

# Simultaneous Determination of Cadmium (II) and Zinc (II) by Molecular Fluorescence Spectroscopy and Multiple Linear Regression Using an Anthrylpentaazamacrocyclic Chemosensor

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**Abstract** The work that is reported here concerns a method that allows the simultaneous determination of cadmium (II) and zinc (II) in aqueous solution by molecular fluorescence spectroscopy using 9-(1',4',7',10',13'-pentaazacyclopentadecyl)-methylanthracene. For this chemosensor, the fluorophore  $\pi$ -system is insulated from an azacrown donor by one methylene group. A self-quenching mechanism, resulting from an electron transfer from the nitrogens of the azacrown to the excited aromatic system, essentially precludes fluorescence emission. Fluorescence is restored when cadmium (II) or zinc (II) are chelated by the macrocycle. The difference between the emission spectra profiles of the free chemosensor, the cadmium and the zinc chelates is such that the concentration determination of the two metals and the remaining free chemosensor is possible

at the nanomolar scale in only one experiment using a multiple linear regression algorithm. Usefulness and convenience of this simple method is proven by steady state and kinetic quantitative determination experiments.

**Keywords** Cadmium · Zinc · Molecular fluorescence spectroscopy · Chemosensor · Multivariate analysis

## Introduction

The topic of environmental and health effects of the heavy metals is in its infancy, but developing fast. Cadmium (II) is known to be teratogen and antagonist to some essential elements. The competition of cadmium (II) with zinc (II) can induce biological diseases. However, in open-ocean areas with extremely low heavy-metal concentration and lack of essential elements, cadmium assisted enzymatic processes can be found in some marine organism [24]. A better understanding of these effects requires heavy metal determination by analytical techniques endowed with the necessary sensitivity and accuracy. While analytical techniques, with the necessary sensitivity and accuracy, are available, but are often time-consuming off-line methods (ICP-MS/GF-AAS) [1], optical techniques based on molecular spectroscopy can offer distinct advantages given their simplicity, high sensitivity, selectivity, ease of automation and the absence of physical contact between the detection system and the analytes.

The pioneering work of de Silva and de Silva [2] has offered a new approach to the determination of metal cations using molecular fluorescence spectroscopy. Although developed on methanolic solutions, the authors introduced a novel sensing method based on the alteration

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of anthracene luminescence via the chelation state of a pendant azacrown ether group.

It is in 1990 that Czarnik and his co-workers adapted this approach by synthesizing water soluble anthrylazamacrocycles [3] (Fig. 1). These compounds display fluorescence intensity variations upon addition of various metal cations (cadmium, zinc, lead, mercury...). In alkaline conditions, the fluorescence yield of anthrylazamacrocycles is low due to electron transfer from the amines to the excited fluorophore [4]. Fluorescence can be restored by involving the amine lone pairs in bonding either by protonation or by metal cation chelation (Chelation-Enhanced Fluorescence—CHEF). Besides metal chelation, some cations such as lead and mercury can quench fluorescence by heavy atom effect (Chelation-Enhanced Quenching—CHEQ).

Among the anthrylazamacrocycles, 9-(1',4',7',10',13'-pentaazacyclopentadecyl)-methylanthracene (Fig. 1, with  $x=4$ ) uniquely demonstrates a perturbation of the fluorophore emission spectrum when chelated to cadmium. In this particular case Czarnik revealed the presence of more than one chelate in aqueous solution by NMR studies [5].

The present paper describes the application of multiple linear regression (MLR) to the steady state fluorescence spectrometry of 9-(1',4',7',10',13'-pentaazacyclopentadecyl)-methylanthracene for the simultaneous determination of cadmium and zinc at the nanomolar scale. Flexibility of this approach is further proved and illustrated by using this method to understand an experimental drawback encountered during sampling optimization.

## Experimental

### Instrumentation

Fluorescence measurements were recorded on a Shimadzu RF-5001 PC spectrometer. The excitation source was a 150-W steady-state Xenon lamp. The molar coefficient of extinction for the chemosensor and its metal chelates is about  $8700 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$  at 370 nm. Absorbance below 500 nm is thus linear as a function of concentration. Fluorescence excitation was performed at 370 nm and emission was collected from 380 to 600 nm with 1 nm

steps. Methacrylate fluorescence cells ( $1 \times 1 \times 4 \text{ cm}^3$ ) were purchased from Aldrich Chemical.

