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Nonionic micellar liquid chromatography coupled to immobilized enzyme reactors

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Abstract

Immobilized enzyme reactors are used as post-column reactors to modify the detectability of analytes. An immobilized amino acid oxidase reactor was prepared and coupled to an immobilized peroxidase reactor to detect low level of amino acids by fluorescence of the homovanilic dimer produced. A cholesterol oxidase reactor was prepared to detect cholesterol and metabolites by 241 nm UV absorbance of the enone produced. The preparation of the porous glass beads with the immobilized enzymes is described. Micellar liquid chromatography is used with non-ionic micellar phases to separate the amino acids or cholesterol derivatives. It is demonstrated that the non ionic Brij 35 micellar phases are very gentle for the enzyme activity allowing the reactor activity to remain at a higher level and for a much longer time than with hydro-organic classical chromatographic mobile phases or aqueous buffers. The coupling of nonionic micellar phases with enzymatic detection gave limits of detection of 32 pmol (4.8 ng injected) of methionine and 50 pmol (19 ng injected) of 20α-hydroxy cholesterol. The immobilized enzyme reactors could be used continuously for a week without losing their activity. It is shown that the low efficiency obtained with micellar liquid chromatography is compensated by the possibility offered by the technique to easily adjust selectivity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Micellar liquid chromatography; Immobilized enzymes; Detection LC; Amino acids; Cholesterol; Surfactants

1. Introduction

There is a continually increasing need for characterization and quantification of analytes in complex biotechnological and clinical matrices, e.g., amino acids, carbohydrates, bile acids, cholesterol and many others. One of the most commonly used techniques to separate samples from biological matrices is reversed-phase liquid chromatography (RPLC) [1,2]. RPLC is popular and successful because of the high efficiency of the analytical columns and the ability to choose organic modifiers and to perform gradients. These features lead to optimal selectivity and resolution of the compounds of interest. A drawback is that RPLC lacks a sensitive universal detector. The convenient UV detector senses accurately many classes of important compounds. The fluorescence detector is very selective and one of the most sensitive detectors. Unfortunately most solutes do not fluoresce and many biologically important solutes do not absorb UV light.

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Several alternative detection modes have been developed following two paths: (1) detection is done using a specific property of the molecule (electrochemical, refractive index or mass spectrometry detection) or (2) the molecule is modified so that a UV detector or a fluorescence detector can be used. Chemical derivatization can be done before or after the analytical column [3,4]. Brown first proposed to use enzyme reactions for solute detection as early as 1970 [5]. She used an enzyme reaction in a post-column treatment of collected fractions of the effluent. In the 1980s, immobilized enzymes were proposed for online selective reaction detection [6–9]. Enzyme reactions were also used in sample preparation. A tandem RNase T_1 capillary enzyme reactor–capillary zone electrophoresis system was described [10]. The pre-column enzyme reactor was able to split dinucleotides making the single adenosine, uridine or cytidine easily separated by the electric field and UV detected [10].

Enzymes are natural polypeptidic molecules that work well in buffers. It was demonstrated that the activity of enzymes decreased rapidly and irreversibly (denaturation) when organic modifiers are present [9,11]. This denaturation problem renders the online use of enzyme reactors in RPLC expensive due to the frequent replacement of the reactor [12]. It has been proposed to work with a minimum amount of organic solvent in the mobile phase or to dilute the mobile phase before it enters the post column enzyme reactor. These solutions result in either prohibitively long retention times or loss in sensitivity [9,11,12].

Micellar liquid chromatography (MLC) uses micellar solutions as mobile phases. Most micellar solutions are made of more than 95% (v/v) water. The remaining 5% are surfactant molecules associated in the form of micelles and a small amount of organic solvent. The full capabilities of MLC were recently described [13]. Ionic surfactants bind to charged groups on proteins and/or enzymes denaturing them. Nonionic surfactants lack charged head groups and are more mild with proteins, often not denaturing them [14]. It seems that nonionic micellar phase would be more compatible with enzyme reactors than classical RPLC hydro-organic mobile phases. The aim of this work is to demonstrate that MLC with nonionic micellar mobile phases is able to separate and allow highly sensitive detection for non UV-absorbing biomolecules with a dedicated enzyme reactor that is not rapidly destroyed by the mobile phase.

