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Nota Técnica

EVALUATION OF SAMPLE PROCESSING METHODS FOR THE POLAR CONTAMINANT ANALYSIS OF SEWAGE SLUDGE USING LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (LC/MS)

Rênnio F. de Sena*, José L. Tambosi, Regina F. P. M. Moreira e Humberto J. José
Departamento de Engenharia Química e Engenharia de Alimentos, Universidade Federal de Santa Catarina, 88040-970 Florianópolis - SC, Brasil
Wilheim Gebhardt e Horst Fr. Schröder
Institute of Environmental Engineering (ISA), RWTH Aachen University Templergraben 55, D-52056 Aachen, Germany

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Monitoring of sewage sludge has proved the presence of many polar anthropogenic pollutants since LC/MS techniques came into routine use. While advanced techniques may improve characterizations, flawed sample processing procedures, however, may disturb or disguise the presence and fate of many target compounds present in this type of complex matrix before analytical process starts. Freeze-drying or oven-drying, in combination with centrifugation or filtration as sample processing techniques were performed followed by visual pattern recognition of target compounds for assessment of pretreatment processes. The results shown that oven-drying affected the sludge characterization, while freeze-drying led to less analytical misinterpretations.

Keywords: sample processing; LC/MS; polar pollutants.

INTRODUCTION

Sewage sludge is an undesired, but inevitable residual solid waste generated during biological wastewater treatment, and its sustainable management is one of the most critical environmental issues of today.[1] In the biological treatment plant, bacterial biocoenosis degrades – metabolizes or even mineralizes - the dissolved and/or emulsified solid pollutants contained in municipal or industrial wastewater by both, aerobic and anaerobic processes, building up bacterial mass. In North America, this dried by-product of biological wastewater treatment, the excess sludge, is often referred to as biosolids, to distinguish it from raw untreated forms of sewage.[2]

These biosolids commonly in the past were applied to agricultural land treatment, however, environmental and health issues today more and more have arisen regarding to this regulated disposal, especially due to the inorganic, organic and microbial contaminants present in sewage sludge. Many reports have studied the risk and environmental effects of this practice, since analytical results proved that conventional wastewater treatment is not always able either to metabolize or mineralize contaminants persisting biodegradation and therefore will be still present in the treated wastewater and/or excess sludge.[3,4]

Currently, under optimized conditions, more than 90-95% of pollutants can be eliminated from wastewater by conventional biological wastewater treatment, and besides biodegradation, i.e., metabolisation and mineralization, adsorption onto the sludge is one of the most effective elimination process.[5] Qualitative and quantitative detection and assessment of pollutants in sludge had been improved substantially in recent years, mainly due to the increased ecological consciousness of the people. In parallel analytical methods also were improved and the capability to apply selective and sensitive experimental and operational conditions for the characterization of the organic pollutant content of sludge, especially the identification and quantification of polar compounds by liquid chromatography – mass spectrometry (LC/MS), increased.[4]

Although the improvement of many analytical tools for the characterization of a vast range of pollutants in very low concentrations has been reported, the processing of samples applying non-standardized extraction, separation and concentration techniques, nevertheless, may lead to different types of erroneous results and consequently interpretations after substance specific analyses. In fact, solely both drying-methods, freeze-drying and oven-drying pre-treatment can heavily influence the sample morphology, because these drying procedures might modify the structures of the mixtures resulting in distinct complexes, which behave differently during extraction. Furthermore extracts may contain precipitates also separated differently by filtration or centrifugation and therefore favoring or discriminating compounds during sample handling prior to analysis. Moreover, the comparison of results obtained by the time saving pressurized liquid extraction (PLE)[6] and the liquid/solid Soxhlet extraction proved that for reliable results standardization is overdue for a long time.

