+M treatment a manure slurry was prepared by adding 33 g of manure to 100 ml distilled water to get a 33% slurry solution. The slurry was stirred for 40 minutes to break up large chunks, and 0.6 ml of slurry was added to each replicate giving the treatments a murky appearance compared to the treatments without the manure amendment. The autoclaved treatment was assembled and analyzed in a laminar flow hood using sterile equipment to maintain sterile conditions. All treatments were maintained in a 24° C environmental chamber with a 12:12 photoperiod. The pH of water in all treatments was monitored weekly and did not vary significantly throughout the course of the study.

Mineralization of [^{14}C]-erythromycin was tracked throughout the study by using sodium hydroxide solution traps for CO_2 evolution. A 25-ml high-density polyethylene vial was glued onto the inner surface of each jar and was filled with 10 ml of 0.5 M sodium hydroxide. Traps were changed on Day 3, 7, 14, 21, 28, 35, 42, 49, and 56 of the study. Three milliliters of each sodium hydroxide sample was mixed with 12 ml Ultima Gold cocktail, mixed, and was counted for radioactivity on a Beckman Coulter 6500 liquid scintillation counter ((LSC), Fullerton, CA).

After 7, 14, 28, and 63 days of incubation the distribution of [^{14}C] in water and sediment was determined. Treatment water was removed from each replicate jar and [^{14}C]-erythromycin radioactivity was counted on the LSC using 1 ml of water with 15 ml Ultima Gold cocktail. Next, the water samples were filtered through 0.2-µm, 47-mm diameter nylon filters (Fisher Scientific). Following filtering, water samples were extracted using Oasis® HLB cartridges (6 cc, Waters Corp., Milford, MA). After extraction of water samples, radioactivity was assessed with 1 ml of water and 15 ml Ulotima Gold cocktail . Cartridges were conditioned using the Kolz et al., (2006) solid phase extraction method. Recovery of ^{14}C-residues of applied [^{14}C]-erythromycin was determined to be 94.7% ± 4.9 from pond water utilizing this method.

Sediment was extracted with 100 ml of acetonitrile: 0.3 M ammonium acetate at pH 4.2 (85:15, v/v), and each sample was shaken on an orbital shaker for 85 minutes at 300 rpm. Samples were allowed to settle overnight at room temperature followed by siphoning off the liquid extract. A second 100-ml aliquot of acetonitrile: 0.3 M ammonium acetate at pH 4.2 (85:15, v/v) was

added to each sediment sample and shaken on an orbital shaker for 15 minutes at 300 rpm followed by centrifuging and decanting. Each sediment extract sample was concentrated to a volume of 1 ml under nitrogen flow at 15 psi, at 50°C and reconstituted to a final volume of 10-ml with acetonitrile. A 3-ml aliquot of sediment extract for each sample was mixed with 12 ml Ultima Gold cocktail and counted for radioactivity on the LSC. Extracted sediment samples were allowed to dry in a fume hood for 24 hours. Dried sediment was sieved through a 5-mm sieve, followed by a 2.5-mm sieve to remove any large non-combustible material. Sieved sediment samples were ground using a mortar and pestle. Next, sediment pellets were constructed with 0.5 g dried, ground sediment and 0.5 g ashless cellulose powder (1:1 ratio). Sediment pellets were oxidized using a Packard Model 307 oxidizer (Perkin Elmer, Waltham, MA) with a two-minute combustion time. Following oxidation, sediment sample vials containing reagents were counted for radioactivity on the LSC to determine bound [^{14}C]-erythromycin residues.

Aged Sediment Experimental Design

Two metal pans were filled with 2.87 kg of pond sediment, and to one of the pans a manure slurry was then added. The manure slurry contained 57.4 g of manure dissolved in 50 ml of distilled water and was stirred for 50 minutes until thoroughly mixed, followed by addition to one container of sediment. Treatment spiking solution was prepared with 24.2 mg of non-labeled erythromycin to obtain a concentration of 0.121 mg ml^{-1} in a 200 ml volumetric flask and 303 µl of 0.1 mCi ^{14}C-radiolabeled erythromycin (specific activity of 55 µCi mmol^{-1}). Each treatment replicate received 97.5 ml of the treatment spiking solution yielding final concentrations of 3.775 mg L^{-1} and 0.19 µCi per jar.

