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Partial characterization of biosolids: Hydrophilic organic components

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ABSTRACT

Biosolids from a municipal sewage treatment plant were studied from the standpoint of partially characterizing the polar components in the matrix as well as targeting specific substances. Targeted analytes were determined by HPLC/fluorescence detection in some cases and confirmed through high resolution mass spectrometry (accurate mass determination) or both. The bacterial endpoints consisting of surfactants and products from surfactants were present as expected and were generally greater than mg/kg levels meaning that they could pose significant interferences to target analytes at low μg/kg levels. Thus, one goal in characterization was to identify some of these major components as an aid in formulating cleanup strategies. Target analytes consisted of fluoroquinolone antibiotics. These occurred in the μg/kg levels for some compounds. The target analytes and matrix characterization were further studied using accurate mass UPLC/TOF-MS which revealed many components containing polyethylene glycol moieties and a group of quartenary ammonium surfactants. Anionic components remain to be characterized more fully by UPLC/TOF-MS but some major components were identified using derivatization (trimethylsilylation) and GC/MS.

KEYWORDS

Biosolids;
Polar;
Surfactants;
Antibiotics;
Accurate mass;
Fluoroquinolone antibiotics.

INTRODUCTION

Biosolids (sewage sludge) represent the end product of bacterial digestion and applied treatments of raw sewage in a municipal sewage treatment facility[1]. The precipitated material from the aqueous solution consists of a relatively intractable agglomeration made up of both inorganic and organic substances that have reached an environmental sink. The characterization of this material is a complex task[2].

In recent times the interest has shifted from disposal of the biosolid material in landfills to the preferred use of processed material as a soil amendment[3]. The question of course arises as to the potential exposure to biota that would result from such an application in the environment as well as effects on human health that might arise indirectly from eating crops exposed to the amended soil or animals eating such crops before going to market. Additionally, concern centers on the likelihood of substances from biosolids entering into groundwater or ultimately into drinking water due to leachate[4].

The biosolids have usually undergone additional treatment to destroy pathogens either by heat or irradiation after the considerable bacterial degradation and

additional process treatment that has taken place. Thus, the biosolids are designated Class A or Class B depending on the extent of elimination of pathogens[5]. Some of these treatments may destroy susceptible contaminants and eliminate them from consideration. Certain proponents advocate the additional step of composting to remove/degrade the remainder of the objectionable compounds that are currently known to remain in biosolids[6].

A number of papers have now started filling in some of our questions about the types of contaminants in biosolids. The review by Rogers[1] included mention of a variety of compounds including organochlorine pesticides (e.g., aldrin), PCBs, and chlorophenols.

A large presence for nonyl phenols and surfactants was also mentioned. The large and diverse class of pharmaceuticals and personal care products (PPCPs) was also mentioned and most of these compounds (e.g., antibiotics) would be found in the polar fraction of biosolids components. The synthetic musks are one group of PPCPs that partition with the lipophilic fraction. Methods were given with extraction and cleanup procedures also included.

Oberg et al. described the occurrence of PBDEs in over 100 sludge samples from Sweden where the predominant tetra, penta, and hexa congeners were found as well as the decabromodiphenyl ether[7]. Ying and Kookana pointed out that high levels of triclosan in biosolids could be a concern in soil applications[8]. Synthetic musks were determined in biosolids[9]. Nonyl phenols, pthalates, and PCBs were determined in biosolids and soil in an effort to follow the fate of such contaminants after biosolids were used for soil amendment[10].

A number of papers have focused on the polar analytes (contaminants) found in biosolids. Giger et al. reported methodology for extraction and determination of antibiotics including the fluoroquinolone antibiotics. Extraction difficulties and relatively low recoveries were noted[11]. Mottaleb and Brumley reviewed the separations used in determining PPCPs in a variety of environmental matrices[12].

In this work we partially characterize the hydrophilic components of biosolids to yield some perspective on the relative contributions of major components and provide more specific determination on select contaminants.

EXPERIMENTAL

Chemicals

Ofloxacin and norfloxacin were obtained from Sigma and orbifloxacin was obtained from a veterinary drug.

Method for FQs

Extraction/Cleanup

Biosolid samples were air-dried in a hood and then initially ground by mortar and pestle. Later work relied on a ball mill grinder (Reutsch) at 20 Hz for 2 min using a glass ball and glass-lined sample vessel.

