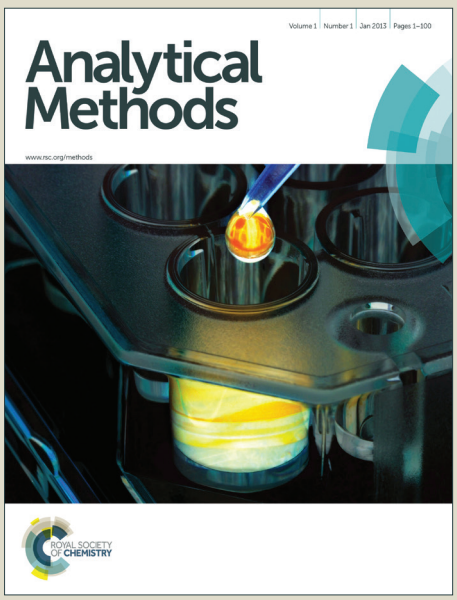
Analytical Methods

Accepted Manuscript



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The analysis of faecal sterols in sediment samples by HPLC-UV using ultrasound-assisted treatment

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ABSTRACT

A method was developed for the analysis of faecal sterols in sediment samples by ultrasound-assisted extraction and quantification by HPLC with a UV detector. A reversed-phase C18 column was used to develop the method and the optimal conditions employed elution under isocratic conditions using a methanol/THF/water (83:9:8) as the mobile phase. Direct detection of sterols by HPLC is difficult, so a derivatization procedure using benzoyl chloride was performed. A sample treatment using ultrasound-assisted extraction with 40 mL of methylene chloride-methanol (1:1) for 30 min without a clean up step was developed. The method recoveries obtained ranged from 65 to 89 % for the sample spike and from 67 to 101% for the blank spike. The detection limits ranged from 1.90 mg L^{-1} for β-sitosterol to 4.17 mg L^{-1} for epicoprostanol. Seven streams of Juiz de Fora city were analysed and the concentrations of coprostanol in the sediment samples varied between 0.01 and 14.48 μg g^{-1}. Except for the Farm Forest sample stream, all others were considered contaminated by faeces using the parameters and evaluation criteria for this type of contamination.

KEYWORDS: Faecal sterols, benzoyl chloride, HPLC, sewage

1. INTRODUCTION

Sewage discharge is the single largest source of pollution of aquatic environments in the world, since the main sources of pollution originate from human activities. The main way of detecting sewage pollution in aquatic environments is through the presence of microbiological indicators such as faecal coliforms. However the analysis of faecal coliforms has some limitations, including short life spans and little resistance to temperature variations; thus, it requires analysis immediately after collection and is often unprofitable. Therefore, a negative faecal coliform test does not always guarantee the absence of organisms harmful to the environment.[1-3] A more reliable way of analysing this type of pollution is by chemical indicators such as faecal sterols.[4,5]

Sterols have been widely used to indicate the relative abundance of sewage pollution in lacustrine and estuarine environments.[4,6,7] These compounds have a hydrophobic character and low solubility in water and, consequently, are associated with particulate material present in sediments. Thus, they can be incorporated into the sediment and preserved for a long time in the anaerobic environment, where it has negligible biodegradation.[6,8,9]

Among the main studied sterols, epicoprostanol, cholesterol, cholestanol, β-sitosterol and coprostanol are prominent. However, due to the specific faecal origin of coprostanol, this compound has been the main sterol investigated in sediment samples, being used as a reliable marker of faecal pollution. This is based on the occurrence of coprostanol in human faeces resulting from the biohydrogenation of cholesterol in the intestines of humans and other higher animals.[3,5] In Brazil, most reports in the literature on the evaluation of contamination by faecal sterols are related to estuarine ecosystems.[6,10,11]

Analytical methodologies for sterols in sediment samples are frequently time consuming and laborious because they involve multi-step procedures.[11] In general, sterols are extracted from sediments by liquid solvent extraction in a Soxhlet apparatus or by sonication, both followed by a

step of extract clean up. According to studies reported in the literature, several reports related faecal sterol extraction using a Soxhlet apparatus with dicloromethane as an extraction solvent for 48 hours, but this method is time-consuming and requires large volumes of organic solvents. To reduce the extraction time and solvent consumption, ultrasound-assisted extraction has been applied, since this is a technique that has low cost, requires small volumes of organic solvents and has simplicity of operation.[12] Isobe *et al* (2002)[8] and Biache and Philp (2013)[13] developed a method using an ultrasound bath with methanol and dichloromethane for 45 minutes of extraction. These studies presented recovery values between 82 and 106 %, respectively, indicating that the extraction method is also efficient and faster than a Soxhlet apparatus.

