



Downscaling conventional methods for the spectrophotometric study of metal ions complexation

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ABSTRACT

One of the major challenges in a chemistry laboratory is the continuous search for a more sustainable practice, following Green Chemistry guidelines. The present work was devoted to the development of a high throughput and miniaturized strategy for chemistry conventional protocols for the study of complex formation of metallic compounds. In this scenario, the spectrophotometric calibration curve method and the Job's method of continuous variation were adapted to the microplate format. This work involved several metal ions (Co(II), Cu(II), Cd(II), Fe(III), Mn(II), Ni(II), Pb(II) and Zn(II)) and two different complexing agents, 2-Carboxy-2'-hydroxy-5'-sulfoformazyl-benzene (Zincon) and 4-(2-Pyridylazo)resorcinol (PAR). Microplate assays demonstrated to be useful tools for the investigation of the equilibrium process of metal ion complexes. Additionally, these methods involved lower volume of reagents and the analysis throughput was augmented when compared with conventional strategies.

1. Introduction

Spectrophotometric assays play an important role in everyday laboratory practice. High throughput assays became indispensable at a laboratory of any field of interest such as environment, biochemistry, biotechnology, food quality or safety. Therefore, it is no longer practicable to use large size laboratory glassware for routine assays when hundreds of samples are needed to be analyzed within a short time frame. However, implementing these classical manipulations on a smaller scale, with less operator manipulation (miniaturization and automatization), is a challenge nowadays. Additionally, one of the main goals in a chemistry laboratory is to reach a more sustainable practice by decreasing the usage of resources and, if possible, resort to low toxicity compounds, which can also be achieved by miniaturization.

So, it is of high interest to address the challenges associated with Green Chemistry [1]. In this scenario, the main objective of this work was to demonstrate the possibility to develop low reagent consumption protocols for classical chemistry methods applied to the study of metal ions complexes.

The present work summarizes some spectrophotometric-based experimental protocols for the study of colored complex formation of metallic cations. In this scenario, the calibration curve method and the

Job's method of continuous variation were adapted to the microplate format.

In biological-related analysis, for miniaturized spectrophotometric assays, a microplate reader is an indispensable equipment. Therefore, it could be of high interest to use this type of platform as a tool for the spectrophotometric study of chemical reactions (stoichiometry studies) or in conventional analytical chemistry methodologies such as calibration curve, in order to minimize the consumption of reagents, time spent per analysis, and minimize the use of lab material.

A calibration is a comparative protocol based on the preparation of standard solutions or calibration standards, with a well-known analyte quantity or concentration. In a spectrophotometric calibration, the absorbance/transmittance of each standard solution is measured, and related to the analyte concentration. By plotting the signal against the standards concentration, the calibration curve graph with the respective linear regression is established. The linear regression is then used to determine, by interpolation, the concentration of test samples. It is recommended that the concentration of the test samples fit the standards concentration range of the calibration curve, and also the matrix composition of the standard solutions and test samples should be similar [2–4].

The Job's method, also known as the method of continuous variation

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Table 1
Listing of the metal standard solutions.

Metal		Concentration (mg L ⁻¹)
Cd	Merck	1000 ± 2
Co	BDH	1000 ± 2
Cu	Aldrich	1002 ± 2
Fe	Spectrosol	998 ± 2
Mn	Spectrosol	1000 ± 2
Ni	Spectrosol	1000 ± 2
Zn	Merck	999 ± 2

Table 2
Conditions used for a 96-well microplate assay for the calibration curve method study. Concentrations of complexing agent (PAR or Zincon - R) and metal (Me) are displayed. To each well, 50 µL of complexing agent, 100 µL of metal solution and 100 µL of buffer solution, was added (final well volume of 250 µL).

