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Development, and Ground Truthing, of Analytical Tools for Measuring Select Emerging Contaminants (ECs) in a Subset of Biosolids Collected During OW's 2006 – 2007 National Biosolids Survey Project

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Development, and Ground Truthing, of Analytical Tools for Measuring Select Emerging Contaminants (ECs) in a Subset of Biosolids Collected During OW's 2006 – 2007 National Biosolids Survey Project

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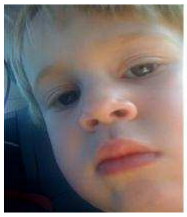

FOREWORD

The U.S. Environmental Protection Agency (EPA) is charged by Congress to protect the nation's natural resources. Under the mandate of national environmental laws, the EPA strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development (ORD) provides data and scientific support that can be used to solve environmental problems, build the scientific knowledge base needed to manage ecological resources wisely, understand how pollutants affect public health, and prevent or reduce environmental risks.

The National Exposure Research Laboratory (NERL) is the Agency's center for investigation of technical and management approaches for identifying and quantifying exposures to human health and the environment. Goals of the laboratory's research program are to: (1) develop and evaluate methods and technologies for characterizing and monitoring air, soil, and water; (2) support regulatory and policy decisions; and (3) provide the scientific support needed to ensure effective implementation of environmental regulations and strategies.

This report presents the experimentation, results, findings, and recommendations of biosolids research conducted from 2006 to 2009. The data from this report will be transmitted to the Office of Water in support of EPA's statutory requirements under the Clean Water Act, Section 405(d)(2)(C), to conduct a review of the 40 CFR 503 standards not less than every two years for purposes of regulating new pollutants where sufficient data exist.

The author acknowledges the tremendous support of Mr. Thomas Moy, Senior Environmental Employment Program (SEEP) and Mr. Reza Kazemi (Student Services Contractor) for all of their laboratory technical support and day-to-day operations, without whom this research would not have been possible.

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EXECUTIVE SUMMARY

On February 19, 1993, EPA promulgated the CFR 40 Part 503 *Standards for the Use or Disposal of Sewage Sludge*, resulting in numerical standards for ten metals and operational standards for microbial organisms. The 1993 rule established requirements for the final use or disposal of sewage sludge when it is: (1) applied to land as a fertilizer or soil amendment; (2) placed in a surface disposal site, including sewage sludge-only landfills; or (3) incinerated. These requirements apply to publicly and privately owned treatment works that generate or treat domestic sewage sludge and to anyone who uses or disposes of sewage sludge. Under Section 405(d), of the Clean Water Act, EPA establishes numerical limits and management practices that protect public health and the environment from the reasonably anticipated adverse effects of chemical and microbial pollutants in sewage sludge. EPA's statutory requirements under the Clean Water Act, Section 405(d)(2)(C), is to conduct a review of the 40 CFR 503 standards not less than every two years for purposes of regulating new pollutants where sufficient data exist. The purpose of such reviews is to identify additional toxic pollutants and promulgate regulations for those pollutants consistent with the requirements set forth.

The development and standardization of state-of-the-art science techniques can give regulators another tool to track, monitor, and measure levels of emerging contaminants in biosolids that are either land-applied, used as biofuels, or landfilled. The research presented in this report is a reflection of a lack in the literature regarding a comprehensive method for the recovery and accurate identification of macrolide antibiotics and other drugs from biosolids.

A pressurized liquid extraction (PLE), using an *in-situ* incorporation of cleanup materials (i.e., fluorosil, alumina) into the PLE cell for biosolids extraction, and high performance liquid chromatography-electrospray ionization-ion trap mass spectrometer (HPLC-ESI-ITMS) analytical method, were optimized and tested on several biosolids matrices. In this report, not only are the positive results presented, but also negative results are presented and discussed, offering these as precautionaries to other environmental analysts.

LIST OF ACRONYMS AND ABBREVIATIONS

API	active pharmaceutical ingredient
AZI	azithromycin
CID	collision induced dissociation
CLA	clarithromycin
CLI	clindamycin
CWA	Clean Water Act
EC	emerging contaminant
EDC	endocrine disrupting compound
EPA	Environmental Protection Agency
ESD	Environmental Sciences Division
ESI-ITMS	electrospray ionization-ion trap mass spectrometer
g	gram
HPLC	high performance liquid chromatography
kg	kilogram
L	liter
LC	liquid chromatograph
LOD	limit-of-detection
LOQ	limit-of-quantitation
MDL	minimum detection limit
MDMA	3,4- methylenedioxymethamphetamine, Ecstasy
mL	milliliter
MS	mass spectrometer
MTBE	methyl tertbutyl ether
n,n'-DMPEA	n,n'-dimethylphenethylamine
NERL	National Exposure Research Laboratory
NIST	National Institute of Standards and Technology
NRC	National Research Council
ORD	Office of Research and Development
OTC	over-the-counter
OW	Office of Water
PBDE	polybrominated diphenyl ether
PEC	predicted environmental occurrence
PLE	pressurized liquid extraction
ppb	part-per-billion
PPE	personal protective equipment
PPCP	pharmaceutical and personal care product
ppt	part-per-trillion
PSEU	pseudoephedrine
RXY	roxithromycin
SIM	single ion monitoring
SEEP	Senior Environmental Employment Program
SPE	solid phase extraction
TNSSS	Targeted National Sewage Sludge Survey
µL	microliter
UPLC	ultra performance liquid chromatography
USE	ultrasonic extraction
WWTP	wastewater treatment plant

1.0 INTRODUCTION

Biosolids are defined, for this report, as the solid residue byproducts from wastewater treatment plants (WWTPs). This biosolids material is usually comprised of human waste which is a mix of excreta containing bacterial microflora, fats, proteins, pigments, as well as ingested xenobiotics, such as pharmaceuticals and illicit drugs (excreted unchanged or as metabolites), personal care products (e.g., shampoos, detergents, cosmetics), along with other domestic, hospital, and industrial wastes. Some WWTPs mix in municipal organic solid waste (e.g., yard trimmings, other greenwastes) along with the WWTP-produced biosolids before composting, landfilling, or marketing. The United States Environmental Protection Agency (EPA) asked the National Research Council (NRC) to conduct an independent evaluation of the technical methods and approaches used to establish the standards for biosolids.(*1*) Among other things this report identified pharmaceutical and personal care products (PPCPs) as one category of diverse compounds that had not yet been studied in biosolids and were especially likely to be present in domestic biosolids. In response, EPA developed a strategy that while emphasis was being placed on pathogens to address areas of uncertainty and public interest, selected new chemicals, of which pharmaceuticals were a set of, would also be addressed to help determine significant issues and identify information gaps that remain to be addressed.

