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Artículo original

Spectrophotometric characterisation of the Cu(II):PPi system: implementation as a method for measuring pyrophosphate (PPi) in solution

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ABSTRACT

The interaction of Cu(II) with pyrophosphate (PPi) in solution modifies the absorption spectrum of the cation. We provide here a proper description of the chemical interactions involved in the absorbance shift, identify the absorption coefficients of the species in solution and afford a model of the Cu(II)-PPi chemical system. Since the changes in the absorption spectrum are concentration dependent, we describe a new method for quantifying PPi in aqueous solutions, based on the modification of the Cu(II) absorption spectrum in the presence of PPi. Changes in absorptivity can be monitored and used to quantify PPi in solution. The determination is simple, fast and cheap. The detection limit of the method is 0.1 μmol of PPi in the assay conditions. The presence of orthophosphate does not interfere in the determination of PPi. Furthermore, it is possible to use this method in biological systems containing proteins, nucleotides, EDTA or magnesium.

Keywords: Absorption spectra.—Bioinorganic chemistry.—Spectroscopy.—Pyrophosphate.—Copper.

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RESUMEN

Caracterización espectrofotométrica del sistema Cu(II):PPi, su implementación como un método para la valoración de pirofosfato (PPi) en solución

El pirofosfato (PPi) tiene una importante función en numerosos procesos biológicos. Participa en muchas reacciones enzimáticas catalizadas por transferasas, hidrolasas, ligasas o sintetasas.

La interacción en solución del Cu(II) con pirofosfato (PPi) modifica el espectro de absorción del catión. Se describen las interacciones químicas involucradas en el cambio de absorbancia, se identifican los coeficientes de absorción de las diferentes especies en solución y proponemos un modelo del sistema químico Cu(II)-PPi. Ya que los cambios en el espectro de absorción son dependientes de la concentración, nosotros proponemos un método para cuantificar PPi en soluciones acuosas basado en la modificación del espectro de absorción de Cu(II) en presencia PPi. Los cambios en la absorción pueden ser monitorizados y utilizados para cuantificar PPi en solución. La determinación es simple, rápida y barata. El límite de detección del método es 0,1 µmol de PPi en las condiciones del ensayo. Además es posible usar este método en sistema biológicos que contienen proteínas, nucleótidos, EDTA o magnesio.

Palabras clave: Espectro de absorción.—Química bioinorgánica.—Espectroscopía.—Pirofosfato.—Cobre.

INTRODUCTION

Pyrophosphate (PPi) plays an important role in many biological processes. It participates in many enzymatic reactions catalysed by transferases, hydrolases, ligases or synthetases. Phosphoric ester bound is highly energetic, and most of the reactions that require PPi make use of it for driving biochemical pathways on the appropriate direction. PPi is involved for instance in:

i) the biosynthesis of nucleic acids: the enzymes DNA polymerase (EC 2.7.7.7) and RNA polymerase (EC 2.7.7.6) catalyse the transfer of a nucleotide monophosphate to a polynucleotide chain, with the release of PPi;

ii) the biosynthesis of carbohydrates: cytidylyltransferases transfer CMP from CTP to activate certain acceptors such as 3-deoxyoctulosonate (EC 2.7.7.38), N-acylneuraminate (EC 2.7.7.43) (1) or choline (EC 2.7.7.15), (2) releasing PPi;

iii) the biosynthesis of proteins: certain transferases such as glutamine synthetase adenylyltransferase (EC 2.7.7.42), which participates in the regulation of glutamine synthetase (EC 6.3.1.2), transfer one monophosphate nucleotide from a triphosphate nucleotide, with the release of PPi; the enzymes called aminoacyl-tRNA synthetases catalyse the binding of each aminoacid with the corresponding tRNA, with the concomitant release of PPi from ATP;

iv) the recovery of nitrogenous bases: hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) catalyses the transfer of a residue of ribose-5-phosphate from phosphoribosyl-PPi to a purine such as hypoxanthine or guanine, releasing a nucleotide and PPi; other enzymes of this group work in the same way to recover other bases.

In all the examples cited, further hydrolysis of PPi by pyrophosphatase (EC 3.6.1.1) drives the equilibria in these reactions towards the corresponding anabolic sense.

The quantification of PPi is important not only to assess its presence and concentration in a sample, but also to determine enzymatic activities. Thus, the measurement of PPi released by a DNA polymerase can be used to quantify viral charge (3) or to determine the presence of PPi in animal (4) or plant (5) tissues. PPi is usually excreted in urine, and the PPi concentration is usually monitored as an indicator of renal function (6). Accordingly, the measurement of increased of PPi excretion in urine is the basis for confirmation of the diagnosis of the rare inherited disorder hypophosphatasia (7). Furthermore, in the industry PPi is often used as a additive in dentifrices, making it necessary to develop processes able to assess its concentration (8).

Several methods have been developed for the determination of PPi. The classical chemical method quantifies total phosphates by the generation of ammonium phosphomolybdate (9). Other approaches resolve the components of a sample by chromatography (10) or capillary electrophoresis (11) and quantify the corresponding compounds. Finally, the PPi-mediated quenching of fura-2 fluorescence can also be used to determining phosphate polymers of different lengths (12). In the biochemistry laboratory, PPi is

commonly determined by following the progress of enzymatic activities, coupling the hydrolysis of PPi either to the oxidation of NADH (13), to the emission of light by a luciferase (14) or to the formation of certain purines (15).

The PPi anion forms complexes with metallic cations, mainly divalent ones. This process is rapid and thermodynamically favoured. Accordingly, PPi is often used for the extraction and quantification of cations in soils (16), and for stabilizing Cu in plating and the manufacture of printed circuits (17). Likewise, PPi interference in the colorimetric determination of orthophosphate can usually be avoided by forming complexes with Cu or nickel (18).

We have extensively characterised the spectrophotometric changes in the Cu(II):PPi system, and modelled the absorption shift as a function of the interactions of the involved chemical species. On this basis, we report a new method for the determination of PPi, based on the modification of the Cu(II) absorption spectrum in the presence of PPi due to the formation of complexes between these two species. The method avoids most of the drawbacks usually encountered in the usual ways of PPi determination; it is simple, rapid and inexpensive, and allows the evaluation of enzymatic activities with a sensitivity limit of 0.1 μmol of PPi in the assay conditions.

EXPERIMENTAL PROCEDURES

Reagents and chemicals

All solutions were prepared with deionized water.

Copper sulphate pentahydrate, sodium hydroxide, hydrochloric acid, trichloro acetic acid and glycine were purchased from PANREAC (Barcelona, Spain).

