Non-aqueous enzymatic flow injection determination of cholestanol in sediments

The Analyst

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A procedure was developed for the spectrophotometric determination of cholestanol in sediments based on its extraction with chloroform–methanol (2 + 1 v/v), dissolution of the extracts, after preconcentration, in pH 7.0 buffer-saturated toluene containing 10^{-3} M *p*-anisidine and enzymatic determination in non-aqueous media using a bienzymic reactor consisting of 1 mg of cholesterol oxidase and 1 mg of horseradish peroxidase non-covalently co-immobilized on controlled pore glass beads. A limit of detection of 2.0×10^{-6} M was obtained under the optimum experimental conditions and recoveries of 95–118% were obtained in the analysis of water and beach sediment samples spiked with cholestanol at concentration levels of 0.4–1.0 mg g^{-1}. This method can also be employed for the determination of cholesterol in sediment extracts, the analytical sensitivity for cholestanol being half that for cholesterol, and studies were carried out in order to determine accurately both compounds in the same sample.

Introduction

The continuous discharge of sewage effluents and sludge in coastal waters and potable water supplies is hazardous to public health, hence it is essential to monitor the extent and source of sewage-related pollutants throughout the water environment.

The determination of pathogenic micro-organisms together with many chemical pollutants present in sewage is an important objective in the assessment of this type of pollution. Bacteria, such as coliform and streptococci, have normally been used as sewage pollution indicators, but their determination has been questioned owing to the extreme variations in their survival under different environmental conditions.[1,2]

Sterols and related compounds have been used as molecular markers for tracing sewage pollutants in waters.[3] Cholesterol (5-cholesten-3β-ol) is the precursor of environmentally interesting sterols. The double bond between atoms 5 and 6 in the B ring of cholesterol is enzymatically hydrogenated, resulting in the formation of three stereoisomers, coprostanol [5β(*H*)-cholestan-3β-ol], cholestanol [5α(*H*)-cholestan-3β-ol] and epicoprostanol (5β-cholestan-3α-ol). The first two compounds have been widely used as an alternative for the analysis of sewage pollution, because they are the main sterols found in human faeces.[4]

The most commonly used method for the determination of cholestanol in natural samples involves Soxhlet extraction, column separation and detection by thin-layer chromatography (TLC),[5] high-performance liquid chromatography (HPLC),[6] capillary gas chromatography[7] or gas chromatography coupled with mass spectrometry,[8–11] the overall analytical procedures being complex, laborious and time consuming.

In this work, we explored the potential of the use of a co-immobilized bienzymic reactor consisting of cholesterol oxidase and peroxidase to monitor cholestanol in sediments as a faecal pollution indicator. A linear relationship was obtained between cholestanol dissolved or extracted in toluene and the absorbance at 458 nm, which corresponds to the oxidized form of *p*-anisidine, used as a test molecule to monitor the enzymatic reaction spectrophotometrically.

Experimental

Apparatus and reagents

Spectrophotometric measurements were carried out on a Hewlett-Packard (Waldbronn, Germany) Model 8452 A diode-array UV/VIS spectrophotometer, equipped with a quartz flow cell of 1 cm pathlength and 30 μl internal volume.

Two flow injection (FI) systems, similar to those previously employed for peroxide determination,[12] were employed (Fig. 1), one consisting 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 id), which was employed for the evaluation of the experimental parameters, and a forked system that includes two alternative modes which was employed to determine alternately the cholestanol and the zero absorbance in natural sample analysis.

A Rheodyne (Cotati, CA, USA) Model 50 Teflon rotary valve was employed for standard and sample injection, and 22 × 5 mm id tubular Teflon reactors with 100 μm nylon filters at each end[13] were packed with glass beads on which enzymes were adsorbed. In all the experiments the carrier was phosphate buffer (pH 7.0)-saturated toluene containing 10^{-3} M *p*-anisidine.

A Goldstar (Manchester, UK) MS-1706 domestic microwave oven with a maximum exit power of 700 W and laboratory-made PTFE reactors of 115 ml internal volume, were employed for the microwave- assisted saponification of sediment extracts.

A Büchi (Geneva, Switzerland) R124 rotary evaporator was used to evaporate chloroform–methanol and light petroleum solutions.

