Surfactant Assisted Separation-Spectrophotometric Procedure for the Trace Analysis of Copper (II) in Drug and Water Samples Using a Heterocyclic Pyridyl Azo Dye

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Abstract

4-(2-pyridyl-Azo) resorcinol mono sodium mono hydrate (NaPAR), a heterocyclic azo dye, was investigated for the flotation of copper (II). The metal ion forms a faint red complex with NaPAR in aqueous solution. An intense clear red layer was formed in the scum, after flotation, by adding an oleic acid (HOL) surfactant. The composition of the float is 1:2 (Cu(II): NaPAR). A highly selective and sensitive spectrophotometric procedure was proposed for the determination of micro-amounts of Cu(II) as its floated complex in the pH range 3.0-5.0. Beer’s law was obeyed up to 5x10⁻⁵ mol l⁻¹. The Interferences from various foreign ions were avoided by adding excess NaPAR. The molar absorptivities of Cu-NaPAR and Cu-NaPAR-HOL systems are 5.3x10⁴ and 6.1x10⁴ mol⁻¹cm⁻¹ for the colored complexes in the aqueous and scum layers, respectively. The Cu-NaPAR complexes formed in aqueous solution and in the HOL were characterized by infrared spectral studies. The method was successfully applied to the analysis of Cu(II) in water and drug samples with a recovery >95% and a RSD <1.5%. The separation mechanism is explained.

Keywords: Copper; 4-(2-pyridyl-azo) resorcinol mono sodium mono hydrate (NaPAR); Flotation; Spectrophotometry; Drugs

Introduction

Heavy metals are of the great importance for the life. Some of these such as Cu, Zn, Co, Fe are essential to humans [1]. Others such as Hg, Cd and Pb are toxic following occupational and environmental exposure [2].

Copper is one of the several metal ions that play an important role in the biological system. It performs a key role during cell respiration, in the blood of invertebrate animals and in the formation of haemocyanin [3]. Apart from the biological utility of copper, it also finds applications in industries [4]. It is used in the electrical industry as fine wires, commuter bars and high conductivity tubes. It is also used in pipe making, roof sheeting, bronze paints and insecticides. In addition, it is a hazardous pollutant in the environment, resulting from the industrial effluents, in the form of particulate or soluble copper waste from electroplating, chemical and textile industries. As a pollutant, copper is of particular concern, because of the high degree of toxicity to aquatic organisms. In view of this, separation and determination of copper from associated elements is indispensable.

Due to the low concentration of the heavy metals in the environmental and biological samples and interfering effects, a pre-concentration/separation technique is generally necessary prior to the determination. For this purpose, various analytical procedures have been used, such as adsorption on activated carbon [5,6] co-precipitation [7,8] column extraction [9,10] ion-selective electrode [11,12] liquid-liquid extraction (LLE) [13], cloud point extraction [14] and flotation [15-26].

Flotation has attracted considerable attention mainly because it complies with the “Green Chemistry” principle [27], as the amount of organic solvent is much less than that of traditional liquid extraction. Moreover, It is simple, cheap, highly efficient, fast, and of lower toxicity than those extractions that use organic solvents. Flotation has been applied for separation and pre-concentration of some metal ions such as Hf and Zr in real samples [15,16], Cu in environmental, human blood and drug samples [17,26], Cd in environmental samples [18], lanthanum and yttrium in some geological and environmental samples [19,20], vanadium(IV) in wastes of Power Stations [21], Selenium(IV) in Food Stuffs [22], Zinc(II) in Human Biofluids and Environmental Samples [23], iron(III) in water, real samples and wastes of power stations [24] and Sc³⁺ in certified materials and different water resources [25].

Since 1960, derivatives of 2-pyridylazo have been extensively studied for analytical purposes. A very important example is 4-(2-pyridylazo)-resorcinol, PAR. This is an excellent metallochromic indicator and also very useful as a chromogenic agent for the quantitative determination of over 50 elements, including at trace levels [28,29]. PAR, C₁₁H₉N₃O₂, is commercially available as the free dye in the protonated form (represented as H₂L) or as the monosodium (NaHL.H₂O) or disodium salt (Na₂L₂H₂O). PAR behaves as a tetradentate or a bidentate ligand to form soluble or insoluble colored complexes with cations of different heavy metals at specific pH values.

