การตรวจวัดเมลามีนทางอ้อมโดยอะตอมมิกสเปกโทรเมตรีของคอปเปอร์(II) ไอออนที่มากเกินพอ

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INDIRECT DETERMINATION OF MELAMINE BY ATOMIC SPECTROMETRY OF EXCESS COPPER(II) ION

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วิธีแอบซอร์บชันสเปกโทรเมตรีทางอ้อมได้พัฒนาขึ้นเพื่อการตรวจวัดเมลามีนหลังจากการเกิดสารเชิงซ้อนกับคอปเปอร์(II) ที่มากเกินพอ สารเชิงซ้อนคอปเปอร์-เมลามีนสังเคราะห์ได้จากปฏิกิริยาของสารละลายคอปเปอร์และเมลามีน โดยให้ความร้อนและเขย่าอย่างต่อเนื่อง ศึกษาพารามิเตอร์ที่ส่งผลต่อการเกิดสารเชิงซ้อน ได้แก่ ชนิดของเกลือคอปเปอร์ ตัวทำละลาย อุณหภูมิ และระยะเวลา เพื่อหาภาวะที่เหมาะสมที่สุดสำหรับการตรวจวิเคราะห์ นั่นคือ ระบบของเกลือคอปเปอร์(II) คลอไรด์ในเมทานอลที่อุณหภูมิ 80 °C เป็นเวลา 14 ชั่วโมง สารเชิงซ้อนที่สังเคราะห์ได้นำไปหาลักษณะเฉพาะด้วยเทคนิคการเลี้ยวเบนของรังสีเอ็กซ์ การวิเคราะห์เชิงความร้อน และอินฟราเรดสเปกโทรเมตรี นอกจากนี้ การศึกษาปริมาณสัมพันธ์โดยวิธีของ Job พบว่าอัตราส่วนคอปเปอร์ต่อเมลามีนเป็น 1:2 การศึกษาทางจลนศาสตร์เคมีได้บ่งชี้ว่า ปฏิกิริยาการเกิดสารเชิงซ้อนนี้เป็นปฏิกิริยาอันดับหนึ่งเมื่อเทียบกับทั้งคอปเปอร์และเมลามีน พลังงานก่อกัมมันต์ที่ได้จากการประมาณด้วยสมการอาร์เรเนียส มีค่า 19.60 กิโลจูลต่อโมล การตรวจวัดปริมาณคอปเปอร์ที่เหลือในสารละลายด้วยวิธีเฟลมอะตอมมิกแอบซอร์บชันสเปกโทรเมตรีแสดงให้เห็นว่าปริมาณคอปเปอร์ลดลงอย่างเป็นสัดส่วนโดยตรงกับปริมาณเมลามีนที่เพิ่มขึ้น กราฟเทียบมาตรฐานที่สร้างขึ้นระหว่างอนุกรมความเข้มข้นของเมลามีนกับแอบซอร์แบนซ์ของคอปเปอร์ได้แสดงความเป็นเส้นตรงด้วยค่าสัมประสิทธิ์ 0.9943 และค่าต่ำสุดของการตรวจวัดเท่ากับ 0.60 มิลลิโมลาร์ จากการใช้วิธีเอ็กซ์เทอนอลสแตนดาร์ด และสแตนดาร์ดแอดดิชันในการหาการได้กลับคืนของเมลามีนพบว่าอยู่ในช่วงที่ยอมรับได้คือ 70-93% และมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ 3.0-10%

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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The indirect atomic absorption spectrometric method was developed for the determination of melamine after forming complexes with excess copper(II) ions. The copper-melamine complex (Cu-Mel) was synthesized by constantly heating and stirring the solution of copper(II) and melamine. Parameters affecting the complex formation including types of copper salt, solvents, temperature, and curing time were investigated in order to optimize the conditions for analytical assay. The optimal conditions for the copper-melamine synthesis were the system of $CuCl_2$ in methanol at 80 °C for 14 hours. The synthesized complex was characterized by X-Ray powder diffraction spectrometry, thermogravimetry, and Fourier transform infrared spectrometry. In addition, stoichiometric study by Job's plot revealed 1:2 copper-melamine. The complexation reaction was first-order with respect to both copper and melamine, and the activation energy of the reaction estimated by Arrhenius equation was 19.60 kJ/mol. Flame atomic absorption spectrometry used for measuring the amount of copper remaining in the solution indicated that the amount of depleting copper was proportional to the amount of melamine. The calibration curve built between melamine concentration series and copper absorbance showed a linearity with the coefficient of 0.9943 and the limit of detection of 0.60 mM. The external standard and standard addition used for melamine determination in fish sample showed acceptable recoveries between 70-93% and 3.0-10% RSD.

Department : Chemistry Student's Signature .....
Field of Study : Chemistry Advisor's Signature .....
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LIST OF ABBREVIATIONS

A	absorbance
Cu-Mel	copper-melamine complex
E_a	activation energy
Eq.	Equation
Exp.	Experiment
FAAS	Flame atomic absorption spectrometry
FT-IR	Fourier transform infrared spectrometry
JCPDS	Joint Committee on Powder Diffraction Standards
K	Kelvin
k	rate constant
K_f	formation constant
K_{sp}	solubility product
ln	natural logarithm (to the base *e*)
log	logarithm (to the base 10)
Mel	melamine
rpm	round per minute
R^2	regression correlation coefficient
TGA	thermogravimetry
XRD	X-ray powder diffraction spectrometry
°C	degree Celsius

CHAPTER I

INTRODUCTION

1.1 State of the Problem

Melamine, a white solid powder, has become a subject of interest since the last recent years as a result of its adulteration in food and feed. The contamination of melamine caused a renal epidemic in cats and dogs. Besides, hundreds of thousands people were impacted from melamine contamination, tens of thousands children must be hospitalized because of blockages of excretory system, and some of which having severe conditions had died. All of these cases were resulted from consumption of melamine-tainted food. There was a presumption that some of fraudulent entrepreneurs deliberately admixed melamine in their products. Whereas melamine contained lots of nitrogen (about 66 % by mass), it was attractive to add melamine into some food and feed, because protein analysis would be done only simple nitrogen determination which could not distinguish between nitrogen contents from protein sources and those from non-protein sources. Thus, the addition of melamine into food and feed could misunderstand of high protein food or feed. Not only did the addition of melamine cause incorrectly report in protein analysis, but also it reduced certainly costs of production.

Toxicity of melamine would not instantaneously affect, but its accumulation was able to damage the excretory system. Since human body could not totally eliminate the consumed melamine, it would gather or combine with its analogues or stone forming agents in urine, such as calcium oxalate, uric acid, phosphate to rapidly form large complexes which would become kidney stones. This might lead to reproductive damage, bladder cancer and abrupt renal failure. With aforementioned seriousness, melamine determination in food was significantly important in order to prevent such severe conditions.

Determination of melamine in food has continually developed since there was the epidemic. In general, it has been mostly conducted by chromatographic methods. Both gas and liquid chromatography coupled with mass spectrometer have been used for this

purpose. Such methods, however, need expensive instrument, high operating cost, and complicated process. Spectrometry is a generally-used technique for analytical applications, having a low operating and instrumental cost, simple and rapid process. With such intriguing benefits, spectrometry is thus an alternative method for melamine determination.

Being a nitrogen-rich compound, melamine is considered as a high affinity ligand to bind with various metal ions especially copper ion. In this work, copper-melamine complex would be synthesized by a reaction of melamine and excess copper(II) solution. After the completion reaction, an amount of copper in the solution would proportionally decrease upon the amount of melamine. Hence, if the amount of initial copper is known, melamine could be quantitatively determined.

1.2 Objectives

This research aimed at (1) development of an indirect method for determination of melamine using atomic spectrometry and (2) determination of melamine-tainted food by such developed method.

1.3 Scope of the Research

Types of copper salts and solvents were initially investigated in order to obtain the most suitable system for copper-melamine complex formation. Then, effects of temperature and curing time were evaluated; the optimal conditions for copper-melamine synthesis would be selected for analytical application. Chemical kinetics parameters including an order of complexation reaction and activation energy were also estimated. Using the chosen conditions, calibration graph was built by fixing an amount of copper, varying an amount of melamine. After complete reaction, the synthesized copper-melamine complex would be characterized, the solution would be analyzed for the amount of remaining copper. This study was based upon an assumption that as the amount of melamine increases, the amount of copper would decrease proportionally.

Finally, the developed method would be validated, and applied for the determination of melamine in food or feed.

1.4 The Benefits of this Research

The developed atomic absorption spectrometric method, as the alternative one, could be used for indirect determination of melamine in food samples.

CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Melamine

2.1.1 Chemistry of melamine

Melamine or 1,3,5-triazine-2,4,6-triamine (structure shown in Figure 2.1) is an organic substance which is a white solid. Melamine is generally insoluble in common solvents, but somewhat dissolve in hot water [1].

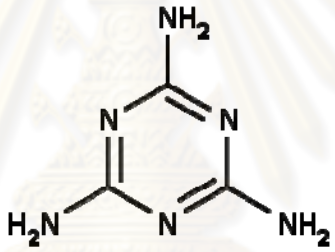


Figure 2.1 Structure of melamine.

Melamine can be produced by several pathways. In general, three substances: urea, dicyadinamide and hydrogen cyanide are used as starting materials [2]. In the preparation process, melamine may be hydrolyzed to yield its derivatives, *e.g.* ammiline, ammelide, and cyanuric acid as shown in Figure 2.2 [3].

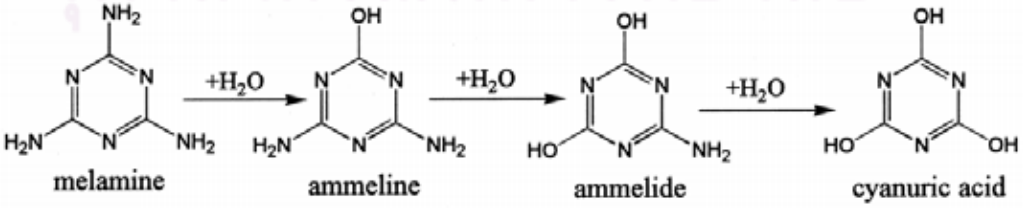


Figure 2.2 Formation of ammeline, ammelide, and cyanuric acid from hydrolysis of melamine [3].

Melamine is usually used as reactant in production of melamine-formaldehyde resins for various manufactures, such as surface coatings, laminates, Formica™, dishware and kitchenware. Some products of melamine also used as flame retardant and smoke suppressing agents [1-3].

2.1.2 Toxicity

Melamine has quite low acute toxicity. It has been reported that melamine has a median lethal dose (LD_{50}) of 3161 mg/kg based on rat data [4].

Melamine and cyanuric acid ingested in the body can accumulate and form huge complexes, and they may block the excretory system. Besides, consumption of melamine may result in reproductive damage, kidney stones, bladder cancer, and renal failure [2].

However, many countries have launched the regulation that allows melamine contaminated in food no more than 1 mg/kg, and dairy products no more than 2.5 mg/kg [2].

2.1.3 Determination of melamine

Since there had been reports of infant deaths and renal epidemic in cats and dogs in 2007 [5-6], the determination of melamine in food and feed has taken into serious consideration. Research in developing method for melamine determination has been widely published. Mostly melamine determination is conducted by chromatographic methods coupled with mass spectrometry [6-11]. Herein, the developed methods for detection of melamine are reviewed.

Xia *et al.* [8] developed sensitive methods for confirmation and detection of melamine in egg by gas chromatography–mass spectrometry (GC-MS) and ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS).

Ibáñez *et al.* [9] developed the liquid chromatography–electrospray tandem mass spectrometry (LC-ESI-MS/MS) for the determination of melamine in milk-based products and other food matrices.

Xu *et al.* [11] developed a coupled capillary column used in gas chromatography-mass spectrometry for both qualitative and quantitative determinations of melamine in dairy products.

Yan *et al.* [12] reported that they was the first ones who had developed capillary zone electrophoresis with diode array detection (CZE-DAD) for the determination of melamine in dairy products, fish feed, and fish.

Sun *et al.* [5] developed a reversed phase high-performance liquid chromatography-diode array detection (RP-HPLC-DAD) with solid-phase extraction (SPE) for the determination of melamine residue in liquid milk.

