



## Evidence of Ternary Inclusion Complexes Formation Using Factorial Design and Determination of Their Formation Constant

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### Abstract

Evidence of the interaction among the  $[\text{PbAc}]^+$  and  $[\text{PbEDTA}]^{-2}$  with the  $\beta$ -cyclodextrin ( $\beta$ -CD) is shown, using a  $2^4$  factorial design. The factors used were the  $[\text{Pb(II)}]$ ,  $[\beta\text{-CD}]$ ,  $[\text{Ac}^-]$  and  $[\text{EDTA}]$  concentrations at pH = 6.2. The statistical results indicate that the changes in chemical response for these species are associated to the interaction among these factors. Spectrophotometric and electrochemical studies were undertaken to assess the interactions. The equilibrium constants for inclusion complexes were determined For  $\beta\text{-CD-}[\text{PbAc}]^+$  and  $\beta\text{-CD-}[\text{PbEDTA}]^{-2}$ .

### Introduction

Recently, the study of cyclodextrins (CDs) has grown steadily, mainly because they are characteristically interesting molecules: it is worthy of mention, their capacity to form inclusion complexes of the “host–guest” type [1–3]. Such a capacity of the CD's ( $\alpha$ ,  $\beta$ , and  $\gamma$  CD's) is attributed to their molecular structure, which resembles a truncated cone with a cavity capable of acting as receptor. This characteristic gives rise to some interactions with the guests, which may take place at two levels of interaction: the first one, within the hydrophobic cavity and the other one on the hydrophilic plane of the CD OH groups [4].

The interest to study inclusion complexes using CDs, stems from a number of important changes that are induced on the properties of the guest molecules, such as: increments of solubility, of chemical reactivity and structural conformation in solution while promoting a decrease in volatility, among others. That is why the cyclodextrins have a wide field of application in the chemical industry, for the enzymatic catalysis in the biochemical industry and as biosensors, to name but a few ones [1–9]. The CDs have also been used for soil remediation, because they are effective in eliminating contaminant species, apart from being non polluting and biodegradable [10].

In view of such characteristics it is important to obtain information related to the physicochemical properties of inclusion complexes, like their stoichiometry, molecular geometry and thermodynamics, and in particular the formation constants ( $K_{\text{CD:Guest}}$ ). These are necessary to explain the stability of such systems, basically because of their high inherent application potential in diverse branches of chemistry [11].

The  $K_{\text{CD:Guest}}$  usually have low values ( $10^{0.5}$ – $10^8 \text{ M}^{-1}$ ) and the interactions which predominate on the association processes of these complexes are: (1) electrostatic interactions, (2) hydrogen bonds, (3) Van der Waals-type forces, (4) hydrophobic effect, among others [12–13].

The chemical interactions in inclusion complexes are weak and it is common to find small variations in the chemical response of the host–guest system [14, 15]. Therefore, it becomes necessary to insure that such changes are not related to random or experimental errors.

Optimisation conditions for different experiments, with liquid chromatography or capillary electrophoresis [16–23], the validation of analytical methods [24–26], the identification of factors that largely influence the reversibility of redox reactions [27], among others, have been successfully undertaken using statistical methods for experimental design and factorial design optimisation, because they are capable of rendering relevant information from a limited number of experiments.

For the reasons above, in order to confirm that the changes in chemical responses are directly related to the analytes' chemical interactions with the  $\beta$ -cyclodextrin, the aim of the present work is to study this system using the factorial design. The assessment of the factors is carried out with a spectrophotometric study of the  $[\text{Pb(II)}]$  system, varying its chemical environment by modifying the coordination shell in order to favour the interaction between the CD's hydrophobic cavity and the guest, so that the stability of the inclusion complex increases. After this study the inclusion complex constant was determined.

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Table 1. 2<sup>4</sup> factorial design to study inclusion complexes

[Pb(II)]	Factors			Experimental values	
	[ $\beta$ -CD]	[Ac]	[EDTA]	Response (R)	A <sup>241 nm</sup>
-1	-1	-1	-1	y1	0.9649
+1	-1	-1	-1	y2	1.2953
-1	+1	-1	-1	y3	1.0037
-1	-1	+1	-1	y4	0.9520
-1	-1	-1	+1	y5	1.0875
-1	-1	+1	+1	y6	1.0025
+1	-1	-1	+1	y7	1.9409
-1	+1	+1	-1	y8	0.9670
+1	+1	-1	-1	y9	1.2268
+1	-1	+1	-1	y10	1.3803
-1	+1	-1	+1	y11	1.0679
+1	+1	+1	-1	y12	1.4170
+1	+1	-1	+1	y13	1.8831
+1	-1	+1	+1	y14	1.8259
-1	+1	+1	+1	y15	0.9914
+1	+1	+1	+1	y16	2.1956