Well*	[R] µM	[Me] µM
A1 - D1	20.0	0.00
A2 - D2	20.0	1.00
A3 - D3	20.0	2.00
A4 - D4	20.0	3.00
A5 - D5	20.0	4.00
A6 - D6	20.0	5.00
A7 - D7	20.0	0.00
A8 - D8	20.0	6.00
A9 - D9	20.0	7.00
A10 - D10	20.0	8.00
A11 - D11	20.0	9.00
A12 - D12	20.0	0.00

* Microplate wells from E1 to H12 were used for another metal and the same strategy of volumes was applied.

or Job Plot, is a protocol developed by Paul Job in 1928 [5]. This method is employed to determine the stoichiometry of a given chemical reaction, that is, to assess the ratio of the involved chemical species.

To illustrate the capability of using this technique for the above-mentioned methods, the study in this work involved two different metal complexing agents: 2-Carboxy-2'-hydroxy-5'-sulfoformazyl-benzene (Zincon) and 4-(2-Pyridylazo)resorcinol (PAR) (Figure S1).

Zincon is a well-known and non-selective reagent for the photometric determination of metal ions, that forms a blue complex at different pHs depending of the metal in study [6], and is used on metal ions determination in a variety of samples, such as environmental or biological. PAR is also a non-selective reagent that is also used for the colorimetric determination of different metals, forming a reddish complex [7].

2. Material and methods

2.1. Reagents and chemicals

All solutions were prepared with ultrapure water obtained from Sartorius Arium® pro system. A 100 µM solution of 2-carboxy-2'-hydroxy-5'-sulfoformazyl-benzene (Zincon reagent - C₂₀H₁₅N₄NaO₆S•H₂O, Merck, Germany) was prepared by dissolving the corresponding quantity of the solid in water. A 100 µM solution of 4-(2-Pyridylazo)resorcinol (PAR reagent - C₁₁H₈N₃NaO₂•H₂O, Sigma-Aldrich, Germany) was prepared by dissolving the corresponding quantity of the monosodium hydrate salt in water.

A 0.5 M boric acid buffer (Merck, Germany) was prepared by dissolution of the corresponding quantity of solid in a 0.22 M solution of NaOH (Fluka, Germany) and the pH was adjusted to 9.0 or 10.0 with NaOH solution.

All metal ion solutions were prepared from the corresponding atomic absorption standard solution: nitrate salt form in acidic media (0.5 mol L⁻¹ HNO₃). A stock solution of 100 µM of each metal (Table 1) was

Table 3
Conditions used for a 96-well microplate assay for the study of the stoichiometry of complexometric reaction resorting to the Job's method. The added volumes of complexing agent (PAR or Zincon - R) and metal solution (Me) are displayed; 100 µL of buffer solution was added to each microplate well (final volume of 250 µL).

Well*	Volume R (µL)	Volume Me (µL)	[R] µM	[Me] µM	[Me]/[R]
A1 - D1	150.0	0.0	60.0	0.0	0.00
A2 - D2	140.0	10.0	56.0	4.0	0.07
A3 - D3	135.0	15.0	54.0	6.0	0.11
A4 - D4	125.0	25.0	50.0	10.0	0.20
A5 - D5	115.0	35.0	46.0	14.0	0.30
A6 - D6	105.0	45.0	42.0	18.0	0.43
A7 - D7	100.0	50.0	40.0	20.0	0.50
A8 - D8	95.0	55.0	38.0	22.0	0.58
A9 - D9	90.0	60.0	36.0	24.0	0.67
A10 - D10	85.0	65.0	34.0	26.0	0.76
A11 - D11	80.0	70.0	32.0	28.0	0.88
A12 - D12	75.0	75.0	30.0	30.0	1.00

* Microplate wells from E1 to H12 were used to another metal and the same strategy of volumes was applied.

prepared by dilution of the 1000 mg L⁻¹ atomic absorption standard solution. Working standard solutions were prepared by dilution of the stock solutions.

2.2. Apparatus

A non-sterile and untreated 96-well microplate (Corning Inc., USA) was used. All microplates were prepared with a multichannel pipette and tips (12-well; Gilson, USA). A Multidetector microplate reader (Synergy HT; Bio-Tek Instruments, USA) was used and controlled by Gen5 software (Bio-Tek Instruments, USA).

The microplate reader was set up to run absorption spectra for full plate from 400 to 700 nm at 5 nm intervals at room temperature for Zincon-Metal complexes and from 400 to 550 nm at 5 nm intervals at room temperature for PAR-Metal complexes.