Among various instrumental methods of analysis, spectrophotometry is preferred as a versatile technique in exploring the use of 4-(2-pyridylazo)-resorcinol, (PAR) as an effective reagent to detect and determine copper (II) at micro level after floatation using oleic acid (HOL) surfactant, in diverse materials and substances. Further, the color of the floated metal complex is found to remain stable...
for a considerable period, which sometimes extends even more than "72 h". Hence, the investigators are encouraged to develop a sensitive and rapid spectrophotometric method for copper (II). The results obtained through UV-VIS spectrophotometer have been compared with those obtained through the atomic absorption spectrometer.

In the present study, a simple, rapid and economical flotation - spectrophotometric method has been developed for the pre-concentration and determination of trace Cu(II) in different matrices using HOL as a surfactant and NaPAR as a chelating reagent. This reagent is more sensitive and specific than many of the reagents reported for Cu(II) [30-34]. Moreover, this method involves the determination of trace Cu(II) after selective separation by flotation, thus eliminating the effect of foreign ions and increasing the sensitivity. Also, the direct determination of Cu(II) in the surfactant phase decreases its determination time and loss during the determination.

A literature survey showed that ion-flotation followed by a spectrophotometric determination of Cu(II) is rarely reported [17,26]. Also, no attempt has been found for using NaPAR and oleic acid in this concern. Fortunately, in this work, NaPAR finds its role for a direct spectrophotometric determination of Cu(II) and also gives high performance in the pre-concentration of Cu(II) via its flotation as Cu(II)-NaPAR colloids.

Materials and Methods

Chemicals and solutions

Unless otherwise specified, all chemicals used are of analytical-reagent grade. Doubly distilled water (DDW) was used throughout. A stock solution of Copper (1 mg/ml) was prepared by dissolving CuSO₄ in double distilled water and a few drops of concentrated sulphuric acid were added. The final concentration of Cu(II) was standardized using an atomic-absorption spectrometer. Monosodium salt of 4-(2-pyridylazo) resorcinol, NaPAR, was purchased from Sigma Aldrich. Stock solution (1 x 10⁻³ mol l⁻¹) was prepared by dissolving the requisite amount of NaPAR in DDW. Oleic acid stock solution (6.36 x 10⁻² mol l⁻¹) was prepared by dispersing 20 ml of HOL (food grade) sp.gr. 0.895, provided by J.T. Baker Chemical Co.) in one l of kerosene. One liter of tap, Nile or sea water samples was filtered off, adjusting the pH to 1 with concentrated HCl, to prevent losses by sorption or co-precipitation, and preserved in a high quality clean plastic containers. Vita stress and Titotav tablets, multiminerals general tonics containing copper available in the Egyptian market, were analyzed to determine the copper content in each drug using the proposed methodology. Exactly 40 mg, each of the drug samples was taken, crushed and heated in 5 ml concentrated HNO₃, for dissolution. The cold solution was filtered, collected in a 50-ml calibrated flask and completed to the mark with DDW.

Apparatus

Flotation cells: Two types of flotation cells were used throughout this work, as has already been described [35]. Flotation cell (a) is a cylindrical graduated glass tube of 16 mm inner diameter and 290 mm length with a stopcock at the bottom. Such cell is used to study the different factors affecting the efficiency of flotation. Flotation cell (b) is a cylindrical tube of 6 cm inner diameter and 45 cm length with a stopcock at the bottom and a quick fit stopper at the top. This cell is used to separate copper from 1 liter of different water samples. The spectral data were recorded on Unicam UV 2100 UV/Vis and MATTSON 5000 FTIR spectrometers. The atomic-absorption measurements were recorded using a Perkin-Elmer 2380 Atomic-absorption Spectrophotometer with an air-acetylene flame. The optimum parameters for Cu measurements are 324.7 nm wavelength; lamp current, 4 mA; burner height, 0.9 cm; slit width, 0.7 mm; fuel flow rate, 31 min⁻¹ and air flow rate, 21 min⁻¹. The pH values of all solutions were measured using a Hanna Instruments 8519 digital pH meter.