Sodium dodecyl sulphate (SDS) was purchased from BIORAD (Munich, Germany).

Sodium pyrophosphate decahydrate (3-[1,1-dimethyl-(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid), sodium salt

(AMPSO), N,N-bis(2-hydroxyethyl) glycine (BICINE), 2-[N-cyclohexylamino]ethane, sulfonic acid (CHES), albumin from bovine serum (BSA), 4-morpholinepropanesulfonic acid, sodium salt (MOPS), sodium tetraborate, ethylenediamine-tetraacetic acid (EDTA), adenosine 5'-triphosphate, disodium salt hydrate (ATP) and DOWEX ® 50WX2-200 ion-exchange resin were purchased from SIGMA-ALDRICH (Sant Louis, USA). Before use, the resin was washed sequentially with 2 N sodium hydroxide, 2 N hydrochloric acid and water, and then allowed to dry overnight.

Tris-(hydroxymethyl)aminomethane (TRIS) and sodium hydrogenophosphate were purchased from PROLABO (Asturias, Spain).

Magnesium chloride hexahydrate was purchased from PROBUS (Barcelona, Spain).

Instruments

A Beckman DU-640 spectrophotometer with cuvettes thermostated at 37 ºC was used to measure absorbance at 280 nm (A_{280}). Beckman quartz microcuvettes with 10 mm pathlength, 700 µl capacity and a 300 µl minimum volume for reading were used.

Procedure

Blank: 50 µL 10 mM $CuSO_4$, 100 µl 0.5 M AMPSO, 100 µl 20% SDS, 200 µl 50 mM $Na_4P_2O_7$ and 550 µl H_2O. Final concentrations were: $[Cu]_{tot}$ = 0.5 mM and $[PPi]_{tot}$ = 10 mM. Sample: 50 µl 10 mM $CuSO_4$, 100 µl 0.5 M AMPSO, 100 µL 20% SDS, 500 µl of a solution containing PPi and 250 µl H_2O. The value of A_{280} for the sample can be used directly for reading the concentration of PPi in the calibration curve (Fig. 7) or for calculating it by means of the corresponding expression (equation 1). If the concentration of protein in the sample was higher than 1 mg/mL, the protein was eliminated by precipitation with trichloroacetic acid before the addition of $CuSO_4$. If the concentration of Mg(II) in the sample was higher than 1 mM, it was removed by the addition of DOWEX-50 at

the pH of the assay (AMPSO, NaOH, pH 9.4), centrifugation and recovery of the supernatant, which was treated as described above.

This study has been carried out using two blanks: one with Cu:PPi at a ratio of 1:20 and other with Cu:PPi at 1:5. The choice of one or the other depended on the expected amount of PPi in the problem solution. Here we report the calculations for the first blank.

Software

Curve fitting by non-linear simple and multiple regression analyses were accomplished with the QNFIT module from the SIMFIT, program developed by Prof. W. Bardsley and distributed freely by the author at http://www.simfit.man.ac.uk.

Statistical treatment

Each experimental condition was performed at least twice, and all experiments were replicated at least three times independently. Suspected outliers placed at 1.5 times the interquartile range above the third quartile or below the first quartile were considered carefully before being included in the analysis. Differences were considered significant applying the Kolmogorov-Smirnoff test, and further validated with Student's unpaired t-test when the experimental data were consistent with a normal distribution.

RESULTS

1. PPi forms complexes with Cu(II) in solution, thereby modifying its absorption spectrum.

In aqueous solution, the presence of PPi modified the absorption spectrum of Cu(II) in a concentration-dependent manner. Changes in absorptivity were monitored in the interval between 240 and 330 nm, keeping the concentration of Cu(II) constant while varying the PPi concentration.

Both PPi and Pi interact with Cu(II) in solution and modify its absorption spectrum in a concentration-dependent fashion (Fig. 1). The presence of both Pi and PPi increase the absorbance of Cu(II) in solution with a maximum at *ca* 250 nm. The presence of PPi decreases the absorbance of Cu(II) with a minimum at *ca* 290 nm. The wavelength of 280 nm was chosen for the assay in order to avoid interferences due to the presence of organic molecules, mainly aromatic rings and nitrogenous bases. The aim of this was to minimize the matrix effects in the sample when comparing one measurement with a calibration curve prepared with standards.

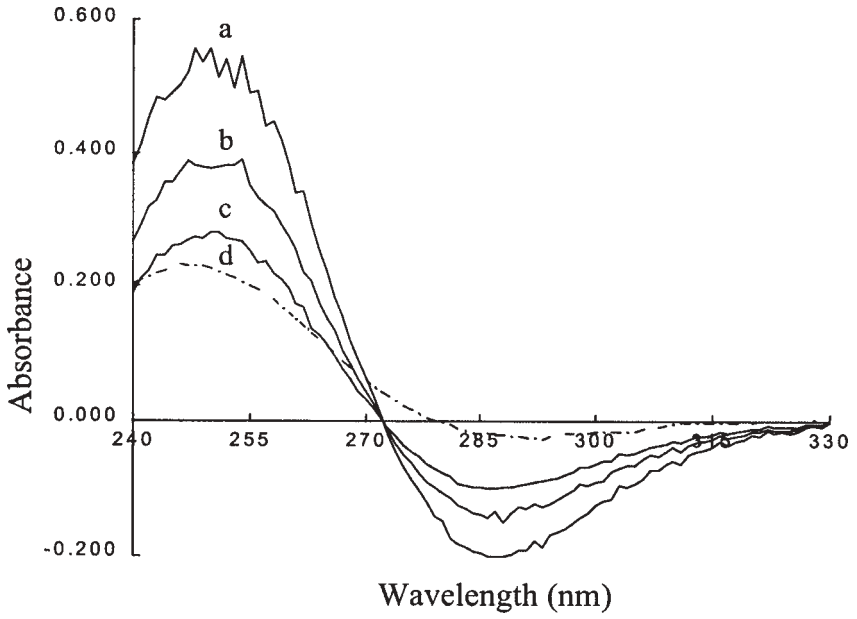

FIGURE 1. ***Influence of pyrophosphate and orthophosphate on the absorption spectrum of Cu(II).*** *All determinations were carried out at pH 9.4 and 37° C. The blank was a solution 0.5 mM of $CuSO_4$ in water. The continuous lines correspond to the spectra of 0.5 mM $CuSO_4$ with 1, 0.5 and 0.1 mM $Na_4P_2O_7$ respectively for* ***a****,* ***b*** *and* ***c****. The broken line* ***d*** *corresponds to the spectrum of 0.5 mM $CuSO_4$ with 10 mM Na_3PO_4. Formation of the Cu(II)-PPi complex decreased the absorption of Cu(II): it was minimum at 290 nm. Formation of the Cu(II)-PPi and Cu(II)-P_i complexes increased the absorption of Cu(II): its maximum was seen at 250 nm.*