Factor	Level (-1)	Level (+1)	Factor	Level (-1)	Level (+1)
[Pb(II)] M	1 × 10 <sup>-4</sup> M	2 × 10 <sup>-4</sup> M	[Ac-]	0.024 M	0.1 M
[ $\beta$ -CD] M	1 × 10 <sup>-3</sup> M	1 × 10 <sup>-2</sup> M	[EDTA]	1 × 10 <sup>-4</sup> M	2 × 10 <sup>-4</sup> M

## Experimental

### Reagents

The solutions were prepared with analytical grade reagents and deionised water (18.2 m $\Omega$ ) from a MilliQ deioniser. The sodium acetate buffer solution (Ac<sup>-</sup>) at pH 6.2 was prepared from CH<sub>3</sub>COOH (J.T. Baker) and the pH was adjusted with NaOH (Sigma). The  $\beta$ -CD and the sodium ethylenediaminetetraacetate (Na<sub>2</sub>EDTA) were supplied by Sigma and the lead acetate Pb(CH<sub>3</sub>COO)<sub>2</sub> by Baker, S. A.

### Instruments

A Tacussel potentiometer LPH430T fitted with a glass counter electrode was used to adjust the solution's pH. A Perkin-Elmer Lambda-20 UV-VIS Spectrophotometer was used to obtain the absorption spectra within the 300-200 wavelength range with a scan rate of 960 nm/s using 1 cm optical path quartz cells.

Cyclic voltammetry was carried out using a BAS-100W potentiostat and a three-electrode electrochemical cell, with a mercury sulphate electrode (SME) reference electrode; a graphite rod was used as auxiliary and a carbon paste electrode as working electrode. The cyclic voltammograms (CV) were obtained applying a potential perturbation in the cathodic direction within the -1.5 to -0.7 V/SMS, potential range starting from the null current potential ( $E_{i=0}$ ) at a scan rate of 0.1 V/s.

The carbon paste electrode (CPE) was prepared from a 1 : 1 ratio mix containing 99.99% purity single crystal grade graphite (Alfa AESAR) and nujol oil (Fluka), having sufficient ease for handling. The paste is inserted into a PVC tube

and is compacted by means of pressure to help eliminating trapped air; afterwards an electronic copper conductor is inserted before the paste sets in [28]. It is relevant to mention, that the surface of the electrode was renovated after each potential scan.

### Thermodynamic study

The existence of Pb(II) complexes with acetate ion and EDTA have been previously reported [29-30]. With this information the distribution diagrams of chemical species were calculated using the program MEDUSA [31].

Figure 1 shows that the [PbAc]<sup>+</sup> and [PbEDTA]<sup>-2</sup> species predominate at pH = 6.2 in the acetates media 0.024 M.

### Experimental design

The experimental design used corresponds to the 2<sup>4</sup> factorial, where the base indicates the two working levels adopted, a low one (-) and a high one (+); in this case the concentration for all factors. The 4 indicates the number of factors or variables to be studied, such as the: [Pb(II)], [ $\beta$ -CD], [Ac<sup>-</sup>] and [EDTA] concentrations. The concentrations selected are shown in Table 1, and so are the responses obtained to a wavelength of 241 nm (A<sup>241</sup>) from the spectrophotometric study for each solution. In all the experiments the pH was maintained constant at 6.2 with acetate buffer.

