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Determination of β-hydroxy fatty acids in sewage sludge by using selected ion monitoring

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Abstract

By using selected ion monitoring, determination of β-hydroxy fatty acids could be easily achieved. These compounds were found as minor components in the acid fraction of lipid extracts. The method developed in the present investigation allowed quantification of 16 hydroxy acids in soils treated with sewage sludge. Also, studies were conducted to determine the accumulative effect of these compounds in soils treated with different doses of sludge. © 1997 Elsevier Science B.V.

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1. Introduction

Lately, the possibility of using sewage sludge as an organic fertilizer has been considered [1,2], since, if performed correctly, this may contribute to the fertility of the soil. In addition, this could solve some of the problems related to the accumulation and disposal of sludge. Previous steps to the re-utilization of sludge as organic fertilizer have been the identification of different components such as heavy metals [3], nutrient, mineral and nitrogen content [4] and organic matter in order to establish its composition. It is also interesting to consider the evolution of these components in soils treated with sludge over a long period of time. Some studies have been conducted over the last ten years in order to understand the effects of sludge application to land as well as the residual effects on some crops [4–6].

One of the fractions that can yield important information is the lipid fraction because it contains many different families of compounds such as fatty acids, hydroxy acids, hydrocarbons, steroids, waxes, etc.

Hydroxy acids constitute a family of compounds widely distributed in nature. They have been detected in sewage sludge [7–9], sediments [10], bacteria [11] and manure [12]. Short chain β-hydroxy acids originating from hydrolysis of polyhydroxyalkanoate (PHA) have been detected in sewage sludge [7,9]. This polyester is a major component of some bacterial membranes.

The origin of long chain α- and β-hydroxy fatty acids in sediments has been associated by some authors with the oxidation of the corresponding α- and β-fatty acids [10]. This conclusion was based on the wide variation in the chainlength (C_{10}–C_{24}) and on the parallel distribution observed among chains of α- and β-hydroxy acids and fatty acids. The presence of both α- and β-hydroxy acids provides evidence of

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microbial oxidation in α- and β-positions as an important step toward degradation of fatty acids in biological [13] and geological systems [10].

The origin of α- and β-hydroxy acids as a first step in the microbial degradation of fatty acids is widely accepted, not only by Eglinton et al. [10], but also by other authors who studied different samples (Wallen and Rohwedder [7], studying sewage sludge).

Taking into account the origin of the sludge and the fact that the fatty acid concentration increases in highly contaminated areas, it is logical to expect β-hydroxy fatty acids in sewage sludge samples. It is not clear though, whether these compounds will accumulate in soils treated with sludge.

Hydroxy acids are part of the acidic fraction of the lipid extract as well as the fatty acids. To analyse them by GC, derivatisation is recommended. An important consideration is that β-hydroxy acids, because of their origin, are minor components of this fraction which makes their quantification extremely difficult.

The presence of a characteristic base peak in the mass spectra of β-hydroxy acid methyl esters at m/z 103, which does not appear in the mass spectra of the fatty acid methyl esters, permits quantification of these compounds using selected ion monitoring (SIM). This technique allows a selectivity superior to that obtained by monitoring the total number of ions (SCAN) and provides improved sensitivity for target compounds in complex samples [14]. The goal of the present investigation was the determination of β-hydroxy fatty acids in soils treated with sewage sludge by using SIM. Also, studies of the accumulation of these compounds in soils were conducted.

2. Experimental

2.1. Reagents

Reagent grade light petroleum, ethyl acetate, KOH, Na_2SO_4 (anhydrous) were obtained from Panreac (Barcelona, Spain). Methanol, hexane and isooctane were obtained from Fluka (Buchs, Switzerland). Internal standard, heptadecanoic acid (C 17:0) was supplied by Merck (Darmstadt, Germany). Diazomethane, used as methylating reagent, was synthesized using the procedure developed by Sempere [15].