After reviewing the literature and various toxicological parameters of some of the most widely prescribed pharmaceuticals in the US, eight emerging contaminants were chosen for methodology development. Chosen were four pharmaceuticals: three

macrolide antibiotics: azithromycin (AZI), clarithromycin (CLA), roxithromycin (RXY); one lincosamide antibiotic: clindamycin (CLI); two illicit drugs: methamphetamine, MDMA (Ecstasy); one industrial food additive: n,n'-dimethylphenethylamine (n,n'-DMPEA) (an isobaric ion to methamphetamine); and one over-the-counter (OTC) drug: pseudoephedrine (PSEU), which is closely related in chemical structure to MDMA. While our list of analytes is diverse, the rationale for developing a method is to ultimately support the EPA's mission to protect the environment and human health.(*2-7*) For example, there are reports in the literature of increasing bacterial resistance to multiple classes of antibiotics.(*6, 8*) One of the most widely prescribed antibiotics (in the United States) is the macrolide azithromycin, the annual sales of this drug, in 2009, were $1,056,715,000 (equating to 49,902,000 prescriptions, see http://drugtopics.modernmedicine.com/Pharmacy+Facts+&+Figures. McCaig et al.(*9*) have reported a correlation between increasing macrolide-resistant pneumococci and the prescription rate for azithromycin and clarithromycin, both of whose prescription rates rose by 388%, during the 1990's. While roxithromycin is not prescribed in the United States, it is widely used in Latin America and Europe; thereby, lending itself as a marker of the importation of drugs by other than traditional means. The two illicit drugs were chosen because of limited environmental occurrence data of illicit drugs in environmental media, and verifiable usage in the United States (last accessed 28-August-2009, http://www.usdoj.gov/dea/statistics.html).(*10, 11*)

There are few studies in the literature regarding the analysis of macrolide antibiotics in WWTP-produced sewage sludges/biosolids. Nieto et al.(*12*) published a

method for the quantitative determination of three macrolides (erythromycin, roxithromycin, and tylosin), five sulfonamides, ranitidine, omeprazole and trimethoprim in sewage sludge samples, using pressurized liquid extraction (PLE) and high-performance liquid chromatography-electrospray ionization-mass selective detector (HPLC-ESI-MSD). One potential issue with this method is that the authors used single ion monitoring (SIM) with source voltage induced fragmentation. Their technique is not as robust, nor definable as other HPLC-ESI methods that use collision induced dissociation mode (CID) in conjunction with ion traps, or triple quadrupoles, or other mass spectrometers that are capable of inducing multiple fragmentation product ions from a precursor ion (referred to as "MS/MS" mode). For example, there are many high molecular weight compounds (e.g., nonylphenol ethoxylates, ionic surfactants) that can give ions at the correct molecular weight range as the macrolides, or other higher molecular weight pharmaceuticals, but without confirmation by MS/MS there can be some doubt as to the correct identification and subsequent quantification of the unknown contaminant.(*13*) Göbel et al.(*14*) developed a PLE for several classes of antibiotics, including macrolides from biosolids. In their method, they first performed a PLE extraction on the biosolids, then a subsequent cleanup of the PLE extract, by passing the extract through a solid phase extraction (SPE) cartridge, concentrating the SPE extract and analysis by LC-ESI-MS/MS (triple quadrupole mass spectrometer). Their average absolute % recoveries (absolute recovery as defined as the amount of material spiked vs the amount of material measured in final extract, without correction of labeled surrogates) of AZI, RXI, CLA were 29, 45, and 33% (n = 4), respectively. Radjenović et al.(*15*) developed a PLE to extract pharmaceuticals, including AZI, from freeze-dried

sewage sludges, followed by SPE cleanup, and analysis by HPLC-MS/MS. In this method, they use both labeled surrogates and labeled internal standards to compensate for matrix effects and ion signal suppression because of co-extracted interfering materials in the biosolids. Their relative % recovery (relative % recovery is defined as labeled surrogate corrected recoveries of the spiked materials, as opposed to absolute % recoveries) of AZI was 81% (n= 3). In another recent publication, Jelic et al.(*16*), using the same method as in Radjenović et al.(*15*), obtained relative % recoveries for RXI and CLA at 146 and 38% (n = 3), respectively.

In this report, an optimized analytical method for the detection of several pharmaceuticals and drugs in biosolids and its application to real-world biosolids samples is presented. The method developed uses a PLE technique, with an *in-situ* extraction cell clean-up (fluorosil and alumina packed into the PLE extraction cell), spike correction for matrix effects, and a definitive detection method using HPLC-ESI-ITMS, in the MS/MS mode.

2.0 SAMPLING

This has been described in greater detail elsewhere.(*17*) Briefly, grab samples of biosolids were collected and composited from various WWTPs around the US (Table 1, Figure 1). Subsets of the samples were split between OW and ORD/NERL-ESD/Las Vegas, with six samples from various capacity and types of WWTPs were sent to ORD/NERL-ESD/Las Vegas. The samples were stored at the Las Vegas laboratory, along with three other biosolids samples collected, by Las Vegas, at an earlier date (*18*). The samples were stored at < -4^{o} C until sample preparation. For most of the samples, there was a lag time of over two years between sample collection and preparation for analysis.

3.0 EXPERIMENTAL

The steps in environmental method development involve assessing: (1) the ability to extract the analytes of interest with some degree of precision and accuracy from an environmental matrix; and (2) the ability to accurately identify and measure at low (environmentally-relevant) concentrations the analytes of interest. The ability to correctly identify and measure the analytes identified in this research has previously been published (*10, 19*); therefore, we will focus the experimental section on the development of the extraction and chromatographic procedures.

3.1.1 Sample preparation. All forms of biosolids have physical properties that pose challenges for analytical chemistry methods development. Biosolids are made up of particles with large surface areas (0.8 - 1.7 m^2 g^{-1}), have negative surface charges, and

have extensive interstitial spaces; of which these physical properties can promote sorption, occlusion into the biomass, and strong bonding between charged species and the particulate surfaces. Further challenges are created by the WWTP-addition of chemical additives, such as, ferric chloride, lime, and cationic polyacrylamide polymers (the most widely used polymers for conditioning) during the biosolids conditioning steps.(*20*) Therefore, due to the complexity and variable sizes of biosolids particulates, they need to be homogenized before extraction.

Batches of biosolids were pre-dried by spreading them around the bottom of a large beaker and leaving it open to air and light in the laboratory before homogenization could proceed. The dried biosolids were placed in 25 ml zirconium oxide/steel jacketed grinding jars, along with one zirconium oxide grinding ball, and ground to a fine powder using a high impact ball mill (mixer mill 301, Retsch Inc, Newtown, PA) for 3 minutes at a frequency of 20 s^{-1}.

3.1.2 Optimization of pressurized liquid extraction (PLE) procedures. Initially, both ultra-sonic extraction (USE) and PLE were investigated as extraction techniques. Lower recoveries were obtained from USE than from PLE; therefore, only the three PLE methodologies (PLE 1, PLE 2, and PLE 3) will be explored in detail. Several PLE variables: temperature, solvents, pressures, and cell matrix materials were examined, and are listed in Table 2.