2. Experimental

2.1. Chemicals

L-Amino acids, decane sulfonate, flavin adenine dinucleotide disodium salt, cholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, sodium cholate, glutaraldehyde grade I, γ-aminopropyltriethoxysilane, controlled pore glass beads with 40–70 μm particle diameter (pore size 38 nm) and 90–120 μm particle diameter (pore size 50 nm) and the enzymes L-amino acid oxidase (type III), cholesterol oxidase (from pseudomonas, 40 units/mg) and horse radish peroxidase (150 units/mg) were purchased from Sigma (L'Isles d'Abeau Chesnes, France and St. Louis, MO, USA). Potassium dihydrogenphosphate, *n*-propanol and tris(hydroxymethyl) amino-methane came from Fisher (Fair Lawn, NJ, USA). Homovanilic acid was from Kodak (Rochester, NY, USA) and Brij® 35 (polyoxyethylene 23 dodecyl ether, M_r 1200 g/mol) was obtained from Fluka (Sigma, L'Isles d'Abeau Chesnes, France). The reagents were used as received. All solutions were made with deionized water purified with a Barnstead Nanopure II system (Boston, MA, USA) and filtered through a 0.45 μm nylon membrane filter (Whatman).

2.2. Instrumentation

Two chromatographic systems were used. The first had a TSK 6010 dual piston pump (Toso Haas, Philadelphia, PA, USA), a 20 μl injection valve model C6W (Valco, Houston, TX, USA), a 15 cm× 4.6 mm I.D. Zorbax CN column, a FD-300 fluorescence detector (Spectrovision, Chemford, MA, USA) with an 8 μl flow cell and a model SP 4290 integrator (Spectra-Physics, San Jose, CA, USA). The column temperature was maintained at 20°C (amino acids) or 35°C (cholesterols) by a Fisher Scientific Isotemp model 9000 circulating bath containing an ethylene glycol–water (50:50) thermo-

static fluid. The second chromatographic system was made with a SP 8800 pump (Spectra Physics), a 6035 Rheodyne valve with a 20 μl loop (Rheodyne, Cotati, CA, USA), a second identical Zorbax CN column, a diode array detector model 1000 S (Applied Biosystems, Ramsey, NJ, USA) with a 10 μl flow cell and a SP 4270 integrator. The enzymatic reactor was inserted between the column and the fluorimeter. An Alitea (Stockholm, Sweden) model C4 peristaltic pump was used to add the peroxidase–homovanilic acid reagent solution when hydrogen peroxide was formed in the enzymatic reactor. The operating temperature of the enzymatic reactors was 35°C. When not in use, they were stored at 4°C in the nonionic surfactant mobile phase.

2.3. Enzymatic reactors

Three enzymatic reactors were prepared. They were made by hand-packing controlled pore glass beads derivatized with the desired immobilized enzyme in 5 cm×4.1 mm I.D. stainless steel tubes. The enzymatic reactor for L-amino acids contained the enzyme amino acid oxidase type III immobilized on 40–70 μm porous glass beads. The second reactor was made to detect by fluorescence the hydrogen peroxide released by the enzymatic oxidation of amino acids. It contained the peroxidase enzyme bonded to the same 40–70 μm porous glass beads. It needed the post-column addition of a 0.5 m*M* homovanilic acid solution at pH 8.5 (0.1 *M* Tris buffer) added with a peristaltic pump and a simple T connector just before the peroxidase enzyme reactor. The third reactor was made to detect cholesterol derivatives. It contained the enzyme cholesterol oxidase immobilized on 90–120 μm porous glass beads. The porous glass beads were first silanized with γ-aminopropyltriethoxysilane in dry toluene under nitrogen. Then, the enzyme oxidase was immobilized on the porous glass bed by the glutaraldehyde method [15,16]: to 1 g of aminopropyl-silanized glass beads, 25 ml of a 2.5% glutaraldehyde solution in 0.05 *M* phosphate buffer (pH 7.0) were added. After 3 h of reaction at room temperature under nitrogen, the activated glass beads were washed with aqueous buffer and the enzymes were added. Azo coupling insures the bonding of the enzymes on the silanized porous glass beads.

2.4. Enzymatic reactions

Enzymes are very specific. They can and will modify a particular molecular structure and nothing else. Three types of enzymes were studied in this work, but the approach can easily be extended to other enzymes.

2.4.1. Amino acid oxidase

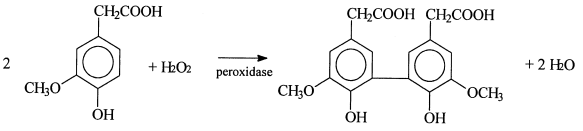
Some amino acids contain a UV-absorbing chromophore such as an aromatic ring (phenyl alanine, tryptophan, tyrosine) or the imidazole ring of histidine. Most amino acids absorb weakly only very short UV wavelengths (200 or 210 nm) and are difficult to detect at low level by a classical UV detector. L-Amino acid oxidase is an enzyme able to use oxygen to convert any amino acid to the corresponding keto acid and producing an equimolar amount of hydrogen peroxide:

$$R{-}CH(NH_2){-}COOH + O_2 + H_2O \xrightarrow[\text{oxidase}]{} C{-}CO{-}COOH + H_2O_2 + NH_3 \quad (1)$$

The keto acid has a better UV absorbance than the original amino acid. It is often preferred to detect the associated hydrogen peroxide [17]. The latter detection can be done by electrochemistry using the oxido-reduction properties of H_2O_2.

