



Analytical Methods

Rapid determination of trace copper in animal feed based on micro-plate colorimetric reaction and statistical partitioning correction

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ABSTRACT

The objective of this study was to develop a micro-plate based colorimetric assay for rapid and high-throughput detection of copper in animal feed. Copper ion in animal feed was extracted by trichloroacetic acid solution and reduced to cuprous ion by hydroxylamine. The cuprous ion can chelate with 2,2'-bicinchoninic acid to form a Cu-BCA complex which was detected with high sensitivity by micro-plate reader at 354 nm. The whole assay procedure can be completed within 20 min. To eliminate matrix interference, a statistical partitioning correction approach was proposed, which makes the detection of copper in complex samples possible. The limit of detection was 0.035 µg/mL and the detection range was 0.1–10 µg/mL of copper in buffer solution. Actual sample analysis indicated that this colorimetric assay produced results consistent with atomic absorption spectrometry analysis. These results demonstrated that the developed assay can be used for rapid determination of copper in animal feed.

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1. Introduction

Copper is a trace element that plays an essential role in animals and is significantly involved in intracellular metabolism (Nath, 1997). It has been widely supplemented in animal feeds as a growth promotant (Coffey, Cromwell, & Monegue, 1994). However, copper is often added in excessive amounts to enhance the growth rate, especially in swine production throughout the world (Dove, 1995). Excessive copper in animal diets can result in serious hazards to animal health and the environment, and consequently poses a potential threat to human health (Lores & Pennock, 1998). Where there is runoff into ground water from animal feeding facilities, excessive copper in animal diets that is carried into the ground water could very well contribute to acute and chronic copper toxicity in humans (Dusek et al., 2015; Yu, Du, & Wang, 2007). Consequently, several organizations throughout the world have limited copper contamination levels in the environment within certain ranges to protect human health. The Environmental Protection Agency of the United States has set the limit of copper in drinking water at 1.30 µg/mL (Yan & Zhou, 2003). With regard to feed, the European Union has set the maximum permitted limit of supplemented copper to 175 mg/kg in animal feeds (European

Commission, 2003), and China authorities have established the maximum limit of copper to no more than 200 mg/kg in animal feeds (AQSIQ, 2010).

Conventional methods for copper determination include atomic absorption spectrometry (AAS) (Ajtony et al., 2016), atomic emission spectrometry (ICP-AES) (Shoae, Roshdi, Khanlarzadeh, & Beiraghi, 2012), and inductively coupled plasma mass spectrometry (ICP-MS) (Martin-Camean et al., 2014). Although these methods possess high selectivity and sensitivity, they generally require cost-prohibitive instrumentation and sophisticated operation, thus they are not suitable for rapid large-scale monitoring in routine laboratory condition. Therefore, there is an increasing interest in the development of simple and low-cost methods for the detection of copper. In recent years, many rapid assays, including electrochemical detection (Chaiyo, Chailapakul, Sakai, Teshima, & Siangproh, 2013; Fu et al., 2013; Cui, Wu, Li, Ge, & Ju, 2014), immunoassay (Liu, Lou, Shi, Wang, & Zhu, 2013; Ouyang et al., 2016; Zhao et al., 2011) and colorimetric assay (Li et al., 2014; Sun et al., 2016; Chaiyo, Siangproh, Apilux, & Chailapakul, 2015; Zhang, Xing, Liu, Zhou & Shi, 2015; Li et al., 2016; Zhao et al., 2016) have been developed (Table 1). Electrochemical detection has very high assay sensitivity. However, the preparation and modification of electrodes and substrates are quite complex, an electrochemical workstation is required, and most analytical laboratories are not routinely equipped with this capability. Additionally, electrochemical detection is very prone to matrix interference and

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Table 1
A brief literature review of copper detection methods.

Method	Key reactive reagent	Detection range	Detection time (min)	Sample type	Reference
Colorimetric assay	Unmodified gold nanoparticle	0.1–10 μM	About 30 min	Drinking water; Tap water; Lake water	Zhang et al., 2015
Colorimetric assay	Polyethylenimine-capped Au nanoparticles	25–300 μM	1 h	River water; Waster water	Zhao et al., 2016
Colorimetric assay	Functional filter paper	33.6 nM–39.8 μM	About 20 min	River water	Li et al., 2016
Colorimetric assay	Silver nanoplates	0.5–200 ppb	Less than 10 min	Mineral water; Ground water; Tomato juices; Rice; Blood	Chaiyo et al., 2015
Fluorimetric and colorimetric analysis	Silver nanoclusters	0.0001–1 μM	About 10 min	Not specified	Sun et al., 2016
Colorimetric assay	TiO ₂ /cellulose paper	0.5–50 ppm	Not specified	Water	Li et al., 2014
Electrochemical detection	Ag nanoparticle	1.0–1000 nM	About 90 min	Tap and lake water	Cui et al., 2014
Electrochemical detection	Gold nanoparticle/single-walled carbon nanotubes	0.1–140 nM	Not specified	River water	Fu et al., 2013
Electrochemical detection	Adsorptive stripping voltammetry	0.1–50 ppb	About 10 min	Orange juice; Vegetable juice; Tea; Honey	Chaiyo et al., 2013
Immunoassa	Antibody	1–1000 ppb	Not specified	Traditional chinese medicine; Water	Ouyang et al. (2016)
Immunoassay	Antibody	0.25–8.13 ppm	About 2 h	Dring water; Human serum	Liu et al., 2013
Immunoassay	Antibody	0.1–10 ppb	About 1.5 h	Water	Zhao et al., 2011
AAS	–	0.03–10 ppm	1.5 min per sample	Alcoholic beverage	Ajtony et al., 2016
ICP-AES	–	5–120 ppb	–	Water sample	Shoae et al., 2012
ICP-MS	–	2.0–100 ppb	–	Oral mucosa cell	Martin-Camean et al., 2014