Our objectives on the one hand were to assess the efficiency and applicability of freeze-drying (lyophilization) or oven-drying steps combined with an extraction followed by filtration or centrifugation, as sample processing techniques prior to the mass and tandem mass spectrometric detection and identification of polar compounds. Applicability of oven-drying, as a time-saving drying method for sludge compared to freeze-drying as time-consuming method was checked, applying the Soxhlet and PLE method using methanol as extractant. With this procedure on the other hand we wanted to check stability of conventional wastewater compounds as well as selected target compounds present in sludge in trace concentrations.

For assessment of results total ion current traces (TIC) after liquid chromatographic separation with mass spectrometric detection (LC/MS), TIC trace shapes and ion patterns recognition in overview spectra (flow injection-MS; FIA/MS) were applied to conventional wastewater constituents. In addition, high resolution selected ion trace analysis after the application of the pre-treatment procedures mentioned before were performed for polar organic micropollutants.

EXPERIMENTAL

Operational conditions of the wastewater treatment plant (WWTP)

Sewage sludge samples were taken from the end of the nitrifica-

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*e-mail: rennio@enq.ufsc.br

tion tank at the municipal WWTP Aachen-Soers, Germany, serving a population of 250,000 inhabitants. The wastewater treatment process consists of a conventional advanced biological multistage treatment process. The sewage sludge is separated from the mixed liquor suspended solids (MLSS) in the final clarification tank. From here the return sludge is recycled while excess sludge is separated and treated anaerobically. Before the wastewater will be discharged into the receiving water, the Wurm river, the effluent of final clarification passes through a biological active filter to remove traces of nitrate and phosphate, besides suspended particles.[7]

Experimental and operational conditions of the WWTP were: average inflow, 88,000 m^3/day; retention time (SRT), 20 h; mixed liquor suspended solids (MLSS), 2.9 g/L; sludge load determined as biological oxygen demand within 5 days (BOD_5): 0.07 kgBOD/kgMLSS/day.

Materials

Ultra-pure water used in all dilution procedures or as liquid chromatography (LC) eluent was prepared by a Milli-Q system (Millipore, Milford, MA, USA). All solvents used for solid-liquid extractions in Soxhlet or PLE extraction process as well as for LC separation were nanograde solvents purchased from LGC Promochem (Wesel, Germany). The polar target compounds, carbamazepine, diclofenac, dodecyl sulfonate, linear benzene sulfonate (LAS), polyethylene glycol, secondary alkyl sulfonate (SAS) and sulfamethoxazole applied for the assessment of sewage sludge pre-treatment methods were analytical grade compounds purchased from Sigma–Aldrich, Taufkirchen, Germany. All other chemicals used were of "Analytical Reagent-" or "Residue Analysis" purity grade (Merck, Darmstadt, Germany). Gases applied were products of Linde, Germany, and were of 99.99% purity.

Sludge sampling and pre-treatment

A representative activated sewage sludge sample from the WWTP-Aachen of 5 L was prepared containing 20% of dry matter after centrifugation. 5 g of sodium azide (NaN_3) were added and mixed vigorously to stop biological activity of wastewater biocoenisis. From this mixture 2 samples of approx. 1 L or 200 g of dry matter were taken and carefully weighed. One sample was determined for the oven-drying step, while the second sample was applied to freeze-drying. Drying of both samples was monitored by weight control.

The oven-dried sludge sample was dried at 105 ± 2 °C in a laboratory furnace. This drying process typically took one to two days. The oven-dried solids were crushed using a porcelain grinder, and stored in brown glass bottles at 4 ºC.

The freeze-dried sludge sample first was frozen at -86 ºC for 6 h prior to freeze-drying in a lyophilization apparatus (Heraeus-Christ, Germany). This procedure, for a 1 L-sample, typically took four to five days. The freeze-dried solids were crushed and also stored in brown glass bottles at 4 ºC as done with the oven-dried sludge.