2.4.2. Horse radish peroxidase

Enzyme peroxidase can be even more sensitive in detecting H_2O_2. A peroxidase–homovanilic acid system was used to detect H_2O_2 produced by the amino acid oxidase reactor according to [17]:



(2)

The homovanilic dimer has a strong fluorescence emission at 425 nm when excited at 315 nm. Coupling the two enzymatic reactors allows the detection of all amino-acids by fluorescence regardless of their attached side groups. The fluorescence signal is directly proportional to the amino acid concentration.

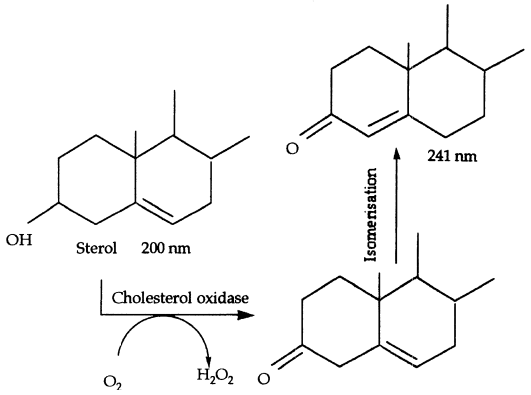


Fig. 1. Enzymatic conversion of sterols to conjugated enones by cholesterol oxidase.

2.4.3. Cholesterol oxidase

Cholesterol and cholesterol metabolites are another class of molecules that are easily separated by HPLC but difficult to detect with a UV detector. The enzyme cholesterol oxidase is able to oxidize a sterol to a conjugated enone that strongly absorbs the 241 nm UV light (Fig. 1) [17]. It is noted that there is also production of a hydrogen peroxide molecule that could be detected with the peroxidase reactor as well.

3. Results and discussion

3.1. Stability of the immobilized enzyme reactors

3.1.1. Effect of surfactant concentration

All chemicals added to the mobile phase can modify the enzyme activity. Since micelles are necessary to perform MLC, it is important to know the effect of the nonionic micelles. The stability of the bonded enzymes was studied by flow injection analysis. Specifically, the Zorbax CN column was removed from the system and replaced by a 15 cm×130 μm I.D. polyether ether ketone (PEEK) tubing directly connected to the enzyme reactor under investigation. The activity of the peroxidase immobilized enzyme reactor was tested by injecting a known amount of hydrogen peroxide (2 nanomoles) and measuring the amount of substrate converted (peak area). Triplicate injections of hydrogen peroxide were made in micellar mobile phases containing amounts of surfactant increasing from 0 to 0.05 *M* (60 g/l) at a flow-rate of 1 ml/min. A 0.5 m*M* homovanilic acid, 0.1 *M* Tris pH 8.5 solution was added using a peristaltic pump at 1 ml/min using a mixing T. The resulting 2 ml/min solution was passed through the peroxidase column reactor maintained at 35°C. The experiments were all done in the same day. It was found that the enzymatic conversion factor, *C*%, decreased almost linearly within the 0–0.05 *M* Brij 35 concentration range. The regression line obtained was:

$$C = 100 - 1640\,[\text{Brij 35}] \qquad n = 5,\ r^2 = 0.83 \tag{3}$$

Eq. (3) indicates that the conversion percentage would be zero for a Brij 35 concentration of 100/1640 or 0.061 *M* or 73 g/l. This is not strictly true because the conversion decrease becomes quadratic for high surfactant concentrations. The viscosity of these concentrated micellar phases is high which would also preclude their use in MLC due to back pressure problems. Micellar concentrations between 0.002 *M* and 0.02 *M* (2.4–24 g/l) give conversion factors between 96% and 70%. This shows that such micellar phases are compatible with the peroxidase enzyme reactor.

3.1.2. Effect of an organic alcohol

The elution strength of micellar phases is limited. The kinetics of mass transfer in MLC is slow due to the surfactant adsorbed layer on the stationary phase surface [13]. It was demonstrated that small amounts of a medium chain alcohol such as propanol could reduce the thickness of the surfactant adsorbed layer and greatly enhance both the efficiency and elution strength of the micellar phase [13,18]. Propanol may be very harmful to the enzyme activity. Its effect on the peroxidase enzyme reactor was studied using the very same experimental set-up previously described, except a new peroxidase enzyme reactor was used. The selected concentration range was 0–10% (v/v). Again, an almost linear decrease of the conversion factor was obtained. The regression line is expressed by:

$$C\% = 100 - 2\,[\text{propanol}\%] \qquad n = 4,\ r^2 = 0.92 \tag{4}$$

in which [propanol%] is the percentage v/v of propanol in the mobile phase before the post-column homovanilic reagent is added. Eq. (4) suggests that a 50% (v/v) aqueous propanol mobile phase would completely and irreversibly denature the peroxidase enzyme reactor. This experiment was not done to save the reactor.

