J. Braz. Chem. Soc., Vol. 13, No. 2, 226-232, 2002.
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0103 - 5053 $6.00+0.00

Article

An Efficient Ethanol-Based Analytical Protocol to Quantify Faecal Steroids in Marine Sediments

Silvia K. Kawakami and Rosalinda C. Montone*

Instituto Oceanográfico, Universidade de São Paulo, Praça do Oceanográfico, 191, 05508-900, São Paulo - SP, Brazil

A maioria dos métodos para análise de esteróides em sedimentos marinhos é laboriosa, consome grandes volumes de solventes orgânicos (muitas vezes clorados) e produz grandes quantidades de material de descarte. Neste trabalho desenvolveu-se um método de extração com etanol e um "clean up" rápido, que oferece uma alternativa de menor custo e minimiza o uso de solventes clorados. As porcentagens de recuperação de esteróides (coprostanol, epicoprostanol, cholesterol, cholestanol, 5α-coprostanona and 5β-coprostanona) ficaram na faixa de 70 a 93%, comparáveis às análises mais tradicionais. A eficiência do método foi avaliada através de material de referência e os resultados obtidos para coprostanol, colesterol e colestanol encontram-se próximos aos valores médios certificados e perfeitamente dentro dos valores aceitáveis. Os desvios-padrão relativos para triplicatas foram baixos (6-12%). Os testes indicaram que o "clean-up" simplificado (sem fracionamento) não produz interferência na quantificação dos esteróides fecais.

The majority of the analytical methods for steroids in marine sediments are laborious and time consuming and involve the use of large volumes of organic (frequently chlorinated) solvents. A method has been developed utilising ethanol extraction with a rapid alumina-ethanol elution clean up, providing an environmentally friendly and inexpensive alternative. Recoveries of steroids (coprostanol, epicoprostanol, cholesterol, cholestanol, 5α-coprostanone and 5β-coprostanone) ranged from 70 to 93% and compared favourably with more traditional analyses. Analytical performance was tested through analysis of reference material and concentrations recorded for coprostanol, cholesterol and cholestanol were close to the mean certified values and were within acceptable values. Relative standard deviations from triplicate analyses were small (6-12%). These initial tests also indicate that the simplified clean up (without fractionation) is not subject to interference in the quantification of the selected faecal steroids.

Keywords*:* analysis of steroids, coprostanol, cholesterol, sewage contamination, marine sediment

Introduction

Steroids are widely distributed in the marine environment as a result of numerous biological syntheses, diagenetic and degradation transformations, and anthropogenic inputs of organic matter. The marine biogeochemistry of steroids has been extensively studied since the mid-1970s.[1] The behaviour of anthropogenic organic matter in marine environment plays an important role in our understanding of the effects of marine pollution. A group of steroids, derived from cholesterol in the digestive tract of higher animals, has often been used as biomarkers of sewage contamination in marine waters and sediments.[2-5]

Despite all the investigations of steroids in the marine environment, the current analytical methodologies for steroids in marine sediments are laborious and time-consuming, involve multi-step procedures and the use of large volumes of solvents, which consequently produces large quantities of wastes. Evaluation and optimisation of methods are essential for both economical and analytical results. Environmental benefits resulting from reduced amounts of hazardous wastes should also be considered. In general, the analytical methods comprise the following procedures: 1) extraction of the steroids from the matrix; 2) fractionation (also called clean up) of the extract by adsorption column chromatography; 3) derivatisation of the steroids into their trimethylsilyl ethers; 4) determination of the steroid by gas chromatography coupled to flame ionization detector, and 5) confirmation of the compounds with mass spectrometry.

* e-mail: rmontone@usp.br

All these steps have to be adapted to meet the needs of specific applications. One must choose the most suitable procedures depending on the type and number of samples, type of analysis (only steroids or steroids and a series of other compounds, such as saturated and polycyclic aromatic hydrocarbons, chlorinated pesticides, poly-chlorinated biphenyls, fatty acids), and equipment available. In this work, a method for analysis of steroids commonly used in investigations of sewage contamination (coprostanol, epicoprostanol, cholesterol, cholestanol, 5α-coprostanone and 5β-coprostanone) has been developed. The method based on ethanol extraction has been compared to the others tested in terms of recoveries of standards and solvent facilities (*i.e.* toxicity, storage, costs). A rapid clean up has also been tested. The analytical performance of the method was assessed through analysis of reference material.

