Using Cuprizone to measure copper concentration

CuRe

iGEM

A comprehensive guide by the EPFL 2021 iGEM team

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EPFL

About this protocol

This protocol uses cuprizone as a copper chelating agent. Cuprizone [oxalic acid bis(cyclohexylidene hydrazide)] is a selective and sensitive copper-chelating agent, typically used in spectrophotometric determination of copper(II). This chemical compound is highly sensitive to copper and, once it interacts with copper ions, it forms a complex that has a peak absorbance between 595nm-600nm, hence rendering the solution blue to the naked eye for concentrations as low as 1mg/L.
Cuprizone is cheap compared to the kits one would normally buy for copper detection experiments. Since it is bought as a highly concentrated powder, it can serve for a great number of experiments whereas the easy to use pre-made kits usually only enable about 20 measurements.
Since we spent hours trying, failing, and learning how to correctly use the agent, we have made an easy-to-use protocol. It is based on calibration experiments, multiple trial and errors, and multiple repeats of the experiment.

We started with following two papers characterizing cuprizone (Messori & al, 2007 and Soares & al 2019). From that, we needed to design an experiment suited to our needs, which are to reproducible copper measurements over time using a spectrophotometer.

To better understand the spectrophotometer and the cuprizone, we completed a few experiments from which we summarized the lessons here so future teams do not make the same mistakes.

Using Cuprizone

Avoid molecular equilibrium between copper and cuprizone:
Our first experiments were done using Cuprizone at molar parity. Cuprizone has a molecular weight of 278.35 (ref 1 Cuprizone | C14H22N4O2 - PubChem (nih.gov)) while copper sulfate has a molecular weight of 159.61(ref 2 Copper sulfate | CuSO4 - PubChem (nih.gov)). The copper in our solution is much less concentrated than the cuprizon. Molar parity means that for 5ml of copper solution, one puts only a few microliters of cuprizone. The results showed no sign of copper. Hence, the cuprizone should not be used at molecular weight equilibrium, but in much higher quantities than the copper.

How to Dissolve Cuprizone:
Cuprizone does not dissolve in water alone. Ethanol is needed and it was found that with 30% ethanol at 50°C, cuprizone remained as a separate phase in the liquid.

Calibration: absorbance to concentration:
Calibration experiments have yielded different results depending on the cuprizone solution. One thing was found in common: the calibration curves show linearity up to 10mg of copper but cuprizone is not a good enough tool for measurements below 0.2mg/l.

Use only Cuprizon on the day it was made:
We have tried using Cuprizone multiple days in a row and have seen an important decrease in absorbance at similar concentrations of copper. Linearity of the calibration measurements was also deteriorated.

Cuprizone is slower than most kits:
Cuprizone kinetics show that a minimum of 16 minutes is needed to have a reliable measurement. That time is extended for concentrations of copper above 5mg/L. Wait that time, as the results will be very variable otherwise.
As was previously mentioned, copper chelating using cuprizone is not instantaneous and the kinetics vary depending on the concentration. This graph should help you choose your time more accurately!