Although the most widely used technique for analysis of faecal sterols in sediment samples is gas chromatography (GC),[6,11,14-18] high performance liquid chromatography (HPLC) can be an alternative technique for this type of analysis. While the GC technique has a high sensitivity, derivatization of the sterols is necessary because of the difficulty of volatilizing these compounds due to their high molecular weight. However, in analysing sterols by HPLC with ultraviolet (UV) detection, a suitable derivatization is also necessary, since these compounds have low ultraviolet absorbance.[19] Besides the increase in the detection limit, derivatization allows the method of analysis to be made selective for the compound of interest, since compounds not reacting with the derivatizing agent will probably not be detected at a wavelength used to monitor the derivative.

Fitzpatrick and Siggia (1972)[20], proposed the use of the benzoylation reaction using p-nitrobenzoate to form an ultraviolet-absorbing derivative in order to improve the detection limit for analysing sterols by HPLC/UV in urinary extract. On the other hand, Piocos and Cruz (2000)[21] put forth the potential application of derivatization by acylation of copostranol with p-nitrobenzoyl chloride to analyse sterols in different samples. Other detection methods for analysing sterols coupled to liquid chromatography are also described in the literature. Hong *et al.* (2007)[22] determined the sterols cholesterol, sitosterol and coprostanol, in addition to bile acid and other lipids, by LC-MS in rat

faecal samples. Wang and Gardinali (2013)[23] have already developed on-line methods employing SPE-HPLC-MS/MS to analyse 72 microconstituents, including cropostanol, in reclaimed water. In the literature methods for the analysis of faecal sterols by HPLC with UV detection in sediment samples have not been described. Thus, the purpose of this work was the development of an extraction method and quantification of faecal sterols (coprostanol, cholesterol, epicoprostanol, cholestanol and β-sitosterol) in sediment samples using ultrasound - assisted treatment and HPLC with ultraviolet detection.

2. EXPERIMENTAL

2.1 Study area

Sediment samples were collected (July and October 2010) from seven tributary streams of the Paraibuna River, all of them located in the urban region of Juiz de Fora, MG, Brazil (Figure 1). The top 5 cm of undisturbed surface sediment was placed in pre-cleaned aluminium foils and then stored at -20 ºC until procedure analysis. All the sediment samples were dried in an oven at 55ºC and then 30 g dry weight were used for the analysis.

INSERT FIGURE 1

2.2 Chemicals and solutions

Sterol standards (purity 95–99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methylene chloride, n-hexane, methanol, tetrahydrofuran (THF) and isopropanol HPLC grade, benzoyl chloride (98%), silica gel (70–230 mesh), anhydrous sodium sulphate and alkaline detergent were purchased from VETEC (Rio de Janeiro, RJ, Brasil).

All glassware was cleaned in a solution of alkaline detergent, washed with deionized water and methanol, and then dried at 150ºC prior to use. The adsorbent silica gel was activated at 120ºC and deactivated with deionized water (5 % w/w). Anhydrous sodium sulphate was heated to 400 ºC.

Stock solutions of the sterols (coprostanol, cholesterol, cholestanol, epicoprostanol and β-sitosterol) were dissolved in methylene chloride at a concentration of 100.0 mg mL^{-1}. After the derivatization procedure, the sterol standards were diluted to concentrations ranging from 0.2 to 1.0 mg mL^{-1} in their benzoate form. These solutions were stored at -4ºC.