### Reagents

Labware was cleaned using a concentrated 1:3  $\text{HNO}_3/\text{HCl}$  mixture then thoroughly rinsed with 18 M $\Omega$ .cm water produced by a Milli-Q water system. The pH of the solutions was measured with an Ankersmit 420 A pH meter calibrated at pH 7.00 and pH 10.01 (buffer solutions purchased from Aldrich Chemical). The fluorescence measurements were made at pH 10.0 using a FIXANAL borate buffer from Riedel de Haen.

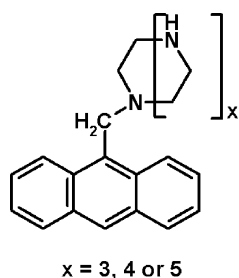
### Synthesis

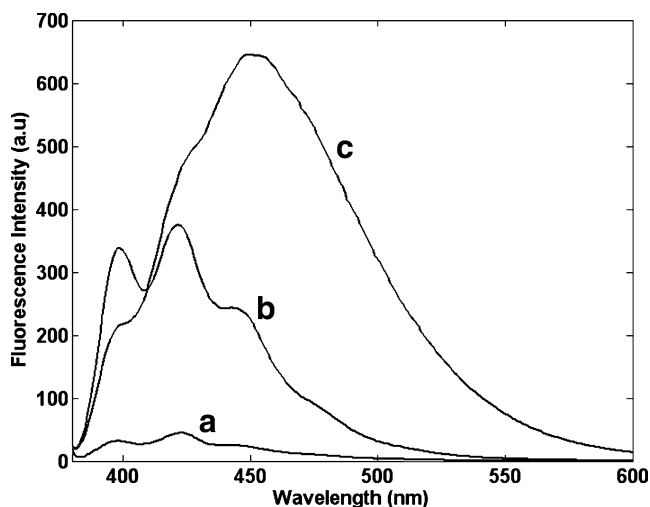
9-(Chloromethyl)anthracene and metal salts were purchased from Aldrich Chemical Co. 1, 4, 7, 10, 13-Pentaazacyclopentadecane was synthesized according to a literature procedure [6–11]. Pentaazacyclopentadecane in its free base form (m.p. 94–96 °C) was obtained with a yield of 42% (molar ratio) when starting from tetratosylated triethylenetetraamine and tritosylated diethanolamine. 9-(1',4',7',10',13'-pentaazacyclopentadecyl)-methylanthracene was synthesized by the reaction of 9-(Chloromethyl)anthracene with an excess of 1, 4, 7, 10, 13-pentaazacyclopentadecane by following the literature procedure [3, 12, 13]. The raw product of this reaction is purified by re-precipitation in distilled ethanol using small amounts of hydrochloric acid 32% pro analysis (Merck). This purification step has to be performed at least twice to significantly reduce the presence of un-reacted pentaazacyclopentadecane. The yield of this reaction is 45% (molar ratio). The product was characterized with mass spectrometry and with NMR  $^1\text{H}$  (250 MHz,  $\text{D}_2\text{O}$ ):  $\delta$  3.06–3.17 ppm (20H, ms,  $\text{CH}_2$ ),  $\delta$  3.35 ppm (s, attributed to the presence of 0.0008 equivalent of 1,4,7,10,13-pentaazacyclopentadecane),  $\delta$  4.42 ppm (2H, s,  $\text{CH}_2$ ),  $\delta$  7.55–7.69 ppm (4H, ms, CH arom.),  $\delta$  8.04 ppm (2H, d, CH arom.),  $\delta$  8.15 ppm (2H, d, CH arom.),  $\delta$  8.42 ppm (1H, s, CH arom.). The melting point could not be determined: the 9-(1',4',7',10',13'-pentaazacyclopentadecyl)-methylanthracene degrades itself at about 180 °C.

