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Enzymatic flow injection analysis of coprostanol in non-aqueous media

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Abstract

An alternative method has been developed for the spectrophotometric determination of coprostanol in sediments, based on its extraction with chloroform-methanol (2+1 v/v) and enzymatic analysis in pH 7.0 buffer saturated toluene, using a bienzymic reactor prepared with 1 mg cholesterol oxidase (23 IU) and 1 mg horseradish peroxidase (220 PU) both noncovalently immobilised on controlled pore glass beads. The method allow us to determine coprostanol in a dynamic range from 2.2×10^{-4} M to 9×10^{-4} M, measuring the absorbance at 458 nm of the oxidised form of *p*-anisidine. The relationship between absorbance (A) and coprostanol concentration (C) expressed in $mol.L^{-1}$ was $A = (0.0059 \pm 0.0005) + (17 \pm 1)(C)$ with a $r^2 = 0.998$ and it was obtained a limit of detection of 7×10^{-6} M in the best experimental conditions. Recovery studies carried out on water and beach sediments, at concentration levels from 0.1 to 0.25 $mg.g^{1}$, provided values from 96 to 112%.

Key words: Coprostanol determination; enzymatic analysis in non-aqueous media; FIA; sediments.

Análisis enzimático por inyección en flujo de coprostanol en medio no acuoso

Resumen

Se ha desarrollado un método alternativo para la determinación espectrofotométrica de coprostanol en sedimentos, que se basa en la extracción del analito con cloroformo-metanol (2+1 v/v) y su análisis enzimático en tolueno saturado con tampón fosfato a pH 7,0, para tal fin se utilizó un reactor bienzimático preparado con 1 mg de colesterol oxidasa (23 UI) y 1 mg de peroxidasa (220 UP) ambas inmovilizadas no covalentemente sobre esferas de vidrio poroso de tamaño de poro controlado. El método permitió la determinación de coprostanol en un intervalo dinámico de $2,2\times10^{-4}$ M a 9×10^{-4} M, midiendo la absorbancia a 458 nm de la forma oxidada de la p-anisidina. La relación entre la absorbancia (A) y la concentración de coprostanol (C) expresada en $mol.L^{-1}$ fue: $A = (0,0059 \pm 0,0005) + (17 \pm 1)(C)$ con una $r^2 = 0,998$, se obtuvo un límite detección de 7×10^{-6} M en las mejores condiciones experimentales Estudios de recuperación realizados en sedimentos de playa y en agua, empleando niveles de concentración de coprostanol de 0,1 a 0,25 $mg.g^{-1}$, proporcionaron valores de recuperación de 96 a 112%.

Palabras clave: Análisis enzimático en medio no acuoso; determinación de coprostanol; FIA; sedimentos.

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Introduction

Sewage contamination of marine water, freshwater, and potable water supplies has been determined traditionally by enumeration of faecal coliform bacteria or streptococci, and by the determination of some inorganic compounds, such as ammonia nitrogen. However the reliability of these proceedings as adequate indicators of sewage contamination has been questioned, because of its great variability (1-3).

Advances in environmental sciences, point out (bio)chemical molecules as alternative analytes for identification and quantification of organic inputs in aquatic systems. Those biochemical markers are mainly lipid molecules, such as sterols; which are related to specific sources. Unlike bacteria colony count, sterol determination seems to be relatively invariable in water environments under determinate anoxic conditions, even at high temperatures or in the presence of toxic substances (such as chlorine) during considerable periods of time, even on order of decades (3-7). This fact has been employed to study faecal material in sediments or soils with archaeological interest, associated to ancient human presence (8).

Sterols, such as coprostanol, have shown to be very sensitive indicators of faecal pollution. Coprostanol (5-β-cholestan-3β-ol) is formed by the enzymatic hydrogenation of cholesterol by intestinal microflora in mammalians including man (5, 9). This faecal specific-origin sterol has been extensively studied as a chemical indicator in many investigations of pollution in sedimentary environments (10-12). Many techniques have been successfully used to trace sewage derived organic matter in a wide range of environments, but any apply coprostanol as a water quality indicator, due mainly of the lack of epidemiological evidence relating the coprostanol concentration and health risks (13).

As the demand of specific analysis which indicate an accurate estimation of the potential environmental impact of sewage outfall on waters, methods should be simple, inexpensive, and efficient. The overall analytical procedures reported in literature are complex, laborious, costly and time-consuming (5, 14-18).