3.1.2.1 Pre-PLE Extraction Procedures.

Sample preparation. For PLE methods 1 and 2, a 1.0 g aliquot of the pre-dried homogenized biosolids was weighed out, placed in a mortar, along with 5 g of Hydromatrix™. The subsequent mixture was homogenized, using a silanized pestle, until a free-flowing powder was achieved. For PLE 3, a 1.0-g aliquot of the pre-dried homogenized biosolids was weighed out, placed in a mortar along with 1 g of Hydromatrix™, and thoroughly mixed with a glass-stirring rod, finally a 5-g aliquot of alumina was added and completely mixed in.

Extraction Cell preparation. A 22-mL stainless steel extraction cell was prepared by inserting a cellulose filter at the bottom of the cell (capped on one end).

For PLE methods 1 and 2: approximately 10 g of Hydromatrix™ was added to the bottom of the extraction cell, the biosolids homogenate was transferred on top of Hydromatrix™ layer, using a teflon lined funnel, tapping the cell to ensure no air pockets were present. The remaining volume was filled with Hydromatrix™. Another cellulose filter was placed on top of the material inside the extraction cell, the cell was capped and the cap was screwed on tightly.

For PLE method 3: a 5-g aliquot of fluorosil was funneled into the cell, tapping the cell to ensure no air pockets were present, followed by 5-g of

alumina, again gently tapping the cell to reduce the air pockets. The biosolids homogenate was transferred on top of the alumina/fluorosil layer, using a teflon lined funnel, tapping the cell to ensure no air pockets were present, and the remaining volume was filled with Hydromatrix™. Another cellulose filter was placed on top of the material inside the extraction cell, the cell was capped and the cap was screwed on tightly.

3.1.2.2 Extraction procedures

For PLE method 1. The extraction cells were loaded into the PLE system and extraction was performed using the following PLE extraction conditions: 99% methanol/1% acetic acid as the extracting solvent; 2-cycles; 2800 psi; extraction temperature: 50°C. After a static period of 15 minutes the eluant (approximately 40 mLs) was purged into a clean collection vial. Leaving the extracts *in-situ* in the Turbovap® tubes, the tubes were removed from the Turbovap® for hexane cleanup.

For PLE method 2 and method 3. The extraction cells were loaded into the PLE system and two extractions were performed using the following PLE extraction conditions. First, a mixture of methyl tertbutyl ether (MTBE):methanol (90:10 v/v) was flushed at 80% of cell volume, at 50° C and 1500 psi; after a static period of 15 minutes the eluant (approximately 40 mLs) was purged into a clean collection vial. A subsequent extraction was performed on the same biosolids sample, with a solvent mixture of methanol:1% acetic acid, flushed at 80% of cell

volume, at 80^{o} C and 2800 psi; after a static period of 15 minutes the eluant (approximately 40 mLs) was purged into a separate, second collection vial. The MTBE:methanol extracts were placed into a Turbovap® (Caliper Life Sciences, MA) evaporation tube and concentrated to 5 mL (Turbovap® settings: 5 psi N_2, 23^{o} C). The methanol:1%acetic acid extract was combined with the MTBE extract and concentrated until a total combined extract volume of 5 mL was reached. Leaving the extracts *in-situ* in the Turbovap® tubes, the tubes were removed from the Turbovap® for hexane cleanup.

Hexane cleanup. Whether using PLE 1, 2, or 3, the resultant PLE extracts were rinsed several times with hexane: 2-mLs of hexane was pipetted into the extract, ensuring mixing occurs, the hexane was allowed to settle out on top of the extract, this hexane layer was removed and discarded (via pipetting), this procedure was repeated until a clear yellowish color was obtained. The number of hexane rinses varies from one biosolids matrix to the next, but this procedure was performed as many times as necessary (up to 6 or 7 times) in order to clean the sample of much of the undesirable compounds, such as fats and waxy materials. The cleaned extract was placed back into the TurboVap® and further concentrated. Solvent exchanging with methanol:1% acetic acid until a 0.5 mL endpoint was achieved; the extract was transferred to a 1.8 mL autosampler vial and ready for analysis by LC-MS/MS. Early in the stages of method development, the loss of the analytes of interest to the hexane was tested and was found to be minimal (< 1%).

3.1.3 Optimization of mass spectrometry detection method

3.1.3.1 Liquid Chromatography. HPLC separations were performed using an Ascentis Express C_{18} (Supelco-Aldrich, Bellefonte, PA), 2.7 µm particle size, 3 cm x 2.1 mm, coupled to a MetaGuard Pursuit XRs 2.0 mm 3µm C_{18} guard column (Varian Inc., Palo Alto, CA). Gradient elution conditions were as follows: Mobile phase A 100%, hold for 2 min, 3 min gradient to 30% A:70% B, hold for 5 min, then a 3 min gradient to 100% A, hold for 2 min, followed by a 5 min equilibration time before the next injection. The mobile phases were composed of the following: mobile phase A: de-ionized water/0.5% formic acid; mobile phase B: 82% methanol/18% acetonitrile/0.5% formic acid.

3.1.3.2 ESI-Ion Trap Mass Spectrometry. Data were acquired with a Varian 500MS ion trap mass spectrometer (Varian Inc., Palo Alto, CA). The 500MS was operated in the positive ionization mode, with the following conditions:

- ESI needle voltage: 5 kV
- Drying gas: 20 psi and 200° C
- Housing chamber: 50^{o} C
- Nebulizer gas at 50 psi
- Spray shield at 600V
- Capillary voltages were individually set per analyte, dependent upon the optimized response of the product ions of interest.

Due to the extremely large amounts of interfering materials that were co-extracted with the pharmaceuticals, the analyses were performed in the MS/MS mode, using CID in the ion trap, for both identification and quantitation of the macrolides and illicit drugs. Two to three product ions were used for identification and the most abundant product ion for quantification. The precursor ions, product ions and limits-of-detection (LODs) used to identify and quantify the analytes have been previously reported.(*10, 19*)

3.1.3.3 Calibration, blanks, and LC-ES-ITMS quantitation. The methods for determining the LODs and limits-of-quantitation (LOQs) have been previously reported.(*10, 19*)

3.1.4 Safety Considerations. The pressurized liquid extractor can rise to very high pressures. The septa on the rinse vials should be changed daily; if not this could possibly lead to an accidental explosion of the rinse vial. All chemicals should be handled with caution and personal protective equipment (PPE) should be used.

4.0 Results and Discussion

The steps in environmental method development involve assessing: (1) the ability to extract the analytes of interest with some degree of precision and accuracy from an environmental matrix; and (2) the ability to accurately identify and measure at low (environmentally-relevant) concentrations the analytes of interest. The ability to correctly identify and measure the analytes identified in this research has previously been published.(*10, 19*) Therefore, we will focus the results and discussion section on the development of the extraction and chromatographic procedures and then on the results of the application of the finalized method to nine different biosolids matrices.