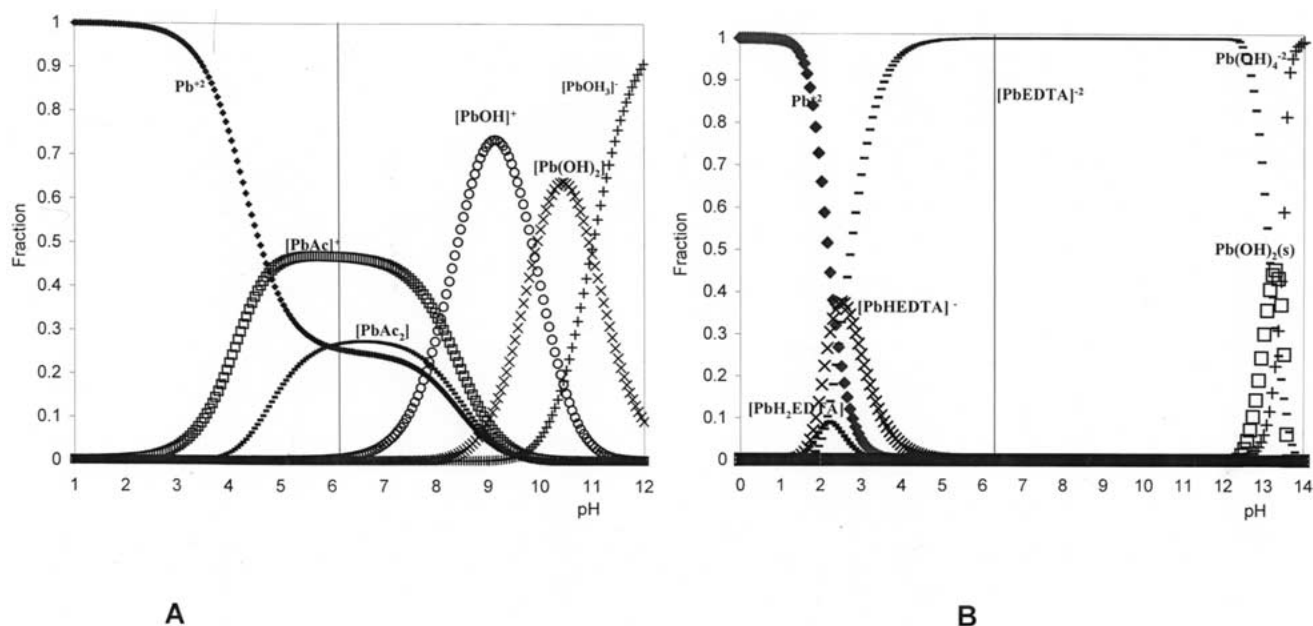


Figure 1. Distributions diagrams of: (A) Pb(II) species in 0.024 M acetate and (B) Pb(II) species with EDTA in 0.024 M acetate.

## Results and discussion

### Analysis of the variance for the 2<sup>4</sup> experimental design

From the spectrophotometric results obtained, an analysis of the variance was carried out for the factorial design, where the Yates' algorithm was used [32, 33]. The F test was applied to the data, thus obtaining a calculated F ( $F_{cal}$ ) for each factor and the interactions, and was compared with the critical F ( $F_{crit}$ ) obtained from the tables, generally at 95% [32].

The results indicate that:

If  $F_{cal} > F_{crit}$ , then the factor is mainly due to the factors involved,

If  $F_{cal} < F_{crit}$ , then the action of the factor falls within the random errors.

The effects associated to the main factors and the effects produced by the interactions between them are shown in Table 2.

In accordance with the spectrophotometric results obtained and from the  $F_{cal}$  values, it was found that factors having a significant effect are: Pb(II), CD, EDTA and the interactions that occur are:  $[Pb][Ac]$ ,  $[Pb][EDTA]$ ,  $[Ac][CD]$ ,  $[Ac][CD][Pb]$  and  $[Pb][CD][EDTA]$ .

As can be observed in Table 2, the larger values of  $F_{cal}$  correspond to Pb(II), CD and EDTA as compared to  $F_{crit}$ . This is associated to the interactions presented by these factors.

The interactions from the most important factors are associated to the  $[Pb][Ac]$  and  $[Pb][EDTA]$  complexes, where the  $F_{cal}$  value for  $[Pb][EDTA]$  is larger than for  $[Pb][Ac]$ . If we compare these results with those reported in the literature, one finds that there exists agreement for the chemical interaction of the species, for  $[Pb][EDTA]$  is  $F_{cal} = 91.1$  and  $pKc = 18.1$ , while for  $[Pb][Ac]$   $F_{cal} = 6.33$  and its is  $pKc$

Table 2. Principal effects and interactions of the factors for factorial design from the responses by  $A^{241\text{ nm}}$

Factor/response	$F_{cal}$	$F_{crit}$
Principal effects		2.352
[Pb(II)]	326.429	
[CD]	32.306	
[EDTA]	136.664	
Interactions between two factors		
[Pb][Ac]	6.330	
[Pb][EDTA]	91.110	
[CD][Ac]	4.070	
Interactions between three factors		
[Pb][CD][Ac]	4.576	
[Pb][CD][EDTA]	2.669	

= 2.68, which would show that  $F_{cal}$  can be associated to the strength of the chemical species interaction [29].

The interaction between  $[Ac][CD]$  is also important as it can be related to formation of an acetate inclusion complex with the  $\beta$ -CD, even if in the literature there does not exist a reported equilibrium constant, there is information regarding the interaction among the carboxylic groups with the CD's [14].

From the interaction among three factors, it was found that  $[Pb][EDTA][CD]$  and  $[Pb][Ac][CD]$  are significant to changes in chemical response. It can be observed that  $F_{cal}$  for these interactions are only slightly larger than  $F_{crit}$  and can be associated to the formation of an inclusion complex. The experimental results that follow, clearly give confirmatory evidence of the statement.

