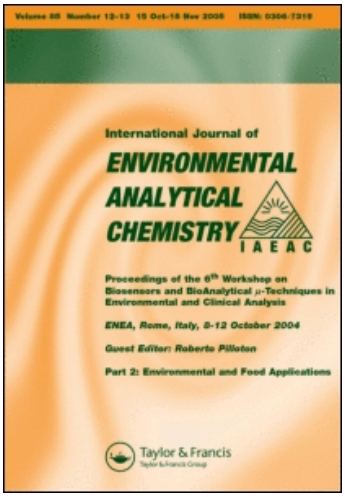

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Thiosteroids in sewage sludge and its post-treatment products

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To cite this Article Mejía, Giovanna , de Nadal, Raquel , Bagó, Bárbara , Broto, Francesc and Comellas, Lluís(2008) 'Thiosteroids in sewage sludge and its post-treatment products', International Journal of Environmental Analytical Chemistry, 88: 11, 803 — 812
To link to this Article: DOI: 10.1080/03067310801942357
URL: http://dx.doi.org/10.1080/03067310801942357

Intern. J. Environ. Anal. Chem.
Vol. 88, No. 11, 15 September 2008, 803–812

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Thiosteroids in sewage sludge and its post-treatment products

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(*Received 30 November 2007; final version received 24 January 2008*)

Different sulphur compound derivatives from steroids were detected and quantified in the analysis of the lipidic fraction of sewage sludge and its post-treatment products, including thermally dried sludge and compost. Some steroid thiols: 5α-cholestane-3β-thiol, 24-methyl-5α-cholestane-3β-thiol and 24-ethyl-5α-cholestane-3β-thiol were detected in the gas chromatography with mass spectrometry detector (GC-MS) analysis. These three compounds, related to the thiostanol family, have only been encountered in previous studies in sea sediments. Gas chromatography with sulphur-selective flame photometric detector (GC-FPD) was used with the objective of detecting other related compounds in sludge. It allowed the detection of other compounds, whose mass spectra could correspond with the one of the steroid enethiols. The research focused on detecting the formation point of these sulphur compounds in the sludge treatment process, as well as their biodegradation resistance in the post-treatment processes. The amount of these products in the sludge was quantified using gas chromatography with flame ionization detector (GC-FID). The result of this study confirms that the identified compounds are not easily biodegradable.

Keywords: thiostanols; enethiols; sewage sludge; GC-MS; post-treatment sludge processes

1. Introduction

In recent years, great interest has been shown in the safe disposal of sewage sludge generated in wastewater treatment plants. The three most common final destinations of sludges are for use in agriculture, energy production and landfilling [1]. Changes in public perception and legislation have made landfilling unpopular. As a consequence, the latest research has been focused on sludge as a conditioner and fertilizer in agriculture and in other recycling outlets (e.g., landscaping).

The use of sewage sludge as an organic-mineral fertilizer in soil implies a dependence on the organic matter and nutrients contained in it and the elimination of residues, as well as minimal environmental impact [2].

Lipids constitute an important fraction of the total organic matter in sewage sludge. They could have an influence on the water retention capacity of amended soils, on their

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ISSN 0306–7319 print/ISSN 1029–0397 online

DOI: 10.1080/03067310801942357
http://www.informaworld.com

structural stability and on the biodegradation-humification balance in soils [3]. Lipid molecular composition is very complex [4] so they do not have common chemical or structural properties able to characterize them. As a consequence, the different families that compose them have to be studied.

The lipidic fraction of sewage sludge depends on the type of wastewater treatment plant and treated water origin. Most of this fraction is composed of hydrocarbons, fatty acids and sterols [5]. These last components are usually called faecal sterols due to the origin of sludge matrix. Coprostanol (5β-cholestan-3β-ol) is the major faecal sterol and it is produced by microbial reduction of cholesterol in an animal's gut [6]. Due to this specific origin, coprostanol has been used extensively as a marker for sewage-related pollution, both in water and in marine sediment [7].

Other potential markers lately used for environmental pollution in marine sediments are the thiosteranes, a series of cyclic compounds formed from Δ-2 sterenes and probably originated from plankton [8,9].