4.1 Extraction methods. Initially, both ultra-sonic extraction and PLE were investigated as extraction techniques. Three materials; sand, Milorganite® (commercially available Class A biosolids from Milwaukee, WI), and a Class A biosolids from the Southern California Los Angeles Hyperion WWTP were tested with the USE and simplified PLE 1. Since lower recoveries were obtained from USE than from PLE, we only pursued further development of the PLE technique.(*18*)

Subsequently, we added several other biosolids materials collected during EPA's 2006-2007 Targeted National Sewage Sludge Survey (TNSSS),(*17*) for a total of nine different biosolids matrices to be studied by PLE 2 and 3 (Table 1). The recoveries were very different dependent upon the materials tested.

4.2 Extraction recovery results. Initially, only the recoveries of AZI, RXY, and CLA from the Milorganite® and Hyperion biosolids were investigated using a simplified PLE. At the time of the initial methods development neither the other emerging contaminants nor the other seven biosolids matrices were available.(*18*) The recoveries from the earlier study, using PLE method 1, were as follows: AZI: 24% (n=6); CLA: 40% (n=6); and RXI: 13% (n=6). While the overall recoveries were low, they were not discouraging. It was interesting to note that when comparing the recoveries between the two different biosolids, they were distinctly different. Milorganite® gave the resulting recoveries: AZI: 28% (n=4); CLA: 54% (n=4); and RXI: 19% (n=4); while the Hyperion biosolids recoveries were: AZI: 16% (n=2); CLA: 13% (n=2); and RXI: 1% (n=2). This difference in recoveries between biosolids matrices is the result of the variations in the unique physical and chemical compositions of each of the biosolids matrices, e.g., varying levels of lipids, de-watering processes, chemical stabilizers and chemical additives. For example, Brumley et al.(*21*) report high levels, part-per-billion (ppb), of surfactants (nonylphenol ethoxylates) and polybrominated diphenyl ethers (PBDEs) in these same set of biosolids, which gave rise to analytical interferences, as the pharmaceutical concentrations were in the part-per-trillion (ppt) range.

In searching for improvements in recoveries to the original PLE (method 1), PLE 2 and subsequently PLE 3, were developed. There were two major differences between method PLE 2 and PLE 3. In PLE 3, a layer of fluorosil and alumina were added to the bottom of the extraction cell and the1-g of biosolids material was mixed with alumina and hydromatrix, not hydromatrix alone as in PLE 2. All other PLE parameters (e.g.,

solvents, pressures, temperatures) were kept the same for PLE 2 and PLE 3 (Table 2).

The spiked recoveries of all seven analytes, from all nine matrices, were compared and the results are presented in Table 3. Overall, the spiked recoveries from PLE 3 were substantially better than those from PLE 2. The average recovery of the three macrolides, AZI, RXI, and CLA were: 17, 8, and 11%; and 57, 16, and 61% recovery, PLE 2 vs PLE 3, respectively.

Better recoveries were obtained for the lower molecular weight emerging contaminants from PLE 3. The recoveries for methamphetamine, MDMA, and pseudoephedrine, and n,n'-DMPEA were: 15, 15, 25 and 26% vs 53, 55, 67 and 57%, PLE 2 vs. PLE 3, respectively. For the lincosamide clindamycin, there was a slight increase in recovery when using PLE 3; 38% (PLE 2) vs 46% (PLE 3).

It is not surprising that PLE 3 provided better recoveries than PLE 2 as fluorosil and alumina were used *in situ* during the extraction procedure; thereby, providing removal of some of the interfering substances. This was evidenced by only one instance of matrix interference from an overlapping ion during the mass spectrometric analyses in comparison to multiple instances of interfering overlapping ions when analyzing extracts produced from PLE 2 (Table 3). Also, there was less drop off in mass spectral sensitivity due to fewer instances of the electrospray shield getting dirty between analyses when analyzing extracts produced from PLE 3. However, the macrolides still had drifting chromatographic retention times no matter which PLE method was used.

4.3 Enhancement of chromatography. The HPLC C_{18} column used for this method incorporated what was termed "fused-core" silica particles. This type of silica particle is a solid particle with the C_{18} moiety bound to the surface; there are no interstitial spaces inside the particle. This type of particle enhanced our chromatographic performance by increasing the number of theoretical plates, on the order of ultrahigh performance liquid chromatography (UPLC) technology, without giving the high back pressures that UPLC gives. Previously, standard particle HPLC C_{18} columns were used in our studies with the total chromatographic analysis times ran upwards of 30 minutes.(*19*) With this newer particle type column, the total chromatographic analyses were shorter at 15 minutes. Another difference between this column and the normal C_{18} was the ability to recover this column's chromatography back to its initial chromatographic conditions even after injecting several biosolids extracts onto the column. The chromatographic recovery was possible by back-flushing the column with 100% organic phase (82% methanol/18% acetonitrile/0.5% formic acid) for 10 min. After several weeks of biosolids analysis, this solid particle column was still operational, whereas the traditional C_{18} column previously used in our studies was not and had to be replaced more frequently.

4.4 Analytical concerns. The biosolids extracts produced with PLE 3 were cleaner than those produced from the other methods. However, there were still residual surfactants and other unidentifiable materials present in some of the extracts. These co-extracted interferents can interact with the C_{18} and silica moieties on the chromatographic column, sometimes irreversibly binding to active sites. These interfering materials can

cause changes in the retention times of the analytes between analyses at the beginning versus those extracts and standards analyzed at the end of an analytical day (typically 8 hrs). As an example, it was observed, in several biosolids extracts, that the azithromycin present appeared as a very small chromatographic peak in the first sample extract injection. In the second injection of the same extract a large peak attributable to azithromycin appeared. A third injection of an instrument blank (methanol) was made immediately after the 2nd injection, and again a large peak attributable to azithromycin appeared (Figure 2). An assumption can be made that the azithromycin in the 1st injection of the extract was actually eluting out in the 2nd sample injection, and that the azithromycin present in the 2nd injection of the extract carries over into the methanol blank injection. This hypothesis was tested and proven by showing that the chromatographic peak carried over into the blank was attributable to azithromycin not only by the large area counts under the peak, but also through spectral confirmation. The azithromycin peak that was present in the 2nd injection and blank chromatogram had the presence of the three product ion masses for azithromycin: 591.4 m/z, the most predominant ion (and quantitation ion), as well as masses 573.4 m/z and 434.4 m/z, two minor product ions. The product ions are in the correct mass isotope ratios to each other, in both the samples and blanks; therefore, this finding confirmed that the chromatographic peak was azithromycin.