Different solutions were prepared to bring an additional reference point for comparison which would support further

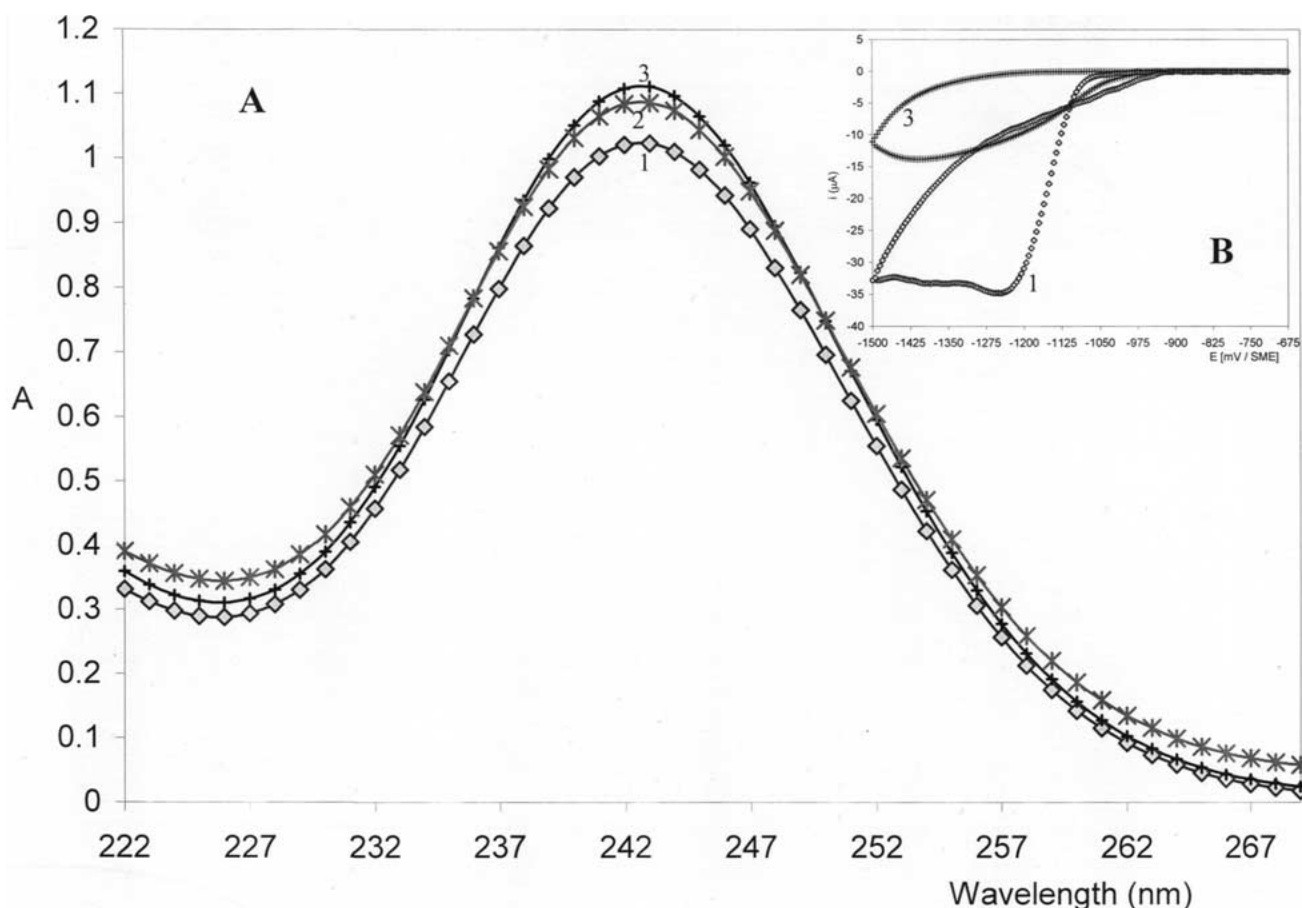


Figure 2. (A) Absorption spectra of the  $1 \times 10^{-4}$  M  $[\text{PbEDTA}]^{-2}$  solution: (1) in the absence of  $\beta$ -CD, (2) with  $1 \times 10^{-3}$  M  $\beta$ -CD and (3) with  $1 \times 10^{-2}$  M  $\beta$ -CD, in acetate media 0.024 M at pH 6.2. (B) Cyclic voltammograms of the  $1 \times 10^{-4}$  M  $[\text{PbEDTA}]^{-2}$  solution: (2) in the absence of  $\beta$ -CD and (3) with  $1 \times 10^{-2}$  M  $\beta$ -CD. Cathodic scan rate: 100 mV/s.

our analysis of the responses obtained in the factorial design. The results obtained for the complex  $[\text{PbEDTA}]^{-2}$  in the absence and presence of  $\beta$ -CD, are shown in Figure 2. The spectra show a well defined absorption band with a maximum wavelength at 242 nm that corresponds to the complex  $[\text{PbEDTA}]^{-2}$  (Figure 2A), while in the presence of  $\beta$ -CD the absorption band maximum increases, which shows the formation of an inclusion complex  $\beta$ -CD.