Erythromycin residues in the sediment were aged for 0, 1, 3, or 8 weeks prior to microcosm assembly and were kept at 25°C using a 16:8 light:dark photoperiod. Microcosms were assembled after the designated timepoints which included 36.75 g (25 g dry weight) of sediment (either with or without manure amendment), and they were topped with 88.25 ml of distilled water in a 250-ml French square bottle. Microcosms were incubated for 0, 1, 3, 7, or 14 days and were performed in replicates of four (n=4). All aged sediment and water columns were maintained in a 25° C environmental chamber with a 16:8 photoperiod.

Water column replicates were sacrificed at the specified timepoints, at which water was removed from treatment containers and SPE was performed as discussed in the environmental fate experimental design and analysis section. Next, sediment was extracted using 50 ml acetonitrile: 0.3 M ammonium acetate at pH 4.2 (85:15, v/v) followed by bound residue analysis with the protocols outlined in the previous experiment environmental fate experimental design and analysis section.

Statistical Analysis

Statistical analysis was performed using SigmaStat 3.0 (Chicago, IL) employing ANOVA analysis with Bonferroni or Dunn's analysis to compare data treatments and time points. Significance level was determined as $P \leq 0.05$

for all analyses. Linear regression and least squares analysis were conducted with SigmaPlot 10 (Chicago, IL) to determine dissipation kinetics in water from treatment samples.

Table 1. Mass balance of [^{14}C]-erythromycin residues in treatment microcosm components[1]

Treatment	*Microcosm System Component*	*Day[2]*			
		7	*14*	*28*	*63*
PW[a]	Treatment Water	87.5 ± 0.7	89 ± 1.8	80.7 ± 1.1	86 ± 0.9
	Mineralization	0.2 ± 0.03	1.1 ± 0.1	1.3 ± 0.05	2.2 ± 0.02
	Total Recovery	87.7	90.1	82.5	88.2
APWS[b]	Treatment Water	33.7 ± 4.4	24.6 ± 1.0	13.9 ± 2.3	10.5 ± 1.3
	Sediment - Extractable	30.1 ± 2.5	29.2 ± 1.7	32.3 ± 1.2	38.3 ± 6.6
	Sediment - Bound	10.6 ± 0.2	12.2 ± 0.8	13.5 ± 1.1	14.2 ± 0.5
	Mineralization	0.06 ± 0.06	0.2 ± 0.03	0.6 ± 0.02	1.1 ± 0.01
	Total Recovery	74.5	66.2	60.3	64.1
PWS[c]	Treatment Water	19.3 ± 1.6	18 ± 1.0	15.4 ± 1.8	2.6 ± 0.2
	Sediment - Extractable	40.2 ± 3.7	30.7 ± 4.5	12.7 ± 1.3	3.7 ± 0.6
	Sediment - Bound	13.1 ± 0.5	16.4 ± 0.8	20.9 ± 2.0	38.2 ± 1.6
	Mineralization	0.2 ± 0.02	1.8 ± 0.3	12.6 ± 0.9	16.1 ± 0.04
	Total Recovery	72.8	66.9	61.6	60.6
PWS+M[d]	Treatment Water	27.5 ± 3.4	24.6 ± 0.3	9.6 ± 0.8	3.7 ± 0.1
	Sediment - Extractable	38.3 ± 5.6	36.1 ± 2.1	9.1 ± 0.2	4.4 ± 0.4
	Sediment - Bound	11.3 ± 0.3	16.1 ± 0.9	32.3 ± 2.6	28.7 ± 1.2
	Mineralization	0.2 ± 0.02	1.3 ± 0.2	8.8 ± 0.6	11.2 ± 0.07
	Total Recovery	77.3	78.1	59.8	48

[1] Values shown are mean percentage of applied radioactivity ± standard error. [2] Day post addition of [^{14}C]-erythromycin added to water portion of microcosm. [a] Pond Water. [b] Autoclaved Pond Water and Autoclaved Pond Sediment. [c] Pond Water and Pond Sediment. [d] Pond Water with Manure Slurry and Pond Sediment.

Results and Discussion

Freshwater Microcosm Study: Mass Balance

Mean percentages of [^{14}C]-residues recovered 63 days after [^{14}C]-erythromycin application to experimental microcosms are listed in Table 1. The pond water (PW) treatment recovery ranged between 89.9% and 81% throughout the course of the study. The APWS treatment displayed a decrease in mean [^{14}C] total recovery throughout the course of the study except with a small increase from day 28 to 63. The APWS water showed a decrease in [^{14}C]-erythromycin recovery and a slight increase in extractable and bound sediment residues. For the PWS treatment, a decrease in total recoverable mean [^{14}C]-erythromycin between day 7 and 63 occurred, 72.8% to 60.6%. Microcosm components for the PWS treatment displayed a decrease in radioactive residues for treatment water and extractable sediment residues, but an increase with sediment bound residues. The PWS+M treatment displayed similar [^{14}C]-erythromycin residue patterns in all microcosm components to the PWS treatment. ANOVA analysis indicated significant differences between the total recovery in the treatments examined ($p = 0.034$).