Experimental

Reagents and glassware

All glassware were soaked in solution of alkaline detergent, washed with distilled water and ethanol, dried in oven, and rinsed with organic solvents prior to use. The adsorbents alumina, Al_2O_3, (70-230 mesh) and silica gel, SiO_2, (70-230 mesh) were heated overnight to 400 °C for activation and, after cooling in dissicator, 5% deactivated with pre-extracted water on a w/w basis as described in Aceves et al.[6] Sodium sulphate was also heated to 400 °C and stored in dissicator. All solvents were pesticide grade except for ethanol, analytical grade, which was distilled. Authentic standards of steroids (Sigma) were dissolved in methylene chloride and diluted in concentrations ranging from 0.25 to 20.0 mg L^{-1}.

A synthetic matrix was prepared for the extraction tests with sodium sulphate (Na_2SO_4) spiked with a mixture of the steroid standards comprising coprostanol (5β-cholestan-5β-ol), epicoprostanol (5β-cholestan-3α-ol), cholesterol (cholest-5-en-3β-ol), 5β-coprostanone (5β-cholestan-3-one), 5α-coprostanone (5α-cholestan-3-one), cholestanol (5α-cholestan-3β-ol), and 5α-cholestane in the concentration range of 10 mg L^{-1}.

Instrumental analysis

The instrumental analysis was performed with a Hewlett-Packard 5890 series II gas chromatograph equipped with flame ionization detector and a splitless injector. The capillary column was of 25 m x 0.32 mm I.D. coated with Ultra-2 (5% phenyl-methyl silicone). The programmed temperature was 40 to 240 °C (1 min) at 20 °C min^{-1}, 240 °C to 260 °C (1 min) at 1 °C min^{-1}, and 260 °C to 310 °C at 50 °C min^{-1}. The injector and detector temperatures were 300 and 325 °C respectively. Hydrogen was used as the carrier gas (2.0 mL s^{-1}) and the injection volume was 1.0 μL. Calibration of the peak area to concentration was done with the steroid standards in the derivatized form within the range of 0.25 to 20.0 mg L^{-1}, linear response for coprostanol was 0.992 (Figure 1). Standards of 5α-cholestane and 5α-androstanol (steroids frequently used as internal standards) and standards of petroleum and polycyclic aromatic hydrocarbons were also injected in the established chromatographic conditions. Data were acquired and processed on an HP Chemstation data system.

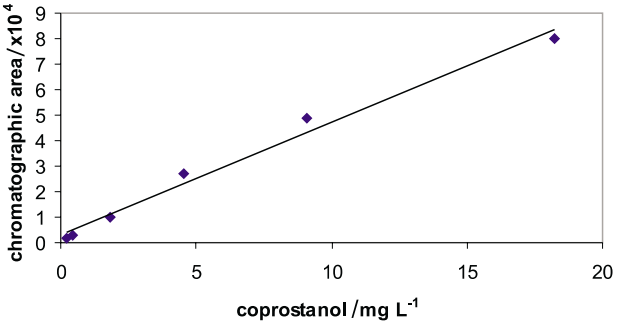

Figure 1. Calibration curve for coprostanol

Detection limits (DL), defined as three times the standard deviations of the lowest steroid concentrations detected[7] were in the range of 1 ng g^{-1} for the steroids.