Analytical procedures: Suitable concentrations of Cu(II) and NaPAR were mixed, 3 ml of DDW was added and the pH was adjusted to the optimum value (4.0). The solution was then transferred quantitatively to a flotation cell and completed to 10 ml with DDW. The cell was shaken well for few seconds to ensure complete complexation. To this solution, 3 ml of HOL (10⁻⁴ mol l⁻¹) was added. The flotation cell was then inverted upside down twenty times by hand. After complete floatation (10 min) the scum layer was separated and taken for the determination of Cu(II) spectrophotometrically at 510 nm and confirmed by flame atomic absorption (AAS) after eluting the analyte with a few drops of HNO₃ or HCl.

The separation efficiency was calculated from the relation:

\[
F = \left( \frac{c_i}{c_f} \right) \times 100\% 
\]

where \(c_i\) and \(c_f\) is the initial and scum concentrations of the analyte, respectively.

Alternatively, Cu(II) was determined directly by AAS in the mother liquor. The separation efficiency (5%) of the analyte was calculated from its concentration in the mother liquor according to the relation

\[
S = \left( \frac{c_f-c_i}{c_i} \right) \times 100\% 
\]

where \(c_i\) and \(c_f\) denote the initial and final concentrations of the analyte, respectively.

Results and discussion

Absorption spectra

The absorption spectra of the Cu(II)-NaPAR system in the aqueous solution and in the scum layer are completely different from that of copper and NaPAR (Figure 1). It is observed that: 1) the \(\lambda_{max}\) of Cu(II)-NaPAR exhibits a red shift (100 nm) from that of NaPAR and 2) the absorbance of Cu(II)-NaPAR system exhibits nearly 10-folds that of Cu(II)-NaPAR. This proves that the species are highly concentrated in the presence of HOL.

Separation and microdetermination of Cu(II)

Effect of pH: A conductive series of experiments was carried out to float Cu(II) using HOL alone. A suitable concentration of the analyte (2 x 10⁻³ mol l⁻¹) was taken in the flotation cell and a sufficient quantity of HOL surfactant (2 x 10⁻³ mol l⁻¹), which is still less than the critical micelle concentration (CMC) was added to float Cu(II) at different pH values. The data in Figure 2 (curve a) prove that not more than 20% of the analyte was separated at any pH. Such a separation percent is not analytically satisfactory in which Cu(II) floats as Cu-NaPAR complex as an intense red color.

Effect of ligand concentration: The data in Figure 2 show that complete separation of the analyte is achieved over a wide range of pH. The direct addition of the reagents acquires the solution pH=4.0.
Effect of HOL concentration: The floatability of Cu(II) at different concentrations of HOL in the presence of NaPAR has been investigated. Figure 5. The floatability of Cu(II) slightly increases and reaches its maximum (100%) over a wide concentration range of HOL, and then slightly decreases. A 2 x 10^-4 mol l^-1 HOL was used throughout this work.

Effect of temperature: A series of experiments was conducted in a wide temperature range (20-80°C), to find out the proper temperature required for maximum flotation of Cu-NaPAR complex. It was found that the flotation efficiency was not markedly affected in the 20-80°C range, (Figure 6). Therefore, subsequent measurements were carried out at room temperature, i.e., ~25°C.

Effect of shaking time: The influence of shaking time of the flotation cell on separation of Cu(II) using NaPAR (4 x 10^-4 mol/L) and 1 x 10^-4 mol/L HOL was investigated (Figure 7). It was found that the flotation efficiency reaches its maximum (~100%) after about 2 minutes and remains constant up to 10 min. Therefore, a shaking time of 3 minutes was used in the subsequent experiments to ensure complete metal separation. This means that the flotation separation procedure is not time consuming. In order to affirm the stability of the metal separation after 10 min, complementary experiments were done from 10 min to 72 hr. It was found that color and the separation efficiency remain constant up to 72 hr.

Effect of ionic strength: Table 1 illustrates the effect of changing the ionic strength of different salts on the flotation efficiency of 2.0 x 10^-5 M Cu(II) ions with 1.0 x 10^-4 M HOL in the presence of 1.0 x 10^-4 M NaPAR at pH 4. The salts used in modifying the ionic strength usually look like individual salts present in natural water samples. As it can be noticed, the ionic strength of the medium has not evidently affected the flotation process or the determination of copper.