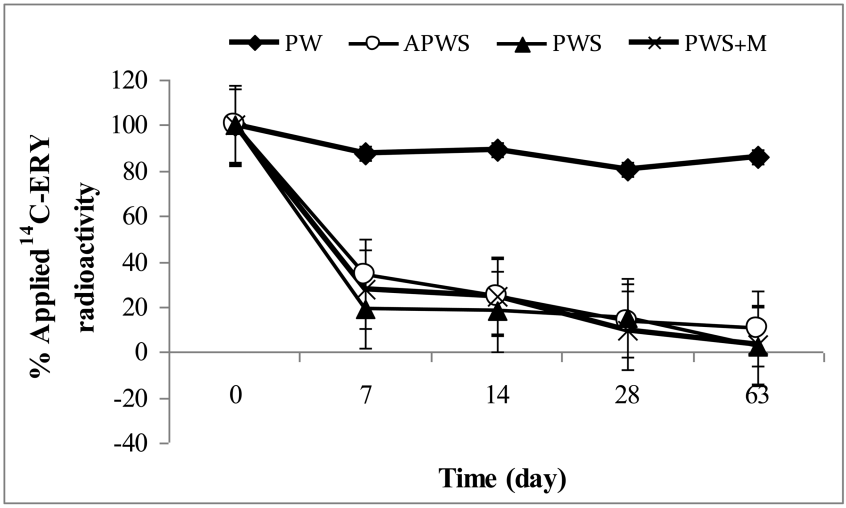

Figure 1. Percentage of [^{14}C]-ERY remaining in surface water.

Freshwater Microcosm Study: Dissipation Kinetics

[^{14}C]-erythromycin residues in surface water remained fairly constant in the PW treatment throughout the study (Figure 1). The PW treatment was significantly different in the quantity of [^{14}C] residue in surface water compared to the sediment-containing systems, with a greater amount present in the PW treatment compared to all other treatments examined. In treatments APWS, PWS, and PWS+M a sharp

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decrease in [^{14}C]-erythromycin was noted between day 0 and day 7, with 33% to 19% remaining in water by day 7 and continued to decrease by day 63 with <10% remaining in the water portion of the microcosm. The pH of the water throughout the course of the study did not vary greatly between day 0 and 63.

Dissipation kinetics in water were examined, and results indicated that erythromycin dissipates from water via a one-compartment model for the PWS and PWS+M treatments. (Equation 1). However, model was not valid for the PW and APWS treatments as poor correlation was observed. A DT_{50}, the time for the concentration of a chemical to reach 50% of applied, was also calculated for the dissipation of erythromycin for the treatments examined, using Equation 2 for all treatments.

$$(1)\ C = C_0 e^{(-k_t)}$$

$$(2)\ DT_{50} = 0.693/k$$

The variables used in the equations above represent the following:
C = erythromycin concentration at time t
C_0 = initial erythromycin concentration
k = first-order rate constant for erythromycin
t = time (days)

Table 2 lists the calculated parameters for the model shown above. Erythromycin dissipates from water to 50% of the applied dose by 5.8 days for APWS and PWS treatments, while it takes 5 days for the PWS+M treatment. The first-order, one-compartment model was used for the APWS, PWS and PWS+M treatments with r^2 values greater than 0.7 and this model has been utilized in pesticide risk assessment (*33*). However, with these treatments better r^2 values were obtained with a three-parameter modified single, exponential decay model for the treatments but these models do not have a mechanistic interpretation as do first-order or two-compartment dissipation kinetic models.