To prove that the methanol blank was not previously contaminated, several experiments were performed. A methanol blank, injected after every calibration standard, was always blank after the standard injection. However, the methanol blank

injected after the biosolids sample injection would show azithromycin eleuting in the blank injection. These two experiments showed that the methanol blank was not contaminated, but instead that the azithromycin was being bound onto something else in the column and not eluted from the column until after a second injection was made. It is believed that this phenomenon is a by-product of the residuals found in biosolids. For example, it has previously been observed that the chromatographic peaks of azithromycin do not drift when environmental water (e.g., wastewater, source water) extracts are injected, nor is this phenomenon observed during multiple injections of calibration standards. This drifting peak phenomenon does not occur with all biosolids, but it did occur with several of the biosolids matrices received from the TNSSS study. The drifting peak phenomenon seemed to be limited to the macrolide antibiotics (i.e., AZI, RXI, CLA), as this phenomenon was not observed to occur with the smaller molecules (i.e., methamphetamine, n,n'-dmpea, MDMA, and clindamycin). The binding of the macrolides to the column is almost certainly due to certain functional groups located on the macrolides (e.g., ethyl aldehyde at C_6 azithromycin, saccharide branch at C_5 azithromycin)(*22*), as well as their cage-like chemical structures that encourages binding (Figure 3).

4.5 Application of optimized method to nine diverse biosolids matrices. Table 4 shows the results of the application of the optimized method to the nine different biosolids matrices, collected from nine different WWTPs across the US. Although sample #7, LVBIO007, was part of the spiking study, we were unfortunately unable to process the unspiked matrix of LVBIO007 at the time of this report. This event was due

to our building being shut down for nine months and LVBIO007 was one of the last unspiked samples to be processed with method PLE 3. However, the other 8 samples are reported in Table 4 and cross-correlated with the data collected from the TNSSS.(*24*)

Although the methods used to study the TNSSS biosolids were different between the EPA contractor (Method 1694) (*24*) and our method (PLE method 3) we tried to make some comparisons between the two methods using samples LVBIO- 004, 005, 006, 008 and 009 (Table 5). Only one of the clarithromycin results corresponded, both being non-detects, perhaps due to the delay between collection and extraction on our part. For roxithromycin, 5 of the 6 results matched up, as non-detects. In only one instance did the TNSSS contractor detect roxithromycin, in sample LVBIO006. However, in this particular biosolids matrix, we were only able to obtain a recovery of 16% for roxithromycin from the spiked matrix. This made it unlikely that our method would detect the low levels, 14 ng/g, that they detected. It should be noted that the amount of roxithromycin detected in the TNSSS study, 14 ng/g, is at, or near, the LOD for Method 1694, as well as our method's LOD for this compound, and should be treated as suspect.

5.0 CONCLUSIONS

The finalized method presented here, PLE 3, provides an efficient *in situ* cleanup of dirty environmental matrices, like biosolids. Overall, the recoveries of the analytes are better and there are less analytical difficulties from interfering matrix ions. In comparing our method to Göbel's et al. (*14*), which uses absolute recoveries like ours, we obtain better recoveries for AZI and CLA: 57% and 61% (n=9) vs. Göbel's, 29% and 33% (n=4).

However, Göbel's method obtained better results for RXI: 45% vs. our 16%. In the US the two most widely prescribed macrolide antibiotics are AZI and CLA, while RXI is not prescribed in the US, it is in Latin America and the European Union. We cannot compare our results to those of Jelic, et al. (*16*), nor Radjenović, et al. (*15*), nor US EPA Method 1694(*23*), as they use a relative % recovery method, a method that uses carbon-labeled standards. While using labeled standards may give a more accurate sense of recoveries and negate the issue of matrix interferences, the disadvantages are that labeled standards are difficult to find, expensive, and/or difficult to have synthesized.

It was not surprising to detect azithromycin in all of the biosolids matrices, as the production levels of the active pharmaceutical ingredient (API) for azithromycin is roughly 219,000 kg/yr. Loganathan et al.(*19*), using this API, calculated a predicted environmental occurrence (PEC) at approximately 3000 ng/L. However, there is a 10-fold difference between what was found in WWTP effluent (water column) and what was predicted (*19*), pointing to other environmental sinks of azithromycin, of which biosolids are one.

The extraction and detection approach presented in this report may not be as precise as an absolute recovery method that uses labeled standards; however, it provides a much simpler and more cost effective approach. The drawback to the method PLE 3, presented in this report, is that in order for it to be effective, every unique batch of biosolids matrices from each WWTP, must include a spiked matrix in order to correct for the extraction efficiency from each distinctive biosolids matrix.

6.0 FUTURE RECOMMENDATIONS

The current standards for the use of biosolids in the U.S. are science-based risk assessments. With respect to emerging contaminants in biosolids, more accurate data on biosolids uses, advances in chemical analytical methodology, survival efficiencies in wastewater treatment facilities, environmental fate and transport, and the potential for effects in humans and the environment are required to conduct reliable exposure and hazard assessments. Sufficient data to conduct an exposure and hazard assessment include unbiased national estimates of concentrations, environmental fate and transport, plausible effects end-points for humans and ecological receptors, and other relevant information for pollutants in biosolids. An even more important question that should be asked is whether these emerging contaminant residues are bioavailable, and if so, then what will be the environmental impact. Therefore, possible future research efforts could be directed towards crop uptake studies from biosolids treated fields, weathering of and *in situ* breakdown of emerging contaminants in biosolids residues, and other studies that would look at the emerging contaminants leaching from, or still available in, the biosolids matrix, dependent upon their final treatment (e.g., landfilling, biofuels, field amendments, composting).

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Tables.

1) Biosolids matrices: identification numbers, WWTP types, mgd, biosolids type, final deposition.
2) Experimental parameters for methods PLE 1, PLE 2, and PLE 3.
3) Spiked biosolids recovery: PLE 2 versus PLE 3.
4) Concentrations of targeted pharmaceuticals and drugs from nine US WWTPs using PLE 3.
5) Comparison of concentrations of targeted pharmaceuticals PLE 3 vs. TNSSS.

Table 1. Biosolids matrices: identification numbers, WWTP types, mgd, biosolids type, final deposition.

Sample ID	WWTP type	mgd	Biosolids type	Final deposition
LVBIO001	Tertiary	91	Class B	Landfill
LVBIO002	Tertiary	250	Class A	Commercial product, land application
LVBIO003	Tertiary	450	Class A	Composting, land application, biofuels on-site use
LVBIO004	Secondary	3.3	Class B anaerobic digestion	Incineration, landfill
LVBIO005	Tertiary	> 100	Class B anaerobic mesophilic digestion	Land application
LVBIO006	Secondary(?)	5	Class B anaerobic digestion	Mixed with green waste, sold as compost to public
LVBIO007	Tertiary	47	Class B anaerobic digestion	Land application
LVBIO008	Secondary	20	Class B activated sludge	Landfill
LVBIO009	Secondary	1	Class B anaerobic digestion	Land application

Table 2. Experimental parameters for methods PLE 1, PLE 2, and PLE 3.