2.3. Calibration curve method

For the calibration curve, the concentration of PAR or Zincon was kept constant, and the concentration of each metal varied from 0 to 9 µM (Table 2). The maximum absorption wavelength of each complex was used for absorbance readings.

2.4. Job's method

The Job's method is an analytical approach used for the determination of the stoichiometry of two interacting chemical species. In this method, the total molar concentration of the mixture was kept constant, while the individual molar concentration ratios of the two components varied (Table 3). For the study of the Job's method, the maximum absorption wavelength of each complex was used.

3. Results and discussion

The main objective of this work was to develop a downscale approach of classical chemistry protocols for the study of metal ions complexes. The study was performed with eight metal ions solutions (cobalt, copper, cadmium, iron, manganese, nickel, lead, and zinc) and two complexing agents (Zincon and PAR).

Some preliminary studies were performed to evaluate and assess the interference of some chemical and physical parameters on each reaction. A spectrum at different pHs, from 5.0 to 10.5, was traced; the influence of the volume of buffer was tested; the order of addition of reagents to the microplate wells was also evaluated.

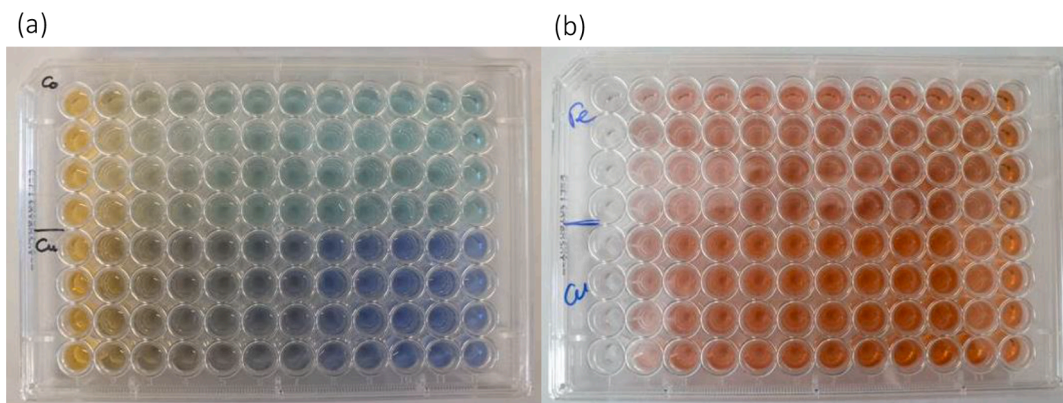


Fig. 1. Typical Microplate assembly for a calibration study using Zincon as complexing agent (a) and using PAR as complexing agent (b).

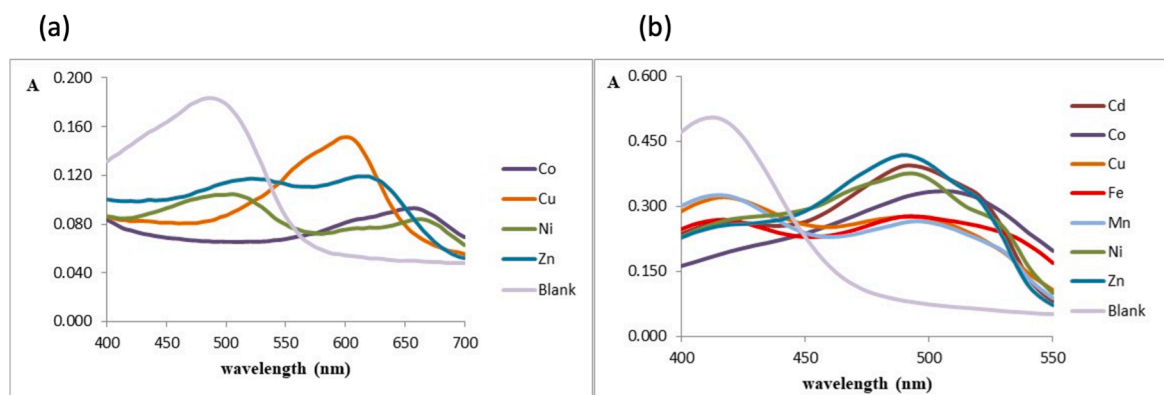


Fig. 2. Schematic representation of the Metal-Zincon complexes spectra at a pH 9 (a), and Metal-PAR complexes spectra at pH 10 (b); the metal ion concentration was 6 μM .