Table 2. Dissipation kinetics for erythromycin in treatment water of surface water microcosm systems

Treatment	*F value*	*Dissipation Model*	*k*	*r^2*	*p-value*	*DT_{50} (days)*
PW	1.9088	$C=C_0e^{(-kt)}$	0.0019	0.32	0.32	—
APWS	44.73	$C=C_0e^{(-kt)}$	0.1187	0.9986	0.007	5.8
PWS	44.73	$C=C_0e^{(-kt)}$	0.1895	0.9891	0.007	5.8
PWS+M	61.54	$C=C_0e^{(-kt)}$	0.1386	0.9889	0.0043	5

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The dissipation of erythromycin from water is mostly due to its partitioning into sediment in the APWS, PWS, and PWS+M treatments. The total recovery of [^{14}C]-residues was examined to determine when the microcosm treatments containing sediment reached 50% of applied by plotting the $\log_{10}$ of total percentage recovered in microcosms at 7, 14, 28, and 63 days versus time and fitting a linear trendline to obtain treatment specific equations listed in Table 3. Results indicate that the DT_{50} of 38 days for the PWS+M treatment is shorter than the 45 day DT50 for PWS treatment. However, the important role microorganisms have in degrading and utilizing erythromycin in the environment may influence erythromycin degradation.

Table 3. Equations for calculating DT_{50} remaining [14C]-applied within sediment containing microcosm treatments including days when 50% is reached

Treatment	*Linear Trendline Equation*	r^2	*DT_{50} of [^{14}C] remaining in microcosm (days)*
APWS	y = -0.0009x + 1.8438	0.3675	—
PWS	y = -0.0037x + 1.8692	0.91	45
PWS+M	y = -0.0057x + 1.9189	0.93	38

Mineralization of Erythromycin in Freshwater Microcosm

The inclusion of sediment increased $^{14}CO_2$ evolution from mineralization in pond water (Figure 2). The PWS and PWS+M treatments displayed similar trends in CO_2 evolution with a lag phase between days 0 and 7, followed by an exponential growth phase between days 7 and 35. After day 35, $^{14}CO_2$ in these treatments began to plateau through day 63. The PWS+M treatment had less mineralization compared to the PWS treatment in total amounts of $^{14}CO_2$ evolved throughout the study, with significant differences seen between the APWS compared to PWS and PWS+M ($P = 0.023$). The PW and APWS treatments were similar in the amount of $^{14}CO_2$ evolved, with <2% of applied ^{14}C-radiolabel detected in these treatments.

Mineralization is a common microbial process and the amount of mineralization occurring in the PWS and PWS+M microcosms may indicate a wide distribution of erythromycin-degrading microorganisms within the pond sediment. This distribution may be due to an increase in the density of microbial populations degrading erythromycin, especially those capable of degrading erythromycin including some gram-negative microorganisms (*34*, *35*). Kim et al., (2004b) demonstrated that the [^{14}C]-radiolabeled methyl group is more readily hydrolyzed compared to [^{14}C]-radiolabeled groups of the macrocyclic lactone ring. The cumulative mineralization rates in this study were 10% to 15%

for PWS+M and PWS treatments, respectively,which were greater than those reported by Kim et al., 2004. Our increased mineralization rates may be due to the position where erythromycin used in our study was labeled.

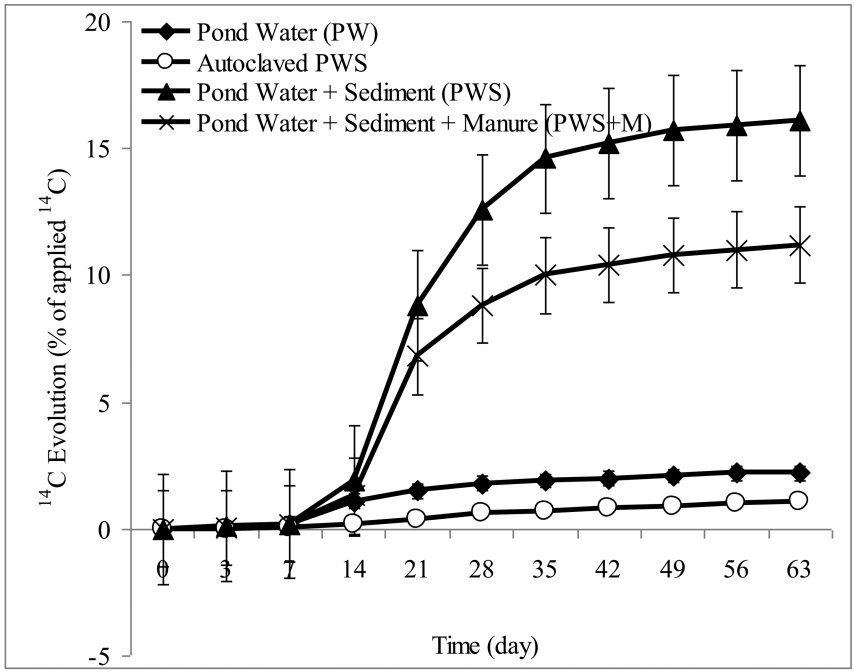

Figure 2. Cumulative mineralization of [^{14}C]-erythromycin from microcosm treatments.