3.1.3. Stability duration

The lifetime of immobilized enzyme reactors is short, often only hours when organic modifiers are used in the HPLC separation [9]. To study the stability duration of the peroxidase enzyme reactor, four 5 cm column reactors were prepared with the same batch of peroxidase bonded porous glass beads. Each was submitted to a 0.4 ml/min flow-rate of one of four different mobile phases, namely (1) a reference 0.1 *M* pH 8.5 Tris buffer mobile phase, (2) 2.5 m*M* Brij 35 in the 0.1 *M* pH 8.5 Tris buffer, (3) 2.5 m*M* Brij 35 and 3% (v/v) propanol in the 0.1 *M* pH 8.5 Tris buffer and (4) a classical hydro-organic mobile phase of acetonitrile–0.1 *M* buffer pH 8.5 (30:70, v/v). The post-column reagent (0.5 m*M* homovanilic acid in 0.1 *M* Tris buffer pH 8.5) was added with a peristaltic pump set at 0.4 ml/min so that a total flow-rate of 0.8 ml/min passed through the enzyme reactor. Two nanomoles of hydrogen peroxide were injected in triplicate to check the reactor activity. The injections were repeated every 12 h for 84 h. The average peak area was divided by the area obtained at time zero with the reference buffer mobile phase. The ratio was used as the indicator of the relative activity of the peroxidase reactor. Fig. 2 shows the results obtained with the four mobile phases over the 3.5 day duration experiments with a total of 2 l of mobile phase passed through the immobilized enzyme reactor.

Fig. 2 shows the denaturing effect of the acetonitrile mobile phase. In 12 h, 65% of the enzyme activity was lost. This result is in agreement with the reported finding of Bowers and Johnson [11]. After a day, less than 20% of the enzyme activity remained. The enzyme activity versus time for the three other mobile phases decreased somewhat linearly as shown