In the present paper we propose a simple and fast strategy for the determination of coprostanol as a model for water faecal pollution indicator, using a noncovalently coimmobilized bienzymic reactor of cholesterol oxidase and peroxidase installed in a flow-injection (FI) device and working in non-aqueous media which is more compatible with the organic extracts than water. The methodology developed is based on that developed previously by us for other sterols (19, 20) and uses the measurement of the oxidised form of p-anisidine at 458 nm as the analytical parameter to be controlled.

Experimental

Apparatus and reagents

Spectrophotometric measurements were made on a Hewlett Packard (Waldbronn, Germany) model 8452 A diode-array UV/visible spectrophotometer, equipped with a 1 cm path length and 30 µL internal volume quartz flow cell.

Two FI systems, similar than those previously designed for the enzymatic peroxide determination (21), were employed through this study. One device consists of a single line manifold with a Gilson (Villiers-le-Bel, France) Minipuls 3 peristaltic pump, equipped with an organic solvent-resistant Viton tube (1.5 mm i.d.), which was employed for the evaluation of the experimental parameters and a forked system that includes two alternative ways which were employed to determine alternately the coprostanol and the corresponding to the natural colour of sample by using a bienzymic reactor and a glass beads container

without enzymes, which provides the same dispersion than the enzymic one but without any reaction.

A Rheodyne (Cotati, CA) Model 50 Teflon rotary valve was employed for standard and sample injection, and 5 mm of internal diameter and 22 mm length tubular teflon-made reactors, with 100 mm nylon filters at each end (22), were used to pack the glass beads on which enzymes were adsorbed. The carrier was for all the experiences phosphate buffer (pH 7.0) saturated toluene containing 10^{-3} M *p*-anisidine.

A Büchi Rotary evaporator R124 (Genève, Switzerland) was used to evaporate the chloroform-methanol solutions. A recirculatory water bath Grant (Cambridge, UK.) was used for the temperature effect experiments using also an HP 890554A thermostatysed cell holder from Hewlett Packard (Waldbronn, Germany).

Streptomyces spiralis cholesterol oxidase (COD) (E.C.1.1.3.6) (23 international units per mg), and horseradish peroxidase (HRP) type II (E.C.1.11.1.7) [220 purpurogallin units per mg, as well as controlled pore glass beads (CPG) (120-220 mesh, 1984 Å mean pore size) were purchased from Sigma (St. Louis, MO).

To obtain enzymatic preparations of HRP and COD noncovalently immobilised on CPG, absorption of the biocatalysts onto the glass beads was carried out basically as previously reported for a batch system by Kazandjian *et.al.* (23) and for a FI system by Braco *et al.* (24) and Piñeiro-Avila *et al.* (19, 25): 1.0 mg HRP and 1.0 mg COD were dissolved in 100 µL of 0.1 M aqueous phosphate buffer (pH 7.0) and this solution was added to 210 mg of CPG, without any pretreatment, spread on a watch glass. The preparation was left to dry for 15 minutes under a gentle cool air drift, and then packed in a tubular PTFE reactor. When it is not in use, the tubular reactor with the noncovalently immobilised enzymes was stored in a refrigerator at 4°C.

Coprostanol (98%), cholesterol (99%), and *p*-anisidine (grade I) were purchased from Sigma (St. Louis, MO), cholestanol (95%) was obtained from Fluka (Neu Ulm, Zwitzerland). Spectroscopic grade toluene was obtained from Scharlau (Barcelona, Spain). All other reagents were of analytical grade.

Sample collection

Samples were collected from 5-10 cm depth sediment zone using a methacrylate tube and filled into plastic containers, then the samples were immediately frozen and kept under these conditions until analysis. Sample 1 was a beach sand collected 5 m from the shore in Port-Saplaya Beach (Valencia, Spain). Sediment 2 was collected from an effluent sewage outfall near the same beach.

Coprostanol extraction from sediments

1 g dry sediment was extracted with three 10 mL portions of a chloroform-methanol (2:1) (v/v) mixture in a separating funnel. After phase separation the organic layer was filtered through a Wathman N° 4 filter-paper. The combined extracts were washed three times with 30 mL distilled water. Then the volume of the organic phase was reduced by rotary evaporation and the concentrated extract treated with anhydrous sodium sulphate to remove any water. After that the extract was reconstituted with 10 mL of buffer-saturated toluene solution with 10^{-3} M *p*-anisidine.

Enzymatic determination of coprostanol

500 µL of coprostanol solutions, prepared from extracts in 0.1 M pH 7.0 phosphate buffer-saturated toluene with 10^{-3} M *p*-anisidine, were injected into the above described FI manifold, using a carrier flow rate of 3 $mL.min^{-1}$.