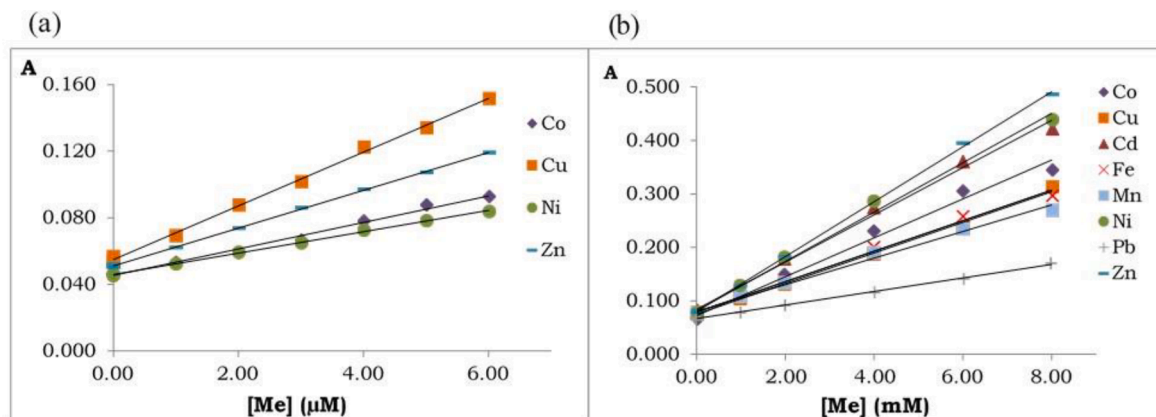


Fig. 3. Calibration curve for each metal with a different complexing agent. a – Zincon as complexing agent at pH 9; b – PAR as complexing agent at pH 10.

Zincon formed a blue coloured complex with cobalt, copper, nickel and zinc; PAR formed red/orange coloured complexes with cobalt, copper, cadmium, iron, manganese, nickel, lead and zinc (Fig. 1).

Overall, as expected, by resorting to the microplate strategy for the study of metal complexes reactions, the time, and reagents volume spent was lower when compared with conventional spectrophotometric methods. The final volume of solution in each microplate well, comprising the metal, complexing agent and buffer solutions, was 250 μL . And so, the solutions volumes needed per assay were low, and consequently the effluent production was also minimized. By resorting to this technique, the analysis of 96 assays in a short time-period was

accomplished.

For the Metal-Zincon complexes, we found that the solution pH that provided the highest absorbance signal was 9.0 and, for the Metal-PAR complexes, the pH was 10 (Fig. 2).

The influence of the order of addition of reagents to the microplate wells was evaluated. The highest absorbance value was reached more rapidly when the order of addition of the solutions was: metal standard, complexing agent and buffer solutions, respectively. The metal-reagent complex was stable for at least 14 h.

For each analysis, the absorbance mean and relative standard deviation was calculated from four consecutive replicates; this value was

Table 4

Calibration curves for each set of Metal-Complexing agent and the respective limits of detection (LOD). λ - maximum absorption wavelength for each set Metal-Complexing agent, A – Absorbance, [Me] – metal ion concentration (μM), r^2 - coefficient of determination.