However, the use of chromatography may be suffered from complicated pretreatment, time-consuming steps, high instrument and operating cost [13-16]. Some other techniques without such limitations were developed for determination of melamine.

Liang *et al.* [16] developed polymeric membrane ion selective electrode based on molecular imprint polymer for selective recognition and determination of melamine in milk. The time consuming for analysis was less than 15 minutes. And this developed method can be used for onsite monitoring.

Rima *et al.* [17] developed a spectrometric method for determination of melamine based on Mannich reaction. Melamine was selectively reacted with formaldehyde and other substances having a ketone functional group. The final product was directly measured by UV-vis absorption spectrometry at 214 nm.

Zhou *et al.* [18] developed a fluorescent technique for melamine determination using cucurbit[7]uril sensor. Melamine could interact with cucurbit[7]uril resulting the increase in fluorescence intensity. The calibration curve constructed between melamine concentration and fluorescence intensity showed a good linearity. The result showed that it was a successful method for the determination of melamine in milk.

Ding *et al.* [19] developed a system of Fe_3O_4 magnetic nanoparticles-H_2O_2-ABTS (ABTS = 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid)diammonium salt) system for colorimetric determination of melamine in dairy products. The magnetic nanoparticles (MNPs) could react to H_2O_2 which generated green solution, and it was then detected by colorimetry. Meanwhile, in the presence of melamine, it could react with H_2O_2 resulting in the paler color. Thus, this method is an

indirect determination of melamine by colorimetry of Fe_3O_4 MNPs- H_2O_2-ABTS without expensive and complex instrument.

Wang *et al.* [20] developed a chemiluminescence method for determination of melamine. Melamine could inhibit the luminescence intensity of luminol and myoglobin. The intensity decreased proportionally to the amount of melamine added. This method could be used in melamine determination in milk products.

Li *et al.* [14], Guo *et al.* [15], and Chi *et al.* [21] have developed a visual detection of melamine using gold nanoparticles. It was based on the concept that melamine can interact with gold nanoparticles resulting in the aggregation of gold nanoparticles, and the color change of colloidal gold nanoparticles. These developed assays could be used for on-site screening of melamine-tainted food due to the clear color change observation.

2.1.4 Sample preparation

Melamine contaminated - food or feed samples are often homogenized and the extraction of melamine is an important process prior to analyze.

Sun *et al.* [5] extracted melamine using 1% trichloroacetic acid (TCA) and lead acetate in order to eliminate protein. After sonication and centrifugation, the supernatant was applied to PCX-SPE cartridge, and melamine was eluted by ammonia in methanol solution. Like Chi *et al.* [21], the mix solution of TCA and acetonitrile was used to extract melamine. After sonication, and centrifugation, the supernatant was filtered and purified using PCX-SPE. In addition, Yan *et al.* [11] proposed the similar sample preparation method; 1% TCA, water, and chloroform were mixed with samples. After sonication, the mixtures were centrifuged before direct injection in a capillary electrophoresis instrument.

Anderson *et al.* [7] used acetonitrile/water and a little amount of hydrochloric acid to eliminate fat. An aliquot of supernatant was removed to another tube. Dichloromethane was then added. After centrifugation, the upper aqueous portion was removed to a glass culture tube, water was added and the sample was re-extracted by

shaking. An Oasis MCX solid-phase extraction was used to cleanup sample extracts. 5% ammonium hydroxide in methanol was used as eluent.

Ibáñez *et al.* [9] extracted samples by shaking with 1% TCA, after centrifugation, tridecafluoroheptanoic acid was added before analysis. In addition, Li *et al.* [14] used TCA to deposit protein. The mixture was centrifuged, and filtered before applying to the developed assay. Ding *et al.* [19] also used the mix solution of acetonitrile and TCA for extraction. The sample was sonicated and centrifuged before using the supernatant for analytical process. Like Wang *et al.* [20], TCA was used to promote protein precipitation. Then, the mixture was sonicated and centrifuged, the clear solution was filtered, and taken for determination.

Besides, Rima *et al.* [17] proposed the mixture of dethylamine, water and acetonitrile as the extraction solvent. After centrifugation, dichloromethane was added in order to eliminate fat in fish sample. The aqueous layer was ready to analyze. And Zhou *et al.* [18] used zinc acetate and diluted hydrochloric acid in extraction process. The mixed solution was filtered, and the filtrate was assayed under the optimized condition.

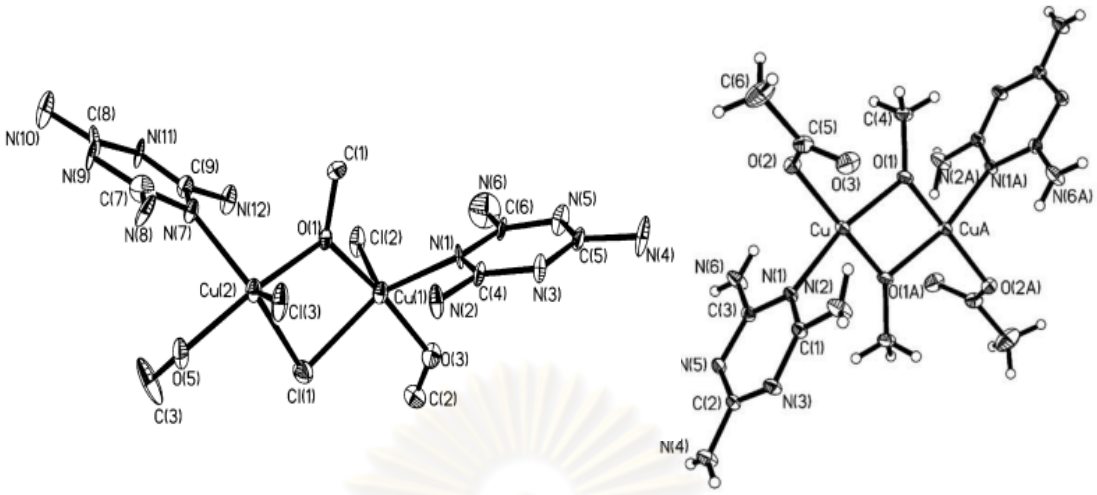
2.2 Copper-melamine complex synthesis

Melamine, a nitrogen-rich heterocyclic triazine compound, is seemed likely to be a promising ligand, however, there were few literatures investigating a coordination chemistry of melamine [1]. This is because of its low solubility as mentioned above. The metal-melamine compounds were almost fallen into three groups: melaminium dication salts coordinated with anions, melamine-co crystallized with inorganic salts, and a few fortuitously formed coordination complexes [1]. For instances, the reaction of $CuCl_2$ in methanol and diacetyl melamine yielded copper-melamine complex (Figure 2.3) [1]. Herein, some literatures reporting the copper-melamine complex synthesis are reviewed [1, 22-24].

Figure 2.3 Diacetyl copper-melamine complex [1].

Wiles *et al.* [1] synthesized copper(I) and (II) complexes of melamine by directly adding melamine into various solutions of copper. The mixed solutions were sealed in thick-walled glass tube, heated and stirred at 100 °C for 20 hours. The complexes were collected by means of filtration and characterized by elemental analysis, X-ray powder diffraction spectrometry, thermogravimetric analysis, and Fourier transform infrared spectrometry. Using CuX (X = Cl, Br, I, NO_3), the synthesized complexex of copper(I) showed stoichiometry of 1:1 copper:melamine, excepted for CuCl which showed the mole ratio of 1:1.1-1.3. Whereas using of CuX_2 (X = Cl, Br, OAc), 1:2 of copper:melamine was shown in the complexes of copper(II), excepted for $Cu(OAc)_2$ showed a 1:1 stoichiometry.

Chen *et al.* [22] synthesized two copper(II)-melamine complexes using $CuCl_2$ and $Cu(OAc)_2$. Such copper salts were added into boiling methanol, and refluxed for 3 hours. The green-yellow crystal obtained from the former salt, and the blue crystal obtained from the latter salt were filtered out, and characterized by spectrometric methods and X-ray crystallography. Besides, thermal properties of these complexes were studied. The structure of products $Cu_2Cl_2(\mu\text{-}Cl)(CH_3OH)_2(\mu\text{-}OCH_3)\text{-}(Mel)_2{\cdot}2Et_2O$ and $Cu(\eta^1\text{-}OAc)\text{-}(\mu\text{-}OCH_3)\text{-}(Mel)_2{\cdot}0.33H_2O$ are shown in Figure 2.4 (a) and (b), respectively.



(a) (b)

Figure 2.4 Structure of (a) $Cu_2Cl_2(\mu\text{-}Cl)(CH_3OH)_2(\mu\text{-}OCH_3)\text{-}(Mel)_2 \cdot 2Et_2O$ and (b) $Cu(\eta^1\text{-}OAc)\text{-}(\mu\text{-}OCH_3)\text{-}(Mel)_2 \cdot 0.33H_2O$ [22].

Xu *et al.* [23] synthesized two co-crystal copper-melamine complexes and studied their electrochemical properties. The complexes *(1)* $[Cu(HBA)_2(Mel)_2][Cu(HBA)_2]$ and *(2)* $[Cu_2(OAc)_4(Mel)\cdot H_2O]_2[Cu(HPE)_2]$ were prepared by slowly dropping of $Cu(OAc)_2$ into melamine-containing ethanol solution, and stirring for 30 minutes. HBA (2-hydroxybenzaldehyde) for complex *(1)* synthesis and HPE (1-(2-hydroxyphenyl) ethanone) for complex *(2)* synthesis were slowly added, then the mixture was refuxed for 1 hour. After solvent evaporation, the dark green crystal *(1)* and light green crystal *(2)* were characterized. Figure 2.5 shows the structure of the two complexes.

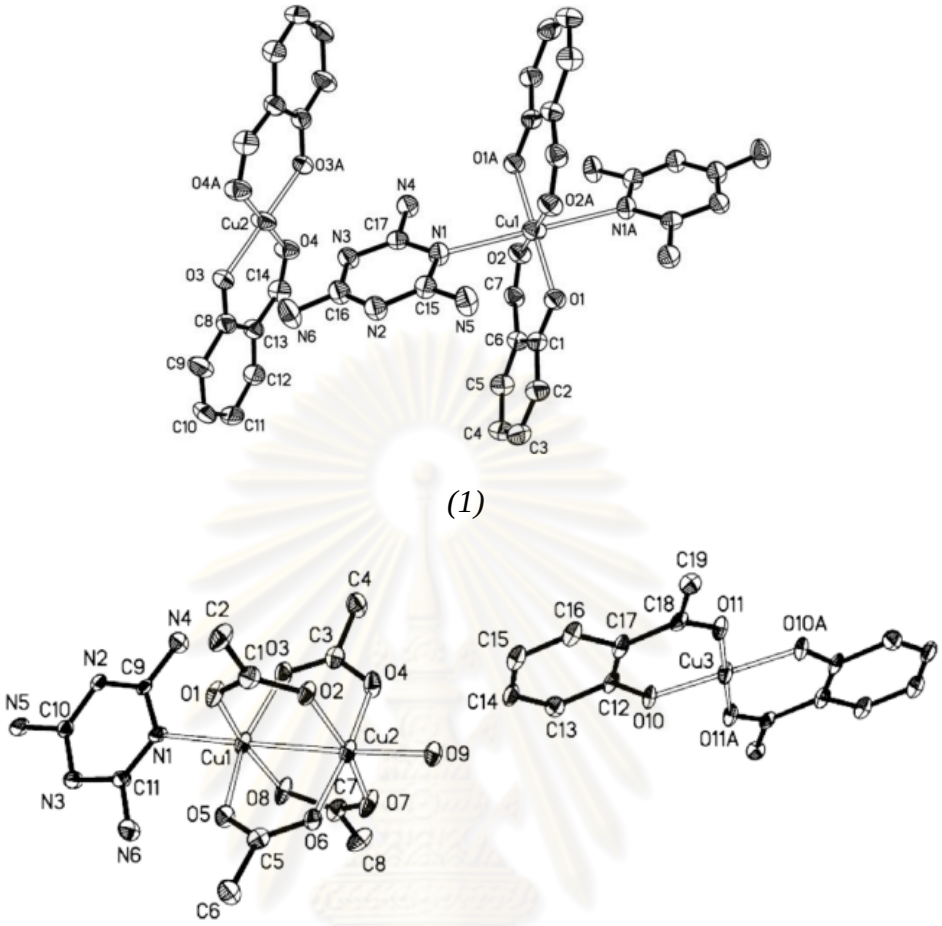


Figure 2.5 Structure of *(1)* $[Cu(HBA)_2(Mel)_2][Cu(HBA)_2]$ and *(2)* $[Cu_2(OAc)_4(Mel)\cdot H_2O]_2[Cu(HPE)_2]$ [23].