Coprostanol was first oxidised by molecular oxygen in a COD-catalysed reaction, releasing stoichiometrically an equivalent

amount of hydrogen peroxide, which was in turn converted to water by HRP. *p*-anisidine yield finally a coloured oxidised product which was monitored in a flow-cell by UV-visible spectrophotometry at 458 nm. The spectra resulting from the oxidised form of *p*-anisidine, in buffer-saturated toluene, were directly related to the coprostanol concentration in standards, ranging from 2.25×10^{-4} M to 1.8×10^{-3} M and results obtained for samples interpolated in this calibration equation.

Results and Discussion

Spectrophotometric determination of coprostanol in nonaqueous media

Coprostanol is recognised by the cholesterol oxidase in a comparable way than that observed for cholesterol and cholestanol as reported in previous studies (19, 20). Spectra obtained after injection of cholesterol, cholestanol and coprostanol standards in a concentration of 4.5×10^{-4} M by reaction in the bienzymic reactor, are indicated in Figure 1. From this figure it is clear that the enzymatic reaction for coprostanol provides a lower yield than that obtained for cholesterol and for cholestanol. However the enzyme COD recognises the two stereoisomers of cholesterol 5 α (cholestanol) and 5 β (coprostanol) in a comparable way than its characteristic substrate but with a low sensitivity.

Effect of FIA parameters

A series of experiences were performed in order to improve the sensitivity of the enzymatic determination of coprostanol by evaluating the effect of flow injection variables such as the injected sample volume and the carrier flow rate.

Figure 2, shows the effect on the absorbance peak height at 458 nm of increasing injected volumes of a coprostanol solution 4.5×10^{-4} M, from 200 to 700 µL, at different carrier flows from 1 to 5 $mL.min^{-1}$.

In general the peak height of signals decreases on increasing the injected volume, thus indicating the uncomplete reaction of coprostanol and some inhibitory ef-

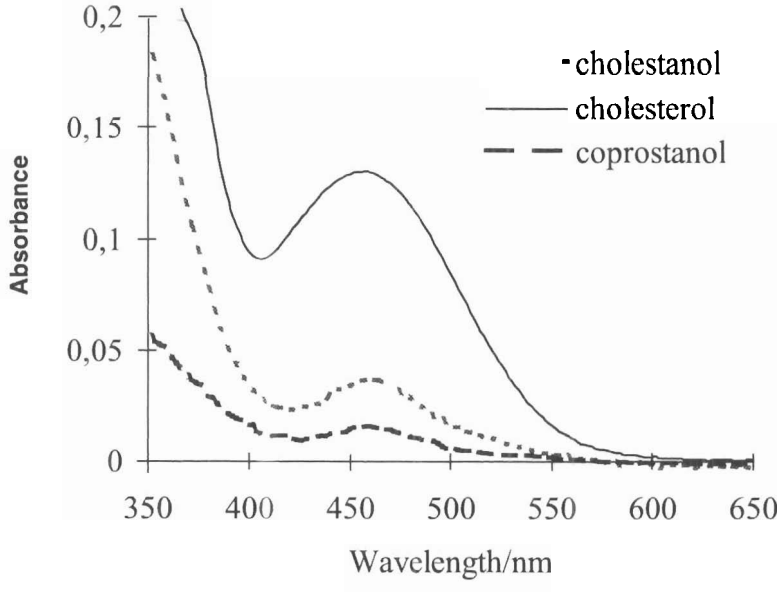

Figure 1. *p*-anisidine absorbance spectra, obtained after enzymatic reaction of 4.5×10^{-4} M cholestanol (- - -), cholesterol (-----), and coprostanol (-- -- --), standards, dissolved in 10^{-3} M *p*-anisidine in pH 7.0 phosphate buffer-saturated toluene.