	PLE 1	PLE 2	PLE 3
Hydromatrix	Yes	Yes	Yes
Fluorosil	No	No	Yes (5 g, bottom layer)
Alumina	No	No	Yes (5 g, 2nd layer)
Sample/hydromatrix mix	Yes	Yes	No
Sample/Alumina mix	No	No	Yes
Extracting Solvent(s)	99% methanol/1% acetic acid	Two extractions: (1) MTBE:methanol (90:10 v/v) is flushed at 80% of cell volume; (2) methanol/1% acetic acid	Two extractions: (1) MTBE:methanol (90:10 v/v) is flushed at 80% of cell volume; (2) methanol/1% acetic acid
Pressure (psi)	2800	1) 1500 2) 2800	1) 1500 2) 2800
Extraction Temp. ^{o}C	50	1) 50 2) 80	1) 50 2) 80
Static time (min)	15	(1) and (2) 15	(1) and (2) 15

Table 3. Spiked biosolids recovery: PLE 2 versus PLE 3.

Sample ID	Spiked biosolids, % recovery: PLE 2 vs PLE 3																	
	AZI		RXI		CLA		CLI		METH		MDMA		d5-MDMA		n,n'-DMPEA		PSEU	
	PLE 2	**PLE 3**	PLE 2	**PLE 3**	PLE 2	**PLE 3**	PLE 2	**PLE 3**	PLE 2	**PLE 3**	PLE 2	**PLE 3**	PLE 2	**PLE 3**	PLE 2	**PLE 3**	PLE 2	**PLE 3**
LVBIO001	4	**41**	33	**1**	6	**35**	9	‡‡‡	18	**92**	18	**87**	40	**72**	15	**101**	25	**94**
LVBIO002	17	**94**	1	*	34	**93**	43	**53**	18	**44**	16	**75**	na	**97**	38	**41**	31	**82**
LVBIO003	26	**55**	**	**39**	27	**48**†	‡‡‡	**37**†	16	**31**	15	**39**	37	**37**	43†	**60**	13	**58**
LVBIO004	7	**46**	5	**6**	1	**58**	29	**29**	27	**39**	25	**54**	57	**50**	64†	**25**†	30	**67**
LVBIO005	11	**37**	16	**2**	11	**41**	64	**51**	12	**46**	9	**68**	31	**65**	9	**74**	23	**64**
LVBIO006	21	**77**	0	**16**	7	**76**	29	**58**	1	**110**	10	**57**	26	**71**	14	**81**	29	**76**
LVBIO007	25	**45**	0	**5**	0	**87**	57	**17**	5	**25**	6	**13**	15	**27**	5	**33**	19	**34**
LVBIO008	14	**86**	1	**5**	2	**51**	37	**79**	5	**36**	8	**45**	13	**62**	7	**50**	25	**64**
LVBIO009	24	**28**	‡	**57**	‡‡	**56**	37	**40**	31	**53**	26	**59**	43	**51**	38	**49**	27	**66**
Average % recovery	17	**57**	8	**16**	11	**61**	38	**46**	15	**53**	15	**55**	33	**59**	26	**57**	25	**67**

*Data unusable due to poor chromatography from interfering unknown compounds. ‡Interference from overlapping ion isotopes from unknown analyte mass 749.8 da; ‡‡Overlapping ion isotopes from unknown mass 589.5 da; ‡‡‡Overlapping ion isotopes from unknown mass 376 da ; †Due to the large amounts (> 100 ng/g) of native analyte found in the original sample the spiked recoveries values were corrected to reflect that. na = labeled standard was not available for spiking for this sample.

Table 4. Concentrations of targeted pharmaceuticals and drugs from nine U.S. WWTPs using PLE 3.

Sample ID	Average amount detected*, ng/g (dry wt)					
	AZI	CLA	CLI	METH	n,n'-DMPEA	PSEU
LVBIO001	150	nd	nd	31	nd	nd
LVBIO002	52	nd	nd	nd	nd	nd
LVBIO003	130	310	2100	nd	nd	nd
LVBIO004	170	nd	nd	nd	630	nd
LVBIO005	200	nd	nd	nd	nd	nd
LVBIO006	53	nd	nd	nd	nd	nd
LVBIO008	180	nd	nd	nd	35	nd
LVBIO009	105	190	nd	nd	nd	140

* (n=2), corrected values from matrix spikes recoveries. nd = not detected. Note: LVBIO007 was not analyzed.

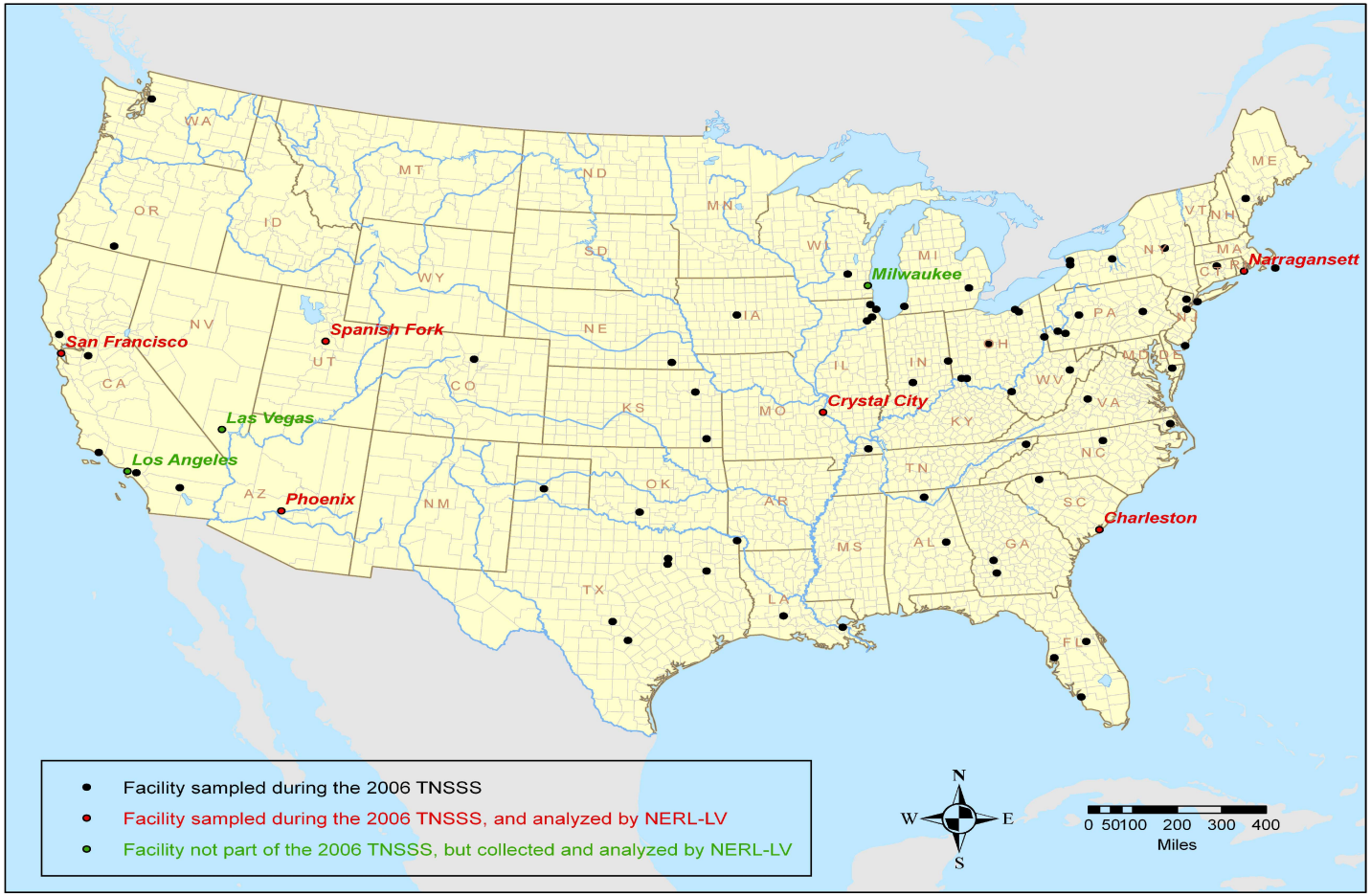
Table 5. Comparison of concentrations of targeted pharmaceuticals PLE 3 vs TNSSS.‡