### Sampling procedure

Sample preparation was found to be a key step for the metal determination at the nanomolar scale. For example, the metal ions have to be chelated by the chemosensor at neutral pH, before rising the pH to 10 to avoid their conversion into oxidized species such as  $\text{HCdO}_2^-$ ,  $\text{Cd}(\text{OH})_2$ ,  $\text{HZnO}_2^-$ ,  $\text{ZnO}_2^{2-}$  and  $\text{Zn}(\text{OH})_2$  [14]. Furthermore, metal precipitation on glass was found to be the most limiting drawback as it

**Fig. 1** Molecular structure of anthrylazamacrocyclic chemosensors





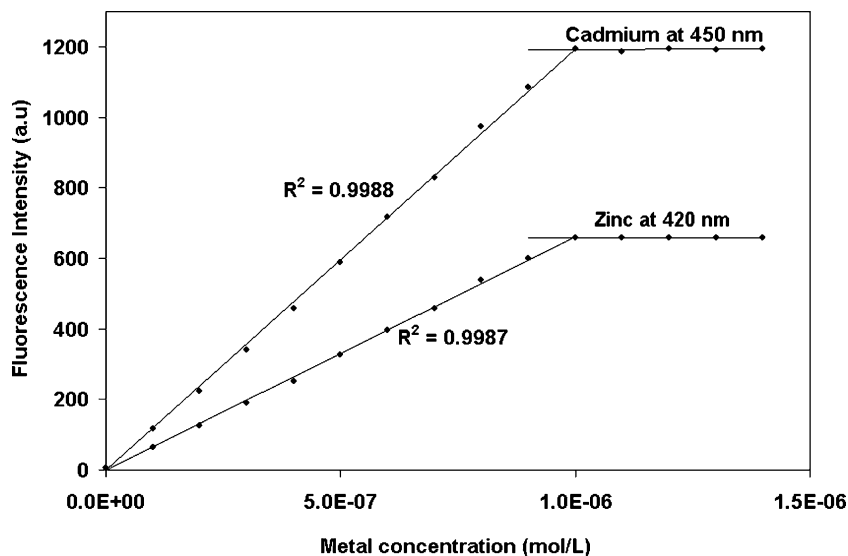
**Fig. 2** Steady-state fluorescence spectrum of **a** 500 nM of anthrylpentaazamacrocyclic, **b** with 500 nM of  $Zn^{2+}$  and **c** with 500 nM of  $Cd^{2+}$ . Excitation wavelength, 370 nm

will be proven later. After many sampling optimizations [12], repeatability of the measurements has been largely improved by using the following procedure: 3 ml samples are directly prepared in disposable methacrylate fluorescence cells using Eppendorf micropipettes (20–200 and 100–1,000  $\mu$ l) and the following sequence: (1) addition of the free chemosensor solution, (2) addition of a metal standard solution, (3) filling to 2.7 ml with MilliQ ultrapure water, (4) addition of 300  $\mu$ l of pH 10 buffer solution, (4) recording of the steady-state fluorescence emission without delays.

#### Programming and calculations

Programming and calculations are performed on an Intel Pentium II 233 MHz computer running under windows 98

**Fig. 3** Titration curves for the chemosensor at 1  $\mu$ M with increasing  $Cd^{2+}$  or  $Zn^{2+}$  concentrations. Excitation wavelength, 370 nm; emission wavelengths, 450 and 420 nm for  $Cd^{2+}$  and  $Zn^{2+}$  respectively



environment and using Matlab 4.0. Multiple linear regression is always performed from 380 to 600 nm with 1 nm step (221 wavelengths).

## Results and discussion

### Emission behaviour at pH 10

The steady-state fluorescence emission spectra of the anthrylpentaazamacrocyclic and its  $Cd^{2+}$  and  $Zn^{2+}$  chelates are shown in Fig. 2. As shown previously [5], the fluorescence intensity of the free chemosensor is low at pH 10 but can strongly be increased when saturated with  $Cd^{2+}$  or  $Zn^{2+}$ .

Since the binding constants of 1, 4, 7, 10, 13-pentaazacyclodecane are high for  $Cd^{2+}$  and  $Zn^{2+}$  ( $\log K_{ML}=19.2$  and  $19.1$  respectively) [15] and even though these values might be affected by the bound fluorophore, it can be observed, in Fig. 3, that at pH 10, the chemosensor fluorescence increases linearly as a function of the metal concentration until equivalence.