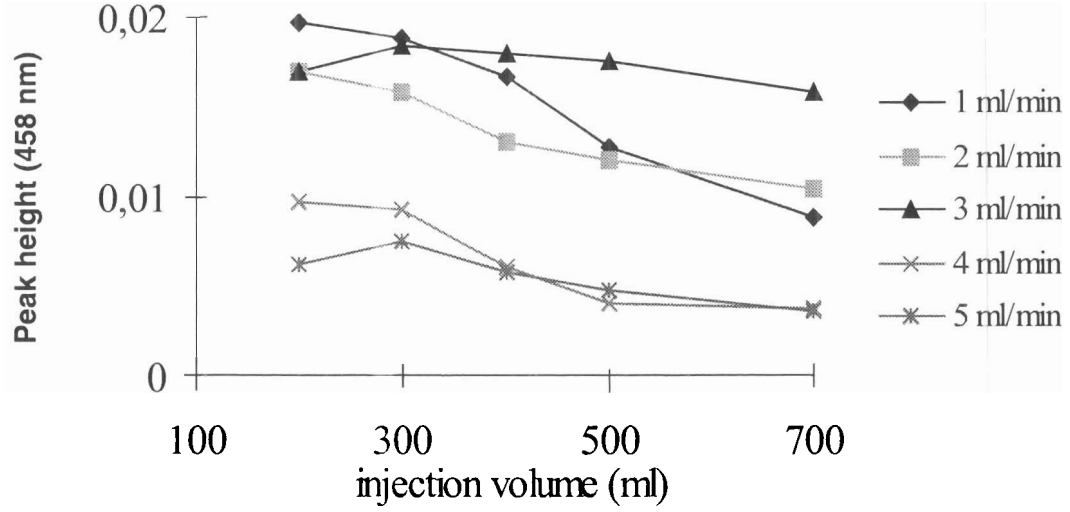

Figure 2. Effect of sample injection volume on peak height absorbance measurements at 458 nm, for a coprostanol concentration of 4.5×10^{-4} M, established at different carrier flow-rates: (◆) 1, (■) 2, (▲) 3, (×) 4, and (✳) 5 mL.min^{-1}.

fect of high amounts of this sterol on the activity of the system COD + HRP. The same effect was obtained on increasing the flow rate which reduces the reaction time and thus the yield of the enzymatic reaction. However, a different but repetitive behaviour was observed at 3 mL.min^{-1} consisting in a stable peak height value obtained as a function of the injected volume. That could evidence a compromise between the use of high coprostanol amounts and a reduced reaction time which could balance the inhibitory effect of a coprostanol excess on the enzyme reactivity. So, a carrier flow of 3 mL.min^{-1} seems the most convenient for coprostanol determination.

On the other hand, the increasing of the injected volume and the decreasing of the flow-rate cause an increase of the peak width, thus providing a reduction of the sampling frequency. Because of that compromise values of 3 mL.min^{-1} and 500 µL were selected in order to obtain a sampling frequency of 60 h^{-1} with an appropriate sensitivity.

Effect of temperature and pH

A study of the effect of temperature on the noncovalently immobilised COD-HRP reactor performance on coprostanol determination was carried in the range from 10 to 45°C. Figure 3-A, depicts, the variation of the peak height of the FIA recordings obtained at 458 nm as a function of temperature and it can be seen that the best values were obtained between 30 and 40°C with a maximum signal obtained at 37°C. For temperatures higher than 40°C the analytical sensitivity decreases.

pH is known to be a critical parameter on enzyme activity and stability in aqueous media however, as it can be seen in Figure 3-B, corresponding to peak height absorbance values obtained for a 4.5×10^{-4} M solution of coprostanol in 10^{-3} M p-anisidine solution in toluene saturated with different phosphate buffers from pH 5 to 8, there is only a small influence of pH on the enzymatic activity. It was obtained a slight increase of the signal around pH 7, being the best results obtained at pH 7.5, which is the

recommended value to obtain a suitable COD activity (26). These results are in a good agreement with those reported in the literature (27, 28), which indicate that enzyme activity was essentially unaltered in toluene, because proteins are kinetically trapped in the organic solvent in a high-rigidity conformation.

In order to made as simple as possible methodology, a pH 7 and room temperature conditions were chosen to do the determination of coprostanol in water and sediments after liquid-liquid extraction.

Analytical figures of merit

The injection into the bienzymic reactor of a series of standard solutions of coprostanol ranging from 2.2×10^{-5} to 9×10^{-4} M in pH 7 buffer-saturated toluene solution with 10^{-3} M *p*-anisidine, provided a satisfactory linearity between absorbance (A) at 458 nm and coprostanol concentration (C), expressed in mol.L^{-1} corresponding to an equation A = (0,0059 ± 0,0005) + (17 ± 1)(C) with a r^2 = 0.998 being the standard deviation of the calibration $Sy/x = 5\times10^{-4}$. In the experimental conditions assayed, the limit of detection corresponds to a coprostanol concentration of 7×10^{-6} M, which is good enough for the determination of this sterol in sediments but, the value is approximately seven times higher than that found for cholesterol, and about three times higher than that reported for cholestanol (19, 24).