The cyclic voltammograms shown in the inset in Figure 2B, depict the electrochemical behaviour of the  $[\text{PbEDTA}]^{-2}$ ; there is one reduction wave that is clearly associated to this complex that decreases significantly when the  $\beta$ -CD is present in solution. The observed shift in the reduction process is attributed to the interaction between the Pb(II) and the  $\beta$ -CD.

Regarding the interaction of the complex  $[\text{PbAc}]^{+}$  with the  $\beta$ -CD, Figure 3 shows the absorption spectra and the cyclic voltammograms in the  $[\text{PbAc}]^{+}$  complex solutions with and without the  $\beta$ -CD. The spectra shown in Figure 3A depict the form of the absorption band having a maximum wavelength at 228 nm, which corresponds to the complex  $[\text{PbAc}]^{+}$ . When the  $\beta$ -CD is present, the maximum for the absorption band of  $[\text{PbAc}]^{+}$  shifts slightly (2 nm) hypsochromically with increased absorbance.

The cyclic voltammograms of the  $[\text{PbAc}]^{+}$  solutions shown in Figure 3B, display two reduction peaks having a cathodic peak potential ( $E_{\text{pc}}$ ) of  $-1.1$  V and  $-1.18$  V, respectively. When the  $[\text{PbAc}]^{+}$  solutions have  $\beta$ -CD, it can be observed that the reduction is shifted to more cathodic potentials, which constitutes evidence that there exists an interaction between the species of the Pb(II) and the CD, thereby forming an inclusion complex. Therefore, the interaction between the  $[\text{Pb}][\text{Ac}][\text{CD}]$  and  $[\text{Pb}][\text{CD}][\text{EDTA}]$  are confirmed. Furthermore, it must be underlined that the  $F_{\text{cal}}$  values for these complexes are only slightly greater than for  $F_{\text{crit}}$ . As can be expected, the results point out at the fact that the forces intervening in the formation of inclusion complexes are weak interactions, as indicated previously.

In order to corroborate the results obtained above, we carried out the determination of the constants value.

#### Determination of the formation constant of the inclusion complex of Pb(II) with $\beta$ -CD

In order to determine the Pb-ligand- $\beta$ -CD interaction, two solutions were prepared in the electrolyte supported 0.024 M at pH = 6.2. The first one was Pb(II) at  $1 \times 10^{-4}$  M and the second contained Pb(II) and  $1 \times 10^{-4}$  M EDTA. Afterwards  $\beta$ -CD  $4 \times 10^{-3}$  M was added successively