2.3. HPLC procedure

The instrumental analyses were performed on a Agilent 1100 Series HPLC with associated software (Agilent Chemstation LC Systems), a multiple wavelength detector (MWD-UV) and a manual injection valve with a 20 μL sample loop. A reversed-phase ZORBAX ODS C-18 column (4.6 mm x 150.0 mm, 5 μm particle size) and a ZORBAX ODS pre-column (4.6 mm x 12.5 mm, 5 μm particle size) were used at room temperature.

Chromatography separations of sterols were optimized using binary and ternary mixtures of solvents composed of the organic solvents methanol, tetrahydrofuran and water in different proportions. To choose the appropriate mobile phase for sterol separation, some chromatographic parameters were evaluated, including retention time, peak symmetry, resolution and separation factor.

2.4. Derivatization

According to Abidi (2004)[24], sterols can be derivatized with benzoyl chloride in pyridine at room temperature overnight. To reduce the reaction time, a new derivatization procedure is proposed in this paper using an ultrasonic bath (Unique, USC2850 model). In glass tubes, 200 μL of benzoyl chloride and 800 μL of pyridine were added to a mixture of sterol standards (epicoprostanol, cholesterol and cholestanol) of 0.20 mg mL^{-1}, for each compound. This derivatization procedure was performed for 30, 45, 60 and 75 minutes of sonication time. After that, a liquid-liquid extraction was performed utilizing n-hexane (3x2 mL) and 0.01 mol L^{-1} HCl (5 mL) under vigorous shaking. Then, after evaporation of the solvent, the residues were re-dissolved in 2 mL of isopropanol. To compare the new procedure's derivatization with those in the literature, the sterols were also derivatized following the method of Abidi (2004)[24] using the same amounts of reagents and the standard mixture. Both studies were performed in duplicate.

2.5. Optimization of the extraction procedure

To optimize the extraction procedure two different sample treatments (n=2) were performed using the São Pedro Reservoir sediment sample (10.0 g) spiked with a mixture of the sterol standards comprising epicoprostanol, cholesterol and cholestanol at a final concentration of 0.80 mg mL^{-1}, for each compound.

In the treatment 1, the extraction (n = 3) was carried out at room temperature with 40 mL of methylene chloride-methanol (1:1, v/v) for 30 min using an ultrasonic bath. Then, the combined extract was reduced by rotary evaporation and cleaned up via silica gel (2.0 g) column chromatography (30 x 0.5 cm I.D.) using 12 mL of methylene chloride-methanol (1:1 v/v) as eluent.

In the treatment 2, after the reducing the volume of solvent by rotary evaporation, the extract was only filtered though a fiberglass prefilter (1 μm porosity) and PTFE filter (0.45 μm).

Prior to HPLC analysis, the extracts obtained were dried by rotary evaporation under a stream of argon, and afterwards derivatized with the benzoyl chloride reagent.

3. RESULTS AND DISCUSSION

3.1. Derivatization procedure for sterols

To select the best conditions of derivatization, the areas under the peaks obtained in the HPLC analysis of sterol derivatives at room temperature overnight and in the ultrasonic bath were evaluated and are shown in Table 1. Comparing the peak area values for the compounds, the derivatization reaction with ultrasound can be performed for a shorter time without reducing its efficiency. Among the peak areas obtained for different sonication times, derivatization for 45 min gave the best result.

INSERT TABLE 1

3.2. Development of the HPLC method

Initially, this study attempted to use binary mixtures composed of water and methanol or water and THF as the solvent phase. None of the binary mobile phase conditions tested showed satisfactory results. The analysis time increased significantly, with values greater than 45 minutes, and the separation was inefficient, with co-eluting compounds. Therefore the compounds were analysed employing ternary mobile phase mixtures composed of methanol, THF and water.

To separate sterol benzoates it was necessary to maintain a high proportion of methanol. When the proportion of THF was increased the run time decreased, but the separation was not efficient. By increasing the proportion of H_2O, the separation improved, but the running time increased significantly. So in order to optimize the mobile phase it was necessary to find proportions of THF, H_2O and methanol that produced good separation of analytes in a shorter analysis time.

In order to determine satisfactory conditions for the mobile phase, the composition of the solvents was varied according to Table 2. Based on results obtained in previous tests, twelve proportions were tested initially with the ternary mobile phase.