2.2. Samples

Samples were kindly supplied by the Barcelona Agriculture School. The experimental design used a square latin model, so, the land was divided in 16 areas of 35.8 m^2 each, separated by corridors 1 m wide. Different kinds of amendments were applied: manure, mineral fertilizer and sewage sludges at two different doses, single and double, assuming that the N mineralization during the crop cycle corresponds, respectively, to 20% and 40% of the organic N in the sludge.

Soils were treated for four years with a previously established annual dose. In the present study, samples used for analysis were those from the original soil (without treatment), from sewage sludge and from soil treated with single or double dose of sludge after four years' treatment.

2.3. Extraction of the lipid fraction

Amounts of sample used in the present study were as follows: 20 g for untreated soils, 10 g for treated soils and 5 g for sewage sludge. The sample was extracted by Soxhlet using 200 ml of light petroleum–ethyl acetate (3:2, v/v). Extraction time was 12 h. The lipid fraction was then evaporated to dryness at reduced pressure.

2.4. Saponification

The lipid fraction was saponified under reflux by using 20 ml of methanolic KOH (10% w/v) for 5 h, at 70°C. After that treatment, 2 ml of water were added in order to favor the two phase separation. The saponified fraction (acid fraction) was acidified to pH 2 with 6 *M* HCl favoring the separation with hexane (3 times with 25 ml each).

2.5. Derivative formation

The acid fraction was dried with Na_2SO_4 (anhydrous) and evaporated to dryness at reduced pressure. After this treatment, 1 ml of a solution containing 160 ppm of heptanoic acid, used as internal standard,

was added. The extract obtained was evaporated to dryness with N_2 (99.9%). Esterification was accomplished by using 1 ml of diazomethane (synthesized in ether solution) for 30 min. The extract obtained was evaporated to dryness with N_2 (99.9%) and 1 ml of isooctane was added. The extract was, then, ready for gas chromatographic analysis.

2.6. Chromatographic analysis

A Hewlett–Packard chromatograph Model HP-5890 (Hewlett–Packard, Avondale, PA, USA) was used equipped with a flame ionization detector. The chromatographic conditions were: column 25 m×0.2 mm, ultra-1 coated with 0.33 μm OV-101 stationary phase. Helium was used as carrier gas at 28 cm s^{-1}. Injector temperature, 260°C (splitless 1 min); detector temperature, 300°C; oven temperature programmed from 130–300°C at 8°C min^{-1}; injection volume, 1 μl.

2.7. Analysis by GC–MS

A Hewlett–Packard chromatograph Model HP-5995 was used equipped with a mass spectrometer detector. The spectra were obtained at 70 eV ionization voltage in SCAN mode. The SIM mode was chosen and the ionization voltage was tuned at 70 eV; the ions selected were the following: *m/z* 103 (β-hydroxy fatty acid methyl esters), *m/z* 87 and *m/z* 74 (typical of the fatty acid methyl esters).

2.8. Quantitative analysis

The internal standard used when either the FID detector and the GC–MS in SCAN mode were employed was heptadecanoic acid at 160 ppm; 1 ml of standard solution was added prior to derivative formation. The chromatographic peaks were normalized to this internal standard.

In order to reduce the quantification errors when the areas obtained by SIM are used, the following procedure was applied: first of all, methyl β-hydroxytetradecanoate (β-OH 14:0) was identified in the chromatograms of the acid fraction obtained by using an FID detector and its quantification was referred to the internal standard methyl heptadecanoate. The result obtained by using this procedure was assigned to the area obtained by SIM and the rest of the peaks were quantified relative to this. The procedure involved the use of two internal standards, the methyl heptadecanoate and the methyl β-hydroxytetradecanoate.

3. Results and discussion

The use of diazomethane as methylating agent allowed satisfactory results to be obtained for all the components of the acid fraction. The methyl esters were, therefore, obtained by using a fast and simple procedure that allowed analysis by GC and identification by the combined technique GC–MS.