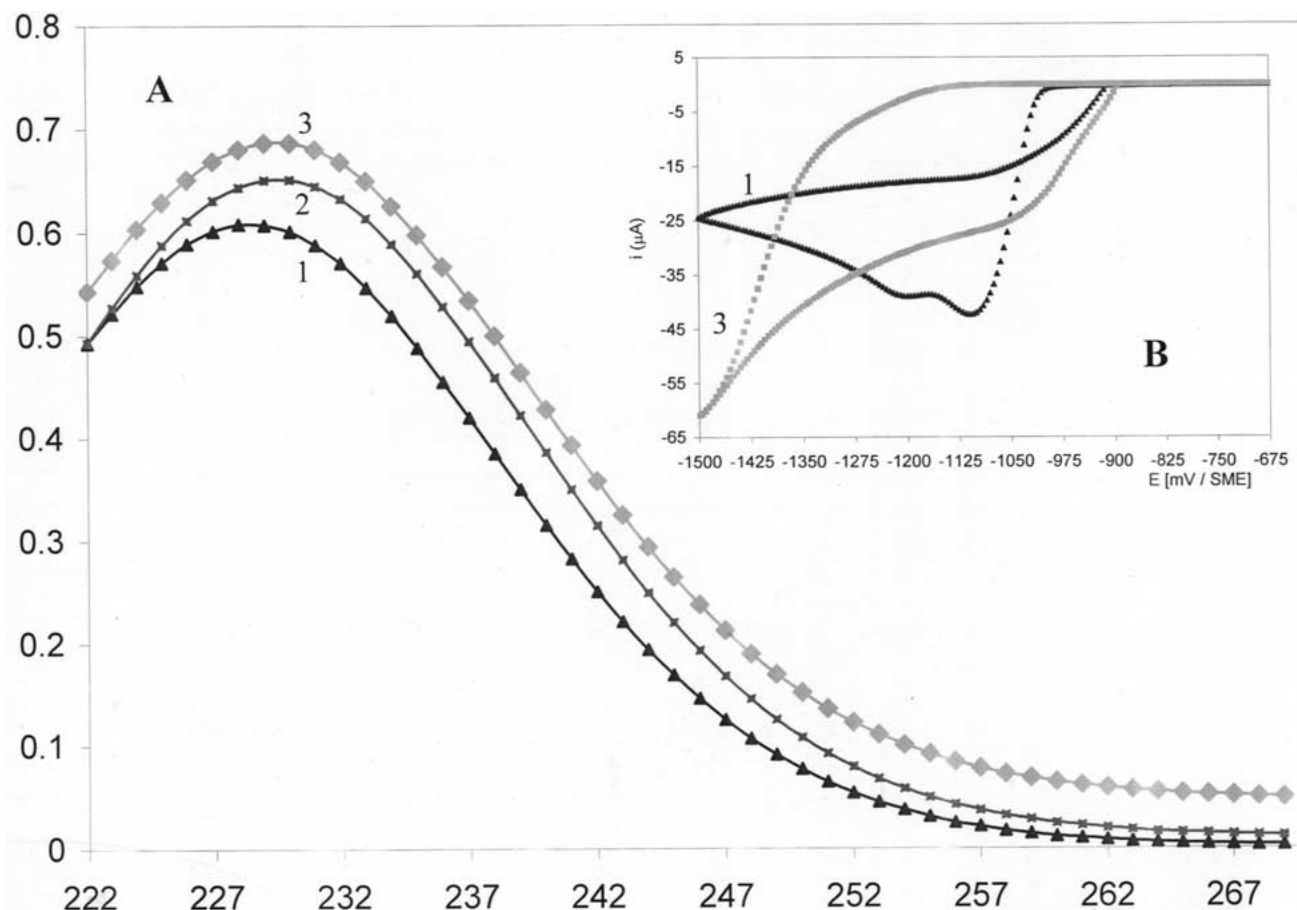


Figure 3. (A) Absorption spectra of the  $1 \times 10^{-4}$  M  $[\text{PbAc}]^+$  solution: (1) in the absence of  $\beta$ -CD, (2) with  $1 \times 10^{-3}$  M  $\beta$ -CD and (3) with  $1 \times 10^{-2}$  M  $\beta$ -CD, in acetate media 0.024 M at pH 6.2. (B) Cyclic voltammograms of the  $1 \times 10^{-4}$  M  $[\text{PbAc}]^+$  solutions: (1) in the absence of  $\beta$ -CD and (3) with  $1 \times 10^{-2}$  M  $\beta$ -CD. Cathodic scan rate: 100 mV/s.

Table 3. Constants of formation of the inclusion complexes

Equilibrium	Log $\beta$	$\text{p}K_{\text{CD:Guest}}$	$\sigma^a$	$U^b$
$[\text{PbAc}]^+ + \beta\text{-CD} \rightleftharpoons [\text{PbAc}]^+\text{-}\beta\text{-CD}$	$3.21 \pm 3.1 \times 10^{-2}$	$1.6 \times 10^3 \pm 1.07$	$6.73 \times 10^{-3}$	$3.1 \times 10^{-2}$
$[\text{PbEDTA}]^{-2} + \beta\text{-CD} \rightleftharpoons [\text{PbEDTA}]^{-2}\text{-}\beta\text{-CD}$	$2.56 \pm 3.1 \times 10^{-2}$	$3.7 \times 10^2 \pm 1.07$	$5.33 \times 10^{-3}$	$3.1 \times 10^{-2}$

<sup>a</sup>  $\sigma$  is standard deviation.

<sup>b</sup>  $U$  is sum of squares.

and the spectrophotometric measurements were undertaken subsequently.

With the spectra obtained and with the computational program Squad [34–37] the formation constant  $\log \beta$  of the inclusion complexes was calculated. The program was fed with 24 absorption spectra in the range 210–250 nm with 1152 wavelength data points for each one.

The best refinement was observed with 1 : 1 stoichiometric equilibria. The values for the formation constants are reported in Table 3.

Sometimes a model which has been well-refined does not ensure a good fit to the experimental results: therefore the absorption spectra were simulated using formation constants and the absorptivity coefficients given by Squad program. Some theoretical and simulated absorption spectra for both inclusion complexes are presented in Figure 4. The theoret-

ical data properly fitted the experimental ones, therefore the constants obtained are reliable.

From a comparison of the constants obtained for  $[\text{PbAc}]^+\text{-}\beta\text{-CD}$  and  $[\text{PbEDTA}]^{-2}\text{-}\beta\text{-CD}$  with the calculated  $F_{\text{cal}}$  for the  $[\text{Pb}][\text{Ac}][\text{CD}]$  and  $[\text{Pb}][\text{EDTA}][\text{CD}]$  a good agreement is observed. The  $F_{\text{cal}}$  for  $[\text{Pb}][\text{Ac}][\text{CD}]$  was 4.576 and the  $\text{p}K_{\text{inc}} = 1.6 \times 10^3 \pm 1.07$  and  $F_{\text{cal}}$  for  $[\text{Pb}][\text{EDTA}][\text{CD}]$  was 2.669 and the  $\text{p}K_{\text{inc}} = 3.7 \times 10^2 \pm 1.07$ . The The aforementioned results confirm that the  $F_{\text{cal}}$  can be associated to the chemical interactions occurring and that in the case of inclusion complexes they are weak.