Effect of interfering ions: The effect of interfering ions on the floatability of Cu(II) with NaPAR has been studied in detail. The obtained results revealed that in spite of the high tendency of NaPAR to form complexes with different transition metal ions, fortunately, most of these complexes are not floated with the Cu(II)-NaPAR complex at pH: 3.0-5.0. The experimental data showed that Na(I), K(I), Ag(I), Ca(II), Mg(II), Sr(II), Pb(II), Cd(II) and Ni(II) as chlorides, sulphates or nitrates have no effect, whereas Al(III), Hg(II), Fe(III), U(IV), V(V) and Os(V) have little interfering effects (~1%). All of these interferences were completely controlled by adding excess NaPAR (2 x 

Accordingly, pH 4.0 is selected as a suitable value for complete separation to facilitate the proposed afford due to the progress in this work. At such pH a conductive series of experiments was carried out to investigate the type of interaction between Cu(II) and NaPAR in solution. It was found that Cu(II) forms a 1:2 complex with NaPAR, and complete floatation-separation was achieved whatever amount of NaPAR was added (Figure 3). This simplifies the procedure for the analytical separation and determination of Cu(II), especially in samples containing unknown amounts of the analyte.

Effect of metal concentration: To confirm the previous data, another series of experimental work was carried out by changing the metal concentration. The data (Figure 4) showed that complete separation occurred up to the previous ratio of 1:2. Above such a ratio of increasing Cu(II), curve (b) in Figure 4 attains a small degradation to a lower separation percentage. This reflects that insufficient ligand is present for complete complexation and indirect separation.

Effect of HOL concentration: The floatability of Cu(II) at different concentrations of HOL in the presence of NaPAR has been
Stoichiometry: Job’s [37] method of continuous variation was applied to identify the stoichiometry of Cu-NaPAR complex formed at pH 4.0 in aqueous solution. The data obtained indicate the formation of 1:2 (Cu: NaPAR) at pH 4.0 with λmax at 510 nm.

Analytical figure of merits: Beer’s law is obeyed up to 5 x 10⁻⁵ mol l⁻¹. The molar absorptivities are 5.3 ×10⁴ and 6.1 ×10⁵ mol⁻¹cm⁻¹ for Cu-NaPAR and Cu-NaPAR-HOL species in the aqueous and scum, respectively. The regular enhancement in the absorbances and the higher molar absorptivity value in the presence of HOL could be attributed to the selective separation of Cu-NaPAR using the HOL surfactant. Although the complex is formed in the aqueous solution and obeys Beer’s law, its absorbance and ε values are very low. Also, Beer’s law is obeyed in a very narrow concentration range. These defects may favor the determination of copper after pre-concentration. The limit of detection, which was calculated as the concentration that gives a reading equal to twice the standard deviation of a series of ten determinations taken with solutions of concentrations, which are close to the level of the blank, was found to be 2.5 x 10⁻⁷ mol l⁻¹. Also, in this concern a fixed quantity of Cu(II) was taken and added to different aqueous volumes. It was found that 7.5 x 10⁻⁷ mol l⁻¹ of Cu(II) was quantitatively separated and determined from different volumes up to 1 L using a definite type of flotation cells. This means that 7.5 x 10⁻⁷ mol l⁻¹ of Cu(II) can be safely separated and spectrophotometrically determined by such a procedure from a one-liter solution into 20 ml of HOL (2 x 10⁻⁴ mol l⁻¹) with a preconcentration factor of 50.

Characterization of Cu(II)-NaPAR complexes

The Cu(II)-NaPAR complexes isolated in the aqueous solution and oleic acid were characterized by infra-red spectral studies. The IR spectra of the free ligand and its metal chelate were carried out in the 4000-400 cm⁻¹ range, Figure 8. The characteristic findings can

Figure 4: Floatability of different concentrations of Cu(II) at pH 4. a) In the absence of NaPAR, b) In the presence of 4 x 10⁻³ mol l⁻¹ NaPAR using 2 x 10⁻⁴ mol l⁻¹ HOL.

Figure 5: Floatability of Cu(II), 2 x 10⁻⁵ mol l⁻¹ vs. HOL concentration: a) In the absence of NaPAR and b) In the presence of 2 x 10⁻⁴ mol l⁻¹ NaPAR at pH 4.

Figure 6: Effect of temperature on the separation efficiency of 2 x 10⁻⁵ mol l⁻¹ Cu(II) at pH 4.0 in the presence of 4 x 10⁻⁴ mol l⁻¹ NaPAR using 2 x 10⁻⁴ mol l⁻¹ HOL.