most of the reported methods were only used for the detection of copper in a simple matrix such as drinking water (Table 1). A further disadvantage is that electrochemical detection can only measure a few sample solutions at one time, which makes it difficult to be used for high-throughput applications. Immunoassay is generally considered as a very specific and sensitive approach for the detection of small and large molecules. However, it requires preparation of monoclonal antibodies against the copper chelating complex and this process generally requires several months. Moreover, the reported immunoassay also encountered serious interference from other metal ions and was only used for measuring copper ions in water (Ouyang et al., 2016) (Table 1). In recent years, many novel colorimetric assays have been developed for copper detection. Most of these colorimetric assays are based on nanomaterials such as gold nanoparticles (Zhang, Xing, Liu, Zhou, & Shi, 2015; Zhao et al., 2016), silver nanoclusters (Sun et al., 2016) and silver nanoplates (Chaiyo et al., 2015), and they were also only used in a simple matrix (Table 1). The ability for colorimetric assays to be applied in a more complicated matrix has not been fully elucidated. For complicated samples such as animal feed, there are sulfur-containing amino acids, peptides, and proteins which can interact with nanomaterials with strong binding affinity and thus could significantly interfere with the detection of copper. Research has been reported using colorimetric assays based on TiO₂/Cellulose paper (Li et al., 2014) or functional filter paper (Li et al., 2016). Unfortunately, these substrate materials were not commercially available and they also require complicated modifications. Based on our review of literature, there are currently no published approaches that can be practically applied in a complex matrix such as animal feed.

In this study, our objective was to develop a new colorimetric assay for the rapid and high throughput determination of copper in animal feed. The basis of this assay involves the chelation reaction between cuprous ions and bichinchoninic acid (BCA), leading to a purple solution in alkaline pH conditions. To enhance detection efficiency, this chelating reaction was performed in micro-plate wells and the reactive solutions were measured by a micro-plate reader. As the sample matrix contains a lot of molecules which have optical densities at the detection wavelength, a statistical partitioning correction approach was introduced to address this problem. To the best of our knowledge, this is the first reported method that can rapidly and accurately determine copper content in a complex sample such as animal feed.

2. Materials and methods

2.1. Reagent and equipment

Standard stock solutions of Fe²⁺, Zn²⁺, Mg²⁺, Ca²⁺, Mn²⁺ and F[–] were purchased from the Institute for Reference Materials of SEPA, Beijing, China. Bichinchoninic acid disodium salt (>98%), sodium carbonate anhydrous, potassium sodium tartrate tetrahydrate, sodium hydroxide, sodium bicarbonate, trichloroacetic acid, copper sulfate pentahydrate, sodium phosphate dibasic, potassium phosphate monobasic, potassium chloride, and sodium chloride were purchased from Sigma Aldrich (MO, USA). All chemicals were analytical grade.

All solutions were prepared with de-ionized water (18 M Ω cm resistance, purified by Millipore Milli-Q purification system). The pH values of solutions were measured by a pH meter (Thermo Scientific™ Orion 8135BN ROSS). The ultra-violet visible spectrum was scanned by a UV–vis spectrophotometer (Evolution 300). The optical densities of each reaction solution in micro-plate well were obtained by Multimode Microplate reader (Synergy™ HT).

2.2. Solution preparation

Trichloroacetic acid (TCA) solution was prepared by dissolving trichloroacetic acid in deionized water to a final concentration of 1% (w/v).

Carbonate-bicarbonate buffer was prepared by dissolving 9.73 g sodium carbonate anhydrous and 9.41 g sodium bicarbonate in 1000 mL ultrapure water, then adjusting pH to 9.8 by sodium hydroxide.

BCA stock solution (10 g/L, 25.8 mM) was prepared by dissolving 0.5 g of bicinchoninic acid disodium salt in 50 mL of carbonate-bicarbonate buffer. This solution is stable for at least 3 months at 4 °C.

BCA working solution (500 µg/mL, 1.29 mM) was prepared by diluting 0.5 mL of the BCA stock solution to 10 mL with carbonate-bicarbonate buffer. This solution is freshly prepared before use.

Hydroxylamine hydrochloride stock solution (100 g/L, 1.44 M) was prepared by dissolving 5 g of hydroxylamine hydrochloride in 50 mL of carbonate-bicarbonate buffer. This solution is stable for at least 3 months at 4 °C.

Hydroxylamine hydrochloride working solution (10 g/L, 144 mM) was prepared by diluting 0.5 mL of the BCA stock solution to 5 mL with carbonate-bicarbonate buffer. This solution is freshly prepared before use.

Working solution was prepared by mixing BCA working solution and the hydroxylamine hydrochloride working solution at the ratio of 5:3 (v/v).

Standard copper solution was prepared by dissolving copper sulfate pentahydrate in TCA solution to 0, 0.1, 1, 2, 4, 6, 8, 10 µg/mL of copper ion, respectively.

2.3. Sample preparation

Animal feed samples were ground and passed through #40 mesh. Subsequently, 1 g of sample was mixed with 100 mL of 1% TCA solution and was vibrated for 3 min. The mixture was then centrifuged at 8000 rpm for 10 min or passed through a filter paper to obtain a clean extract. The clean extract was used in determination of copper concentrations.