INSERT TABLE 2

According to the values of analysis time, peak symmetry, resolution and separation factor, the best results were obtained with mobile phase condition number 12 at a flow of 1.7 mL min^{-1}. These conditions presented efficient separation of the compounds and a good time analysis, as shown in Figure 2. Values for peak resolution of the sterols under these conditions varied from 1.31 to 1.93, which are considered suitable for separation and quantification of analytes. The values of symmetry thereof varied between 0.960 and 1.040 and were also within the acceptable range, while the separation factor ranged from 1.106 to 1.154. The analysis time was 26 minutes and the wavelength of maximum absorption of derivatized sterols was 230 nm.

INSERT FIGURE 2

3.3. Sample preparation

The recoveries of sterols obtained using treatments 1 and 2 are shown in Table 3. The recovery percentages for the treatment 2 ranged from 73 to 90 %, and for treatment 1 between 74 and 89 %. When comparing the results obtained for spiked samples by t-test for the two treatments, it was concluded that there were no significant differences in relation to the percentage recovery of analytes between the two methods, at a 95% level of confidence for all compounds.

INSERT TABLE 3

Therefore, we chose treatment 2, without a clean up step, which proved to be faster but with the same efficiency as treatment 1 and no difference was observed when the chromatographics profiles for both treatments were compared (Figure 3). In this case, there was a decrease in the number of steps in transferring the samples, thus minimizing the possibility of inserting undesirable foreign compounds or even losing portions of samples.

INSERT FIGURE 3

3.4. Features of the analytical method

Linearity was evaluated, taking into account the correlation coefficient (r) using calibrate curves (n=3) of different sterol standards concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1}. The proportionality of peak area and concentration was confirmed for all the analytes (correlation coefficient > 0.994).

The precision of the method was evaluated by extracting and analysing a sediment sample (n=5), and the standard deviation was determined as better than 1.5 % (Table 4). The limit of detection (LOD) was defined as three times the standard deviation of the average signal of five sediment sample injections with sterol at a final concentration of 0.3 mg mL^{-1}, for each and the limit of quantification (LOQ) was ten times the standard deviation. [25]

INSERT TABLE 4

The accuracy of the method was evaluated through recovery using sediment samples spiked with a mixture of sterol standards comprising cholesterol, coprostanol, epicoprostanol, cholestanol and β-sitosterol at the fortification level of 0.3 mg mL^{-1}. Blank spikes were prepared using a synthetic sodium sulphate matrix with the same mixture of sterol standards used in the sample spikes at a final concentration of 0.3 mg mL^{-1}, for each compound. Table 5 shows the results for the accuracy of the method.

INSERT TABLE 5

The percentage recovery was acceptable for the analysis of trace-level compounds, for the sample spikes were the range of 50 % to 150 %, and the values for the percent relative standard deviation (% RSD) between repetitions should be less than 30 %.[26] Thus, according to Table 6, the recoveries of the sterols obtained and the corresponding RSD were satisfactory using this method, with average recovery ranging from 65 to 89 % for the sample spike and from 67 to 101 % for the blank spike.

3.5. Analysis of sterols in sediments

The optimized method was applied to real sediment samples and the concentrations (μg g^{-1} dry weight) of the sterols are reported in Table 6. Concentrations of faecal sterols ranged from 0.005 to 14.48 μg g^{-1}, with the highest concentration was found at site 5. Only at site 6 were the concentrations of cholestanol and epicoprostanol below the LOQ. The site 7 was used as a control sample where no faecal sterols were found. This site is located in a reservoir of JF city's water supply. The concentrations of sterols reported in this paper are consistent with published data for lacustrine sediments in different places as shown in Table 7.