The method uses an ASE (Dionex 200) for extraction of biosolids in acetonitrile/water/formic acid (80/20/0.1) 80°C, 2500 psi pressure. The extract was concentrated to an aqueous phase by nitrogen blow down and then isolated on a strong cation exchange resin under acidic (formic acid) conditions. FQs were eluted using ammonia/methanol (1/3 v/v) and concentrated to an aqueous phase that was devoid of ammonia. The extract was acidified with formic acid, about 0.5 mL methanol added, and filtered for HPLC separation.

HPLC/Fluorescence detection

The HPLC separation was based on a shallow water/acetonitrile gradient under acidic conditions using a Discovery (Supelco) RP Amide C16 5μm column 250 mm×3.0 mm ID with a Discovery RP Amide C16 3.0 mm ID cartridge . The gradient program was as follows:

Time(min)	Acetonitrili%	Water %
0-5	10	90
5-25	15	85
25-35	80	20
35-40	100	0
40-50	10	90

Both solvents contained 0.1% formic acid with flow rate 250 μL/min.

Originally, the column used was a C18 (Luna, Phenomenex) 250 mm x 2 mm ID with the same gradient and flow rate.

A Linear Instruments LC305 fluorescence detector (FLD) was used initially with 280 nm excitation/450 nm emission characteristic of FQs. The detector failed and was replaced with the Spex Fluorolog 2 fitted with an HPLC detector cell but had a poorer de-

tection limit. Later work used an Agilent 1200 series FLD. The HPLC was a Beckman System Gold (168 detector, DAD, 126 solvent module, and 507e autoinjector) operated with Beckman 32 Caret Software. The DAD was used to assess cleanup and to detect higher levels of FQs and assess separations and confirm retention times of standards. An internal standard consisted of orbifloxacin (orbax) which was not detected in the BS samples. Typical retention times for norfloxacin, ciprofloxacin, and orbifloxacin were 13.49, 14.46, and 16.51 min respectively on the RP Amide column and 28.3, 32.09, and 34.45 min respectively on the C18 column.

Recoveries for ciprofloxacin and norfloxacin for processing after the initial extraction were 86.7% and 94.0%, respectively. This represents a nearly complete recovery through the concentration and preparation stages for HPLC/fluorescence. The overall recovery from spiked biosolids at 100 μg/kg was 24.1% and 13.6% from ciprofloxacin and norfloxacin respectively, indicating that the matrix strongly retains these compounds.

Calibration ranged from 7.5 pg/μL to 700 pg/μL in 1 mL volumes. Internal standard was 454 pg/μL for a 1 mL sample extract size; 5 μL sample injections.

UPLC/TOF-MS

Accurate mass measurements were carried out using a Waters-MicroMass LCT Premier XE in the W mode with Leu-enkephalein as the lock mass compound (m/z 556.12345). Separations were carried out with a 5 cm×2 mm C8 BEH (1.7 um particle) column (35°C) operated at a flow of 0.3 mL/min. The solvents were water (0.1% formic acid) and acetonitrile (0.1% formic acid) beginning at a composition of 10% and then following a gradient:

Time(min)	Acetonitrili%	Water %
0	10	90
1	10	90
5	85	15
10	100	0
14	100	0
16	10	90
20	10	90

Accurate mass measurements were used to confirm the presence of target analytes and internal standard as a check compound as follows for the $(M+H)^+$ ions: norfloxacin (320.14104); ciprofloxacin (332.14104); ofloxacin (362.15161); and orbifloxacin (396.15350). Calibrations ranged from about 100 pg/μL to over 1 ng/μL. The internal standard was 687 pg/μL; 2 μL sample injections. Typical retention times for norfloxacin, ofloxacin, ciprofloxacin, and orbifloxacin were 2.25, 2.29, 2.46 and 3.49 min., respectively.

The TOF-MS was operated in the ESI+mode with cone voltage 75V, desolvation temperature 250°C, source temperature 100°C, desolvation flow 450 L/hr nitrogen and cone flow 11 L/hr nitrogen.

GC/MS of anionics

An acidic fraction was isolated using acid/base partitioning (first 1N KOH extraction of the concentrated extract washed with hexane and then acidification by 3 N HCl followed by extraction into methylene chloride). This eliminated most of the neutral lipophilic components and left us with an acid enriched fraction. We then derivatized this fraction with 100μL of BSTFA in 1 mL of toluene at 65°C for at least 2 hours which would react with both carboxylic acids and hydroxy groups.