Extraction of steroids

To check recoveries of steroids, extractions with different solvents were performed in triplicates using the synthetic matrix previously described (25.0 g). In test 1, the extraction was carried out with 80 mL of methanol (MERCK) reflux for 5 h (adapted from LeBlanc et al.[8]). Both tests 2 and 3 were performed for 6 h in a *Soxhlet* apparatus. Extraction of test 2 was done with 80 mL of a mixture of 50% acetone (J.BAKER) in methylene chloride (MERCK) and test 3 was extracted with 80 mL of distilled ethanol (Cia Nacional de Álcool). Test 4 (adapted from Mudge & Bebianno[9]) was done with 90 mL of 0.5 mol L^{-1} KOH in ethanol under reflux for 3 h (saponification and extraction simultaneously). Extracts of test 4 were treated *via* aqueous/organic liquid-liquid partitioning (1 mL of n-hexane/10 mL of pre-extracted water) in a separation funnel. The organic phase was reduced by rotary

evaporation with the same following procedures of the previous tests. The resulting extracts of all tests were taken to the derivatisation procedure, described further on, and then analysed by gas chromatography.

Fractionation procedures

Two clean up procedures were assessed in glass columns of 38 x 0.7 cm I.D. The first test consisted of the glass column packed with 1.0 g of alumina and 2.0 g of silica gel (both 5% deactivated), in the top and bottom of the column respectively. It was introduced 1.0 mL of the steroid standard solution (10 mg L^{-1}). The fractionation was done with the following increasing polarity gradient: 15.0 mL of n-hexane; 15.0 mL of 20% methylene chloride in n-hexane; and 15.0 mL of 50% acetone in methylene chloride, and provided three fractions with the last one comprising the steroids. The second clean up test was carried out with 2.0 g of alumina and elution with 15.0 mL of distilled ethanol, providing one fraction.

Derivatisation of steroids

The fractions obtained from the extraction and cleanup tests, after reduced by vacuum evaporation and transferred to 1.0 mL vials, were taken to dryness under nitrogen blow and submitted to derivatisation with the commercial mixture N,O,-bis(trimethylsilyl)trifluoracetamide and trimethylchlorosilane, BSTFA/TMCS 99:1 (Supelco), 40 μL, 65 ^{o}C/1.5 h (adapted from Green et al.[10]). After reaction, the final residues were redissolved to 1.0 mL with methylene chloride/n-hexane (1:1) and stored in glass ampoules until gas chromatographic analysis.

Analysis of reference material

Reference material (lyophilised marine sediment) provided by the International Atomic Energy Agency (IAEA-383) was also analysed for steroids. The solvent of choice for the extraction was ethanol and the clean up procedure consisted of alumina column/ethanol elution. A summary of the analytical protocol is shown in Figure 2.

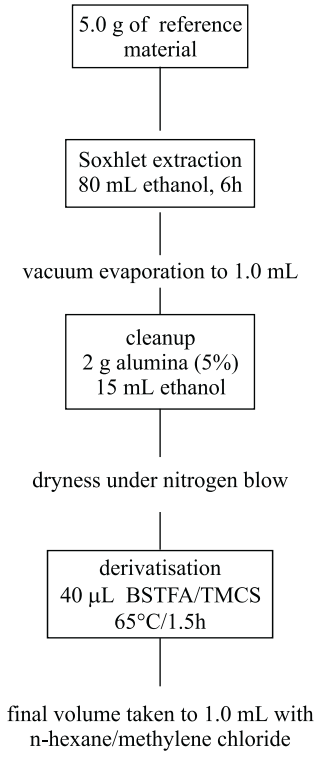

Figure 2. Summary of the analytical protocol to quantify steroids in marine sediments

Results and Discussion

Recovery of steroids

Due to the low concentrations of total and individual steroids in the water column and sediments, these compounds are typically analysed by gas chromatography after forming their trimethylsilyl ether derivatives. In order to perform gas chromatographic analysis, steroids should be transformed in more volatile compounds. In the case of sterols, hydrogen bonds must be avoided using a derivatisation reaction, which also improves chromatographic resolution. Sterol data are presented in the derivatised form throughout this paper.