Streptomyces spiralis cholesterol oxidase (COD) (EC 1.1.3.6) [23 international units (IU) mg^{-1}], horseradish peroxidase (HRP) type II (EC 1.11.1.7) [220 purpurogallin units (PU) mg^{-1}] and controlled pore glass beads (CPG) (120–220 mesh, 1984 Å mean pore size) were purchased from Sigma (St. Louis, MO, USA).

To obtain enzymatic preparations of HRP and COD non-covalently immobilized on CPG, absorption of the biocatalysts on the glass beads was carried out basically as reported previously for a batch system by Kazandjian *et al.*[14] and for an FI system by Braco *et al.*,[15] also employed by Valencia-González and Díaz-García, [16] and Piñeiro-Avila *et al.*[17,18]

A 1.0 mg amount of HRP and 1.0 mg of COD were dissolved in 100 μl of 0.1 M aqueous phosphate buffer (pH 7.0) and this solution was added to 215 mg of CPG, without any pre-treatment, spread on a watch-glass. The preparation was left to dry for 15 min under a gentle flow of cool air and then packed in a tubular PTFE reactor.

Cholestanol (95%) was obtained from Fluka (Neu-Ulm, Switzerland) and cholesterol (99%) and *p*-anisidine (grade I) from Sigma. Spectroscopic grade toluene, chloroform, ethanol, methanol and light petroleum ether (bp 40–60 °C) were obtained from Scharlau (Barcelona, Spain). All other reagents were of analytical-reagent grade.

Sample collection

Samples were collected from the 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 beach sand collected 5 m from the shore in Port-Saplaya Beach (Valencia, Spain). and sediment 2 was collected from an effluent sewage outfall near the same beach.

Direct determination of cholestanol

Sterol extraction. A 1 g amount of dry sediment was extracted with three 10 ml portions of chloroform–methanol (2 + 1 v/v) in a separating funnel. After phase separation, the organic layer was filtered through a Wathman No 4 filter-paper. The combined extracts were washed three times with 30 ml of distilled water, then the volume of the organic phase was reduced by rotary evaporation and the concentrated extract treated with anhydrous sodium sulfate to remove any water. The residue was dissolved in 10 ml of buffer-saturated toluene solution containing 10^{-3} M *p*-anisidine and the solution was analysed for free cholestanol.

Extract saponification. The organic phase obtained after extraction of sediment samples was microwave-assisted saponified with a 1.0 M ethanolic KOH solution, following the procedure developed previously for cholesterol saponification.[17] The chloroform–methanol extract obtained from 1 g of sediment was washed with distilled water and dried and the residue was reconstituted with 10 ml of pure toluene. This solution was introduced inside a PTFE reactor and 40 ml of ethanolic 1.0 M KOH solution were added. The reactor was closed and irradiated at 50% power level for 2.5 min in a domestic microwave oven. After cooling, 40 ml of distilled water were added and the organic phase was extracted with three 40 ml portions of light petroleum (bp 40–60 °C), then washed with three 40 ml portions of ethanol–water (1 + 1 v/v), dried by rotary evaporation and the residue reconstituted with 10^{-3} M *p*-anisidine in toluene.

Enzymatic cholestanol determination

Samples, previously extracted (for free cholestanol determination) or extracted and saponified (for total cholestanol determination) were taken to complete dryness and the residues dissolved in 10 ml of buffer-saturated toluene solution containing 10^{-3} M *p*-anisidine. Volumes of 500 μl of these solutions were injected into a carrier stream of the *p*-anisidine in toluene solution and passed through the bienzymic reactor, monitoring the oxidized *p*-anisidine at 458 nm. To correct for the interference of natural sample colour, samples were alternately passed through a non-enzymic reactor for blank measurement, the dispersion characteristics of the two manifold lines being identical. Corrected absorbance values were interpolated on the corresponding calibration line obtained for standard solutions containing 0.0875–0.7 mg ml^{-1} cholestanol prepared in the same way as the samples.