INSERT TABLE 6 AND TABLE 7

Nichols *et al.* (1996) [31] suggested that values of coprostanol greater than 0.5 μg g^{-1} indicate sewage contamination. A concentration of coprostanol below that value was only found at site 6. In spite of coprostanol being widely used as an indicator of sewage contamination, some authors have questioned the evaluation of faecal contamination using just the concentration of coprostanol as an indicator.[31,32] Therefore, coprostanol can better be used in conjunction with others to indicate faecal contamination.[25,26,31-33]

Cholesterol is the major sterol found in aquatic sediments and can be associated with zooplankton, phytoplankton and aquatic fauna.[25] Therefore, values of the coprostanol/cholesterol ratio greater than 1 can indicate if an area was affected by sewage.[25] This indicator suggests that all the sites contained sewage residues except for site 6. A lower value of the sitosterol/cholesterol ratio was also found at site 6, indicating the source of β-sitosterol in this area is likely to be terrestrial organic matter.[26] The site 6 is located at a farm in a rural area of the town. This place is a preserved environment with the intact riparian zone and free of anthropic activity.

Epicoprostanol is an isomer of coprostanol formed principally during the process of sewage treatment. This sterol in conjunction with coprostanol has been used to indicate the level of sewage treatment that a determined area has received. High values of the coprostanol/epicoprostanol ratio indicate untreated sewage and lower values indicate treated sewage.[26,33] This ratio indicated that sites 1 – 5 received untreated sewage. Such results are in agreement with the fact of less of 20 % of the whole sewage of the city is treated.[34]

Cholestanol has been found in sediments due to biosynthesis by plankton. High cholestanol concentrations compared with coprostanol concentrations suggest sediments without sewage discharge.[5] Using the coprostanol/(coprostanol+cholestanol) ratio, values ($\geq$ 0.70) indicated again that only site 6 was not affected by sewage contamination. As previously mentioned, this site is located in a region away from the city, so more preserved than the other sites samples.

The remaining sites were characterized as contaminated by domestic sewages. These sites are located in an urban area and majority receive direct input of sewage from the houses around. As they are not pre-treatmented for decontamination, these places showed visual characteristics of polluted environments: trash, sediment darkened and bad smelling. All these characteristics are found, mainly at site 5, where also presented both higher coprostanol concentration and rates of faecal contamination.

4. CONCLUSION

This paper demonstrated the potential use of HPLC for the analysis of faecal sterols in sediment samples. A new derivatization procedure using benzoyl chloride was proposed that was faster than methods described in the literature. Sample treatment in this study was performed using an ultrasonic bath without a clean up step and was shown to be fast and efficient. The sterol recoveries from spiked samples and blank spikes varied between 65 to 89 % and 67 to 101%, respectively, with RSD < 30 %, values considered acceptable for environmental samples. The sediment samples were quantified using an external standard and indicators of faecal contamination were used to evaluate the seven streams of Juiz de Fora city. The majority of the streams are contaminated with domestic sewage however some sites are less contaminated than others. The values of the coprostanol/cholesterol and sitosterol/cholesterol ratios indicate that only site 6 was not contaminated with sewage. According to values of the epicoprostanol/coprostanol ratio, it can be concluded that the sewage inputted in the Paraibuna river by this tributary streams is untreated or the treatment is inefficient.

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ACKNOWLEDGEMENTS

The authors would like to thank the FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior) and PROPESQ/UFJF (Pró-Reitoria de Pesquisa da Universidade Federal de Juiz de Fora) for financial support. The Laboratório de Protozoologia da Universidade Federal de Juiz de Fora for their support in collecting samples.

FIGURE CAPTION

Figure 1: Location map of the sampling sites (•) of sediment samples in Juiz de Fora city. Streams: 1. Campo Grande; 2. Humaitá; 3. Carlos Chagas; 4. Tapera; 5. Matirumbide; 6. Floresta; 7. Represa de São Pedro.

Figure 2: Chromatogram obtained for the standard mixture solution of sterols (100 mg L^{-1}). Peaks: (1) coprostanol, (2) epicoprostanol, (3) cholesterol, (4) cholestanol and (5) β-sitosterol. Analytical conditions: column, ZORBAX ODS; flow-rate, 1.7 mL min^{-1}; detection, 230 nm; mobile phase, methanol/THF/H_2O (83:9:8).