The presence of PPi decreases the absorptivity of Cu(II) in solution at 280 nm. Since monitoring an absorbance increase is more sensitive than monitoring a decrease, we chose a blank containing a high concentration of PPi and followed the increase in absorbance in the samples, which contain a lesser amount of PPi. Two blanks with different PPi concentrations were chosen, thus making the method suitable for use at different concentrations ranges of PPi: the first one with a cation: ligand ratio of 1:20 (0.5 mM $CuSO_4$, 10 mM $Na_4P_2O_7$) and the second one with a ratio of 1:5 (0.5 mM $CuSO_4$, 2.5 mM $Na_4P_2O_7$). Absorbance was maximum in the absence of PPi, decreased when the concentration of PPi in the sample increased, and was zero when [PPi] was 10 mM or 2.5 mM respectively.

Cu(II) and PPi form complexes in solution, rendering the species $CuP_2O_7^{2-}$ and $Cu(P_2O_7)_2^{6-}$. The corresponding equilibria are:

$$Cu^{2+} + P_2O_7^{4-} \xleftrightarrow{K_1=1e6.7} CuP_2O_7^{2-}$$

$$Cu^{2+} + 2P_2O_7^{4-} \xleftrightarrow{K_2=1e9.0} Cu(P_2O_7)_2^{6-}$$

Cu(II) and PPi are also involved in different side reactions, which must also be considered. Thus Cu(II) participates in reactions of hydroxicomplexes formation and PPi in protonation reactions, which are considered to be parasites of the main reactions. To deal with such parasite reactions, the constants that describe the main equilibria are redefined taking into account the simultaneous involvement of the chemical species in the different equilibria. For the Cu(II)/ PPi system the conditional constants are:

$$k_1' = \frac{[CuP_2O_7^{2-}]}{[Cu^{2+}]'\ [P_2O_7^{4-}]'} \qquad k_2' = \frac{[Cu(P_2O_7)_2^{6-}]}{[Cu^{2+}]'\ [P_2O_7^{4-}]'^2}$$

where $[Cu^{2+}]'$ represents the concentration of Cu(II) in all the forms in solution, except the complexes with PPi, and $[P_2O_7^{4-}]'$ represents the concentration of PPi in all species except the complexes with Cu(II). The relationship between the conditional constants and the equilibrium constants is given by the coefficient of parasite reactions, defined as:

$$\alpha_{Cu^{2+}} = \frac{[Cu^{2+}]'}{[Cu^{2+}]} \qquad \alpha_{P_2O_7^{4-}} = \frac{[P_2O_7^{4-}]'}{[P_2O_7^{4-}]}$$

Therefore: $$k_1' = \frac{K_1}{\alpha_{Cu^{2+}}\alpha_{P_2O_7^{4-}}} \qquad k_2' = \frac{K_2}{\alpha_{Cu^{2+}}\alpha_{P_2O_7^{4-}}^{2}}$$

The equilibrium constants that describe the parasite reactions of Cu(II) with OH^- and of PPi with H^+ in solution are well documented in the literature. The value of $\alpha_{Cu(II)}$, α_{PPi}, k_1' and k_2' can therefore be expressed as a function of the experimental pH by means of the equilibrium constants. The dependence of these variables on pH is depicted in Fig. 2. Due to the presence of the parasite reactions, the conditional constants k_1' and k_2' for the system Cu(II)/PPi never reach the theoretical values for K_1 and K_2, 1e6.9 and 1e9.0 respectively (Fig. 2).

2. The absorptivity shift of Cu(II) in the presence of PPi is maximum at pH 9.0

The conditional constants of the Cu(II)/ PPi system have maximum values in the pH range between 7 and 10 (see Fig. 2). In the determination of Cu(II) by dipyrophosphatecuprate(II) titration, the pH used is also in that interval (19). In light of this, the effects of pH and of the nature of the buffer solution on the change in the absorbance due to PPi were checked in that pH interval. The results are shown in Fig. 3. A strong dependence of the shift in absorbance on both the pH and the nature of the buffer was observed. These variations might arise as a consequence of further interactions between Cu(II) and side groups of the buffer molecule.

For a Cu(II):PPi ratio of 1:20 and a buffer concentration in the sample of 50 mM, the largest change in A_{280} was observed for AMPSO/NaOH at pH 9.4 (Fig. 3). These conditions were therefore chosen for further experiments. The value of pH 9.4 corresponds to the pK_a of the AMPSO buffer and the pK_{a4} of pyrophosphoric acid. The values for the parasite reaction coefficients and for the conditional constants for the system at pH 9.4 are: $\alpha_{Cu(II)}$ = 4.06e3, α_{PPi} = 2.00, k_1' = 6.15e4 and k_2' = 6.17e2 (Fig. 2). The value 2.0 for

αPPi means that half of the PPi molecules not forming complexes with Cu(II) appear as PPi anion, and nearly all the rest as hydrogenpyrophosphate anion. Regarding the Cu not forming complexes with PPi, only 0.025% appears as free cation, while 98% is in the form of dihydroxicuprate.

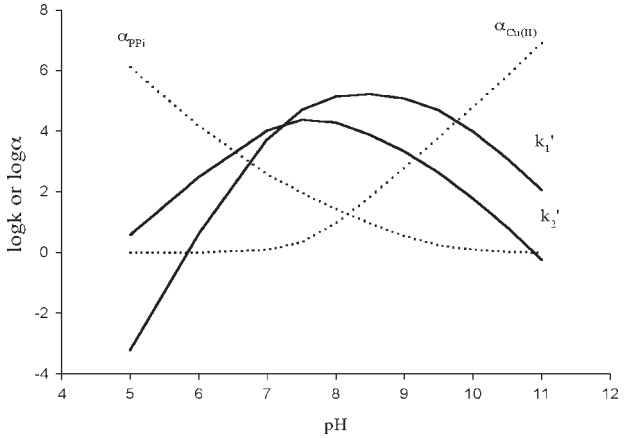

FIGURE 2. ***Dependence of the values of $\alpha_{Cu(II)}$, α_{PPi}, k_1' and k_2' on the pH of the Cu(II)/PPi system.*** *The maximum values of k_1' and k_2' never reach the theoretical values of K_1 and K_2 respectively due to the parasite reactions of Cu(II) and PPi.*