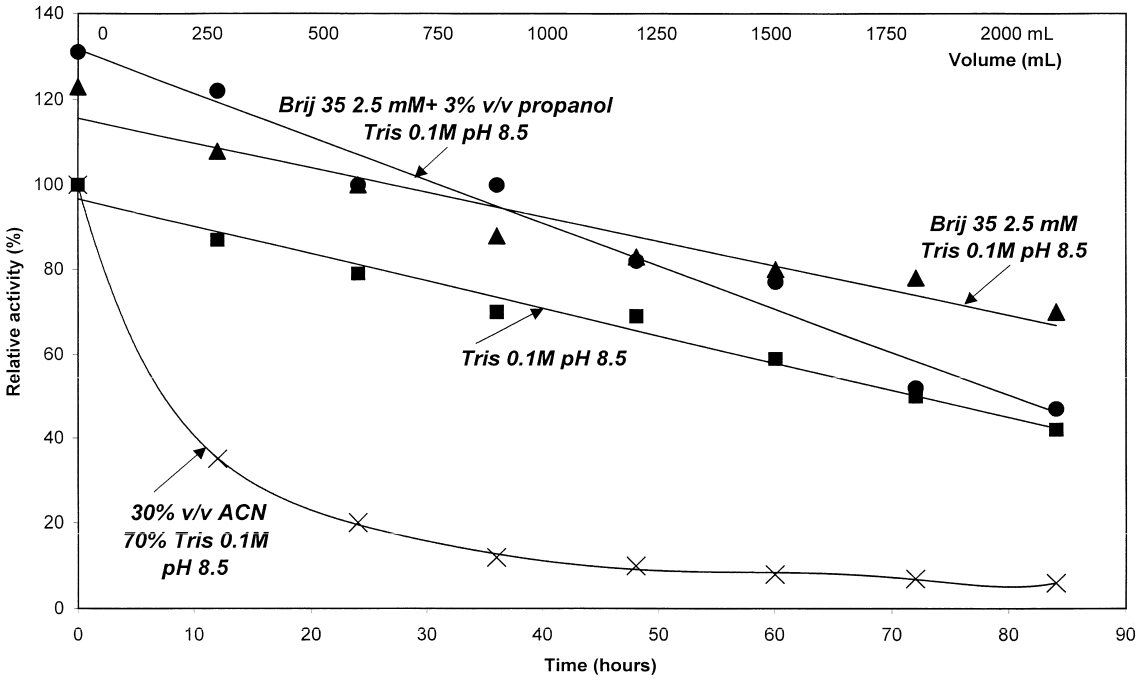


Fig. 2. Relative activity of horse radish peroxidase immobilized on porous glass beads packed in four 5 cm column reactors and submitted to four different mobile phases. Flow rate of the indicated mobile phase: 0.4 ml/min. Post-column addition to the mobile phase of 0.4 ml/min of a 0.5 m*M* homovanilic acid in 0.1 *M* Tris buffer pH 8.5 solution. The straight lines correspond to the regression Eqs. (5) (aqueous Tris buffer), (6) (Brij 35 2.5 m*M*) and (7) (Brij 35 2.5 m*M* + 3%, v/v, propanol). Reference 100% activity: Tris buffer at time 0. Average value of triplicate injections of 2 nmol of H_2O_2.

in Fig. 2. Using the symbol $C\%$ for the relative percent activity and t for the time in hours, the regression lines are: for the reference 100% aqueous Tris buffer:

$$C\% = 97 - 0.65\,t \qquad n = 8,\ r^2 = 0.983 \tag{5}$$

for the micellar 2.5 mM Brij 35 mobile phase:

$$C\% = 116 - 0.58\,t \qquad n = 8,\ r^2 = 0.930 \tag{6}$$

and for the micellar 2.5 mM Brij 35 mobile phase containing 3% (v/v) propanol:

$$C\% = 132 - 1.02\,t \qquad n = 8,\ r^2 = 0.974 \tag{7}$$

The intercepts of the regression lines correspond to the initial enzyme activity. Surprisingly, the initial relative activity of peroxidase in the two mobile phases containing the Brij 35 surfactant was found higher than that observed with the reference aqueous buffer. This could be due either to an activating effect of the nonionic surfactant on the enzyme, or to a spectroscopic enhancement of the fluorescent signal by the Brij 35 micelles [13,19]. The slopes of the regression lines give a measure of the denaturing effect of the mobile phase on the enzyme reactor. The Brij 35 surfactant is slightly less damaging to peroxidase enzyme than the purely aqueous buffer. The addition of 3% propanol increases the slope confirming that organic solvents are very damaging for enzyme activity. It is pointed out, however, that the two mobile phases containing the nonionic surfactant maintained an enzyme activity better than that obtained with the aqueous buffer over the 85 h time span. Clearly, as far as enzyme denaturation is concerned, the nonionic surfactant Brij 35 is far more gentle to the enzyme than any organic solvent.

Equivalent experiments were done with the amino acid oxidase reactor and the cholesterol oxidase reactor. The results were very similar. The non ionic Brij 35 micellar mobile phase is very gentle toward the enzyme and even preserves activity against chemical aggression. For example, the activity of three cholesterol oxidase reactors made from the same batch of bonded glass beads are compared in Fig. 3. The 95% (v/v) methanol mobile phase, necessary to elute the apolar cholesterol compounds,