In the extraction tests, compounds were quantified by comparison with external standards. As adsorbents, solvents and glassware are potential sources of organic contamination, blanks were performed for each series of 10 extractions and interfering peaks were subtracted from all the sample chromatographic runs. The average recoveries of the steroids with the corresponding standard deviations (SD) were acceptable for all the tests, except for coprostanones in test 4 (Table 1). Considering measurements in terms of μg g^{-1}, the variations in recovery percentages are very wide, ranging from 50 to 120%. Examples of such variations were found by LeBlanc *et al.*[8] who obtained coprostanol recoveries varying between 62-96% processing standard mixture and spiked samples with methanol extractions. Eganhouse *et al.*[11] determined 30 to 65% recoveries with spiked blanks through methylene chloride extractions. Tests 1 and 2 using methanol and 50% acetone in methylene chloride respectively, presented steroid recovery ranges from 64 to 90% and 66 to 101%. In test 3, ethanol also showed elevated recoveries, from 70 to 93%,

Table 1. Average recovery percentages and standard deviations for the steroids from the extraction tests performed in triplicates

Steroid	Trivial name	Test 1[(1)] 80 mL methanol, 5h	Test 2 80 mL 50% acetone in methylene chloride, 6h	Test 3 80 mL ethanol, 6h	Test 4[(2)] Reflux 90 mL KOH/ethanol 0.5 mol L^{-1}, 3h
5α-cholestane	Cholestane	84±5	83±4	89±5	80±5
5β-cholestan-3β-ol	Coprostanol	81±4	85±1	93±4	75±20
5β-cholestan-3α-ol	Epicoprostanol	90±2	85±4	81±8	81±12
5β-cholestan-3β-one	5β-coprostanone	77±2	66±5	70±2	13±10
5β-cholestan-3α-one	5α-coprostanone	76±1	65±9	75±1	< DL
cholest-5-en-3β-ol	Cholesterol	64±4	78±6	71±1	72±22
5α-cholestan-3β-ol	Cholestanol	89±2	101±20	83±8	79±14

DL = detection limit, < 1 ng g^{-1}
[1] Adapted from LeBlanc et al., 1992, [2] Adapted from Mudge & Bebianno, 1997

which was expected since ethanol polarity index is between methanol and methylene chloride. Test 4 provided sterol recoveries from 72 to 81%. This test involves saponification and extraction simultaneously which increases the yield of sterols in 30% due to the release of esterified sterols in sediments[8]. However, saponification is not suitable when concerning coprostanone determinations because of reduction reactions leading to low stanone recoveries. Low recoveries in the tests can also be related to loss of compounds during the transference of the extracts, especially for test 4 with the highest SD. Test 4 involves a multi-step process which is laborious to be adopted in analysis with large number of samples.

There is not a preferable solvent and time for the extraction of steroids in the literature so that the analyst must choose the most convenient and efficient procedures. Regarding the steroid recovery results, the methods 1, 2 and 3 presented good results. The most suitable solvent for our purposes showed to be ethanol because it is easier to be acquired and stored in our laboratory conditions and is less toxic and volatile than methanol or methylene chloride. Other advantage is the lower costs of ethanol, *ca*. three times cheaper than the other solvents. In addition, the ethanol distillation is part of our laboratory routine in order to use this solvent for cleaning glassware for trace analyses of PCBs, organochlorine pesticides, aliphatic and polycyclic aromatic hydrocarbons.

Internal standards for faecal steroid analyses

Steroids are more accurate quantified based upon internal standards, which must have the same analyte physical-chemical properties, good resolution within the elution time of the steroids under investigation and cannot be present in the sample. Two common internal standards for steroid analysis are 5α-cholestane and 5α-androstanol. In our tests, the elution of 5α-androstanol (in its derivatised form) occurred at the beginning of the run coinciding with impurities of the ethanol blank, as shown in Figure 3. Although the absence of hydroxyl group in 5α-cholestane, not characterising this compound as an alcohol, it is used as internal standard in several studies[12-17] and presented