In our groups' previous research on sewage sludge and its post-treatment products, concerned with the levels of di-ethylhexyl phthalate (DEHP), the composition of the lipidic fraction was intensively studied using GC-MS [10]. The study showed that different families (hydrocarbons, fatty acids, sterols and stanols) were present in sewage and thermally dried sludge but only one family was found in the compost. This last particular family, which was in the compost, was also encountered in the other two materials. The chromatogram was composed of a number of abundant peaks that eluted at the end. These last family components were identified provisionally as thiostanols. The study of these thiostanols was considered necessary for the environmental risk associated with the degree of persistence of these compounds after its use as amended soil materials.

In this work the identification of these thiols has been carried out using gas chromatography coupled with mass spectrometry and flame photometric (sulphur mode) detection. Quantification has been performed on a flame ionization detector. The analyses were carried out on the lipidic fraction of the sewage sludge of two wastewater treatment plants and its two products of post-treatment: thermally dried sludge; and compost.

2. Experimental

2.1 *Sample collection and preparation*

In order to carry out this study, sludge before digestion, sewage sludge, thermally dried sludge and compost from the same origin was required.

Sewage sludges were collected from two domestic wastewater treatment plants of the Catalan area: Blanes and Manresa (Catalonia, Spain), with an anaerobic stabilization.

Thermally dried sludge was obtained by indirect drying of sewage sludge in the thermally drying plant in Banyoles (Catalonia, Spain) at a temperature higher than 80°C, for ensuring a reduction of water content to less than 10%.

The compost was obtained by mixing sewage sludge and plant residues in a rate 1 : 4 by weight. Thermophilic aerobic stabilization was performed at a temperature of at least 55°C with a retention period of 15 days in the respective wastewater treatment plant. The moisture content was approximately 35%.

The sludge before digestion was decanted and stored at −20°C. Sewage sludge was also stored at −20°C. Thermally dried sludge and compost were kept at room temperature

until use. Before analysis, sludges were dried at 105°C and ground. The compost was passed through a 212 μm sieve to remove any wood chips.

2.2 *Standards, chemicals and solvents*

Dichloromethane residue grade was from Romil (Barcelona, Spain) and anhydrous sodium sulphate was bought from Merck (Barcelona, Spain). Cellulose extraction thimbles (33 × 10 mm) were purchased from Albet (Barcelona, Spain). The standard thiostanol 5α-cholestan-3β-thiol was synthesized and given by Adam *et al*. [11].

2.3 *Sample extraction*

Five grams of sludge were mixed with an amount of anhydrous sodium sulphate in order to obtain a dry mixture. This mixture was extracted with 200 mL of dichloromethane by Soxhlet extraction for 6 hours in cellulose thimbles. The extracts were reduced to a small volume (a few millilitres) using a rotary evaporator. The remaining solvent was evaporated to dryness under a gentle stream of dry nitrogen and was reconstituted with 10 mL of dichloromethane.

2.4 *Gas chromatography-mass spectrometry (GC-MS)*

GC-MS analyses were performed on a HP 5890 – HP 5989 MSD combination. Injection of 2 μL extract was performed in the splitter mode using a splitter flowrate of 30 mL min^{-1}, a column flow of 0.8 mL min^{-1} and split ratio of 37 : 1. Separation was achieved on a 30 m × 0.25 mm i.d. × 0.25 μm TRB-META-X5 column (Teknokroma). Helium at 9 psi constant pressure was used as a carrier gas. The GC injector port, ion source and mass analyser temperatures were set at 280, 250 and 110°C, respectively. The oven temperature programme was started at 120°C for 1 min, then increased by 10°C min^{-1} to 300°C and holding it for 21 min. Spectra were obtained by electron impact at 70 eV in scan acquisition mode.

2.5 *Gas chromatography-flame photometric detection (GC-FPD)*

Extracts analysed by GC-MS were also analysed by HRCG-FPD on a HP 5890 gas chromatograph equipped with a FPD detector. Injection of 1 μL extract was performed in the splitter mode using a splitter flow rate of 30 mL min^{-1}, a column flow of 9.7 mL min^{-1} and split ratio of 3.1 : 1. Separation was achieved on a 30 m × 0.53 mm i.d. × 2.65 μm HP-5 column (Agilent Technologies). The oven temperature programme was 120°C, 1 min hold, 20°C min^{-1} to 300°C, 30 min hold. The detector temperature was set at 280°C. The other chromatographic conditions were identical to those described in the GC-MS analysis.