From a simple equilibrium model involving the metal, the remaining free chemosensor and the formed chelate concentrations, the linearity of the titration curves allow estimating the anthrylpentaazamacrocyclic binding constants,  $K_{ML}$ , for the two metals as being higher than  $10^9 M^{-1}$ .

The spectral intensities, profiles variation and the linear titration curves allow assessing the use of an adequate chemometric method for the simultaneous determination of the two metals using the entire spectral emission range for higher accuracy. Among various methods, multiple linear regression is chosen for its simplicity and because from the beginning, spectral identities are known and measurable.

Orthogonal projection algorithm for multiple linear regression (MLR)

The chosen method should allow extracting the analytes concentrations starting from a fluorescence spectrum obtained on a sample containing both metals and a given amount of chemosensor [16–18]. If the total metal concentration ( $[Cd^{++}] + [Zn^{++}]$ ) is lower than the total chemosensor's one, the obtained measurement is a composite spectrum containing three contribution: fluorescence of the free remaining chemosensor and of the formed  $Cd^{++}$  and  $Zn^{++}$  chemosensor's chelates (Fig. 2).

If a fluorescence spectrum is composite and its spectral components profiles are known and linearly independent, decomposition into weighted spectral components can efficiently be done provided that the original fluorescence spectrum is a linear combination of its spectral components. This condition is frequently encountered in systems governed by Beer–Lambert's law.

The composite spectrum can be formalized as a vector that is belonging to the hyperspace subtended by the spectral components vectors. However, as spectra are experimental measurements containing noise, this situation is practically never met. The composite spectrum will always be slightly out of the spectral components vectors hyperspace. One approach is to find the most similar or the nearest vector that belongs to the spectral components vectors hyperspace. This least square approach is solved by finding the orthogonal projection of the composite spectrum vector into the spectral components hyperspace.

Suppose a composite spectrum in the form of a column vector  $Y(n \times 1)$  containing steady-state fluorescence intensities measured at  $n$  different wavelengths and  $X(n \times p)$  a matrix containing  $p$  column vectors each representing a spectral component profile measured at the same wavelengths. Then  $Y$  is related to  $X$  through Eq. 1 with  $\beta(p \times 1)$  a column vector containing  $p$  weighting factors and  $\varepsilon(n \times 1)$  the error vector (essentially noise) that is the difference vector between  $Y$  and its orthogonal projection  $Z$ .

$$Y = X\beta + \varepsilon \quad (1)$$

Equation 1: Relation between a composite spectrum and its spectral components

If  $X^T X$  is non singular, it can easily be shown that minimizing the modulus of  $\varepsilon$  (the distance between  $Y$  and  $Z$ ) allows to find  $b(p \times 1)$  the least square estimate of  $\beta$  with the orthogonal projection operator  $(X^T X)^{-1} X^T$  in Eq. 2.

$$b = (X^T X)^{-1} X^T Y \quad (2)$$

Equation 2: Orthogonal projection operation

For the spectroscopist this technique is very useful to determine  $b$ , the amount of different contributions (e.g.

molar fractions), when analyzing a composite spectrum  $Y$  in terms of known spectral components  $X$ .

If the model is correct, the mean square of the residual is a good estimation of the residual variance  $\sigma^2$  (Eq. 3).

$$\sigma^2 \approx s^2 = \frac{\sum_{i=1}^n (Y_i - Z_i)^2}{n - p} \quad \text{with } Z = bX \quad (3)$$

Equation 3: Residual variance

One can then estimate  $V(p \times p)$  by Eq. 4 whose diagonal values  $V_{11}$  to  $V_{pp}$  are the variances on each values of vector  $b$ .

$$V = (X^T X)^{-1} \sigma^2 \quad (4)$$

Equation 4: Determination of the variance–covariance on  $b_i$

Simultaneous determination of cadmium (II) and zinc (II)

A concentration of 500 nM has been chosen for the chemosensor to range the measurements from 0 to 500 nM for the total metal concentration ( $[Cd^{++}] + [Zn^{++}]$ ). In this way, three species are emitting: the free remaining chemosensor and the formed  $Cd^{++}$  and  $Zn^{++}$  chelates. Matrix  $X$  is build using the steady-state emission fluorescence spectra of these three species at a concentration of 500 nM (Fig. 2). In this way, analyzing a composite spectrum taken on a sample containing a total metal concentration lower than 500 nM, will give weighting factors (matrix  $b$ ) that corresponds to molar fraction of each emitting species with  $\sum_{i=1}^p b_i \approx 1$ .