Complexing agent	Metal	λ (nm)	Typical calibration curve $A = \text{slope} \times [\text{Me}] + \text{intercept}$	r^2	LOD (μM)
PAR	Cd	490	$A = 0.047 [\text{Cd}] + 0.081$	0.9993	0.1
	Co	510	$A = 0.040 [\text{Co}] + 0.069$	0.9995	0.1
	Cu	495	$A = 0.029 [\text{Cu}] + 0.075$	0.996	0.2
	Fe	495	$A = 0.031 [\text{Fe}] + 0.076$	0.9996	0.2
	Mn	495	$A = 0.027 [\text{Mn}] + 0.078$	0.997	0.1
	Ni	495	$A = 0.060 [\text{Ni}] + 0.071$	0.991	0.2
	Zn	490	$A = 0.051 [\text{Zn}] + 0.080$	0.9995	0.1
Zincon	Co	655	$A = 0.008 [\text{Co}] + 0.045$	0.987	0.7
	Cu	600	$A = 0.015 [\text{Cu}] + 0.055$	0.9987	0.8
	Ni	625	$A = 0.0064 [\text{Ni}] + 0.046$	0.9988	0.6
	Zn	620	$A = 0.011 [\text{Zn}] + 0.051$	0.9994	0.5

below 2.5% for all the studied assays, indicating a good repeatability of these methodologies.

3.1. Calibration curve method

A linear calibration curve was obtained for each set of Metal-Complexing agent at the respective reaction optimized pH and at the wavelength of maximum absorbance for each metal complex (Fig. 3).

The calibration curves, as well the wavelength of maximum absorbance and the limit of detection (LOD) for each set of metal and complexing agent were summarized in Table 4.

The LOD was calculated according to IUPAC recommendations [8,9] as the concentration corresponding to three times the standard deviation of the mean absorbance of ten consecutive blank measurements.

A higher sensitivity/slope in the calibration curve and a lower limit of detection were achieved using PAR as colourimetric agent. However, it displayed less selectivity, forming coloured complexes with all the metals in study, when compared with Zincon.

3.2. Job's method

Resorting to the Job's method of continuous variation, the absorbance signal is plotted against the molar ratio of the two reagents in study ($[\text{Metal}]/[\text{Complexing agent}]$). By analyzing the graph, it is possible to determine the stoichiometry of the complexometric reaction by the interception of the two trendlines (Figs. 4 and 5).

The stoichiometry of each reaction event is summarized on Table 5; the IUPAC reference for the stoichiometry is also presented [10]. The interception of the two trendlines for all sets of Metal-PAR is around the ratio of 0.33, corresponding to a 1Metal:3Complexing agent stoichiometry at the conditions set in this work. For the Metal:Zincon complexes the ratio is around 0.50, meaning that the stoichiometry of this complexing reactions is 1Metal:2Complexing Agent. In this work, at the conditions that were set, the stoichiometry for both complexing agents differ from those referred by IUPAC. The conditions of the study could be