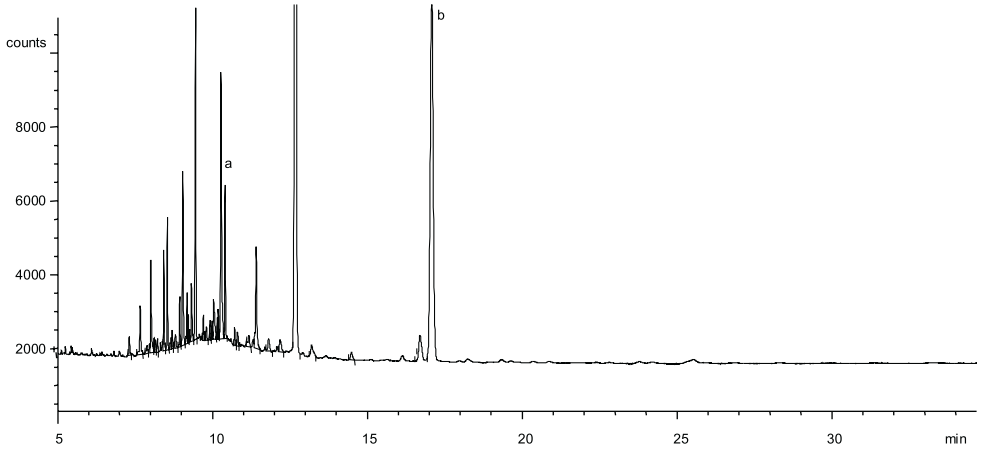

Figure 3. Chromatogram of the ethanol blank and internal standards for steroids analyses: (a) interfering peak for 5α-androstanol; (b) 5α-cholestane

recoveries very close to the steroids as observed in the extraction tests. 5α-cholestane was chosen as internal standard for the tests with reference material because of its good resolution and suitable retention time. This compound was added at the beginning of the subsequent experiments. The most suitable internal standards would be deuterated steroids with combined GC-MS instrumentation, however they were not available during the tests.

Clean up of extracts containing steroids

A number of compounds other than steroids are co-extracted under the extraction conditions and a previous removal of co-extracted compounds may be necessary. The adsorption column chromatography with alumina removes interfering compounds while silica gel can both handle and separate compounds. Some steroid analyses also include determinations of a series of organic pollutants which require long and careful fractionation procedures.[2,13,14,18] At the established gas chromatographic conditions, pollutants such as aliphatic and polycyclic aromatic hydrocarbons do not elute in the retention time of interest (from 15 to 35 min). It implies that these compounds do not interfere in the steroid chromatographic profile. The fractionation with silica/alumina column showed the first two fractions, eluted respectively with n-hexane and 20% methylene chloride in n-hexane, did not contain steroids, except for 5α-cholestane in the first fraction (F1), while the third fraction (F3) comprised all the steroids of the spiked matrix (Figure 4). This silica/