Results and discussion

Spectrophotometric determination of cholestanol in non-aqueous media

Cholestanol is recognized by the COD in the same way as cholesterol regardless of the differences in their chemical

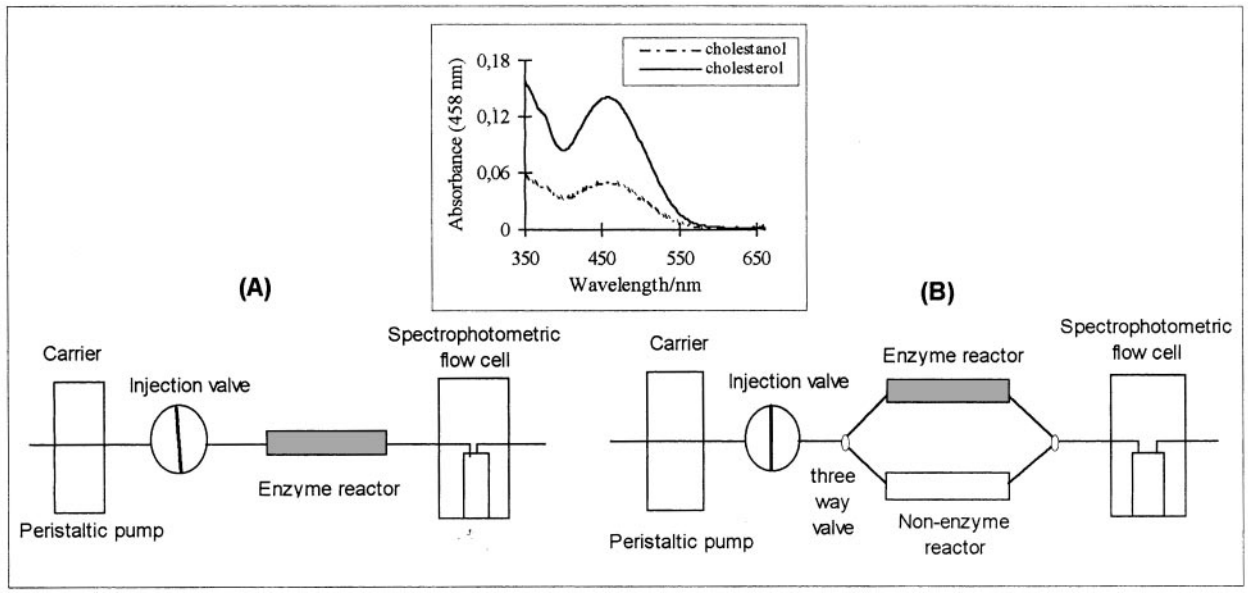

Fig. 1 Schematic diagram of the manifolds employed for the evaluation of the effect of experimental variables (A) and for the determination of cholestanol in natural samples (B). Inset: *p*-anisidine absorbance spectra, obtained after enzymatic reaction of 4.5×10^{-5} M cholestanol (dashed line) and 4.5×10^{-5} M cholesterol (solid line) standards dissolved in 10^{-3} M *p*-anisidine in pH 7.0 phosphate buffer-saturated toluene.

structures. Free cholestanol is oxidized by dissolved oxygen in the presence of the enzyme and H_2O_2 is produced, which then oxidizes *p*-anisidine through an HRP catalysed reaction. This oxidized product can be followed by measuring the spectrophotometric absorbance at 458 nm. The spectrum resulting from oxidized *p*-anisidine in toluene (see the inset in Fig. 1) obtained after this reaction is half as intense as that for cholestanol for the same concentration of cholesterol.

Effects of FIA parameters

The effects of flow injection variables, such as sample volume injected and mobile phase flow rate, on the analytical signal were investigated. In general, an increasing injection volume in the range 100–700 μl provided signals of increasing peak height which tended to stabilize at high injection volumes (Fig. 2). It can be seen that the peak height of the cholestanol signal decreases as the carrier flow rate through the COD-HRP reactor, increases. This indicates that the sterol oxidation reaction does not reach completion, and the extent of reaction clearly increases as the residence time of the injected plug inside the reactor increases. On the other hand, an increase in the injected volume and a decrease in the flow rate cause an increase in the peak width, thus providing a decrease in the sampling frequency. Hence compromise values of 3 ml min^{-1} and 500 μl were selected in order to obtain a sampling frequency of 60 h^{-1} with appropriate sensitivity.