Linear independence of the three spectra of Fig. 2, composing  $X$ , can be checked by calculating their mutual correlation coefficient (Table 1).

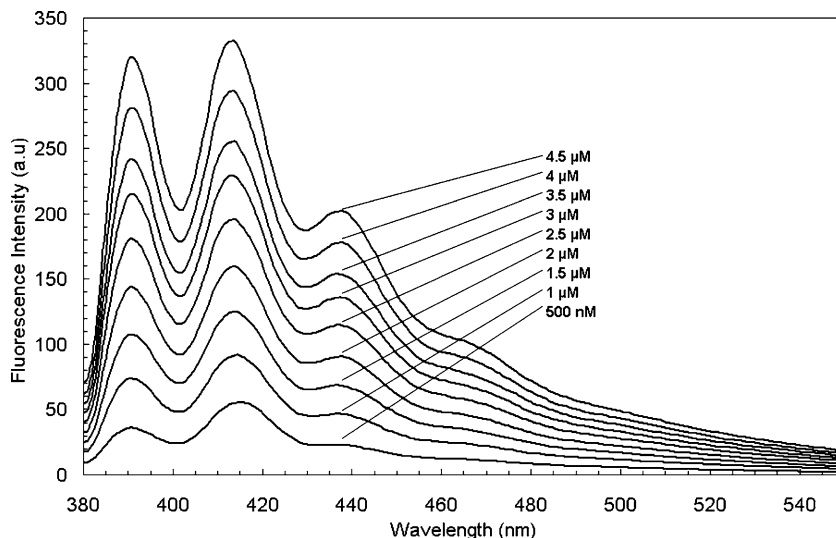
These correlation coefficients give information on the spectral resemblances. Even though there is a strong correlation between the free chemosensor and the  $Zn^{++}$  chelate spectra, the approach will still give an acceptable accuracy for  $Zn^{++}$  determination because of the low fluorescence intensity of the free chemosensor.

Given our experimental procedure, the condition  $\sum_{i=1}^p b_i = 1$  could be imposed to constraint the model. However, this constraint is not considered because it is restrictive in the eventuality of small experimental errors on

**Table 1** Mutual correlation coefficient calculated on spectra of Fig. 2

	Free chemosensor	$Zn^{++}$ chelate	$Cd^{++}$ chelate
Free chemosensor	1	0.9884	0.5376
$Zn^{++}$ chelate	0.9884	1	0.5662
$Cd^{++}$ chelate	0.5376	0.5662	1

**Fig. 4** Steady-state fluorescence measurements of the chemosensor at different concentrations



the total chemosensor’s concentration. Without this constraint, small errors are allowed on the added chemosensor quantity. Moreover, it is observed that this constraint is already slightly imposed by background hidden signals.

Indeed, extrapolation of the signals measured in Fig. 4 at a null chemosensor’s concentration and the measurement of a pH 10 buffered water sample in Fig. 5 only reveal the presence of water’s Raman signal corresponding to the stretching of OH bond at  $3,400\text{ cm}^{-1}$  [19].

As this Raman band is present in every measurements and as we have chosen our experimental procedure in order to obtain molar fractions, this hidden signal slightly imposes the condition  $\sum_{i=1}^p b_i = 1$  on the 400 to 440 nm spectral region.

To illustrate the MLR approach, Fig. 6 shows the spectral decomposition of a sample containing 500 nM of chemosensor, 125 nM of  $\text{Cd}^{++}$  and 125 nM of  $\text{Zn}^{++}$  into weighted components spectra.