Freshwater Microcosm Study: [^{14}C]-Erythromycin in Sediment

Erythromycin movement into the sediment corresponded to an increase in extractable residues and bound [14C]-residues (Fig. 3). The APWS treatment displayed a plateau of bound [^{14}C]-residues throughout the 63-day study A slight increase in bound residues between day 7 and day 63 was seen in the PWS and PWS+M treatments, most likely due to erythromycin interacting with clay and organic matter causing binding to occur through abiotic processes. In addition, this decrease in extractable residues may be attributed to microorganisms utilizing erythromycin and subsequently incorporating it as biomass. In the PWS and PWS+M treatments extractability of erythromycin decreased between day 7 and day 63. Bound [^{14}C]-residues within PWS demonstrated a linear increase from day 0 to day 63, and a decrease in extractable [^{14}C]-residues between day 7 and day 63. In contrast, the PWS+M treatment showed a slight decrease between day 7 and 14 in extractable erythromycin residues, followed by a sharp reduction between day 14 and 63. Bound residue in the PWS+M treatment increased from day 0 to day 28.

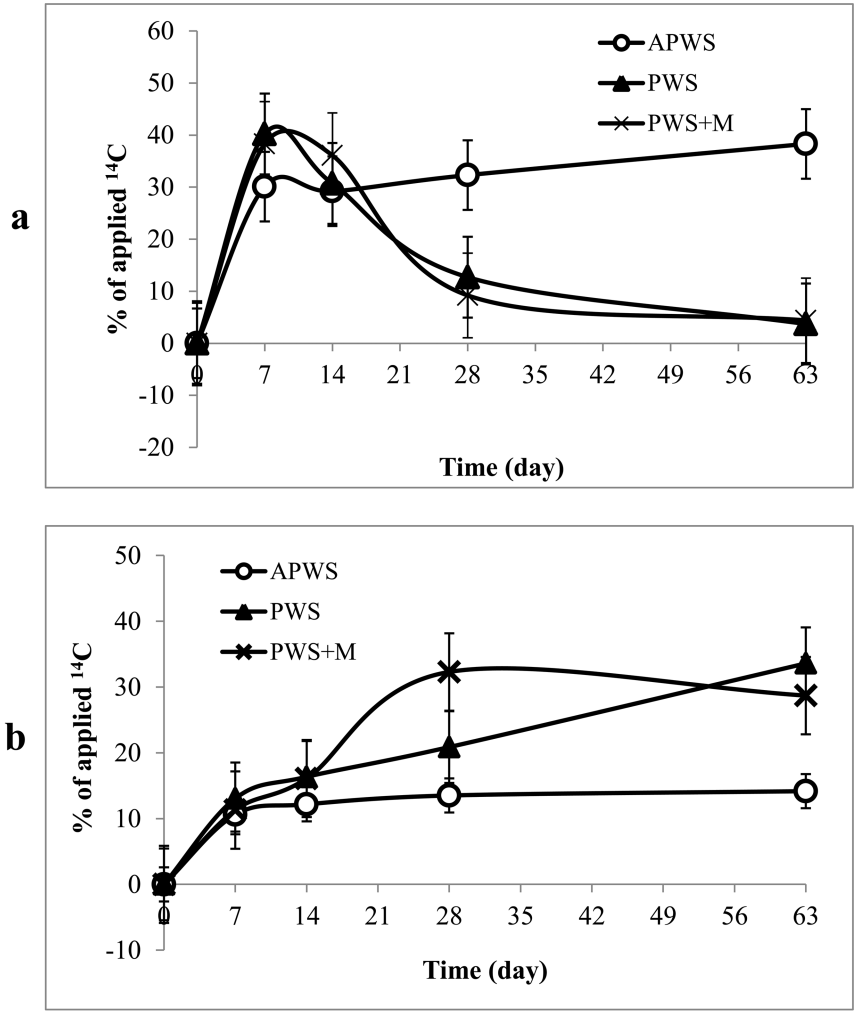

Figure 3. Percentage of extractable and bound [^{14}C]-residues derived from applied [^{14}C]-erythromycin (a) extractable [^{14}C]-residues (b) bound [^{14}C]-residues.