Extraction procedures

After drying, sludge samples were extracted either by Soxhlet or by PLE. For liquid/solid extraction in a Soxhlet 5.0 g of both samples were extracted over a period of 14 h applying 50 mL of methanol. When Soxhlet extraction was finished the cold extracts of the freeze-dried and oven-dried sludge samples either were filtered or centrifuged at ambient temperature. Filtrate or centrifugate were evaporated in a gentle stream of nitrogen to 10 mL and stored at 4 ºC. These methods are partially in accordance to the scope of the U.S. EPA methods for biosolids analysis using high resolution chromatography coupled with high resolution mass spectrometry (HPLC/(HR)MS).[8,9] For PLE the differently dried sewage sludge samples (2 g) were mixed with 5 g of annealed sand (*T*, 450 °C) before they were extracted in a pressurized liquid extraction unit (Dionex ASE 200) using methanol as extraction solvent (*T*, 100 °C; 10 MPa).

Preparation of extracts and concentration for LC/MS analysis

Centrifuged samples were prepared by centrifugation of homogenized, fixed aliquotes (4 mL) of the volume reduced Soxhlet extracts at 10,000 x *g* for 10 min at room temperature. The precipitate was washed with 2 portions of 2 mL of cold (0 °C) methanol. The washing solution and the supernatant were combined before they were reduced in volume in a gentle stream of nitrogen at 50 ºC to dryness. The dry sample kept under gaseous N_2 was stored at -85 °C. Prior to LC/MS analysis the residue was reconstituted in 1 mL MeOH.

Filtered samples were prepared by filtration of homogenized, fixed aliquotes (4 mL) of the volume reduced Soxhlet extracts using glass fiber filters (2.0 µm) without the use of vacuum (approx. 20 min). The filtration residue was washed with 2 portions of 2 mL of cold (0 °C) methanol. The filtrate and the washing solutions were handled as described for the centrifuged samples.

The PLE-extracts of freeze- and oven-dried sludge samples contained very small amounts of solids (< 20 mg) which were removed from the liquids by membrane filtration (0.45 µm) before the samples were dried and stored as described before.

After processing, aliquots of the 6 samples (*Sludge A*, freeze-dried and centrifuged; *Sludge B*, freeze-dried and filtered; *Sludge C*, oven-dried and centrifuged; and *Sludge D*, oven-dried and filtered, *Sludge E*, freeze-dried and filtered, *Sludge F*, oven-dried and filtered) were analyzed by means of LC/MS and LC/MS/MS applying electrospray ionisation both in positive and in negative modes (ESI(+) and ESI(–)) as described by Gebhardt and Schröder.[7] The scheme of sample processing and analysis is shown in Figure 1.

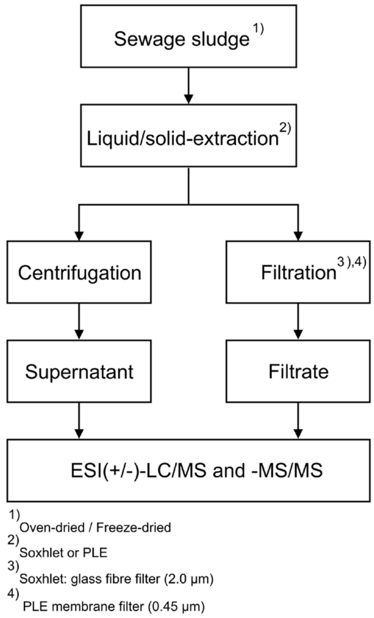

Figure 1. *Scheme of the alternative sewage sludge pretreatment procedures prior to flow injection- (FIA) and liquid chromatographic separation (LC) coupled with mass (MS) and tandem mass (MS/MS) spectrometric detection for the determination and identification of polar organic compounds*

LC/MS system and analytical conditions

Analytical LC-separations were carried out on a Hypersil Gold aQ column (RP5, 5 µm, spherical; 150 x 2.1 mm I.D.) from Thermo

Fisher Scientific (Dreieich, Germany) equipped with a Hypersil GOLD aQ pre-column of 10 x 2.1 mm I.D., filled with 5 µm, spherical material. Gradient elution by means of (A) methanol/water 90:10 (v:v) in combination with (B) Milli-Q-purified water/methanol 90:10 (v:v) was applied both containing 2 mM ammonium acetate and 0.1% acetic acid. The gradient was programmed as follows: Starting with 20% A/80% B the concentration was increased linearly to 90% A/10% B within 12 min. Up to 20 min the composition was kept constant. The overall flow rate was 0.2 mL/min.