Figure 7: Effect of time on the separation efficiency of 2 x 10⁻⁵ mol l⁻¹ Cu(II) at pH 4.0 in the presence of 4 x 10⁻⁴ mol l⁻¹ NaPAR using 2 x 10⁻⁴ mol l⁻¹ HOL.
be summarized as follow: The IR spectrum of the ligand (Figure 8a) [38] shows a broad band at 3446 cm–1, which can be attributed to the phenolic OH group. This band is still broad in complex, which renders it difficult to attribute to the involvement of phenolic OH group in coordination. The involvement of the deprotonated phenolic OH group in chelation is confirmed by the shift of the ν (C–O) stretching band, observed at 1186 cm–1 in the free ligand, to the extent of 1118 cm–1 in the complex. The ν (N=N) stretching band in the free ligand is observed at 1592 cm–1. This band is shifted to lower frequency values upon complexation suggesting coordination via the azo group (M–N) [18,19]. The IR spectrum of the ligand revealed a sharp band at 1632 cm–1 due to ν (C=N) of the N3 pyridyl azo nitrogen. This band is shifted to lower frequencies in the complex indicating that it has been affected upon coordination to the metal ions [39,40]. The pyridine ring vibrations at 1467 cm–1 in ligand disappeared in the spectra of complex, suggesting involvement of pyridine nitrogen in bonding [40,41]. In the far-IR spectra of all complexes, the non-ligand bands observed at 475 and 447 cm–1 region can be assigned to the ν (M–N) stretching vibrations of the azo and N3 pyridylazo nitrogen, respectively. Conclusive evidence regarding the bonding of oxygen to the metal ions is provided by the occurrence of bands at 535 cm–1 as the result of ν (M–O) [38,42,43].

Oleic acid initiates to dissociate at pH ≥ 5.2. Consequently, oleic acid can interact with other system, through hydrogen bonding, whichever by its undissociated or dissociated form dependent on the pH of the medium. The infrared spectrum of the complex formed in oleic acid layer (Figure 8c) is different from that formed in the aqueous solution (Figure 8b). The appearance of the bands at ~ 1820, 2050 and 2400 cm–1 in the spectrum of the Cu-NaPAR-HOL is due to ν (O–H…O) vibrations of the intermolecular hydrogen bonding. The absorption bands corresponding to the oleic acid surfactant at 1710 cm–1 are due to ν (C=O) vibration of carboxylate ion of oleic acid (Figure 7c). Another band is also observed at 1462 cm–1 assigned to νs COO– vibration.

Careful comparison of the IR spectrum of the of the NaPAR complexes, isolated from the aqueous solution and surfactant layer with that of NaPAR indicated that NaPAR may act as a tridentate ligand complexing with the Cu(II) ion through the ortho hydroxyl group, the azo nitrogen nearest to the phenolic ring and the heterocyclic nitrogen atom.

So, the proposed mechanism may be through intermolecular hydrogen bonding. Therefore, the system Cu–NaPAR – HOL become hydrophobic and floated with air bubbles to the surface.

**Flotation mechanism**

The role of surfactant is very important in studies concerning separation via flotation. The nature of the interaction between oleic acid surfactant and the complex formed (Cu-NaPAR) must be studied to approach the actual mechanism of floatation.

The proposed mechanism may proceed through a physical interaction or by forming a hydrogen bond between the hydrophilic part of HOL and the active sites in the ligand complex, or by an interaction between oleic acid and the complex, formed in solution, through a coordinate bond forming a self-floatable (Cu-NaPAR-HOL) species. In every case, the hydrophobic part of the surfactant attaches to air bubbles and floats separating the analyte-containing species.

| Salt  | Concentration, (M) | F (%) |
|-------|-------------------|-------|
| NaCl  | 0.1               | 100   |
|       | 0.01              | 100   |
|       | 0.001             | 100   |
| KCl   | 0.1               | 98.1  |
|       | 0.01              | 100   |
|       | 0.001             | 100   |
| CaCl2 | 0.1               | 92.5  |
|       | 0.05              | 94.5  |
|       | 0.001             | 96.3  |

**Table 1:** Effect of Ionic Strength on the Floatability of 2.0 x 10–5 M of Cu(II) using 1.0 x 10–4 M of NaPAR, 1.0 x 10–4 M HOL at pH 4.