**Fig. 5** Extrapolation of spectra from Fig. 4 to a null chemosensor’s concentration and steady-state fluorescence spectrum of a pH 10 buffered water sample

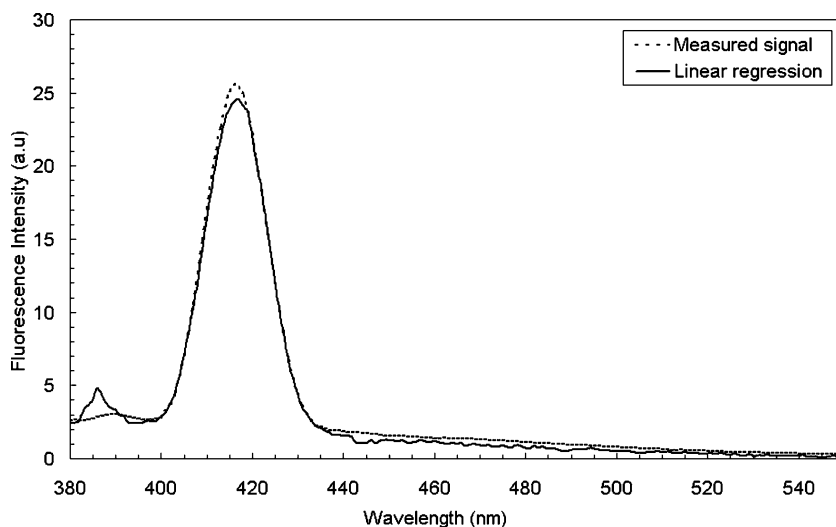
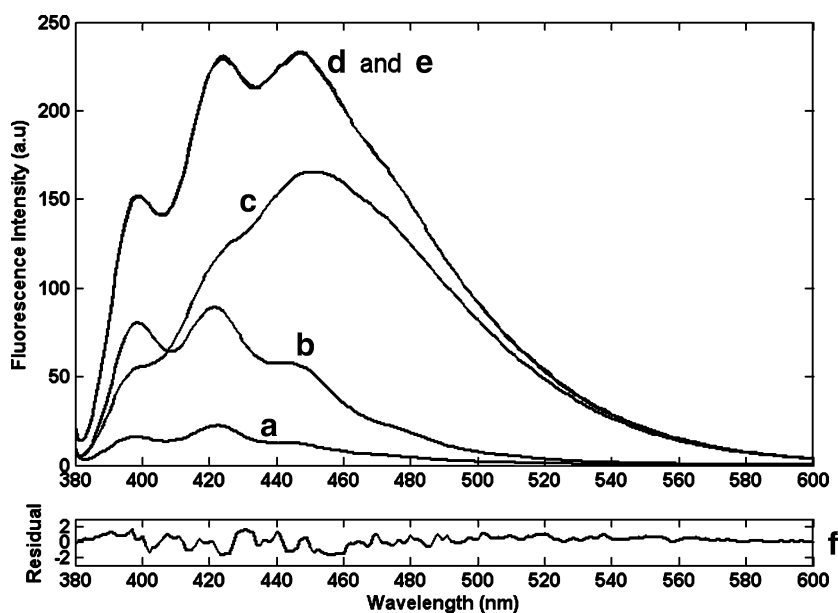


Table 2 gathers determination experiments realized at different contents of  $\text{Cd}^{++}$  and  $\text{Zn}^{++}$ . The example of Fig. 6 is the first example of Table 2.

Experiments from Fig. 6 and Table 2 clearly shows the effectiveness of  $\text{Cd}^{++}$  and  $\text{Zn}^{++}$  simultaneous determination with 9-(1',4',7',10',13'-pentaazacyclopentadecyl)-methylanthracene. It is important to note here that the confidence intervals are derived from variances (Eq. 4) corresponding to uncertainties on the instrumental and measurement spectral decomposition (for example noise) and not on experimental errors encountered during the sampling procedure. This is why some of the analytical concentrations do not belong to the determined confidence intervals. This observation suggests that experimental errors arising from sample preparation are much higher than for the spectral decomposition operation.

The detection limit is then evaluated as three times the standard deviation on ten blank measurements (500 nM of

**Fig. 6** Decomposition of a composite spectrum (**d**) into its weighted spectral components. **a** Free remaining chemosensor, **b** Zn<sup>++</sup> chelate, **c** Cd<sup>++</sup> chelate and **e** sum of **a**, **b** and **c**. **f** residuals calculated by subtracting **d** from **e**



chemosensor at pH 10). These detection limits reach 5.9 and 2.5 nM for Zn<sup>++</sup> and Cd<sup>++</sup> respectively.

Finally, as last example of application, this method was used to investigate an experimental drawback encountered during sampling optimization. The use of quartz fluores-

cence cells at low metal concentration was found to be a major source of instability of the samples.