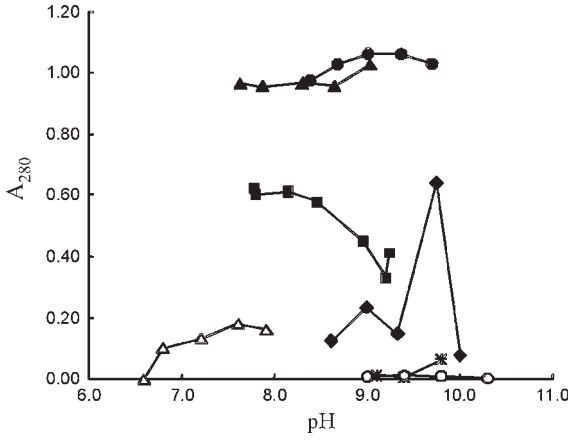

FIGURE 3. ***Dependence of the A_{280} of Cu(II) on the pH and the buffer at 37ºC.*** *In all cases, the blank was a solution of 0.5 mM $CuSO_4$ and 10 mM $Na_4P_2O_7$. All buffers were tested at a concentration of 50 mM and adjusted to the final pH with HCl or NaOH. The different signs correspond to: ●, AMPSO, ▲, BICINE, ■, Tris, ◆, CHES, △, MOPS, ✱, borate and ○, glycine.*

Given the values of the conditional constants, a diagram of species for the system at pH 9.4 for $[Cu(II)]_{tot}$ = 0.5 mM as a function of $[PPi]_{tot}$ can be built (Fig. 4). It can be observed that for values of $[PPi]_{tot}$ lower than 0.50 mM the introduction of PPi into the system leads to a linear increase in both $[CuP_2O_7^{2-}]$ and $[Cu(P_2O_7)_2^{6-}]$. In this range the slope of the straight line representing the variation of the species with two ligands is twice the slope of that representing the variation of the species with one ligand. $CuP_2O_7^{2-}$ predominates for $[PPi]_{tot}$ values lower than 10.7 mM, and the species with two ligands $Cu(P_2O_7)_2^{6-}$ predominates when $[PPi]_{tot}$ increases.

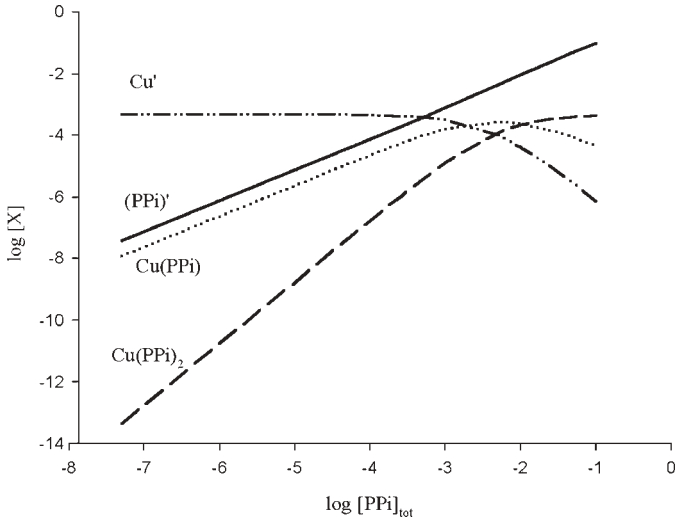

FIGURE 4. ***Diagram of species for the Cu(II)/PPi, system added as $CuSO_4$ and $Na_4P_2O_7$ respectively.*** *Calculations were made for $[Cu(II)]_{tot}$ = 5.0e-4 M, pH 9.4, considering the formation of the $CuP_2O_7^{2-}$ and $Cu(P_2O_7)_2^{6-}$ complexes, with constants of formation log k_1' = 2.79 and log k_2' = 4.79 respectively.*

3. Complexes of Cu(II) and PPi have a lower molar absorptivity than Cu(II) in solution.

In a multi-component system such as the one studied Cu(II)/PPi the Beer-Lambert law is additively applied to all the absorbent species. Thus, assuming that there is no interaction between the different components:

$$A_{280,\,tot} = A_{280,Cu'} + A_{280,CuP_2O_7^{2-}} + A_{280,Cu(P_2O_7)_2^{6-}}$$

$$A_{280,Cu'} = \varepsilon_{Cu'}\,[Cu']\,l$$

$$A_{280,CuP_2O_7^{2-}} = \varepsilon_{CuP_2O_7^{2-}}\,[CuP_2O_7^{2-}]\,l$$

$$A_{280,Cu(P_2O_7)_2^{6-}} = \varepsilon_{Cu(P_2O_7)_2^{6-}}\,[Cu(P_2O_7)_2^{6-}]\,l$$

where $\varepsilon_{Cu'}$ represents the molar absorbance of all the species of Cu(II) in solution except for the complexes with PPi. According to the values obtained previously, it is assumed that virtually all the Cu not complexed with PPi appears as dihydroxicuprate, thus allowing us to consider a unique molar absorbance for all these species.

To determine the molar absorbance of the species in solution, four experimental data series were generated with increasing concentrations of Cu(II) in the 0.01 to 0.3 mM range: one series in the absence of PPi, and three with constant $[Cu]_{tot}$:$[PPi]_{tot}$ ratios of 2:1, 1:1 and 1:10, respectively. For each series A_{280} was measured. The results are depicted in Fig. 5.