By using capillary GC–MS it is possible to detect and identify the most abundant β-hydroxy fatty acids (β-OH 12:0, 14:0, 15:0, 16:0 and 18:0) even though the quantification is difficult due to the overlapping with the fatty acid peaks that constitute the major components of this fraction (Fig. 1). The effort to find specific methods to quantity long chain β-hydroxy fatty acids, is related to the observation of characteristics and main differences between the two kinds of compounds found in the same fraction.

The most important difference is observed in their mass spectra. Comparing the mass spectra of the β-hydroxy fatty acid methyl esters and the fatty acid methyl esters it is possible to find the differences that can direct the study towards specific detection methods such as SIM. For example, β-hydroxy fatty acid methyl esters give a base peak at *m/z* 103 that corresponds to the following fragment:

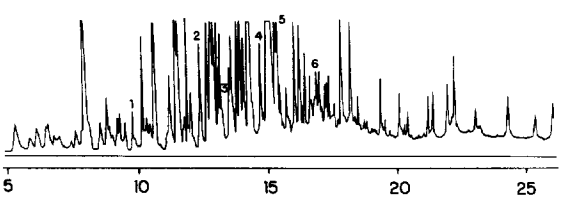

Fig. 1. Reconstructed total ion current chromatogram obtained by GC–MS of a sample of sewage sludge. Peak identification: 1=β-OH 12:0, 2=β-OH 14:0, 3=β-OH 15:0, 4=β-OH 16:0, 5=β-OH 17:0, 6=β-OH 18:0.

$[HO{=}CH{-}CH_2{-}COOCH_3]^+$

m/z 103

This fragment characterizes methyl esters of all β-hydroxy fatty acids when they are ionized by using electron impact. On the other hand, the mass spectra of fatty acid methyl ester show, as base peak, the ion at *m/z* 74 that corresponds to a McLafferty rearrangement as suggested by some authors [16]. This rearrangement is typical of all the esters with the structural element: $R{-}CH_2{-}CO{-}OCH_3$. So, as is evident, the fragment at *m/z* 74 will also appear in the mass spectra of β-hydroxy fatty acids methyl esters. In order to differentiate the fatty acids and β-hydroxy acids, a new ion, at *m/z* 87 was selected. This ion corresponds to the fragment [17]

$CH_2{=}CH{-}C({-}OCH_3){=}\overset{+}{O}H$

m/z 87

and is obtained as an important fragmentation in the mass spectra of the linear fatty acids methyl esters.

Taking into account that all long chain β-hydroxy fatty acid methyl esters display, as it has been mentioned above, a base peak at *m/z* 103, their analysis can be resolved by using the SIM technique. The following ions were chosen to perform the analysis, *m/z* 103, *m/z* 87 and *m/z* 74.

A sample of non-treated soil, a soil treated with a single dose of sewage sludge and sewage sludge were analyzed in triplicate. The resulting selected ion current profiles are shown respectively in Figs. 2–4.

The peaks corresponding to β-hydroxy fatty acid methyl esters were easily recognized. Sixteen compounds of this family were detected but it was not possible to identify all of them because the SIM method does not allow one to obtain the complete mass spectra of the compounds. By comparing retention times with those identified by using the SCAN mode (Fig. 1) it was possible to assign the peaks corresponding to the six most abundant β-hydroxy fatty acids. The other compounds were characterized by their chromatographic retention time.

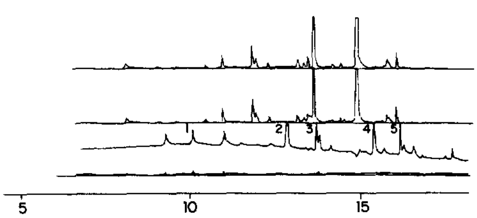

Fig. 2. Selected ion current profiles resulting from analysis of a non-treated soil, ions selected as follows: *m/z* 74 (top), *m/z* 87 (middle) and *m/z* 103 (bottom). Chromatographic conditions as described in Section 2.6.