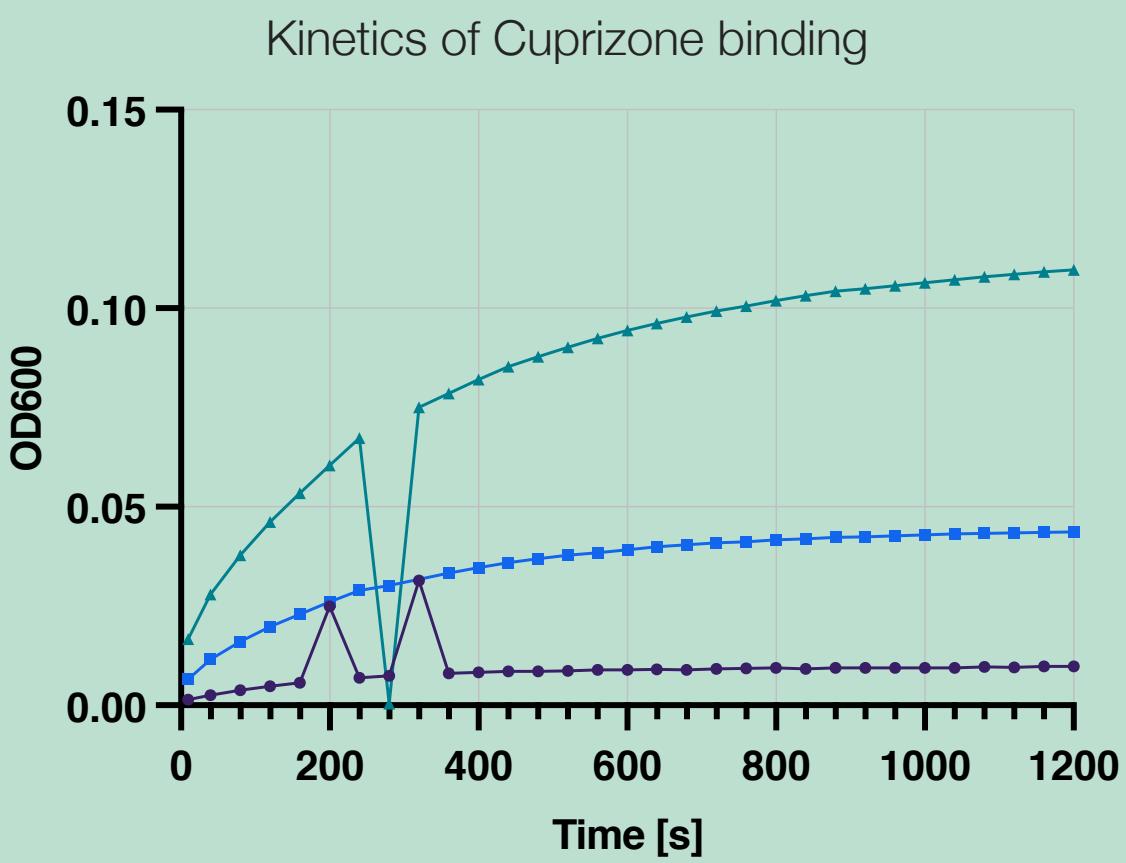

Figure - Kinetics of Cuprizone binding to Copper. Binding kinetics are monitored by changes in OD600, which depends on copper-cuprizone complex formation. Three different conditions were tested, from top to bottom: 10 mg/L, 5 mg/L and 2 mg/L copper in presence of 5g/L of cuprizone. Stability is reached after 16 minutes for all conditions. The lower the copper concentration, the faster stability is achieved.

The protocol

Cuprizone Preparation (250ml, enough for at least 95 tests)
Purpose: This solution is used for chelating copper, and hence detecting it with a 600nm absorbance measurement. See wavelengths you can use on this document (soares & co 2019).

Materials:

- 1x 250ml bottle
- 1.25g cuprizone 99%
- 125ml deionized water
- 125ml ethanol 99%

Setup & protocol:

- Add Cuprizone, water and ethanol into the bottle
- Stir with a magnetic stirrer at 70°C for for 20minutes or until cuprizone is dissolved
- Let the bottle cool to about 35°C before use

Copper Concentration measurement
Materials:

- Blanks (450ul per blank)
- Samples (450ul per sample)
- Cuprizone solution (2.55ml per sample)
- NaOH 0.2M (3 drops per sample)
- pH paper
- 1x spectrophotometer
- 4 ml cuvettes (1 per sample)

The protocol

Setup & protocol:

- Make the appropriate blanks using the same media as for the samples
- Align samples and cuvettes in their respective boxes so that for each cuvette, the position of the corresponding sample is known.
- Put 450ul of each sample into its corresponding cuvette
- add 3 drops of NaOH 0.2M
- Set up a timer
- Every 40 seconds, pipette 2.55ml of cuprizone per cuvette into 3 empty cuvettes until all cuvettes are full. Keep track of the cuvettes as you will have to follow the same order when measuring.
- At the 16 minutes time point measure the first samples. Proceed by measuring 3 samples at a time every 40 seconds.

Making a Calibration Curve

Purpose: This measurement is needed for accurately calculating the relationship between copper concentration and absorbance.
Comment: This part can be done simultaneously with the samples.
Warning: Make sure you use the same blank for the calibration and for the samples!

The calibration curves are made using exactly the same protocol as for the rest of the samples.

References

Messori, Luigi & Casini, Angela & Gabbiani, Chiara & Sorace, Lorenzo & Muniz-Miranda, Maurizio. (2007). Unravelling the chemical nature of copper cuprizone. Dalton transactions (Cambridge, England : 2003). 2112-4. 10.1039/b701896g.

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ref 1 Cuprizone | C14H22N4O2 - PubChem (nih.gov)
ref 2 Copper sulfate | CuSO4 - PubChem (nih.gov)