Effect of temperature and pH

A study of the effect of temperature on the non-covalently immobilized COD–HRP reactor performance was carried out over the range 10–45 °C. Fig. 3 depicts the temperature dependence of the peak height in the FI determination of cholestanol in toluene. The highest peak height values were obtained between 30 and 40 °C, the optimum temperature being 37 °C. For temperatures higher than 40 °C the analytical sensitivity decreased.

pH is known to be a critical parameter with respect to enzyme activity and sometimes enzyme stability. In order to investigate the effect of the pH of the aqueous solution saturating the organic phase on the non-covalently co-immobilized COD–HRP activity, the enzymic reactor was studied at pH values ranging from 5 to 8. In Fig. 4, it can be seen that an increase in pH increases the enzymic reactivity, but no important changes in the peak height are observed. The best results were obtained for a pH of the aqueous phase saturating toluene near 7.5, the optimum value found in the literature for COD activity.[19,20] These results indicate that the enzymic activity was essentially unaltered in toluene, as reported, because proteins are kinetically trapped in the organic solvent in a highly rigid conformation.[21,22]

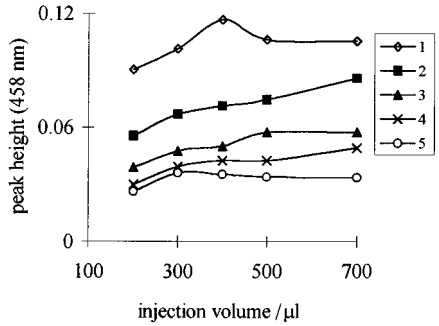

Fig. 2 Effect of sample injection volume on peak height absorbance measurements at 458 nm for a cholestanol concentration of 4.5×10^{-5} M, established at different carrier flow rates: (◇) 1, (■) 2, (△) 3, (×) 4 and (○) 5 ml min^{-1}.

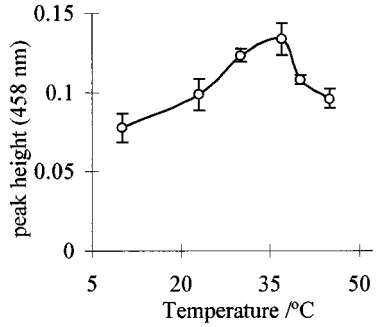

Fig. 3 Temperature dependence of the peak height of FI recordings obtained from the absorbance at 458 nm for a cholestanol concentration 4.5×10^{-5} M determined in toluene using the COD–HRP reactor. Conditions: injection volume, 500 μl; flow rate 3 ml min^{-1}; carrier pH 7.0 buffer-saturated toluene containing 10^{-3} M *p*-anisidine. Bars indicate the standard deviation of the mean value of three independent determinations.

Analytical figures of merit

From the injection of 500 μl volumes of cholestanol standards ranging from 2.2×10^{-5} to 1.8×10^{-4} M dissolved in buffer-saturated toluene solution containing 10^{-3} M *p*-anisidine, a typical linear relationship between absorbance (A) at 458 nm and cholestanol concentration in mol l^{-1} (C) was obtained, $A = (144 \pm 1)C - 0.014$ (±0.001) with $r^2 = 0.9999$. The standard deviation of calibration corresponded to $s_{y/x} = 0.0013$. Under these conditions, a limit of detection of 2.0×10^{-6} M of cholestanol was obtained for a probability level of 99.86%.

Analysis of sediments

In order to determine both free and total cholestanol in natural sediments, two assays were performed on real samples: (i) direct analysis of raw extracts obtained from sediment samples and (ii) analysis of extracts after microwave-assisted saponification. Two sediment samples were analysed and results obtained are summarized in Table 1. It can be seen that free cholestanol represents only 25–50% of the total cholestanol, indicating that the natural formation of cholestanol esters occurs in the beach sediments. On the other hand, it is clear that sediments collected in the sweet water effluent contain a lower sterol concentration than the sand sediments, indicating the absence of faecal contamination of this effluent.

However, it must be taken into consideration that cholesterol is ubiquitous in natural sediments and because of this and also owing to the activity of COD–HRP with respect to both

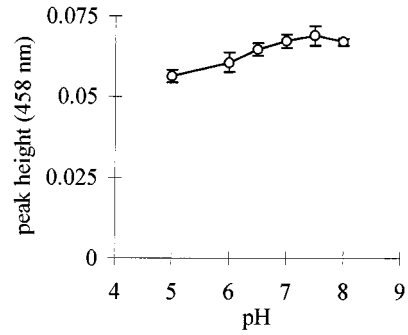

Fig. 4 Effect of pH of the aqueous solution saturating toluene on peak height of the FI recordings obtained for a cholesterol standard of 4.5×10^{-5} M in 10^{-3} M *p*-anisidine in toluene solution. Conditions: injection volume, 500 μl; flow rate, 3 ml min^{-1}; room temperature. Bars indicate the standard deviation of the mean value of three independent experiments.

substrates, cholesterol and cholestanol, the values reported in Table 1 correspond to free and total sterols, respectively.