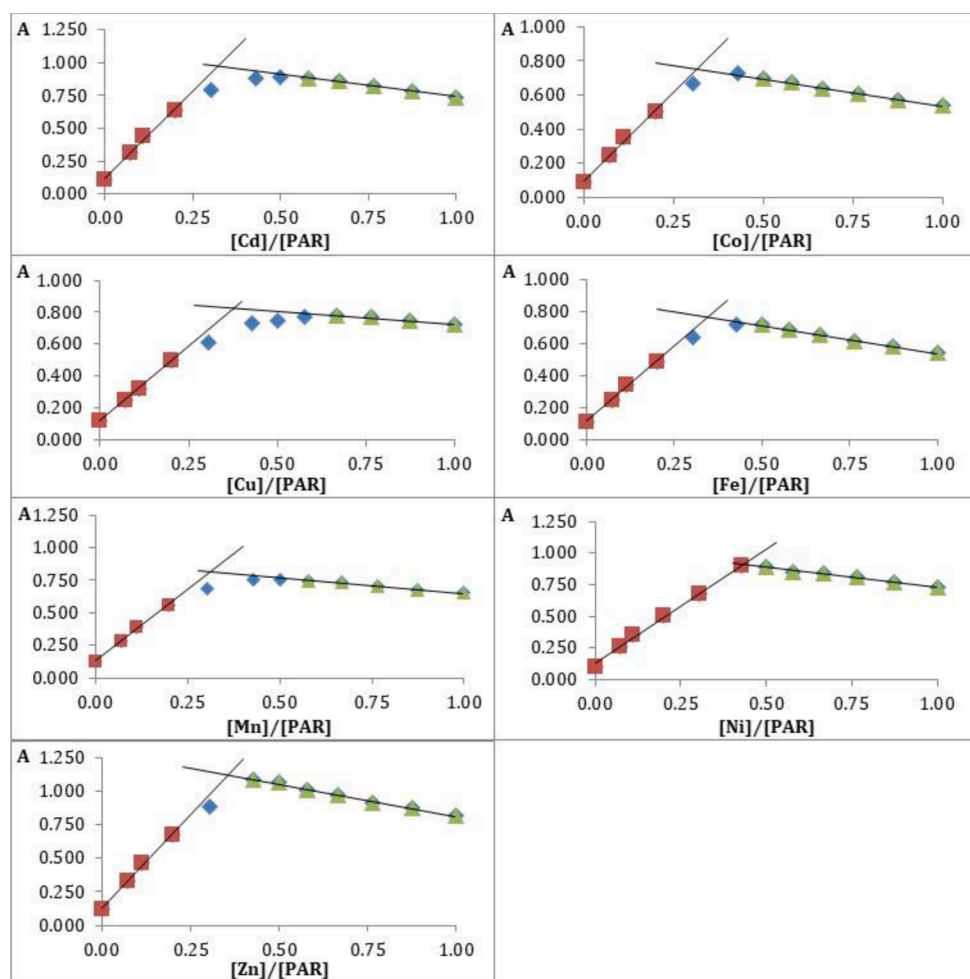


Fig. 4. Graphs for the Job's method of continuous variation for the reaction of PAR with the different metal ions (- all tested $[\text{Me}]/[\text{Complexing agent}]$ ratio values; - values before the $[\text{Me}]/[\text{Complexing agent}]$ stoichiometric ratio; - values after the $[\text{Me}]/[\text{Complexing agent}]$ stoichiometric ratio).

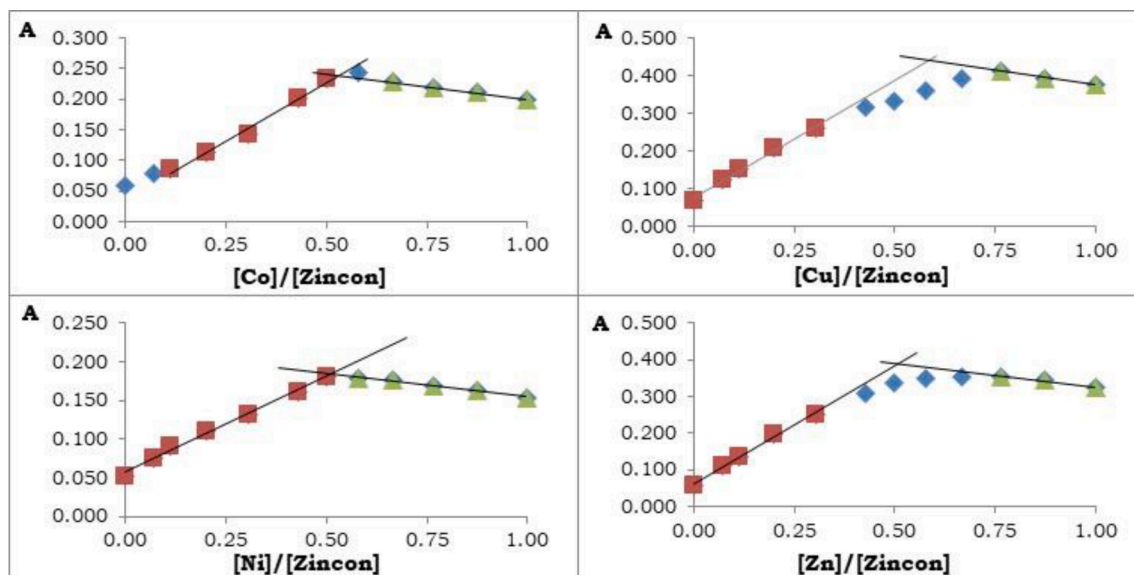


Fig. 5. Graphs for the Job's method of continuous variation for the reaction of Znicon with the different metal ions (- all tested [Me]/[Complexing agent] ratio values; - values before the [Me]/[Complexing agent] stoichiometric ratio; - values after the [Me]/[Complexing agent] stoichiometric ratio).