Figure 3: Chromatogram obtained for a sample using (A) treatment 1and (B) treatment 2. Peaks: (1) epicoprostanol, (2) cholesterol and (3) cholestanol. Analytical conditions: column, ZORBAX ODS; flow-rate, 1.7 mL min^{-1}; detection, 230 nm; mobile phase, methanol/THF/H_2O (83:9:8).

TABLE CAPTIONS

Table 1: Average values for peak areas of derivatized sterols under the reaction conditions used with ultrasound treatment and at room temperature.

Derivatization time	Epicoprostanol		Cholesterol		Cholestanol	
	Average area (mAU s)	RSD (%)	Average area (mAU s)	RSD (%)	Average area (mAU s)	RSD (%)
30 min at US	1961.5	0.1	2043.6	0.6	2485.2	1.2
45 min at US	2025.9	2.2	2029.6	3.9	2559.1	3.3
60 min at US	1956.1	1.5	1950.9	2.1	2479.8	1.7
75 min at US	1889.3	3.3	1861.9	1.6	2390.9	2.4
12 hours at room	1990.9	0.1	1451.1	15.9	2388.2	5.4

RSD = Relative standard deviation
US = ultrasound bath

Table 2: Compositions of ternary mobile phase tested.

Condition	Methanol (%)	THF (%)	H_2O (%)	Flow (mL min^{-1})
1	90	5	5	1.0
2	85	10	5	1.0
3	75	20	5	1.0
4	83	10	7	1.5
5	81	12	7	1.5
6	79	12	9	1.5
7	77	14	9	1.5
8	75	14	11	1.5
9	81	10	9	1.5
10	81	10	9	2.0
11	83	9	8	1.5
12	83	9	8	1.7

Table 3: Recovery and relative standard deviations achieved for samples spiked with a mixture of sterol standards (0.8 mg mL^{-1}) using sample treatments 1 and 2.

Replicate	Recovery of sterols (%)					
	Sample treatment 1			Sample treatment 2		
	Epi	Chor	Chol	Epi	Chor	Chol
1	68.8	80.4	87.7	70.9	79.7	85.7
2	76.9	75.0	92.9	76.9	73.6	92.9
Average	72.9	77.7	90.3	73.9	76.6	89.3
Sd	5.7	3.8	3.73	4.3	4.3	5.1
RSD	7.8	4.9	4.1	5.8	5.6	5.7

Epi= epicoprostanol; chor= cholesterol; chol= cholestanol; Sd= standard deviation; RSD= relative standard deviation

Table 4: Limit of detection (LOD), limit of quantification (LOQ) and repeatability.

Compound	LOD (mg L^{-1})	LOQ (mg L^{-1})	RSD (%)
Coprostanol	2.18	7.28	0.73
Epicoprostanol	4.17	13.90	1.39
Cholesterol	3.35	10.82	1.08
Cholestanol	3.14	10.48	1.05
β-sitosterol	1.90	6.32	0.63

RSD = relative standard deviation

Table 5: Mean recovery and relative standard deviation achieved for a sample and a blank spiked with a mixture of sterol standards.

Compound	Blank spike		Sample spike	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Coprostanol	70.4	9.0	64.7	2.1
Epicoprostanol	80.7	5.9	69.1	5.1
Colesterol	87.9	4.7	66.6	3.7
Colestanol	100.6	5.6	89.1	8.1
β-sitosterol	66.9	1.5	74.1	14.0