Colombo *et al.* [24] synthesized melaminium hexachlorodicuprate(II), $(MelH_2)Cu_2Cl_6$. The compound was prepared by the reaction of $CuCl_2$ and melamine in concentrated hydrochloric acid. After some hours, the red crystals precipitated, and were studied some physical properties.

2.3 Characterization of complex

Commonly used techniques for complex characterization are summarized below.

2.3.1 X-ray powder diffraction spectrometry

X-ray powder diffraction or XRD provides diffraction patterns of crystalline materials. Observed diffraction pattern can be compared with those of known substances in the Joint Committee on Powder Diffraction Standards (JCPDS) database. The thin layer of the powders was exposed to an X-ray beam. The instrument plots the intensity of diffracted beam against the scattering angle (2θ). Component of mixtures can be identified from their characteristic patterns [25].

2.3.2 Thermogravimetry

Measurement of mass changes during heating are made with thermo-balance. The output of thermogravimetric curve is a plot between percentage of the original mass and temperature, and provides information about drying processes, loss of solvent, thermal decomposition reaction, and oxidation reaction [25].

2.3.3 Fourier transform infrared spectrometry

IR method has a potential for identification and confirmation of molecular structure [26]. The most common application of infrared spectrometry is qualitative analysis, and compilations of characteristic functional group frequencies are available [25]. Molecular species can interact with applied electromagnetic radiation in infrared region, which causes changing the electronic state. The molecule can vibrate or rotate in many ways relating to its bonds and functional groups. IR spectra are a plot of wavenumber (usually 400-4000 cm^{-1}) against percentage transmittance.

2.4 Physico-chemical study of complex

2.4.1 Chemical kinetics

Chemical kinetics is a study of rates of chemical reactions. It is closely to an investigation of reaction mechanisms [27]. Such study can help understand steps of reaction progress. The equation of a net chemical reaction represents the overall stoichiometric transformation of reactants to products without addressing its mechanism.

2.4.1.1 Rate law

A common chemical reaction is represented by Eq. 2.1.

$$aA + bB \rightleftharpoons cC + dD \qquad (2.1)$$

Reaction rate (expressed by Eq. 2.2) is defined as a change with time in concentration of one of the reactants or products; that is,

$$\text{Rate} = \nu = -\frac{1}{a}\frac{d[A]}{dt} = -\frac{1}{b}\frac{d[B]}{dt} = \frac{1}{c}\frac{d[C]}{dt} = \frac{1}{d}\frac{d[D]}{dt} \qquad (2.2)$$

The rate law expresses the rate of a reaction in terms of concentrations of reactants and other species in solution affecting the rate. Supposing that the reaction rate of the above reaction depends only on the concentrations of reactants, the rate law is given by Eq. 2.3;

$$\nu = k\,[A]^m\,[B]^n \qquad (2.3)$$

where k is a rate constant. The value of *m* and *n* in Eq. 2.3 determines the order of the reaction in A and B, respectively. The overall order of the reaction is (*m* + *n*), and cannot be obtained simply by looking at the net chemical reaction. The order of reaction is not

necessarily related to the stoichiometry; it can be determined only by experiment. Table 2.1 shows the expression of rate law of each reaction order.

Table 2.1 The rate expression of each reaction order

Reaction order	Differential form	Integrated form
Zeroth	$\nu = -\frac{d[A]}{dt} = k[A]^0 = k$	$[A]_t = [A]_0 - kt$
First	$\nu = -\frac{d[A]}{dt} = k[A]$	$[A]_t = [A]_0 e^{-kt}$
Second	$\nu = -\frac{d[A]}{dt} = k[A]^2$	$\frac{1}{[A]_t} = \frac{1}{[A]_0} + kt$

2.4.1.2 Experimental determination of reaction orders

Integral method

Testing for simple first-order or second-order kinetics by plotting method as integrated form can be done quickly and easily [27]. Graphical characteristics of the zeroth, first and second are shown in Figure 2.6.

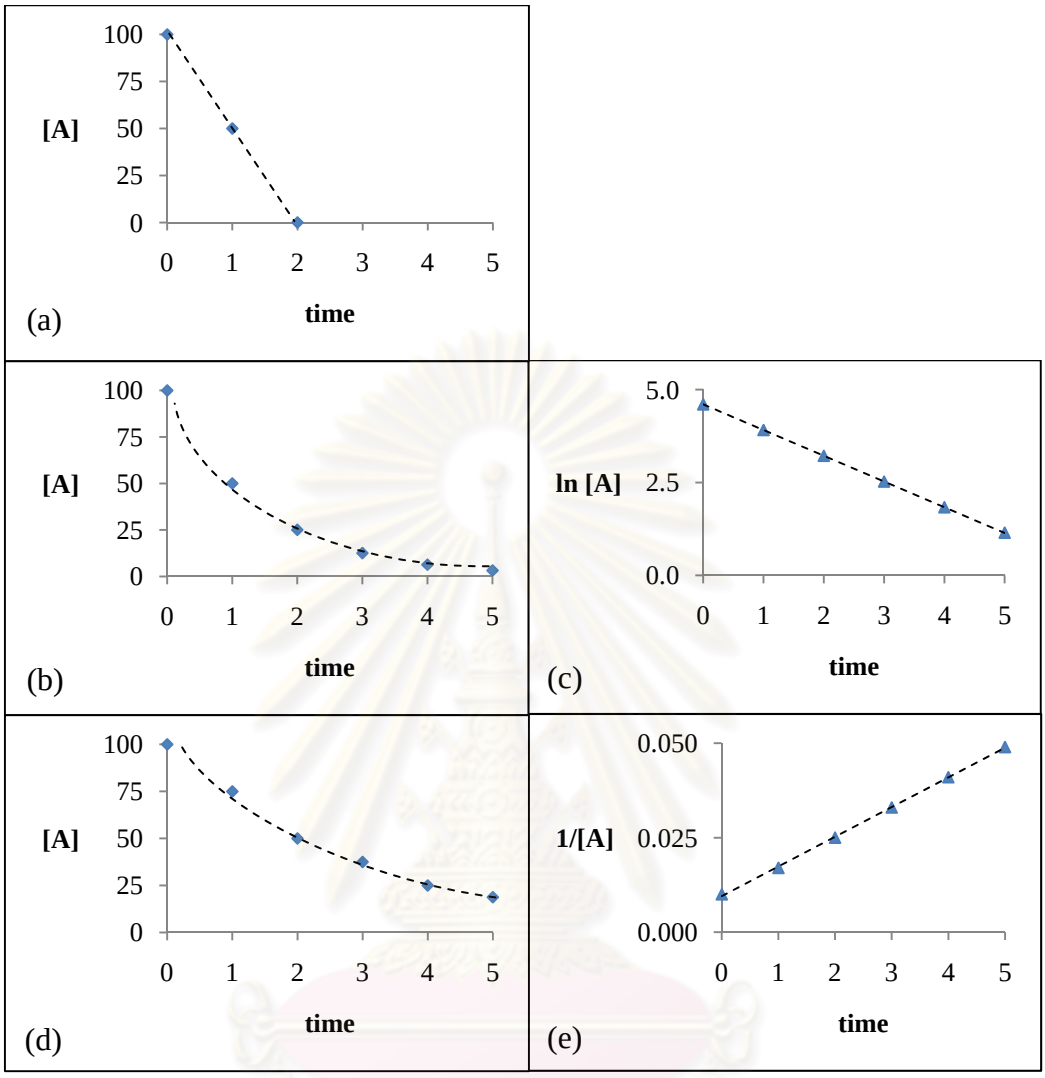


Figure 2.6 Graphical characteristic (a) zeroth order,
(b) and (c) first order
(d) and (e) second order (adapted from [28]).

Initial rate method (differential method)

Method of initial rate was done by measuring of rate of reaction with known reactants concentrations at the beginning of reaction [28].

$$\nu = k\,[A]^m\,[B]^n \qquad (2.3)$$

$$\log \nu = \log k + m \log [A] + n \log [B] \qquad (2.4)$$

By varying $[A]_0$ with holding $[B]_0$ as constant and measuring the initial rate, logarithmic plot of rate versus a concentration of $[A]_0$, the order *m* can be estimated from the slope, and vice versa, the order *n* can be also found.

Flooding method (Isolation method)

In case of reaction rate depends on several substances, flooding method can help mathematically simplify complicated rate law to pseudo-first order. The experiment can be done by jumping other species to rather high concentration, compared to low concentration of one species of interest, so the others are served as constant [27].

Fractional times (half-life method)

Fractional time method is based on the concept that the time required to convert a given function of limiting reagent is a characteristic of the rate equation. A comparison of successive half-time can reveal what simple integral order of rate equation that reaction follows. The ratio of time required for 75% reaction to that for 50% reaction is an own characteristic reaction order. Table 2.2 showed each half-time value of reaction order [27].

Table 2.2 Half time value (adapted from [29])

Reaction order	Half time	$t_{¾}$ / $t_{½}$
Zeroth	$a_0/2k$	1.5
First	*ln* 2/k	2
Second	$1/ka_0$	3
Third	$3/2ka_0^2$	5

Extent of reaction (Wilkinson's method)

The extent of reaction, defined by Eq. 2.5, represents the fractional progress of reaction from beginning to end.

$$E = 1 - \frac{A}{A_0} \qquad (2.5)$$

The plot between t/E and t, according to Wilkinson relation (Eq. 2.6) where *n* is reaction order, can found the variation *n* from the slope [27].

$$\frac{t}{E} \approx \frac{1}{k\,[A]_0^{n-1}} + \frac{n}{2}t \qquad (2.6)$$

2.4.1.3 Activation energy

Activation energy is the least energy that can make any chemical reaction occurs. In general, it has been known that temperature can affect on the rate of reaction and rate constant is a value of its own temperature.

According to Arrhenius equation (Eq. 2.7), if natural logarithm is taken throughout Eq. 2.7, Eq 2.8 is obtained;

$$k = Ae^{-E_a/RT} \qquad (2.7)$$

$$ln\,k = ln\,A - \frac{E_a}{RT} \qquad (2.8)$$

where *A* is Arrhenius constant, R is the gas constant and T is the absolute temperature in Kelvin. A plot of *ln* k versus reciprocal temperature can reveal the activation energy from its slope.

2.4.2 Solubility equilibrium

Complexation reaction involves a metal ion (M) reacting with a ligand (L) to form complex, ML, as shown in Eq. 2.9;

$$M + L \rightleftharpoons ML \qquad (2.9)$$

where the ion charges have been omitted so as to be general.

The complexation reaction occurs in a stepwise process. If the completed reaction has two ligands reacting with dication, The Eq. 2.9 is followed by Eq. 2.10.

$$ML + L \rightleftharpoons ML_2 \qquad (2.10)$$

Formation constant (K_f) indicating the stability of the complex can be expressed as

$$K_f = \frac{ML_2}{[M^{2+}][L^2]} \qquad (2.11)$$

In case of insolubility complex is formed (Eq. 2.12), K_f can be calculated by Eq. 2.13;

$$M^{2+} + 2L \rightleftharpoons ML_2(s) \qquad (2.12)$$

$$K_f = \frac{1}{[M^{2+}][L^2]} \qquad (2.13)$$

In contrast to complexation equilibrium treated as formation reaction, solubility equilibrium is usually treated as dissociation reaction [26]. The reaction can be written as Eq. 2.14;

$$ML_2(s) \rightleftharpoons M^{2+} + 2L \qquad (2.14)$$

Solubility product constant (K_{sp}) can be expressed by Eq. 2.15;

$$K_{sp} = [M^{2+}][L]^2 = \frac{1}{K_f} \qquad (2.15)$$

2.4.3 Continuous variation method (Job's method)

The method of continuous variation or Job's plot is widely used to study the stoichiometry of complexes. A series of solution containing the fix total mole number, but systematically varied mole ratio of reactants is prepared. The products are then measured the analytical signal. Plotting of the signal against the mole ratio of one of reactants which are metal or ligand yields the curve that can be used to determine the stoichiometry of the complex by taking into account the mole fraction (*x*-axis) of the highest signal or mass product (*y*-axis), as shown in Figure 2.7.