In order to test the accuracy of the procedure developed for cholestanol determination, samples were spiked with known amounts of cholestanol and recovery studies were carried out at different concentration levels. As can be seen from Table 2, the recovery varied from 95–118%, which agrees with the repeatability of the results obtained.

The proposed enzymatic method provides a good idea of sediment pollution by faeces, but it is unable to discriminate between cholestanol and cholesterol in sediment samples, because both molecules react with non-covalently co-immobilized COD–HRP, providing the same effect on *p*-anisidine. In order to solve this problem and to enhance the selectivity of enzymic determination, additional assays, based on the differences between the analytical sensitivity obtained for cholestanol and cholesterol, were performed.

Table 1 Spectrophotometric determination of sterols in natural sediment samples by enzymic reaction in non-aqueous media using both raw extracts and microwave-assisted saponified extracts

	Concentration[a]/mg g^{-1}	
Sample	Non-saponified extract	Saponified extract
Sediment 1	0.11 ± 0.01	0.44 ± 0.02
Sediment 2	0.042 ± 0.002	0.083 ± 0.001

[a] Each result is the mean ± standard deviation of three independent determinations.

Table 2 Recovery studies on the spectrophotometric enzymic determination of cholestanol in water and natural sediment samples spiked with known amounts

Samples	Added/mg ml^{-1}	Found[a]/mg ml^{-1}	Recovery (%)
Water	0.4	0.38 ± 0.04	95
	1.0	0.98 ± 0.02	98
Sediment 1	0.4	0.39 ± 0.02	97.5
	0.5	0.59 ± 0.02	118
Sediment 2	0.3	0.34 ± 0.03	113
	0.5	0.57 ± 0.02	114

[a] Each result is the mean ± standard deviation of three independent determinations.

Analysis of binary mixtures of cholestanol and cholesterol

The determination of both cholestanol and cholesterol in the same sample was carried out taking into consideration the absorbance obtained at 458 nm for standards of each of the compounds considered at different flow rates. Flow rates of 1.5 and 3 ml min^{-1} were chosen for this purpose and, based on the proportional equations approach, the following equations, which relate the absorbance at each flow rate and the sensitivity coefficients corresponding to each compound considered under each set of conditions, were obtained:

$$\begin{pmatrix} A_{q=1.5} \\ A_{q=3} \end{pmatrix} = \begin{pmatrix} 603 & 210 \\ 340 & 132 \end{pmatrix} \begin{pmatrix} [\text{cholesterol}] \\ [\text{cholestanol}] \end{pmatrix}$$

where A_q = 1.5 is the absorbance at 458 nm obtained for a sample containing cholesterol and cholestanol, measured using a carrier flow rate of 1.5 ml min^{-1} of pH 7.0 buffer-saturated toluene containing 10^{-3} M *p*-anisidine passing through a bienzymic non-covalently immobilized COD–HRP reactor, and A_q = 3 the absorbance value for a carrier flow rate of 3 ml min^{-1}. The numerical values are the slopes of the calibration curves obtained for a series of standard solutions of each compound measured at the aforementioned flow rates. Using these equations, a series of synthetic mixtures of cholesterol and cholestanol and a natural sediment sample spiked with mixtures of these compounds were analysed. Table 3 summarizes data found for binary mixtures of cholesterol and cholestanol and, as can be seen, accurate results were obtained in all cases even for those samples which contained only one of two sterols.

For spiked sediment sample 2, satisfactory recoveries were obtained for both compounds (Table 4).

Many studies on sterols as pollution indicators are based on the 5α- to 5β-stanol ratio, suggested to be a more reliable criterion for assessing faecal input in sediments.[4,13] The mean ratio of cholestanol and cholesterol concentrations found in sample 2 is 0.68 ± 0.01, which is in a good agreement with that reported by Jeng and Han,[4] for cholestanol to cholesterol ratios in superficial sediment samples.