Full mass scans from m/z 50 to 550 were used under EI conditions on an Agilent 5973 fitted with a 40 m ×0.18 mm ID DB5 column with 0.18μm film thickness. GC program was 1 min at 105°C, then temperature program to 300°C at 15°C/min.

RESULTS AND DISCUSSION

Fluoroquinolones

Levels of ciprofloxacin and ofloxacin were found at various times in the range of less than 10 g/kg to about 20 μg/kg by HPLC/FLD. Good confirmative data for ciprofloxacin by LC/MS/MS were not obtained but were supportive of its presence.

Accurate mass data by UPLC/TOF-MS supported the presence of norfloxacin (30:1 S/N) and ciprofloxacin (10:1 S/N). This is explained by noting that the HPLC work began with three standards norfloxacin, ciprofloxacin, and the internal standard orbifloxicin. The orbifloxacin is a veterinary drug and was not detected in biosolids. The later UPLC/TOF-MS work added the additional compound ofloxacin that has a retention time very close to that of norfloxacin. Thus, the confirmative accurate mass data together with peak

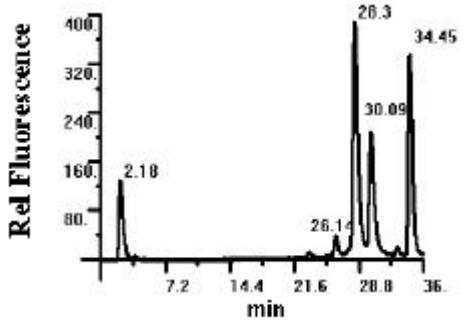

Figure 1: HPLC/FLD chromatogram (280 nm excitation/ 450nm emission) of fluoroquinolones: norfloxacin RT=28.3, ciprofloxacin RT=30.09 and orbifloxacin RT=34.45 on C18

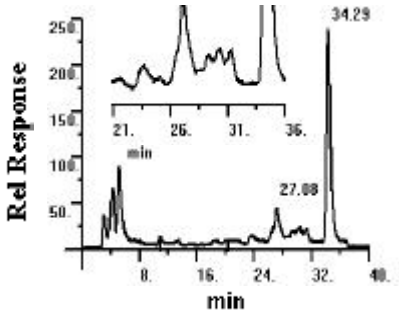

Figure 2: HPLC/FLD chromatogram (280 nm excitation/ 450 nm emission) of fluoroquinolones in biosolid extract. with approximate retention times norfloxacin RT=28, ciprofloxacin RT=30, unknowns RT=29 and RT=31 and orbifloxacin RT=34.29 on C18

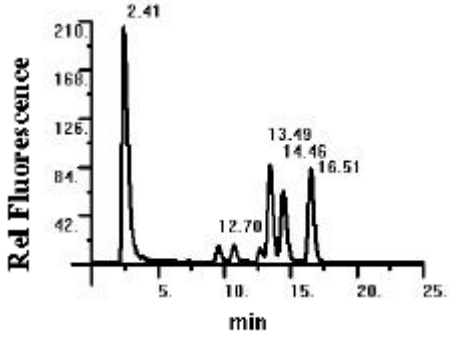

Figure 3: HPLC/FLD chromatogram (280 nm excitation/ 450 nm emission) of fluoroquinolones: norfloxacin RT= 13.49, ciprofloxacin RT=14.46 and orbifloxacin RT=16.51 on RP Amide C16.

shape and retention time indicated that ofloxacin rather than norfloxacin was actually the major component. This points up the difficulty in establishing certainty in identification without confirming data from mass spectrometry or other technique.

Data for the fluorescence detection are shown in figure 1 for standards and in figure 2 for a biosolids

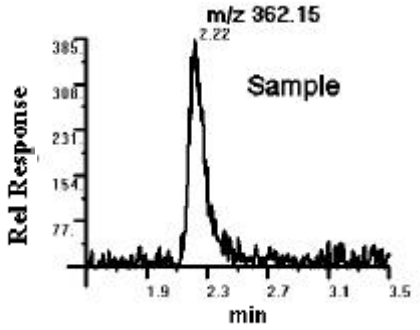

Figure 4: Ion profile from UPLC/TOF-MS for m/z 362.15 ± 0.050 in biosolid extract

sample extract using the C18 column. The three compounds are nearly baseline separated but the sample extract contains a number of responses from the matrix that could be fluroquinolones or matrix interferences. The four small peaks in the vicinity of the retention times of norfloxacin and ciprofloxacin (between 28 and 31 min) provide the basis for the tentative identification/ quantitation. A comparison of figure 1 using the C18 column for separations to figure 3 using the RP Amide C16 column for separations reveals an overall similarity. Giger et al. used the RP Amide C16 column for the separation of a number of fluoroquinolone compounds, but only norfloxacin and ciprofloxacin were found in biosolids[11].