For LC-separations prior to the LTQ Orbitrap hybride MS detector (Thermo Electron, Bremen, Germany), a Surveyor MS Plus pump (Thermo Electron, San Jose, CA, USA) was applied coupled with a HTC-PAL LC autosampler system (CTC Analytics, Zwingen, Switzerland). An LTQ Orbitrap mass spectrometer (Thermo Electron) was used for research work in ESI-LC/MS(+/-) and –MS/MS(+/-) mode as described in detail by Gebhardt and Schröder.[7] In brief, the Orbitrap was operated in full scan MS- and MS/MS-mode with a resolution of 15,000. To obtain high resolved mass traces the mass resolution was set to 60,000 at *m/z* 400 before spectra were accumulated. Product ion MS/MS-data of sulfamethoxazole and polyethylene glycol were recorded under collisionally induced dissociation conditions (CID) with 30 or 50% of normalized collision energy. Instrument control, data acquisition and data processing were performed using Xcalibur 2.0 software (Thermo Electron, San Jose, CA, USA).

RESULTS AND DISCUSSION

Our aim was to assess different sewage sludge sample processing techniques prior to analysis and to compare each one with respect to qualitative, as well as quantitative determinations of selected polar organic pollutants of relevance. Before starting here a differentiation was necessary related to the concentrations of pollutants. On the one hand pollutants present in wastewater and sewage sludge in conventional concentrations - µg to mg per L or kg – on the other hand, the trace and ultra-trace compounds present in ng or µg-range per L or kg in the matrix. Both types of pollutants can be relevant, the way to monitor them, however, is quite different. The information about the analytical approaches for their determination should provide help for engineers, biologists and chemists in their research to apply efficient, reliable procedures.

The efficiency to give relevant evidence about the spectrum of pollutants in the sludge samples differently handled during the pretreatment steps - drying, extraction and concentration prior to separation, characterization and identification - was assessed by choosing first a mass spectrometric pattern recognition approach. So compounds selected as target pollutants using LC/MS and MS/MS, covering a wide range of polarity and therefore known because of their different behaviour under heat stress and under extraction conditions applied, were determined to judge stability and extractability as indirect criteria for sample pre-treatment applying different drying processes.

According to Li *et al.,*[5] the chromatograms and overview spectra obtained for qualitative check-up of the pollutant load can be used for visual comparison of the TIC shapes as well as ion patterns of flow injection overview spectra (FIA). The comparison of the ion patterns allowed us to find or to miss the ions of those compounds in the different sludge sample extracts. Reasons for missing ions would be: volatilisation or destruction of compounds by heat during drying process applying 105 °C in laboratory-furnace and/or poor extraction efficiency. Reasons for finding new ions can be the destruction of compounds resulting in new ionizable compounds or a better extraction efficiency, e.g., using PLE.

Following this pattern recognition approach for the assessment of sewage sludge pre-treatment steps in the analysis of polar organic pollutants by LC/MS first the TICs of the different extracts were examined before ion patterns were used for assessment. So extracts recorded under identical conditions using positive ESI ionization are displayed in Figure 2.

In Figures 2a-f it is possible to recognize that each sludge sample, dried differently and pretreated or not pretreated by centrifugation or filtration, exhibited a distinct chromatographic behavior, differing one from another as shown in the TICs. From these results obtained from the same sludge, but differently treated and extracted, correct and reliable or false and inaccurate interpretations and conclusions can be drawn.