## Conclusions

It has been shown that the experimental design can be applied not only to optimized experimental conditions, and also be used to determine if there exist interactions among

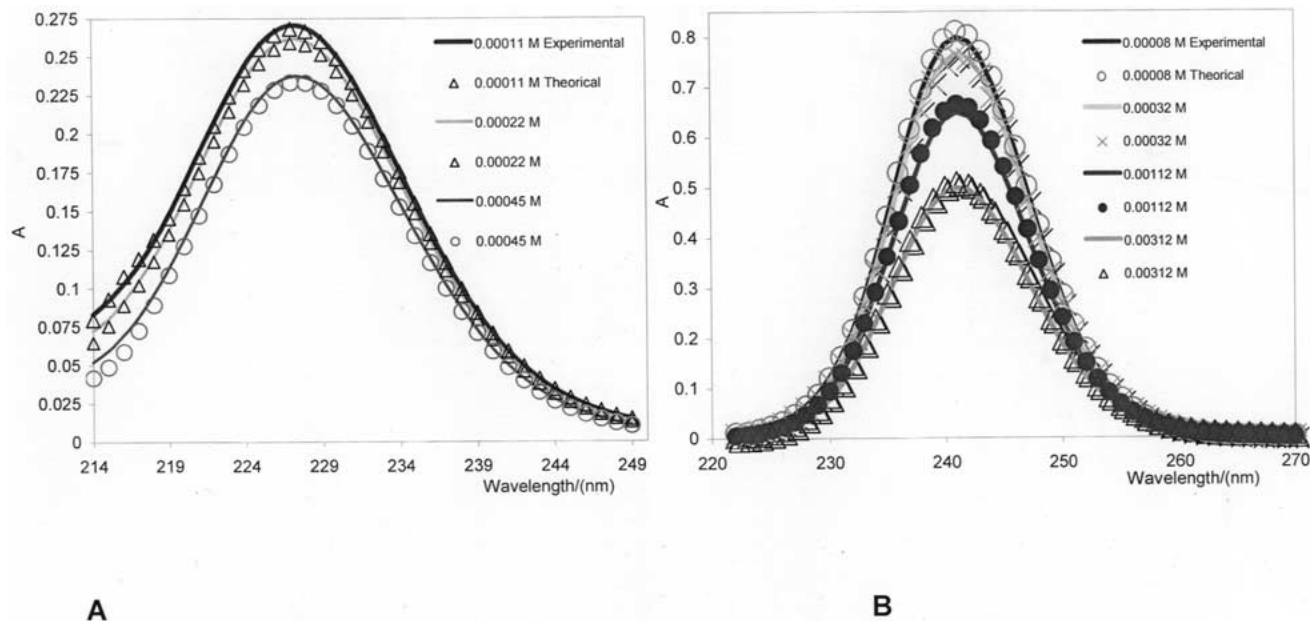


Figure 4. Absorption spectra experimental (continuous line; —) and simulated (with the marking) for (A)  $1 \times 10^{-4}$  M  $[PbAc]^+$  solutions for different  $\beta$ -CD concentrations. (B)  $1 \times 10^{-4}$  M  $[PbEDTA]^{-2}$  solutions for different  $\beta$ -CD concentrations. In acetate media 0.024 M at pH 6.2.

chemical species. The results obtained in the variance analysis for the interactions among the factors were associated to interaction forces for the formation of complexes, comparing them with the complexes pK formation values, in the case  $F_{cal} > F_{crit}$ .

From to the factorial design results and the electrochemical study it was shown the existence of the  $[Pb][Ac]$ ,  $[Pb][EDTA]$ ,  $[Ac][CD]$ ,  $[Pb][Ac][CD]$  and  $[Pb][EDTA][CD]$  interactions.

The  $[Pb][Ac]$ ,  $[Pb][EDTA]$  and  $[Ac][CD]$  have previously been studied, but the  $[Pb][Ac][CD]$  and  $[Pb][EDTA][CD]$  were not known calculated. The aforementioned interactions were evidenced by the  $pK_{CD-Guest}$  for the ternary inclusion complexes by a spectrophotometric study and the Squad program. The stoichiometric relation that fitted best the experimental results was 1:1. For the  $[PbAc]^+-\beta$ -CD complex a  $pK_{CD:Guest} = 1.6 \times 10^3 \pm 1.07$  was obtained and for the  $[Pb][EDTA][CD]$  it was  $pK_{CD:Guest} = 3.7 \times 10^2 \pm 1.07$ .