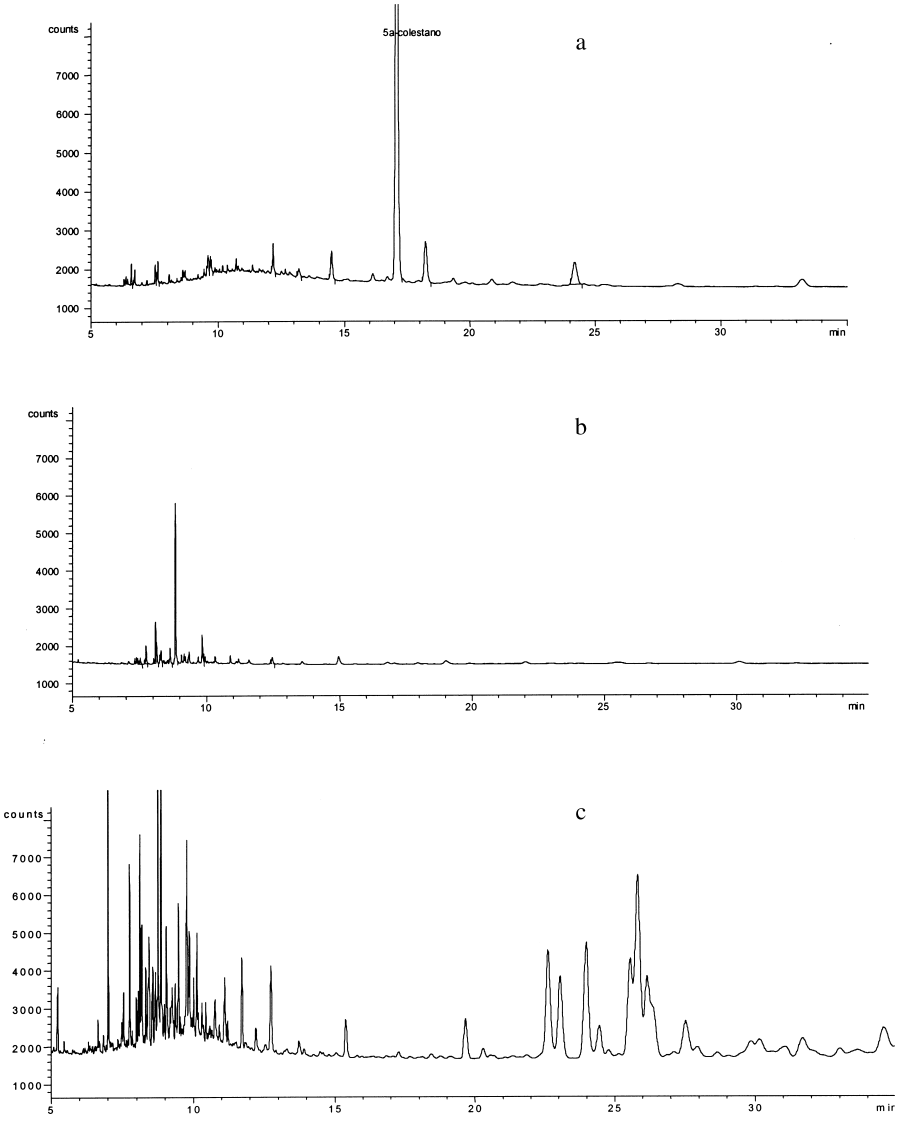

Figure 4. Chromatograms of the cleanup fractions of a synthetic matrix: a) F1 - eluted with n-hexane, 5α-cholestane is eluted in this fraction; b) F2 - eluted with a mixture of 20% of methylene chloride in n-hexane; c) F3 - eluted with a mixture of 50% of acetone in methylene chloride, fraction that contains steroids

alumina clean up appear to be useful in fractionating steroids from other compounds, however another internal standard is needed for this clean up since 5α-cholestane is eluted apart from the other steroids. The clean up with alumina column and ethanol elution provided suitable results, mainly because of the reduced time for the analysis. This clean up can retain fine sediment particles and precipitates eventually formed during extraction as observed for reference material. As this study was concerning only steroid analysis it was preferred to perform the last test consisting on a simplified clean up – alumina column and ethanol elution – in the following experiments.

Analytical performance of the method based on ethanol

Synthetic matrices are useful and practical to provide preliminary results to evaluate methods in terms of recoveries although they are not a perfect approach for marine sediments due to the matrix effects. One has to consider that organic compounds added to the sample may be more easily extractable than the ones incorporated into samples through normal biological processes. The performance of the method relies upon the efficiency with which the solvent systems breakdown the environmental matrices to release the steroids. To validate the method using ethanol extraction and alumina/ethanol clean up, the analytical performance was tested through analysis of reference material. Figure 5 shows the chromatogram of the reference material. The steroid concentrations (based on the internal standard 5α-cholestane) presented satisfactory results. Coprostanol, cholesterol and cholestanol concentrations were close to the mean certified values and well within acceptable values (Table 2). Analytical errors were between 18 to 34% and relative standard deviations ranged from 6 to 12%, values comparable to similar studies.[8,19-21]

Optimisation and evaluation of analytical methods are essential to improve both analytical and economical results and should minimise hazardous wastes (as chlorinated chemicals) as well. This is especially important for studies involving environmental monitoring, which require analyses of large number of samples resulting in large volumes of wastes. This work demonstrates that extraction of faecal steroids using ethanol followed by rapid clean up with alumina-ethanol elution can be applied successfully in routine laboratory analyses. It showed to be a rapid, quantitative and reproducible analytical method for steroids, offering an environmentally friendly and inexpensive alternative in comparison to methods based on methanol or methylenechloride extractions.