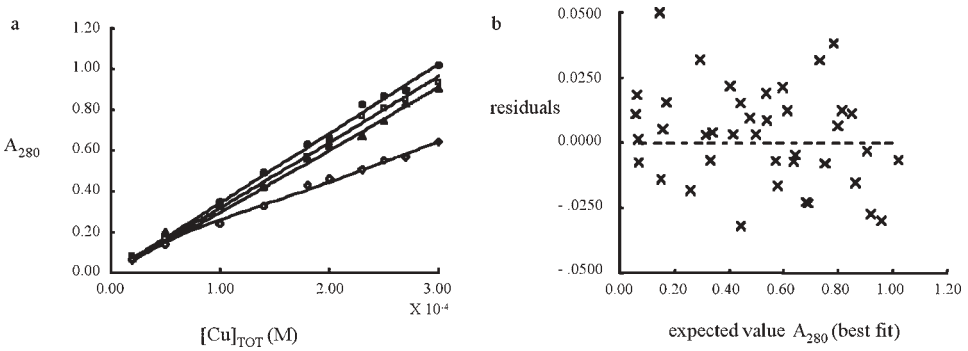

FIGURE 5. ***Spectrophotometric caracterisation of the CuII/PPi system.*** ***(a)*** *Plot of A_{280} against $[Cu(II)]_{tot}$ in 50 mM AMPSO/NaOH pH 9.4 and 37º C. The four data series correspond to: ●, absence of PPi; □, Cu(II):PPi 2:1; ▲, Cu(II):PPi 1:1; ◇, Cu(II):PP_i 1:10. The fittings correspond to the best model with $\varepsilon_{Cu(P_2O_7)_2^{6-}} = \varepsilon_{CuP_2O_7^{2-}} = 1373.8\ M^{-1}cm^{-1} \pm 107.3$, $\varepsilon_{Cu'} = 3372.6\ M^{-1}cm^{-1} \pm 43.9$, expressed as value ± gap of 95% confidence.* ***(b)*** *Plot of residuals against the expected value of A_{280} for the best fit considered. The distribution of the residuals is random and there is no bias in the distribution of the positive and negative strings ($P > 0.05$).*

Even though the $[Cu]_{tot}$:$[PPi]_{tot}$ ratios were kept constant in the experimental design, the ratios [Cu]':[PPi]':$[CuP_2O_7^{2-}]$:$[Cu(P_2O_7)_2^{6-}]$ were not constant, as a further manifestation of the complexity of the system. In order to determine values of the molar absorptivities for each of the components of the system it was necessary to previously estimate the individual concentrations as a function of $[Cu]_{tot}$. Since there is no simple expression representing this dependence, the values were calculated analytically for each data, by using sequentially the expressions shown in Table 1.

TABLE 1. *Expressions used for the analytical calculation of the concentration of each component of the system as a function of* $[Cu]_{tot}$ *and* $[PPi]_{tot}$

Fixed	Used	Obtained
$[Cu]_{tot}$ and° $[PPi]_{tot}$	$\frac{[Cu]_{tot}}{1+k_1'[PPi]'+k_2'[PPi]'^2}=\frac{[PPi]_{tot}-[PPi]'}{k_1'[PPi]'+2\,k_2'[PPi]'^2}$	[PPi]'
$[Cu]_{tot}$, $[PPi]_{tot}$ and [PPi]'	$[Cu]'=\frac{[PPi]_{tot}-[PPi]'}{k_1'[PPi]'+2\,k_2'[PPi]'^2}$	[Cu]'
[Cu]' and [PPi]'	$k_1'=\frac{[CuP_2O_7^{2-}]}{[PPi]'[Cu]'}$	$[CuP_2O_7^{2-}]$
[Cu]' and [PPi]'	$k_2'=\frac{[Cu(P_2O_7)_2^{6-}]}{[PPi]'^2\,[Cu]'}$	$[Cu(P_2O_7)_2^{6-}]$

The data were fitted empirically to polynomial or rational expressions of the lowest order necessary to provide acceptable fits and with comparable goodness. The expressions used are given in Table 2.

The empirical expressions were applied to four different models of the behaviour of the absorption of the system. Each one was fitted by non-linear multiple regression to the experimental data of the four series using the QNFIT module of the SIMFIT program. The four models assayed and the results of the respective fittings are given in Table 3. In all cases, the molar absorptivity of the Cu(II) species not complexed with PPi was assumed to be the same. Underlying this

assumption is the fact described above, with nearly all the Cu(II) not complexed with PPi being in the form of dihydroxycuprate. In the first model, the molar absorptivity of the Cu-PPi complexes is negligible, and absorbance is due exclusively to dihydroxycuprate. In the second model, the molar absorptivity of $Cu(P_2O_7)_2^{6-}$ is negligible, and $CuP_2O_7^{2-}$ and dihydroxycuprate have the same molar absorptivity. In the third model, the molar absorptivity of $[CuP_2O_7^{2-}]$ and $[Cu(P_2O_7)_2^{6-}]$ is the same, and different from that of dihydroxycuprate. Finally, in the fourth model the molar absorptivities of $[CuP_2O_7^{2-}]$, $[Cu(P_2O_7)_2^{6-}]$ and dihydroxycuprate are different. The chemical description of these models is given in Table 3.

Table 2. *Empirical expressions representing the dependence of [Cu]', $[CuP_2O_7^{2}]$ and $[Cu(P_2O_7)_2^{6-}]$ on $[Cu]_{tot}$ for the $Cu^{2+}/P_2O_7^{4-}$ system at pH 9.4 for different ratios of cation: ligand, $[Cu]_{tot}$:$[PPi]_{tot}$, and with $[Cu]_{tot}$ in the 0 to 0.30 mM range.*

Cu(II): PPi 2:1	$[Cu]' = 0.9212\ [Cu]_{tot}$ $[CuP_2O_7^{2-}] = 1.660e-3[Cu]_{tot} + 285.0[Cu]_{tot}^2 + 1.580e5[Cu]_{tot}^3$ $[Cu(P_2O_7)_2^{6-}] = -1.167e-4\ [Cu]_{tot} + 1.665[Cu]_{tot}^2 - 5.986e3[Cu]_{tot}^3$
Cu(II): PPi 1:1	$[Cu]' = 0.8505\ [Cu]_{tot}$ $[CuP_2O_7^{2-}] = 4.908e-3[Cu]_{tot} + 546.2[Cu]_{tot}^2 - 3.579e5[Cu]_{tot}^3$ $[Cu(P_2O_7)_2^{6-}] = -4.476e-4\ [Cu]_{tot} + 6.915[Cu]_{tot}^2 + 2.080e4[Cu]_{tot}^3$
Cu(II): PPi 1:10	$[Cu]' = \frac{1.361e-4\ [Cu]_{tot}}{1.248e-4 + [Cu]_{tot}}$ $[CuP_2O_7^{2-}] = 0.4311e[Cu]_{tot} + 479.0[Cu]_{tot}^2$ $[Cu(P_2O_7)_2^{6-}] = -0.02649[Cu]_{tot} + 576.2[Cu]_{tot}^2$

The results of the changes in absorbance as a function of the PPi concentration, for the four experimental series with different ratios Cu(II):PPi were fitted to the four models described above by non-linear multiple regression, using the same boundary conditions and initial limits. The best fit corresponded to the third model, in which the molar absorbance of the two complexes of Cu(II) with PPi was the same, and different from that of Cu(II) in solution. These values are $\varepsilon_{CuP_2O_7^{2-}} = \varepsilon_{Cu(P_2O_7)_2^{6-}} = 1373.8\ M^{-1}cm^{-1} \pm 107.3$ and

$\varepsilon_{Cu'}$ = 3372.6 M^{-1} cm^{-1} ± 43.9 respectively, expressed with the 95% confidence interval. The corresponding fit is depicted in Fig. 5a, and the plot of the residuals against the expected value is displayed in Fig. 5b, showing a random dispersion and an unbiased distribution of strings (P > 0.05).