Figure 7 shows the variation of the steady-state fluorescence for a sample initially containing 140 nM of remaining free chemosensor, 120 nM of Cd<sup>++</sup> chelate and 240 nM of Zn<sup>++</sup> chelate placed in a quartz fluorescence cell.

Each spectrum from Fig. 7 can be decomposed through multiple linear regression to inspect each specie concentration variation as a function of time in Fig. 8.

Figure 8 clearly shows a significant metal chelates decomposition with a release of the chemosensor during the experiment. This effect was checked several times and found not to be photochemical. It was interpreted as a slow equilibrium displacement with metal precipitation on the cell's walls [20]. This instability was then avoided using methacrylate fluorescence cells.

**Table 2** Comparison between analytical and determined Cd<sup>++</sup> and Zn<sup>++</sup> concentrations

Analytical concentration (nM)			Determined concentration (nM) with 95% confidence intervals		
Remaining chemosensor	Zn <sup>++</sup>	Cd <sup>++</sup>	Remaining chemosensor	Zn <sup>++</sup>	Cd <sup>++</sup>
250	125	125	250±29	119±3	128±0.3
250	0	250	240±40	1±4	264±0.3
250	250	0	250±29	258±3	-0.5±0.3
375	62.5	62.5	400±26	68.7±2.8	62.22±0.24
440	30	30	415±15	41±5	28.40±0.13
250	187.5	62.5	253±20	192.1±2.1	65.03±0.22
375	93.75	31.25	373±19	91.6±2.1	30.72±0.21
437.5	46.87	15.63	428±11	55.4±1.1	14.82±0.17
250	62.5	187.5	260±40	64±5	178.5±0.4
375	31.25	93.75	380±30	30±3	85.7±0.3
437.5	15.63	46.87	467±14	12.2±1.5	42.10±0.14
300	200	0	311±22	209.7±2.1	-0.44±0.22
300	0	200	340±50	0±5	190.6±0.5
300	100	100	320±40	114±4	96.3±0.3
490	10	0	489±8	12±8	0.12±0.13
300	200	0	333±21	197.4±2.1	-0.20±0.21
490	0	10	489±10	-3±13	11.42±0.12
80	400	20	100±50	394±4	18.1±0.3
100	200	200	120±50	193±4	196.7±0.4

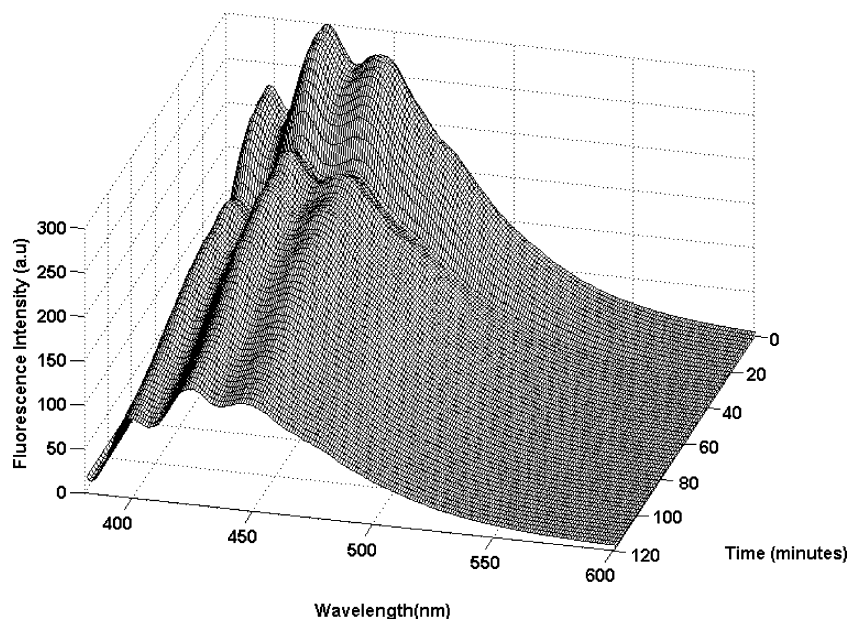
Confidence intervals are determined from Eq. 4