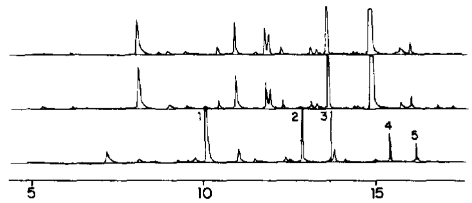

Fig. 3. Selected ion current profiles resulting from analysis of a soil treated with a single dose of sewage sludge, ions selected as follows: *m/z* 74 (top), *m/z* 87 (middle) and *m/z* 103 (bottom). Chromatographic conditions as described in Section 2.6.

Results shown in Table 1 were obtained using the SIM method and were quantified using the internal standard as described in Section 2.8.

The major β-hydroxy fatty acids detected in sewage sludge were β-hydroxyhexadecanoic acid (3.8 mg/100 g), β-hydroxytetradecanoic acid (3.7/100 g) and β-hydroxyheptadecanoic acid (3.4 mg/100 g). An accumulation of β-hydroxy acids in soils treated with sewage sludge was observed which was greater in soils treated with a double dose of sludge. As can be seen in Fig. 5, this accumulation is higher in the lower components of the series.

The detection of appreciable amounts of β-hy-

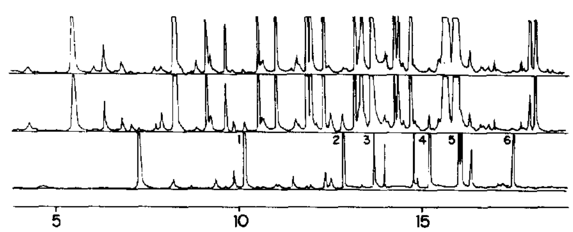

Fig. 4. Selected ion current profiles resulting from analysis of a sewage sludge, ions selected as follows: *m/z* (top), *m/z* 87 (middle) and *m/z* 103 (bottom). Chromatographic conditions as described in Section 2.6.

Table 1
Concentration (mg/100 g) of β-hydroxy fatty acids detected in sewage sludge, non-treated soil, soil treated with a single dose of sewage sludge and soil treated with a double dose of sewage sludge, quantified by using the SIM technique

Compound	Retention time (min)	Sewage sludge	Non-treated soil	Soil + single dose	Soil + double dose
Unknown	7.19	1.2	0.002	0.071	0.099
Unknown	9.24	0.16	0.007	0.013	0.012
β-OH 12:0	10.02	2.3	0.009	0.15	0.31
Unknown	10.91	0.064	0.006	0.022	0.042
Unknown	11.40	0.15	0.001	0.011	0.018
Unknown	12.26	0.16	0.002	0.010	0.015
β-OH 14:0	12.72	3.7	0.026	0.16	0.27
β-OH 15:0	13.68	0.96	0.005	0.024	0.036
Unknown	14.01	0.43	0.002	0.011	0.017
β-OH 16:0	15.24	3.9	0.016	0.091	0.13
Unknown	15.54	0.037	0.005	0.015	0.003
β-OH 17:0	16.01	3.4	0.009	0.074	0.12
Unknown	16.12	1.4	0.002	0.018	0.026
Unknown	16.42	0.36	0.002	0.008	0.013
β-OH 18:0	17.56	0.70	0.004	0.014	0.028

droxy acids in the original soil is related to the possible source of these compounds. Some authors described them as degradation products of fatty acids in biological systems [13]. The formation path would be through a β-oxidation of the fatty acids.