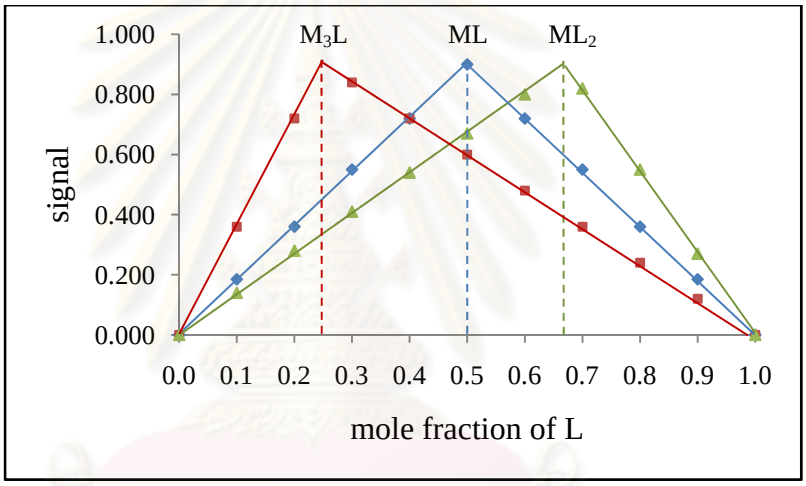


Figure 2.7 Job's plot for the M_3L, ML, and ML_2 complex formaton (adapted from [30]).

2.5 Factors influencing complex formation

There are several factors affecting the formation of complex. Various metals may differently react with a ligand. Sizes and charges of the components are important factors towards forming the complex. If a ligand is a molecule, its dipole moment will be important among the factors determining its complexing power [31]. Complex formation ability of metal ions usually depends on three factors; the charge, the size, and the ionization potential [31]. Chelation is also the effect of complex formation [31]. Ligand containing two or more donor groups can affect to the arrangement of the complex. The complex usually adjusts its orientation to the higher stable form [32].

Besides, when ligand allowing to interact with the same metal in different solvent to form complex, the solvent systems often play a vital role in complex formation and its structure [33]. In addition, formation of complex often depends upon temperature. Complex formation is promoted by negative enthalpy (ΔH) and positive entropy (ΔS), according to the Gibbs energy (ΔG). Generally, it is widely known that complexation reaction is the exothermic reaction, in thermodynamic aspect. However, that some reactions may produce large quantity of adducts when raising of temperature can be explained by its kinetically favor [32].

2.6 Methodology in spectrochemical analysis

One of the very important parts in analytical procedures is the calibration [34]. Analytical methods are most often calibrated with external standards, nevertheless, it used to calibrate instruments and procedures having no interference effects and matrices [34-35]. Standard addition is one of alternative choices for minimizing errors from matrix effects [35]. Furthermore, for the developed analytical methods, the consistent, reliable, and accurate data are the summit purpose. Any developed technique should be made sure that it can measure and report reliable and accurate data [36]. Validation of the methods is thus significantly important.

2.6.1 External standard calibration

An external calibration curve between analytical signal S and known analyte concentration C_s is established from fitting the results to a suitable mathematical function using of least-squares method. Then, S obtained from the sample is used to determine unknown analyte concentration C_x from the calibration graph. However, the basic assumption of the external standard procedures is that standard and sample with same analyte concentration will yield the same analytical signal. After applying the appropriate sample preparation factors, the analyte concentration in sample can be found [34-35].

2.6.2 Standard addition method

A standard addition method is particularly useful for analyzing samples in which matrix effect is substantial [34]. The procedures for going through the standard addition method is as follows: (1) several aliquots V_x of unknown with a concentration C_x of sample are transferred to a series of volumetric flask having a volume V_t; (2) a small volume of standard V_s with known concentration C_s is spiked in a series in another aliquot of analytical samples; (3) suitable reagents are added, and each solution is adjusted to the marked volume; (4) each of solutions is measured to yield a signal S (Eq. 2.16) [34-35],

$$S = \frac{V_s C_s}{V_t} + \frac{V_x C_x}{V_t} \tag{2.16}$$

Plotting between S and V_s, the slope $m = \frac{C_s}{V_t}$ and intercept $b = \frac{V_x C_x}{V_t}$ can be found from the graph. C_x can then be obtained from Eq. 2.17-2.18;

$$b/m = \frac{\frac{V_x C_x}{V_t}}{\frac{C_s}{V_t}} \tag{2.17}$$

$$C_x = \frac{bC_s}{mV_x} \tag{2.18}$$

When the straight line is extrapolated to the x-axis, as shown in Figure 2.8, the difference between added standard volume at the origin and the volume at the intersection or x-intercept $(V_s)_0$ is the volume of standard reagent equivalent to the amount of analyte in the sample. Besides, the x-intercept corresponds to null instrument response. C_x can then be obtained from Eq. 2.19-2.20 [34];

$$S = \frac{V_s C_s}{V_t} + \frac{V_x C_x}{V_t} = 0 \tag{2.19}$$

$$C_x = -\frac{(V_s)_0 C_s}{V_x} \quad (2.20)$$

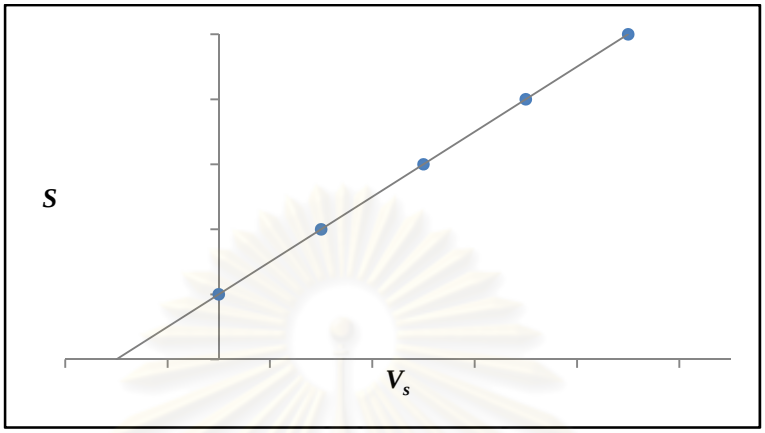


Figure 2.8 Linear calibration graph for standard addition method.

2.6.3 Validation of spectroanalytical method

Method validation is the process used to assure that the developed analytical technique or procedure applied for analysis is appropriate. Results from validation of the method are used to judge the consistency, reliability, and accuracy of analytical results [36].

2.6.3.1 Validation using certified reference material

A certified reference material (CRM) serves multiple purposes, one of which is to demonstrate equivalency of a developed method. Certified reference materials are available as pure solution for single component calibration, mixtures in solutions for multicomponent calibration, and solids with components and matrices as close as possible to the matrix of the unknown sample [36].

A use of CRM has to realize an accuracy of data. Therefore laboratories should aware of requirements for CRM. The compound and concentration should be as closed as possible to the sample. In addition, matrix of reference sample should be also similar to that of unknown sample. The reference material should be

homogeneous. The use of any portion in a container should be the same, and it should rehomogenize if there is a risk of segregration during storage or transport. The properties of reference material and the stability of matrix are also important. Lastly, the uncertainty value of certified reference material should be estimated [36].

2.6.3.2 Parameters for method validation

Accuracy

The accuracy of the method is the extent to which test results generated by the developed method and the true value agree [36]. It can be obtained in several ways, one of which is to compare the result with that from certified reference material. The other is obtained by examination of recovery from spiked sample. This is used when CRM is not available, a sample matrix of interest will be spiked with a known amount of analyte. After extraction process and running by the instrument, its recovery can be determined.

Precision

The precision of the method is the extent to which the individual test results of multiple injections agree [36]. Precision can be expressed in both repeatability and reproducibility. Repeatability is obtained when the analysis is carried out using the same equipment over a relative short time span. The relative standard deviation (R.S.D.) can be calculated. The R.S.D. can be fluctuated depending on type of analysis. For example, an analysis of food or environment will yield the high value of R.S.D., because of largely matrix dependent. As for reproducibility, it represents the precision obtained between different laboratories. Laboratories may have different surroundings and conditions, but there are same specific parameters to be investigated.

Limit of detection and Limit of quantitation

The limit of detection (LOD) is the minimum quantity of analyte in a sample that can be detected but not necessarily quantified. The limit of quantitation (LOQ) is the minimum amount that can be detected with acceptable precision [36]. In

general, LOD and LOQ are estimated by 3 and 10 times, respectively, of a blank signal or the standard deviation of the blank signal.

Method detection limit

Method detection limit (MDL) is the lowest concentration or smallest quantity of an analyte that can be detected based on measurements of a blank real sample or a low-level spike that has been processed through all of the steps of the developed method [37-38].

Linearity and range

The linearity is the ability of analytical method to elicit results that are proportional to the analyte concentration in samples within a given range. The range of analytical method is the interval between the upper and the lower levels having been demonstrated to be analyzed with accuracy, precision and linearity [36].

CHAPTER III

EXPERIMENTAL SECTION

3.1 Apparatus

3.1.1 Flame Atomic Absorption Spectrometer

A copper concentration was determined by an AAnalyst 100, Perkin Elmer, Flame Atomic Absorption Spectrometer (FAAS). A 15-milliampres Cu Hollow Cathode Lamp at a wavelength of 327.4 nm was used for this purpose. C_2H_2 and air flow rates were 3 and 10 mL min^{-1}, respectively.

3.1.2 X-ray diffractometer

A DMAX 2200/Ultima+, Rigaku, X-Ray Powder Diffractometer was used to examine the XRD pattern of the copper-melamine complex. The instrument using Cu Kα radiation source was operated at 40 kV and 30 mA. XRD data was collected from 2θ of 20-70 degrees.

3.1.3 Fourier Transform Infrared Spectrometer

Infrared spectra recording from 4000 to 400 cm^{-1} in transmission mode by KBr pellet technique were obtained by a 6700, Nicolet, Fourier transform infrared spectrometer. All absorptions were reported in wavenumber (cm^{-1}).

3.1.4 Thermal Gravimetric Analyzer

Studying a thermal behavior of the copper-melamine complex was done by a 761 Connecticut 06859, Perkin Elmer, Thermal Gravimetric Analyzer. The

temperature program was set up from 50 °C to 800 °C with the rate of 10 °C/min under air atmosphere.

3.1.5 Orbital mixer incubator

An OM11, Ratek, was used to heat and stir mixture solutions for a complex synthesis. A stirring rate was 250 rpm.

3.1.6 Centrifuge

After complete complex synthesis, solid precipitate and supernatant phase were separated by a centrifuge, model CENTAUR 2 from SANYO.

3.2 Chemicals

All Chemicals were ACS grade listed in Table 3.1.

Table 3.1 Chemicals

Chemicals	**Supplier**
Copper(II) chloride dihydrate	BDH
Copper(II) nitrate trihydrate	Merck
Lead acetate trihydrate	FARMITALIA CARLO ERBA
Melamine	Fluka
Methanol	Merck
Trichloroacetic acid	Sigma-Aldrich

3.3 Preparation of Chemicals

a) Stock Standard Solutions

Stock solutions of 1.00 M $CuCl_2$ and $Cu(NO_3)_2$ in both methanol and water were prepared by dissolution of an exactly weighed amount of metal salts.

b) Working Standard Solutions

Stock standard solutions of chloride and nitrate salts of copper were used to prepare working solutions by stepwise dilution to the required concentrations.

Melamine solution was daily prepared as needed by dissolution of an exactly weighed amount of melamine in 1% trichloroacetic acid.

Trichloroacetic acid and lead acetate solutions were prepared as needed using deionized water.

3.4 Study of copper-melamine complexation reaction

3.4.1 Optimization of copper-melamine complexation reaction

A 50-time diluted centrifugate was analyzed by FAAS for determination of remaining copper and investigation for the optimal system for copper-melamine complex formation. All experiments were done in triplicate.