2.6 *Gas chromatography-flame ionization detection (GC-FID)*

The same extracts were also analysed by HRCG-FID. These analyses were performed on a HP 5890 gas chromatograph equipped with a FID detector. Injection of 2 μL extract was

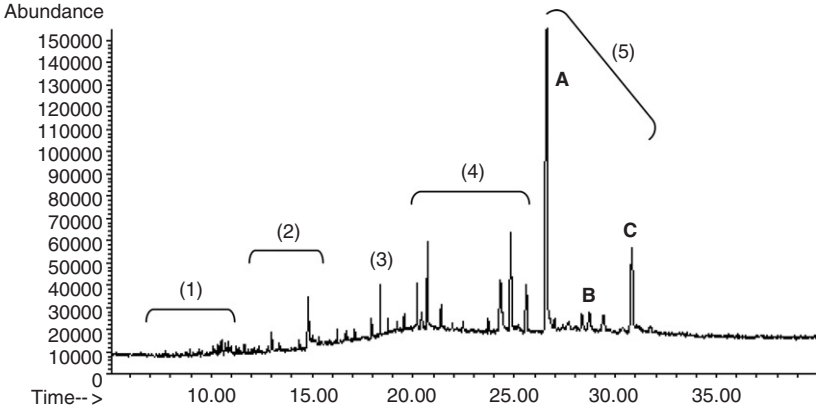

Figure 1. The typical total ion chromatograms (TIC) of sewage sludge from Blanes obtained by GC-MS in SCAN mode.
Notes: 1: hydrocarbons; 2: fatty acids; 3: di-(2-ethylhexyl)phatalate; 4: cholestanols; 5: thiostanols (compounds **A**: 5α-cholestane-3β-thiol, **B**: 24-methyl-5α-cholestane-3β-thiol and **C**: 24-ethyl-5α-cholestane-3β-thiol).

performed in the splitter mode using a splitter flowrate of 30 mL min^{-1}, a column flow of 0.5 mL min^{-1} and split ratio of 60 : 1. Separation was achieved on a 30 m × 0.25 mm i.d. × 0.25 μm HP-5 column (Agilent Technologies). The oven temperature programme was 120°C, 1 min hold, 20°C min^{-1} to 300°C, 30 min hold. Other chromatographic conditions were identical to those described in the GC-MS analysis.

3. Results and discussion

3.1 *Analysis by GC-MS*

The dichloromethane extracts of the sludge from Blanes wastewater treatment plant were first analysed by GC-MS. The typical total ion chromatograms (TIC) are shown in Figure 1.

The lipidic fraction of sewage sludge mainly consisted of hydrocarbons C_{14}–C_{30}, fatty acids, phthalate (DEHP), sterols and thiostanols at the end of the chromatogram (26–31 minutes). Thiostanols were labelled in the chromatogram with A, B, and C (Figure 1).

The composition of the aliphatic fraction of the thermally dried sludge was very similar to that of sewage sludge. Only some small differences could be detected in the content of fatty acids and sterols. Compost sample only presented DEHP in a very low concentration similar to the level of the blank and of the thiostanols at the end of the chromatogram.

Thiostanols were identified by comparison of their mass spectra with the published ones for such compounds in marine sediments [9]. The compound **A** was identified as 5α-cholestane-3β-thiol, compound **B** as 24-methyl-5α-cholestane-3β-thiol, and compound **C** as 24-ethyl-5α-cholestane-3β-thiol with molecular ions at m/z 404, 418, and 432, respectively (Figure 2).

The sludge extracts in dichloromethane from Manresa wastewater treatment plant showed the same composition as the Blanes case, with the exception of thiostanols. However, in compost analysis 5α-cholestane-3β-thiol was detected (Figure 3).