It is noticeable that the response was linear up to 9×10^{-4} M coprostanol. However, when coprostanol standards were prepared at concentrations higher than 1.8×10^{-4} M, it was obtained a saturation of the absorbance signals which evidences, once again, a negative effect of high amounts of coprostanol on the COD activity. Additional efforts carried out in order to improve the determination of coprostanol by increasing the number of COD units from 23 to 46, thus immobilising 2 mg of COD, do not improved the sensitivity and reduced the dynamic range, indicating

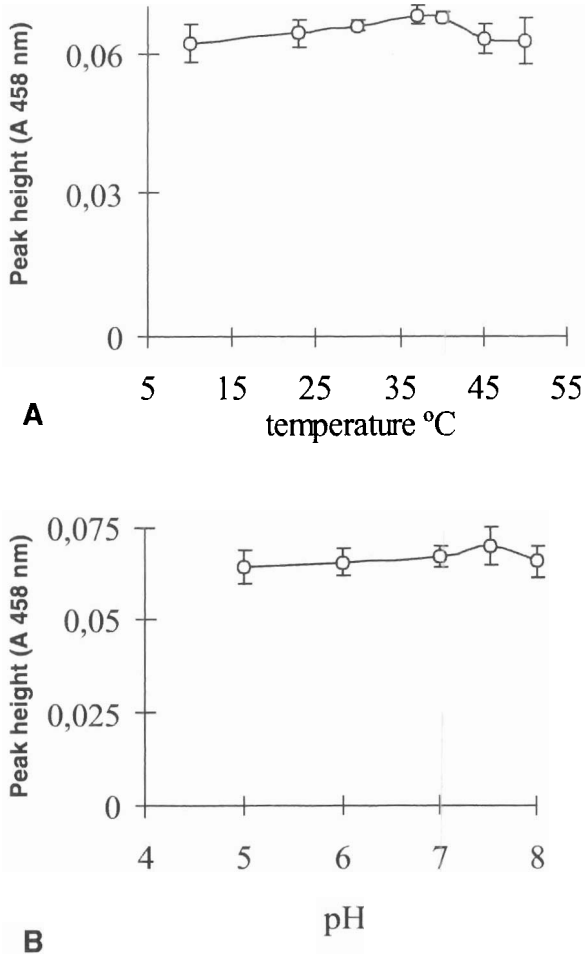

Figure 3. Effect of temperature and pH on enzymatic determination of coprostanol in non-aqueous media. (**A**) Temperature effect on the peak height of FIA recordings obtained from absorbance at 458 nm for a coprostanol concentration of 4.5×10^{-4} M. (**B**) Effect of the pH of the aqueous solution saturating toluene on peak height of the FIA recordings obtained for coprostanol. Conditions: Injection volume 500 µL, flow-rate 3 mL.min^{-1}. Bars indicate the ± *s* interval of the mean value of three independent determinations.

Table 1
Recovery studies on spectrophotometric enzymic determination of coprostanol in water and natural sediment samples spiked with known concentrations of this compound

Samples	Added/mg.mL^{-1}	Found/mg.mL^{-1}	Recovery (%)
Water	0.11	0.11 ± 0.1	100
	0.15	0.14 ± 0.08	96
Sediment 1	0.16	0.18 ± 0.02	112
	0.25	0.28 ± 0.01	112
Sediment 2	0.1	0.11 ± 0.02	110
	0.22	0.24 ± 0.04	109

Each result is the mean ± the corresponding standard deviation of three independent determinations.

the difficulties to obtain better figures of merit than those reported.

Analysis of spiked sediment samples

In order to evaluate the accuracy of the enzymatic procedure developed for coprostanol determination, a series of recovery experiments were performed by spiking water and natural sediment samples with known concentrations of coprostanol, carrying out the coprostanol extraction and enzymatic determination using the procedure indicated in the experimental part.

Table 1 summarises results found for the recovery of coprostanol added to beach sediment samples, and to 10 mL of water. As can be seen the recovery varied from 93-112%, and the mean recovery was 106% which guarantees the quantitative extraction of free coprostanol from natural matrices and its quantitative determination by the developed procedure.

Conclusions

It has been evidenced through this study that the developed procedure of enzymatic flow injection determination of coprostanol in nonaqueous media, is suitable to be applied for sewage pollution determination in water and sediments. Also it evidences that COD can recognise in nonaqueous media other substrates than cholesterol. Despite of the relative low analytical sensitivity of the developed procedure for coprostanol determination as compared with those found for cholesterol, and cholestanol, the proposed method could be advantageous in tracing the presence of faecal pollution of waters and sediments.

Attempts to improve the sensitivity to coprostanol by the proposed method failed, thus evidencing a deleterious effect of this substract on activity of both coimmobilised enzymes (COD, and HRP) but additional studies must be carried out in order to clearly establish the reasons of this behaviour.

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