The HPLC/FLD calibration went to about 10 pg/ μL per component so that the fluorescence detection is able to detect low μg/kg levels easily. Although a large set of 15 or more fluoroquinolones are known, only a small number occur in large enough use to be detected in biosolids. The UPLC/TOF-MS calibration went to about 100 pg/μL so that the MS detection limit was not as good as the FLD limit.

The accurate mass chromatogram of m/z 362.1516 is shown in figure 4 for the biosolids extract and comparison can be made to the similarly shaped peak from the standard in figure 5. Clearly, the peak shape and mass correspond to the standard at the same retention time. In these accurate mass chromatrograms a window of 0.050μ is used to produce the ion current profile centered at the theoretical exact mass. The agreement of measured to calculated exact mass for ofloxacin was 362.1514 to 362.1516, respectively, indicating an error of 0.6 parts-per-million (ppm). The measured mass is obtained by averaging all spectra over the ion chromatogram and subtracting an averaging of back-

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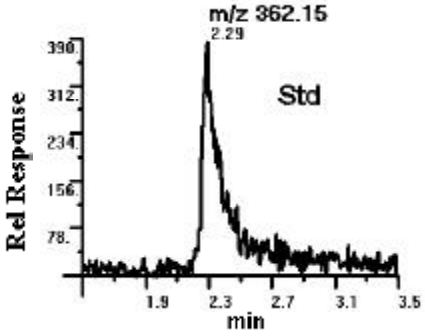

Figure 5: Ion profile UPLC/TOF-MS for m/z 362.15 ± 0.050 of a standard containing ofloxacin

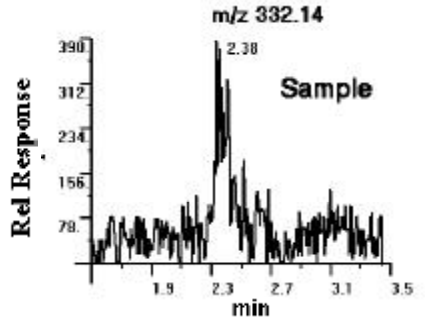

Figure 6: Ion profile for UPLC/TOF-MS m/z 332.14 ± 0.050 in biosolid extract

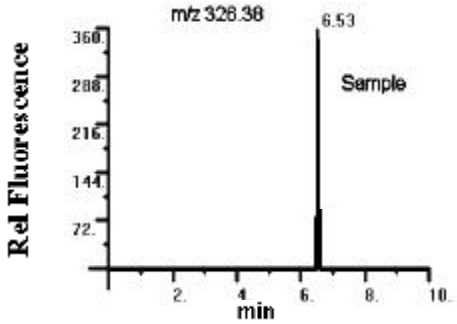

Figure 7: Ion profile for a quartenary amine compound (presumably didecyl, dimethyl ammonium ion) m/z 326.38 ± 0.050, RT=6.53

ground scans. A significant S/N ratio was obtained with ofloxacin and a poorer S/N resulted in figure 6 for ciprofloxacin (m/z 332.1410). Norfloxacin could not be detected by UPLC/TOF-MS and was, additionally, obscured by a matrix ion at a different but unresolvable mass.

In general, the instrument specification is 3 ppm or better so that is the accuracy sought. In practice, the accuracy can vary somewhat depending on mass in an unpredictable way. One might want to allow up to a 3 mmu error (10 ppm for mass 300) in considering unknowns for determination of elemental composition or a particular value suggested by determining known compounds very close in mass to the unknown.

TABLE 1: Tentatively identified quartenary ammonium surfactants in biosolids

Quartenary ion	Exact mass	Exper mass	Error ppm
Dioctyl,dimethyl am	270.3161	270.3160	0.4
Didecyl,dimethyl am	326.3787	326.3773	4.3
Benzyl,myristyl,dimethyl am	332.3317	332.3329	3.6
Decyl,octyl,dimethyl am	298.3474	298.3476	0.7
Benzyl,decyl,dimethyl am	276.2691	276.2708	6.2(weak ion)
Benzyl,cetyl,dimethyl am	360.3630	360.3630	< 0.3

The HPLC/FLD method reported here found presumptive evidence of norfloxacin and ciprofloxacin. Later work with LC/MS including ofloxacin supported its presence rather than norfloxacin. Considering the non-exhaustive recovery found for these compounds, the actual level present could be 4 to10 times higher. Survey work could use a recovery surrogate to generate corrected recovery of target analytes. Future research may uncover useful chemistry that improves the overall recovery of polar analytes from biosolids.