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### References

- J. Clarke, J. Coates, and S. Lincoln: *Adv. Carbon. Chem. and Biochem.* **46**, 205 (1988).
- F. Scharinger and Z. Unter: *Nahr: V Genusm* **6**, 865 (1903).
- K. Freunderberg, E. Schanf, G. Dumpert, and T. Ploetz: *Naturwissenschaften* **27**, 850 (1939).
- N. Schaschke, S. Fiori, E. Weyher, Ch. Escrieut, D. Fourmy, G. Müller, and L. Moroder: *J. Am. Chem. Soc.* **120**, 7030 (1998).
- R. McCandless and S.H. Yalkowsky: *J. Pharm. Sci.* **87**(12), 1639 (1998).
- L. Azente, J. Szejtli, and G.L. Kis: *J. Pharm. Sci.* **87**, 778 (1998).
- B. Bhesh, B.R. D'Arcy, and L. Thi Bich: *J. Agric. Food. Chem.* **46**, 1494 (1998).
- K. Suzane, Schreyer and S.R. Mikkelse: *Bioconjugate Chem.* **10**, 464 (1999).
- A. Kaifer, P.A. Quintela, and J.M. Schuette: *J. Inclusion. Phenom.* **7**, 107 (1989).
- Seok-OH Ko, M.A. Schlautman, and E.R. Carraway: *Environ. Sci. Technol.* **33**, 2765 (1999).
- Á. Buvári and L. Barcza: *J. Incl. Phenom.* **7**, 379 (1989).
- I. Seiji, S. Hada, S. Neya, and N. Funasaki: *J. Phys. Chem. B* **103**, 1208 (1999).
- M.V. Rekharsky and Y. Inoue: *Chem. Rev.* **98**, 1875 (1998).
- S. Miertus, V. Frecer, E. Chiellini, F. Chiellini, R. Solaro, and J. Tomasi: *J. Incl. Phenom.* **32**, 23 (1998).
- R. Retna and R. Ramaraj: *Electrochemical Acta* **44**, 2685 (1999).
- M. Nechar, M.F. Molina, and J.M. Bosque-Sendra: *Analytica Chimica Acta* **382**, 117 (1999).
- B. Izgi, C. Demir U, and S. Gücer: *Spectrochimica Acta Part B* **55**, 971 (2000).
- M.A.L. de Oliveira, G.A. Micke, R.E. Bruns, and M.F.M. Tavares: *Journal of Chromatography A* **924**, 533 (2001).
- A.G. González: *Analytica Chimica Acta* **36**, 227 (1998).
- R. Ragonese, M. Mulholland, and J. Kalman: *Journal of Chromatography A* **870**, 45 (2000).
- S.A. Wood, J.M. Long, R.J. Simmonds, J.W. Bridges, and D. Stevenson: *Journal of Pharmaceutical and Biomedical Analysis* **16**, 231 (1997).
- S. Mikaeli, G. Thorsen, and B. Karlberg: *Journal of Chromatography A* **907**, 267 (2001).
- C. Reis, J.C. de Andrade, R.E. Bruns, and R.C.C.P. Moran: *Analytica Chimica Acta* **369**, 269 (1998).
- Y.V. Heyden, F. Questier, and D.L. Massart: *Journal of Pharmaceutical and Biomedical Analysis* **17**, 153 (1998).
- E. Cagiga, L. González, R.M. Alonso, and R.M. Jiménez: *Talanta* **54**, 1121 (2001).
- A. Nijhuis, H.C.M. Van der Knaap, S. de Jong, and B.G.M. Vandeginste: *Analytica Chimica Acta* **391**, 187 (1999).

27. R. Rocha, S. S. Rosatto, R.E. Bruns, and L.T. Kubota: *J. Electroanalytical Chem.* **433**, 73 (1997).
28. R. Martínez, M. T. Ramírez, and I. González: *Electroanalysis* **10**(5), 336 (1998).
29. M.R. Smith, Martell: *Critical Stability Constants*, 1st edn, Plenum Press (1974).
30. C. Baes and R. Mesmer: in Robert E. Krieger (ed.), *The Hydrolysis of Cations*, Malabar, FL (1986) pp. 362–365.
31. I. Puigdomenech: *Hydrochemical Equilibrium Constants Database (MEDUSA)*, Royal Institute of Technology, Stockholm, Sweden (1997).
32. J.C. Miller and J.N. Miller: *Estadística para química analítica*, 2nd edn, Addison-Westey Iberoamericana (1993).
33. C. Montgomery, Douglas: *Design and Analysis of Experimental*, 3rd edn, John Wiley & Sons (1991).
34. D. Legget: *Computational Methods for the Determination of Formation Constants*, Plenum, New York (1995).
35. M.A. Villegas, D. Bonilla, M.T. Ramírez, and A. Rojas: *Recent Res Devel. In Pure & Applied Anal. Chem.* **1**, 1 (1998).
36. M.T. Ramírez, A. Rojas, and I. González: *Talanta* **44**, 31 (1997).
37. P. Balderas, M.T. Ramírez, A. Rojas, and A. Gutiérrez: *Talanta* **46**, 1439 (1998).