RSD = relative standard deviation

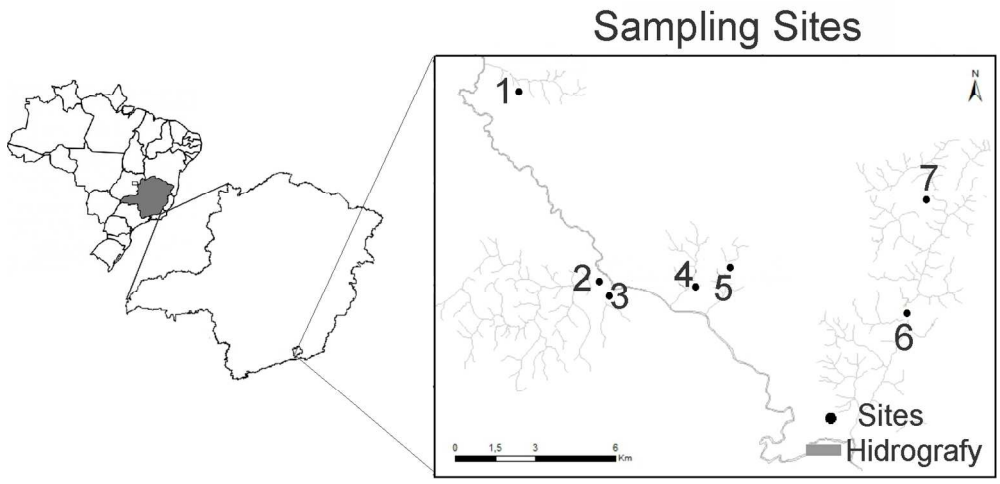
Table 6: Content (μg g^{-1} dry weight) of sterols in sediment samples of streams of Juiz de Fora city.

Sample	Content μg g^{-1} dry weight				
	Coprostanol	Epicoprostanol	Cholesterol	Cholestanol	Sitosterol
Site 1	2.08	0.19	1.01	0.11	0.23
Site 2	0.62	0.12	0.45	0.27	0.17
Site 3	3.46	0.31	2.07	0.17	0.17
Site 4	3.41	0.31	1.89	0.08	0.44
Site 5	14.48	1.40	4.11	0.67	0.77
Site 6	$5.0x10^{-3}$	<LOQ	0.07	<LOQ	0.45
Site 7	nd	nd	nd	nd	nd

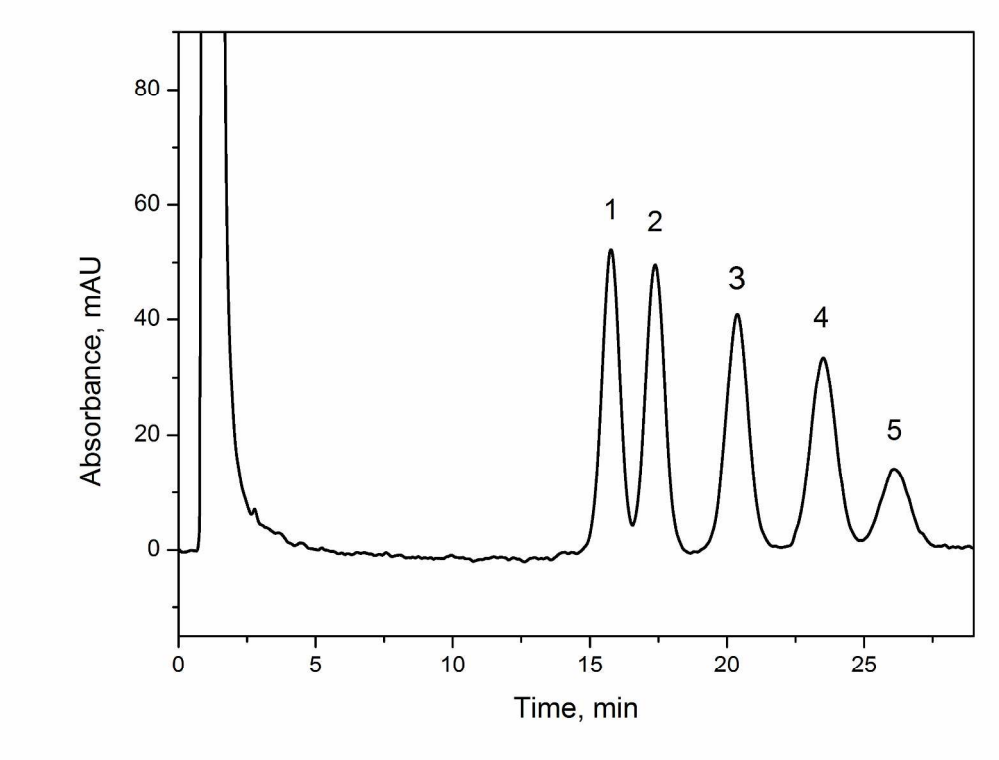
<LOQ= below of quantification limit; nd= not detected

Table 7: Comparison of the coprostanol concentration in samples of lacustrine sediments from different areas of study.