2.4. Analytical procedure

To enhance detection efficiency, this assay was performed in micro-plate wells and reactive solutions were measured by a micro-plate reader. For the construction of standard curves, eight different concentrations (0, 0.1, 1, 2, 4, 6, 8, 10 µg/mL) of copper standard solutions were added into micro-plate wells with 150 µL per well, then 100 µL of the working solution was added into each well. For the determination of copper concentration in sample solutions, two micro-plate wells were used for each sample. One well was used as a background well and the other one was used as a detection well. The sample extract solution (150 µL/well) was added into both wells. Subsequently, 100 µL of carbonate-bicarbonate buffer was added into the background well, whereas 100 µL of the working solution was added into the detection well. All the micro-plate wells were then kept at room temperature for 15 min, followed by reading at 354 nm by a micro-plate reader.

2.5. Result calculation

The absorbance value resulting from each copper standard solution was corrected based on the following equation:

$$A_c = A - \frac{\beta_{BCA} V_{BCA} C_{BCA_0}}{(V_{BCA} + V_{Cu})}$$

where A_c is the corrected absorbance value, A is the original absorbance value at 354 nm, V_{BCA} and V_{Cu} are the added volumes of working solution and copper standard solution respectively, C_{BCA_0} is the BCA concentration in working solution, M_{BCA} and M_{Cu} are the molecular weights of BCA and copper respectively. β_{BCA} is the slope of the BCA calibration curve, which was calculated as 0.00442 as shown in Section 3.

The calibration curve of copper standard solutions was then constructed by plotting the corrected absorbance values (A_c) against the concentrations of copper standards (C_{Cu-std}). The calibration curve was then linearly fitted and the slope of the curve (β_{Cu}) was obtained according to the equation as below.

$$A_c = \beta_{Cu} C_{Cu-std} + a$$

The β_{Cu-BCA} value was then calculated by the following equation:

$$\beta_{Cu-BCA} = \frac{\beta_{Cu} M_{Cu} (V_{BCA} + V_{Cu}) + 2\beta_{BCA} M_{BCA} V_{Cu}}{M_{Cu-BCA} V_{Cu}}$$

Copper concentrations in sample supernatants were then calculated based on the following equation:

$$C_{Cu-sample} = \frac{(A_2 - A_1)(V_{Cu} + V_{BCA})M_{Cu} - V_{BCA} C_{BCA_0} \beta_{BCA} M_{Cu}}{V_{Cu} \beta_{Cu-BCA} M_{Cu-BCA} - 2V_{Cu} \beta_{BCA} M_{BCA}}$$

where $C_{Cu-sample}$ is the copper concentration in sample supernatant, A_1 and A_2 are the absorbance values of the background well and detection well, respectively, and β_{Cu-BCA} is calculated as described above.

Copper content in animal feed samples was then calculated by the following equation:

$$\omega_{Cu} = \frac{C_{Cu-sample} \times V_{TCA}}{m}$$

where ω_{Cu} is the copper content in the sample, $C_{Cu-sample}$ is the copper concentration in the sample supernatant, calculated as shown above, V_{TCA} is the volume of TCA extraction solution, and m is the sample weight.

3. Results and discussion

3.1. Selection of detection wavelength

The assay principle was as shown in Fig. 1A. Copper ion was reduced to cuprous ion by hydroxylamine hydrochloride, and then the cuprous ions were bound with BCA to form a BCA-Cu complex. The BCA-Cu complex, along with BCA alone can contribute to significant absorbance in several wavelength regions. As shown in Fig. 1B, BCA and BCA-Cu complex presented three major absorbance peaks at 257, 354 and 562 nm, which is in agreement with the report of a previous study by Fransted et al. (2014). The optical density (OD) value at 257 nm is the highest among the three peaks, but at this wavelength, the feed sample supernatant also has strong absorbance which would interfere with the detection of Cu-BCA complex (Fig. 1C). The OD value at 562 nm is relatively low in comparison to that at 354 nm (Fig. 1D), which results in lower sensitivity. Therefore, 354 nm was selected for the determination of copper in this research.

3.2. Optimization of reaction conditions

The effect of pH value on formation of Cu-BCA complex was investigated (Fig. 2A). It was found that when pH was within the range of 5–11, the absorbance value of the formed complex were similar throughout this range. This result is consistent with previous results (Brenner & Harris, 1995). Also, it was reported that the BCA complex was stable in carbonate-bicarbonate buffer