Erythromycin accumulated in sediment with a decrease in extractable residues and an increase in bound residues observed throughout the course of the study in the non-autoclaved treatments. We found 40% to 50% erythromycin in sediment components after 7 days, with slightly more erythromycin in the manure-containing treatment. These studies demonstrate that erythromycin accumulates in sediment with the potential for degradation to occur through biotic processes. However, additional experiments are needed to better understand the degradation pathway and the influence of chemical and biological parameters (pH, temperature, etc…) on sorption.

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Aged Residues Study: Sediment

Our results and those of others (*9*, *34*, *36*, *37*) show that erythromycin and other antibiotics partition from the overlying water into stream and pond sediments. We examined the dissipation of erythromycin residues in sediment that had been previously aged for 1, 3 or 8 weeks. Extractability of [^{14}C]-erythromycin from aged sediments was assessed prior to the assembly of the microcosms (Figure 4), and show a decrease over the 1 week, 3 weeks, and 8 weeks of aging, but no difference was seen between the two matrices examined. After microcosms were established and the non-aged (fresh) and aged residues were submerged, the [14C]-erythromycin degraded slowly (Figure 5). There was little change in extractable residues freshly added to sediment over the 14 day period. Residues aged for 1 week and 8 weeks decreased to about 50% of their levels at the start of the incubation, but the 3 week aged residues showed little decline over the 14 day period, similar to the freshly added residues.

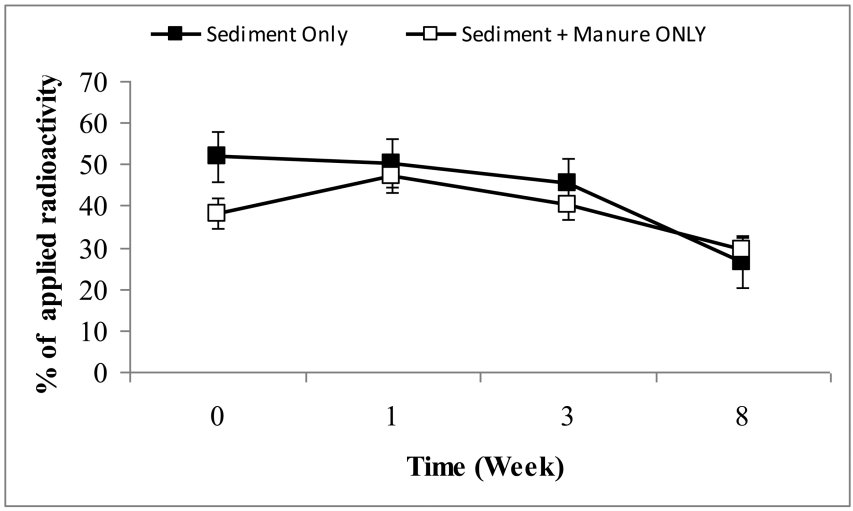

Figure 4. Extractable aged [^{14}C]-erythromycin residues (% of applied [^{14}C]) in sediment matrices with and without manure amendment prior to assembly of surface water columns. The time on the x-axis represents the number of weeks the microcosms containing water, sediment were incubated.

Statistically significant differences were found in extractable residues including fresh sediment compared to 3 and 8-week aged sediment ($P < 0.001$ and $P = 0.008$). Week 1 aged sediment extractable residues were statistically different from week 8 aged sediment extractable residues ($P = 0.029$). No significant differences were seen in sediment with manure amendment at the various time points examined (0, 1, 3, and 8 weeks). Comparison between sediment and sediment with manure amendment treatments aged 0, 1, 3, and 8 weeks showed a significant difference ($P=0.006$). The extractable [^{14}C]-residue results from the aged study are increased compared to the fate study, which may be due to the

incorporation route of the manure and erythromycin in each study. In the aged study the manure was mixed directly into sediment versus the fate study which utilized a manure slurry added to water representing manure runoff from rainfall. The manure incorporation route utilized in this experiment could also represent antibiotics which enter river sediment that is overlain with fresh stream water. This difference in manure and residue environmental entry routes could suggest erythromycin's potential to be more bioavailable in sediment.