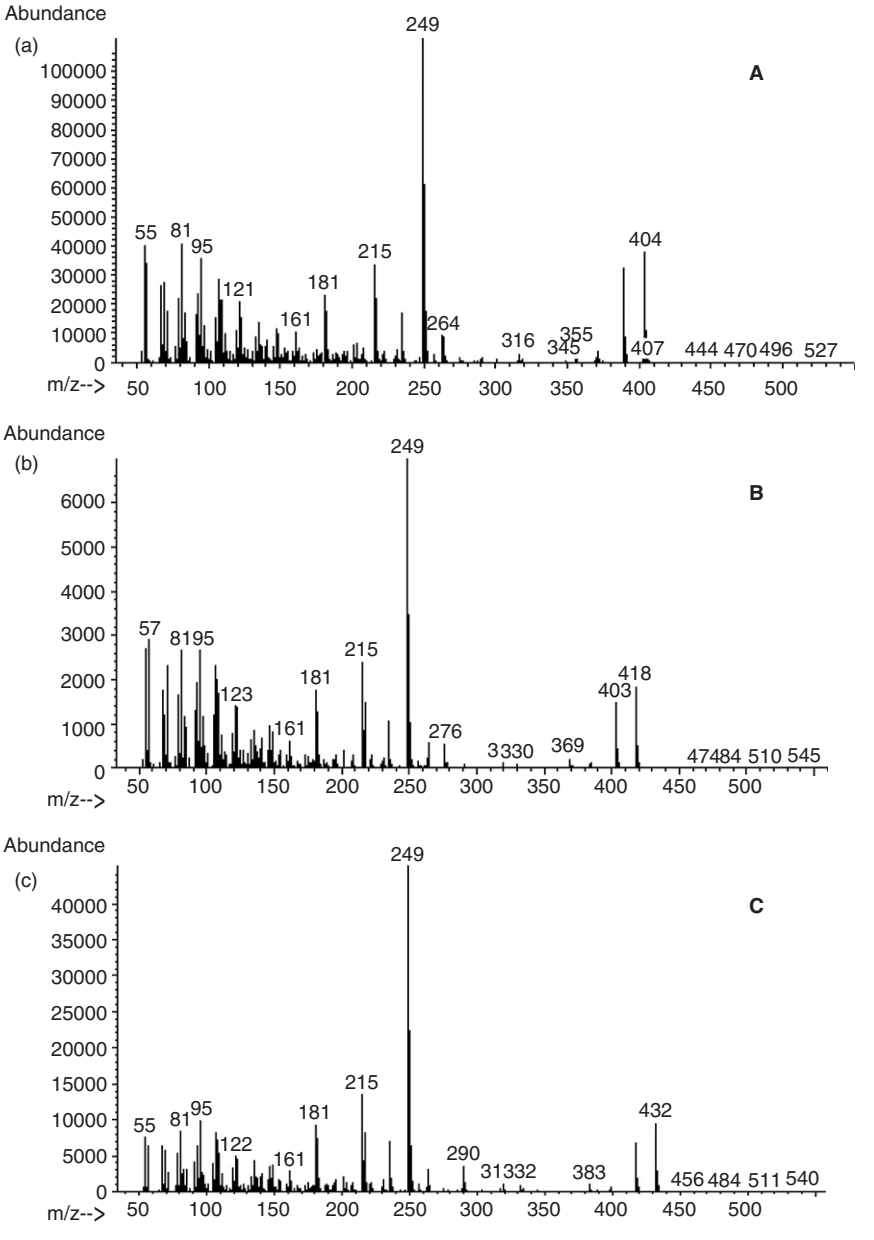

Figure 2. Mass spectra of compounds.
Notes: (a) **A**: 5α-cholestane-3β-thiol, (b) **B**: 24-methyl-5α-cholestane-3β-thiol and (c) **C**: 24-ethyl-5α-cholestane-3β-thiol.