3.4.1.1 Effect of types of copper(II) salts and solvents

5.00 mL of 0.00, 0.05 and 0.10 M melamine solution were added into 5.00 mL of 0.05 M $CuCl_2$ in methanol, $CuCl_2$ in water, $Cu(NO_3)_2$ in methanol, and $Cu(NO_3)_2$ in water. After heating at 80 °C and stirring for 14 hours, the concentrations of remaining copper from four systems were compared in order to obtain an appropriate copper salt/solvent system.

3.4.1.2 Effect of temperature

5.00 mL of 0.00, 0.05 and 0.10 M melamine solution were added into 5.00 mL of 0.05 M of copper(II) ion solution using the copper salt system chosen from section 3.4.1.1. An effect of temperature towards complex formation was studied by varying incubation temperature from ambient temperature to 80 °C using the same incubation time of 14 hours.

3.4.1.3 Effect of curing time

5.00 mL of 0.00, 0.05 and 0.10 M melamine solution were added into 5.00 mL of 0.05 M of copper(II) ion solution using the optimal copper salt system. Using the chosen temperature, an investigation of curing time was conducted by varying from 2 to 20 hours.

3.4.2 Stoichiometric study

Job's method was used to study the stoichiometry of the copper-melamine complex. Varied amounts of copper and melamine were shown in Table 3.2. Using the optimum conditions from section 3.4.1, each series was done in triplicate.

Table 3.2 Varied amounts of copper and melamine for Job's plot

Mole fraction of melamine	Mole fraction of copper	Volume (mL)	
		0.10 M melamine	0.10 M copper
0.00	1.00	0.00	20.00
0.10	0.90	2.00	18.00
0.20	0.80	4.00	16.00
0.30	0.70	6.00	14.00
0.40	0.60	8.00	12.00
0.50	0.50	10.00	10.00
0.60	0.40	12.00	8.00
0.70	0.30	14.00	6.00
0.80	0.20	16.00	4.00
0.90	0.10	18.00	2.00
1.00	0.00	20.00	0.00

3.4.3 Kinetics study

Chemical kinetics focusing on determination of reaction order of the complexation was studied. Two methods were taken into account as follows:

Graphical method: Plotting of *ln* [Cu] versus time based on the data of effect of curing time was used to study a reaction order with respect to copper.

Initial rate method: A copper concentration was fixed, a melamine concentration was varied according to Table 3.3. The reaction rate was estimated in order to attain a reaction order with respect to melamine.

Table 3.3 Concentration of copper and melamine for initial rate study

Experiment	Concentration (mM)	
	copper	melamine
1	20.00	10.00
2	20.00	20.00
3	20.00	30.00
4	20.00	40.00
5	20.00	50.00

3.5 Synthesis and characterization of copper-melamine complex

0.10 M melamine in 1% trichloroacetic acid (10.00 mL) was added into 0.10 M $CuCl_2$ in methanol (10.00 mL). The mixed solution was sealed in a 40-mL glass tube before constantly stirring and heating by an orbital mixer incubator. After 14 hours, the greenish precipitate was obtained, and a blue or green copper solution became paler. The mixtures were centrifuged at 3,000 rpm before collection of the solids by means of filtration.

The complex was characterized by an X-ray powder diffractometer, a Fourier-transform infrared spectrometer and a thermal gravimetric analyzer.

3.6 Standard calibration curve

The plot between melamine concentration and copper absorbance was built by adding various melamine concentrations into a series of solutions with fixed copper concentration. After the reaction had completed, the absorbance of 4-fold dilution of remaining copper was examined. The investigation was done in three series; each series was done in triplicate.

Series A: 5.00 mL of 0.00, 2.00, 4.00, 6.00, 8.00 and 10.00 mM melamine was added into 5.00 mL of 10.00 mM $CuCl_2$ in methanol.

Series B: 5.00 mL of 0.00, 1.00, 2.00, 3.00, 4.00 and 5.00 mM melamine was added into 5.00 mL of 5.00 mM $CuCl_2$ in methanol.

Series C: 5.00 mL of 0.00, 0.40, 0.80, 1.20, 1.60 and 2.00 mM melamine was added into 5.00 mL of 2.00 mM $CuCl_2$ in methanol.

3.7 Application to real sample

3.7.1 External standard method

Melamine (5.0, 7.0, 8.0, and 10.0 ± 0.1 mg) was fortified and homogenized with 1.00 ± 0.05 g of fish samples (Giant Seabass fish). 10.00 mL of 1% trichloroacetic acid was pipetted into the mixtures to extract melamine. The mixtures were sonicated for 10 minutes and heated for another 10 minutes. 5.00 mL of 2.2% lead acetate was added to deposit and precipitate protein matrices. The mixtures were centrifuged at 3,000 rpm for 10 minutes. 5.00 mL of supernatant was pipetted to react with 5.00 mL of 0.0100 M $CuCl_2$ in methanol. After the reaction had completed (under optimal conditions previously obtained), the absorbance of 4-fold dilution of remaining copper was measured. Each quantity of melamine was done in triplicate.

Blank experiment was done as the same procedure mentioned above, but no melamine was added.

All experiments were done in triplicate.

3.7.2 Standard addition method

Melamine (4.0, 5.0, and 10.0 ± 0.1 mg) was fortified and homogenized with 1.00 ± 0.05 g of fish samples. 10.00 mL of 1% trichloroacetic acid was pipetted into the mixtures to extract melamine. The mixtures were sonicated and heated for 10 and 10 minutes, respectively. 5.00 mL of 2.2% lead acetate was added to deposit protein matrices. The mixtures were centrifuged at 3,000 rpm for 10 minutes. Each 2.00 mL of supernatant was pipetted into five glass tubes. Standard melamine and copper solutions were added in each glass tube. The amounts of substances were shown in Table 3.4.

Table 3.4 Volume of substances for standard addition method

Experiment	Volume (mL)			
	Extracted melamine	0.0100 M melamine	1% TCA	0.0100 M $CuCl_2$/MeOH
1	2.00	0.00	2.00	4.00
2	2.00	0.50	1.50	4.00
3	2.00	1.00	1.00	4.00
4	2.00	1.50	0.50	4.00
5	2.00	2.00	0.00	4.00

Blank experiment was done as the same procedure mentioned above, but no melamine was added.

All experiments were done in triplicate.

CHAPTER IV

RESULTS AND DISCUSSION

This study made use of copper-melamine complexation in analytical application. The copper-melamine complex (Cu-Mel) was synthesized by reaction of copper and melamine in a solution, as shown in Eq. 4.1.

$$Cu^{2+}(\text{excess}) + 2\text{Mel} \rightleftharpoons [Cu(Mel)_2] + Cu^{2+}(\text{remaining}) \qquad (4.1)$$

greenish precipitate

$CuCl_2$ in methanol was the best system for copper-melamine synthesis (section 4.1.1). The increase of greenish precipitate product with raising of temperature suggested that the rate of reaction increases kinetically. However, in thermodynamic point of view, a complex formation is generally an exothermic reaction. The complex obtaining from the optimized condition would be characterized by various techniques for studying its structural information including XRD, TGA, and FT-IR (section 4.2). Besides, stoichiometry of copper and melamine in the complex was 1:2 (section 4.1.2). According to kinetics study, the reaction was first order with respect to both copper and melamine, and the activation energy (E_a) of the reaction was 19.60 kJ/mol estimated by Arrhenius equation (section 4.1.3). Interestingly, the rather high value of formation constant (K_f) at 80 °C also exhibited the good complexation ability of Cu and melamine which was useful for the determination of melamine by this complexation method (section 4.3 and 4.4).

4.1 Study of copper-melamine complexation reaction

4.1.1 Optimization of copper-melamine complexation reaction

4.1.1.1 Effect of types of copper salts and solvents

By visual observation (data not shown), a quantity of complex obtaining from each system was different. A maximum amount of precipitate could be observed in the system of $CuCl_2$ in methanol, then the order was $CuCl_2$ in water, $Cu(NO_3)_2$ in methanol, and $Cu(NO_3)_2$ in water, respectively.

The measurement of the amount of copper remaining in the solution after complex formation by Flame atomic absorption spectrometer showed that $CuCl_2$ in methanol gave the least amount of remaining copper. A linear plot of remaining copper versus amount of melamine added also yielded the maximum slope (Figure 4.1).

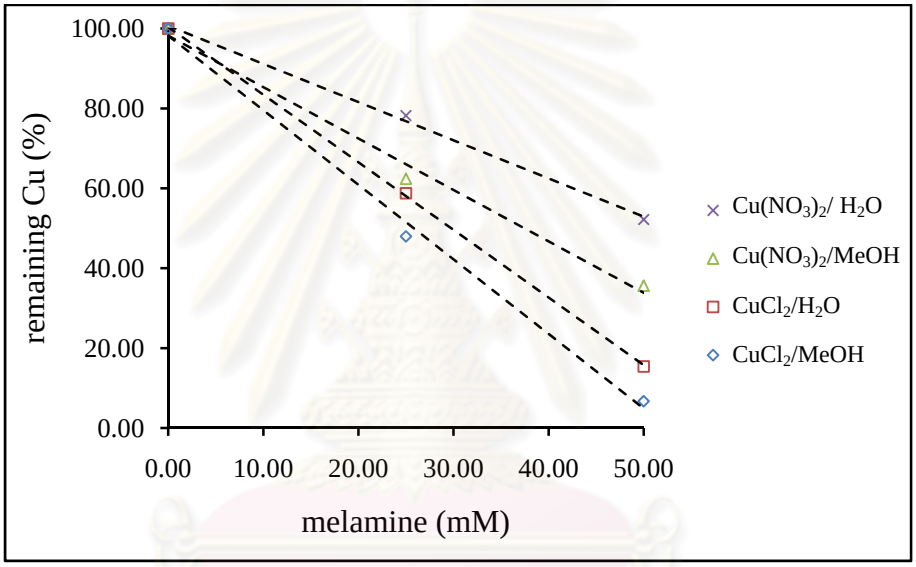


Figure 4.1 Remaining graph of copper with varied melamine in the systems of $CuCl_2$/MeOH, $CuCl_2/H_2O$, $Cu(NO_3)_2$/MeOH, and $Cu(NO_3)_2/ H_2O$.

Besides, according to the copper and melamine stoichiometry of 1:2 (section 4.1.2), the amount of both copper and melamine at equilibrium were used to calculated formation constant (K_f) and solubility products (K_{sp}), as Eq. 4.2 and 4.3, respectively (detailed shown in Appendix);

$$K_f = \frac{1}{[Cu^{2+}][Mel]^2} \quad (4.2)$$

$$K_{sp} = [Cu^{2+}][Mel]^2 = \frac{1}{K_f} \quad (4.3)$$

According to the Table 4.1, K_f from $CuCl_2$ in methanol was the highest, and K_{sp} was the lowest. This indicated $CuCl_2$ in methanol showed the greatest complexation ability for Cu-Mel complex. $CuCl_2$ in methanol, therefore, was the most optimal system for the complex formation and would be used for further investigation.

Table 4.1 Calculated solubility products of Cu-Mel in each system

Copper(II) salt/solvent	K_f	K_{sp}
$CuCl_2$/MeOH	5.37×10^{-2}	1.86×10^{1}
$CuCl_2/H_2O$	3.53×10^{-4}	2.83×10^{3}
$Cu(NO_3)_2$/MeOH	4.39×10^{-3}	2.28×10^{2}
$Cu(NO_3)_2/H_2O$	1.13×10^{-4}	8.88×10^{3}

4.1.1.2 Effect of temperature

By varying the incubation temperature, it was found that the higher temperature, the more observable greenish precipitate. A quantity of synthesized complex was maximum at 80 °C. Figure 4.2 showed the effect of temperature towards amount of remaining copper.