For the analysis of saponified extracts, the equations mentioned above could be also suitable for differentiating between α- and β-stanols owing to the efficacy of the microwave-assisted treatment for the complete saponification of extracts of cholesterol and cholestanol, and the different kinetics of the two substrates.

Table 3 Analysis of synthetic mixtures of cholesterol and cholestanol by enzymic reaction in non-aqueous media

	Concentration added/mg ml^{-1}		Concentration found[a]/mg ml^{-1}		Recovery (%)	
Mixtures	Cholesterol	Cholestanol	Cholesterol	Cholestanol	Cholesterol	Cholestanol
A	—	0.5	0.006 ± 0.005	0.56 ± 0.08	—	112
B	0.25	0.81	0.28 ± 0.05	0.83 ± 0.09	112	102.5
C	0.45	0.4	0.47 ± 0.01	0.41 ± 0.01	104	102.5
D	0.46	0.3	0.49 ± 0.04	0.33 ± 0.11	106.5	111
E	0.5	—	0.55 ± 0.02	0.01 ± 0.02	111	—

[a] Each result is the mean ± standard deviation of three independent determinations.

Table 4 Analysis of a natural sediment sample spiked with known concentrations of synthetic mixtures of cholesterol and cholestanol

	Concentration added/mg g^{-1}		Concentration found[a]/mg g^{-1}		Recovery (%)	
Mixtures	Cholesterol	Cholestanol	Cholesterol	Cholestanol	Cholesterol	Cholestanol
1[b]	—	—	0.028 ± 0.004	0.019 ± 0.003	—	—
2	0.5	—	0.46 ± 0.02	0.02 ± 0.05	92	—
3	0.5	0.5	0.46 ± 0.03	0.47 ± 0.08	92	94
4	0.25	0.7	0.23 ± 0.03	0.73 ± 0.06	92	104

[a] Each result is the mean ± standard deviation of three independent determinations. [b] Sample spiked corresponds to unsaponified sediment 2.

Stability of the bienzymic reactor

In order to study the presumably long storage stability of the non-covalently immobilized bienzymic reactor in toluene, a reactor was prepared containing 24 IU of COD and 220 PU of HRP, which was intermittently tested in water-saturated toluene. Fig. 5 shows the effect of the number of injections of cholestanol solutions on the sensitivity of its determination and it can be seen that the slope of the calibration curve follows approximately a first-order decay process during storage and multiple use of the reactor. It is clear from Fig. 5 that the analytical sensitivity decreases exponentially as a function of time, and it can therefore be recommended to use freshly prepared reactors for the determination of extremely low cholestanol concentrations. However, it must be taken into consideration that during a period of approximately 3 months, more than 100 accurate analyses were carried out with only 1 mg of COD and 1 mg of HRP, which provides an inexpensive methodology.

As has been found in earlier studies, the reason for the enzyme activity loss of non-covalently immobilized reactors could be explained by, in addition to the enzyme sensitivity to temperature changes, the effect of partial leaching of enzymes due to the continuous passage of small volumes of aqueous buffer which saturate the toluene phase.[12,15] However, it is clear that, compared with the enzymatic stability in aqueous media, the developed reactor can be considered to have high stability.

Conclusions

These studies carried out on cholestanol determination *via* an enzymatic reaction with non-covalently immobilized COD and HRP demonstrate the possibility of using this type of enzyme reaction for the determination of sterols other than cholesterol, thus offering exciting possibilities for the use of this reactor in environmental studies in order to evaluate rapidly and easily the presence of faecal contamination. The different sensitivity of this system as a function of the nature of the sterol permits one to obtain, in addition to global information on the sterol content, quantitative data about both cholesterol and cholestanol. On the other hand, the fact that previous extraction with an organic phase is required as a sample preparation step offers excellent compatibility between the final analyte environment and the medium employed to perform the enzymic reaction.

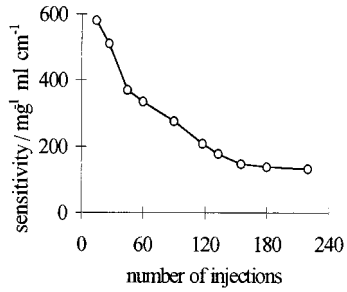

Fig. 5 Stability of a non-covalently co-immobilized bienzymic reactor of COD and HRP (24 IU COD and 220 PU HRP) determined from the variation of the slope of the cholestanol calibration curve as a function of the number of injections.

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