3.2 *Analysis by GC-FPD*

3.2.1 *Sewage sludge and posttreatments products*

GC-FPD analysis confirmed the presence of sulphur in molecules A, B and C. The sulphur specific chromatogram obtained for compost is shown in Figure 4. The major compounds

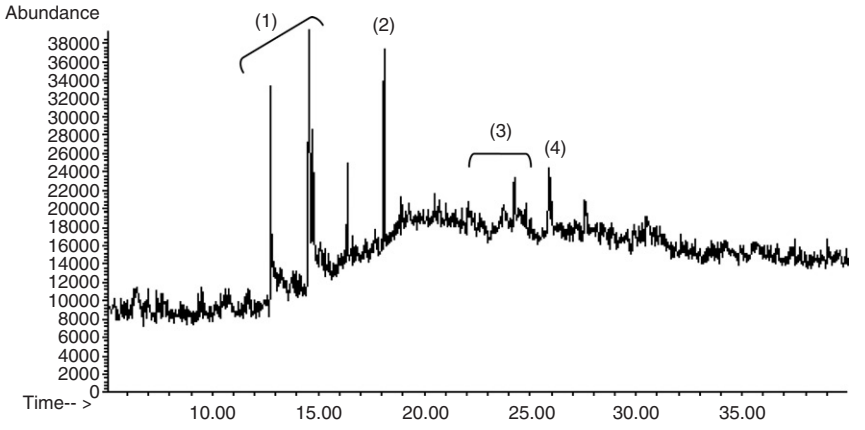

Figure 3. The typical total ion chromatograms of compost from Manresa obtained by GC-MS in SCAN mode.
Notes: 1: fatty acids; 2: di-(2-ethylhexyl)phatalate; 3: cholestanols; 4: 5α-cholestane-3β-thiol (compound **A**).

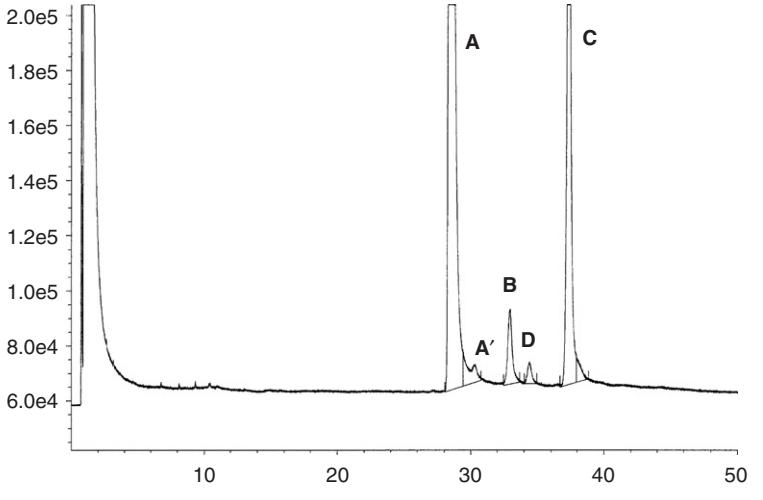

Figure 4. Chromatogram of compost from Blanes obtained by GC-FPD.
Notes: Thiostanols: **A**: 5α-cholestane-3β-thiol, **B**: 24-methyl-5α-cholestane-3β-thiol and **C**: 24-ethyl-5α-cholestane-3β-thiol and possible ene-thiostanols **A**′ and **D** related to compounds **B** and **C**, respectively.

containing S correspond to peaks **A**, **B** and **C**. The high selectivity of this detector for sulphur compounds offered the detection of a minor sulphur compound in the elution zone of the thiostanols family (28–38 minutes). This compound was labelled **D** in the chromatogram. Also, it is possible to detect the presence of another compound in the tail of **A** labelled **A**′.

The analysis of sewage sludge and compost from the Manresa treatment plant by GC-FPD allowed the detection of thiostanols. The chromatogram profile was the same as the Blanes chromatogram profile, but its signals were less intense.