Sample ID	Average amount detected ng/g (dry wt)		
	AZI PLE vs TNSSS	RXI PLE vs TNSSS	CLA PLE vs TNSSS
LVBIO004	170 – 1180	nd - nd	nd - 141
LVBIO005	200 – 392	nd - nd	nd - 19
LVBIO006	53 – 63	nd - 14	nd - nd
LVBIO008	180 - 548	nd - nd	nd - 53
LVBIO009	105 - 157	nd - nd	190 - nd

‡ EPA TNSSS results were taken from reference (*24*). nd = not detected.

Figures

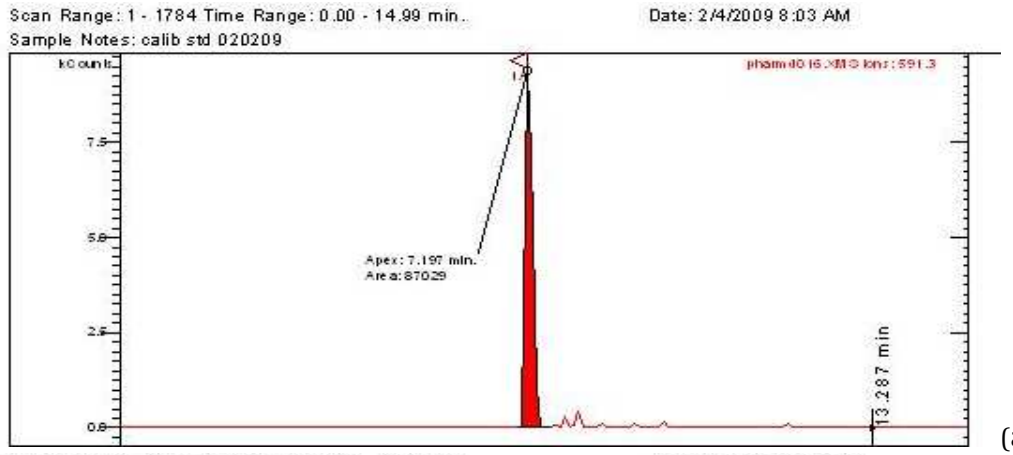
Figure 1. TNSSS Survey sites.



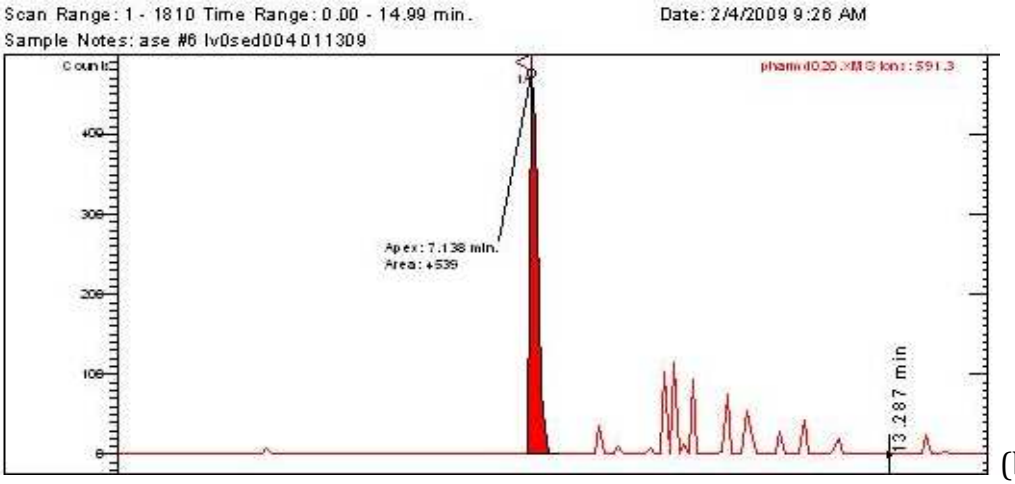
Map provided by USEPA/OW

Figure 2. Chromatograms of drifting retention times of azithromycin.

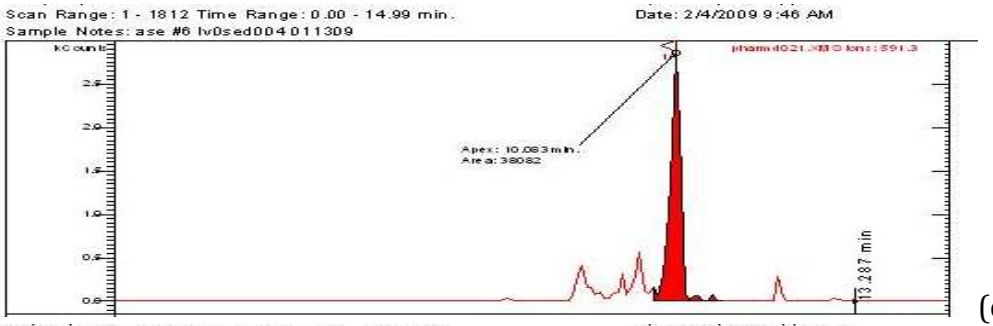
(a) Calibration standard: injection time: 8:03 am; apex of peak: 7.2 min
(b) Biosolids sample 1st inj: injection time: 9:26 am; apex of peak: 7.1 min
(c) Biosolids sample 2nd inj: injection time: 9:46 am; apex of peak: 10.1 min
(d) Methanol blank inj: injection time: 10:07 am; apex of peak: 9.1 min
(e) Calibration standard, end of day: injection time: 1:29 pm; apex of peak: 7.2 min