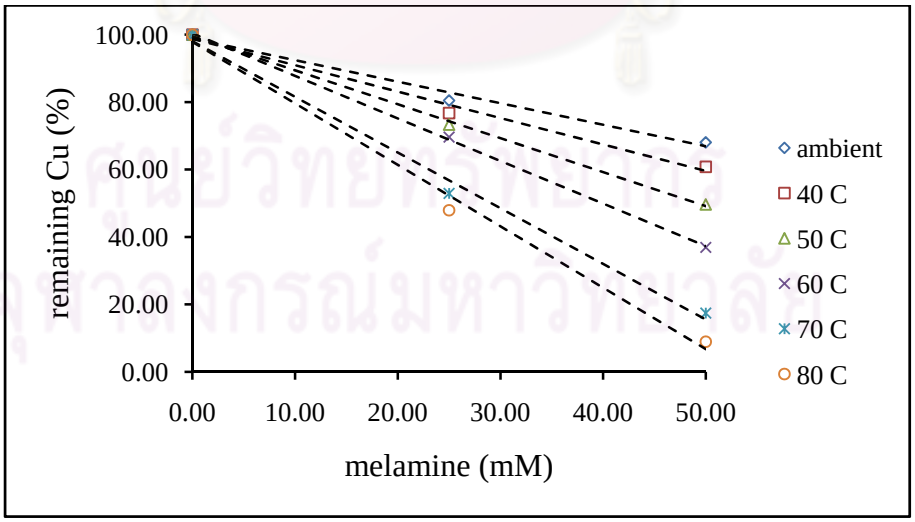


Figure 4.2 Remaining graph of copper with varied temperature from ambient temperature to 80 °C.

According to the remaining graph, the amount of copper remaining in the solution was the least at 80 °C, and this temperature also showed the maximum slope. It was due to a limitation of the incubator that it could not heat up more than 80 °C. Therefore, 80 °C was the chosen temperature used for copper-melamine synthesis.

4.1.1.3 Effect of curing time

The study of the complex formation from 2-20 hours exhibited that copper was continuously consumed along curing time (Figure 4.3). The amount of remaining copper was minimum at 20 hours; however, it was not significantly different since 14 hours onwards. According to the graph, copper was not totally consumed. Its concentration at 14-20 hours was rather constant. This indicated that there was a chemical equilibrium, and the system might reach the equilibrium at 14 hours. The slope of the remaining copper versus melamine concentration, as shown in Figure 4.4, was maximum and rather similar at 14-20 hours. Thus the chosen curing time for complexation was 14 hours because of energy conservation.

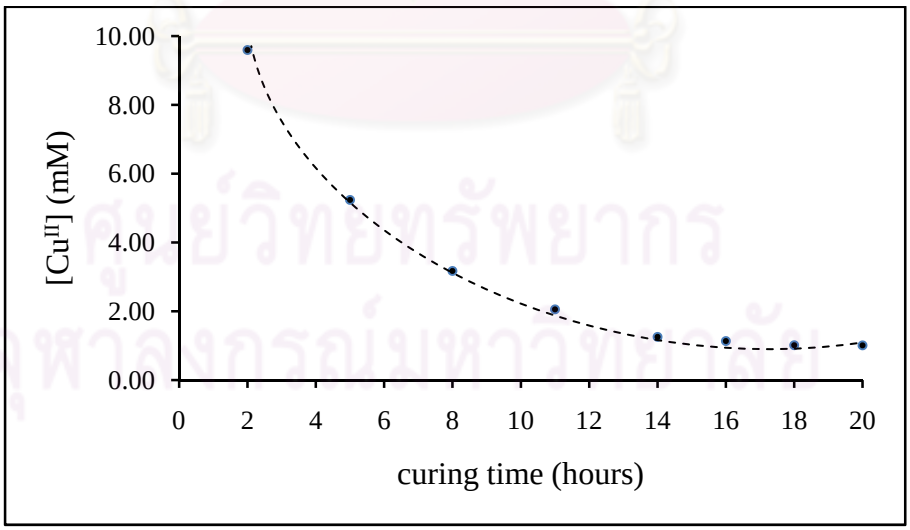


Figure 4.3 Remaining copper versus curing time.

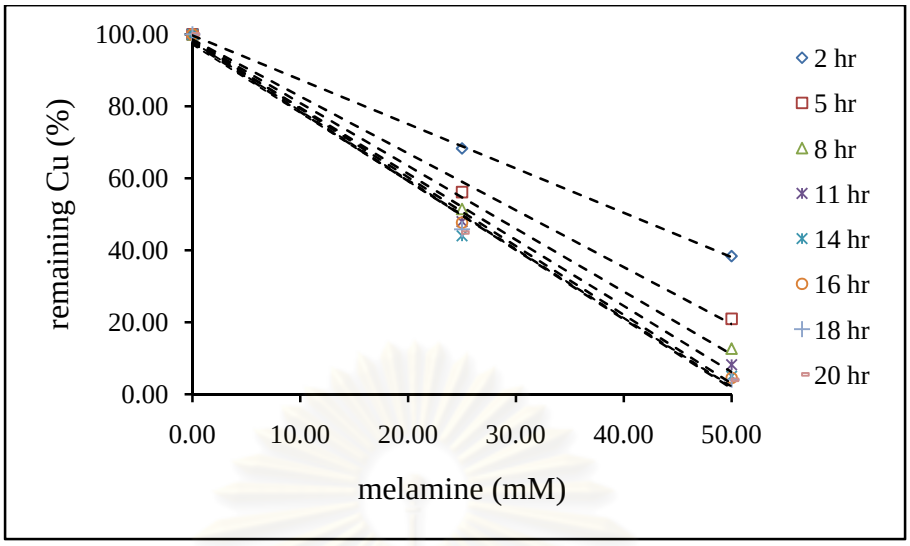


Figure 4.4 Remaining graph of copper with varied curing time from 2 to 20 hours.

4.1.2 Stoichiometry of copper and melamine in the complex

After each reaction in Table 3.2 was complete, the Flame atomic absorption spectrometer was used to measure the amount of copper remaining in the solution. This investigation was based on the assumption that copper formed complex only with melamine. By subtracting an absorbance of remaining copper from the initial one, signal A_0-A assuming that it related to the amount of copper in the complex were obtained. Job's plot (Figure 4.5) was built between mole fraction of melamine ($x_{melamine}$) and A_0-A, where A_0 was initial copper absorbance without complexation, and A was remaining copper absorbance after complexation. The plot indicated copper and melamine bound in the ratio of 1:2 (corresponding to the maximum A_0-A at mole fraction of melamine of 0.67).

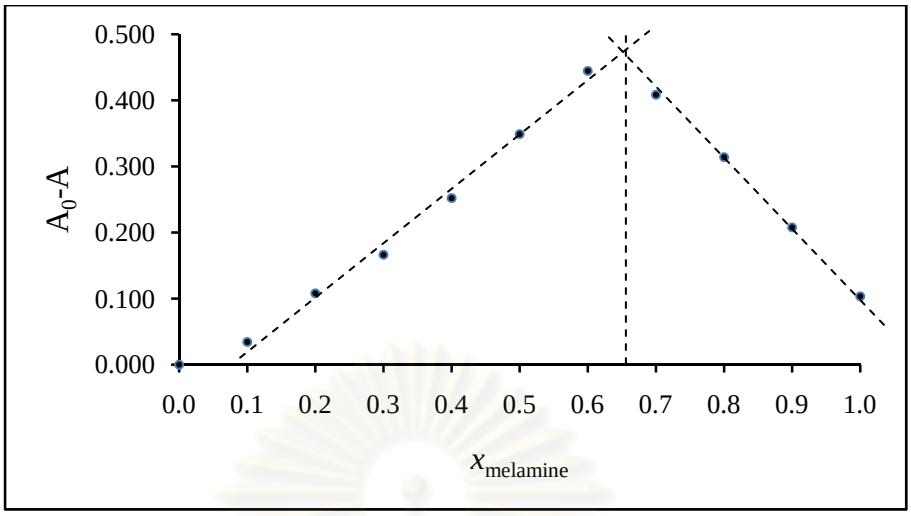


Figure 4.5 Plot between A_0-A and mole fraction of melamine.

4.1.3 Kinetics study

4.1.3.1 Reaction order

Figure 4.6 showed a graphical integral method of *ln* Cu concentration versus curing time plot. The graph showed a linear plot in a range of 2-14 hours. After 14 hours onwards, the cause of tiny amount of remaining copper was that *ln* Cu concentration had closed to zero. The linear graph of *ln* Cu concentration versus time in the first 14-hour range could confirm that the reaction was first order with respect to copper.

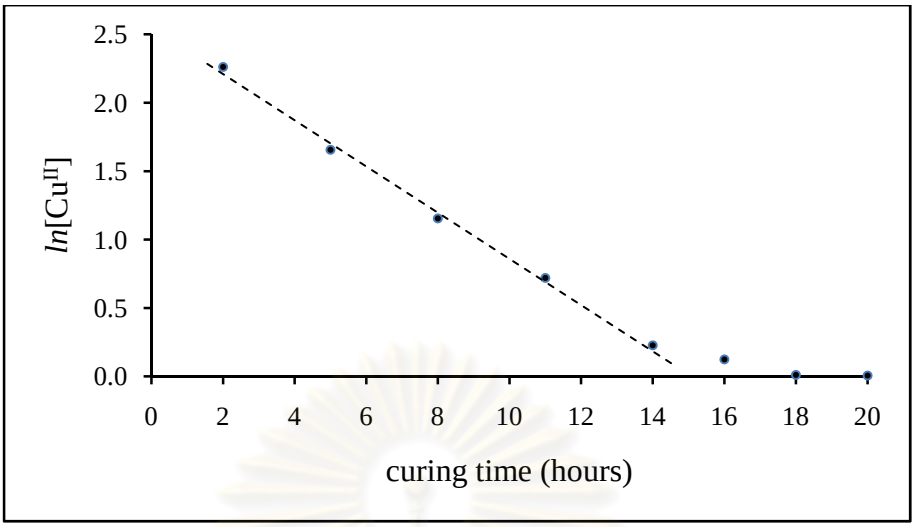


Figure 4.6 *ln* Cu concentration as a function of time.

Besides, the order of reaction with respect to melamine was also studied by using a method of initial rate. Table 4.2 showed rates of reaction in each experiment.

Table 4.2 Concentration-time data for reaction rate for 4 hours

[Cu] (mM)	[Melamine] (mM)	Rate ($mM^{-1}s^{-1}$)
20.00	10.00	0.640
20.00	20.00	1.154
20.00	30.00	1.730
20.00	40.00	2.621
20.00	50.00	2.999

A logarithmic plot of rate and melamine concentration (Figure 4.7) showed a linear which had a slope of 0.99. This could indicate that the complexation reaction was also first order with respect to melamine.

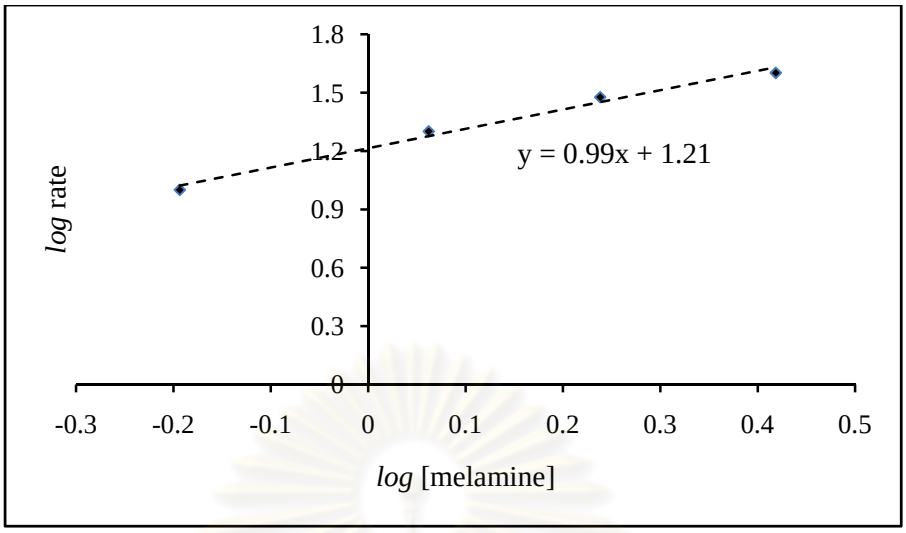


Figure 4.7 *log* rate as a function of *log* melamine concentration.