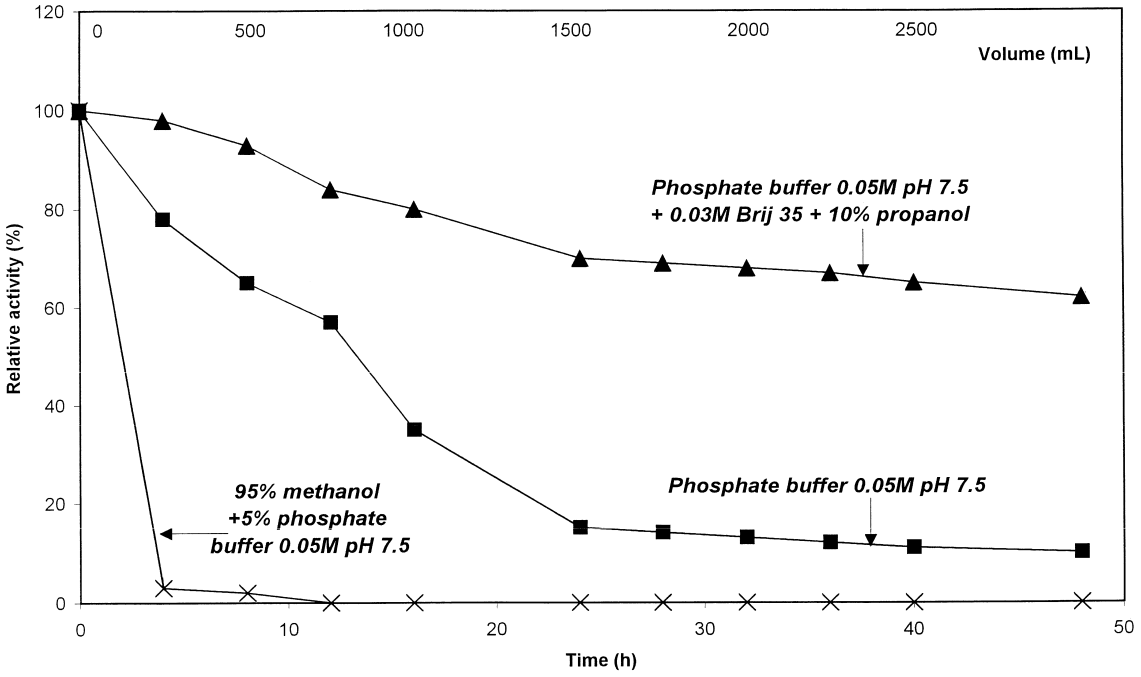


Fig. 3. Relative activity of cholesterol oxidase immobilized on porous glass beads packed in three 5 cm column reactors and submitted to three different mobile phases. Flow-rate of the indicated mobile phase: 1 ml/min. Reference 100% activity; phosphate buffer at time 0. Average value of triplicate injections of 5 μg cholesterol.

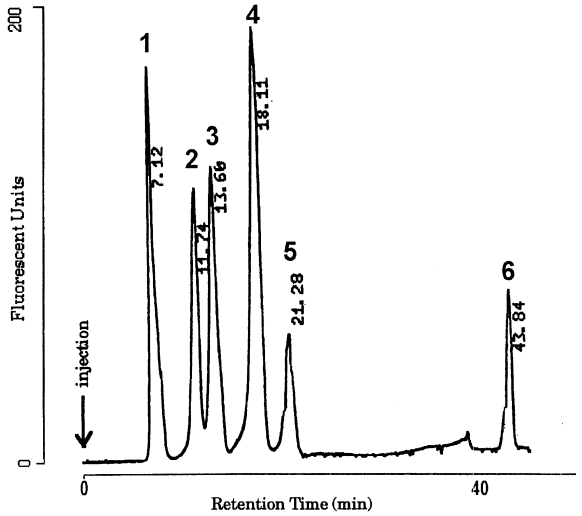


Fig. 4. Fluorescence chromatograms of amino acids after enzymatic reaction. 1=Methionine, injected mass: 0.5 μg; 2=leucine (0.5 μg) (or isoleucine); 3=tyrosine (0.5 μg); 4=phenylalanine (2 μg); 5=histidine (10 μg), 6=tryptophan (25 μg). Mobile phase with a double gradient: for the first 16 min, the decane sulfonate ion-pairing concentration decreased from 25 m*M* to 0, with a constant 0.125 m*M* Brij 35 micellar concentration; from 16 min to 45 min, a gradient of micellar concentration was run from 0.125 m*M* to 1.25 m*M* Brij 35, flow-rate 0.6 ml/min. Column Zorbax CN, 15 cm, at room temperature. Detection by two enzymatic reactors, addition of 0.6 ml/min of homovanilic acid 0.5 m*M* in 0.2 *M* tris buffer, pH 8.5 before the second peroxidase immobilized column reactor maintained at 35°C.

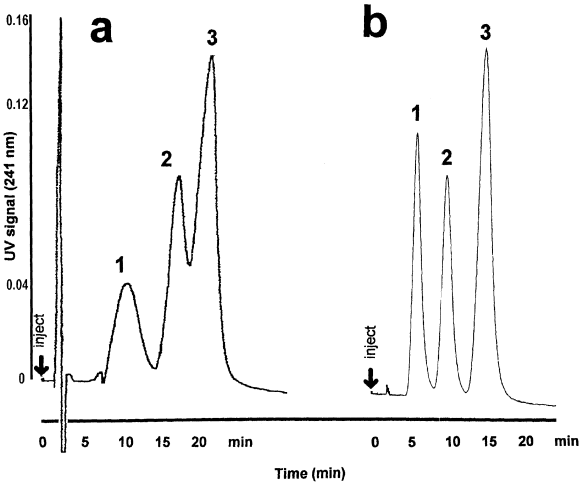


Fig. 5. Chromatogram of cholesterol and two hydroxy derivatives. 1=Cholesterol (1 μg); 2=25-hydroxycholesterol (1 μg); 3=20α-hydroxycholesterol (1 μg). (a) Mobile phase: 0.5 *M* phosphate buffer pH 7.5, 0.03 *M* Brij 35, 10% (v/v) propanol, 1 ml/min, peak efficiencies: Peak 1=45 plates, 2=220 plates, 3=280 plates; (b) same as (a) plus 0.025 *M* sodium cholate, peak efficiencies: Peak 1=120 plates, 2=280 plates, 3=400 plates. Column Zorbax CN, 15 cm×4.6 mm I.D. Detection UV at 241 nm after enzymatic oxidation at 35°C.

kills the enzyme in less than 4 h (240 ml of mobile phase). When a 0.05 *M* pH 7.5 phosphate buffer is passed over the enzyme at 1 ml/min, its activity is reduced by about 90% in 1 day (1.4 l). With the same phosphate buffer and 10% propanol, a concentration of 0.03 *M* Brij 35 (36 g/l) protected the enzyme so well that, after 2 days or 3 l of mobile phase passed through it, 65% of the initial activity was preserved.