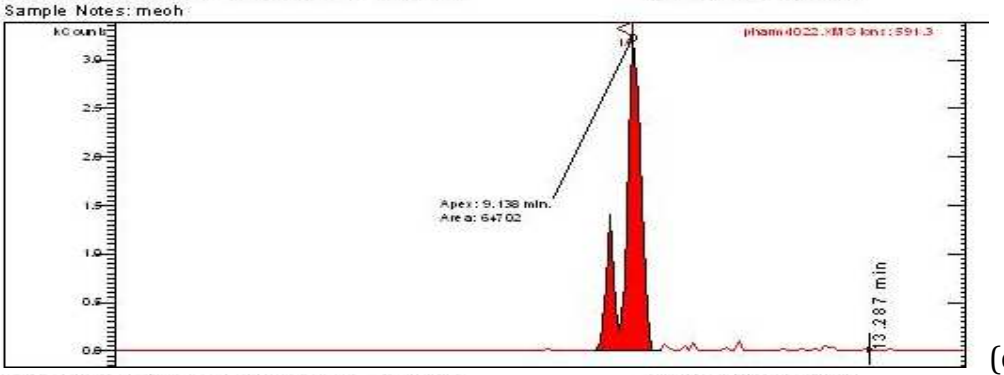
(a)



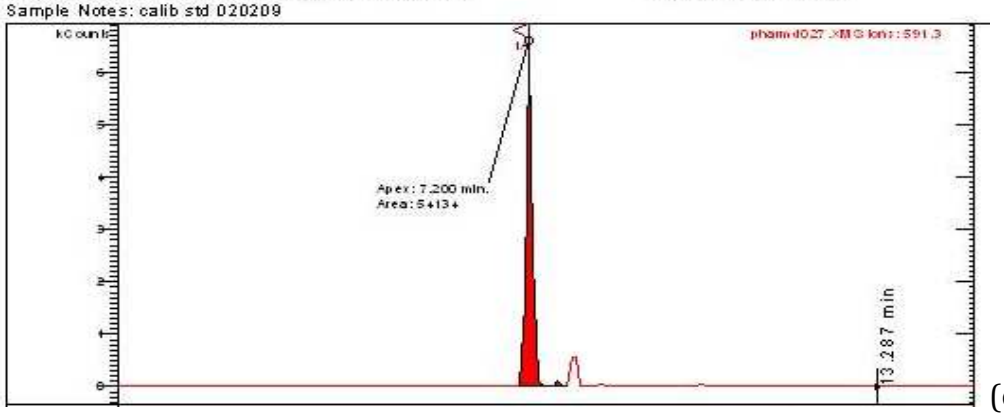
(b)


Scan Range: 1 - 1812 Time Range: 0.00 - 14.99 min.
Date: 2/4/2009 9:46 AM
Sample Notes: ase #6 lv0sed004011309
kCounts
pham4021.XMIG Ion: 591.3
Apex: 10.083 min.
Area: 38082
13.287 min

(c)

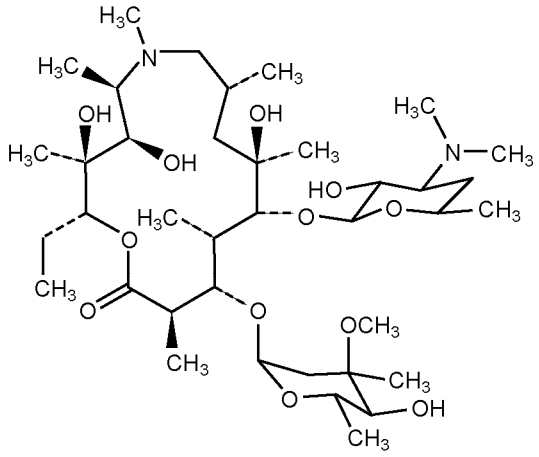

Scan Range: 1 - 1793 Time Range: 0.00 - 14.99 min.
Date: 2/4/2009 10:07 AM
Sample Notes: meoh
kCounts
pham4022.XMIG Ion: 591.3
Apex: 9.138 min.
Area: 64702
13.287 min

(d)

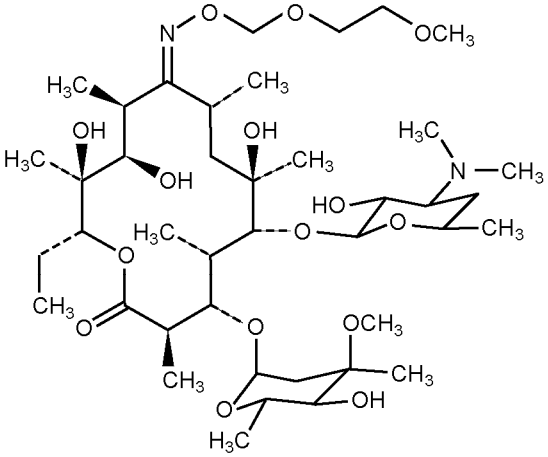

Scan Range: 1 - 1781 Time Range: 0.00 - 14.99 min.
Date: 2/4/2009 1:29 PM
Sample Notes: calib std 020209
kCounts
pham4027.XMIG Ion: 591.3
Apex: 7.200 min.
Area: 54134
13.287 min

(e)

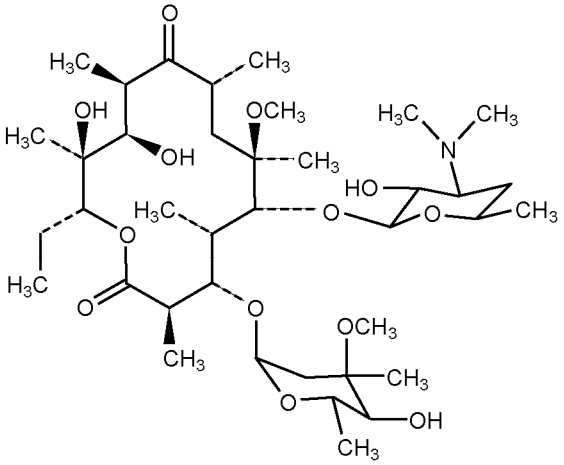
Figure 3. Chemical structures of three antibiotics and two illicit drugs



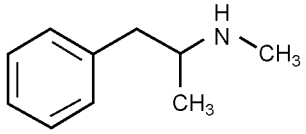
Azithromycin mw = 748 Da



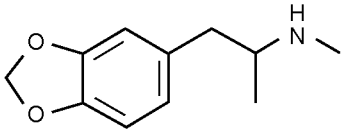
Roxithromycin mw = 837 Da



Clarithromycin mw = 747 Da



Methamphetamine mw = 149 Da



MDMA mw = 193 Da

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