4.1.3.2 Activation energy

An activation energy was determined by means of *Arrhenius plot.* Based on the data of effect of temperature, rate constants for each temperature could be estimated from a rate law (Eq. 4.4),

$$\nu = k\,[Cu]\,[Mel] \qquad (4.4)$$

where ν is a rate of reaction and k is a rate constant. Then, plotting of *ln* rate constant versus reciprocal temperature could indicate activation energy of the reaction.

According to the proposed rate law, rate constants estimated from Eq. 4.4 were shown in Table 4.3.

Table 4.3 Rate constant (k) and *ln* k of various temperatures

Temperature (K)	Rate constant (k) ($M^{-1} \cdot s^{-1}$)	*ln* k
303	4.56×10^{-4}	-7.69
313	5.60×10^{-4}	-7.49
323	7.20×10^{-4}	-7.24
333	9.01×10^{-4}	-7.01
343	1.18×10^{-3}	-6.74
353	1.30×10^{-3}	-6.64

A study of activation energy (E_a) was conducted based upon Arrhenius equation (Eq. 4.5),

$$ln\ k = ln\ A - \frac{E_a}{RT} \quad (4.5)$$

where k is the rate constant, A = Arrhenius factor, R = gas constant and T = the absolute temperature.

Plotting of *ln* k versus 1/T, as shown in Figure 4.8, yielded a linear which its slope could be calculated the E_a. It was found that the activation energy of the copper-melamine complexation was 19.60 kJ/mol.

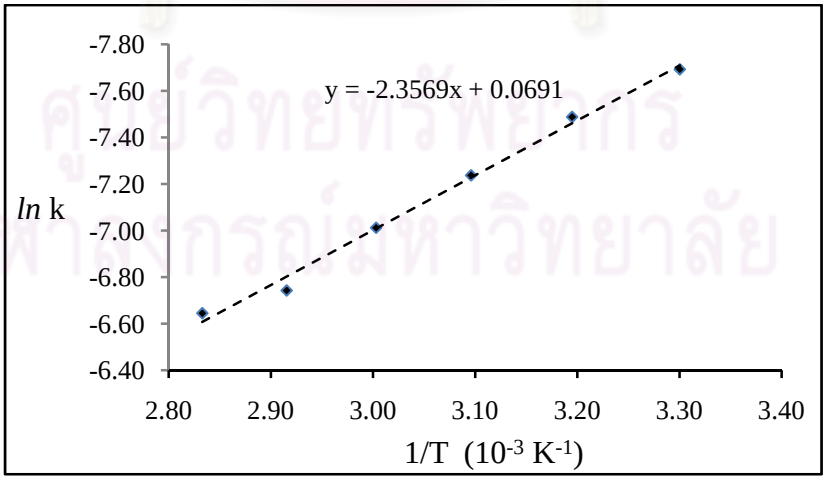


Figure 4.8 Arrhenius plot between *ln* k *vs* 1/T.

4.2 Characterization of copper-melamine complex

4.2.1 X-ray powder diffraction spectrometry

Figure 4.9 shows the XRD patterns of Cu-Mel complex and reactants; $CuCl_2$ and melamine. It could be seen that the XRD pattern of the complex product contained with medium sharp peaks did not correspond to any pattern of the reactants. This indicated that the complex was a pure phase product which was well crystalline. And it also confirmed that Cu^{II} and melamine could completely react at 80 °C for 14 hours which was suitable for using in melamine determination. However, the exact crystal structure of the complex could not be identified by the obtained XRD data because there was no standard pattern matching in the JCPDS database.

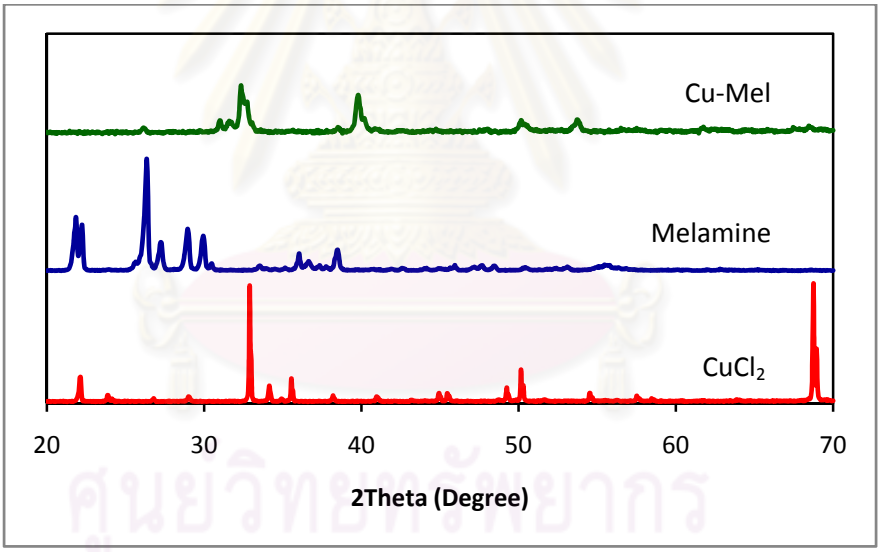


Figure 4.9 X-ray powder diffractograms of copper-melamine complex compared with $CuCl_2$ and melamine.

4.2.2 Thermogravimetry

The thermogravimetry was used to study the thermal decomposition of the complex product. As shown in Figure 4.10, the thermogram exhibited two important weight loss regions corresponding to the decomposition of melamine and chlorine from

the complex at 200-350 °C and 350-600 °C, respectively [2]. These decompositions also reflected that the obtained product was the Cu complex possessing melamine molecule as ligand. The weight of the residual solid remained constantly around 70% after heating at 600-800 °C. This residual solid may attribute to copper(II) oxide which is thermally stable.

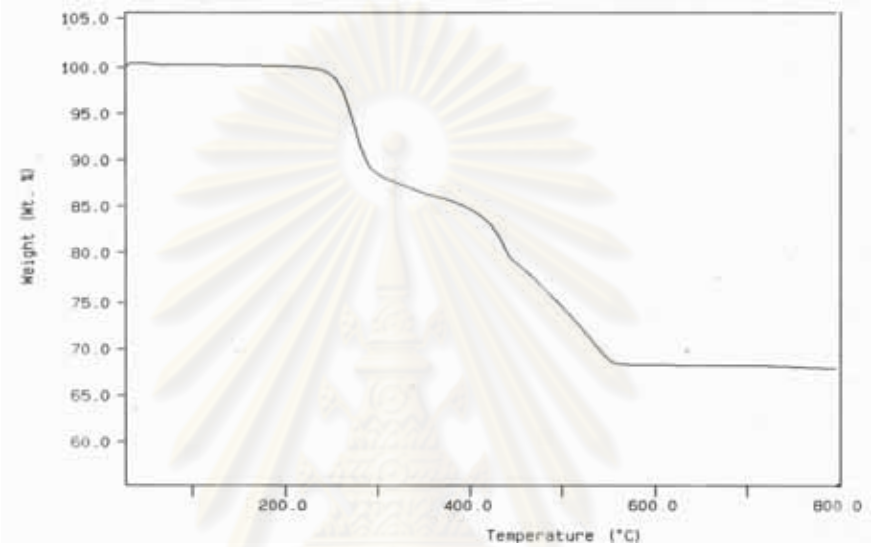


Figure 4.10 Thermogram of Cu-Mel complex.

4.2.3 Fourier transform infrared spectrometry

The results from FT-IR could be an evidence of the existing of melamine in the complex. Figure 4.11 showed the infrared spectra of Cu-Mel complex (Figure 4.11 a) and melamine (Figure 4.11 b). It could be seen that almost absorption peaks in the complex spectrum corresponded to the characteristic absorption peaks of melamine.

In the Cu-Mel complex spectrum, there were two main absorption regions. Firstly, the group of strong bands around 2800-3500 cm^{-1} was assigned to the N-H stretching. The other important bands around 1400-1700 cm^{-1} correlated with the ring distortion and N-H bending. However, the interesting absorption region of Cu-Mel complex spectrum was the frequencies of N-H stretching. They showed a bit red shift indicating the formation of Cu-Mel complex [2].

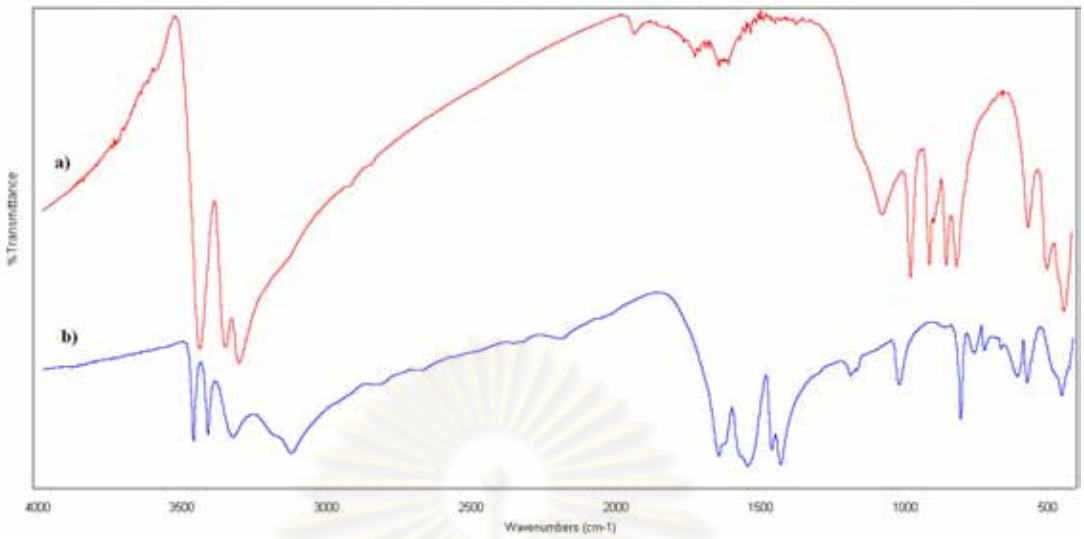


Figure 4.11 FTIR spectra of a) Cu-Mel complex and b) melamine.

The copper-melamine complex structure was proposed as Figure 4.12. Such structure was refered from molecular formular which Wiles *et al*. [1] had reported as $[CuCl_2(Mel)_2{\cdot}2MeOH]$ [2]. The proposed structure corresponded to the characterization results and Job's study.

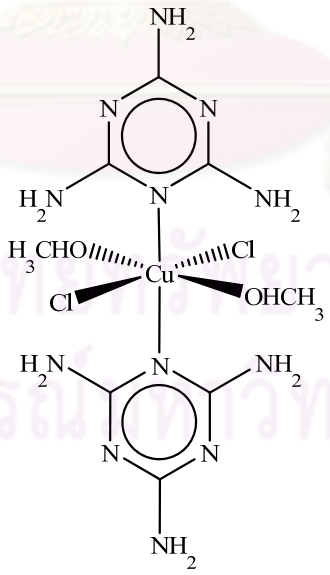


Figure 4.12 Proposed copper-melamine complex structure.

4.3 Standard calibration curve

The absorbance of remaining copper was plotted with concentration series of melamine. The plot is shown in Figure 4.13.

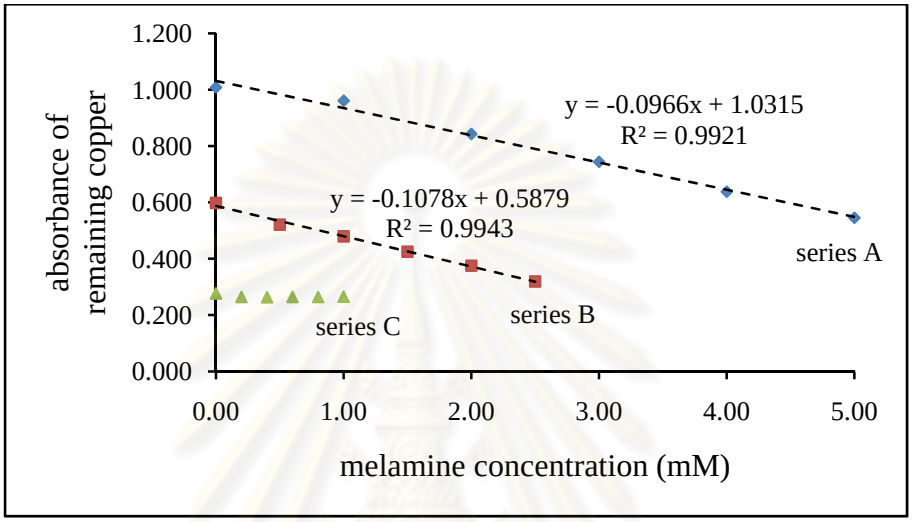


Figure 4.13 Relationship between absorbance of remaining copper and concentration series of melamine (n=3).