## Conclusions

It was found that the simultaneous determination of Cd<sup>++</sup> and Zn<sup>++</sup> can efficiently be performed using 9-(1',4',7',10',13'-pentaazacyclopentadecyl)-methylanthracene using MLR on a wide spectral region (from 380 to 600 nm). The detection limits were evaluated at 5.9 and 2.5 nM for Zn<sup>++</sup> and Cd<sup>++</sup> respectively. Even though the instrumental confidence intervals and the experimental detection limit are higher for Zn<sup>++</sup> than for Cd<sup>++</sup> this situation is not limiting this approach especially considering that Zn<sup>++</sup> content is generally higher than Cd<sup>++</sup> in natural waters.

The important correlation between the free chemosensor and the Zn<sup>++</sup> chelate spectral profiles was not found to be

**Fig. 7** Kinetic evolution of steady-state fluorescence for a sample prepared in a quartz fluorescence cell. [free chemosensor]=140 nM, [Cd<sup>++</sup> chelate]=120 nM, and [Zn<sup>++</sup> chelate]=240 nM



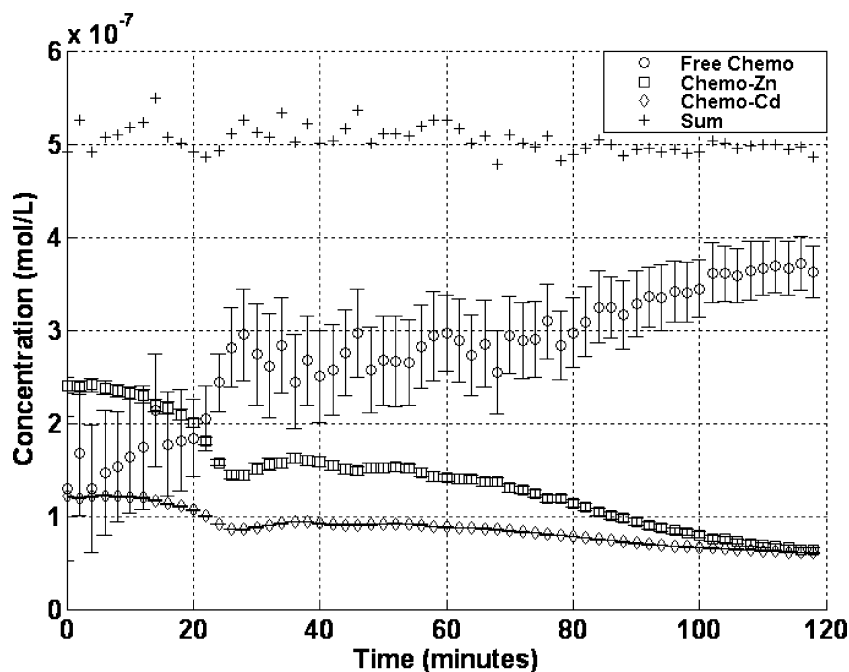
limiting for their respective determination. This situation can however be optimized by performing the multiple linear regression on a limited number of wavelengths that decreases the correlation between these two spectra or using other methods like PCR (principal component regression). Another optimization for MLR is to refine the spectral components profiles each time an unknown spectrum is decomposed into its components [16].

It was found in Table 2 that regression coefficients can reach negative values, however this situation arose only when the content of one of the analytes was null.

The kinetic measurements of Figs. 7 and 8 have shown that adequate sampling is of crucial importance when determining metal concentration at the nanomolar level. These experiments have not only given valuable information concerning sampling optimization. This type of approach could be adapted to follow for example metal speciation in natural waters [21].

While it was considered useless to use the same strategy for other chemosensors presenting only low profile variations, application of this method to 9-(1',4',7',10'-tetraazacyclododecyl)-methylanthracene (Fig. 1, with  $x=3$ ) and to

**Fig. 8** Temporal evolution of the different emitting species found by MLR of spectra from Fig. 7 with 95% confidence intervals



9-(1',4',7',10',13',16'-hexaazacyclooctadecyl)-methylantracene (Fig. 1, with  $x=5$ ) was also found to be efficient for the same analytes [12].

This method has numerous interesting perspectives such as coupling to metal preconcentration methods [22] and inclusion into an automated flow injection analysis [23] or microfluidic device.

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