The signals in the LC/MS TICs of the freeze-dried samples recorded in positive ESI-mode (Figures 2a,b,c) and independent of the extraction method applied, seemed to be clearer and better separated than the TIC signals of oven-dried sample extracts (Figures 2d,e,f). Besides, the comparison of the peak shapes of the sludge samples dried by the same method, i.e., lyophilisation, however, extracted by Soxhlet or PLE and processed differently by means of centrifugation (Figure 2a), filtration (Figure 2b), or without any treatment for the PLE-extract, shows that the peak shapes of the TICs also differ one from another. Both PLE-extracts from sludge, freeze or oven-dried before, contained more signals of distinct compounds distribute over the whole time axes in the TICs (Figures 2c,f) than in the other TICs (Figures 2a,b,d,e) originating from Soxhlet extraction.

The comparison of the respective TICs of the furnace-dried sludge (Figures 2d,e,f) with the freeze-dried samples (Figures 2a,b,c), however, leads to more obvious changes. So many signals present in the positively generated TICs of freeze-dried samples (Figures 2a,b,c) at $t_R > 15$ min now are missing in the oven-dried sludge samples (Figures 2d,e,f). In addition, the peak shapes of the chromatograms of furnace-dried sludge extracts are less structured and more disturbed. The reason for this behavior can be seen in the thermal destruction of the compounds during drying process applying the oven-drying method.

So, the sample pre-treatment, "sludge drying process" we applied, resulted in significant differences in the spectrum of extracted compounds as recognizable by the peak shapes of the TICs. While in the TICs the compounds were separated because of their retention behaviour on the analytical LC-column, in the FIA/MS spectra the accumulated ions under the TICs give a total survey about the ionizable compounds in the extracts.

Taking this into account we have two possibilities to assess reliability und reproducibility for the generation of sludge sample extracts: Peak shapes of LC/MS(+) total ion chromatograms (TIC) of extracts of freeze-dried and oven-dried samples and, in addition, FIA/MS overview spectra, as described before, presenting a survey of the ionizable compounds. Besides both assessment approaches – number and structure of the signals in the TICs and number of ions in the overview spectra - the large variations in the TIC itensities as well as in overview spectra intensities also have to be taken into account.

The next variable influencing the analytical results was the choice of the extraction step, therefore, we assessed the standard liquid/solid extraction applying Soxhlet and the more hyphenated pressurized liquid extraction method (PLE). Extraction in a Soxhlet apparatus is a very common technique in the analysis of solids. The extraction temperature is higher than room temperature and the sample is repeatedly brought into contact with fresh extraction solvent refluxed in a closed loop-cycle.[10] The main advantage of this method is the exhaustive extraction of the dissolvable analytes. The disadvantage of this method is, to require relatively long extraction periods, large amounts of solvents and therefore an additional long-lasting evaporation step for sample concentration compared, e.g., to PLE.

After Soxhlet extraction the solutions were cloudy and contained precipitated solids, therefore both, the freeze- and oven-dried extract samples were submitted to a further purification step, to separate the