According to the graph, both series A and B exhibited that copper absorbance decreased linearly and proportionally to the concentration series of melamine. Meanwhile, the absorbance of remaining copper in series C did not show significant difference. This indicated that the added melamine concentrations was too low and insufficient for forming complex with copper(II) ions. Since the concentration of melamine in series B was less than that in series A, thus it was chosen to use in the method of external standard.

Furthermore, the limit of detection was also studied. Based on copper(II) concentration in series B, varied melamine concentration in the range from 0.00 to 1.00 mM was added. It was found that melamine concentration of 0.60 mM did not change the absorbance of remaining copper (shown in Figure 4.14). Therefore, the limit of detection of this method was 0.60 mM melamine.

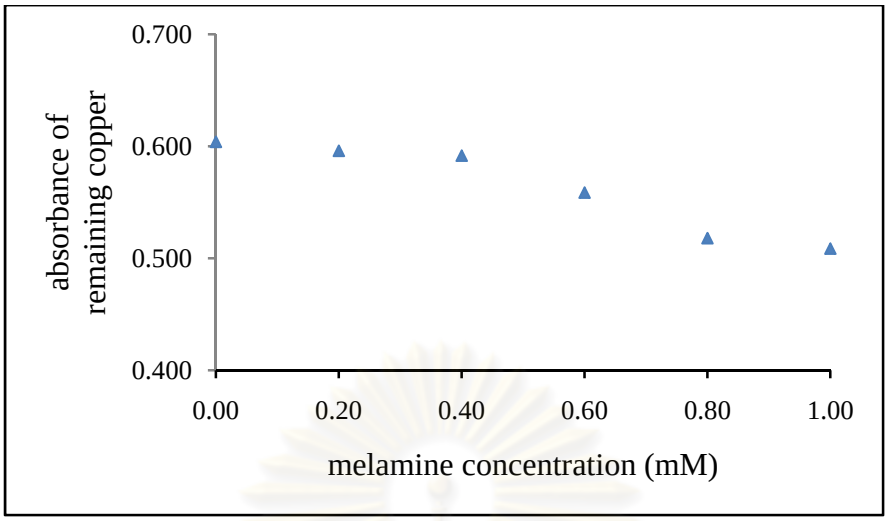


Figure 4.14 Relationship between absorbance of remaining copper and concentration of melamine between 0.00-1.00 mM (n=3).

4.4 Application to real sample

It was due to unavailable certified reference material for checking traceability of melamine in samples, Giant Seabass fish used as a sample was fortified with melamine in order to validate the developed method by examination of the recovery.

4.4.1 External standard method

Table 4.4 shows the melamine amount found in fortified samples using external standard curve series B in section 4.3. The percentage recovery was calculated by Eq. 4.6;

$$\% \text{ recovery} = \frac{(\text{sample found} - \text{blank found})}{\text{weighed melamine}} \times 100\% \qquad (4.6)$$

Table 4.4 The recovery result using external standard method

Exp.	Weighed melamine (mg)	Blank found (mg)	Sample found (mg)	Subtracted blank (mg)	Recovery* (%)
1	4.9	1.0	3.0	2.0	40 ± 7.7
2	7.1	1.0	5.5	4.5	64 ± 2.6
3	8.0	1.0	7.0	6.0	70 ± 2.4
4	10.0	1.0	8.4	7.4	74 ± 2.2

*mean ± SD (n=3)

The results showed that recoveries of melamine in samples of 8.0 mg and 10.0 mg were 70% (3.4% R.S.D.) and 74% (3.0% R.S.D.), respectively. That this recovery was not close to a hundred might result from incomplete melamine extraction. It might be caused by matrix interference. The lower amounts of melamine were trial. The melamine amounts of 4.9 mg and 7.1 mg showed unsatisfied recovery which was lower than that of 8.0 and 10.0 mg. This might result from few quantity of melamine, so it cannot form complex with copper. According to the table, it was suggested that this method could apply to analyze melamine in fish sample at the amount of melamine from 8.0 mg up. Method detection limit was 8.0 mg/g.

4.4.2 Standard addition method

The standard addition graph (Figure 4.15) was plotted between added standard melamine concentration versus A_0-A; where A_0 was remaining copper absorbance from the extraction process of sample without melamine spiking and A was remaining copper absorbance.

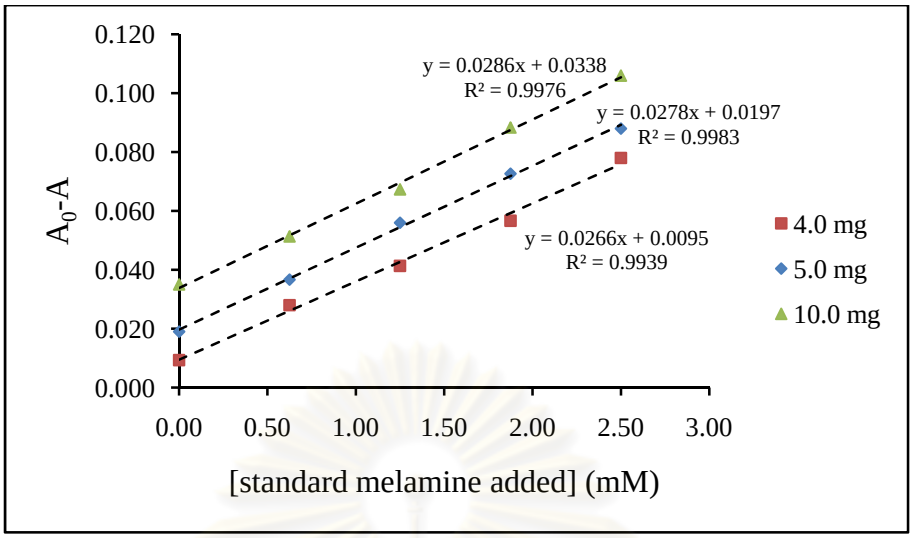


Figure 4.15 Standard addition graphs.

The amount of melamine in fortified sample was calculated by extrapolation of linear lines in standard addition graph to y = 0 point. This point was the concentration of melamine forming complex with copper. By multiplying with dilution factor and converting concentration to weight, the amount of melamine in fortified sample was found.

Table 4.5 shows the recovery result from standard addition method. The recovery of fortified melamine of 4.9 g and 10.0 g samples were 93% (4.1% R.S.D.) and 90% (10% R.S.D.), respectively. It decreased significantly when melamine weight was 4.0 mg. This revealed that the method detection limit was 4.9 mg/g.

Table 4.5 The recovery result using standard addition

Exp.	Weighed melamine (mg)	Sample found (mg)	Recovery (%)
1	4.0	2.7	36 (9.4)[a]
2	4.9	5.4	93 (5.4)[a]
3	10.0	8.9	90 ± 9.2[b]

[a]mean (range) (n=2),[b]mean ± SD (n=3)

Comparing the external standard with standard addition method, it was found that method of standard addition was able to analyze melamine which had lower concentration than method of external standard. The external standard might be affected from matrix interference, so there was an error when calibrated with the calibration graph derived from standard substances. Besides, the recovery using external standard was less than 80%, while the use of standard addition could recover melamine closed to 100%. This was due to similar interference effect of standard addition method, thus it could determine more accurate than external standard.

The relative standard deviation might not follow as the AOAC proposed, because it can be varied depending on types of analysis and samples. For biological, environmental, and food samples, the R.S.D. largely depends on matrices, analyte concentration, equipment performance, and analytical techniques [36].

CHAPTER V

CONCLUSIONS

The copper-melamine complex was synthesized by constantly heating and stirring a mixed solution of copper(II) and melamine. The copper(II) salts and solvents were studied to obtain the most suitable system for copper-melamine synthesis. The result showed that the complex obtained from the $CuCl_2$ in methanol system was maximum with naked-eye observation. According to the FAAS measurement, $CuCl_2$ in methanol system also gave the least amount of copper remaining in the solution. The minimum solubility products (K_{sp}) and the maximum formation constant (K_f) could reveal the great complexation ability of copper and melamine from this system. Besides, temperature and curing time which might affect on the complex formation were investigated. It was found that the temperature of 80 °C was the best one for the reaction. The quantity of synthesized complex was maximum at this temperature. And the curing time of 14 hours was the optimum time for the synthesis. The copper remaining in the solution was closed to zero, and the reaction start reaching equilibrium at this point. By using the chosen conditions, the Job's plot revealed the stoichiometry of copper and melamine that was 1:2. In addition, the investigation of reaction order of the copper-melamine complexation using integral and initial rate methods was found that the complex formation reaction was first-order with respect to both copper and melamine. The activation energy of the reaction estimated by Arrhenius equation yielded 19.60 kJ/mol.

The greenish precipitate of copper-melamine complex obtained from the optimized system was characterized by X-Ray powder diffraction spectrometry, thermogravimetry, and Fourier transform infrared spectrometry. The XRD pattern of the complex showed the difference from that of reactants. The TGA result showed the weight loss region corresponding to the decomposition of melamine. Besides, FTIR spectra also exhibited that the complex spectrum had characteristic peaks which similar to that of melamine. All of these evidences could confirm that copper-melamine complex was successfully synthesized.

More importantly, this complexation method shows a potential for analytical application. The calibration curve between melamine concentration and copper absorbance was efficaciously built. Its linearity showed the promising method for potential determination of melamine based upon a Flame atomic absorption spectrometry of copper(II). Fortified melamine in fish sample was done in order to validate the proposed method. It was found that the limit of detection of this method was 0.60 mM, and the method detection limit was 8.0 mg/g for external standard, and 4.9 mg/g for standard addition.

Suggestion for future work

1. Find out an appropriate solvent to dissolve the complex for benefits of exact structural study.
2. Study more on the sample preparation for food samples in order to analyze a lower concentration of melamine.
3. Validate the proposed method using real melamine-contaminated samples and compare the validated result with a reference method already published.
4. Investigate a preconcentration of extracted melamine solution prior to formation of copper(II) complex.
5. Study a wide variety of melamine-tainted samples.

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Appendix

Formation constant (K_f) and solubility products (K_{sp}) calculation

According to the stoichiometric investigation, it was found that copper bound with melamine in the ratio of 1:2. Thus, the reaction might occur as Eq. A.1;

$$Cu^{2+}(\text{excess}) + 2\text{Mel} \rightleftharpoons [Cu(\text{Mel})_2]\,(s) + Cu^{2+}(\text{remaining}) \qquad (A.1)$$

$$K_f = \frac{1}{[Cu^{2+}][\text{Mel}]^2} \qquad (A.2)$$

$$K_{sp} = [Cu^{2+}][\text{Mel}]^2 = \frac{1}{K_f} \qquad (A.3)$$

Example of calculation for $CuCl_2$ in methanol system was shown below;

5.00 mL of 0.10 M melamine was added into 5.00 mL of 0.05 M $CuCl_2$ in methanol. After heating at 80 °C and stirring for 14 hours, the measurement of remaining amount of copper showed that it was 1.67 mM.

	Cu^{2+}(excess)	+ 2Mel	$\rightleftharpoons$	$[Cu(\text{Mel})_2]$
[initial]	25.00	50.00		
[change]	x	$2x$		
[equilibrium]	1.67	50.00-2x		

The variable x calculated from equilibrium equation was 23.33 mM, and melamine concentration at equilibrium was 3.34 mM.

$$K_f = \frac{1}{[1.67][3.34]^2} = 5.37 \times 10^{-2} \qquad (A.4)$$

$$K_{sp} = [1.67][3.34]^2 = \frac{1}{5.37 \times 10^{-2}} = 18.6 \qquad (A.5)$$

K_{sp} and K_f of $CuCl_2$ in water, $Cu(NO_3)_2$ in methanol, and $Cu(NO_3)_2$ in water could be calculated as same as the above approach.

VITA

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