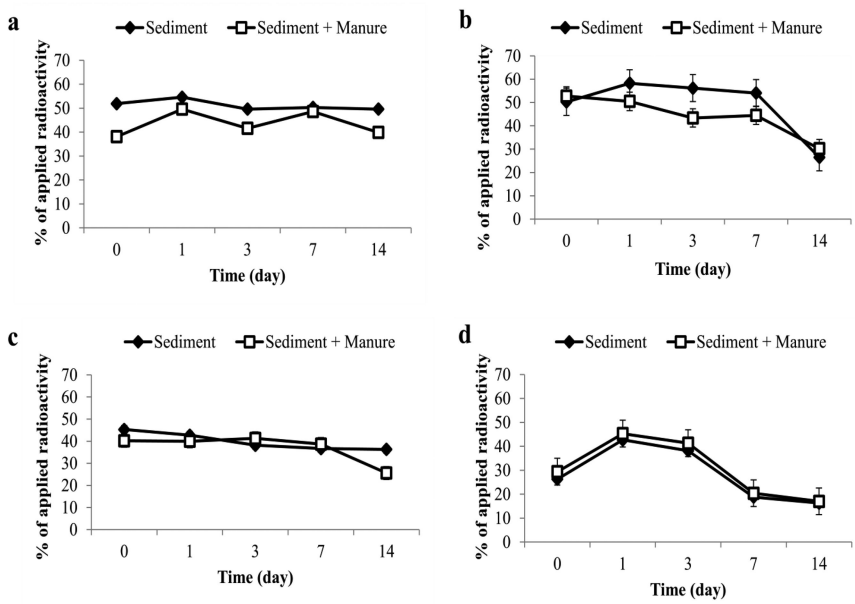

Figure 5. Extractable [^{14}C]-ERY (% of applied [^{14}C]) in sediment and sediment with manure amendment (50:1, v/v) followed by addition of distilled water and incubation for 1, 3, 7, or 14 days; (a) fresh (b) Aged 1 week (c) Aged 3 weeks (d) Aged 8 weeks.

Aged Residues Study: Water

The percentage of [^{14}C]-erythromycin moving into surface water from the aged erythromycin residues in sediment is shown in Figure 6. More [^{14}C]-erythromycin was released into surface water from the manure-containing sediment treatment compared to the sediment only matrix. In the sediment only system, fresh and 3-week-aged [^{14}C]-residues yielded higher amounts of erythromycin in surface water compared to 1 and 8-week aged [^{14}C]-residues by day 14 (Figure 6a). The sediment with manure amendment showed more erythromycin in water in the fresh and 1 week aged treatments at day 14 compared to 3 and 8-week aged samples. No significant differences were seen between aged [^{14}C]-residue treatments in the water component of sediment containing microcosms (without manure) for 1, 3, 7, and 14 days.