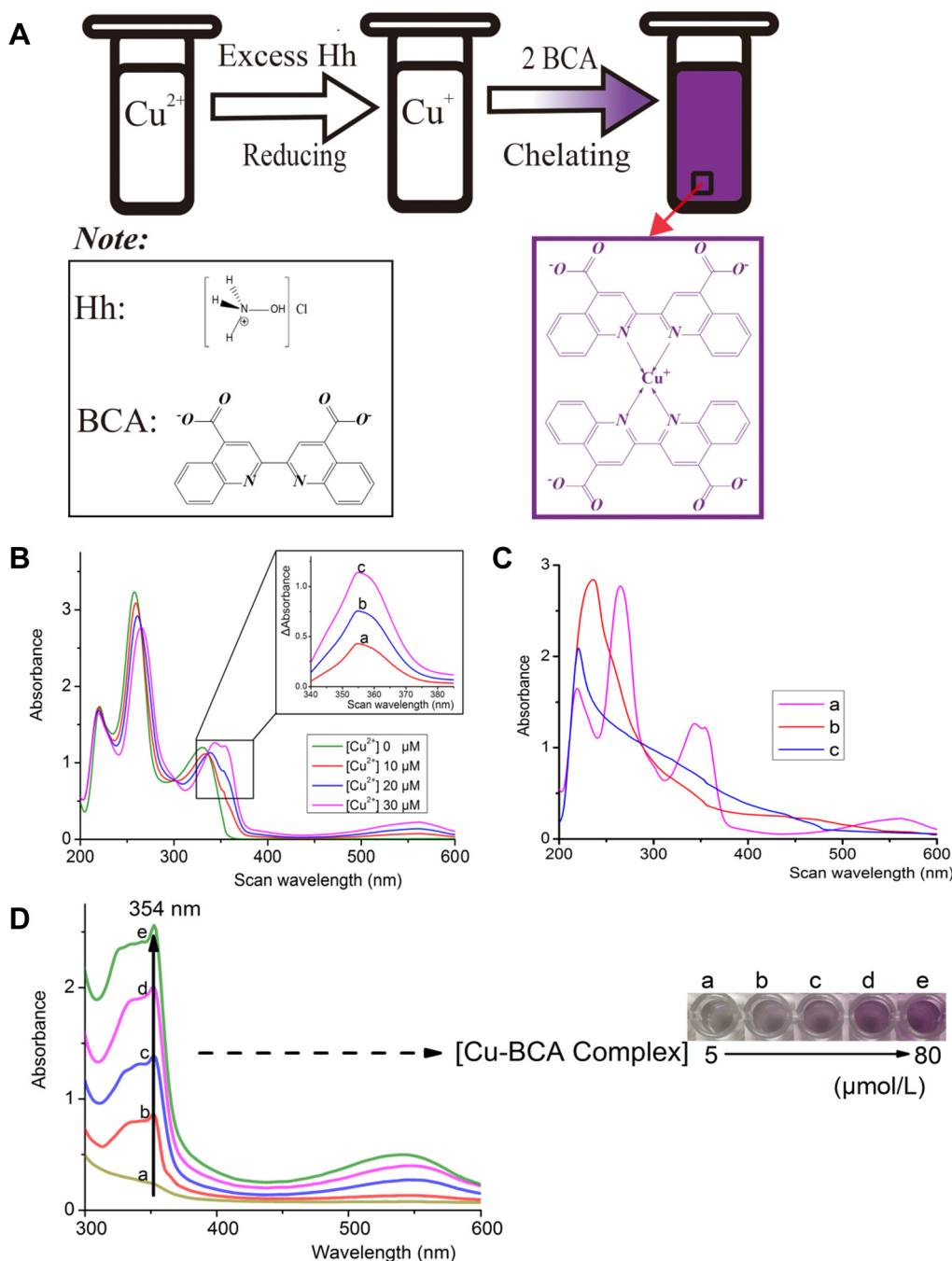


Fig. 1. BCA assay principle and UV-vis wavelength scan. (A) BCA assay principle. (B) UV-vis scan of BCA and BCA-Cu complex in solutions containing 70 μM BCA, with concentrations of $[\text{Cu}^{2+}]$ mixed as: (a) 10 μM , (b) 20 μM , (c) 30 μM . $\Delta\text{Absorbance}$ means readings were calibrated against carbonic acid buffer (pH = 9.31) with 70 μM BCA. (C) UV-vis scan of (a) solution containing 70 μM BCA and 30 μM copper, (b) and (c) are two different feed samples randomly selected, diluted in TCA at the ratio of 1:100 (g/mL) after centrifugation at 8000 rpm for 10 min. (D) UV-vis scan of BCA-Cu complex in various solutions with Cu, Cu and excess Hh: (a) 5 μM Cu and 10 μM BCA, (b) 15 μM Cu and 30 μM BCA, (c) 45 μM Cu and 90 μM BCA, (d) 65 μM Cu and 130 μM BCA and (e) 80 μM Cu and 160 μM BCA.

(Braun, Wiechelman, & Gallo, 1989). Thus, carbonate-bicarbonate buffer at pH 9.8 was chosen in the present work.

The effects of reaction time and temperature were also investigated. As shown in Fig. 2B, for a high concentration of copper (6 $\mu\text{g/mL}$), the formation of Cu-BCA complex is very rapid independent of reaction temperature. When the incubation time was less than one hour, both reaction time and temperature had little impact on the formation of the BCA-Cu complex. Trials demonstrated that the complex could be stable for 60 min at 37 $^{\circ}\text{C}$ and stable for 6 h at 4 $^{\circ}\text{C}$ and 20 $^{\circ}\text{C}$ for the higher concentration of copper.

For low concentrations of copper (0.4 $\mu\text{g/mL}$), the formation of Cu-BCA complex was much slower and was influenced by both time and temperature. The time for sufficient reaction between BCA and copper was 15, 30 and 75 min for 37 $^{\circ}\text{C}$, 20 $^{\circ}\text{C}$ and 4 $^{\circ}\text{C}$ respectively. Because the objective of our study was to develop an on-site detection method, the operation of all procedures at room temperature was preferred. Fig. 2B illustrates that the absorbance values for reaction at room temperature for 15 and 30 min were similar, thus 15 min was chosen as the optimal reaction time.