The presence of higher quantities of β-hydroxy fatty acids in soils treated with sludge could have two possible origins: by oxidation of the fatty acids added to the soil with the sewage sludge or by addition of the β-hydroxy fatty acids contained in the sludge. The first hypothesis is not very suitable given the fact that the treatment was applied for a relatively short period of time and microbial oxidation could not be so intense. In addition, if that were the main formation path, no big accumulation of fatty acids would be observed. The second proposal more readily explains the high accumulation of β-hydroxy fatty acids in soils fertilized with sludge. These type of residues showed a high concentration of fatty acids and, as it has been suggested previously, β-hydroxy acids can be formed through β-oxidation of the fatty acids. Therefore, appreciable amounts of β-hydroxy acids are expected to be found as is shown in Table 2.

The results obtained in the present research are comparable to those achieved by Eglinton et al. in

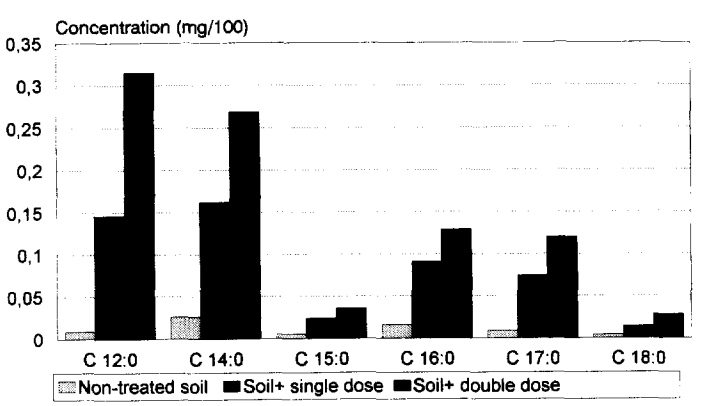

Fig. 5. Graphic representing the concentration (mg/100 g sample) of β-hydroxy fatty acids found in non-treated soils, and soils treated with both single and double dose of sewage sludge.

Table 2
Concentration (mg/100 g) of fatty acids and β-hydroxy fatty acids detected in sewage sludge

Fatty acids	Concentration	β-hydroxy fatty acids	Concentration
C 12:0	27.1	β-OH 12:0	2.3
C 14:0 iso	8.7	β-OH 14:0	3.7
C 14:0	47.6	β-OH 15:0	0.96
C 15:0	0.68	β-OH 16:0	3.9
C 15:0 ante	26.6	β-OH 17:0	3.4
C 16:0 iso	8.9	β-OH 18:0	0.69
C 16:1	43.3		
C 16:0	288.7		
C 18:2	18.4		
C 18:1	178.6		
C 18:0	112.4		
C 20:0	14.6		
C 21:0	15.1		
C 22:0	4.4		
C 23:0	0.25		
C 24:0	0.93		
C 25:0	1.3		
C 26:0	1.5		

1968 [10] in their study of accumulation on sediments, even though a parallel distribution in fatty acids and β-hydroxy acids was not observed. In this case, fatty acid that can be found is hexadecanoic acid (C 16:0) that has been described as typical of purifying plant disposal [18]. The fact that the distribution in fatty acids and β-hydroxy acids is not parallel can be better explained by the presence of β-hydroxy acids in the sewage sludge rather than by oxidation of the fatty acids of the sewage sludge.

4. Conclusions

Following the ion at m/z 103 (base peak of all β-hydroxy fatty acid methyl esters) using the SIM technique, 16 compounds of this family have been detected. All of them have been quantified although only six have been identified (from C_{12} through C_{18}) by using GC–MS (SCAN mode).

The major β-hydroxy acids in sludge are β-hydroxyhexadecanoic acid, β-hydroxytetradecanoic acid and β-hydroxyheptadecanoic acid. An accumulation of β-hydroxy fatty acids in samples treated with sludge was observed even though this accumulation was produced mainly at the lower components of the series.

The presence of β-hydroxy fatty acids in samples treated with sludge can be explained by addition of compounds of this family that are present in the sewage sludge.

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