Table 5

Stoichiometry of the reaction between metals and the complexing agent (Me:Reagent). The stoichiometries were calculated based on the Job's method for each reaction between the metal and the complexing agent (PAR and Znicon).

Complexing agent	Metal ion	Trendline regression 1*	Trendline regression 2*	Experimental ratio**	Me:Reagent Stoichiometry (this work)	Reference Me:Reagent Stoichiometry [10]
PAR	Cd	$A = 2.65x + 0.129$ $r^2 = 0.994$	$A = -0.34x + 1.02$ $r^2 = 0.996$	0.32	1:3	1:2
	Co	$A = 2.10x + 0.096$ $r^2 = 0.993$	$A = -0.32x + 0.856$ $r^2 = 0.992$	0.31	1:3	1:2
	Cu	$A = 1.88x + 0.118$ $r^2 = 0.9999$	$A = -0.17x + 0.891$ $r^2 = 0.993$	0.38	1:3	1:2
	Fe	$A = 1.89x + 0.113$ $r^2 = 0.996$	$A = -0.35x + 0.883$ $r^2 = 0.993$	0.34	1:3	1:2
	Mn	$A = 2.20x + 0.130$ $r^2 = 0.995$	$A = -0.26x + 0.907$ $r^2 = 0.99$	0.31	1:3	1:2
	Ni	$A = 1.80x + 0.139$ $r^2 = 0.994$	$A = -0.31x + 1.039$ $r^2 = 0.997$	0.43	1:3	1:3
	Zn	$A = 2.78x + 0.130$ $r^2 = 0.994$	$A = -0.48x + 1.294$ $r^2 = 0.997$	0.36	1:3	1:2
	Znicon	Co	$A = 0.38x + 0.037$ $r^2 = 0.99$	$A = -0.084x + 0.284$ $r^2 = 0.99$	0.53	1:2
Cu		$A = 0.62x + 0.078$ $r^2 = 0.991$	$A = -0.16x + 0.531$ $r^2 = 0.995$	0.58	1:2	1:1
Ni		$A = 0.25x + 0.058$ $r^2 = 0.993$	$A = -0.061x + 0.216$ $r^2 = 0.992$	0.51	1:2	1:1
Zn		$A = 0.64x + 0.064$ $r^2 = 0.993$	$A = -0.13x + 0.452$ $r^2 = 0.99$	0.51	1:2	1:1

r^2 - coefficient of determination.

* Absorbance (A) = slope * [Me]/[Complexing agent] (x) + intercept; Trendline regression 1 (squares in Figs. 4 and 5) and trendline regression 2 (triangles in Figs. 4 and 5) correspond to the trendlines before and after the change at the slope signal, respectively.

** The stoichiometry between the two reacting species of each reaction was determined based on the ratio value at the interception between the two trendlines.

pointed out as the determining factor of this difference.

4. Conclusions

Microplate assays demonstrated to be useful tools for the investigation of the equilibrium process of metal ion complexes. As expected, these automatic methods involved lower volumes of reagents (in the range of microliters) when compared with the classical method (usually in the range of milliliters). Overall, by implementing these methods in a microplate format, the amount of chemicals needed (less than 10 μ g per

well plate), and the production of effluents (250 μ L of solution per microplate well) were minimized. Additionally, by using this type of strategy in a laboratory routine, the throughput of analysis was augmented, performing 96 different absorbance measurements in a short timeframe (less than 5 min). This was attained without compromising the study fundamentals for this type of methods when compared with classical chemistry protocols, which are commonly more laborious and time consuming. Furthermore, the microplate assay features also meet some of the goals of the Green Chemistry recommendations.

Supplementary Information

Figure S1. Chemical structure of free Zincon and metal-Zincon complex (a) and free PAR and metal-PAR complex (b), and the corresponding chelation reactions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.talo.2021.100074](https://doi.org/10.1016/j.talo.2021.100074).

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