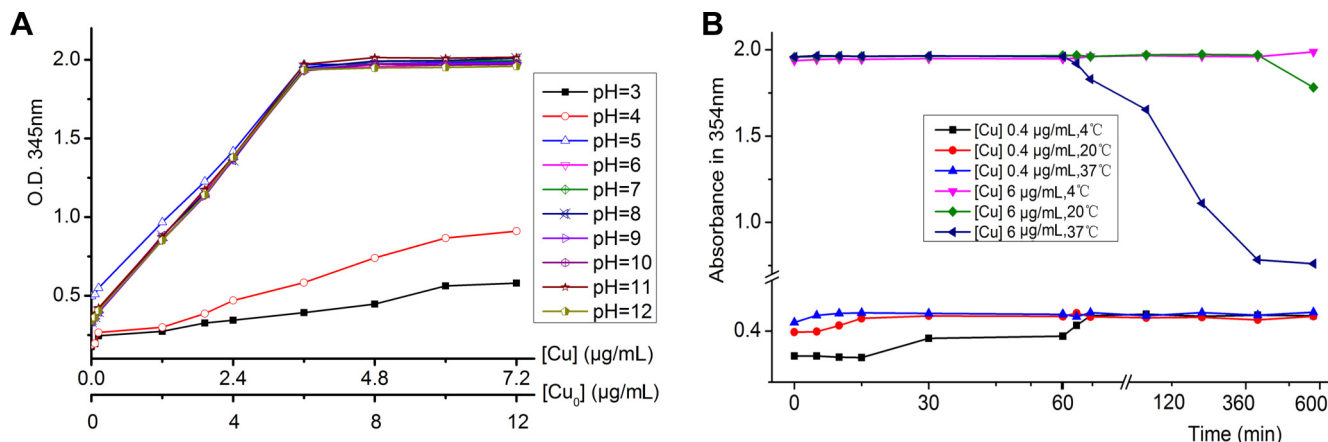


Fig. 2. Optimization of assay conditions. (A) Effect of pH on the absorbance of Cu(II)-BCA solutions containing 62.5 μg/mL BCA, measured in micro-plate wells, where [Cu] and [Cu₀] represent the actual (after eventually mixed in micro-plate wells) and added (in standard copper solutions) copper concentration. (B) Effect of time and temperature upon the solutions of cuprous ion and BCA solution, in which [Cu] = 0.4 μg/mL and 6 μg/mL, respectively.

3.3. Derivation of equations

Several factors could contribute to the whole absorbance of sample solution at 354 nm. For example, BCA contributes to the OD value at 354 nm, and the amount of interference depends heavily on amount of BCA in the solution. Moreover, sample solutions contain complex ingredients such as vitamins, amino acids and other additives, some of which may contribute to the OD value at 354 nm (Højberg, Knudsen, Canibe & Jensen, 2001). In addition, the micro-plate well contributes to the OD value at 354 nm because of its material composition (Atkar et al., 2013). Thus, to obtain an accurate OD value of the Cu-BCA complex, the partitioning of the total OD value is necessary.

According to Beer-Lambert's law, the whole absorbance (OD value) of a certain solution can be partitioned into several parts. For background and detection well as above, their OD values can be expressed as Eqs. (1) and (2),

$$A_1 = A_s + A_w + A_e \quad (1)$$

$$A_2 = A_{Cu-BCA} + A_{BCA-excessive} + A_s + A_w + A_e \quad (2)$$

by subtracting (1) from (2), we can get Eq. (3):

$$A_2 - A_1 = A_{Cu-BCA} + A_{BCA-excessive} \quad (3)$$

where A_s stands for absorbance value of sample ingredients, A_w is absorbance caused by micro-plate wells, A_e is random detection error that follows the normal distribution (Fig. S1), A_{Cu-BCA} and $A_{BCA-excessive}$ are the absorbance values of Cu-BCA complex and excessive BCA, respectively. Eq. (3) demonstrated that the difference between A_2 and A_1 is influenced by the absorbance values of Cu-BCA complex and excessive BCA.

As the absorbance values of Cu-BCA complex and BCA follow the Beer-Lambert's law, we can get Eqs. (4) and (5) as follows:

$$A_{BCA-excessive} = k_{BCA} C_{BCA} d = \beta_{BCA} C_{BCA-excessive} \quad (4)$$

$$A_{Cu-BCA} = k_{Cu-BCA} C_{Cu-BCA} d = \beta_{Cu-BCA} C_{Cu-BCA} \quad (5)$$

where k is absorption coefficient ($\text{g}^{-1} \text{L cm}^{-1}$) and C represents concentration (g/L), d stands for optical length (cm). With unit's transformation and cumulative, k and d are combined to get β as the slope of absorbance-concentration equation. $C_{BCA-excessive}$ and C_{Cu-BCA} represent the concentration of excessive BCA and Cu-BCA complex in micro-plate wells, respectively. Because of excess hydroxylamine hydrochloride and the reported stability constant of Cu-BCA complex at $\beta_2 = 10^{17.67}$ (Bagchi, Morgan, Bacsá, &

Fahrni, 2013), all copper ions were reduced to cuprous ions and formed to Cu-BCA complexes, thus

$$C_{Cu-BCA} = \frac{M_{Cu-BCA}}{M_{Cu}} C_{Cu-well} \quad (6)$$

Combining Eqs. (3)–(6), we can get Eq. (7):

$$\begin{aligned} A_2 - A_1 &= \beta_{Cu-BCA} C_{Cu-BCA} + \beta_{BCA} C_{BCA-excessive} \\ &= \frac{M_{Cu-BCA}}{M_{Cu}} \beta_{Cu-BCA} C_{Cu-well} + \beta_{BCA} C_{BCA-excessive} \end{aligned} \quad (7)$$

When the BCA working solution and sample supernatant were mixed in micro-plate wells, the actual concentration of BCA and copper in the sample well is as shown in Eqs. (8) and (9) based on their initial concentrations:

$$C_{BCA-well} = \frac{V_{BCA} C_{BCA_0}}{V_{BCA} + V_{Cu}} \quad (8)$$

$$C_{Cu-well} = \frac{V_{Cu} C_{Cu-sample}}{V_{BCA} + V_{Cu}} \quad (9)$$

where V_{BCA} and V_{Cu} are the added volumes of BCA working solution and sample solution (feed supernatant or copper standard solution), C_{BCA_0} and $C_{Cu-sample}$ are BCA concentration in BCA working solution and copper concentration in sample solution, respectively.