Surfactants/Antimicrobials

The samples also contained large amounts of polyethylene glycol polymers (PEG) that act to obscure the presence of other components of the extracts due to their ubiquitous presence and their enhanced surface activity relative to ionization by ESI.

Many oligomers and types of PEG- containing substances were observed whose mass extended beyond the 1000 da range used for target analysis. It is conservative to say that PEGs constitute a major component of the polar fraction. No further effort was made to pursue this class of compounds that are a major component of detergents and soaps that enter the sewage system.

An additional class of compounds found in large abundance were a series of quartenary ammonium salts consisting of varying alkyl and aryl components. Specific compounds tentatively identified are given in TABLE 1 together with calculated and observed accurate mass. The compounds were not confirmed using standards so that the identities have not been confirmed definitively. The retention times (6 to 7 min) and extremely sharp peaks (<3 sec peak width at half height) for these substances conform to the pattern observed with quartenary ammonium compounds, and the accurate mass chromatogram of the m/z 326.3787 ion is given in figure 7. Quartenary ammonium surfactants are

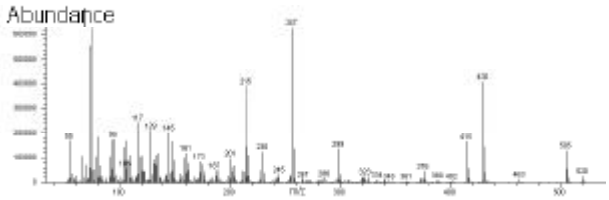

Figure 8: Bistrimethylsilyl-lithocholic acid standard GC/MS (EI) RT=21.34 min

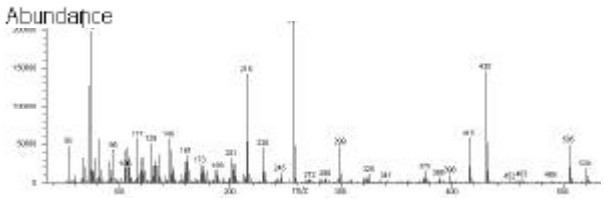

Figure 9: Bistrimethylsilyl-lithocholic acid identified in acidic fraction of biosolids, GC/MS (EI) RT=21.41 min

expected components within the matrix because they are commonly used antimicrobial disinfection products that work by disrupting bacterial cell walls for example.

Anionics

There existed a number of responses within the anionic fraction run by UPLC/TOF-MS but these responses have not been correlated with expected components and their tentative masses encompass a relatively large potential class that could benefit from obtaining MS/MS data to narrow down possibilities. No evidence was found for sulfates, sulfonic acids, and other expected acids within the fraction we isolated. This class is subject to further study in order to clarify the types of anions present in this matrix from an LC/MS perspective.

However, in order to make some headway into the issue of organic acids in biosolids apart from LC/MS identifications, we isolated an acidic fraction using acid/base partitioning. This eliminated most of the neutral lipophilic components and left us with an acid-enriched fraction. We then derivatized this fraction with which, presumably would react with both carboxylic acids and hydroxy groups.

The results of this analysis revealed hundreds of compounds and many could be tentatively identified by GC/MS. Prominent responses were noted for 4-hydroxybenzoic acid and a number of fatty acids including members from C16 through C24. The full significance of all of these anionic compounds is not entirely clear, but the presence of salts of carboxylic acids (that would be the likely natural form in biosolids) would indicate a significant presence of detergent-like compounds.

In consonance with this idea, we suspected that the presence of bile acids would also be likely and would further add to the surfactant content of the material. Among the compounds tentatively identified was the bis-trimethylsilyl ether of lithocholic acid. To confirm this compound we derivatized a standard of lithocholic acid and found complete agreement of retention time (24.37 versus 24.44) and mass spectra that are given in figures 8 and 9.

CONCLUSION

Biosolids constitute an extremely complex matrix containing substances of widely different polarities. Surfactants make up a major component of the polar fraction, and fluoroquinolone antibiotics also are present. We have confirmed the presence of a bile acid within the acidic fraction.

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