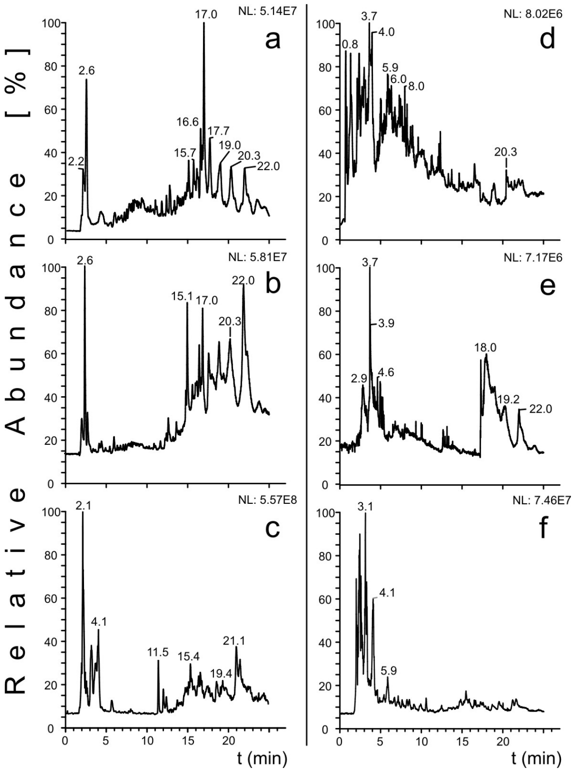

Figure 2. ESI(+)LC/MS total-ion current (TIC) traces and flow injection mass spectra (FIA/MS) of sewage sludge samples, dried and extracted under different conditions: (a) Sludge A, freeze-dried, Soxhlet extracted and centrifuged; (b) Sludge B, freeze-dried, Soxhlet extracted and filtered; (c) Sludge C, freeze-dried, extracted by pressurized liquid extraction (PLE); (d) Sludge D, oven-dried, Soxhlet extracted and centrifuged; and (e) Sludge E, oven-dried, Soxhlet extracted and filtered; (f) Sludge F, freeze-dried, extracted by PLE

suspended and emulsified solids by centrifugation or filtration to obtain clear extracts for LC/MS analyses. This handling monitored in the TICs and FIA/MS overview spectra influenced the peak shapes or ion patterns; however, their influence on the intensities of TICs and FIA/MS overview spectra logically seemed to be marginal. Polar compounds will not precipitate but stay dissolved in methanol, quite different to unpolar compounds, which would crystallize and precipitate under these circumstances. Deeper insights would be possible when the TICs as well as the overview spectra would be normed to the TIC or spectrum with the highest intensity as presented in literature.[5]

According to Schröder[11] the multitude of isomeric and isotopic constituents is separated from one to another by LC and therefore under optimized circumstances can be distinguished in TICs by the retention time of standards - if available. The mass spectra hiding behind the time-dependent eluting peaks in the TIC could provide the information on the molar masses of the ions - ammonium adduct-, protonated- or de-protonated ions - separated as molecules by LC prior to ionization. Thus, comparison of ESI(+)- and/or ESI-(–)LC/MS traces of the sludge samples provided outstanding information about the compounds recorded under both ESI ionization modes. Comparing ionization efficiency for the extracts of lyophilized or oven-dried sludge the ionization efficiency for the extracts of freeze-dried sludge samples, positively or negatively ionized, was observed to be better than for furnace-dried sample extracts.

CONCLUSION

LC-MS examinations confirmed that different sample processing procedures permanently modify the chromatographic results regarding to separation and identification of target masses. The quality overview of the chromatograms and their intensity were hardly affected by the different sample preparation steps. Especially for the oven-dried sludge samples, LC-MS results showed that target compounds that could be easily determined using liquid chromatography were destroyed or suppressed, and also, the effects of this process over target ion monitoring was broadly prejudiced. These findings should be used for the development of modified methods with improved characterization performances.

The analysis of sewage sludge for a sustainable assessment how to dispose it off – deposition on a waste disposal, incineration or its use as fertilizer in biological agriculture – needs comprehensive examinations of the material “sewage sludge” by experienced analytical staff as also applied for the spectrum of unpolar organic pollutants. The high capacity of sludge to adsorb also polar pollutants was expected and now could be confirmed with the results of these examinations. Therefore a standardized pre-treatment of sludge was overdue. LC/MS and FIA/MS examinations proved that the application of different sample processing procedures to identical samples might modify the molecular structure of the target compounds, with the result that their chromatographic separation and mass spectrometric detection changed. With the visual pattern recognition approach applied to TICs or FIA/MS-overview spectra qualitative and quantitative information will be provided. So, appearance and intensities which were affected by different sample preparation steps, can be used for the evaluation of reproducibility and reliability.

Especially for the extracts of the oven-dried sludge samples, LC/MS and FIA/MS results proved, that compounds easily determined in the freezed-dried sludge extracts were destroyed or suppressed, while influence of centrifugation or filtration as pretreatment on the MS results is not worth mentioning. Again the freeze-drying method provided the reliable results compared to oven-drying process, however, applying PLE (not shown) to the freeze-dried samples the recovery of the selected target compounds in addition increased by 12, 22 and 29% for PEG, sulfamethoxazole and carbamazepine, respectively.