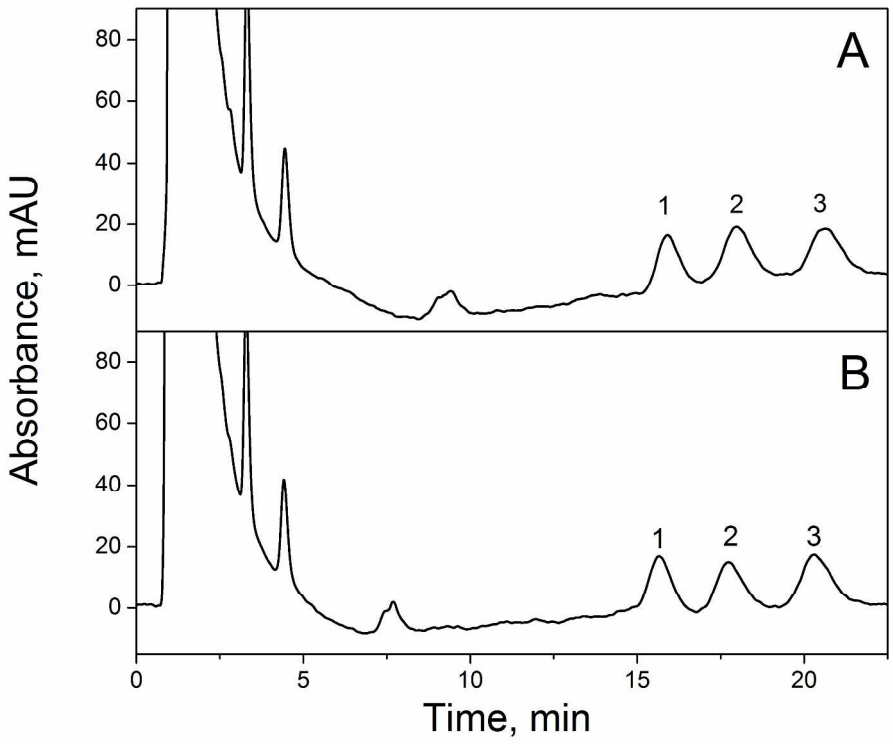
Study area	Coprostanol concentration (μg g^{-1} dry weight)	Reference
Streams of Juiz de Fora city	0.0050 to 14.48	This paper (2014)
Mississipi river. USA	0.05 to 1.60	Barber and Writer (1998)[27]
Han river. North Korea	0.00034 to 3.8	Li *et al*. (2007)[28]
Patos Lake. Brazil	0.00014 to 0.0918	Martins *et al*. (2007)[29]
Capibaribe river. Brazil	0.52 to 7.32	Fernandes *et al*.(1999)[30]
Formosa river. Portugal	1.10 to 41.80	Mudge and Bebianno (1997)[4]
Rivers of Malaysia	0.037 to 41.80	Isobe *et al*. (2002)[8]



Location map of the sampling sites (•) of sediment samples in Juiz de Fora city. Streams: 1. Campo Grande; 2. Humaitá; 3. Carlos Chagas; 4. Tapera; 5. Matirumbide; 6. Floresta; 7. Represa de São Pedro.
451x222mm (96 x 96 DPI)



Chromatogram obtained for the standard mixture solution of sterols (100 mg L-1). Peaks: (1) coprostanol, (2) epicoprostanol, (3) cholesterol, (4) cholestanol and (5) β-sitosterol. Analytical conditions: column, ZORBAX ODS; flow-rate, 1.7 mL min-1; detection, 230 nm; mobile phase, methanol/THF/H2O (83:9:8).
272x208mm (300 x 300 DPI)



Chromatogram obtained for a sample using (A) treatment 1and (B) treatment 2. Peaks: (1) epicoprostanol, (2) cholesterol and (3) cholestanol. Analytical conditions: column, ZORBAX ODS; flow-rate, 1.7 mL min-1; detection, 230 nm; mobile phase, methanol/THF/H2O (83:9:8).
330x304mm (300 x 300 DPI)