The actual concentration of copper and excessive BCA can be determined because of the stoichiometric ratio of this reaction (Cu:BCA = 1:2), which is shown as Eq. (10),

$$C_{BCA-excessive} = \left(\frac{C_{BCA-well}}{M_{BCA}} - \frac{2C_{Cu-well}}{M_{Cu}} \right) M_{BCA} \quad (10)$$

$C_{BCA-excessive}$ represents the concentration of excessive BCA in micro-plate well. Combining Eqs. (10)–(7), the formula to calculate copper content in sample supernatant is shown as Eq. (11):

$$A_2 - A_1 = \frac{M_{Cu-BCA}}{M_{Cu}} \beta_{Cu-BCA} C_{Cu-well} + \beta_{BCA} \left(\frac{C_{BCA-well}}{M_{BCA}} - \frac{2C_{Cu-well}}{M_{Cu}} \right) M_{BCA} \quad (11)$$

Combining Eqs. (8), (9) and (11), we can get Eq. (12) as below:

$$C_{Cu-sample} = \frac{(A_2 - A_1)(V_{Cu} + V_{BCA})M_{Cu} - V_{BCA} C_{BCA_0} \beta_{BCA} M_{Cu}}{V_{Cu} \beta_{Cu-BCA} M_{Cu-BCA} - 2V_{Cu} \beta_{BCA} M_{BCA}} \quad (12)$$

where C_{Cu_0} stands for copper concentration in sample supernatant. Consequently, to calculate copper content in animal feed, β_{Cu-BCA} and β_{BCA} have to be calculated.

3.4. Calculation of β_{BCA}

The partitioning of total absorbance values of BCA solutions can be shown as Eq. (13):

$$A = A_{BCA} + A_w + A_e \quad (13)$$

Combining Eqs. (4) and (13), we can get Eq. (14):

$$A = \beta_{BCA} C_{BCA-std} + (A_w + A_e) = \beta_{BCA} C_{BCA-std} + a \quad (14)$$

where a is the intercept of this linear equation. To obtain the β_{BCA} value, series concentrations of BCA standard solutions were measured at 354 nm. The calibration curve of BCA standard solution was then constructed by plotting the absorbance value of BCA against the respective BCA concentration (Fig. 3A). Thus β_{BCA} can be considered the slope of the calibration curve. The measurement of β_{BCA} was then repeated 10 times and the average value was calculated as 0.00442 with low coefficient of variation (0.085%).

3.5. Calculation of β_{Cu-BCA}

The total absorbance of Cu-BCA complex can be partitioned as equation (15):

$$A = A_{Cu-BCA} + A_{BCA-excessive} + A_w + A_e \quad (15)$$

Combining Eqs. (4)–(6), (8)–(10) and (15), we can get Eq. (16):

$$A - \frac{\beta_{BCA} V_{BCA} C_{BCA0}}{(V_{BCA} + V_{Cu})} = \frac{(\beta_{Cu-BCA} M_{Cu-BCA} V_{Cu} - 2\beta_{BCA} M_{BCA} V_{Cu}) C_{Cu-std}}{M_{Cu}(V_{BCA} + V_{Cu})} + (A_w + A_e) \quad (16)$$

We define that

$$A_c = A - \frac{\beta_{BCA} V_{BCA} C_{BCA0}}{V_{BCA} + V_{Cu}} \quad (17)$$

$$\beta_{Cu} = \frac{\beta_{Cu-BCA} M_{Cu-BCA} V_{Cu} - 2V_{Cu} M_{BCA} \beta_{BCA}}{M_{Cu}(V_{BCA} + V_{Cu})} \quad (18)$$

Eq. (16) can be transferred as:

$$A_c = \beta_{Cu} C_{Cu-std} + a \quad (19)$$

where β_{Cu} and a represent the slope and intercept of standard curve of Cu-BCA complex. C_{Cu-std} stands for the concentration of copper standard solution. As a consequence, by plotting A_c and C_{Cu-std} , β_{Cu} can be calculated as shown in Fig. 3B.

Meanwhile, Eq. (18) can be transferred as:

$$\beta_{Cu-BCA} = \frac{\beta_{Cu} M_{Cu}(V_{BCA} + V_{Cu}) + 2\beta_{BCA} M_{BCA} V_{Cu}}{M_{Cu-BCA} V_{Cu}} \quad (20)$$

Then β_{Cu-BCA} can be calculated according to Eq. (20).

To obtain the β_{Cu} value, series concentrations of copper standard solutions were mixed with BCA solution and then measured at 354 nm. The calibration curve of Cu-BCA solution was then be constructed by plotting the correcting absorbance (A_c) against the copper concentration in sample supernatant $C_{Cu-sample}$. In the present study, the measurement of β_{Cu} was repeated 10 times and the average value was 0.30501 with a relatively high coefficient of variation (10.76%). Substituting $\beta_{Cu} = 0.30501$ into Eq. (18), the β_{Cu-BCA} value was calculated at 0.04235 in the present study. Therefore, for each copper determination, a series of copper standard solutions were also involved to obtain more accurate results. In the meantime, the molar extinction coefficients of Cu-BCA complex at various pH values were also calculated based on β_{Cu-BCA} in Eq. (18). The values were in the range of $4.27\text{--}4.91 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ as shown in Table S1, which are consistent with previous reports (Brenner & Harris, 1995) and this result indicates that the BCA-based colorimetric assay is highly sensitive.

With the parameters β_{BCA} and β_{Cu-BCA} determined, the absorbance of detection and background well (A_2 and A_1) of a sample could be substituted into Eq. (12) to calculate the copper content in the sample.