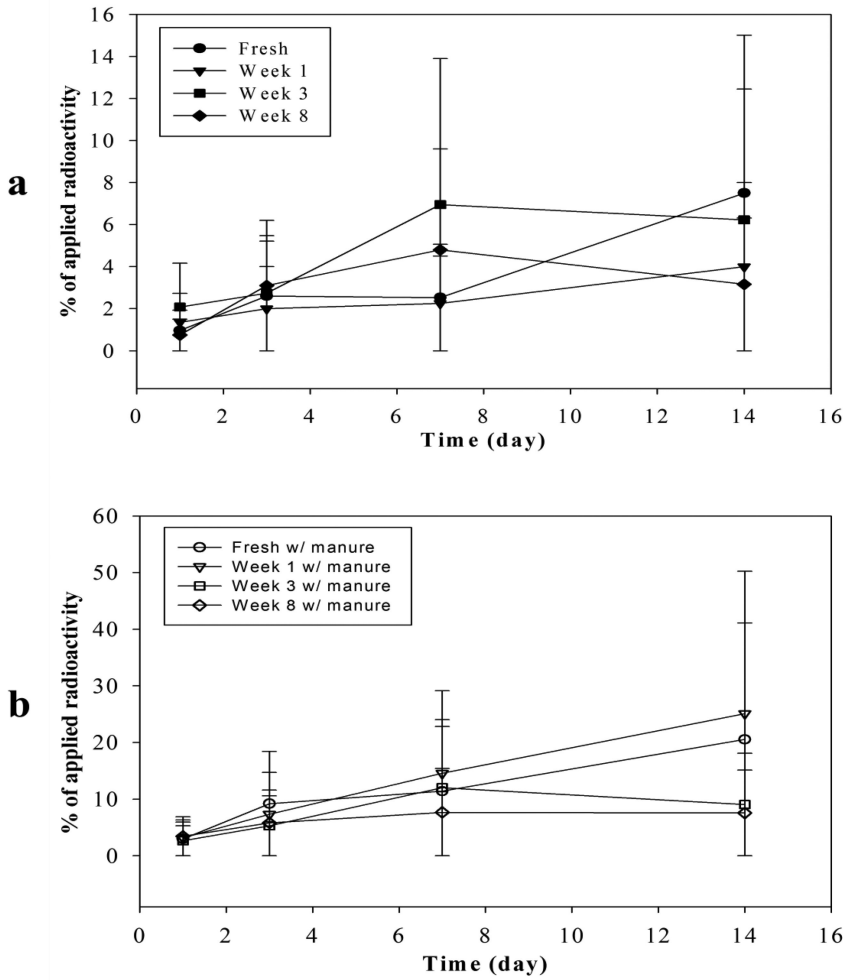

Figure 6. Percentage of [^{14}C]-ERY in surface water released from sediments treated with [^{14}C]-erythromycin at zero (fresh), 1, 3, and 8 weeks previously (a) Pond sediment system (b) Pond sediment + manure (50:1, v/v by weight) system.

Addition of manure to sediment influenced the availability of [^{14}C]-erythromycin residues in surface water with greater amounts seen in the water portion for fresh and 1-week aged treatments. Overall, aged residues in sediment with manure showed an increase in [^{14}C]-erythromycin into water between day 0 and 7 for all treatments (Figure 6b). A statistically significant difference was observed with erythromycin released from aged residues in sediment at 7 and 14 day incubation times with surface water ($P = 0.021$; $P = < 0.001$). By day 14 a decrease in erythromycin from water was seen with 3 and 8-week aged sediment with manure amendment, compared to an increase in fresh and 1 week aged treatments. Examination of extractable ^{14}C-residues from sediment with

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manure amendment incubated for 0, 1, 3, and 8 weeks indicated no significant difference between surface water incubation timepoints (1, 3, 7, and 14 days) with ANOVA analysis (P = 0.08). Statistical analysis indicated that there is a significant difference between aged erythromycin in sediment with manure after 7 and 14 days of water incubation in fresh and 1-week aged compared to 3 and 8-week aged treatments (P = < 0.001; P = < 0.001).

Conclusions

Erythromycin dissipates slowly in surface water half-life in the pond water of 365 days compared to the water with underlying sediment (APWS, PWS, and PWS+M) with 5 to 6 day half-lives. The quick dissipation in water is due to the rapid partition of erythromycin into the sediment. In contrast, when the total erythromycin residues in the microcosm are considered, the time for 50% erythromycin loss was 38 to 45 days in non-autoclaved treatments, demonstrating the tendency of sediment to sequester erythromycin. Biodegradation of erythromycin in sediment systems was observed as increased $^{14}CO_2$ evolution in non-autoclaved sediment-containing systems. The PWS and PWS+M treatments displayed higher mineralization rates compared to PW and APWS treatments due to microorganisms present in the sediment.

Erythromycin partitioned back into water from aged sediment, with manure influencing the partitioning in fresh and 1-week aged sediment samples. Erythromycin was found to be extractable from aged sediment and manure-containing sediment samples with a slight decrease observed with an increase in water incubation time. Further studies are needed to understand the bioavailability of erythromycin in the environment to non-target organisms due to this compound adsorbing into sediment and also movement into water from aged residues in sediment. Additional studies to better recognize the potential for metabolites to form in environmental components are needed, which may aid in a better understanding of erythromycin's fate in water and sediment.

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George Cobb is a Professor of Environmental Science at Baylor University. Prof. Cobb received a BS in Chemistry from The College of Charleston ('82) and a PhD in Chemistry from The University of South Florida ('89). Prof. Cobb's group develops analytical techniques to assess contaminant presence and movement. Many of these techniques have been deployed to evaluate contaminant presence, transport, and transformation in organisms and environmental systems. Major research efforts have coupled ultra-trace analytical methods with non-lethal monitoring techniques as a critical component of comprehensive risk assessments. Prof. Cobb serves on the Editorial Board for *Environmental Toxicology and Chemistry*.

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Dr. Smith is an Associate Professor of Environmental Toxicology at Texas Tech University. He received a BS in biology from Murray State University (1988) and a doctorate in environmental toxicology from Texas Tech University (2000). Dr. Smith's interests include contaminant exposure and responses among ecological receptors. His research emphasizes pathways of contaminant exposure among organisms, and trophic transfer of environmental contaminants. Physiological and population-level responses to contaminant exposure are of particular interest as they relate to ecological risk assessment. Dr. Smith serves as Editor for *Environmental Toxicology and Chemistry*, Associate Editor for *Herpetology,* and Editorial Board member for *Environmental Pollution*.

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