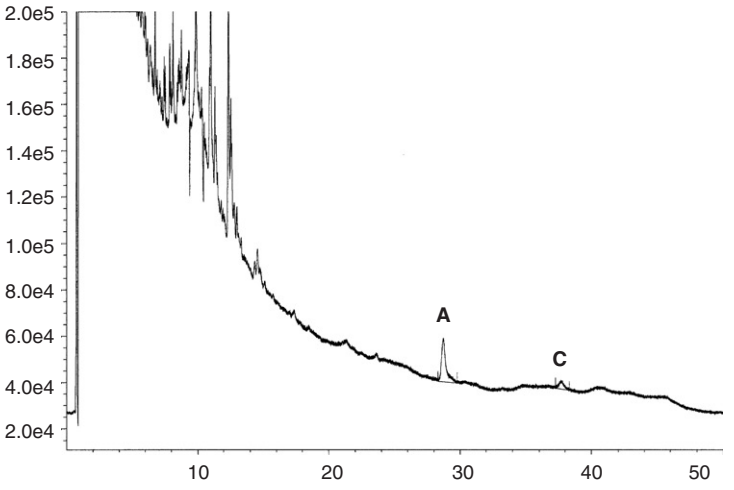

Figure 5. Chromatogram of sludge before digestion from Blanes obtained by GC-FPD.
Notes: Thiostanols **A**: 5α-cholestane-3β-thiol and **C** 24-ethyl-5α-cholestane-3β-thiol.

3.2.2 *Sludge before anaerobic digestion*

The use of the FPD detector was not enough to increase the sensitivity in the analysis of sludge before anaerobic digestion. The injection volume was, therefore, increased.

For both (Blanes and Manresa) samples, two thiostanols, 5α-cholestane-3β-thiol and 24-ethyl-5α-cholestane-3β-thiol were detected (Figure 5). The output signals were less than those originated by sewage sludges. This fact might lead to the conclusion that the anaerobic digestion process is responsible for the increase in the concentration of these thiostanols. As already mentioned, similar organo-sulphur compounds were found in sulphur rich sediments. These compounds were formed by the reaction of reduced organic sulphur species, produced by bacterial processes, with functionalised lipids of biological origin [11,12]. A similar process could be occuring here between the steroid derivatives in the sludge and H_2S or hydrogen sulphide or polysulfide ions formed by the anaerobic digestion process or bacterial sulphate reduction.

The study of the fourth minor compound detected in Blanes sewage sludge was performed by GC-MS in the splitless mode, with the purpose of increasing sensitivity. In the elution zone of compounds identified like thiostanols, a compound very well separated and another compound overlapped with thiostanol 5α-cholestane-3β-thiol were eluted. These compounds would correspond to compounds **D** and **A′**, respectively, in Figure 4.

The mass spectra of compound **D** showed molecular ion at m/z 430. This molecular ion has a difference of two mass units with regard to the molecular ion m/z 432 of the compound 24-ethyl-5α-cholestane-3β-thiol. This difference, which corresponds to two hydrogen atoms, may be due to the presence of a double bond in the 24-ethyl-5α-cholestane-3β-thiol structure. The mass spectra of compound **D** was compared with the mass spectra of standard cholesterol (cholest-5-en-3β-ol). Both showed a similar fragmentation, but the position of a double bond in C-2 or C-5 was not possible to determine.

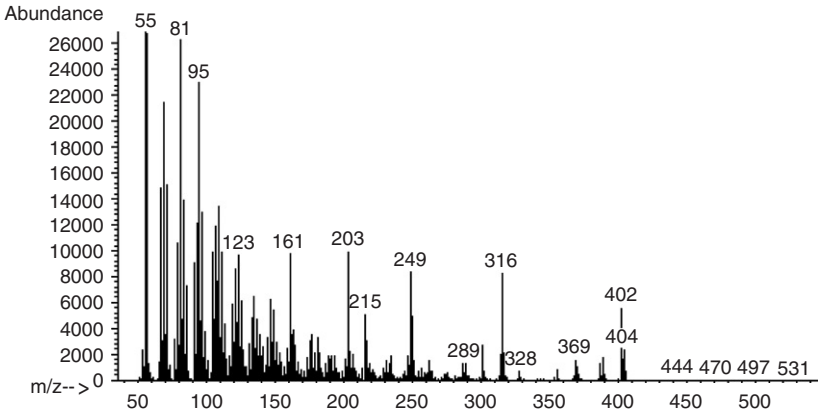

Figure 6. Mass spectra of compound 5α-3-thio-cholest-2-ene with molecular ion *m*/*z* 402.