3.6. Interference of foreign ions

The effect of co-existing metal ions on the determination of copper ion was investigated. Several ions with high abundance in animal feed were tested for their possible interferences with copper determination. Briefly, $4 \mu\text{g/mL}$ of copper standard solution was applied to the colorimetric assay and the absorbance value

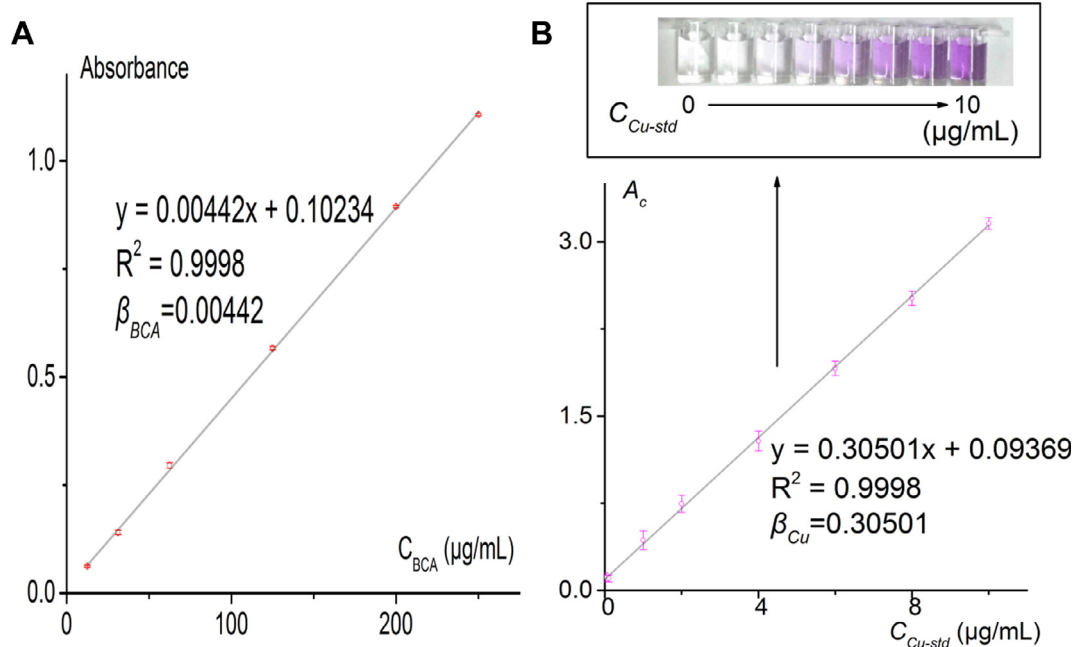


Fig. 3. Correction equations of BCA assay. (A) corrected linear regression curve and equation of BCA to calculate β_{BCA} . (B) linear regression equation between $[Cu]$ and A_c to calculate β_{Cu} , where C_{Cu-std} represents the copper concentration in standard copper solutions. The error bar represents standard deviation; experiments were repeated ten times.

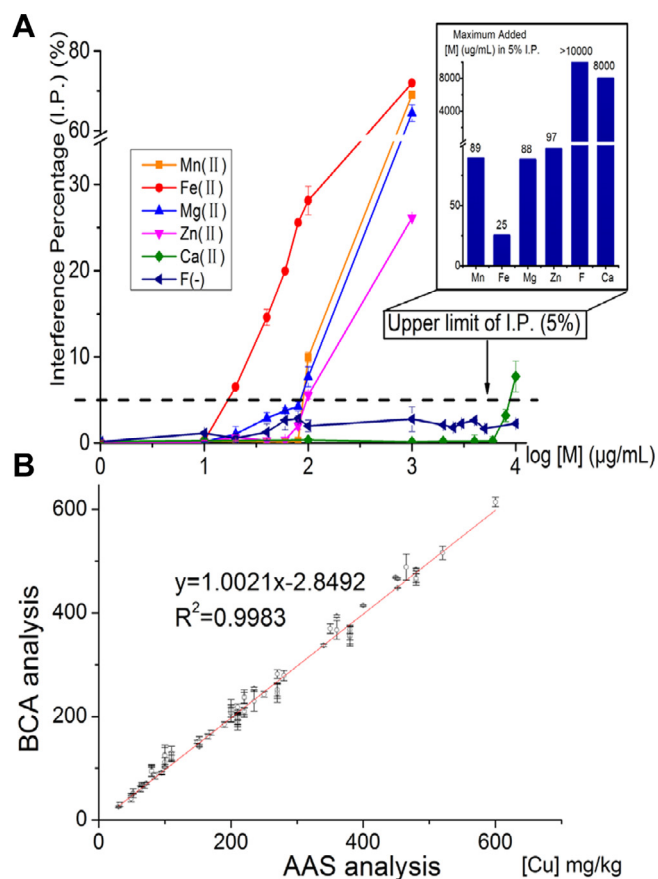


Fig. 4. Selectivity and accuracy of BCA assay. A: Comparison between the *I.P.* of 4 μg/mL standardized copper solutions with and without foreign ions added. The error bar represents standard deviation of two repeats. B: Linear regression equation for data analyzed by both standard AAS method and proposed BCA method for 78 actual feed samples. (x axis, standard AAS method; y axis, proposed BCA method.) The error bar represents standard deviation, in which the analysis of BCA method is repeated four times.

was set as the background value (A_b : 4 μg/mL [Cu] without foreign ions), whereas the same solution supplemented with Fe^{2+} , Zn^{2+} , Mg^{2+} , Mn^{2+} from 1 to 1000 μg/mL, respectively, and Ca^{2+} , F^- from