TABLE 3. *Description of the four models considered for the absorptivity of the $Cu^{2+}/P_2O_7^{4-}$ system at pH 9.4 together with the results of the corresponding non-linear multiple regression fittings to the experimental data*

Model	Number of parameters	Number of imprecise parameters	Sum of deviations*
$\varepsilon_{Cu'} \neq 0$ $\varepsilon_{Cu(P_2O_7)_2^{6-}} = \varepsilon_{CuP_2O_7^{2-}} = 0$	1	–	0.302
$\varepsilon_{Cu'} = \varepsilon_{Cu(P_2O_7)_2^{6-}} \neq \varepsilon_{CuP_2O_7^{2-}}$	2	1	0.235
$\varepsilon_{Cu'} \neq \varepsilon_{Cu(P_2O_7)_2^{6-}} = \varepsilon_{CuP_2O_7^{2-}}$	2	–	0.0166
$\varepsilon_{Cu'} \neq \varepsilon_{Cu(P_2O_7)_2^{6-}} \neq \varepsilon_{CuP_2O_7^{2-}}$	3	1	0.0139

*The sum of deviations gathers the residual sum of squares

4. The influence of PPi on the absorbance of Cu in solution can be described by a mathematical model

Changes in the absorptivity of Cu(II) in aqueous solution in the presence of PPi were monitored for concentrations of $[PPi]_{tot}$ ranging from 0.005 to 10 mM. The results are shown in Fig. 6. The experimental data and the estimated values using the molar absorbance coefficients calculated previously are represented. The correspondence between both series reflects both the goodness of the response of the method and that of the estimated values found for $\varepsilon_{Cu'}$ and $\varepsilon_{CuP_2O_7^{2-}} = \varepsilon_{Cu(P_2O_7)_2^{6-}}$. The mathematical expression used to calculate A_{280} is:

$A_{280,tot}=\varepsilon_{Cu'}([Cu]'sample-[Cu]'blank) + \varepsilon_{CuP_2O_7^{2-}}([CuP_2O_7^{2-}]sample-[Cu(P_2O_7)_2^{6-}]blank+[Cu(P_2O_7)_2^{6-}]sample-[CuP_2O_7^{2-}]blank)$

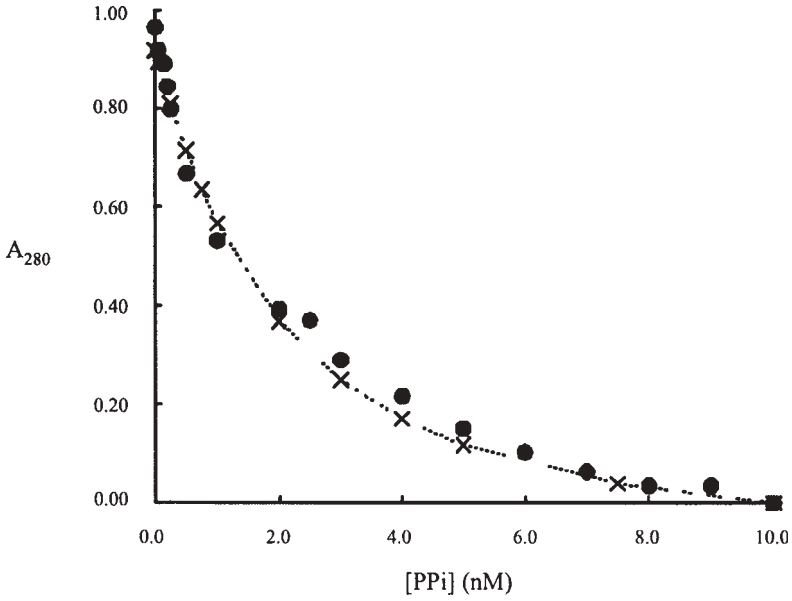

Figure 6. ***Correspondence between expected and real behaviour for the Cu(II)/PPi system.*** *Plotting of the experimental (●) and calculated (×, dotted line) values of A_{280} for the addition of PPi, with $[Cu]_{tot}$ = 0.50 mM, and containing the blank 0.50 mM $CuSO_4$ 10.0 mM $Na_4P_2O_7$ in 100 mM AMPSO/NaOH pH 9.4 at 37° C.*

where the values of the concentrations of the absorbent species are $[Cu]'_{blank} = 4.14e\text{-}5$, $[CuP_2O_7]_{blank} = 2.38e\text{-}4$ and $[Cu(P_2O_7)_2^{6-}]_{blank} = 2.21e\text{-}4$.

Owing to the impossibility of finding an easy mathematical expression affording A_{280} as a function of $[PPi]_{tot}$ we decided to describe the behaviour of the system by means of an empirical equation. The simplest expression providing the best fit with a minimum number of parameters was a modified hyperbolic decay:

$$A_{280} = p(1)[PPi]_{tot} + \frac{p(2)\ p(3)}{p(3) + [PPi]_{tot}}$$

where $p(1)$ = 1.269e-2 ± 1.9e-3, $p(2)$ = 0.9539 ± 1.13e-2, and $p(3)$ = 1.4866 ± 9.29e-2 expressed as data ± standard error of the mean. The fitting of the experimental data to this expression and the 95% confidence limits are shown in Fig. 7. Other equations were also tested, and both hyperbolic decay and exponential decay rendered acceptable fits. However, according to the F criterion defined by Mannervik (20) the former equation provides the best fit, considering both the goodness of the fit and the number of parameters needed, and the current availability of non-linear fitting software does not justify the use of over-simplified mathematical approximations (21). On the other hand, we are aware that analytical methodology tends to prefer linear relationships. The inset in Fig. 7 shows the linear relationship between absorbance shift and [PPi] after an exponential decay fit (r^2 = 0.9904) for small [PPi] values.