3.2. Chromatographic separations

3.2.1. Amino acids

Amino acids are zwitterions too polar to be

Table 1
Chromatographic data and figures of merit of the detection of amino acids using two coupled immobilized enzyme reactors

L-Amino acid	Retention time (min)	Retention factor (k')	Dynamic range[a] (μg injected)	Limit of detection (ng injected)
Methionine	7.1	0.8	1.5–0.015	4.8
Leucine–isoleucine	11.7	1.9	(2–0.02[b])	(6[b])
Tyrosine	13.6	2.5	2.5–0.02	6.7
Phenylalanine	18.1	3.5	10–0.1	11
Histidine	21.3	4.3	150–1	390
Tryptophan	43.8	9.8	250–2	1200

Mobile phase: 0.1 *M* tris buffer, pH 8.5, 0.0025 *M* Brij 35, 3% (v/v) propanol, 0.4 ml/min.
[a] Relative standard deviation for phenylalanine at 2 μg injected (n=6) was 2.2%, correlation coefficients equal or higher than 0.985.
[b] Leucine and isoleucine coeluted, leucine injected alone.

retained by the Zorbax CN stationary phase. It was necessary to reduce their polarity by forming ion pairs. Decane sulfonate was the anionic ion pair additive selected. The separation procedure imposed a double gradient: for the first 16 min, the decane sulfonate concentration decreased from 25 m*M* to 0, the micellar concentration was 0.125 m*M* Brij 35 (0.15 g/l). From 16 min to 45 min, a gradient of micellar concentration was run from 0.125 m*M* to 1.25 m*M* Brij 35 (0.15 g/l to 1.5 g/l) to speed the elution of tryptophan. We found that no reequilibration of the column was necessary as the ion-pairing agent does not adsorb strongly on the cyano bonded phase and the Brij 35 has an adsorption that changes only little with concentration above the critical micellar concentration (CMC) [13,20,21]. Fig. 4 shows that no propanol addition was necessary to obtain good resolution between peaks.

The reactor response was linear over almost two orders of magnitude, but the limit of detection (LOD) depended strongly on the amino acid nature. It was as low as 4.8 ng injected (32 pmol or 20 μl of $1.6\cdot10^{-6}$ *M*) for methionine reaching 1.2 μg injected (5.8 nmol or 20 μl of $2.9\cdot10^{-4}$ *M*) for tryptophan. Table 1 lists the LODs obtained with the fluorescence detection of the hydrogen peroxide produced by the enzyme reactor. These values were comparable or slightly better than previously published LODs [9]. Isoleucine and leucine were not separated by the chromatographic column and micellar phase. The LOD of hydrogen peroxide directly injected in the peroxidase detector was 30 times lower (1 pmol injected or 20 μl of $5\cdot10^{-8}$ *M*), a value comparable to that obtained by Kurth et al. using a similar enzyme reactor [22]. This shows that the kinetics and/or yield of the amino acid oxidase reactor are critical. Phenylalanine and histidine have similar retention factors. The LOD of histidine is 35 times higher than that of phenylalanine which means that the amino acid reactor oxidized 35 times less histidine than phenylalanine in the same amount of time and under the same conditions. Concerning peak width, no efficiency study could be done since gradient conditions were used.

3.2.2. Cholesterol derivatives

High levels of cholesterol in blood have been demonstrated to be a factor related to high blood pressure and increased risk of cardiovascular disease. The determination and quantitation of cholesterol in serum and food samples are very frequently performed assays [23]. More than 30 oxidation products have been reported; many of which were proved active in the arteriosclerotic process and/or angiotoxic [24]. Some of the oxidation products are 20α-hydroxycholesterol, 25-hydroxycholesterol and 7α-hydroxycholesterol. All these compounds have a weak UV absorption at 200 nm. They are all oxidized by the enzyme cholesterol oxidase to a conjugated enone easily detected at 241 nm (Fig. 1). However, cholesterol and hydroxycholesterols are very hydrophobic. In RPLC, they require a high percentage of organic solvent in the mobile phase to be eluted in a reasonable amount of time. As shown in Fig. 3, these methanol rich mobile phases are deadly for any enzyme reactor. It was demonstrated that enzyme reactors could be, however, used with the post column addition of a high flow-rate of buffer that decreased the organic solvent percentage in the enzyme reactor below 20% (v/v) [9]. Of course, such post column addition decreased the solute concentrations, and consequently, the LODs as well.