The compound **A′** overlapped to thiostanol 5α-cholestane-3β-thiol had molecular ion *m*/*z* 416. For the same reasons stated for compound **D**, it was identified as a steroid enethiol related to 24-methyl-5α-cholestane-3β-thiol. The presence of both hydrogenated compounds related to two of the thiostanols, identified as 24-ethyl-5α-cholestane-3β-thiol and 24-methyl-5α-cholestane-3β-thiol, suggested the presence of a third compound related to 5α-cholestane-3β-thiol.

The ion extraction of the molecular ion *m*/*z* 402 becomes useful to find the third hydrogenated compound. This compound totally overlapped the 5α-cholestane-3β-thiol. It is, however, more abundant than both previous ones and therefore its mass spectrum is better defined. The mass spectrum is characterised by fragments typical for Δ-2 sterenes that result from a retro-Diels Alder type cleavage of ring A (*m*/*z* 316; *m*/*z* 203; *m*/*z* 161) (Figure 6). The presence of these fragments would indicate that the position of the double bond is in the position C-2. Therefore, the compound could be assigned as 5α-3-thio-cholest-2-ene.

Adam, Schneckenburger and colleagues [11,12] have conducted experiments that demonstrate a relation between enethiols and thiols in natural environments. The enethiols would be intermediate compounds in the formation of thiols by reductive sulphurization of ketones. The authors have shown that steroid ketones, when treated with hydrogen sulphide ions, are first transformed into the corresponding enethiols (or thioketones) probably by addition of hydrogen sulphide ions followed elimination of water. Then, these enethiols are further reduced to thiols by hydrogen sulphide ions.

3.3 *Analysis GC-FID*

Once having identified all thiosterols, major thiostanol 5α-cholestan-3β-thiol was quantified by external pattern using a synthesised standard by Adam and colleagues [11]. The results obtained for sludges from the two analysed wastewater treatment plants are presented in Table 1.

The results show that the lipidic fractions of sludges from Blanes wastewater treatment plant contain a percentage of thiostanols much greater than those coming from Manresa wastewater treatment plant, where they are only quantifiable in compost.

Table 1. Ratio of thiostanols vs. lipidic fraction and dry sample in two wastewater treatment plants.

Sample	Ratio 5α-cholestan-3β-thiol/lipidic fraction (%)	Ratio 5α-cholestan-3β-thiol/d.m (%)
Sludges from Blanes		
Sludge before digestion	<0.1	<0.01
Sewage sludge	6.6	0.2
Thermally dried sludge	4.9	0.2
Compost	14.2	0.2
Sludges from Manresa		
Sludge before digestion	<0.1	<0.01
Sewage sludge	<0.1	<0.01
Compost	0.6	0.01

The lipidic fraction of Blanes sludge before digestion show a much smaller percentage of thiostanols than its corresponding digested sludge. This suggests that the formation of these compounds would be favoured during the anaerobic digestion process. In the case of the thermally dried sludge the proportion of thiostanols is similar to its corresponding digested sludge. Moreover, these compounds represent an important concentration in the lipidic fraction of the compost.

Considering the results on dry samples, the concentration of thiostanols in Blanes sewage sludge and in its two post-treatment products remains constant. The concentration of thiostanols in Blanes sludges is greater than the one of Manresa sludges.

4. Conclusions

GC-MS analysis of the lipidic fraction of sewage sludge and its post-treatment products has allowed detection of a family of thiostanols. GC-FPD analysis has enabled the authors, for the first time in these type of samples, to obtain complementary data to HRCG-MS that led to the identification of enethiols related to thiostanols. The concentration of identified compounds does not vary during any of the post-treatment processes. In the case of compost, the identified compounds are the only components of the lipidic fraction resistant to the biodegradation during the composting process. The concentration of thiostanols varies from one wastewater treatment plant to another, both with a process of anaerobic digestion. The process of anaerobic digestion would favour the formation of the identified compounds.

Acknowledgements

The authors are grateful to the Generalitat de Catalunya - CIRIT (Project ECOFANGS Utilization of sewage sludge, compost and thermally dried sludge in soil amendment) and to Ministerio de Ciencia y Tecnología (Project LODOTOX Ecotoxicological risk assessment of sewage sludge and pig slurries) for financial support. The authors wish to thank specially J. Grimalt, J.P. Adam and J.P. Albrech for providing the standard 5-α-cholestan-3-β-thiol.

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