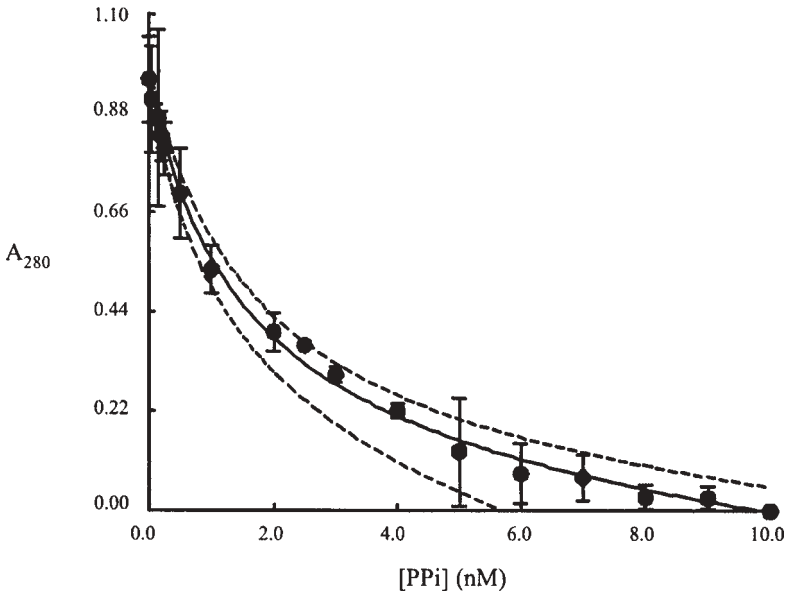

FIGURE 7. ***Variation in the A_{280} of Cu(II) in the presence of PPi.*** *The blank contained 0.5 mM $CuSO_4$, 10 mM $Na_4P_2O_7$ in 100 mM AMPSO/NaOH, pH 9.4, at 37° C. The error bars encompass the gap of 95% confidence for each experimental value. The continuous line corresponds to the best fit for the empirical expression of A_{280}, where p(1) = –1.269e-2 ± 1.9e-3, p(2) = 0.9539 ± 1.13e-2 and p(3) = 1.4866 ± 9.29e-2 (values expressed as data ± standard error of the mean). The broken lines delimit the gaps of 95% confidence for the best curve of fitting.*

In our system, the signal at zero was 0.9539 ± 0.0632, corresponding to a relative detection limit (3σ-criterion) of 0.233 mM, corresponding to 0.116 μmol of PPi. The sensitivity of the method is defined by the slope of the calibration line. In this case, between [PPi] = 0.50 and 2.0 mM there was a change of 0.30 absorbance units for each change of concentration unit (mM), corresponding to a change of 0.60 A_{280} units per mmol of PPi.

The sensitivity of the method proposed here can be compared with the sensitivity of enzymatic methods: it is thus higher than PPi spectrophotometric determination by NADH oxidation (13), similar to that of the formation of purines (15) and lower than that of the chemiluminescence quantification (14). A deeper comparison with other methods for measuring PPi is given below.

5. PPi can be determined spectrophotometrically in the presence of phosphate, SDS, Mg(II) and proteins

The interference of phosphate, SDS, proteins, EDTA, nucleotides and Mg(II) in the response of the analytic method proposed was investigated. Values are given as concentrations of interferent in the final mixture. For the estimation of the true interference values it must be noted that the sample represents half the volume of the final mixture (see Material and Methods).

Interference by orthophosphate

The interference produced by the presence of orthophosphate at final concentrations of 1, 10 and 50 mM was studied. No significant difference was found in the response of the method in the presence of orthophosphate, up to 50 mM ($P > 0.05$). The absence of interference produced by orthophosphate is the main difference between the method proposed here and methods that titrate total phosphates, monitoring either the formation of ammonium phosphomolybdate (9) or the formation of 2-amino-6-mercapto-7-methyl-purine (15). These chemical methods can not differentiate orthophosphate and PPi, and only those based on the enzymatic hydrolysis of PPi are able to discern between both species. The

spectrophotometric method reported here has sensitivity comparable to that of enzymatic methods but, in contrast, is inexpensive and less time-consuming. Furthermore, it avoids the competitive inhibition by orthophosphate in the enzymatic reactions coupled to the hydrolysis of PPi.

Interference by SDS

The interference produced by the presence of SDS in the medium was studied. Final concentrations of 0.50%, 1.0%, 5.0% and 10% w/v were assayed. No statistically significant differences were found ($P > 0.05$) up to 5% SDS upon comparison with the values observed without SDS (Fig. 8). The absence of interference by SDS allows working with a high number of samples without immediately processing them, and also permits measuring PPi in cell extracts.

Interference by proteins

Interference by protein at final concentrations of 0.1, 1.0 and 2.5 mg/mL was studied using bovine serum albumin. The presence of protein quenches the change in the A_{280} of Cu(II), possibly due to the reaction of the lone electron pair on the nitrogen of the peptide bond with Cu(II). For protein concentrations below 1 mg/mL, the behaviour of the system is restored in the presence of 1% SDS (Fig. 8). For concentrations of protein above 1 mg/mL, the addition of 1% SDS does not restore the behaviour of the system, although it does decrease the intensity of the interference. In these cases, protein can be removed to eliminate the interference by precipitation with trichloroacetic acid prior to the addition of $CuSO_4$, as described under material and methods. This treatment does not affect the determination of PPi with $CuSO_4$.

Interference by EDTA

Interference by EDTA at final concentrations of 0.1, 0.5 and 1 mM was studied. In all the cases, a decrease in the absorbance of

Cu(II) at 280 nm was observed, possibly due to the formation of complexes between this cation and the EDTA. The presence of 2% SDS restores the behaviour of the system for the concentrations tested (data not shown).