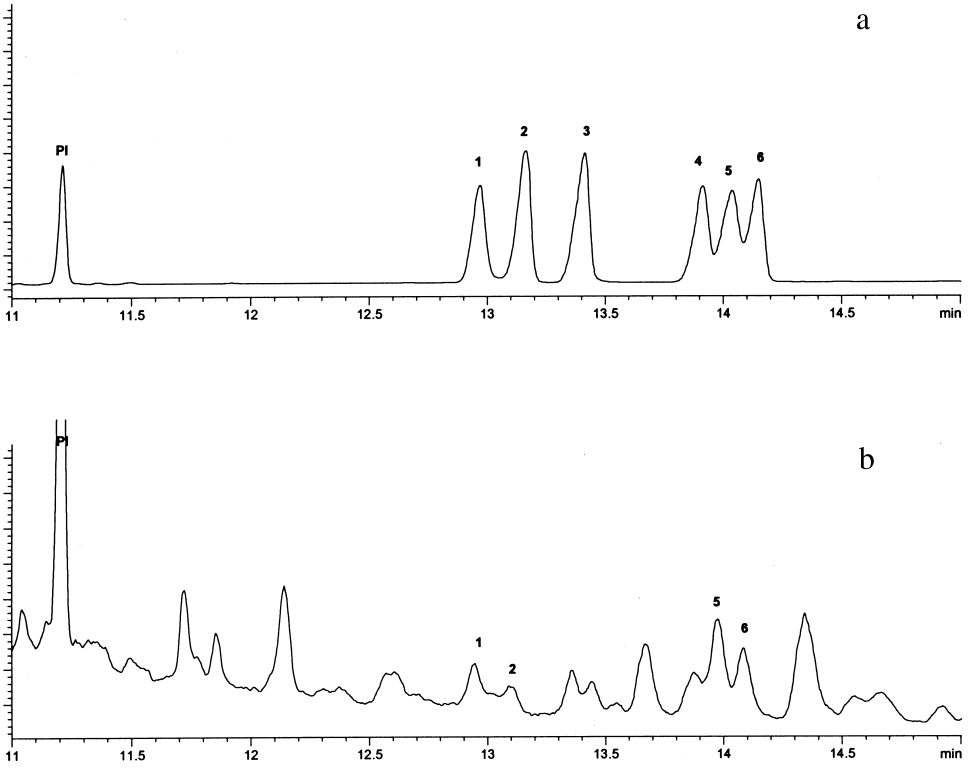

Figure 5. Chromatograms of steroids standards (a) and reference material IAEA-383 (b). PI = 5α-cholestane, 1 = coprostanol, 2 = epicoprostanol, 3 = 5β-coprostanone, 4 = 5α-coprostanone, 5 = cholesterol, 6 = cholestanol

Table 2. Steroid concentrations in the reference material IAEA 383 ($\mu g\ g^{-1}$ of dry sediment weight) and statistical parameters

Replicate	Coprostanol	Cholesterol	Cholestanol
1	0.63	1.17	0.84
2	0.68	1.02	0.84
3	0.80	1.09	0.94
Mean value	0.70	1.09	0.87
Mean deviation	0.06	0.05	0.04
Standard deviation	0.09	0.08	0.06
Relative standard deviation (%)	12.4	6.9	6.6
Total error (%)	34.0	18.4	18.3
Acceptable values			
Min. value	0.34	1.05	0.58
Max. value	0.84	1.25	1.10
Standard deviation	0.25	0.10	0.26

Acknowledgements

The authors would like to thank the financial support provided by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, proc. 97/09690-5. The authors are grateful for the helpful comments of the two anonymous reviewers.

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Received: March 13, 2001
Published on the web: February 25, 2002

FAPESP helped in meeting the publication costs of this article.