Figure 8: Infrared spectra of a) NaPAR in KBr, b) Cu(II)-NaPAR isolated in aqueous solution in KBr and c) Cu(II)-NaPAR-HOL isolated in oleic acid in KBr.
In the present study, the flotation mechanism is proposed to proceed through hydrogen bonding between HOL and Cu-NaPAR system. This confirmation is extracted from the following experimental data and observations:

1) The floated species (Cu-NaPAR-HOL) have the same \( \lambda_{\text{max}} \) as those formed in aqueous solution (Cu-NaPAR).

2) The color intensity of the scum layer was not affected by time. It should be noted that the color fading of the sub late begins after at least 72 hrs after flotation; such a time is more than enough for our determination. 3) The data in Fig. 6 show that increasing the temperature up to 80 °C does not affect the separation percentage, reflecting that the hydrogen bond between HOL and the Cu-NaPAR complex is not destroyed by heating. 4) The IR spectra of the Cu-NaPAR complex isolated in the absence and presence of HOL are not identical (Figure 8b and 8c), reflecting the fact that there is some hydrogen contribution between HOL and the Cu-NaPAR complex.

In conclusion, all of the mentioned data confirm the formation of hydrogen bonding between HOL and Cu-NaPAR system forming a self-floating (Cu-NaPAR-HOL) species. In such cases, the hydrophobic part of the surfactant attaches to air bubbles and floats, thus separating the analyte-containing species.

**Application**

**Water samples**

The recovery of definite amounts of Cu(II) added to different water samples was studied. To 100 ml aliquots of pure uncontaminated and filtered water samples definite concentrations of Cu(II) were added and the pH was adjusted to ~ 4. After flotation, the concentration of Cu(II) was determined spectrophotometrically and confirmed by atomic absorption spectrometry (AAS). The recovery % is >95%, Table 2.

| Type of water (location) | Cu(II) added (mM) | AAS Recovery, % | RSD, % | Spectrophotometry Recovery, % | RSD, % |
|-------------------------|------------------|-----------------|--------|-------------------------------|--------|
| Distilled w ater         | 0.0              | 0.0             | 0.0    | 0.0                           | 0.0    |
|                         | 5.0              | 99.8            | 1.80   | 99.7                          | 1.6    |
|                         | 10.0             | 95.8            | 1.5    | 99.0                          | 2.0    |
|                         | 15.0             | 100.0           | 2.0    | 98.5                          | 1.0    |
| Tap w ater (Our laboratory) | 0.0              | 0.0             | 0.0    | 0.0                           | 0.0    |
|                         | 5.0              | 99.6            | 1.6    | 99.6                          | 2.3    |
|                         | 10.0             | 99.8            | 2.8    | 100                           | 1.5    |
|                         | 15.0             | 99.4            | 1.4    | 100                           | 2.0    |
| Nile w ater (Mansoura City) | 0.0              | 0.0             | 0.0    | 0.0                           | 0.0    |
|                         | 5.0              | 99.9            | 2.3    | 99.7                          | 1.6    |
|                         | 10.0             | 99.4            | 1.5    | 99.8                          | 2.1    |
|                         | 15.0             | 99.6            | 2.0    | 100                           | 1.8    |
| Sea w ater (Demiatle City) | 0.0              | 0.0             | 0.0    | 0.0                           | 0.0    |
|                         | 5.0              | 99.8            | 2.3    | 99.7                          | 2.5    |
|                         | 10.0             | 100.0           | 1.5    | 99.8                          | 1.9    |
|                         | 15.0             | 100.0           | 2.00   | 100                           | 1.4    |
| Sea w ater (Ras El-Barr) | 0.0              | 0.0             | 0.0    | 0.0                           | 0.0    |
|                         | 5.0              | 99.6            | 2.5    | 99.7                          | 2.3    |
|                         | 10.0             | 100             | 1.9    | 99.8                          | 1.5    |
|                         | 15.0             | 100             | 1.4    | 100                           | 2.00   |

Table 2: Recovery of Cu(II) spiked in natural water samples after flotation using 1.0 x 10-3 M NaPAR and 1.0 x 10-4 M HOL at pH ~ 4 at 25°C (n=3).

**Drug samples**

Analysis of Cu(II) in real drug samples under the recommended conditions is shown in Table 3 with a relative standard deviation, RSD, <1.0%.

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