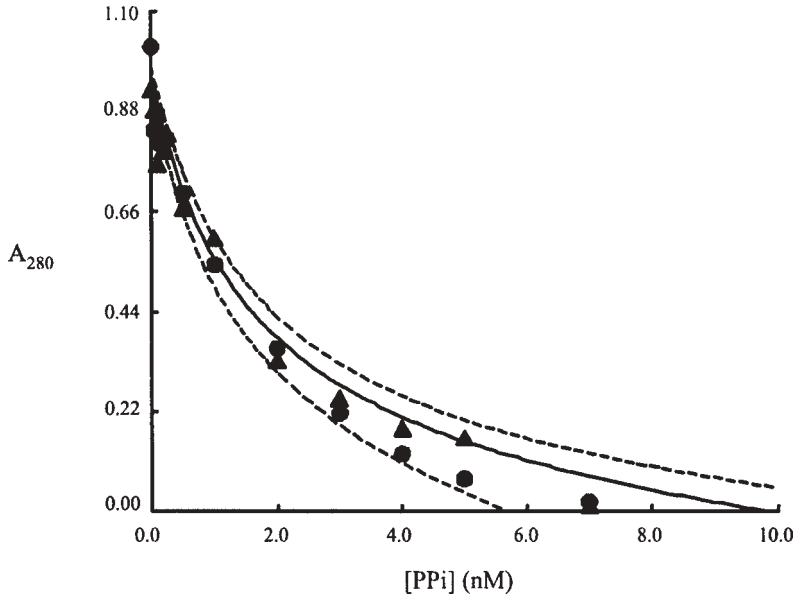

FIGURE 8. ***Variation in the A_{280} of Cu(II) in the presence of PPi, and interferences produced by the presence of 5% SDS or 50 mM Na_3PO_4.*** *The blank contained 0.5 mM $CuSO_4$ and 10 mM $Na_4P_2O_7$ in 100 mM AMPSO/NaOH, pH 9.4, at 37º C. The continuous line corresponds to the best fit in the absence of interferences. The broken lines delimit the margins of confidence for 95% of the curve. The presence of 5% SDS ● or 50 mM Na_3PO_4 ▲ did not significantly change the behaviour of the system (P > 0.05), which can be described by the same empirical equation.*

Interference by nucleotides

The influence of nucleotides in the medium at concentrations of 1.0 and 5.0 mM was studied using ATP. The presence of ATP decreases the absorbance of Cu(II), possibly by forming complexes with the polyphosphate tail. The addition of SDS 2% allowed this

interference to be controlled up to a concentration of 5 mM ATP (data not shown).

Interference by Mg(II)

The presence of Mg(II) is essential for the catalytic activity of many enzymes whose reaction product is PPi. Accordingly, interference by Mg(II) at concentrations of 1.0, 5.0 and 10.0 mM was studied. It resulted in a decrease in the absorbance of Cu(II) that was proportional to the concentration of $MgCl_2$ in the sample, probably due to the competition between Mg(II) and Cu(II) for PPi. The presence of 2% SDS allowed this interference to be overcome up to a concentration of 1 mM Mg(II) (data not shown). For higher concentrations of Mg(II), it was necessary to remove this cation from the reaction medium. This was accomplished by the addition of DOWEX-50 at the pH of the assay (AMPSO/NaOH pH 9.4), followed by centrifugation. The interference is thus abolished and the supernatant can then be treated as formerly described.

DISCUSSION

The interaction of PPi with Cu(II) in water solution modifies the absorption spectrum of the cation. We have spectrophotometrically characterised the Cu(II):PPi system and modelled the interaction between the different chemical species. We have provided here a proper description of the system, the equilibria, and the spectrophotometrical behaviour of the absorbent species. Since the changes in absorptivity are concentration-dependent, we have implemented the knowledge of the system into a spectrophotometric method for determining PPi in solution. The main advantages of the method for PPi determination here presented are its speed, simplicity, price, and relative high sensitivity. In comparison with methods based on chromatography (10) or capillary electrophoresis (11), titration with Cu(II) is more sensitive and the procedure is simpler, regarding both sample preparation and data reading.

Methods based on chemical reactions are less sensitive than the assay reported here. Another important difference with the method

based on the generation of ammonium phosphomolybdate (9), is its selectivity: Cu(II) titration allows measurement of 0.5 mM PPi in the presence of 50 mM orthophosphate.

The spectrophotometric method reported here is especially useful for quantifying PPi in the biochemistry laboratory. In this environment, enzymatic methods are the standard, but they fail to achieve direct detection of PPi; instead, they follow the progress of enzymatic activities such as the oxidation of NADH (13), the emission of light by a luciferase (14) or the formation of purines (15), all of them coupled to the hydrolysis of PPi. With the titration with Cu(II) it is possible to measure PPi directly, therefore decreasing the error in the measurements. Regarding sensitivity, the proposed method is more sensitive than following NADH oxidation, similar to the monitoring of purine synthesis, and less sensitive than quantification of light emission by luciferase. Before applying these enzymatic methods, moreover, the activities of many reagents must be monitored to ensure that PPi is in fact the rate-limiting component in the assay. Furthermore, the components of such assays often contain compounds that may hamper the proper development of enzyme activity. It is necessary therefore to spend time establishing the optimum assay conditions before using these methods (22). Additionally, enzymatic protocols are usually cumbersome, they waste reagents and are time-consuming. In comparison with this, the method here described is rapid and these complexes are stable at the assay temperature used.

Regarding the robustness of the assay, the use of SDS makes it possible to stop enzymatic reaction and collect a large number of samples or cell extracts with no need of immediately reading each of them. Interferences by proteins up to 1 mg/mL, EDTA up to 1 mM, ATP up to 5 mM and $MgCl_2$ up to 1 mM can be overcome by addition of SDS to the sample. Regarding the presence of nucleotides, enzymatic methods such as that based on the formation of purines permit the presence of ATP only up to 0.5 mM; others, such as that employing luciferase, cannot be used if ATP is a component of the sample. Simple modifications of the general procedure are also provided for cases with higher concentrations of proteins or $MgCl_2$ in the sample.

An enzymatic method for measuring PPi coupled to NADH oxidation is commercially available, but is expensive: each determination has an average cost of 10€ and needs *ca* 30 min *per* point to be completed. Compared with this, Cu(II) titration is inexpensive and the only additional reagent is inorganic, $CuSO_4$, thus allowing low assay costs.

CONCLUSIONS

Here we present a comprehensive chemical and spectrophotometric description of the Cu(II):PPi, implementable as spectrophotometric method for the quantification of PPi. This method is based on modification of the Cu(II) absorption spectrum in the presence of PPi by following the formation of complexes between these two species. The sensitivity limit of the method is 0.1 μmol of PPi, such that it is possible to titrate small amounts of this product and work with microsamples.

The determination is rapid. The formation of complexes between Cu(II) and PPi is immediate and these complexes are stable at the assay temperature, so it is possible to analyse many samples in a short period of time.

The procedure to quantify PPi is also simple and a general procedure is described including preparation of the blank and the sample at the assay pH. SDS is added to avoid interferences by proteins, nucleotides, EDTA and Mg(II). Modifications of the method in the presence of high concentrations of proteins and Mg(II) do not represent a significant change in this general procedure and are also properly detailed.

The determinations achieved with the proposed method are inexpensive. The only additional reagent is $CuSO_4$, and hence the assays can be considered to have virtually zero cost.

These characteristics make the Cu(II) titration appropriate for the determination of PPi in chemical and biochemical reactions. The method also allows the measurement of PPi in the presence of orthophosphate. Accordingly, it is possible to quantify mixes of these two analytes.

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