1 to 10,000 μg/mL, were also tested and the absorbance value was set as test value (A_t : 4 μg/mL [Cu] with foreign ions added). The interference percentage (*I.P.*) was defined as equation below to quantify the interference of foreign ions.

$$I.P. = \left| \frac{A_t - A_b}{A_b} \right| \times 100\%$$

The result was as shown in Fig. 4A, where the concentrations of Fe^{2+} , Zn^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} , and F^- resulting in 5% *I.P.* were about 25, 97, 89, 88, 8000 and >10,000 μg/mL, respectively. These concentration of ions in the sample solution were equal to 2500, 9700, 8900, 8800, 800,000 and >1,000,000 mg/kg of Fe^{2+} , Zn^{2+} , Mg^{2+} , Mn^{2+} , Ca^{2+} , and F^- in animal feed, respectively assuming that all the interferent ions were totally extracted. It is obvious that animal feed would not contain such high concentrations of these ions, thus the existence other ions in the sample solution would not pose significant effects on the detection of copper. These data also demonstrated that BCA is a highly specific chelating reagent for copper detection.

3.7. Assay validation

The calibration curve for copper determination was established by plotting the corrected absorbance values (A_c) against the concentrations of copper standards (C_{Cu-std}) and was then fitted linearly. As shown in Fig. 3B, the linear range for copper concentration covered from 0.1 to 10 μg/mL. The limit of detection (LOD) was calculated according to the equation as below (Uhrovčík, 2014).

$$LOD = \frac{3s - a}{k}$$

where s is the standard deviation of blank sample (micro-plate with 0 μg/mL copper standard solution), and k and a are the slope and intercept of the calibration curve of copper standard solutions (Fig. 3B). Applying the values $a = 0.09369$ and $k = 0.30501$, the LOD was calculated at 0.035 μg/mL in standard solution.

The assay accuracy and precision were evaluated by the spiked recovery and coefficients of variation (CVs). Four batches of feed samples including two chicken (Sample 1 and 2) rations and two swine rations (Sample 3 and 4) were used as the spiked matrix. The original copper content in these feed samples were measured

Table 2
Spiked recoveries and coefficients of variation (CVs) of copper in animal feed.

Samples	BCA detection Cu^{2+} (mg/kg)		Mean of recovery (%)	CV (%)
	Added	Detected ^a	Mean ± SD	
Sample 1 (30 mg/kg copper content by AAS)	0	30.12	100.40 ± 6.19	6.17
	20	51.68	103.37 ± 7.24	7.01
	100	128.72	99.02 ± 2.87	2.90
	400	448.36	104.27 ± 5.40	5.18
Sample 2 (49 mg/kg copper content by AAS)	0	47.40	96.74 ± 8.05	8.32
	20	66.55	96.45 ± 8.00	8.30
	100	151.13	101.43 ± 2.12	2.09
	400	468.82	104.41 ± 0.29	0.28
Sample 3 (52 mg/kg copper content by AAS)	0	50.90	97.88 ± 6.30	6.44
	20	70.93	98.51 ± 10.21	10.36
	100	153.45	100.95 ± 0.44	0.43
	400	496.26	109.79 ± 0.05	0.05
Sample 4 (65 mg/kg copper content by AAS)	0	65.81	101.24 ± 1.21	1.20
	20	81.98	96.44 ± 2.16	2.24
	100	172.69	104.66 ± 4.46	4.26
	400	471.98	101.50 ± 0.34	0.33

^a Value of copper content is the average of four repeated subsamples.

by AAS analysis and the values were 30, 49, 52 and 65 mg/kg from sample 1 to sample 4, respectively.

These samples were considered as “blank” matrix and were then spiked with 0, 100, 200 and 400 mg/kg of copper ion. The copper ions in these spiked samples were then extracted and the contents were measured by the developed colorimetric assay as described above. As shown in Table 2, the recoveries were found to be in the range of 96.44% and 109.79% with CVs less than 11%, which suggests that the accuracy and precision of the proposed BCA assay are acceptable.

Seventy-eight actual feed samples were analyzed by both the BCA-based colorimetric assay and AAS analysis, and results were compared. For AAS analysis, sample is treated by acid digestion, which would take more than 3 h to complete digestion. Animal feed is mainly composed of corn, soybean meal, amino acid additive, mineral additive, and vitamin additive. Copper ion in animal feed is mostly artificially added through mineral premix, so it can be directly extracted by suitable solution. In this study, 1% TCA solution was used to extract copper ion before BCA assay. It is observed that the extraction ratio (volume of extract solution vs sample weight) had significant impact on recovery test. The optimal extraction ratio was chosen as 100:1 as it can produce the best recovery efficiency (Table S2). Although different sample preparation procedures were employed for the two methods, they can produce similar results. As shown in Fig. 4B, the copper contents in all tested samples were in the range of 30–600 mg/kg by both methods. By plotting the analysis results of BCA assay and AAS analysis, the linear regression equation was calculated as $y = 1.0021x - 2.8492$ ($R^2 = 0.9983$). These results indicated that the two methods provided comparable results and demonstrated the developed assay in this study can be used for real sample analysis.

4. Conclusions

In this study, we developed a BCA-based colorimetric assay for rapid determination of copper content in animal feed. This assay has three advantages: First, it can rapidly detect copper within 20 min with high assay accuracy and precision. Second, it can effectively eliminate the matrix effect by the proposed statistical partitioning approach. Third, it can be performed in micro-plate wells which can be used in high-throughput detection. Furthermore, real sample analysis demonstrated that this assay can produce consistent results with AAS analysis and thus it can be a useful tool for the determination of copper in animal feed.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.11.012>.

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