Between the two sample separation steps applied for analytical characterization, filtration also present losses regarding to relative intensity and overview LC separation, important attributes when an accurate characterization and quantification of the target compounds identified in the sludge sample is desirable.

The analytical techniques and sample processing procedures presented herein provided that application of reliable methods that should help analysts to gain deeper insights into the characterization and identification of anthropogenic specific compounds in sewage sludge. The utilization of the right materials and specific processing methods may be the most powerful tool for monitor the development of various solid wastes and for assessing LC-MS as a sophisticate, sensitive and selective technique for the determination of a wide range of pollutants in complex solid matrices.

SUPPLEMENTARY MATERIAL

On Supplementary Material, available on http://quimicanova.sbq.org.br as a PDF file, with access free, are presented the Figures 1S to 4S. Figure 1S shows the respective TICs in negative ESI-mode, and some particular aspects of the polar pollutants determinations and the TIC shapes of the negatively ionized extracts (Figures 1S,a-f) and their respective mass spectra (Figures 1S,g-l). The target compounds recorded in high resolution scan mode in the TICs as shown before with a resolution (FWHM) of 1 mmu can be easily recognized as displayed with their selected mass traces in Figure 2S. The MS/MS-spectra of the protonated precursor $[M+H]^+$-ion of sulfamethoxazole (*m/z* 254) and the ammonium adduction ion ($[M+NH_4]^+$) of the precursor PEG homologue (*m/z* 476) are presented in the Figures 3Sa and 3Sb. Figure 4S shows the chemical structures of the target compounds from Figure 3S.

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Rênnio F. de Sena*, José L. Tambosi, Regina F.P.M. Moreira e Humberto J. José
Departamento de Engenharia Química e Engenharia de Alimentos, Universidade Federal de Santa Catarina, 88040-970 Florianópolis - SC, Brasil
Wilheim Gebhardt e Horst Fr. Schröder
Institute of Environmental Engineering (ISA), RWTH Aachen University Templergraben 55, D-52056 Aachen, Germany