Micellar nonionic mobile phases were able to elute cholesterol from the cyano column in less than 10 min (1 ml/min, 35°C). Fig. 5a shows the separation of cholesterol and two hydroxy cholesterols with a 0.03 *M* Brij 35 (36 g/l), 10% propanol micellar mobile phase. The two cholesterol derivatives are isomers differing only by the hydroxyl group position (on carbon 20 or 25). To increase the resolution, the efficiency could be increased or the selectivity could be modified. It was first tried to increase the efficiency by raising the temperature of the HPLC column. At 50°C, the efficiency doubled, which was not enough to obtain a good resolution. The cyano column also had a short life time at elevated temperature due to enhanced silica solubility. Attempts to work with 20% propanol nonionic micellar mobile phase did not improve the efficiency. This was not surprising since it was recently shown that short chain alcohols have little effect on the chromatographic efficiency in MLC when using nonionic micellar phases [25]. It was chosen to act on the chromatographic selectivity.

Bile salts can modify the eluting power of nonionic surfactants [13]. They are natural com-

Table 2
Chromatographic data and figures of merit of the detection of cholesterol and derivatives using a cholesterol oxidase enzyme reactor

Solute	Retention time (min)	Retention factor (k')	Peak efficiency (plates)	Dynamic range[a] (μg injected)	Limit of detection (ng injected)
Cholesterol	5.7	1.9	150	20–0.1	75
25-Hydroxycholesterol	9.5	4.1	300	20–0.1	35
20α-Hydroxycholesterol	14.8	5.6	400	20–0.1	19

[a] Relative standard deviation for cholesterol at 4 μg injected ($n=6$) was 1.9%, correlation coefficients (r^2) equal to or higher than 0.975. Mobile phase: 0.05 *M* phosphate buffer pH 7.5+0.03 *M* Brij 35+10% (v/v) propanol+0.025 *M* sodium cholate.

pounds with a rigid steroid structure having a hydrophobic side and a hydrophilic side. Beside the carboxylate anion, sodium cholate has three hydroxyl groups on its hydrophilic side. This steroid surfactant seems the logical additive to enhance the selectivity of the Brij 35 micelles toward the cholesterol derivatives, other members of the steroid family. After optimization, the three cholesterol derivatives were baseline separated by the mobile phase containing 0.03 *M* Brij 35 (36 g/l), 10% *n*-propanol and 0.025 *M* sodium cholate (10.8 g/l). Fig. 5b shows that besides baseline separation, the elution time was reduced by 30%. The sodium cholate addition did not have any adverse effect on the enzyme reactor activity. Table 2 lists the chromatographic figures of merit for the separation. The linear range was two orders of magnitude and the LOD of cholesterol was 19 ng injected (50 pmol or 20 μl of 1 mg/l solution). These LODs, obtained with classical UV detection of the enzymatically produced derivative, are comparable to the values obtained with amino acids (fluorescence detection of hydrogen peroxide). The peroxidase detector is particularly well suited to work with nonionic micellar mobile phases.

Table 2 lists also the peak efficiency obtained for each solute. Efficiencies of 300–400 plates with a 15 cm column corresponds to plate heights between 0.4 and 0.5 mm, or more than 100 particle diameters. These values are really low for a modern HPLC system. This is partly due to MLC that is known to produce low efficiencies [13] and to post-column band broadening by the immobilized enzyme reactor volume. This is particularly evident on the efficiency of the most retained peak which is higher than that of the less retained peaks. The added variance due to the enzyme reactor is constant. Its weight decreases as the column variance, time depending, increases for the most retained compounds. This results in an increase of the plate count for the most retained compounds.

In conclusion, micellar liquid chromatography with nonionic micellar phases is the technique of choice to work with immobilized enzyme reactors and biological samples. Although this feature was not used in this work, it is possible to inject biological samples directly in the chromatographic system [13]. It was demonstrated that the nonionic micelles were not only very gentle for the selected enzymes but they even maintained their activity longer than with aqueous buffers. The LODs obtained for amino acids and cholesterol derivatives were comparable or lower than those obtained using classical hydro-organic mobile phases. These results prove that MLC should not be systematically turned down because it is not able to generate a high number of chromatographic plates. Selectivity (thermodynamics) is often more important than efficiency (kinetics). MLC is able to separate solutes widely differing in hydrophobicity such as methionine and tryptophan. In the particular example described here, it is not required to have a very highly efficient chromatographic system since the enzyme reactor and the detector cell produced some inherent post column band broadening.

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