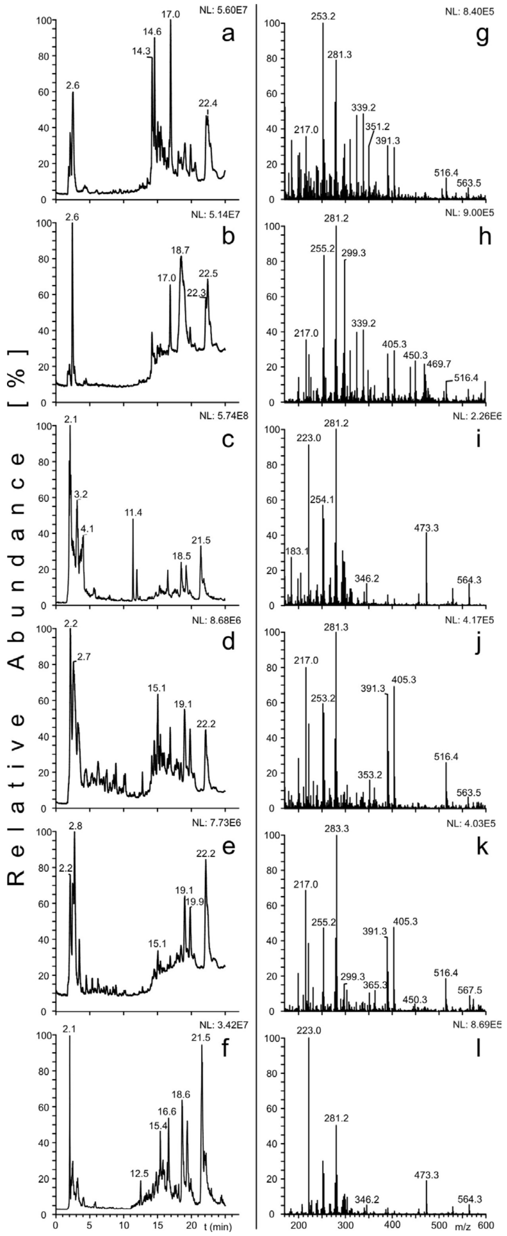

Figure 1S. *ESI(-)LC/MS total-ion current (TIC) traces and flow injection mass spectra (FIA/MS) of sewage sludge samples, dried and extracted under different conditions: (a,g) Sludge A, freeze-dried, Soxhlet extracted and centrifuged; (b,h) Sludge B, freeze-dried, Soxhlet extracted and filtered; (c,i) Sludge C, freeze-dried, extracted by PLE; (d,j) Sludge D, oven-dried, Soxhlet extracted and centrifuged; (e,k) Sludge E, oven-dried, Soxhlet extracted and filtered; (f,l) Sludge F, freeze-dried, extracted by PLE*

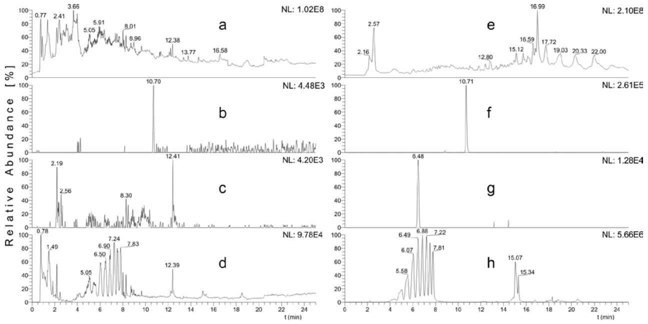

Figure 2S. *ESI(+)LC/MS (a,e) total-ion current (TIC) traces as in Figure 2d (Sludge D, oven-dried, Soxhlet extracted and centrifuged) and 2a (Sludge A, freeze-dried, Soxhlet extracted and centrifuged) and extracted mass traces (b,f) carbamazepine, (c,g) sulfamethoxazole and (d,h) HO-(CH$_2$-CH$_2$-O)$_{10}$-H-homologue of polyethylene glycol in those samples recorded under high resolution mass spectrometric conditions*

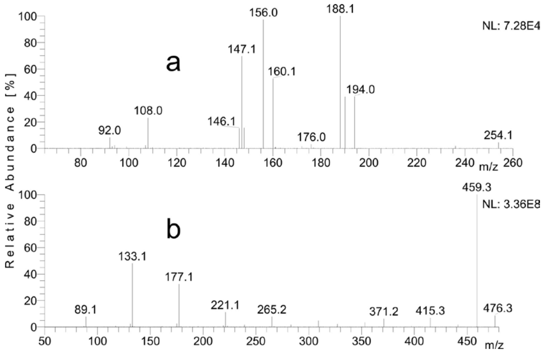

Figure 3S. *ESI(+)LC/MS/MS product-ion mass spectra of (a) sulfamethoxazole (m/z 254.1; [M+H]$^+$) and (b) ammonium adduct ion of polyethylene glycol homologue HO-(CH$_2$-CH$_2$-O)$_{10}$-H (m/z 476.3; [M+NH$_4$]$^+$). Product ions were generated by collisionally induced dissociation (CID) to compounds in Figure 2S,g and Figure 2S,h (t_R 6.48 and 7.22 min)*

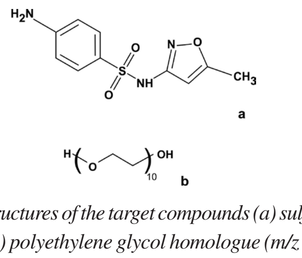

Figure 4S. *Chemical structures of the target compounds (a) sulfamethoxazole (m/z 254.1; [M+H]$^+$) and (b) polyethylene glycol homologue (m/z 476.3; [M+NH$_4$]$^+$)*

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*e-mail: rennio@enq.ufsc.br